SEROPREVALENCE, MOLECULAR AND CLINICAL EPIDEMIOLOGY OF AVIAN METAPNEUMOVIRUS INFECTION IN THREE SELECTED CLIMATIC ZONES IN NIGERIA

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A Thesis in the Department of Veterinary Medicine,
submitted to the Faculty of Veterinary Medicine in
partial fulfilment of the requirements for the Degree of

DOCTOR OF PHILOSOPHY

of the

UNIVERSITY OF IBADAN

MAY 2023

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DEDICATION

I dedicate this Thesis to God, my Creator, for giving me the chance to complete this project. Also, thanks to my family for their patience and sacrifice throughout this education.

ACKNOWLEDGEMENTS

I truly thank the Almighty God, the beneficent and compassionate, for his unfathomable generosity and kindness throughout this investigation.

Prof. Omolade A. Oladele, my wonderful supervisor, mentor, mother, and Head of the Avian Diseases Unit, compassionately supervised my work, meticulously perused this Thesis, and fruitfully evaluated it in order to make a positive difference in it and in me. I will also like to thank my co-supervisor, Prof. D. O. Oluwayelu, for his keen interest and guidance throughout this project. He truly is a father figure. I wish you and your family God's blessing.

Dr. Clement Meseko, Assistant Director, National Veterinary Research Institute (NVRI, Vom), Dr. Shittu Ismaila, Head of Avian Diseases Laboratory, NVRI, and Dr. I. Bitrus, Mr. N. Daspan, Mr J.I. Stephen, and Dr. Damilola Arowolo provided technical assistance, laboratory facilities, reagents, and training during this study. Dr. O.D. Ishola, Dr. Hauwa'u Umar Mugadi, Dr. I. L. Peter, Mr. Dare Aiyepolu, Mr. Ini Akpan, and Dr. Dotun Oladele were all very helpful throughout sample collection.

My profound gratitude goes to the Head of Department of Veterinary Medicine, Prof. O. T. Jeremiah as well as late Prof. C. A. O. Adeyefa, Prof. T. O Omobowale, Dr. G. A. Oladosu, Dr. R. A. M. Adedokun, Dr. O. O. Omotosho, Dr. J. O. Abiola, Dr. S. C. Olaogun, Dr. O. A. Adejumobi and Dr. O. O. Esan for their encouragement and support. I am also grateful to Dr. O.R. Anifowose and Dr. Bukola A. Alaba for their love and genuine interest in the progress of this work. I appreciate Mrs M.N. Ofarn, Mrs Joy. I. Akoma, Mrs. Dupe Awoyemi, and other members of staff for their assistance and encouragement during the course of this study. I also appreciate Prof. J. K. Olopade, Dr Afusat Jagun-Jubril, Dr Olamide Adebiyi, Dr. A. S. Akinrinde, Dr O. I Azeez and Dr. Bisi Adeoye for their immense encouragement and support. I am grateful to my teachers and entire staff of the Faculty of Veterinary Medicine.

I am grateful for the support of my family, which I acknowledge. My parents, Mr. Kolade I. and Mrs. Salewa L. Akindele, My mother-in-law, Alhaja N. Bakre as well as my cherished aunts, Mrs. Biodun Olagunju and Mrs. Morufat Badmos, my uncle and his wife, Dr. and Dr. R.O. Folorunsho, for their love and care throughout this program. Prof. and Mrs. Olayinka Aderinto have my sincere gratitude for their prayers and deep love. I am grateful for the constant support I have had from my siblings, Mr. and Mrs. Adetoyese Akindele, Mr. Dare and Mrs. Adetunmise Salisu, Dr. Adetunwase Akindele and Mr. Raji Olagunju. All of you, may God reward you for your excellent acts.

I am appreciative of the support of my friends and well-wishers, who are too many to list, for their numerous encouragements, fervent prayers, and steadfast faith, which gave me the hope and confidence I needed to successfully complete the programme.

My deepest thanks to Olukayode Bakre, my best friend and husband who stuck with me through it all. I thank God for the day I met you. Thank you for your patience and love, Oyinkansola, Omorinsola, and Taiwo, my darling children.

I am grateful to the Vice Chancellor and the administration of University of Ibadan for accepting my research leave and providing me with the opportunity to realize my dream. God bless you all in the future. University of Ibadan, long live.

In addition, I want to express my gratitude to everyone else who did something to contribute to the success of my Thesis that time and space did not permit me to express.

ABSTRACT

Avian metapneumovirus (aMPV) causes an immunosuppressive disease of the upper respiratory tract of chickens and turkeys, leading to substantial economic loss in poultry production. Despite the significant global burden of this disease, little is known about its endemicity, distinguishing clinical features, circulating virus subtypes and the role of climate in its occurrence in Nigeria. This study was designed to investigate aMPV seroprevalence, circulating subtypes, clinical presentation and predisposing factors in Nigeria.

Using a cross-sectional study design and simple random sampling technique, blood was collected from 480 apparently healthy commercial chickens from states within three climatic zones of Nigeria: near-temperate (Plateau, n=160), rainforest (Oyo, n=160) and semi-arid (Sokoto, n=160) during the dry and wet seasons between December 2018 and September 2019. Harvested sera were tested for aMPV antibodies using indirect ELISA. A total of 168 tissue samples including conjunctivae, turbinates, tracheae and lungs (n=42 each) were collected from carcasses from chicken flocks with signs of respiratory distress presented at Veterinary diagnostic facilities in the study areas between December 2019 and April 2020 for virus detection using RT-PCR to amplify the N- and G-genes of the virus. Amplicons were sequenced using Sanger's method and phylogenetic analysis was performed with appropriate software. Pretested questionnaires were administered to 42 owners of the sampled flocks to access information on clinical presentations and antibiotic usage during respiratory disease outbreaks. Thereafter, RT-PCR-positive samples were processed for virus isolation in Specific-Antibody-Negative embryonated chicken eggs. Bacteria associated with aMPV-positive tissues were isolated and identified using standard methods. Data were analysed using descriptive statistics and ANOVA at $\alpha_{0.05}$.
The aMPV seroprevalence rates were 100.0, 48.8 and 56.2% for Plateau, Oyo and Sokoto states, respectively, during the dry season and 52.5, 36.2 and 65.0%, in the wet season. Mean antibody titers were significantly higher in the dry season (4757.9±223.5, 1414.0±158.0 and 2800.9±313.1) than wet season (670.7±74.9, 499.4±55.8 and 548.8±61.4) for Plateau, Oyo and Sokoto states, respectively. Turbinate and conjunctiva samples from five flocks (11.9 %) of layer chickens of all age groups were positive for aMPV in Plateau State with significant association between near temperate zone and the occurrence of the disease. Phylogenetic analysis revealed that the Nigerian aMPV strain clustered with European and Asian subtype B strains with unique mutations (T12I, G223E and A238V) in the G-gene. Clinical signs presented by aMPV-positive flocks included rales, coughing, sneezing and dyspnoea while the commonly used antibiotics by farmers were tylosin (71.4%), doxycycline (66.7%) and enrofloxacin (59.5%), without prescription. Virus isolation from aMPV-positive tissues was unsuccessful while secondary bacteria isolated included *Escherichia coli, Pseudomonas aeruginosa* and *Klebsiella pneumoniae*.

Avian metapneumovirus infection associated with a more virulent Subtype B strain was widespread in commercial layers in the study areas, with the turbinate and conjunctivae being the predilection sites. Associations with *Escherichia coli, Pseudomonas aeruginosa* and *Klebsiella pneumoniae* were established, while transmission was aided by low environmental temperature and humidity. Routine vaccination of commercial chickens using homologous virus strains is recommended.

Keywords: Avian metapneumovirus, Seroprevalence, Nigerian climatic zones.
Word count: 486

TABLE OF CONTENTS

CHAPTER SIX SUMMARY, CONCLUSION AND RECOMMENDATIONS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
AIV	Avian Influenza Virus
aMPV	Avian Metapneumovirus
BLAST	Basic Local Alignment Search Tool
CAM	Chorioallantoic membrane
CFU	Colony-forming unit
Cj	Conjuctiva
CMI	Cell Mediated Immunity
CRBC	Chicken red blood cells
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
ELISA	Enzyme linked immunosorbent assay
EMB-Agar	Eosin Methylene Blue Agar
EXPASY	Expect Protein Analysis System
FAO	Food and Agricultural Organization
FTA cards	Flinders Technology Associates cards
G-PROTEIN	Glycoprotein
HMPV	Human Metapneumovirus
HA	Haemaglutination Assay
IB	Infectious Bronchitis
IFA	Immunofluorescence assay
IFN-γ	Interferon-Gamma
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IgA	Immunoglobulin A
ILT	Infectious laryngotracheitis
IP	Immunoperoxidase
Lg	Lung
LPAI	Low Pathogenic Avian Influenza
mAbs	Monoclonal antibodies
MRVP	Methyl- Red-Voges- Proskauer
NCBI	National Center for Biotechnology information

NIMET	Nigerian Meteorological Agency
NOIF	Nitric oxide-inducing factor
NVRI	National Veterinary Research Institute
RNA	Ribonucleic acid
RT-PCR	Reverse Transcriptase- Polymerase Chain Reaction
SEM	Standard error of mean
SHS	Swollen head syndrome
SIAS	Sequence identity and similarity
SPF	Specific pathogen free
TAE	Tris base, acetic acid
Tr	Trachea
TRT	Turkey rhinotracheitis
T-cells	Thymus-derived cells
TSA	Trypticase Soy Agar
Tu	Turbinate
UK	United Kingdom
USA	United State of America
VNDV	Virulent Newcastle disease virus
VNT	Virus neutralization test
VTH	Veterinary Teaching Hospital
VTM	Virus transport medium

LIST OF APPENDICES

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND TO THE STUDY

Agriculture remains the largest sector of economy in Nigeria presenting an average of 24% to the Nation`s Gross Domestic Product (GDP). The sector employs more than 70% of the country's work force, a feat that ranks it as the largest employer of labour in the country (NBS, 2019; FAO, 2019). Chicken, turkeys, ducks as well as guinea fowl, pheasant and more notably Ostriches raised for egg and meat production are referred to as Poultry birds (OAHN, 2016; FAO, 2018). Among all types of poultry, chicken is the commonest and it is found in every part of the world (Padhi, 2016). Nigeria, after South Africa, has the second largest chicken population in Africa, producing 650 000 tonnes of eggs and 300 000 tonnes of meat in 2013 (FAOSTAT, 2019). The poultry industry is a major agricultural sub-sector in Nigeria, providing high-value lipids, vitamins, and proteins to humans (Makinde *et al*., 2017). Poultry diseases are one of the greatest risks to profitable poultry farming in Nigeria (Balami *et al*., 2014). Poultry diseases are significant because they have a high prevalence and are related to morbidity and mortality in tropical environmental conditions (Asfaw *et al*., 2021). A few decades ago, the major diseases of poultry which were recognized, were due to bacterial, parasitic and viral agents (Batista *et al*., 2020).

Most diseases caused by bacterial and parasitic agents have been controlled or in some cases, eradicated in developed agricultural countries where more and newer viral diseases have subsequently emerged (Adene, 2004).

Viral diseases are less amenable to common therapeutic and other control measures. The respiratory viral diseases including infectious bronchitis, Newcastle disease, infectious laryngotracheitis, avian metapneumovirus infection and avian influenza are currently posing problems for the poultry sector (Batista *et al*., 2020).

Advancements in poultry health management technologies in developed countries have continued to benefit the poultry industry in developing countries. Furthermore,

importation into the poultry industry in the developing countries has inevitably acquired some new problems in the process. However, some of the so-called emerging poultry diseases are indeed exotic to Africa. Findings in the past have shown that many of our native chickens and guinea fowls, have little or no contact with imported chickens and still show antibodies to most of the viral diseases (Adene, 2004). Therefore, many diseases that exist undetected in our poultry flocks and continue to wreck our poultry industry are due to lack of facilities to monitor and control them.

As an emerging poultry pathogen, avian metapneumovirus (aMPV) affects the poultry industry globally (Jardine *et al*., 2018). It belongs to the Paramyxoviridae family, the Pneumovirinae subfamily, and the Metapneumovirus genus, and it is one of the causes of upper respiratory diseases in poultry, especially turkeys and chickens (Kaboudi and Lachheb, 2021). Other avian species may be affected, including duck, pheasants, guinea fowls (Legnardi *et al*., 2021), wild water fowls and ostriches (Jardine *et al.*, 2018). Seagulls as well as other Wild birds are also possible carriers of aMPV (Rizotto, 2019). The clinical signs of avian metapnemovirus infection in poultry are pathognomonic respiratory signs which include torticollis, coughing, nasal discharge and watery eyes, which are typically found in broiler flocks and their breeding stock (Hartmann *et al*., 2015). Decreased production of eggs are also observed in laying birds due to this infection (Sun *et al*., 2014). These clinical manifestations might result in considerable financial losses, especially when they are linked to secondary microbial infections like *Mycoplasma gallisepticum, Bordetella avium* and *Escherichia coli* (Ball *et al*., 2018). The propagation of aMPV on the farm is largely dependent on various factors, such as biosecurity within or between the poultry flock, hygiene requirements, age, breed and population density of the flock. It is rapidly transmitted horizontally by aerosol or by contact with polluted materials (Ali *et al*., 2019). South Africa was the first country to recognize aMPV in 1978, and since then, researches have been conducted in a number of nations, including the United Kingdom (Anon, 1985; Hafez *et al*., 2000), Spain (Anon, 1985) France (Giraud, 1986), Hungary (Lantos, 1990), Germany (Hafez, 1992), Italy (Catelli *et al*., 2006) and recently, in almost all poultry-grown areas of the world including China and Nigeria (Bakre *et al*., 2020). Avian metapneumovirus (aMPV) has four subtypes based on nucleotide similarity to protein attachment: A, B, C, D, and the new subtypes (Retallack *et al*., 2019). Subtypes A and B have been identified in almost every part of the world, including Europe (Mescolini *et al*., 2021) and Nigeria (Owoade *et al*., 2008), also, in

some countries such as the United States, France, South Korea and Italy, Subtype C has been detected (Legnardi *et al*., 2021). Subtype D has been identified in Europe, Italy and France (Cecchinato *et al*, 2013). New subtypes of avian metapneumovirus have recently been identified in North America and California (Retallack *et al*., 2019). Due to lack of distinct clinical symptoms, aMPV infection is difficult to detect and isolate (Cook and Cavanagh, 2002).

Transmission of infectious diseases exhibits temporal and spatial incidence variations representing seasons, interannual weather variability, extreme weather events and other natural disasters (Moriyama *et al.*, 2020). Infectious diseases are profoundly influenced by extreme weather conditions and temperature variations (Bello *et al*., 2017).

Significant changes in global temperature, wind patterns, precipitation, weather patterns such as drought, storms, insect infestation and other climate indicators that occur over several decades or more are referred to as climate change (Tiruneh and Tegene 2018). Climate change is high risk for poultry flocks, as it affects their behaviour, physiological activities and disease outcomes due to the range of thermal conditions (Liverpool-Tasie *et al*., 2019). Seasonal variations related to climate change affects bird migration, influence avian influenza virus transmission, epidemiology/trend and the cycles of transmission of other respiratory viruses. Seasonal variations can also have a direct impact on the survival of these viruses outside their host, thus promoting the outbreak of most viral diseases that inevitably affect the health and productivity of poultry (Hassan and Abdul-Careem, 2020). In the United States, turkey rhinotracheitis outbreaks have been observed to follow seasonal influence, with approximately 80% of cases happening in the spring and fall, implying that climatic conditions may influence disease transmission (Bennett *et al*., 2005). Nevertheless, disease and climate related conditions or the synergism of both have been described by Tiruneh and Tegene, (2018) as a contributing limitation to modern animal husbandry in tropical areas. In Nigeria, there are only two seasons, and they have been related to disease outbreaks (Olumade *et al*., 2020).

1.2 Statement of Problems

In particular, the Nigerian poultry industry has expanded significantly in recent years, becoming one of the country's most commercialised agricultural subsectors (Shapouri *et al*., 2001). Furthermore, poultry meat is exceptionally tender, and consumers, regardless of religious convictions, accept it. (Akintunde *et al*., 2015). Poultry diseases,

particularly viral infections such as avian metapneumovirus, remain a major hazard to increasing poultry output (Brown *et al*., 2014). Disease outbreaks have resulted in the closure of several poultry operations, costing millions of naira each year in treatment, prevention, and control costs, as well as mortality rates. Early disease detection and treatment/prevention are critical to ensuring a viable chicken sector that is responsive to the nation's animal protein demands and nutrition security. Poultry is one of the most complex and interwoven aspects of the livestock industry and production subsector in agriculture, with the potential for quick and efficient production and supply of animal protein, it is therefore critical to refocus efforts on disease surveillance and management (Owoade *et al*.,2008).

In Nigeria, little research has been done on the seroprevalence, detection, and characterisation of strain types, as well as whether there are chicken types or seasonal influence in the incidence of avian metapneumovirus (aMPV) in chickens and the secondary bacteria associated with its proliferation. Human metapneumovirus (HMPV) has recently been identified as a leading cause of respiratory infection in newborns, piqueing the public's interest in the genus because HMPV is strongly linked to subtype C-aMPV (Brown *et al*., 2014).

The symptoms of avian metapneumovirus are almost identical to those of other viral infections, notably infectious bronchitis and Newcastle disease, which are well-known among farmers and Veterinarians. It is possible that it may be mistaken for any of them, especially in a country like Nigeria, where facilities for confirmatory diagnosis are limited. Although, Owoade *et al*. (2008) documented the seroprevalence and identification of the virus in apparently healthy commercial chickens in South western part of Nigeria, awareness of the existence of this virus in the country among veterinarians and poultry farmers is low. The current situation with respect to aMPV in Nigeria is unknown including the circulating subtypes

The influence of climate on the proliferation of the virus in Nigeria has also not been reported. According to Goraichuk *et al*. (2020), aMPV being an RNA virus is liable to variation in its genetic make-up leading to the occurence up of new subtypes which are distinctive and definitive to each region. Since the report of Owoade *et al.* (2008) thirteen years ago, that Subtypes A and B were circulating in apparently healthy commercial poultry birds in Nigeria, there is possibility of emergence of a new subtype in other parts of Nigeria because of mutation. The most successful strategy for containing the disease is vaccination and vaccines are produced based on the

knowledge of available strains. To the best of my knowledge there is no vaccine available in Nigeria against this virus.

1.3 Justification of the Study

To signify aMPV infection as an important viral respiratory disease of poultry birds in Nigeria, it is imperative to determine the current prevalence status. The only study on seroprevalence in Nigeria was that of Owoade *et al*., (2006) which indicated a rate of 40% in chicken in Southwestern, Nigeria. Knowledge of the characteristics of circulating aMPV in Nigeria is critical for the recommendation and establishment of vaccination to control the disease. Aside from the work of Owoade *et al*. (2008), who discovered Subtypes A and B of the virus in apparently healthy birds raised in Southwestern Nigeria, there is no other information on the circulating virus, particularly from other parts of the country. In addition, it is important to relate the actual outbreak of a respiratory disease to aMPV as the causal agent rather than the mere detection of infection. Thus, the need to sample poultry showing clinical signs unlike Owoade *et al.* (2008)'s work , which involved only apparently healthy birds.

Furthermore, the recognition of aMPV infections in association with its epidemiological factors on the field is still very challenging to Veterinarians, therefore remaining grossly underreported. While vaccines for the control of most viral respiratory diseases are readily available in Nigeria, that of avian metapneumovirus (aMPV) is not. This is because there is dearth of knowledge on the presence and occurence of the disease in Nigeria, as well as the characteristics of the available strains. Furthermore, the influence of seasonal variation on virus distribution has not been studied to complement regional diagnostic and control effects.

1.4 Aim of the Study

To investigate the occurrence, genetic diversity and clinical epidemiology of avian metapneumovirus (aMPV) infection in chickens from three climatic zones of Nigeria

1.5 Specific Objectives

1. To determine the seroprevalence of aMPV infection in commercial chickens from three climatic zones of Nigeria.
2. To detect and characterise aMPV strains circulating in three climatic zones of Nigeria.
3. To elucidate the epidemiological factors and clinico-pathological presentation associated with avian metapneumovirus in Nigeria.

4. To isolate detected aMPV in specific antibody negative embryonated chicken eggs.
5. To determine the secondary bacterial pathogens associated with aMPV infection in three climatic zones of Nigeria.

1.6 Research Questions

i. What is the seroprevalence of aMPV infection in commercial chickens in Nigeria and how does it vary with seasons and climatic zones?
ii. What is the genetic diversity of the circulating strains of aMPV from three different climatic zones of Nigeria?
iii. What epidemiological factors and clinico-pathological presentations are associated with aMPV infections in Nigeria?
iv. Can detected Nigerian strains of aMPV grow in specific-antibody-negative embryonated chicken eggs?
v. What are the secondary bacteria associated with aMPV infection in chickens in three climatic zones of Nigeria?

CHAPTER TWO

LITERATURE REVIEW

2.1 Poultry Production in Nigeria

Poultry is the most commonly kept livestock and its population is 140-160 million in Nigeria (Aminu and Hermmans, 2021). Poultry production in Nigeria has increased in recent years, with its share of Gross Domestic Product (GDP) increasing in absolute terms (FAOSTAT, 2018). This generates market prospects for entrepreneurs and provides job opportunities and thereby alleviating poverty (Omodele and Okere, 2014). Chickens, guinea fowl and turkeys, among which chickens predominate, are the types of poultry that are of economic significance. Chicken (*Gallus domestica*) is a member of the Phasiendae family and represents around 69 per cent of all poultry reared in Nigeria (FAO, 2019). A daily minimum of 65 g to 75 g of total protein, 40 per cent of which should be obtained from animal sources, was requested by the Food and Agriculture Organisation (Anosike *et al.*, 2018). However, the roughly measured intake of animal protein by an average adult in Nigeria is around 17 g, suggesting a gross deficit (FAO, 2015). The poultry industry is very important for the economy because it increases the production of high-protein foods such as eggs and meat, which are known to provide the most nutritious foods that are rich in all the vital amino acids, minerals and vitamins required for human health, strength and productive capacity (FAO, 2015). Compared to other livestock production operations, commercial egg and meat production is arguably the most significant and inexpensive source of excellent protein and income generation (Hafez and Attia, 2020). The availability and use of highly enhanced vaccinations that aid in disease control, lower tariffs on imported day-old chicks and parent stocks, and access to well-compounded feed made from local feedstuffs have all contributed to considerable increase in poultry production (Adedeji *et al*., 2014; World Bank, 2017).

2.1.1. Management Systems in Poultry Production in Nigeria

In Nigeria, there are three management systems: intensive, extensive, and semi-intensive, which are distinguished by flock size and the relationship between output and input (Zheng *et al*., 2021). Intensive production system includes flock sizes of thousands of birds raised in either battery cages or deep litter management systems and about 40 million chickens are raised in this system (ASL 2050, 2018), while semi-intensive production system includes flock sizes ranging from 2000 to 5000, often increased in some instances with about 60 million chickens raised in this system in Nigeria. The birds are housed in a fixed building but are allowed to roam about within a fenced area during the day, a good example is a fold unit system (ASL 2050, 2018). Extensive management is mainly local and backyard poultry production where birds are required to scavenge food for survival, which is an advantage but they are often exposed to predators and unfavourable weather conditions (Dal Bosco *et al*., 2021).

Excellent housing, early culling of unproductive birds, proper disease and predator management, good record keeping, and high egg and meat output are all advantages of the deep litter and battery cage system of chicken rearing (Omodele and Okere, 2014; Maduka *et al*., 2016). Despite all of its advantages, its drawbacks include cannibalism, disease outbreaks, and a significant capital investment (Okpokiri *et al.*, 2018).

2.1.2. Constraints in Poultry Management

Despite the economic potential of the poultry industry, many factors are limiting poultry production system, such as accommodation, types of poultry, socioeconomic history of farmers, source of feed and feeding, sales and disposal, health and diseases (Adedeji *et al*., 2014; Hafez and Attia, 2020). The biggest challenge to improving poultry production in Nigeria remains diseases caused by various species of organisms such as viruses, bacteria, helminths, ectoparasites, fungi and rickettsia (Akintunde *et al*., 2015).

Among the diseases reported in the Nigerian poultry industry were avian influenza, infectious bronchitis, avian pox, Newcastle disease, infectious bursa disease, avian metapneumovirus, colisepticemia, coccidiosis, and worm infestations (Adedeji *et al*., 2014; Mahendra, 2016). Important avian diseases affect various systems, some affecting multiple systems such as the nervous, gastro-intestinal, circulatory and respiratory systems (Bande *et al*., 2016).

2.2. Respiratory Diseases of Avian Species

Respiratory diseases are common cause of high mortality rates in livestock production around the world because of their multifactorial attributes (Sid *et al*., 2015), but largely understudied in most African countries (Akanbi and Taiwo, 2014). Respiratory diseases have different aetiologies including viral organisms (Umar *et al.,* 2017). Viruses such as Infectious Laryngotracheitis (ILT), infectious bronchitis (IB), avian metapneumovirus (aMPV), avian influenza (AI) and virulent Newcastle disease virus (vNDV) (Table 2.1) are responsible for respiratory diseases associated with high mortality in chickens (Hichem *et al.,* 2015). Except for infectious laryngotracheitis (ILT), which usually affects chickens before 3 weeks of age, the aetiology of these diseases affects chickens of all ages (Umar *et al*., 2019). Aside from these infectious organisms, noninfectious factors such as environmental conditions (e.g., poor ventilation, high ammonia levels, extreme temperature) can also play a part to the disease progression (Sid *et al*., 2015). The length of the disease, mortality, and severity of clinical symptoms are highly varied and determined by a variety of factors, including the infectious agent's virulence and pathogenicity, as well as environmental conditions (Marchewka *et al*., 2013).

The cost of professional veterinary services, as well as medications to treat respiratory infections, raise operational costs (Marien *et al*., 2005). The emergence of new virulent genotypes as a result of national outbreaks, as well as recurring alterations detected in the genomic sequences of the organisms have resulted in insufficient diagnostic and control strategies (Umar *et al*., 2019). Many pathogens are known to complicate poultry respiratory diseases (Table 2.1), including avian metapneumovirus, Newcastle disease virus (NDV), avian influenza virus, infectious bronchitis virus, *Mycoplasma iowae, Mycoplasma meleagridis, Mycoplasma gallisepticum, Mycoplasma synoviae, Esherichia coli, Avibacterium paragallinarum, Bordetella avium, Pasteurella multocida, Ornithobacterium rhinotracheale and Chlamydophila psittaci* which are associated with huge loss of revenue to the poultry sector (Van de Zande *et al*., 2001).
Over the last several years, an increase in avian respiratory disease has been observed in Nigerian commercial chickens, with low to medium mortality and high morbidity rates. clinical presentations (Akanbi and Taiwo, 2014). Avian Metapneumovirus (aMPV) could play as a contributory aetiology, therefore requires investig

Table 2.1: Respiratory pathogens and related diseases of poultry

Disease	Aetiology	Major clinical Signs/lesions	Prevention/Control
Swollen head syndrome/ turkey rhinotracheitis	Avian metapneumovirus	Swollen head, tracheitis, airsacculitis, conjunctivitis, pneumonia	Vaccine available Good biosecurity measures
Avian Influenza (AIV)	Influenza virus	Moderate to severe respiratory signs depending on the subtype of the virus	Vaccine available Good biosecurity measures
Infectious bronchitis	Coronavirus	Tracheitis, airsacculitis, pneumonia, nephritis	Vaccine available Good biosecurity measures
Newcastle disease	Avian paramyxovirus	Variable: moderate to severe respiratory clinical symptoms and lesions	Vaccine available Good biosecurity measures
Infectious laryngotracheitis (ILT)	Gallid Alphaherpesvirus-1	Tracheitis	Vaccine available Good biosecurity measures
Chronic respiratory Disease	*Mycoplasma gallisepticum*	Chronic tracheitis; chronic Airsacculitis	Mycoplasma free progeny.
Mycoplasmosis	*Mycoplasma synoviae*	Airsacculitis and moderate tracheitis, arthritis.	Vaccination Possible Mycoplasma free progeny
Infectious coryza	*Avibacterium paragallinarum*	Sinusitis, conjunctivitis, airsacculitis	Vaccines available
Collibacillosis	*Escherichia coli* often associated with other respiratory pathogens	Fibrinous, pericarditis, airsacculitis, tracheitis	Vaccine available against *E. coli* serotypes.
Pasteurellosis	*Pasteurella multocida*	in chronic form e.g., conjunctivitis, tracheitis; in acute form septicaemia	Reduce dust in shed. Vaccination possible Good biosecurity measures.
Ornithobacteriosis	*Ornithobacterium rhinotracheal*	Tracheitis, Airsacculitis	Vaccination possible Good biosecurity measures.

Source: Umar *et al*., 2017

2.2.1 Overview of Avian Metapneumovirus

Avian metapneumovirus (aMPV) is a worldwide pathogen that infects the upper respiratory system of chickens and turkeys, resulting in clinical symptoms and significant economic losses (Catelli *et al*., 2010), especially in association with secondary bacterial infections (Cecchinato *et al*., 2016). The disease is said to be clinically similar to *Bordetella avium* (Seal, 2000), but under experimental conditions, the presence of aMPV is mainly associated with infection with *Escherichia coli* and *Mycoplasma gallisepticum* (Jirjis *et al*., 2009), and field findings have also indicated that other respiratory viruses such as infectious bronchitis virus and Newcastle disease viruses (Villareal *et al*., 2007b) can contribute to the disease. aMPV also affects the lower part of the respiratory tract, decreases the production of eggs, reduces the quality of eggs, and is close to the clinical signs of infectious bronchitis (Nagy *et al*., 2018). The clinical signs displayed by chicken and turkey in response to the sudden onset of aMPV infection and the rapid spread of the infection across flocks are also very close to those of Newcastle disease (Catelli *et al*., 2010).

2.2.2 History of Avian Metapneumovirus

Buys and Du preez (1990) detected the very first case of aMPV infection in South Africa in turkeys in 1970 and shortly thereafter the disease was reported by Giraud *et al*. (1986) in France, then UK, where the causative agent was first characterized by Mc Dougall and Cook (1986). The infection appeared in England in mid-1985 and spread rapidly through England and Wales to turkeys of all ages (Anon, 1985). The disease was identified by Listorti *et al*. (2014) in broiler parent flocks in Europe, United States of America (Retallack *et al*., 2019), Africa, Middle East and Asia (Nguyen *et al*., 2021), Brazil (Santos *et al*., 2012) as well as other places of the globe (Cook and Cavanagh, 2002). Based on seroprevalence studies, avian metapneumovirus has also been observed in Israel (Banet-Noach *et al*., 2005), Morocco (Fellahi *et al*., 2015), Zimbabwe (Cadman *et al*., 1994), Japan (Otsuki *et al*.,1996), Brazil (D'Arce *et al*., 2005), USA (Cecchinato *et al*., 2014), Taipei China (Wei *et al*., 2013), Pakistan (Ahmad *et al*., 2005), Saudi Arabia (Al-Ankari *et al*., 2004; Al-khalaf, 2010), Egypt (Abdel-Azeem *et al*., 2014), Iran (Homayounfar *et al*., 2015) and in Canada (Jardine *et al*., 2018).

2.2.3. Aetiology of avian metapneumovirus disease

The avian metapneumovirus (aMPV) in the family of paramyxoviridae belongs to the subfamily pneumovirinae (Adams *et al*., 2016; Rima *et al*., 2017) and order Mononegavirales (Cecchinato *et al.,* 2016). The subfamily is divided into two genera; *Pneumovirus* consisting of metapneumovirus that involves human metapneumovirus (hMPV) and avian metapneumovirus (aMPV) and mammalian respiratory syncytial viruses (Lamb *et al*., 2009; Amarasinghe *et al*., 2019). The aMPV is a respiratory virus that affects chickens and turkeys and other avian hosts. It causes turkey rhinotracheitis (TRT) in turkeys and swollen head syndrome (SHS) in chickens (Cecchinato *et al.,* 2016).

2.2.4. Structure of Avian Metapneumovirus

The virus genome is unsegmented and consists of a single-strand negative sense RNA of approximately 13.4 kilobases (Cecchinato *et al.,* 2016). The chromosome consists of eight viral polypeptide encoding genes, two of which are glycosylated and three of which encode proteins that are specified by unstructured viruses. The highly variable G-attachment gene, which codes for conjugated protein and a key major immunogen of aMPV, is a strong candidate for epidemiological studies and differentiation of subtypes (Homayounfar *et al*., 2015). As indicated in Figure 2.1, viral polypeptides include small hydrophobic protein (SH), viral RNA-dependent RNA polymerase (L), phosphoprotein (P), nucleoprotein (N), fusion protein (F), matrix protein (M), surface glycoprotein (G), and second matrix protein (M2) (Gough and Pedersen, 2016). A genome schematic diagram of aMPV is given in Figure 2.2 which shows the avian metapneumovirus virion contains a phospholipid sheath generated from the host cell's cytoplasm through which the, fusion (F), attachment (G) glycoprotein, and small hydrophobic (SH) proteins are attached (Wei *et al.,* 2013). The virus nucleoprotein (N) envelopes the viral genome and protects it against nuclease action. The F-protein mediates the fusion between the cell membrane and the viral envelope, while the G-protein facilitates binding to the target cell (Cecchinato *et al*., 2010; 2016). Viral RNA-dependent RNA polymerase (L) and Phosphoprotein (P) are required for RNA genome replication (Easton *et al.,* 2004; Wei *et al.,* 2013). Furthermore, the primary membrane-associated structural proteins of aMPV, which are the Fusion protein(F) and the glycoprotein (G), play essential roles in viral pathogenicity and immunogenicity (Yu *et al*., 2010).

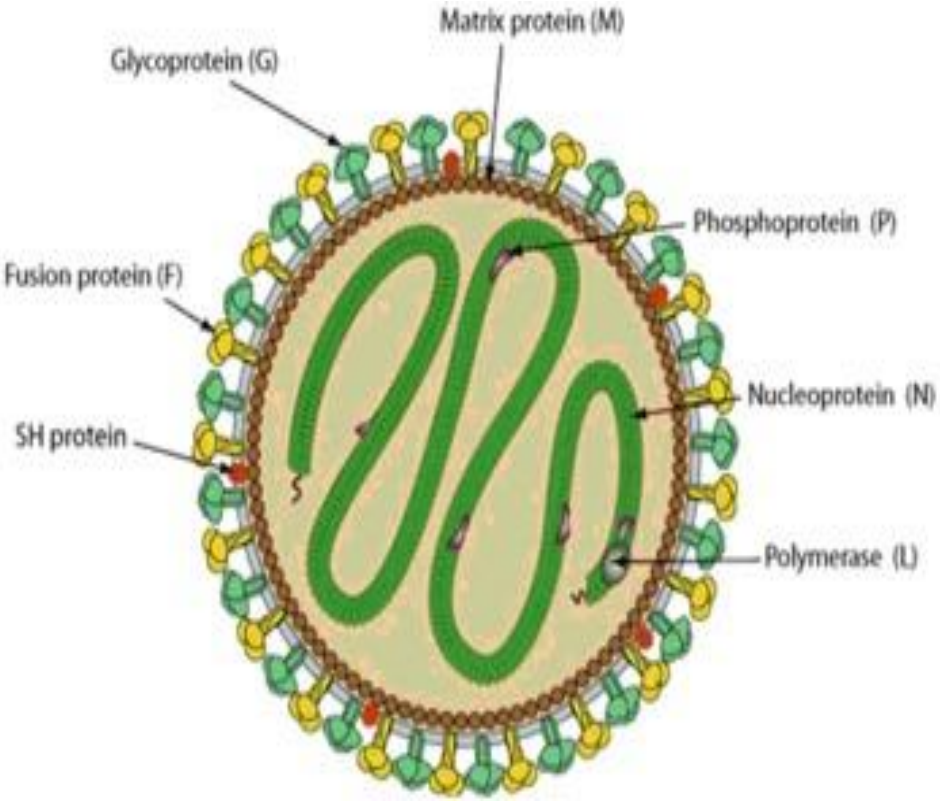

Figure 2.1: A framework for avian metapneumoviruses. The viral membrane shields the single-stranded RNA genome, which is encased in nucleocapsid protein and surrounded by phosphoprotein and massive polymerase protein. Under the membrane is a layer of matrix proteins (Umar *et al*., 2016.).

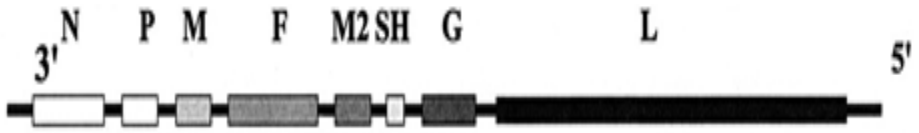

Figure 2.2: A schematic representation of the aMPV-gene sequence (Easton *et al.*, 2004)

N- Nucleoprotein

P- Phosphoprotein

M and M2-Matrix protein

F- Fusion protein

SH- Small hydrophobic protein

G- Attachment protein

L- Large polymerase protein

2.2.5. Electron Microscopic appearance of the avian metapneumovirus

Negative polarity electron microscopy of avian metapneumovirus shows multinucleated frayed elements of 80-200 nm in size, generally spherical but seldomly disc-like particles of 500 nm or more in diameter, as depicted in Plate 2.1. Beaded flagellated structures with diameters of 80-100 nm and length reaching 1000 nm have been seen, most notably in organ culture preparations. The surface projections were 13-14 nm long and the spiral nuclear envelope was 14 nm in size with an average pitch of 7 nm per turn, according to Gough and Jones (2008).

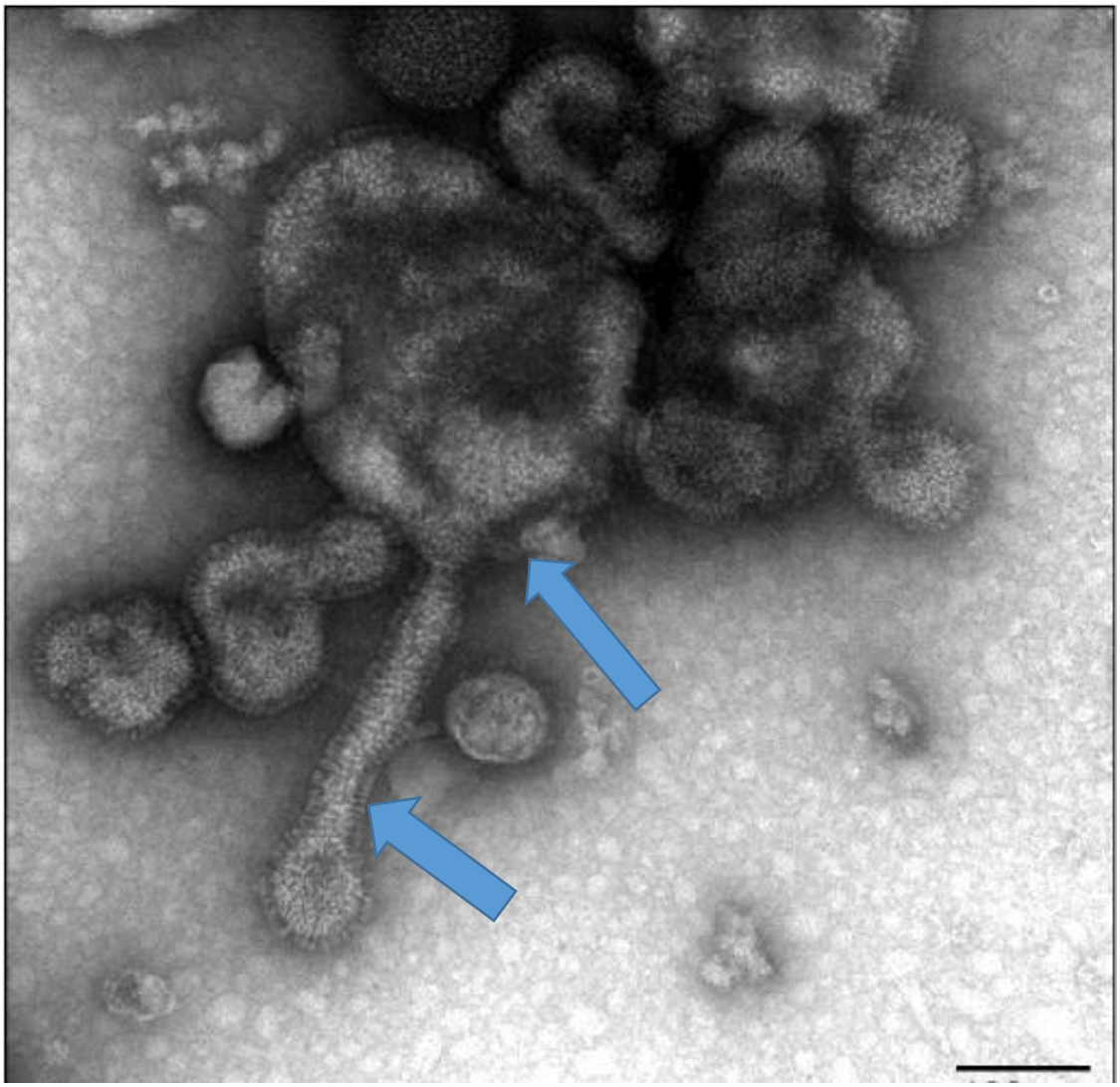

Plate 2.1: Electron microscopy picture of avian metapneumovirus (aMPV) particles in the chicken turbinate, revealing intact particles and various surface projections (Gough and Jones, 2008)

2.2.6. Susceptibility of avian metapneumovirus to chemical and physical agents

Avian metapneumovirus can endure temperatures as low as -70 degrees Celsius and as high as -20 degrees Celsius for more than 26 weeks, 4 degrees Celsius under 12 weeks, 20 degrees Celsius under 4 weeks, 37 degrees Celsius for 48 hours, and 50 degrees Celsius under 6 hours (Sun *et al*., 2014). After 12 cycles of frozen and defrost, the virus remained active, and it is impervious to pH changes of 5 to 9 for 1 hour. Various antimicrobials are efficient in suppressing the virus life, inclusive of quaternary ammonia, iodophors, a phenol derivative, ethanol and sodium hypochlorite (Townsend *et al.*, 2000). The virus exhibited remarkable resilience after seven days of desiccating at ambient temperature and can be recovered in culture medium. The presence of an envelope in virus is observed to explain its highly sensitivity to lipid solvents (Hartmann *et al*., 2015).

2.2.7. Geographic distribution of avian metapneumovirus

There is only one known serotype, which is further subdivided into A, B, C, and D, in addition to newly discovered subtypes (Retallack *et al*., 2019). Recent worldwide circulation subtypes of avian metapneumovirus are presented in Figure 2.3. Subtypes A and B have been identified in Israel (Banet-Noach *et al.*, 2009), Mexico (Rivera-Benitez, 2014), Jordan (Roussan *et al.*, 2008), Brazil (Santos *et al*., 2012), Japan (Mase *et al*., 2003), Nigeria (Owoade *et al.*, 2008), the United Kingdom (UK) and, continental Europe (Franzo *et al*., 2020; Mescoini *et al*., 2021). Subtype A has been identified in South Africa (Cook, 2009), Subtype C aMPV in China (Sun *et al*., 2014), the United States (Wei *et al*., 2013), and Canada (Jardine *et al.*, 2018). Subtype D was discovered and described in Muscovy ducks in France by Bayon-Auboyer *et al*. (2000). The new subtypes were reported in California and North America (Canuti *et al*., 2019).

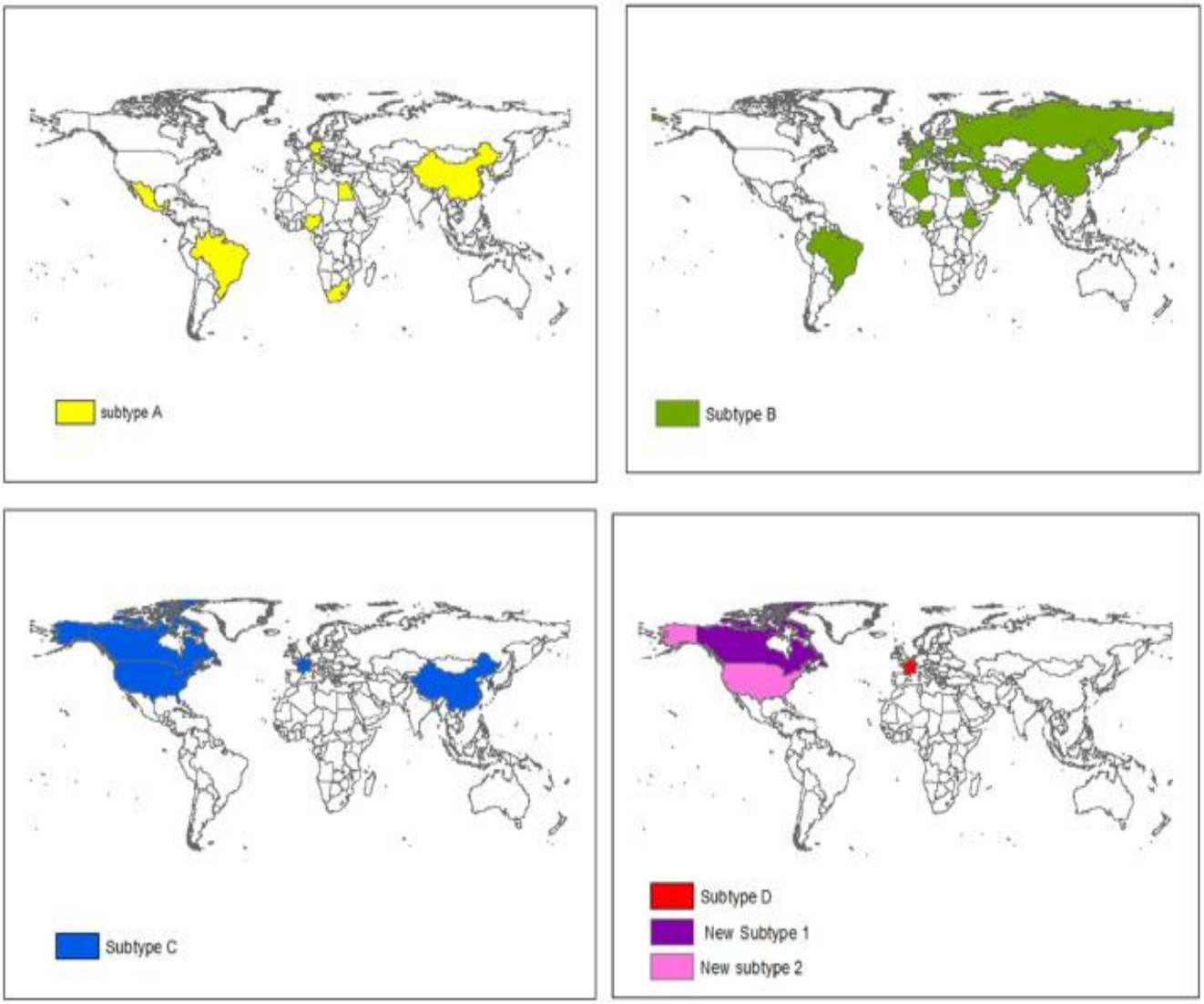

Figure 2.3: Geographic distribution of avian metapneumoviruses subtypes around the world

Index: Regions with yellow indicates countries showing aMPV* A, *countries with green colour indicates aMPV subtype B while those with blue shows countries with subtype C, Red dot indicates countries with subtype D while pink and purple regions indicate countries with new aMPV novel subtype observed (Kaboudi and Lachheb, 2021)

2.2.8 Transmission of avian metapneumovirus

Virus transmission occurs when two animals come into direct contact, but the virus's fast development in many nations shows that it can also happen in other ways. There have been records of indirect spread by shipping, supplies, polluted water and airborne propagation, as shown in Plate 2.2 (Tucciarone *et al*., 2018a). Viruses have been identified in the reproductive tracts of layer birds, but no evidence of vertical transmission has been found (Hassan and Abdul-careem, 2020). The importance of wild bird transmission has also been emphasised (Umar *et al*., 2016), which might explain why aMPV has spread so quickly throughout the world after the initial epidemic in South Africa (Cook and Cavanagh, 2002). Seasonal drift of aMPV outbreaks in turkeys in the United States was approximately 80 % in the spring and fall, indicating that roving birds may contribute to the virus's spread (Bennett *et al*., 2002). The emergence of avian metapneumovirus in wild birds is critical for understanding the epizootiology of the disease (Felippe *et al*., 2011).

2.2.9 Pathogenesis of Avian metapneumovirus

The cloning of the organism occurs majorly in the upper respiratory system, although it further occurs in the air sacs and lungs (Gough and Jones, 2008). It takes up to 14 days after inoculation to extract the virus from the nasal lamina of birds, and the genetic material can be observed up to 17 days after inoculation using polymerase chain reaction (Jones, 2001). Cells of epithelioid nasal and tracheal cells are also affected by avian metapneumoviruses, resulting in ciliostasis, ciliary death, and necrosis, which encourages incidental bacterial colonization (Gough and Jones, 2008). Chickens seem to excrete aMPV for a few days after infection. Under experimental conditions, this brief duration of moulting shows that there is no carrier status, although there is proof that aMPV may persist for extended periods of time on farms (Aung *et al*., 2008). Re-convalescent flocks may become infected with the virus from one period to the next (Jones, 2001).

Avian metapneumovirus has the ability to induce disease in field experiments, resulting in significant morbidity and, in certain cases, high mortality rates. However, in experimentations under controlled environment in which birds are displaying symptoms of milder form of rhinotracheitis, the situation is somewhat different. The magnitude of clinical symptoms varies according to the environment of the farm where

the poultry are raised, as well as the availability or nonavailability of secondary infection-causing microorganisms (Gough and Jones, 2008).

Several bacteria, including *Mycoplasma spp*, *Staphylococcus spp*, *Escherichia. coli* (*E. coli*) *and Pasteurella multocida* were isolated in poultry during outbreak of swollen disease syndrome, although it was noted that *Esherichia coli* was the most significant agent (Seifi and Boroomand, 2015). Clinical symptoms were exacerbated with the co-infection of aMPV and *E. coli*, suggesting a synergistic effect on clinical presentation in domesticated birds (Gough and Jones, 2008). Also, Van Loock *et al*. (2005) reported that turkeys with aMPV infection showed severe infection when exposed experimentally to *Chlamydia psittaci*. Given the significant incidence and occasionally elevated death rates associated with avian metapneumovirus in the field, determining the virulence of avian metapneumovirus in research laboratories has been challenging. Rhinotracheitis symptoms in infected birds are typically milder than those seen during disease surveys in laboratories (Van de Zande *et al*., 1999). The existence or absence of aggravating pathogens, as well as the environment in which the birds are housed, are most likely to blame for the differences in pathogenicity between laboratory and field infections (Turpin *et al*., 2008; Catelli *et al.*, 2010).

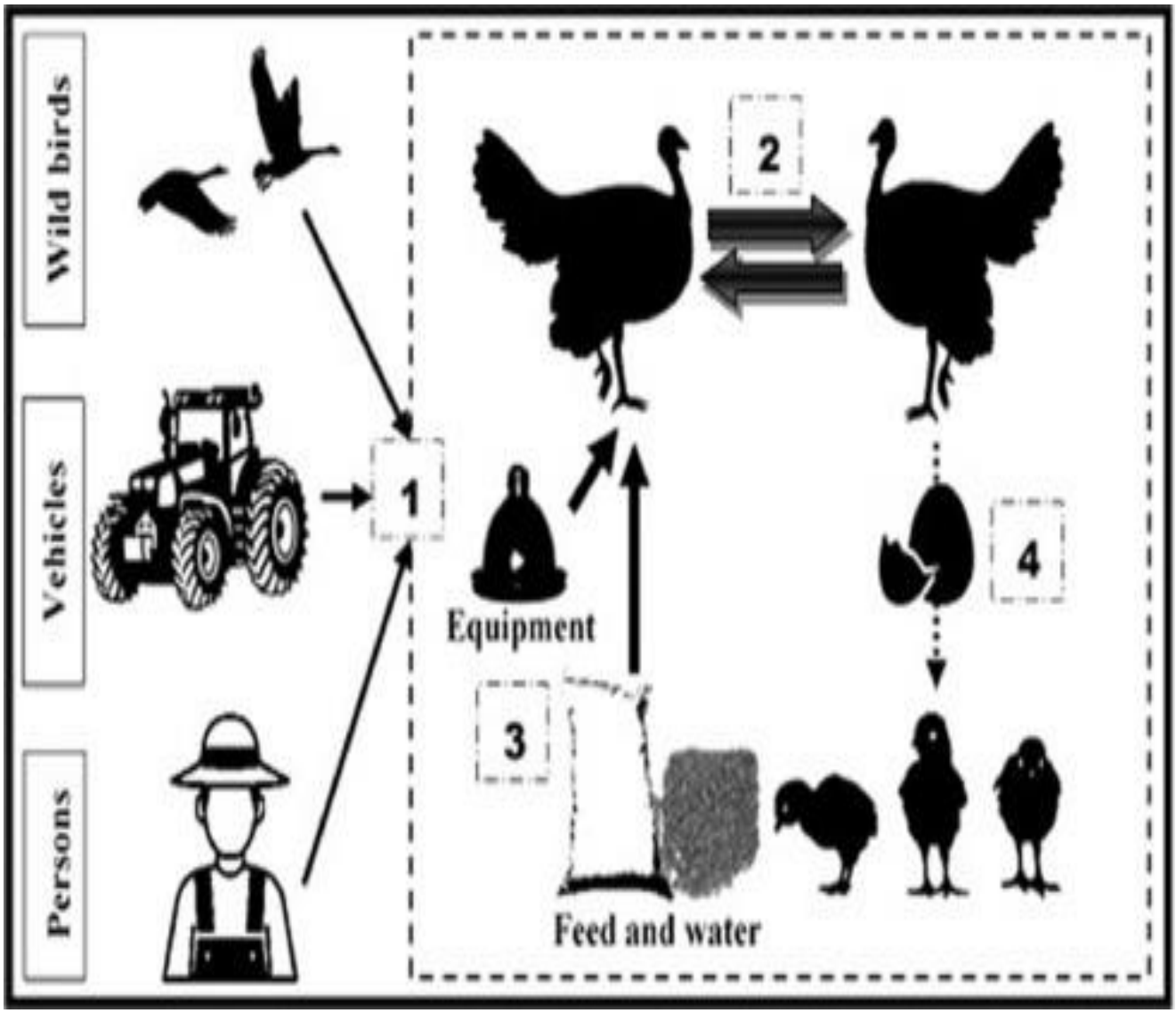

Plate 2.2: Dissemination of avian metapneumovirus in chicken and turkey flock (Kaboudi and Lachheb, 2021)

Index: 1. Environmental source. 2. Direct transmission between birds. 3. Indirect transmission. 4. Possible vertical transmission has been reported

2.2.10 Avian metapneumovirus Infection in Poultry

The incubation period for avian metapneumovirus is 3-7 days, and mortality can range from 1% to 50% depending on the age of the birds and the composition of the flock, and also, secondary infections. Birds with strong immunity and no subsequent disease may be able to recover in seven to ten days (Falchieri, 2016).

2.2.10.1 Avian metapneumovirus infection in turkeys

In young growing turkeys, aMPV infection of the respiratory tract can occur. Clinical symptoms of this disease include tracheal rales, nasal and ocular discharge, swollen infraorbital sinuses, and sneezing (Ganapathy *et al*., 2007; Ali *et al.,* 2019). Conjunctivitis, clear and watery nasal discharge which becomes concentrated and mucopurulent, frothy ocular discharge, coughing and head tremors have also been observed (Catelli *et al*., 2010). In a turkey house, clinical symptoms appear quickly and the virus spreads within 12 to 24 hours (Gough and Jones, 2008). Mortality can exceed 15% in fully susceptible flocks (Ali *et al*., 2019). When farm management is poor or secondary infection with bacteria occurs, pneumonia, pericarditis, airsacculitis, and perihepatitis may be protracted, with associated morbidity and death (Giovarnadi *et al*., 2014). Secondary bacterial infection that raises the severity of clinical symptoms does not affect the length or degree of replication of the virus (Cook *et al*., 1991). However, under laboratory conditions, the presence of aMPV can aggravate a *Mycoplasma gallisepticum* infection rather than the other way around (Ball *et al*., 2018). Similarly, field observations have shown that aMPV can increase infection with *Ornithobacterium rhinotracheale* (Babosa *et al*., 2020), while *Escherichia coli* has been found to be the most effective agent in clinical infection with aMPV (Seifi and Boroomand, 2015). Normally, respiratory infections in laying turkeys are milder; however, a substantial reduction in egg production with an increased incidence of thin-shell and egg-peritonitis affecting egg quality can be due to aMPV disease (Gough and Jones, 2008). In breeding turkeys, excessive tussis caused by a lower respiratory tract illness can lead to uterine prolapse (Cook, 2000).

2.2.10.2 Avian metapneumovirus in chickens

The significance of aMPV as an important disease producing agent in chickens is less clear, but the organism is frequently associated with swollen head syndrome (SHS) (Rautenschlein *et al*., 2020). Avian metapneumovirus affects both turkeys and chickens with the production of specific antibody response (Catelli *et al*., 2010). It has

been isolated from all ages of diseased chickens (Jones and Rauntenschlein, 2013). There have also been records of chickens experimentally infected with aMPV (Catelli *et al*., 2004). The distribution of aMPV in chicken has been shown to be similar to that of growing turkeys and is of short time (Cecchinato *et al*., 2016). Avian metapneumoviruses were found only in respiratory tract tissues using virus isolation and immunochemical techniques, with the highest concentrations observed in turbinate tissue (Majo *et al*., 1995; 1996; Ali *et al*., 2019). It may not be the main pathogen in broilers, but it may be involved in the complicated respiratory disease in conjunction with other agents (Gough and Jones, 2008). Strong evidence exists among breeders to indicate that it could be one of SHS's aetiological agents; in addition, in chickens with SHS, aMPV antibodies have been reported (Santos *et al*., 2012). The SHS has been reported as a complicated disease in which the initial lesion is mainly caused by aMPV, while the clinical signs are a consequence of bacterial complications and the severity of the disease depends on environmental factors (Bao, 2020). Respiratory problem, apathy, infraorbital sinus enlargement, unilateral or bilateral swelling of the face that extends throughout the head, as presented in Plate 2.3. Cerebral disorientation, torticollis, and opistothonus are all symptoms of the condition (Aung *et al.*, 2008; Suarez, 2020). Although mortality typically does not exceed 1 or 2%, morbidity can hit 100% (Gough and Jones, 2008). The development of eggs is often impaired and causes a number of anomalies in the reproductive system, such as malformed eggs, folded shell membranes in the oviduct, peritonitis, regression of the oviduct and ovary (Villareal *et al.*, 2009).

2.2.10.3 Avian metapneumovirus in other poultry species

In guinea fowls, swollen head disease has been reported (Cecchinato *et al*., 2018). Susceptibility of ducks and pheasants has been shown to clinical disease in experimental infections with turkey metapneumovirus isolate (Brown *et al*., 2019). Antibodies to the virus was also observed in guinea fowls (Cecchinato *et al*., 2018), farmed ostriches in Zimbabwe (Cadman *et al*., 1994), Baltic seagulls (Heffels-Redmann *et al*., 1998) and game birds (Rizzoto *et al*., 2017). The virus tends to be refractory in pigeons, geese and Muscovy ducks (Tocquin *et al*., 2006 a, b; Sun *et al*., 2014). The virus was seen for up to 14 days in mice and 6 days in rats in transmission studies involving mice, rats, and waterfowl using aMPV turkey isolate from Minnesota and even outside the state in Central America. (Bennette *et al*., 2004). However,

aMPV- Subtype C has recently been reported in waterfowl (Jardine *et al.*, 2018), geese, wild sparrows, starlings and swallows (Cecchinato *et al*., 2018). Recently, new novel subtypes of the virus have been discovered in monk parakeet (Retallack *et al*., 2019) and in a gull (Canuti *et al*., 2019).

Plate 2.3: A chicken with a severe case of enlarged head syndrome (Atterby, 2012)

2.2.11 Immunity to avian metapneumovirus

Laboratory studies revealed that the cell-mediated immune (CMI) response was primarily responsible for controlling the infection during aMPV infection (Liman *et al*., 2007). In contrast, the humoral immune (HI) response does not play an important role in protection against the infection (Worthington *et al.*, 2003).

Jones *et al*. (1992) demonstrated that aMPV-vaccinated, chemically bursectomized poults were still resistant to challenge with a pathogenic strain of aMPV. In chickens and turkeys, virus neutralization (VN), enzyme linked immunosorbent assay (ELISA), and indirect immunofluorescence will detect antibodies to aMPV as soon as 5-7 days post-exposure (Gough and Jones, 2008). Much more extensive observational research on humoral immune responses to vaccination with aMPV-A and repeated challenge with pathogenic aMPV-A was conducted by Khehra and Jones (1999). Following vaccination and challenge, he found IgA and IgG antibodies in lacrimal fluid, tracheal washes, and blood. For (re-)infections with aMPV-C, the reported early local B cell response, notably of IgA+ B cells in the mucosa of the respiratory tract, was confirmed. In the latter investigation, IgG+ and IgM+ B lymphocytes were seen infiltrating the mucosal layer. Jones *et al*. (1992) administered cyclophosphamide to turkeys prior to vaccination and challenge with aMPV. This therapy has been shown to result in a significant irreversible deficit in humoral response but only a temporary depression of the thymic system. The therapy had no effect on aMPV resistance after vaccination. It was previously assumed that protection from aMPV challenge is not dependent on humoral immunity (Worthington *et al.*, 2003). aMPV-C infections were also found to increase the accumulation of local CD4+ T cell populations groups, as well as the generation of CMI-related cytokines (Sharma *et al*., 2004b).

Sharma and Gerbyshakszudy, (2002) showed increased levels of IFNγ transcript levels in the spleen up to 7 days after aMPV-C infection using real-time PCR. Ex-vivo excitation of spleen tissues with Concavalin A and lipopolysaccharide caused the spleen tissues of diseased birds to produce nitric oxide-inducing factors (NOIF), but spleen cells from control birds did not release NOIF. The significance of CMI in preventing aMPV infection was validated by these findings (Chary *et al*., 2002b). It is possible to establish that cellular immune responses were involved in the development of damages (Naylor *et al*., 2010). A report of Zanvit *et al*. (2005) shows that mucosal

immunity is regarded as a critical component of defense against major respiratory virus infections also, Immune responses, including humoral and cellular have been linked to the disease. According to Zanvit *et al*. (2005), mucosal immunity is a critical component of defence against major respiratory virus infections. Immune responses, both humoral and cellular, have also been linked to the disease. Ganapathy *et al.* (2005) also reported that local IgA synthesis was identified in sick birds. Furthermore, an increase in CD4+ T cells has been detected in the spleen and the Harderian glands (Liman and Rautenschlein, 2007). Various immunoglobulin populations (IgG+, IgA+ and IgM+ cells) as well as IgA against the disease were raised in the nasal tract as a result of aMPV infection (Cha *et al*., 2007). According to IgA-ELISA testing, turkeys exposed to the subsequent virus (Avian metapneumovirus /Minnesota/ turkey/ 1a/1997) had an elevated level of IgA in their bile and nasal secretions (Liman and Rautenschlein, 2007).

2.2.12 Pathology of Avian Metapneumovirus Infection

2.2.12.1 Gross pathological lesions

Watery to mucoid exudates were contained in the turbinate for one to nine days after infection with excess mucus in the trachea following the infection of laying turkeys and chickens (Velayudhan *et al*., 2005). There have also been reports of egg yolk peritonitis, folded oviduct shell coverings, malformed embryos, ovarian and oviduct regression, as well as inspissated albumin and rock-hard yolk (Umar *et al*., 2016). During the laying season, chickens may have swollen up oviducts due to extreme coughing. Several macroscopic lesions have been found during natural field epidemics exacerbated by secondary infections, including air sacculitis, pericarditis, pneumonia, and perihepatitis (Cook, 2000; Gough and Jones, 2008; Giovarnadi *et al.*, 2014). Swollen head syndrome (SHS) in broilers is the only significant lesion associated with aMPV infection in chickens. The macroscopic afflictions throughout the percutaneous membranes of the head, neck and wattles include inflammation of the lateral orbital orifices can be visible in various degrees and severe yellow viscoelastic to purulent effusion (Giovarnardi *et al*., 2014).

2.2.12.2 Microscopic lesions

In experimentally infected turkey poults, histological studies of the turbinate showed increased glandular development, focal cilia loss, congestion, and mild sub mucosal mononuclear cell infiltration within one to two days (Gough and Jones, 2008). There

was disruption of the epithelial layer and abundant penetration of mononuclear cell into the sub mucosa within three to five days. Similar histological results were observed in experimentally infected chickens using isolates from turkeys and chickens (Catelli *et al*., 2001). In summary, the damage is localized and temporary, aMPV may cause destruction to the upper respiratory tract of chickens causing lymphoid cell infiltration of the mucosal layers of the turbinate and trachea (Cha *et al*., 2013)

2.2.13 Diagnosis of Avian Metapneumovirus infection

Although aMPV has already been detected in the lung, trachea and viscera diseased poultry, including the ovary and uterus (Ali *et al*., 2019). Choanal swabs, ocular, and nasal secretions, as well as tissue taken from the turbinates and sinuses, have been shown to be more efficient sources of organism detection (Aung *et al*., 2008). Since avian metapneumovirus can only survive for 6–7 days in the sinuses and turbinates, it's imperative to collect samples immediately post-infection. (Cook, 2000; Aung *et al.,* 2008). It is rare to isolate a virus from birds that exhibit severe symptoms; the severe clinical indications are most likely the consequence of subsequent bacterial infections in birds susceptible to aMPV infection. Because severe symptoms are usually caused by secondary pathogens, this probably explains the adversity in recovering the virus from chickens, mostly with swollen head disease. However, due to the virus's labile nature, viral isolation samples must be delivered to the laboratory on ice as soon as possible (Cook and Cavangh, 2002; Jones and Rautenschlein, 2013). Samples should be stored on dry ice or preserved at –50 °C to –70 °C if delays are inevitable. FTA cards have appeared to be capable of receiving and transferring aMPV isolates, such as respiratory tissue homogenate supernatant and smears, for the molecular characterisation of viral RNA (Awad *et al*., 2015).

2.2.13.1 Virus isolation

Multiple approaches have been used to diagnose the disease with the probability of successfully isolating the virus (Franzo *et al*., 2020). Turkey tracheal organ cultures and chicken embryos could be used. After being inoculated with samples, they are examined for ciliostasis, which may take several passages before a clear result is seen. Rivera-Benitrez *et al.* (2014) have also reported the use of primary culture of the trachea and lung for viral isolation. Tracheal organ cultures have been shown to be ineffective in characterising subtype C viruses due to the fact that the isolate does not possess ciliostasis (Cook *et al.*, 1999; Lee *et al*., 2007). The virus was isolated by

inoculation of the yolk sac of 6–8-day-old embryonating turkey and chicken eggs, as well as 11-day-old duck eggs from flocks' negative for aMPV (Rivera-Benitrez *et al.*, 2014). The organism frequently causes stunted growth and embryo death after repeated passages. The first aMPV subtype C strains from the US and, recent years, China were also discovered using this technique, which was first used to detect the first aMPV strain in South Africa in 1980 (Awad *et al*., 2014; Sun *et al*., 2014). Avian metapneumovirus is produced to significant antibody response in a variety of avian and mammalian cell lines once it has been modified to develop in embryonating eggs and Trachea Organ Culture. For primary isolation, Vero cells, chicken embryo cells, and QT35 cells have all been used successfully in the past (Sun *et al*., 2014). Multiple blind passages are required to obtain a clear cytopathic effect which suggests the presence of the virus. The biological characteristics of subtype C aMPV are quantified and evaluated using a direct plaque test in rhesus monkey kidney cells (LLC-MK2) and Vero cells. This cytopathic effect is associated with the production of syncytia cells after 7 days. (Zhang *et al*., 2012).

2.2.13.2 Virus detection

The identified virus has a structure that resembles that of a paramyxovirus when observed using negative contrast electron microscopy (Dongdong and Bo, 2021). It is possible to look into the physicochemical properties to help identify the virus. Molecular techniques are now more frequently used to identify strains than monoclonal antibodies (mAb), which were once used (Pedersen and Gough, 2009).

2.2.13.2a Molecular identification

Numerous RT-PCR techniques have already been carried out, analysed, and extensively documented (Rautenschlein, 2020). Molecular methods are quick and sensitive, and can detect the equivalent of around 0.5 infectious doses (Franzo *et al*., 2014). Positive or negative internal controls are used (Falchieri *et al*., 2013). When using PCR techniques, it is necessary to consider utilizing subtype specific RT-PCRs or generic RT-PCRs that can detect numerous aMPV subtypes (Van Boheemen *et al*., 2012). RT-PCR methods that target the SH, M, G and F genes have been designed, but they may not be able to detect all subtypes due to their low specificity. N gene-targeting RT-PCR primers were found to be effective in identifying common aMPV isolates of subtypes A, B, C, and D (Andreopoulou *et al*., 2019). All four subtypes can be detected using commercial quantitative RT-PCR (Brown *et al.*, 2019). Positive

results are used to identify the genotype via subtype-specific RT-PCR, genotyping, and component length analysis, which can help differentiate between subtypes such as the aMPV/B field and the VCO3 vaccine strain, which has a distinctive Tru 9I dormain. This approach is extremely valuable for viral subtyping, diagnostics, and epidemiological research (Andreopoulou *et al*., 2019).

2.2.13.2b Serology

Avian metapneumovirus protein was identified using heterologous and recombinant antibodies (Hartmann *et al*, 2015). Antibodies that react with a conserved area of the aMPV nucleoprotein (N) have been reported to be sensitive with the N protein hMPV as well (Alvarez *et al*., 2003; 2004). Immunoperoxidase (IP) and immunofluorescence (IF) staining have also been used to identify the presence of aMPV antigen in each of the preserved and unresolved tissue samples and smears. The most popular serological test for detecting aMPV antibodies is the ELISA test (Jones and Rautenschlein, 2013). Several commercial and in-house kits were used and it has been demonstrated that when recombinant isolates of aMPV have been employed in generating the coated protein for the immunoassay plates, vaccinal antibodies may not be identified (Eterradossi *et al*., 2001). A particular monoclonal antibody from aMPV is included in several competitive immunoassay kits, allowing testing of serum from many species of birds. Antibodies to the Colorado strain of aMPV were shown to be rather insensitive in testing using ELISA kits containing genotype A or B antigens (Cook *et al*., 1999). ELISA kits that include entire viral antigens produced from aMPV isolates from the United States have recently been developed. Sandwich capture ELISA kits with antigens expressed in M and N proteins have been developed to be more sensitive and specific to discover subtype C aMPV antibodies (Jones and Rautenschlein, 2013). Antibodies produced from egg yolk have been used in laying chickens (Cecchinato *et al*., 2012). Standard viral neutralisation techniques can also be used to identify aMPV antibodies (Pedersen and Gough, 2009). There is immunological reaction between avian metapneumovirus subtypes A and B, and there is a strong link between ELISA and indirect immunofluorescence testing (Ali *et al*., 2019). Sera should be collected from both acute and convalescent individuals and heat treated for 30 minutes at 56°C to eliminate nonspecific inhibitors, then stored at –20°C if testing delays are unavoidable (Hu *et al*., 2017).

2.2.14 Differential diagnoses of avian metapneumovirus Infections

Infectious bronchitis and influenza viruses, as well as paramyxoviruses, notably Newcastle disease and avian paramyxovirus-3, can induce respiratory illness and egg production complications in hens and turkeys that are similar to avian metapneumovirus (aMPV) infection (Gough, 2003; Gough and Jones, 2008). The shape of paramyxoviruses and low pathogenic avian influenza (LPAI) virions is similar, but the latter has hemagglutinin and neuraminidase activity, which distinguishes it from aMPV. Morphological and molecular (PCR) characteristics differentiate infectious bronchitis virus from aMPV (Gough and Jones, 2008).

2.2.15 Prevention and control of avian metapneumovirus

It became evident that rigorous sanitary measures with excellent biosecurity were unable to control aMPV infections (Ali *et al*., 2019). The most effective technique of managing the disease soon became live attenuated and killed vaccines (Umar *et al.,* 2015). The disease is difficult to eradicate due to the size and unpredictability of the chicken industry in most nations, however, this has been accomplished in Colorado, USA. Colorado's achievement may have been due to the outbreak's modest size and secluded geography (Cook, 2009). Jones (2010) identified Sweden as another country that has effectively eradicated aMPV infection, owing to the country's low poultry size combined with rigorous monitoring and stringent biosecurity measures. Young turkey poults are not protected from infection by maternal immunity in turkeys (Worthington *et al.,* 2003).

2.2.16 Vaccination

Effective vaccines developed for turkeys and chickens provide excellent protection against avian metapneumovirus infection in birds of all ages, as well as their laying and breeder birds (Bao *et al*., 2020). Live attenuated and inactivated vaccines have been shown to promote local and systemic immunity against avian metapneumovirus infection (Youn *et al*., 2021). Vaccination is often administered by spray or, preferably, eye drop, although the in ovo method may prove to be more effective in the future (Cecchinato *et al*., 2010). A single vaccination could be enough to confer immunity in chickens and turkeys for the rest of their lives, but latent infection could happen at older age, therefore birds raised for longer than 10 to 12 weeks are sometimes revaccinated at the same age (Cecchinato *et al*., 2012). Vaccine failure, on the other hand, is not uncommon and may be ascribed to a variety of factors, including

overattenuation of viral strain, which results in a poor immunity, underattenuation of viral strain, which results in an excessive response, or insufficient vaccine delivery (Cook, 2009). Numerous examples of after-vaccinal epidemics, and vaccine instability may be a contributing cause (Catelli *et al.*, 2010). However, due to the presence of multiple or unpredictable subtypes, failure of the vaccine may be noticed (Van de Zande *et al*., 2000). A further reason could be the virus's ability to evolve quickly, allowing it to avoid the immunological response induced by vaccination (Catelli *et al.,* 2010). Despite these possible issues, vaccination is considered very helpful, provided that the administration technique is carefully considered (Youn *et al*., 2021).

The difficulties in diagnosing aMPV in hens have raised questions about whether the vaccinations are specifically designed for broilers. A proper diagnosis is required to assess the effectiveness of the vaccine. According to Cook (2009), an aMPV vaccination will not help the condition in a flock when the virus is present. However, the anticipated benefit of using chicken-origin strains prompted the development of vaccines containing poultry derived aMPV isolates used in that species, owing to the likelihood that chicken isolates would replicate more successfully in that species (Sugiyama *et al*.,2006). Furthermore, because these pathogens attack the same cells in the respiratory system, there is a potential that aMPV and IB or NDV vaccines will interact. Therefore, it is advisable to allow approximately one week between infectious bronchitis, Newcastle disease and aMPV vaccine delivery (Chacón *et al*., 2011). While live vaccines successfully reduce general illness, virulence reversion of vaccine virus has been proven on diverse instances (Cecchinato *et al*., 2014). Different studies have shown vaccine breakdowns and vaccine isolates changing into live avian metapneumoviruses, raising concerns about disease vaccination among farmers. Field reversion from live vaccination pathogenicity is more common in avian species than in other animals, due to the larger potential of horizontal vaccine transfer (Cecchinato *et al*., 2010; 2014). The ease of administering a large number of live vaccines enhances the danger of vaccine virus spread in birds (Listorti *et al*., 2014). In addition to live attenuated vaccines, effectively killed avian metapneumovirus vaccines are commonly used for egg-laying birds. Both experimentally and in the field, the use of inactivated vaccines has been shown to provide adequate protection against the impact of aMPV on egg development and quality (Catelli *et al*., 2010). Although some clinical symptoms could be observed for a brief duration after the challenge. As a result, in

some nations where obtaining a licenses for a live attenuated aMPV vaccination is challenging, monitoring devices are used while administering inactivated vaccines (Chacón *et al*., 2011). The most practical route of vaccine administration in the field is spray and drinking water (Kaboudi and Lachheb., 2021).

2.2.16.1 Future development of new vaccines

Recombinant vaccines containing unique immunogens, such as fusion (F) glycoprotein, have also been studied in fowl poxviruses. It was determined that the vaccines produced aMPV antibodies and offered some resistance in experimentally challenged turkeys and hens (Yu *et al*., 1994). There have also been reports on research on protection of avian metapneumovirus vaccine the in turkeys after in-utero vaccinations. According to these findings, this vaccination technique has several benefits above older methods of vaccination, and subunit vaccines can be created and tested (Worthington *et al*., 2000).

2.2.17 Economic and Public Health Significance of Avian Metapneumovirus Infection

Avian metapneumovirus remains the most significant respiratory disease (next to avian influenza) in large turkey and chicken populations, sometimes in nations where aMPV vaccination is standard, it causes considerable economic losses (Márcia *et al*., 2012). Avian metapneumovirus infections in turkeys and chickens have caused significant economic problems as a result of production losses since the initial outbreaks in 1997 in South Africa and have replaced avian influenza as the main respiratory disease of turkeys (Jirjis *et al*., 2009). Its global health importance has been linked to a modern development investigated by De Graaf *et al*. (2008), who stated that genetic studies have shown that human metapneumovirus (hMPV), which originated around 200 years ago from subtype C avian metapneumovirus, is successful in terms of also producing upper respiratory infection in humans and has begun to attract attention since it was reported as a major aetiology of recurrent pneumonia in small children. These changes related to specie crossing of different respiratory viruses have been associated with many factors, one of which is climate change, a trending global environmental influence (Van den Hongen *et al*, 2001; Morin *et al*., 2018).

2.3 Climate change in Nigeria

Climate change is a long-term transition in the changes in climatic conditions that can be recognized by modifications in its average or variation (for a period of 30 years) (Tiruneh and Tegene, 2018), where the degree of deviation from standard values and the effect on the environment are paramount (Tsilini *et al*, 2014). Many parts of Nigeria have been affected by the constant warming and heating of the earth, especially those in the northern part of the country, those far from the cooling impact of the sea along the coastline to the south, this results in the area experiencing continuous climate change characterised by decreased rainfall, high dryness rate, and heat (Olaniyi *et al*., 2013). The environment can be defined based on the microclimate or macroclimate levels relative to the selective perspectives (Tsilini *et al.*, 2014; Opoku *et al*., 2021). Mankind faces many challenges due to climate change that has deleterious effects on global food security and animal health (Besada and Sewankambo, 2009; Godde *et al.*, 2021).

Nigeria is located within the tropical zone with climatic variations across the country (Olaniyi *et al*., 2013). Seasons are not clearly delineated, and temperatures seldom exceed 90 °F. The moisture content is often elevated, and most evenings are warm. There are basically two weathers in Nigeria: a wet weather from April to October, with usually lesser temperatures, caused through an air mass emanating in the South Atlantic Ocean, poularly called the southwest breezes, or by its official name the tropical maritime (MT) airmass. The harmattan period (dry season), which occurs from November to March, is characterized by winds blowing from the Sahara Desert and it is officially named, the tropical continental airmass, with the during the day temperatures exceeding 100 º F but relatively cool nights dropping as small as 54 º F (Ugbah *et al*., 2020). There have been noticeable changes in the timing structure and intensity of the seasons attributed to climate change (Shiru *et al.*, 2020). Temperatures are more moderate in the Plateau state (Olagunju *et al.*, 2021). In Nigeria, there are two significant wind systems known as trade winds (Renaudeau *et al*., 2012; Weli *et al*., 2016). The average rainfall along the coast ranges from 180 cm (70 in) in the west to 430 cm (170 in) in some areas of the east. On the ground, it is around 130 cm (50 in) over most of central Nigeria and only 50 cm (20 in) in the far north (Ogunsote, 2003). The climate of Nigeria in West Africa is associated with high latitudinal zones that become drier as one travels from north to south.

2.3.1 Rainy (wet) season

Rain is one of the most important climatic elements. It often starts in February or March, when humid Atlantic weather, also called the Southwest monsoon, sweeps across the entire region, mainly along Nigeria's coast and in the country's southeastern parts (Odekunle, 2006). The arrival of rain is usually signaled by high gusts and scattered squalls; rain is plentiful in some regions while other parts are absolutely dry. Most years, the dispersed character of rainfall, particularly in the north, may be detected by April, or even earlier in certain years (Oguntunde *et al*., 2014). This season is mainly in the south of the Niger and Benue River valleys; in most regions of Northern Nigeria, it is normally in June or July before the rains arrive, and it peaks in August, when the Atlantic air covers the whole nation (Ogunsote, 2003).

2.3.2 Harmattan (Dry season)

The northeast trade winds blow strong from December to February, bringing a load of fine Saharan dust with them (Aweda *et al*., 2017). These dust-laden winds, locally called monsoon season, appear as a thick cloud and conceal everything in fine particles. With the exception of a tiny band along the southwest coast, the harmattan is more widespread in the north but impacts the entire country. A strong harmattan, on the other hand, can encroach as far south as Lagos, bringing respite from the extreme humidity of the and blowing dust clouds out to sea (Ochei and Adenola, 2018).

2.4 Climate zones in Nigeria

According to NIMET (2017), Nigeria has four climatic zones, including the Tropical Monsoon Climate, which is discovered in the country’s south. Warm humid sea-to-land seasonal winds with high humidity characterize this climate and give it a high tendency to generate abundant rainfall. Its temperature variations are consistent throughout the year (IPCC, 2014). As early as March, the first precipitation comes, with a peak in June, and the dry season lasts until late February, with its peak months being early December and late February (Aweda *et al*., 2017). Wet and dry conditions prevail in the western part of Nigeria, with a clearly defined rainy season and equally distinct dry weather with a single peak described as Summer Maximum (Eludoyin *et al*., 2013). During the year, the solitary dry season encountered begins in December and ends in March. It is heated with the harmattan wind, a tropical load of sand that blows over the Sahara Desert throughout this time frame. The rainy season is distinguished by elevated humidity, dense cloud cover, and significant rainfall, which

lasts until September. Total rainfall in central Nigeria ranges from 1,100 mm in the lowlands along the Niger-Benue River to more than 2000 mm above the southwest escarpment of the Plateau (Daniel, 2015). Northern Nigeria has a Sahel climate, or tropical dry climate; annual rainfall totals are lower than in the south and central parts of the country, and the rainy season only lasts three to four months (June-September); the rest of the year is hot and dry, with temperatures exceeding 40°C (Brousse *et al*., 2019). At elevations of more than 1,520 metres (4,987 feet) above sea level, the alpine climate, also known as the mountain climate, can be observed in Nigeria's mountains. Due to its location in the tropics, this elevation is massive enough to approach the temperate climatic line in the tropical regions, providing a cold mountain climate above the highlands, mountains, and peak regions (Brousse *et al.,* 2019).

2.5 Climatic variations and diseases

Variations in average temperatures, climate extreme and rainfall caused by climate change will affect not only the animals and plants, but also the distribution and pressure of infection and disease that afflict these hosts (Moriyama *et al.,* 2020). Certain infectious diseases, such as infectious bronchitis, avian influenza, avian metapneumovirus, infectious bursal disease and Newcastle disease thrive during cold temperatures (Bello *et al*., 2017). These viruses would be able to persist longer at cooler temperatures, raising the risk of disease and making management more difficult. As the temperature drops, birds will become more huddled together, increasing the risk of disease transmission (Moriyama *et al*., 2019). Warmer temperatures may aid in the destruction of these viruses, but higher humidity levels may exacerbate respiratory and gastrointestinal infections. Temperatures that are too high or low, as well as low relative humidity, can create stress in birds, making them more susceptible to disease and affecting their productivity (Fouque and Reeder, 2019).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Preamble

The research was designed to proffer solution to five important questions through five objectives: The first objective was to conduct a serological study of avian metapneumovirus in commercial chickens in Nigeria from selected climate zones. The second objective was to detect and characterize the avian metapneumovirus isolates from different climatic zones in the country to determine if the circulated serotypes or strains are unique to climatic zones within Nigeria or similar to strains from other countries. The third objective was to evaluate the epidemiological factors and clinical presentation associated with outbreaks of avian metapneumovirus infection via a cross-sectional survey using questionnaire in different climatic zones in Nigeria. The fourth objective was to isolate the virus in embryonated chicken eggs, to propagate the virus for clinical studies. The fifth objective was to determine secondary bacterial infections associated with avian metapneumovirus infections in the study area. To achieve these objectives, different approaches and methods were used.

3.2 Ethical Considerations

The procedures used in this research were authorized by the Animal Care and Use Research Ethics Committee (ACUREC) of the University of Ibadan with the approval number UI-ACUREC/App/05/2017/0091 (Appendix I). In addition, the consent of the farmers was obtained before their chickens were sampled for these studies. The consent of various Veterinary Clinics and Veterinary Teaching Hospitals used as data collection centres for this research was also sought and received.

3.3 Study Locations

Blood and tissue samples were collected from chickens in commercial farms, Veterinary Teaching Hospitals and Clinics in three States located in three different climatic zones of Nigeria. These states were Plateau, Sokoto and Oyo located in near-temperate, semi-arid, and rainforest climatic zones, respectively (Oguntunde *et al*., 2014) (Figure 3.1a). The weather conditions of the three states are shown in Figure 3.1b.

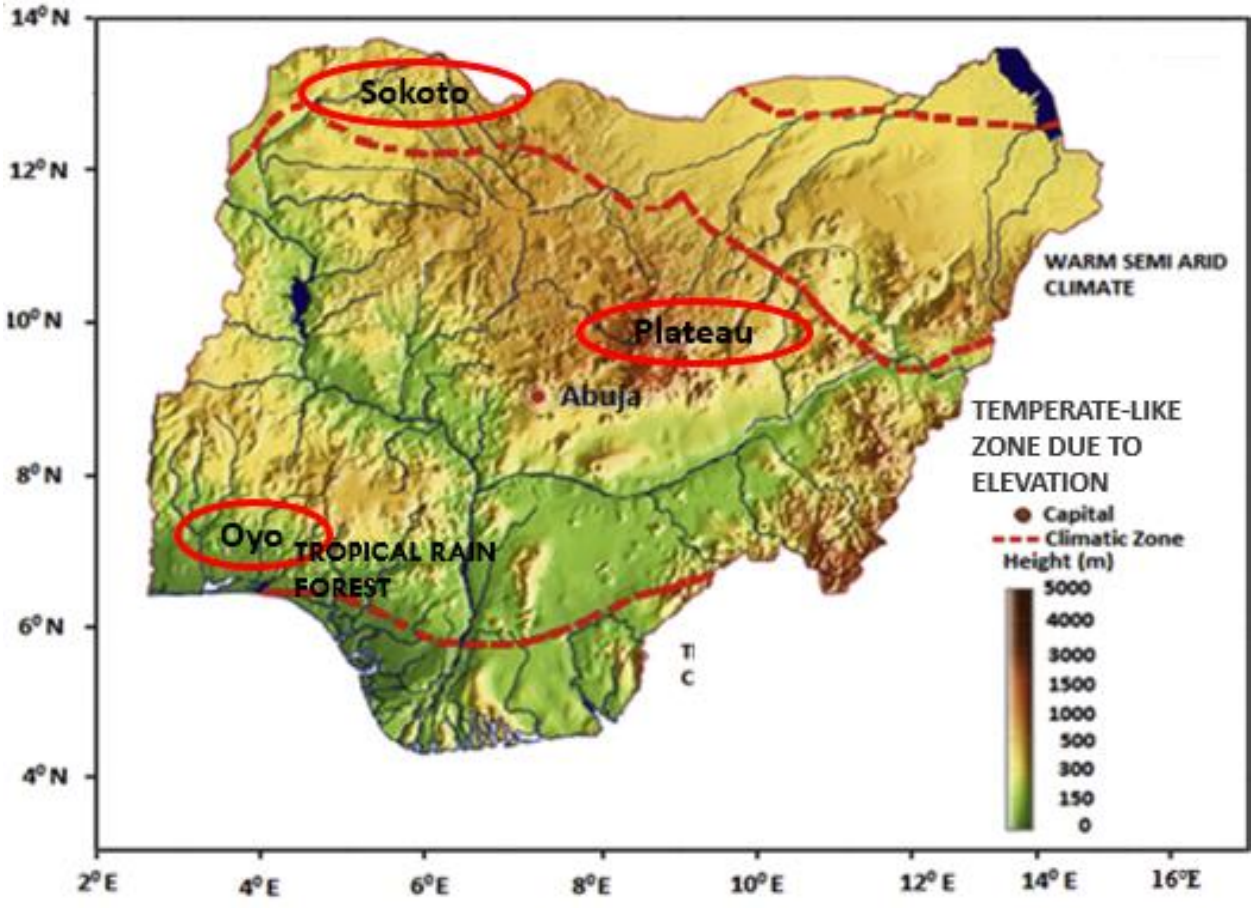

Figure 3.1a: A map of Nigeria depicting the various study areas and their climatic zones (Ibrahim *et al*., 2021)

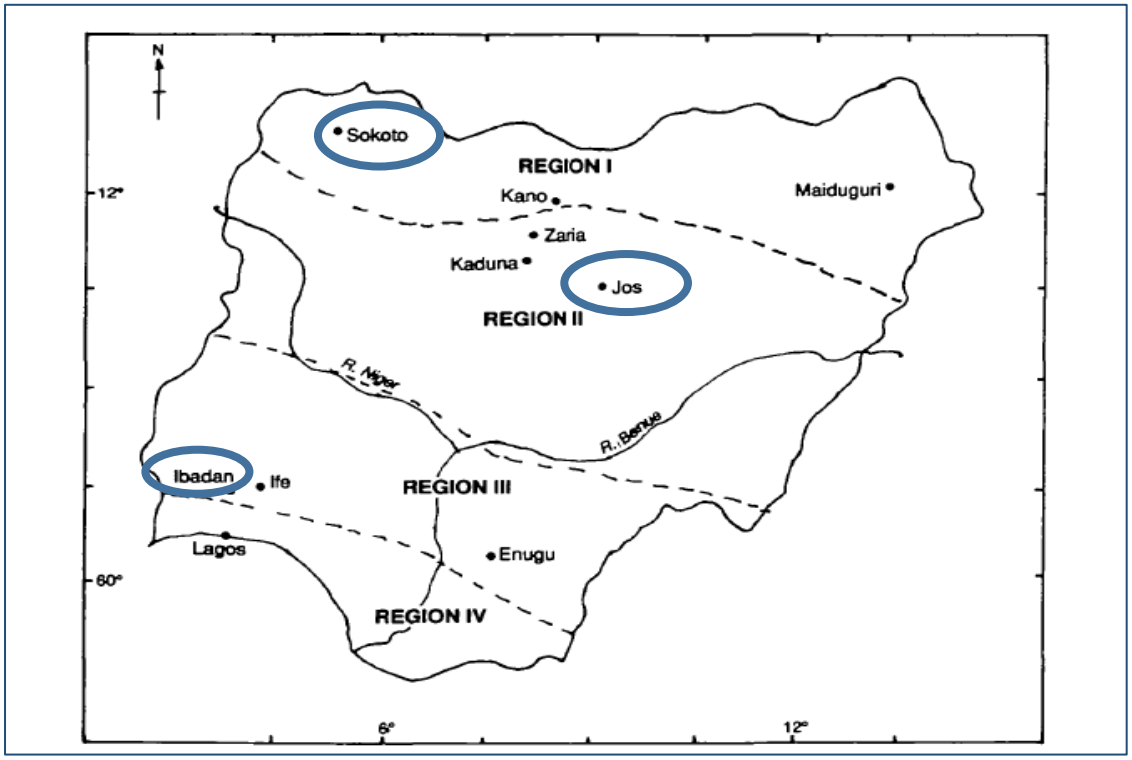

Figure 3.1b: Map of Nigeria showing the weather conditions in the study areas. *Region I: There is little rain, with scorching days (35–40°C) and cold evenings (18–21°C). Region II: 5-8 months of rain, mostly dry conditions, warm nights, and scorching days. Region III: A typical midday vapour pressure of higher than 20 mb indicates seasonal humidity. Region IV: No month goes by without rain, with a daily average humidity of more than 77 percent (Oguntunde *et al*., 2014)

3.4 Objective 1: Seroprevalence of Avian Metapneumovirus Infection in Commercial Chickens from three Climatic Zones of Nigeria

3.4.1 Study Area

The study area comprised Plateau, Sokoto and Oyo States of Nigeria. The three States were selected on the basis of their geographical areas in various climatic regions of nation. Using the climate classification of Koppen shown on the Nigerian map. Plateau State lies on latitude 9.2182° N and longitude 9.5179° E. Despite being in the tropical savannah climate region, its 4,200-feet (1,280 meters) elevation gives it a climate that is almost temperate. Sokoto State has a hot and semi-arid climate, and is placed on latitude 13.0059 ° N and longitude 5.2476 ° E while Oyo State is situated on latitude 8.1574 ° N and longitude 3.6147 ° E. It has the climate of a tropical rainforest (Ugbah *et al*., 2020).

3.4.2 Sample Size Determination

The sample size for this study was calculated according to the formula of Thrusfield (2005)

$$n=Z_{\alpha}^{2} \times p \times (1-p)/d^{2} \quad \text{.....3.1}$$

Where: -

n= sample size

Z_{α} = value of the standard distribution corresponding to a significance level of α (1. 96 for a 2-sided test at 0.05 level)

P_{exp}= expected prevalence in the population.

d^2 = Desired absolute precision

- Using a prevalence rate of 40% according to Owoade *et al*. (2006).
- Thus 480 commercial chickens were sampled (Table 3.1).

3.4.3. Blood Sample Collection

Four millilitres of blood was drawn from randomly selected chickens via jugular venipuncture and placed in plain bottles to clot at room temperature. The samples were then taken to the Avian Medicine Laboratory at the University of Ibadan's Faculty of Veterinary Medicine. Clotted blood samples were vortexed at 1,500 g for 5 minutes,

after which serum was extracted and stored at -20^{o}C in a deep freezer. The samples were collected between December 2018 and February 2019, as well as between July and September 2019, depicting both the dry and wet seasons. There were 480 blood samples in all, 160 of which were from each of the three states (Plateau, Oyo, and Sokoto State). A Local government area was sampled in each of the three senatorial districts of each state as shown in Table 3.1. The samples were collected from commercial chickens aged between 6 and 70 weeks on various farms with intensive and semi-intensive management systems and flock sizes ranging from 189-22,000 and 4000-6000 chickens, respectively. The flocks were not vaccinated against avian metapneumovirus infection.

Table 3.1: Samples collected from different Local Government Areas in the States

Location	Senatorial district	Local Government Area	No of samples collected	Dry season	Wet season
Plateau State	Plateau Central	Mangu	60	30	30
	Plateau North	Jos North	40	20	20
	Plateau South	Langtang North	60	30	30
Oyo State	Oyo Central	Egbeda	60	30	30
,	Oyo North	Iseyin	60	30	30
	Oyo South	Ibarapa central	40	20	20
Sokoto State	Sokoto East	Rabah	40	20	20
	Sokoto North	Wamakko	60	30	30
	Sokoto South	Bodinga	60	30	30
Total			480	240	240

3.4.4 Determination of Avian metapneumovirus (aMPV) Antibody Titres

3.4.4.1 Procedure

Each serum sample was tested using a commercial enzyme-linked immunosorbent assay (ELISA) kit specific for the identification of immune response to aMPV subtypes A, B, C, and D (ID Screen® Avian Metapneumovirus Indirect Antibody Test Kit, rue Louis Pasteur, Grabels, France). Prior to use, reagents were allowed to warm up to room temperature of 26° C. After homogenizing the reagents, serum samples were tested in the dilution buffer at a final dilution rate of 1:500. In the predilution plate, wells (A1, B1, C1, and D1) were set aside for controls. Serum samples (5 µl) and 245 µl of dilution buffer were dispensed and mixed into each well of an uncoated microtiter plate. Dilution buffer (90 µl) was applied to as many wells as there were samples to be examined before adding prepared pre-diluted samples (10 µl). The plate was sealed with foil paper and incubated for 30 minutes at 26 ° C. Following that, the wells were vacuumed and washed three times with 300 µl of washing solution. Each well received 100 µl of conjugate, which was then sealed and incubated at 26° C for 30 minutes. The wells were then vacuumed and washed three times with the wash solution (300 µl). Substrate solution (100 µl) was added to each well, and the plate was covered and incubated at room temperature for 15 minutes. Stop solution (100 µl) was then dispensed into each well as shown in Plate 3.1. In an ELISA plate reader (Optic Ivymen® System, Model 2100C, Biotech SL, Madrid, Spain), the optical density (OD) was measured at 450 nm.

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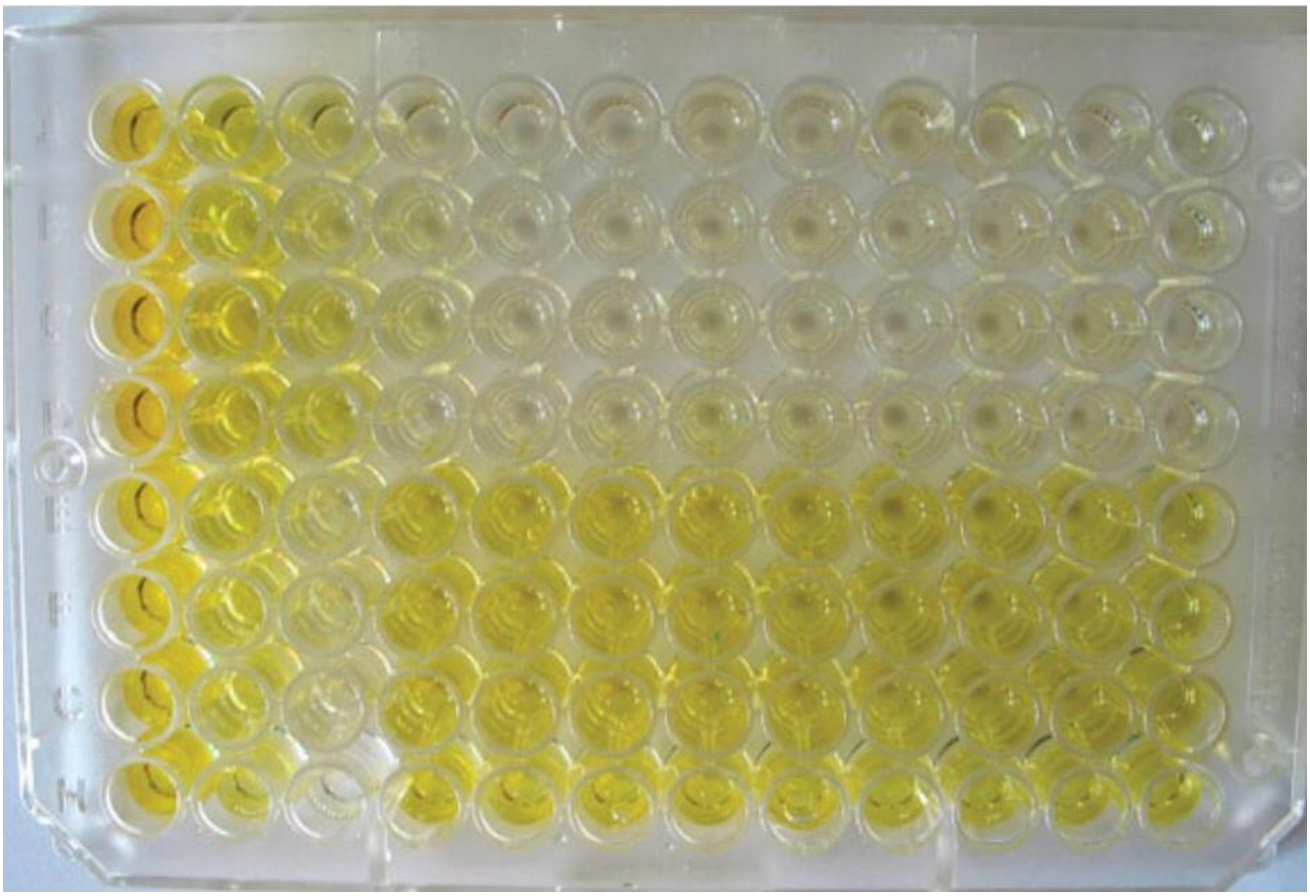


Plate 3.1: Arrangement of the serum samples and controls on the microtitre plate

3.4.4.2 Interpretation of Results

The test results were interpreted in accordance with the manufacturer's instructions for the ELISA kit. When the mean OD value of the positive control (ODpc) was greater than 0.250 and the mean ratio of the positive and negative control values (ODpc and ODnc) was greater than 3, the sample was considered positive. Furthermore, when the S/P ratio and antibody titre were 0.2 and 396, respectively, the results were considered negative. They were considered positive when the S/P ratio was 0.2 and the antibody titer was 396 as shown in Table 3.2.

The ratio of S/P and antibody titer were determined as follows:

1. S/P ratio $= \frac{OD_{sample} - OD_{nc}}{OD_{pc} - OD_{nc}}$ 3.2

2. Antibody titre

$$Log_{10}(Titre) = 1.09 \times Log_{10}(S/P) + 3.360$$

$$Titre = 10_{log\ 10(Titer)} \text{.....3.3}$$

(Pc- Positive control, Nc- Negative control)

Table 3.2: Antibody titre result interpretation according to the ELISA kit manufacturer

S/P value	ELISA Antibody titer	Immune status
S/P ≤ 0.2	Titre ≤ 396	Negative
S/P > 0.2	Titre >396	Positive

3.4.4.3 Statistical Analyses

Data was compiled into Microsoft Excel® and tabulated with descriptive statistics using IBM SPSS version 20.0 Software (Scientific Package for Social Sciences, Inc., Chicago, USA). In each State and season, seroprevalence was expressed as a percentage of the total number of chickens screened. The mean± SEM of aMPV virus antibody titer was calculated, and ANOVA and the Chi-square test were used to determine whether there was a significant difference between States and seasons. Significant values were considered at $\alpha<0.05$.

3.5 Objective 2: Detection and Molecular Characterization of Avian Metapneumovirus in three Climatic Zones in Nigeria

3.5.1 Study Locations

The study was carried out in Plateau, Sokoto, and Oyo States during the harmattan period (November, 2020-March 2021). Three Veterinary Teaching Hospitals at University of Jos, Usman Danfodio University and University of Ibadan as well as three Veterinary Clinics i.e., Marie Veterinary Clinic in Plateau State, Peter Veterinary clinic in Sokoto State and CHI in Oyo State as shown in Figure 3.2, were used as sample collection points for this study

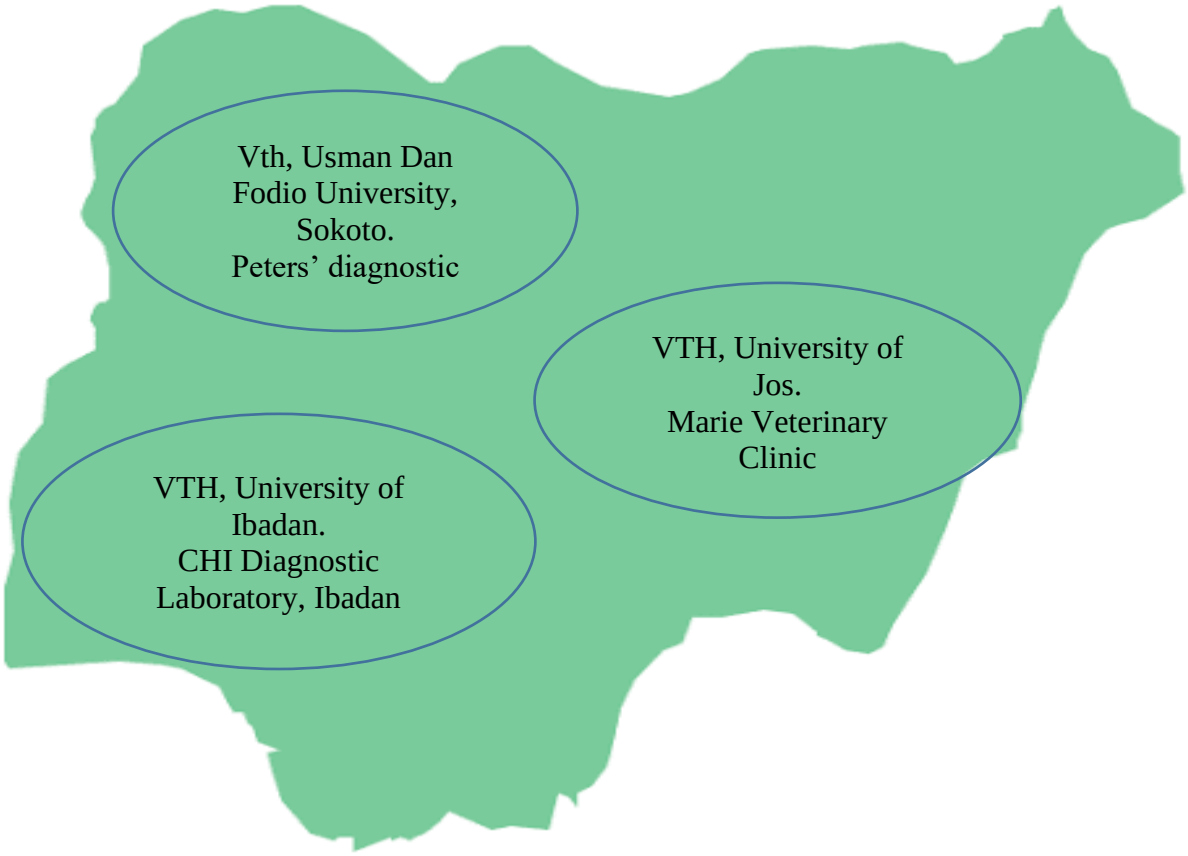


Figure 3.2: Map of Nigeria showing the different Veterinary teaching hospitals and Veterinary clinics in the study area

3.5.2 Tissue Samples Collected and Sampling Procedure

A total of 42 disease outbreaks of birds showing respiratory signs and presented for diagnosis were sampled in the three Veterinary Teaching Hospitals and three Veterinary clinics. The respiratory signs included mild to severe sneezing, coughing, nasal and ocular discharges.

However, from the cases presented, gross pathological lesions were noted and conjunctivae (Cj), lungs (Lg), tracheae (Tr) and turbinates (Tu) were sampled using sterile swab sticks which were transferred into separately labeled plain bottles containing virus transport medium (VTM).

A total of 294 birds pooled into 168 tissue samples were obtained in the study area between November 2020 and March 2021, through the harmattan season. None of the birds tested was vaccinated against aMPV. The samples were then transferred on dry ice to the Avian Disease Laboratory of the National Veterinary Research Institute (NVRI), Vom, where they were stored at -80^{o} C until assayed for the presence of aMPV.

3.5.3 Viral RNA extraction

Viral RNA was isolated from the 168 samples utilizing the RNeasy® Mini kit (Qiagen®, U.S.A), according to the manufacturer's instructions**.** Four volumes of (96-100%) ethanol were added to buffer RPE to produce a standard solution i.e., 260 ml of ethanol to 65 ml of buffer RPE concentrate. Dithiothreitol (DTT) (50 µl) was mixed into 1 ml of buffer RLT and stored at room temperature. Ethanol (70%) was also prepared in desired volume and one volume of ethanol (70%) was applied to the lysis buffer and mixed by pipetting. The sample including the precipitate (700 µl) was added to a RNeasy® mini spin column positioned in the supplied 2 ml collection tube. The lid was sealed and vortexed for 30 seconds at 8000 ×g (10,600 rpm) after which the flow through was poured away. Buffer RW1 (700 µl) was added to the spin column, the lid was closed and centrifuged for 30 seconds at ≥8000 ×g (10,600 rpm). The flow through was then removed. Buffer RPE (500 µl) was also append to the RNeasy spin column, the lid was sealed and centrifuged for 2 minutes at ≥8000 ×g (10,600 rpm). Buffer RPE (500 µl) was again introduced to the spin column also for another 2 minutes at ≥8000 ×g (10,600 rpm). It was then positioned in a new 2 ml collection tube and vortexed at full momentum for 1 minute to dry the membrane. The spin column was then transferred to a new 1.5 ml microcentrifuge tube and RNase free

water (50 µl) was poured directly onto the spin column membrane. The lid was sealed and vortexed for 1 minute at ≥8000×g (10,600 rpm) to extract the RNA which was stored at -20^{o} C until needed.

3.5.4 Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) Assay

RT-PCR was performed using a one-step RT-PCR kit (Qiagen), primers designed by Bayon-Auboyer *et al*. (1999), nuclease- free water and the RNA template in a 25 µl reaction mix (Tables 3.3a and 3.3b). The samples were screened using the primer pair Nd/Nx which amplify a 115 bp fragment of the nucleocapsid (N-gene) of aMPV and primer pair GaG/GyG which is specific for a 448 bp fragment of the aMPV glycoprotein (G) gene.

3.5.5 Preparation of Primer Stock and Working Solutions

For this study, the primers (Table 3.4) were obtained from African Biosciences Limited, Ibadan (Appendix V). Primer stock of 100 mM solutions was prepared by diluting with sterile buffered solution, TE (10 mM Tris, pH 7.5-8.0, 1 mM EDTA). A 10 µM primer working solutions was then prepared from the primer stock by diluting one part of the stock solution in nine parts of the nuclease free water, vortexed and aliquoted in sterile Eppendorf tubes and stored at -20^{o} C until used.

3.5.6 Polymerase Chain Reaction Optimization

Conventional RT-PCR was observed using the Qiagen one-step RT-PCR kit (Figure 3.4). The PCR machine C1000 Touch (Bio-Rad, Foster City) used for the amplification has different thermal protocol for different targeted gene as stated in Table 3.5a and 3.5b according to Bayon- Auboyer *et al.* (1999).

3.5.7 Gel Preparation and Electrophoretic Analysis of PCR Products

The amplicons obtained were analysed on 2% (w/v) agarose gel produced by adding 2 g agarose powder (Sigma, St. Louis, Missouri, USA) to 100 ml of TAE buffer (Tris base, acetic acid and, EDTA) in a conical flask. The mixture was boiled for few seconds in a microwave oven to allow dissolution of the agarose powder. Ethidium bromide (5 µl of 5 mg/ml) was applied to the molten agar and swirled gently to mix. The agar was cooled to about 45^{o}C before it was ran into the electrophoresis trough with appropriate comb placed and allowed to thicken. A 100 bp DNA ladder (New England Biolabs) was employed to approximate the band size. Thereafter, 3 µl of dye mixture (New England Biolabs) followed by 5 µl of PCR product were placed on a

clean parafilm and mixed. The amplicon –dye mixture and the 100 bp marker were then loaded into the wells. Gel electrophoresis was done at (120 volts) for 30 minutes in 1X TBE buffer (Promega) and the gel was visualised under UV light (Alpha Imager, Alpha Innotech, San Leandro, CA) (Plate 3.2). Amplicons with an expected band size of 115 bp for the N gene and 448 bp for the G-gene were considered positive for avian metapneumovirus.

Table 3.3a: Composition of the reaction mix used for RT-PCR assay for amplification of the aMPV N-gene (Bayon-Auboyer *et al*., 1999)

Reagent	*Volume required per sample (µl)*	*Final concentration*
Nuclease free water	12	
5X buffer	5	
DNTPs	1	
$MgCl_2$	1	
ND-F	1	20 mMol
NX-R	1	20 mMol
Enzyme	1	
RNA template	3	
Total	25	

Table 3.3b: Composition of the reaction mix used for RT-PCR assay for amplification of aMPV G-gene

Reagent	*Volume required Per sample (µl)*	*Final concentration*
Nuclease free water	12	
5X buffer	5	
DNTPs	1	
$MgCl_2$	1	
GaG-F	1	20 mMol
GYG-R	1	20 mMol
Enzyme	1	
RNA template	3	
Total	25	

Table 3.4: Sequences of primers used for aMPV RNA amplification

Primer name	Sequences	Size (bp)	Reference
Nd-F	5'AGC AGG ATG GAG AGC CTC TTG 3'	115	Bayon Auboyer *et al*., 1999
NX-R	5'CAT GGC CCA ACA TTA TGT T 3'		
GaG-F	5' CCG GGA CAA GTA TCT CTA TGG 3'	448	Bayon-Auboyer *et al*.,1999
GyG-R	5'TCT CGC TGA CAA ATT GGT CCT GA 3'		

Table 3.5a: Thermocycling conditions used for RT-PCR assay of N-gene (Listorti *et al*., 2014)

Step	Temperature	Duration	No of cycles
Reverse transcription	50^{o} C	30 min	
Initial denaturation	95^{o} C	15 min	
Denaturation	95^{o} C	30 sec	40
Annealing	51^{o} C	1 min	
Extension	72^{o} C	1 min	
Final extension	72^{o} C	5 min	
Holding	4^{o} C	∞	
Total		2-hour 46 min	

Table 3.5b: Thermocycling conditions used for RT-PCR assay of G-gene (Listorti *et al*., 2014)

Step	Temperature	Duration	No of cycles
Reverse transcription	50^{o}C	30 min	
Initial denaturation	95^{o} C	15 min	
Denaturation	94^{o} C	1 min	39
Annealing	55^{o} C	1 min	
Extension	72^{o} C	1 min	
Final Extension	72^{o} C	10 min	
Holding	4^{o} C	∞	
Total		3 hours 15 min	

3.6 DNA purification for sequencing

The PCR products (amplicons) obtained from amplification of the glycoprotein of G-gene of aMPV PCR product (amplicon) from field samples were refined using the Wizard® SV Geland PCR clean up system (Promega, Cat. No. A9281/2/5) based on the manufacturer's procedure as follows:

Equal volume (40 µl) of membrane binding solution was mixed with each of the amplicons, which were transferred into Wizard® SV columns and incubated for 1 minute at room temperature (24^{o} C). The Wizard® SV columns were then vortexed at 14,000 rpm for 1 minute and the effluent was disposed. The column was then washed with 700 µl membrane washing buffer and centrifuged for 5 minutes at 14,000 rpm. The column was removed, dried, spun for 1 minute and finally eluted with 25 µl RNase- free water. The purified cDNA product (25 µl) and aliquot of 50 µl of 10 µm of each primer (GAG-F/GYG-R) were sent for sequencing at Macrogen Incorporation, Seoul, South Korea.

3.6.1 Sequencing using ABI Capillary Sequencer

The Sequencing of the avian metapneumovirus segment and analysis were carried out at the Macrogen, Incorporation, Seoul, South Korea.

3.6.2a DNA quantification Methods

The volumes necessary for the assay were calculated as 100 µl diluted test sample volume + 100 µl diluted Pico green. Kit components were then brought to room temperature and 20xTE was diluted to 1x TE in autoclaved ddH_2O. Stock solution (2 µg/ml) of positive control dsDNA (10 µl with 490 µl TE) in 96 well plate for standard was prepared. For best accuracy, a serial predilution of the standard was made in advance and stored in aliquots of 1 ml, mixed well, incubated for 5 minutes in the dark and measured in the Tecan Genios with ex-480 nm and em. 520 nm filter set (Pico green protocol in the Magellan software).

3.6.2b Cycle Sequencing PCR

The necessary template for the reaction was calculated. For templates of 300 to 1000 bp, 10 ng was used as the standard amount. The ready mix was thawed at 4^{o} C and vortexed before use. For each sample 8 µL of the following mix: 50% Big Dye, 25% ddH_2O, 25% 5X TE buffer from ABI was prepared. Columns Forward (F) and Columns Reverse (R) were labelled and reaction per PCR product (Forward and a

Reverse reaction in the 96 well plate) was prepared. In each well, 4 µl of reaction mix, 1µl primer, template (max 5 µl), H_2O to 10 µl were dispensed in columns and closed with strip lids. The plate was placed in centrifuge with a balance plate, spun down for 10 seconds without vacuum drying and the following program was run in the master cycler (Table 3.6).

Table 3.6: Master cycler protocol for sequencing (Listorti *et al*., 2014)

Step	Temperature	Time
1	96 ^{O}C	2 min
2	Pause-push enter	
3	96 ^{O}C	1 min
4	96 ^{O}C	10 sec
5	50 ^{O}C	5 sec
6	60 ^{O}C	4 min
7	Go to step 4	Repeat 25x
8	10 ^{O}C	Hold

The samples were kept at 4 ^{O}C for post cycle reaction purification

3.6.2c Post Sequencing PCR Purification

The strip lids were removed from the PCR plate. The following mixture was prepared: 1/3 125 Mm EDTA; 2/3 ddH_2O (15 µl), was dispensed per well and thoroughly mixed. Wells were closed with strip-lids, mixed again by inverting the plates several times making sure each well had a sufficient mix and incubated for 15 minutes at 25^o C in the dark. This was then centrifuged for 30 minutes at a speed of 3,000 rpm at 4^o C. Afterwards, the strip-lids were removed and the plate was inverted on tissue paper in the centrifuge plate holder. It was again centrifuged for 15 minutes at 3,000 rpm. 60 µl of 70% ethanol was mixed in each well and further centrifuged at 900 rpm for 1 min. At 15 min, post-centrifugation drying was carried out. Wells were then closed with strip-lids and stored at 4^{o}C before loading on the sequencer.

3.6.2d Sample Preparation for Electrophoresis

The strip-lids were removed and 10 µl HI-DI was added to each sample. One well was filled with DNA standard from ABI and other wells were closed with strip-lids. The lids were set at above 100 ^{o} C before the plates were loaded. These plates were further boiled for 5 minutes at 95 ^{o} C and thereafter, placed on ice and loaded into the sequencer.

3.6.3 Data analyses

3.6.3.1 Statistical Analysis

The Chi-square test was used to determine whether there was a significant difference between the occurrence of the disease and the climatic zone. A value at $\alpha<0.05$ was considered significant.

3.6.3.2 Multiple Sequence Alignment and Phylogenetic Analysis

The completed sequences were aligned utilizing Bioedit software version 7.2.5 to produce a consensus sequence, and the nucleotide sequences were translated to protein using the EXPASY translate tool (http://web.expasy.org). Multiple alignments of nucleotide and deduced amino acid sequences were performed using the Cluster W software, while phylogenetic analysis was performed using the Neighbor- Joining method (Saitou and Nei, 1987) of the Molecular Evolutionary Genetics Analysis (MEGA X) software (Kumar *et al*., 2016; 2018) with a total of 1000 bootstrap replicates (Tamura *et al*., 2011). For the phylogenetic analyses, previously published avian metapneumovirus sequences from GenBank were used as the reference strain.

3.6.3.3 Sequence Identity and Similarity (SIAS)

This was done on line using the Immunomedicine group tool: imed.med.ucm.cs. It was used to calculate pair wise sequence identity and similarity from multiple sequence alignment.

Plate 3.2: Electrophoretic Analysis of PCR Products using PCR machine C1000 Touch (Bio-Rad, Foster City) at Avian Disease Laboratory, National Veterinary Research Institute (NVRI), Vom

3.7 Objective 3: Clinical Presentation and Epidemiology Factors Associated With Avian Metapneumovirus Infection in Nigeria

3.7.1 Study Design

A cross- sectional study was conducted with questionnaires (Appendix IV) designed and distributed to farmers and Veterinarians in the selected Veterinary Teaching Hospitals and Veterinary Clinics in the study area for data collection on respiratory disease outbreak in commercial chickens (Study 2 population)**.** Information contained in the questionnaire were name of the farm, farm address and phone number, age of flock, type of flock, flock size, mortality pattern (onset, number dead per day and in the last one week), vaccination records, medications administered since outbreak started, clinical signs presented, postmortem findings, tentative diagnosis and antibiotics used. The inclusion criteria for this study were chickens showing respiratory signs before death, while the exclusion criteria were chickens not showing respiratory signs.

3.7.2 Data analyses

The data from the retrieved questionnaire were structured using Microsoft Excel® 2016 and IBM- SPSS version 21 (Statistical Program for Social Sciences (SPSS) Inc., Chicago, USA). Descriptive analysis was used to evaluate data in the form of frequencies and percentages, which were presented as Tables and Charts.

3.8 Objective 4: Isolation and Identification of Avian Metapneumovirus

3.8.1 Embryonated Chicken Eggs

Eighteen- 7- day-old specific-antibody-negative (SAN)- embryonated chicken eggs acquired from the hatchery of the National Veterinary Research Institute, Vom, were candled to check for their viability; non-viable eggs were discarded. The eggs were also visually inspected for cracks (Plate 3.3).

3.8.2 Preparation of Samples for Egg Inoculation

3.8.2.1 Egg inoculation

The aMPV positive tissue (conjunctivae and turbinate) samples confirmed by RT-PCR were used for this study. Fifteen eggs were placed on a tray with the air cell uppermost and labelled with sample identification (A, B, C, D, E and F). The eggs were sterilized with cotton wool and 70% alcohol before a small hole was drilled through the shell at the top of the air cell at the marked inoculation site. The syringe (1 mL) was pushed straight down through the hole up to the hilt of the needle (23G×12) and 0.2 ml of the inoculum was drawn into the yolk sac of the egg (Plate 3.4a). The hole was then sealed with varnish (Plate 3.4b) and the eggs were incubated at 37 ^{o} C. Three embryonated eggs from the same batch were inoculated with sterile PBS and served as the negative control. The eggs were examined daily by means of a candling lamp and non-viable eggs after the first 24 hours post-inoculation were discarded. The eggs that showed cell death after 48 hours after the three-passage period in accordance with internationally recognized standards (Stear, 2005) were ruptured. The deposit of the embryo, yolk sac, embryonic fluid and membrane of the eggs were harvested in Petri dishes. Five millilitres of the embryonic fluid and yolk sac per egg were collected and examined for haemagglutinating activity (HA) using chicken red blood cells and aMPV genome using RT-PCR.

3.8.3 Haemagglutination Assay

Phosphate buffered saline (pH 7.4) (25 µl) was appended to each well in a row of a microtitre plate for each of the embryonic fluid and yolk sac membrane samples. Two-fold dilutions of the embryonic fluid and yolk sac membrane samples were prepared by adding 25 µl of the samples in well 1 of the appropriate row and 25 µl was titrated across the microtitre plate. 25 µl was discarded from column 12. 1% chicken red blood cells (CRBC) (25 µl) was also added to all wells. After gently mixing the contents of

each well, they were kept at room temperature for 35 minutes. The plates were tilted towards the operator and observed for haemagglutination. The findings were noted appropriately.

3.8.4 Ribonucleic Acid Extraction

Ribonucleic acid (RNA) was extracted from the embryonic fluid and yolk sac membrane samples utilizing the RNeasy® Mini kit (Qiagen®, U.S.A), according to the manufacturer's instructions**.** Four volumes of (96-100%) ethanol were added to buffer RPE to produce a standard solution i.e., 260 ml of ethanol to 65 mls of buffer RPE concentrate. Dithiothreitol (DTT) (50 µl) was mixed into 1 ml of buffer RLT and stored at room temperature. Ethanol (70%) was also prepared in desired volume and one volume of ethanol (70%) was applied to the lysis buffer and mixed by pipetting. The sample including the precipitate (700 µl) was added to a RNeasy® mini spin column positioned in the supplied 2 ml collection tube. The lid was secured and vortexed for 30 seconds at 8,000 ×g (10,600 rpm) after which the flow through was discarded. Buffer RW1 (700 µl) was filled into the spin column, the lid was closed and centrifuged for 30 seconds at ≥8,000 ×g (10,600 rpm). The flow through was then removed. Buffer RPE (500 µl) was also appended to the RNeasy spin column; the lid was sealed and vortexed for 2 minutes at ≥8,000 ×g (10,600 rpm). Buffer RPE (500µl) was again added to the spin column also for another 2 minutes at ≥8,000 ×g (10,600 rpm). It was then placed in a new 2 ml collection tube and centrifuged at full momentum for 1 minute to dry the membrane. The spin column was then transferred to a new 1.5 ml collection tube and RNase free water (50 µl) was added straight to the spin column membrane. The lid was then sealed and vortexed for 1 minute at ≥8,000×g (10,600 rpm) to extract the RNA which was stored at -20^{o} C until needed.

3.8.5 Reverse Transcriptase-Polymerase Chain Reaction Assay

RT-PCR was performed using a one-step RT-PCR kit (Qiagen), primers designed by Bayon-Auboyer *et al*. (1999), nuclease- free water and the RNA template in a 25 µl reaction mix (Tables 3.3a and 3.3b). The samples were screened using the primer pair Nd/Nx which amplify a 115 bp fragment of the nucleocapsid (N-gene) of aMPV and primer pair GaG/GyG which is specific for a 448 bp fragment of the aMPV glycoprotein (G) gene.

3.8.6 Polymerase Chain Reaction optimization using Reverse Transcriptase-Polymerase Chain Reaction

The allantoic fluid (ALF) positive for aMPV (Instituto Zooprofilattico Sperimentale delle Venezie (IZSVe), Padova, Italy) serves as the positive control for the primer optimization since to the best of our knowledge this is the first major work on avian metapneumovirus in Nigeria. Non template control (with no primer and antigen) was the negative control. Conventional RT-PCR was performed using one-step RT-PCR kit (Qiagen). The PCR machine C1000 Touch (Bio-Rad, Foster City) used for the amplification has different protocol for different targeted gene as stated in Table 3.5a and 3.5b above according to Bayon- Auboyer *et al.* (1999).

3.8.7 Gel Preparation and Electrophoretic Analysis of PCR Products

The amplicons obtained were analysed on 2% (w/v) agarose gel produced by adding 2 g agarose powder (Sigma, St. Louis, Missouri, USA) to 100 ml of TAE buffer (Tris base, acetic acid and, EDTA) in a conical flask. The mixture was boiled for few seconds in a microwave oven to allow dissolution of the agarose powder. Ethidium bromide (5 µl of 5 mg/ml) was applied to the molten agar and swirled gently to mix. The agar was cooled to about 45^{o}C before it was ran into the electrophoresis trough with appropriate comb placed and allowed to thicken. A 100 bp DNA ladder (New England Biolabs) was employed to approximate the band size. Thereafter, 3 µl of dye mixture (New England Biolabs) followed by 5 µl of PCR product were placed on a clean parafilm and mixed. The amplicon –dye mixture and the 100 bp marker were then loaded into the wells. Gel electrophoresis was done at (120 volts) for 30 minutes in 1X TBE buffer (Promega) and the gel was visualised under UV light (Alpha Imager, Alpha Innotech, San Leandro, CA). Amplicons with an expected band size of 115 bp for the N gene and 448 bp for the G-gene were considered positive for avian metapneumovirus (Bayon- Auboyer *et al.*, 1999).

Plate 3.3: Candling of 7-day old Specific Antibody Negative (SAN)-embryonated chicken eggs

Plate 3.4a: Inoculation of aMPV positive samples into the yolk sac

Plate 3.4b: Sealing of eggs with varnish (Black arrow)

3.9 Objective 5: Isolation and Identification of Bacteria Associated with Avian Metapneumovirus Infection in three States of Nigeria

3.9.1 Preparation of Media Used

I. Nutrient agar (BIOLAB)

In 100 ml of distilled water, 2.9 g of nutrient agar was liquefied. It was autoclaved at a pressure of 15 lbs (121^{o} C for 15 minutes). The agar was cooled to 45 ^{o} C. It was then poured into sterile plates to solidify.

II. Tryptone soya agar (BIOLAB)

Melted tryptone soy agar (4.5 g) was autoclaved for 15 minutes at 15 lbs of pressure in 100 ml of distilled water (121^{o} C). It took the agar 45 ^{o} C to cool. After that, it was put onto sterilized Petri dishes to set.

III. Blood agar

100 ml of distilled water were used to liquefy 2.9 g of nutrient agar, which was then autoclaved for 15 minutes at 15 lbs of pressure (121^{o} C). After allowing the agar to cool to about 40 ^{o} C, 5 ml of blood was aseptically added and carefully mixed before being put into sterile petri dishes and allowed to solidify.

IV. Nutrient broth (HIMEDIA)

100 ml of distilled water was used to melt 1.3 g of nutrient broth before it was autoclaved for 15 minutes at 15 lbs of pressure (121^{o} C).

V. MacConkey agar with salt (LAB M)

Melted MacConkey agar with salt (5.2 g) was autoclaved for 15 minutes at 15 lbs of pressure in 100 ml of distilled water (121^{o} C). Before being placed into sterile Petri dishes to set, the agar was cooled to 45 ^{o} C.

VI. Starch agar (1 %)

1 g of soluble starch and 2.9 g of nutrient agar were melted in 100 ml of distilled water. It was 121° C autoclaved for 15 minutes at 15 lbs of pressure, chilled, and then flooded onto sterile Petri dishes to solidify.

VII. Triple sugar iron agar (TSI)

100 ml of distilled water were used to liquefy 6.5 g of TSI agar, which was then autoclaved for 15 minutes at 15 lbs of pressure (121° C). After cooling, it was put into sterile test tubes and angled or inclined to create a 1-inch-long butt.

VIII. Methyl Red Voges Proskeur (M.R.V.P) broth (OXOID):

In 100 ml of distilled water, 1.7 g of methyl-red-voges-proskeur (M.R.V.P.) broth was liquefied before being autoclaved for 15 minutes at 15 lbs of pressure (121°C).

IX. Phenol red agar

After being solubilized in 100 ml of distilled water, nutrient agar (2.9 g) and phenol red (2.5 mg) were autoclaved at 15 lbs of pressure (121° C) for 15 minutes before being put onto sterile petri dishes to solidify.

X. Simmons citrate agar (LAB M)

After being solubilized in 100 ml of distilled water and autoclaved at 15 lbs of pressure (121° C) for 15 minutes, nutrient agar (2.9 g) and phenol red (2.5 mg) were put onto sterile petri dishes to solidify.

XI. Urea broth (BIOLAB)

Nutrient agar (2.25 g) was dissolved in 220 ml of distilled water, autoclaved at 15 lbs pressure (121^{o} C) for 15 minutes, and chilled to approximately 50 ^{o} C. The broth was then treated with a 20% urea solution (5 g of urea crystals + 25 ml of sterile distilled water).

3.9.2 Reagents used for Biochemical Tests

a. Kovac's reagent

10 g of para-dimethyl aminobenzaldehyde was dissolved in 150 ml of pure amyl alcohol, and 50 ml of strong hydrochloric acid was progressively poured and stored at 4^{o} C.

b. 6 % 1-naphthol: 94 ml of absolute ethanol was mixed with 6 g of 1-naphthol.
c. KOH crystals were dissolved in 60 ml of distilled water at 40% concentration.
d. 0.12 g of methyl red was diluted in 180 ml of ethanol, and the pH was adjusted to 5.0.
e. 3 ml of 6% hydrogen peroxide solution + 3 ml of sterile distilled water = 3 ml of hydrogen peroxide (3%) solution.
f. 0.1 g of oxidase reagent (1%) was dissolved in 10 ml of distilled water.

3.9.3 Study Location

The research was conducted in Plateau, Sokoto and Oyo States of Nigeria during the harmattan period (November, 2020 to March 2021). Three Veterinary Teaching Hospitals at University of Jos, Jos, Usman Dan Fodio University, Sokoto and University of Ibadan, Ibadan as well as three Veterinary Clinics i.e., Marie Veterinary

Clinic, Plateau State, Peter Veterinary Clinic, Sokoto State and CHI in Ibadan, Oyo State, were used as sample collection points for this study.

3.9.4 Samples and Sampling Procedures

A total of 42 disease outbreak of birds showing respiratory signs and presented for diagnosis at the three Veterinary Teaching Hospitals and the three Clinics were sampled from November 2020 to March 2021 during harmattan. The respiratory signs included mild to severe sneezing, coughing, nasal and ocular discharges. From the selected cases presented, gross pathological lesions were noted and conjunctiva (Cj), lung (Lg), trachea (Tr) and turbinate (Tu) were sampled using swab sticks which were immersed into plain bottles containing 3 ml of peptone water. A total of 42 swab samples i.e., 14 swab samples per State were therefore collected in the study area. The samples were then transported on dry ice and stored at 4^{o}C with constant electricity at the Animal Care Diagnostic Laboratory, Ring Road Ibadan, until assayed.

3.9.5 Bacterial Isolation and Identification

To obtain pure cultures of bacterial organisms, individual swabs of the conjunctivae, lungs, tracheae, and turbinates were used to inoculate nutrient agar plates (HIMEDIA). A sterile wire loop was then used to streak the plates with parallel overlapping strokes. After 24 hours, the plates were incubated at 37 ^{o} C overnight and bacterial growth was assessed. The growth plates were placed on Trypticase Soy Agar (TSA) and MacConkey agar and incubated at 37 ^{o} C. Bacteria growth was observed and inoculated into other media, including Starch agar for the detection of organism capable of hydrolysing starch, triple sugar iron agar based on sucrose fermentation, lactose, dextrose and hydrogen sulphide production for Gram negative bacilli identification. Biochemical tests such as Methyl-Red-Voges-Proskauer broth, Phenol red agar for fermentation, Simmons citrate agar (Lab M) are used for characterisation of Enterobacteriaceae based on the ability to use citrate as sole carbon source, and urea broth (BIOLAB) for bacterial differentiation based on urease production (Plate 3.5).

3.9.6 Biochemical Tests

The bacterial isolates were identified with the following biochemical tests

i. Catalase test

Catalase is found in the majority of aerobic microorganisms. This test determines the organism's ability to produce catalase, which breaks down hydrogen peroxide to produce water and oxygen bubbles.

Procedure: A wire loop was used to transport colony growth to the edge of a clean, dry glass slide, and a drop of 3 per cent hydrogen sulphide was placed on it. The evolution of oxygen bubbles could be seen right away.

ii. Oxidase test

It is used to determine bacteria that generate cytochrome C oxidase, an electron transport chain enzyme.

Procedure: A drop of a 1% aqueous solution of tetramethyl-p-phenylenediamine dihydrochloride (oxidase reagent) was added to a piece of No 1 Whatman filter paper in a Petri dish. A few colonies were smeared onto the filter paper with a glass slide. Within 5 seconds, a purple color indicates satisfaction. A purple coloration obtained within 10-16 seconds indicates a delayed positive result, whereas any subsequent reaction is considered negative.

iii. Indole test:

The ability of an organism to synthesize indole from tryptophan is determined by this test. Indole combines with Kovac's reagent to form a deep rose colour.

Procedure: The organism was incubated for 20 hours after being injected into nutrient broth. After incubation, 0.5 ml of Kovac's reagent was gently placed on the tube and allowed to stand. A deep rose colour developed in the presence of indole, which separates out in the alcohol layer.

iv. Citrate test:

It establishes whether an organism can use citrate as its only carbon source.

Procedure: The test organism was inoculated onto Simmon citrate agar, which was then left to incubate for 24 hours. Positive reactions were indicated by an agar's color changing from green to blue, while negative reactions were indicated by no colour change.

v. Methyl Red Test

Detects when an organism produces acid as a result of fermentation.

Procedure: The organism was incubated in MRVP broth for 24 hours after being injected into it. Following incubation, the broth was divided into two parts and labeled M and V, respectively (M for methyl red and V for Voges Proskauer). After adding five drops of methyl red solution, the colour reaction was observed. While yellow indicated a negative reaction, red indicated a positive reaction, or the formation of acid.

vi. Voges- Proskauer Test

The test measures the organism's ability to produce acetylmethylcarbinol or acetoin, a byproduct of dextrose metabolism.

Procedure: With a light shake of the tube, 0.5 ml of the 6%-naphthol solution and 0.5 ml of KOH were added to the tube labeled V (as stated in the methyl red test). The appearance of red colouration, which usually occurred within 30 minutes, indicated a positive reaction.

vii. Urease test

The capacity of a biological organism to produce the urease enzyme, which helps break down urea and releases ammonia, is ascertained.

Procedure: The organism was incubated for 24 hours after being inoculated in urea broth. Urease hydrolyses urea to release ammonia, raising the pH of the broth if it is produced. The medium's color will alter as a result. Positive results were indicated with a pink or red color, while negative results were indicated with a yellow colour.

viii. Sugar fermentation

Determines whether the organism is capable of fermenting the sugars present in the medium.

Procedure: After adding the sugar disc and incubating for four hours, phenol red agar was inoculated with the organism. Positive results were demonstrated by a red to yellow colour change in the agar, while negative results were shown by a lack of colour change.

ix. Starch hydrolysis

Determines whether an organism can hydrolyse starch.

Procedure: With a light shake of the tube, 0.5 ml of the 6 percent -naphthol solution and 0.5 ml of KOH were added to the tube labeled V (as stated in the methyl red test). The appearance of red colouration, which usually occurred within 30 minutes, indicated a positive reaction.

x. Triple sugar iron

The organism's ability to ferment 1% sucrose, 1% lactose, and 0.1 % glucose, as well as sodium thiosulfate and ferrous sulfate, is demonstrated. It regulates the synthesis of hydrogen sulfide.

Procedure: The bacterial culture was placed in triple sugar iron agar and allowed to grow for 24 hours. When the medium's color changed from orange red to deep red

and hydrogen sulfide was produced and appeared as a black substance at the bottom of the tube, it was a favorable reaction.

xi. Gram Staining

On a clean, grease-free microscope slide, a droplet of saline solution was inserted. With the use of a sterile inoculating loop, an organism colony was selected. Then a suspension smear was created, and it was allowed to air dry. A few drops of crystal violet were applied to the smear after it had been heated fixed. After rinsing the discoloured slide with water and applying a few drops of Lugol's iodine for 30 seconds, the smear was cleaned. The slide was gently rinsed with water before being decoloured for 10–20 seconds using an acetone–alcohol solution. After an instant water washing, a drop of safranine were applied, and left for a minute. After being wet and blot dry, the slide was air dried and viewed under a light microscope (Chessbrough, 2006).

The results were entered on Gideon at www.gideononline.com (a software for confirmation and differential diagnosis of infectious diseases) to confirm the isolated bacteria. This software is a one-stop resource for data on Infectious Diseases for research, education and diagnostics.

3.9.7 Statistical analysis

The Statistical Package for Social Sciences software version 21 (SPSS Inc., Chicago, IL, USA) was used to interpret the data. Descriptive statistics was used to estimate the prevalent bacterial disease in the study areas.

Plate 3.5: Isolation and identification of different bacteria organisms at the Diagnostic Laboratory, Ibadan

CHAPTER FOUR

RESULTS

4.1 Seroprevalence of Avian Metapneumovirus in Commercial Chickens from Three Climatic Zones in Nigeria

The overall seroprevalence of avian metapneumovirus in chickens in the study area was 59.79% (297/480). Plateau State had 76.25% (122/160), Sokoto State 60.63% (97/160) while Oyo State had 42.50% (68/160) (Table 4.1).

The highest seroprevalence was recorded in dry season 68.33% (164/240) (Table 4.2) with mean antibody titre of (2990.9 ± 231.6) as shown in Figure 4.1, while the seroprevalence during wet season was 51.25% (123/240) (Table 4.2) with mean antibody titre of 572.9 ± 64.1 which was significantly lower ($\alpha<0.05$) than that of the dry season (Figure 4.1). Also, out of the 80 sera collected per state during the dry season, the highest seroprevalence was recorded in Plateau State i.e., 80/80 (100%), while Sokoto State had 56.25% (45/80) and Oyo State had 48.75% (39/80) as shown in Table 4.2. Plateau State had the highest mean antibody titre of 4757.9 ± 223.5 compared to Sokoto State with 2800.9 ± 313.1 and Oyo States with 1414.0 ± 158.1 (Figure 4.2), with a statistical difference ($\alpha<0.05$).

During wet seasons, of the 80 sera collected per state, the highest seroprevalence was recorded in Sokoto State i.e., 65% (52/80) while Plateau State had 52.50% (42/80) and Oyo State 36.25% (29/80) (Table 4.2). The mean antibody titres were 670.7 ± 74.9, 548.8 ± 61.4 and 499.4 ± 55.8 (Figure 4.2) in Sokoto, Plateau and Oyo State, respectively.

Table 4.1: Seroprevalence of aMPV antibodies in chickens from the three states (Plateau, Sokoto and Oyo) in Nigeria

State	Total No. of Samples tested (N)	Seroprevalence	
		% Positive	% Negative
Plateau	160	76.25[a]	23.75
Sokoto	160	60.63[b]	39.38
Oyo	160	42.50[c]	57.50
Total (Overall)	**480**	**59.79**	**40.20**

Table 4.2: Seroprevalence of avian metapneumovirus infection in commercial chickens in Plateau, Sokoto and Oyo States during dry and wet seasons

State	Total No. of Samples tested (N)	Seroprevalence	
		Dry Season	**Wet Season**
		n=80	n =80
Plateau	160	100.00[a] (80)	52.50[b] (42)
Sokoto	160	56.25[b] (45)	65.00[a] (52)
Oyo	160	48.75[c] (39)	36.25[c] (29)
Total (Overall)	**480**	**68.33[a] (164)**	**51.25[b] (123)**

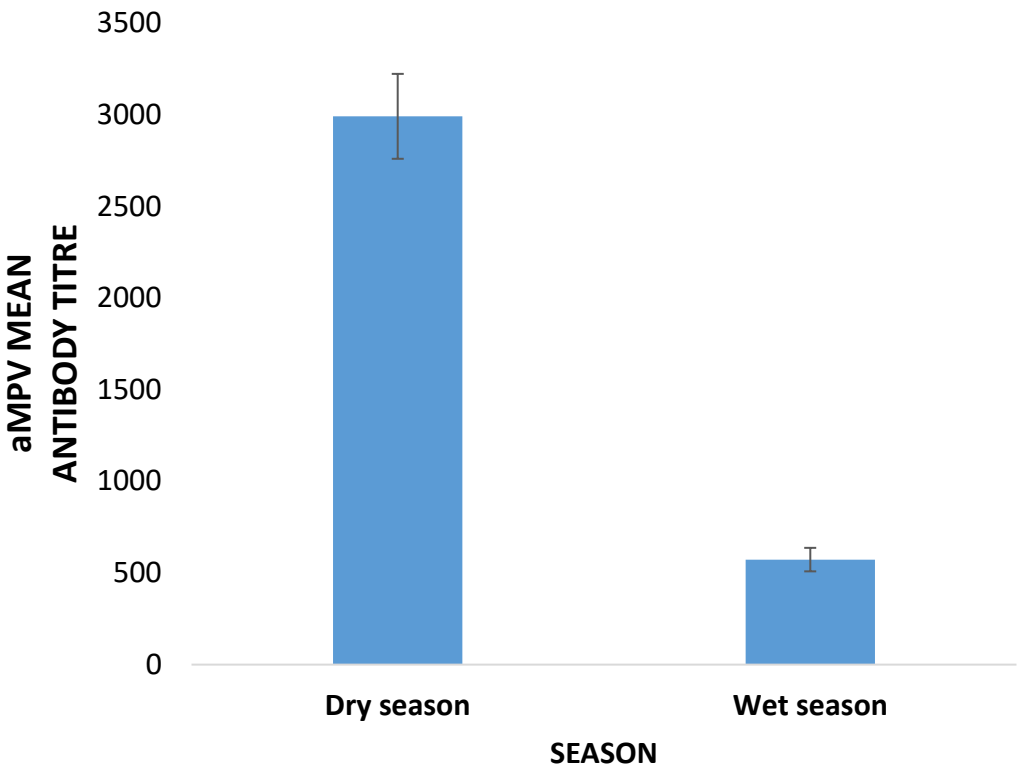


Figure 4.1: Mean ± SEM avian metapneumovirus antibody titres in commercial chickens during the dry and wet season

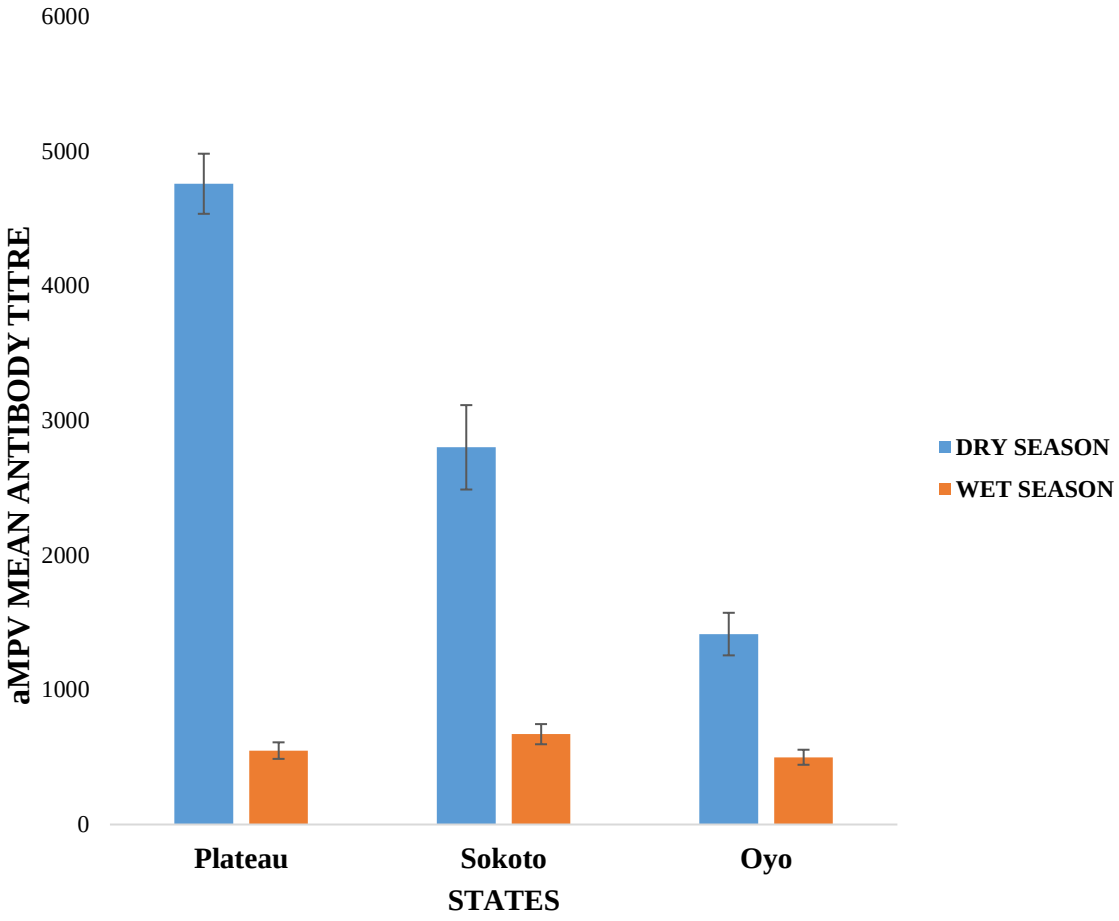


Figure 4.2: Mean ± SEM avian metapneumovirus (aMPV) antibody titers in the commercial chickens in Plateau, Sokoto and Oyo States, Nigeria

4.2 Presence and Molecular Characteristics of Avian Metapneumovirus in Three Climatic Zones in Nigeria

4.2.1 Presence of Avian metapneumovirus (aMPV) Strains in Three Climatic Zones in Nigeria

Out of forty-two cases (14 each from Plateau, Sokoto and Oyo States) that were subjected to RT-PCR technique, only 5/42 (11.90 %) samples were positive for aMPV (Plate 4.1 and 4.2) and all the five positive samples were from Plateau State (Figure 4.3). The five positive samples were detected with the primer (N-gene; 115 bp). Out of these five samples, one sample was equally detected as positive for the G-gene (448 bp) (Plate 4.2).

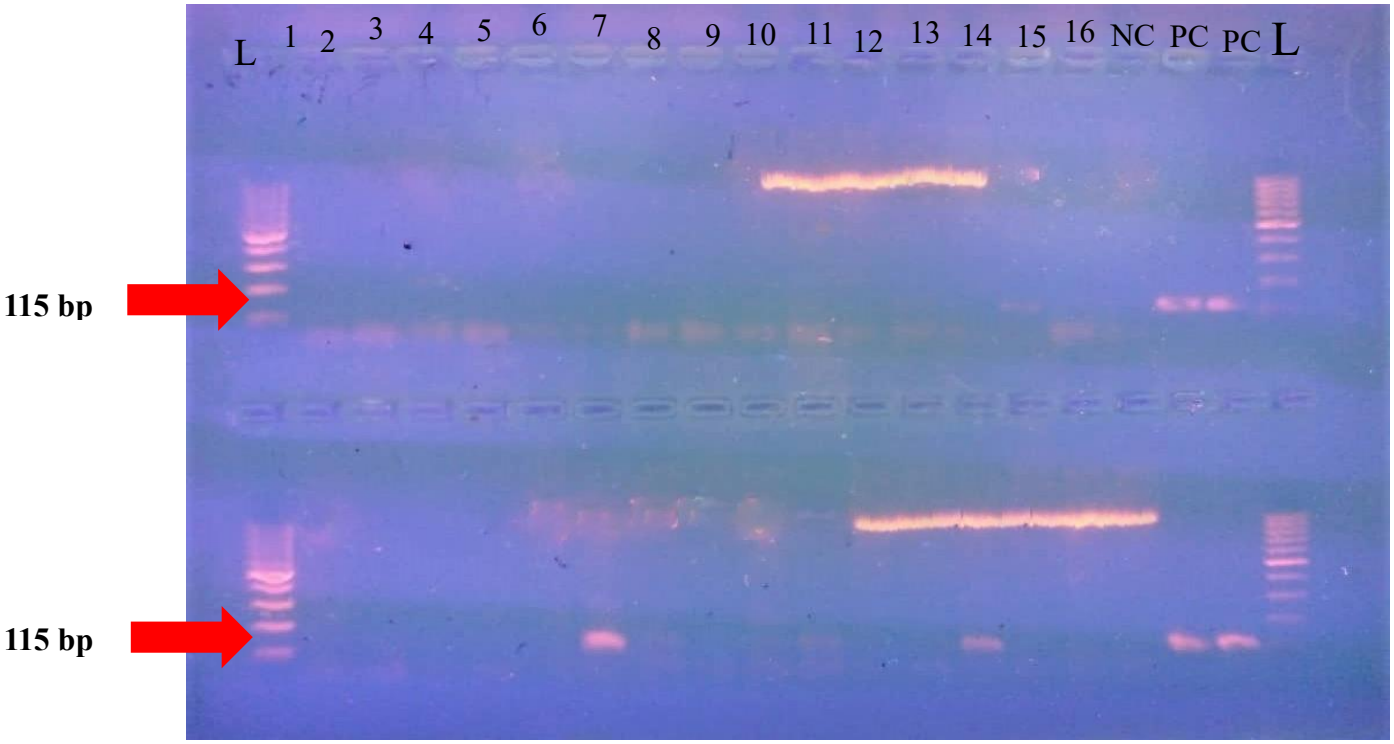


Plate 4.1: Agarose gel picture of the PCR products in well 15 (up), wells 7 and 14 (down) using the N-gene specific primers

PC: Positive control

NC: Negative control

L: Ladder

Lane 1-16: Test samples

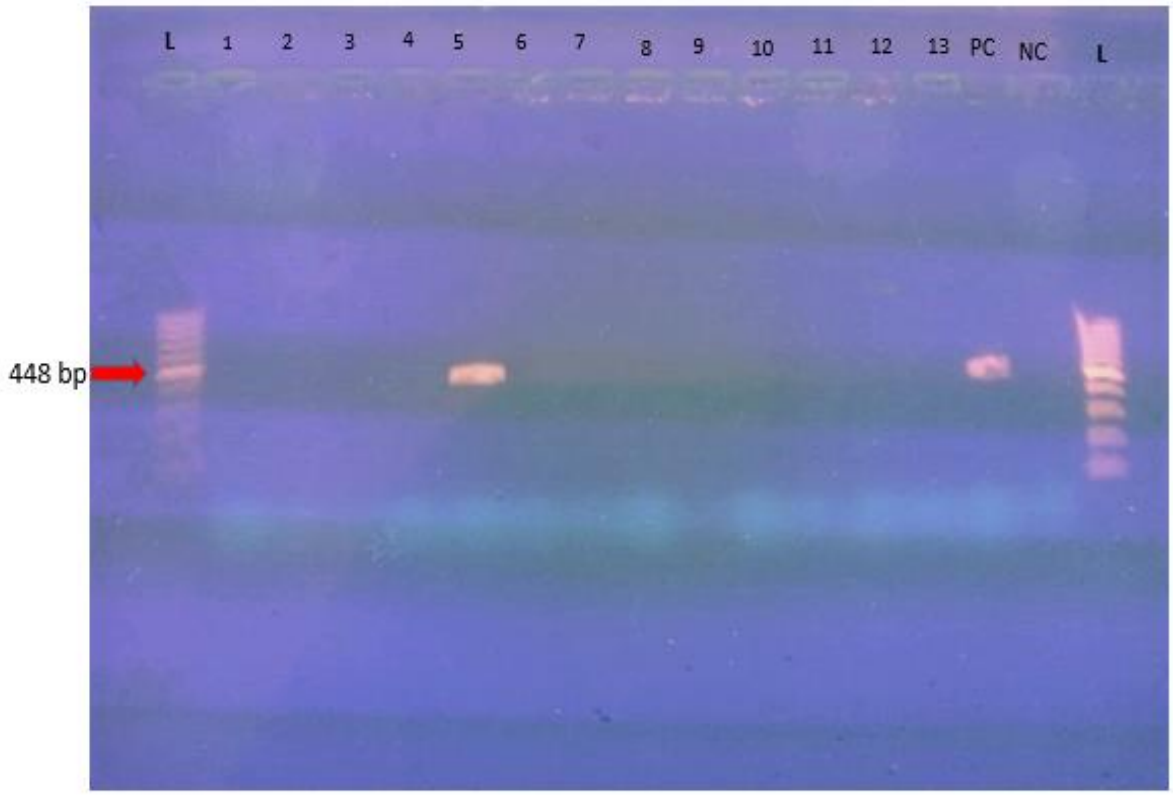


Plate 4.2: Agarose gel picture of the PCR product in well 5 using the G-gene specific primers

NC: Negative control

PC: Positive control

L: Ladder

Lane 1-13: Test samples

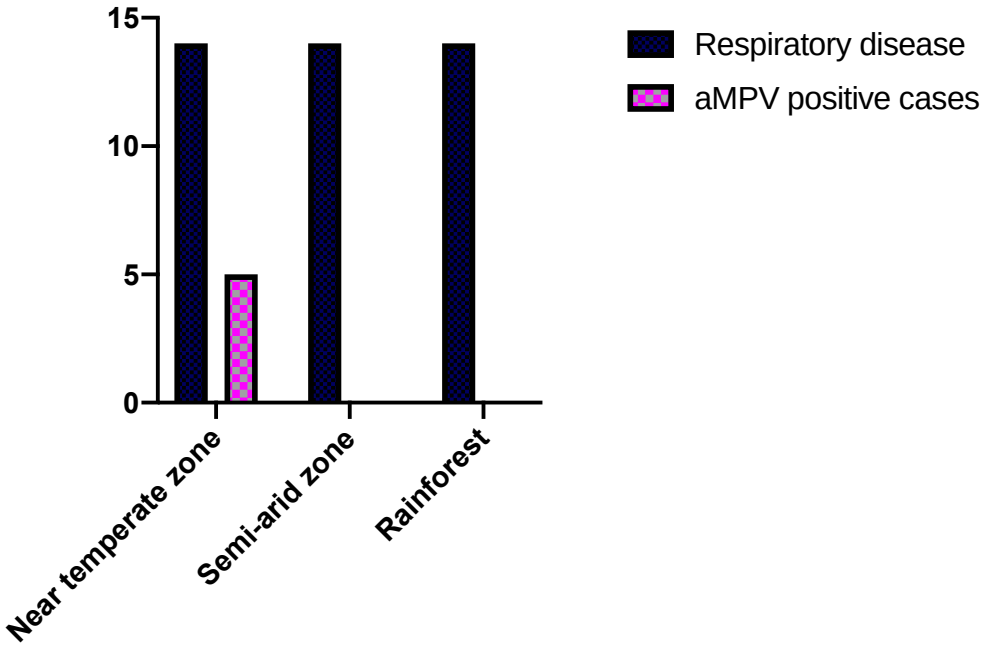


Figure 4.3: Distribution of avian metapneumovirus positive samples based on disease occurrence and climatic zones with level of significance at p values < 0.05 and df=8.246,2.

4.2.2 Molecular Characterisation of Avian metapneumovirus from Clinical Samples in Three Selected Climatic Zones in Nigeria

4.2.2.1 Phylogenetic grouping

The blast result showed that the G-gene of avian metapneumovirus (aMPV) positive sample sequenced belonged to the Subtype B group of aMPV with range of 96.4-97.3% homology to already deposited aMPV in the GenBank and named avian metapneumovirus Nigeria/plateau/aMPV/B/NGA03/2021 with accession number MZ408311 (Table 4.3). Phylogenetic tree showed a largely similar grouping to Subtype B avian metapneumovirus already deposited in the GenBank regardless of whether nucleotide or amino acid sequences had been utililised for alignment. Based on phylogeny and amino acid mutation in the G-gene attachment protein capsids, the Nigeria aMPV isolate studied carries genetic signatures that are homologous to strains from a distant geographical region, particularly those of European and Asian strains (Figure 4.4).

4.2.2.2 Multiple alignment of nucleotide and deduced amino acid sequences of avian Metapneumovirus

The sequence is contrasted to a group of other related sequences in this study using multiple sequence alignment, so all positions with less than 70% site accessibility were eliminated. Less than 30% alignment gaps, missing data, and unclear bases were allowed in any position (partial deletion option), with 387 places in the data set. Multiple nucleotide alignment showed areas of insertions and point mutations in different regions when the detected isolate was compared for similarities and differences for protein analysis with aMPV strains already deposited in the GenBank. AM490057 aMPV Nigeria/B, AM490058 aMPV Nigeria/B, AM490059 aMPV Nigeria/A, FJ828954 (aMPV/A/2007; vaccine strain), AJ811993 aMPV 00094/C, AY734531 Turkey RV/A, KC954643 aMPV 31/2011/B and AJ251085 aMPV/D as indicated in Figure 4.7 a, b and c. The isolate from this study has possibly significant substitutions found in some amino acid sequence locations. While substitutions K183R and H224Y at positions 183 and 224, respectively, were similar to Subtype B previously reported in Nigeria, the substitution A8S present is similar to the other subtypes A and B previously reported in Nigeria, but not in Subtype C and D from other countries. Also, substitution V13I at position 13 was similar to Subtypes B and D. The substitutions H235R and S181T at positions 235 and 181 seen in the isolate

from this study were similar only to the subtype A from Nigeria. Substitutions T195V and G294S at positions 195 and 294 respectively were similar to only the subtype D, also, substitution N24K at position 24 was similar to subtypes B, C and D and different from subtype A. The substitution R66Q at position 66 is similar to strain of Subtype B vaccine previously deposited in the GenBank. The non-synonymous substitutions T12I, G223E and A238V at positions 12, 223 and 238, respectively, occurred only in the isolate detected in this study and were different from the subtypes A, B, C and D previously deposited in the GenBank (Figure 4.5 a, b, and c).

Table 4.3: Accession number of sequence of avian metapneumovirus detected in Plateau State (Temperate zone)

BANKIT NUMBER	NAME	SEQUENCE NUMBER	ACCESSION NUMBER
2473006	NGA1	Seq1	MZ408311

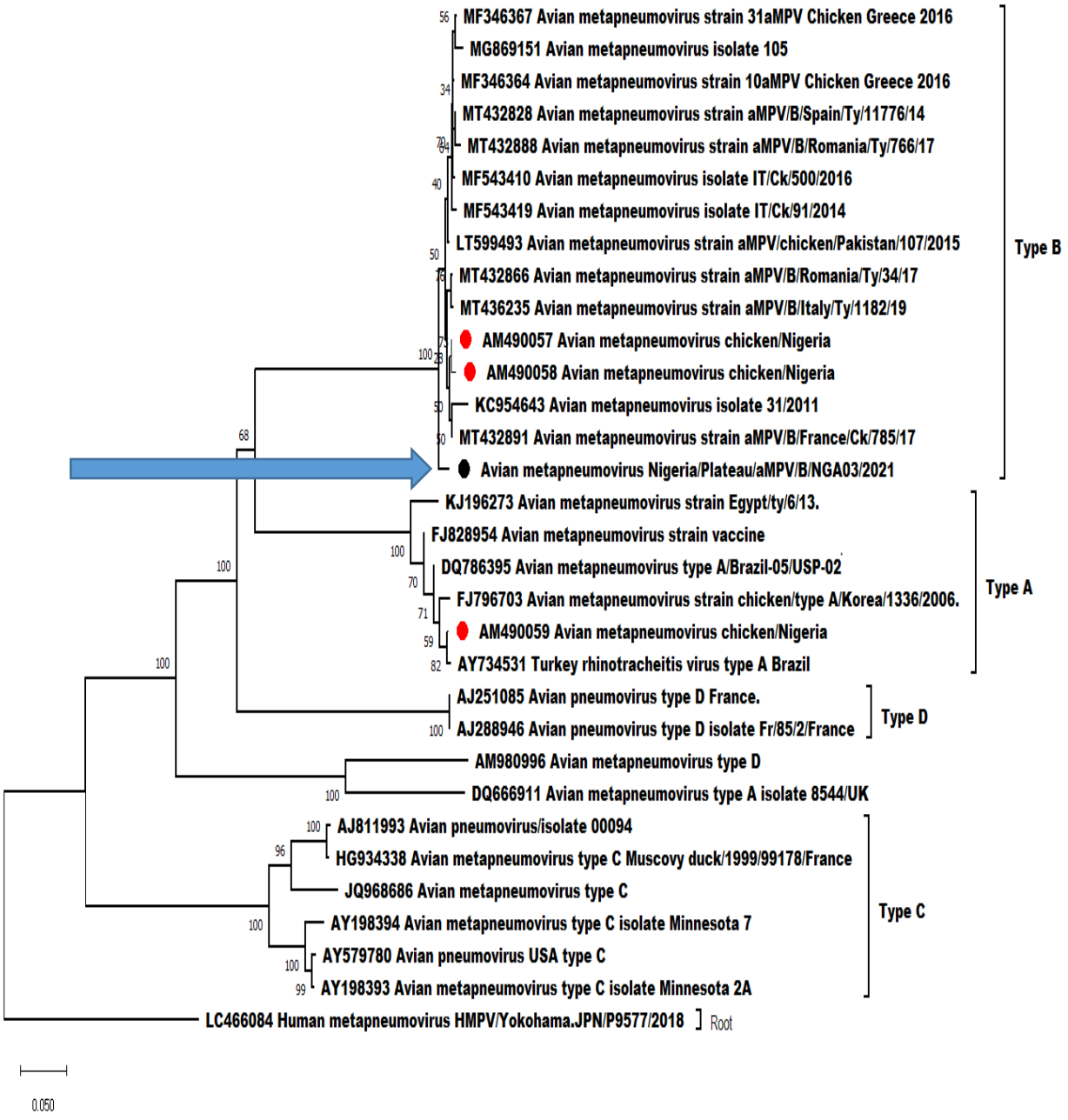


Figure 4.4: Phylogenetic grouping of Nigerian aMPV isolate

Index: The aMPV isolate in this study is represented by black circle. Short branches connect very similar sequences; longer branches suggest decreasing sequence homology

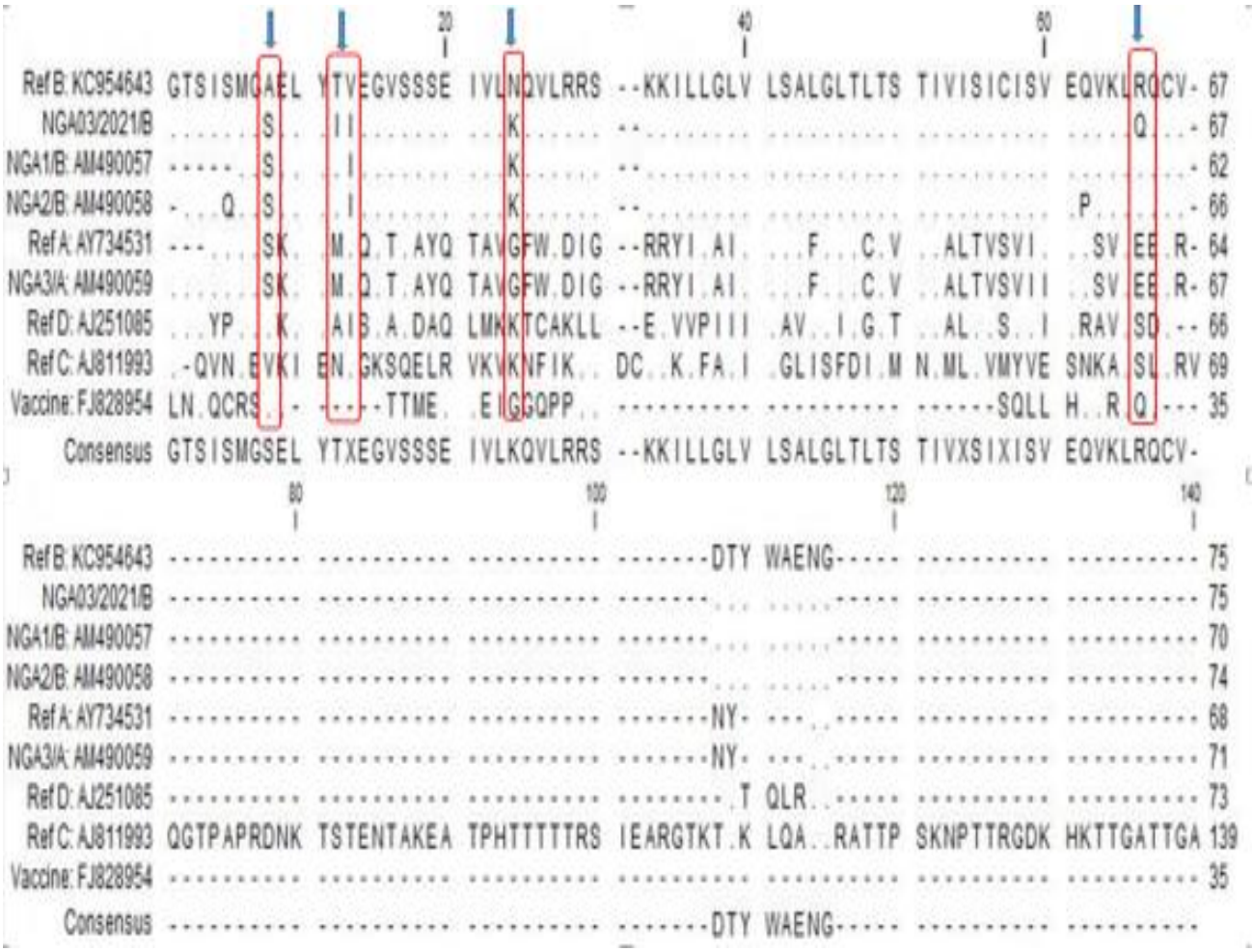


Figure 4.5a: Predicted alignment of the amino acids in the G-attachment glycoproteins of the identified metapneumoviruses. The consensus sequence contains the amino acid residues that are completely conserved throughout the pneumovirinae subfamily. A dash indicates the absence of an amino acid similar to the majority of the sequences, while a dot shows the presence of an amino acid relative to the consensus

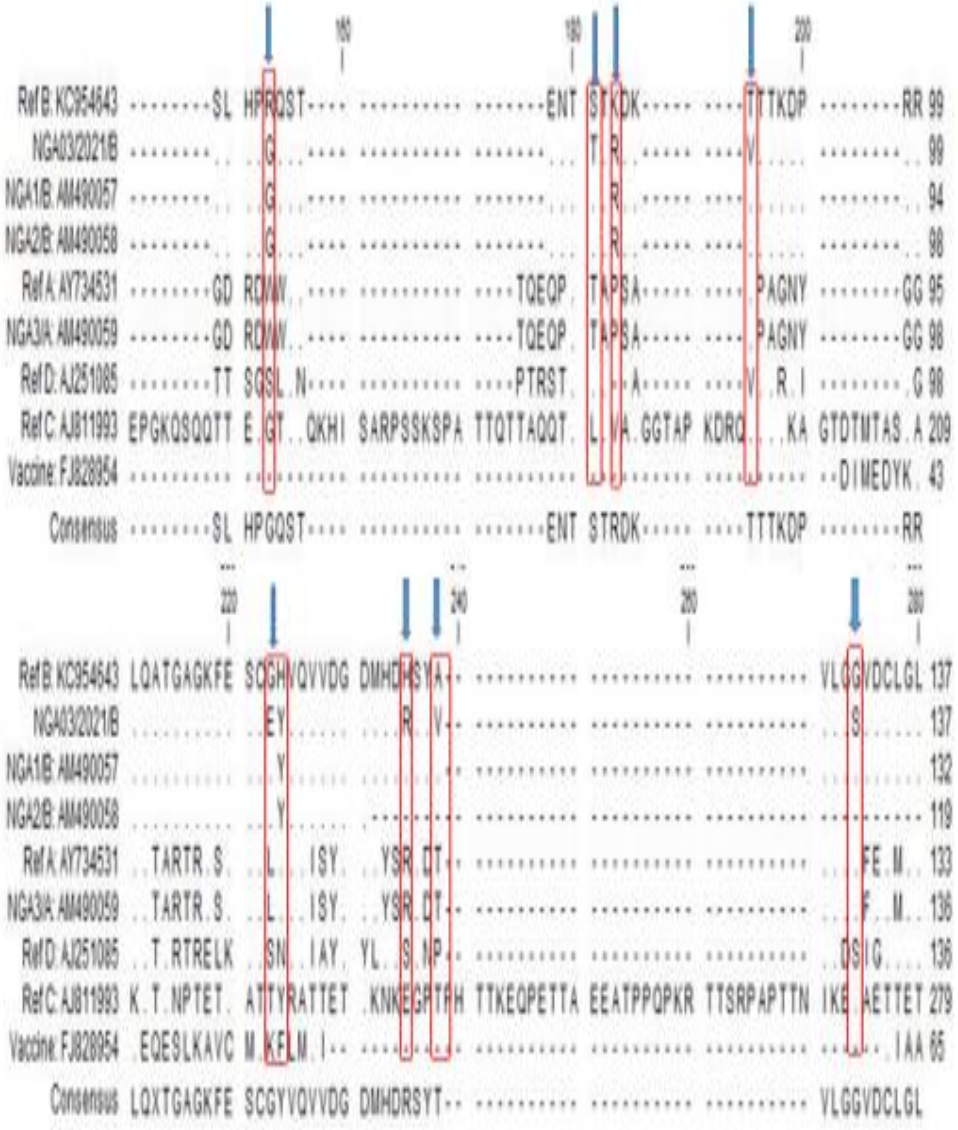


Figure 4.5b: Predicted alignment of the amino acids in the G-attachment glycoproteins of the identified metapneumoviruses. The consensus sequence contains the amino acid residues that are completely conserved throughout the pneumovirinae subfamily. A dash indicates the absence of an amino acid similar to the majority of the sequences, while a dot shows the presence of an amino acid relative to the consensus

Figure 4.7c: Predicted alignment of the amino acids in the G-attachment glycoproteins of the identified metapneumoviruses. The consensus sequence contains the amino acid residues that are completely conserved throughout the pneumovirinae subfamily. A dash indicates the absence of an amino acid similar to the majority of the sequences, while a dot shows the presence of an amino acid relative to the consensus

Index: A-Alanine, H-Histidine, R-Arginine, I-Isoleucine, N-Asparagine, P-Proline, D-Aspartic acid, C-Cysteine, T-Threonine, E-Glutamic acid, Q-Glutamine, G-Glycine, V-Valine, Y-Tyrosine, L-Leucine, K-Lysine, M-Methionine, F-Phenylalanine, S-Serine, W-Tryptophan,

Site of mutations in the samples at positions 8, 12, 13 24, 66, 153,181, 183, 195, 223, 224, 235, 238 and 274

4.3. Clinical presentation and Epidemiological Factors Associated with Avian Metapneumovirus Infections in Nigeria

4.3.1. Epidemiological factors associated with Avian metapneumovirus in different climatic zones in Nigeria

All five aMPV positive samples were obtained during the harmattan period (dry season) with one sample obtained in December 2020 and the other four samples obtained in February 2021 (Table 4.4). The five RT-PCR positive samples comprised of 4 turbinate samples (i.e., 80%) and 1 conjunctiva sample (i.e., 20%) (Figure 4.6).

Table 4.4: Detection rate of aMPV infection by month during the harmattan period in the study areas

Month	No. of samples tested	No. Positive (%)	No. Negative (%)
December, 2020	11	1 (9.09)	10 (90.9)
January, 2021	15	0 (0.00)	15 (100)
February, 2021	8	4 (50.00)	4 (50.00)
March, 2021	1	0 (0.00)	1 (100)
Total (Overall)	42	5 (11.91)	37 (88.09)

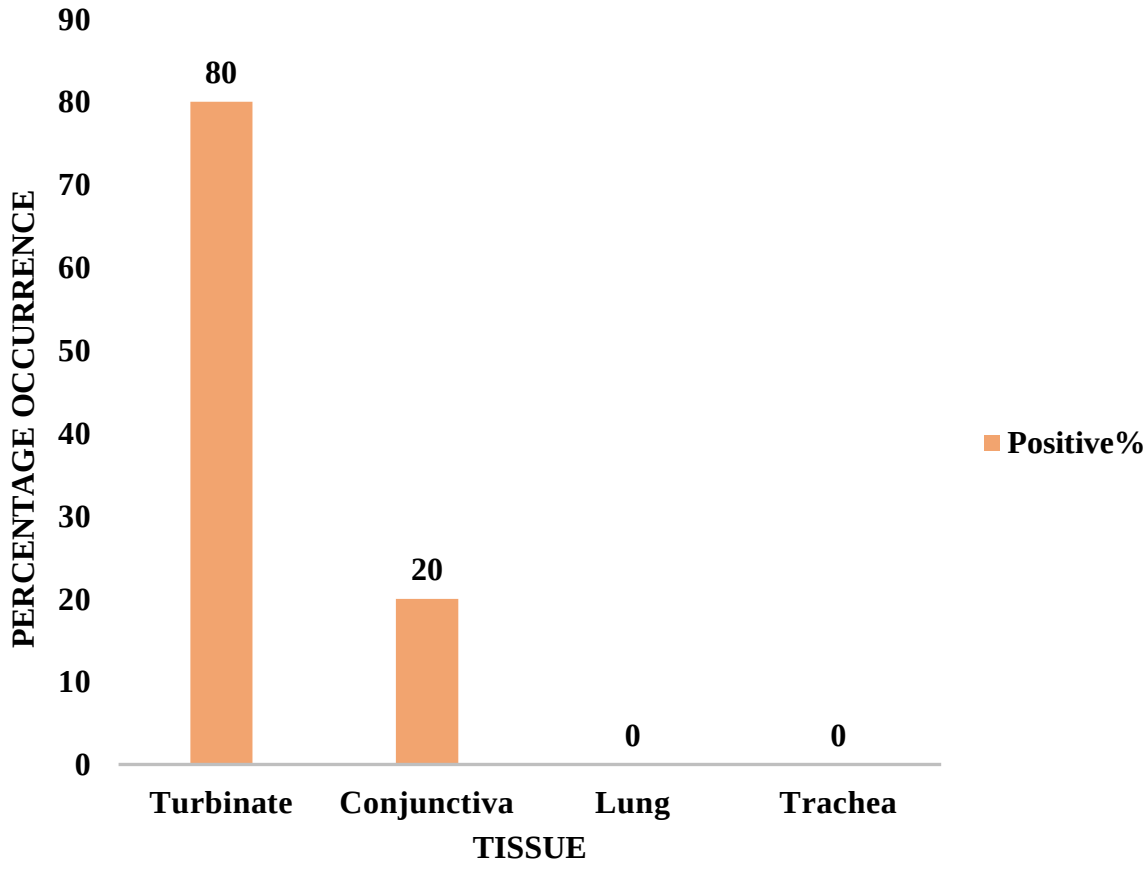


Figure 4.6: Distribution of avian metapneumovirus by tissue affected during sampling

All five positive samples from the 42 cases presented and investigated at the Veterinary Teaching Hospitals and Clinics of the three States (Plateau, Sokoto and Oyo State) were from layer flocks i.e., 11.90 % (Table 4.5). Three out of the 19 cases i.e., 15.79%, were from flock sizes of less than 1000 chickens, one, out of seven cases (5.88%) was from flock size in the range of 1001 – 5000 chickens and one out of three cases (33.33%) was from flock size in the range of 5001 – 10000 chickens (Table 4.6). All the positive samples were from flocks on intensive management.

With regards to age distribution of positive samples, one sample out of 22 (4.54%) was obtained from age group 0-10 weeks old, one sample out of 7 (14.30%) was obtained from age group 11-20 weeks old, one sample out of 6 (16.70 %) was obtained from 21-30 weeks old while two positive samples out of 7 (28.60 %) were obtained from age group 31 weeks old and above (Table 4.7).

Table 4.5: Detection rate of avian metapneumovirus infection based on chicken type during sampling in the study area

Flock type	Number of samples tested	Positive (%)	Negative (%)
Layers	**16**	**5 (31.25)**	**11 (68.75)**
Broilers	**26**	**0 (0.00)**	**26 (100)**
Total (Overall)	**42**	**5 (11.90)**	**37 (88.09)**

Table 4.6: Detection rate of avian metapneumovirus infection in affected chickens based on flock size

Flock size	Number of Flocks tested	Number of Positive Flocks (%)	Number of Negative Flocks (%)
≤1000	19	3 (15.79)	16 (84.21)
1001 - 5000	17	1 (5.88)	16 (94.11)
5001 - 10000	3	1 (33.33)	2 (66.67)
≥10001	3	0 (0.00)	3 (100)
Total (Overall)	42	5 (11.9)	37 (88.09)

Table 4.7: Age group distribution in the incidence of avian metapneumovirus infection

Flock age (weeks)	Number of flocks tested	No of Positive flocks (%)	No of Negative flocks (%)
0-10	22	1 (4.54)	21 (95.45)
11-20	7	1 (14.28)	6 (85.71)
21-30	6	1 (16.67)	5 (83.33)
≥31	7	2 (28.57)	5 (71.42)
Total (Overall)	42	5 (11.91)	37 (88.09)

The pattern of antibiotic usage recorded in this study was 100% (42/42) with all the farmers accepting they had used drugs on their farm, 35.71% (15/42) was prescribed by the Veterinarian and 64.29% (27/42) was prescribed by farmer. Most farmers seek information about drugs used through friends 35.71% (15/42), through the seller 35.71% (15/42) and lowest through an animal health worker 28.57% (12/42).

Most of the farmers reported they used antibiotics mainly during sickness 59.52% (25/42) while 23.81% (10/42) reported they used drug daily once in a week and 16.67% (7/42) reported once in week-once in a month use of antibiotics. 47.61% (20/42) of the farmers reported using antibiotics for the prevention and treatment of disease, 11.90% (5/42) use antibiotics to prevent disease only, while, 28.57% (12/42) of the farmers use antibiotics to treat disease only, and 11.90% (5/42) use antibiotics as growth promoters Table 4.8.

The antibiotics used come in a variety of commercial forms with numerous trade names (not disclosed in this study for ethical reasons). Tylosin was the most used antibiotics (71.4%), followed by doxycycline (66.7%) and enrofloxacin (59.5%) and amoxicillin (4.76%) (Table 4.9).

Table 4.8: Trend of antibiotic usage during sampling in the research area.

Variables		Frequencies	Percentage (%)
Use of drugs on Farm	Yes	42	100
	Veterinary doctors	15	35.71
	Self	27	64.29
Source of information about antibiotics used	Animal health worker	12	28.57
	Through the seller	15	35.71
	Through a friend	15	35.71
Frequent use of antibiotics	Daily-once in a week	10	23.80
	Once in a week-once in a month	7	16.67
	During period of sickness	25	59.52
Reason for Antibiotic usage	To prevent and treat diseases	20	47.61
	To prevent diseases	5	11.90
	To treat diseases	12	28.57
	As growth promoters	5	11.90

Table 4.9: Commonly used antibiotics in the treatment of respiratory diseases in commercial chicken farms in the Plateau, Sokoto and Oyo State, Nigeria

Antimicrobial	Frequency N=42	Percentage (%) of usage
Tylosin	30	71.23
Doxycycline	28	66.7
Enrofloxacin	25	59.5
Oxytetracycline	8	19.04
Neomycin	7	16.67
Tyvalosin	7	16.67
Erythromycin	7	16.67
Cefquinor	7	16.67
Ciprofloxacin	5	11.90
Tiamulin	5	11.90
Streptomycin	5	11.90
Cephalosporin	5	11.90
Gentamycin	3	7.14
Amoxycillin	2	4.76

All farmers indicated that their flocks had received vaccinations against Egg Drop Syndrome, Infectious Bronchitis, and Newcastle disease.

4.3.2 Clinical Presentation associated with Avian Metapneumovirus Infections in different Climatic Zones in Nigeria

Information on clinical signs presented by commercial chickens from the 42 cases presented at the sample collection sites i.e. Three Veterinary Teaching Hospitals and Clinics, were collected via questionnaires. These cases were acute and severe respiratory disease outbreaks characterised by mortality 100% (42/42), rales/coughing 40.48 % (17/42), sneezing 30.5 % (13/42), dyspnea 26.19 % (11/42), anorexia 19.05 % (8/42), drop in egg production 14.29 % (6/42), shell-less eggs 9.52 % (4/42), mucoid nasal discharge 14.29 % (6/42), purulent ocular discharge 11.90 % (5/42), somnolence 2.38 % (1/42), swollen infraorbital sinus 7.14 % (3/42) (Plate 4.4), stunted growth 4.76 % (2/42), weight loss 2.38 % (1/42), torticollis 9.52 % (4/42), sudden death 2.38 % (1/42), drooping wings 2.38 % (1/42) and greenish diarrhea 4.76 % (2/42) (Table 4.10).

However, the clinical observations presented by cases that were RT-PCR positive were mortality 100% (5/5), drop in egg production 80% (4/5), shell-less egg 80% (4/5), rales and coughing 80 % (4/5), sneezing 80 % (4/5), dyspnea 40% (2/5), facial swelling 60% (3/5) (Table 4.11), reduce feed and water intake 60% (3/5), mucoid nasal discharges 60% (3/5), stunted growth 40% (2/5), torticollis 60% (3/5), purulent ocular discharge 40% (2/5) as shown in Plate (4.3) and swollen head (Plate 4.4). Mortality rates recorded in aMPV positive chicken flocks were 2-50% (Table 4.12)

Table 4.10: Common clinical signs of respiratory diseases observed during sampling in commercial chicken flocks in Nigeria

Clinical observation	Frequency	Percentage (%)
Mortality	42	100
Sneezing	13	30.95
Dyspnea	11	26.19
Anorexia	8	19.05
Drop in egg production	6	14.29
Mucoid nasal discharges	6	14.29
Ocular discharge	5	11.90
Torticollis	4	9.52
Shelless eggs	4	9.52
Swollen infraorbital sinus	3	7.14
Stunted growth	2	4.76
Greenish diarrhoea	2	4.76
Weight loss	1	2.38
Sudden death	1	2.38
Drooping wings	1	2.38
Somnolence	1	2.38

Table 4.11: Clinical signs associated with avian metapneumovirus positive flock

Clinical observations	Frequency	Percentage (%)
Rales and coughing	4	80.00
Sneezing	4	80.00
Dyspnea	2	60.00
Anorexia	3	60.00
Drop in egg production	4	80.00
Mucoid nasal discharges	3	60.00
Ocular discharge	2	40.00
Torticollis	3	60.00
Shelless eggs	4	80.00
Swollen infraorbital sinus	3	60.00
Stunted growth	2	40.00
Greenish diarrhea	2	40.00
Weight loss	1	20.00
Sudden death	1	20.00
Drooping wings	2	40.00
Somnolence	1	20.00
Mortality	5	100

Table 4.12: Mortalities recorded in commercial layer flock from Plateau State with aMPV positive samples

No		**1**	**2**	**3**	**4**	**5**
Flock type		Pullets	Layers	Layers	Layers	Pullets
Flock age(weeks)		14	23	30	33	11
Mortality	Onset	7	3	1	7	8
	Per day	8	3	1	1	5
	Past one week	30	15	2	8	27

Plate 4.3: Swollen conjuctiva in a clinical case of avian metapneumovirus infected chicken

Plate 4.4: Swelling of the infraorbital sinuses and subcutaneous tissues of the head, neck, and wattles in avian metapneumovirus infected chicken

The gross lesions observed in the cases of respiratory disease outbreaks that were presented and investigated during this study were grouped by systems affected with the respiratory system 100% (42/42) being mostly affected and comprised of congested lungs with frothy exudates, dirty brown colouration with meaty appearance, air sacculitis, haemorrhagic trachea, congested trachea with both mucoid and catarrhal exudate. Thirty-four out of 42, (80.95%) of the cases had lesions in the digestive system comprising petechial haemorrhages and erosion with areas of suffusion on the proventricular mucosa and/or colorectal mucosa, petechial haemorrhages on caeca tonsils, mucoid enteritis, haemorrhages in the duodenal mucosa, visceral gouts, mucoid caeca content and caeca cores and engorged gallbladder. Fourteen out of 42 cases, (33.3%) had lesions in the urinary system comprising nephritis, congested kidneys, pale kidneys and distended ureters. Lymphatic system, 26.19% (11/42) of the spleen were enlarged/congested, necrotic, congested and/or atrophic and pale spleen with diffused haemorrhagic foci. Furthermore, 8 out of 42 (19.04%) cases showed lesions in the reproductive system comprising malformed, congested, and pedunculated ovarian follicles with some regressed, thin-shelled eggs in the oviduct (Table 4.13).

Gross lesion shown in aMPV positive cases were also grouped by system with the respiratory system being involved in all cases 100% (5/5) comprising lesions such as congested lung with frothy exudate and fibrin formation (Plate 4.5) and haemorrhage with caseous plug and cheesy exudate in the trachea (Plate 4.6) and cloudy air sac. Four out of five cases (80%) had involvement of the digestive system comprising of petechial hemorrhages and erosion on proventriculus mucosa and colorectal mucosa, petechial haemorrhages with mucoid exudate on caeca tonsils, haemorrhages on the ileocecal junction and engorged gallbladder. Two out of five cases (80%) had involvement of the reproductive system comprising of malformed and pedunculated ovarian follicles with regressed, thin-shelled eggs in the oviduct. One out of five cases (20%) had involvement of the urinary system comprising of nephritis, congested kidneys and distended ureters (Table 4.14).

Table 4.13: Gross lesions observed in the respiratory disease diagnosed during sampling in the three climatic zones of Nigeria

Organ system		Frequency n (%)
Respiratory tract	Congested lungs with/out frothy exudates	
	Hemorrhagic tracheitis	
	Cloudy air sac/air sacculitis	
	Congested tracheal with catarrhal exudate	
	Mucoid exudates in the trachea/tracheitis	
	Congested tracheal with whitish cheesy exudate	
	Congested and consolidated lungs covered with fibrins	
	Congested lungs with cheesy exudates	
	Caseous plug in the trachea	
	Caseous attachment to the lung	
	Nodular lesions in the lungs	
	Foamy and congested lungs	
	Dirty-brown discolored meaty lungs	
	Sub-total	**42 (100%)**
Digestive system	Congested/Petechial hemorrhages on proventriculus mucosa and/or colorectal mucosa	
	Mild/caseous peritonitis	
	Petechial hemorrhages on caeca tonsils	
	Mild /mucoid enteritis	
	Congested intestinal/duodenal mucosa	
	Visceral gouts	
	Mucoid caeca content	
	Caeca cores	
	Eroded periventricular mucosa with area of suffusion	
	Hemorrhages on the ileocecal junction	
	Bleached intestinal serosa	
	Sub-total	**34 (80.95)**
Kidney/urinary	Nephritis	
	Congested kidneys	
	Engorged gallbladder	
	Pale kidneys	
	Distended ureters	
	Sub-total	**14 (33.33)**
Reproductive system	Misshapen and pedunculated ovarian follicles	
	Congested ovarian follicles	
	Regressed ovarian follicles	
	Thin-shelled eggs in oviduct	
	Sub-total	**8 (19.04)**

Table 4.14: Post mortem lesions observed in the aMPV positive samples

Organ system		Frequency (%)
Respiratory tract	Congested lung with frothy exudate	
	Hemorrhagic tracheitis	
	Cloudy air sac/Air sacculitis	
	Mucoid exudate in the trachea	
	Consolidated lungs with fibrin	
	Consolidated trachea with cheesy exudate	
	Caseous plug in the trachea	
Sub-total		**5 (100%)**
Digestive system	Congested/Petechial hemorrhages on proventriculus mucosa and/or colorectal mucosa	
	Petechial hemorrhages on caeca tonsils	
	Mucoid caeca content	
	Eroded periventricular mucosa with area of suffusion	
	Engorged gall bladder	
	Hemorrhages on the ileocecal junction	
Sub-total		**4 (80%)**
Reproductive system	Misshapen and pedunculated ovarian follicles	
	Congested ovarian follicles	
	Regressed ovarian follicles	
	Thin-shelled eggs in oviduct	
Sub-total		**2 (40%)**
Urinary system	Nephritis	
	Congested kidneys	
	Distended ureters	
Sub-total		**1(20%)**

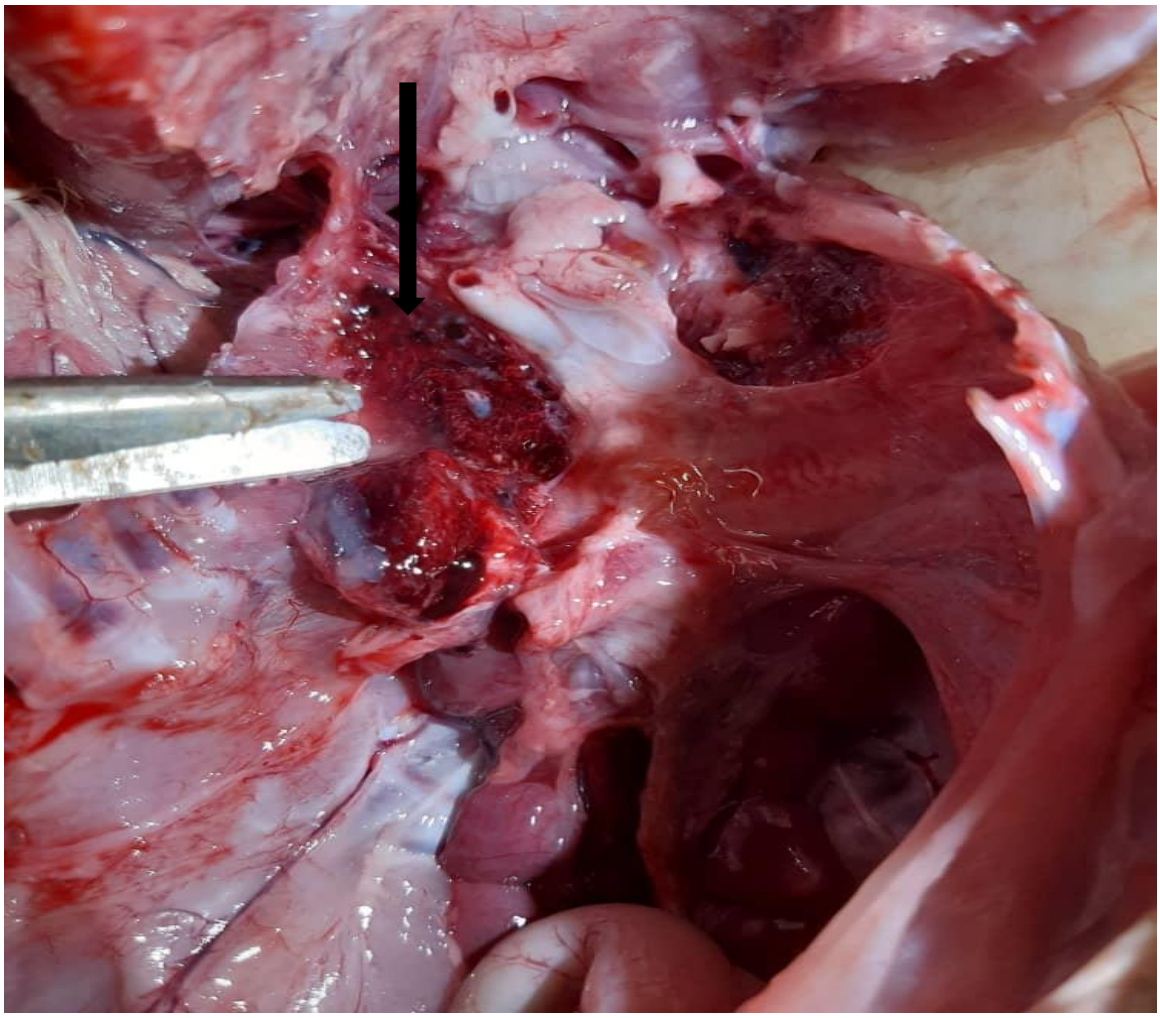


Plate 4.5: Lung congestion with cheezy exudate in typical avian metapneumovirus infection

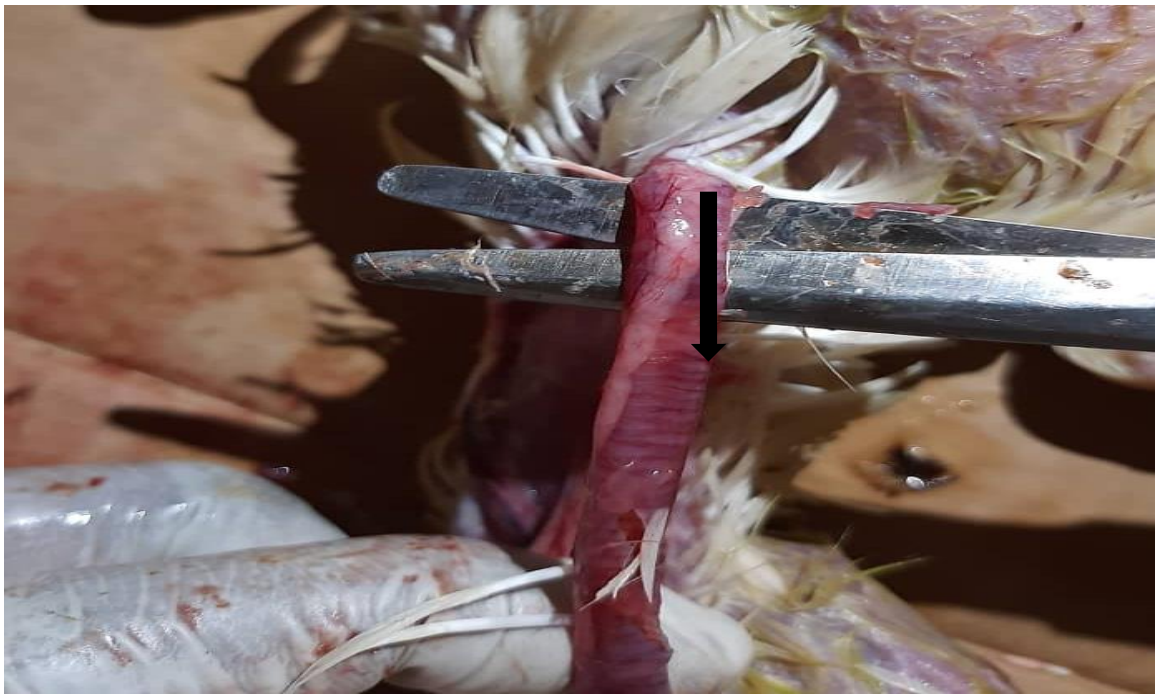


Plate 4.6: Congested trachea in avian metapneumovirus infected layer chicken

4.4 Isolation and Identification of Avian metapneumovirus

The chorioallantoic membrane (CAM), the deposit of embryo, yolk sac and extra embryonic fluid, as well as the membrane of eggs harvested from the three sets of specific antibody negative (SAN) - embryonated chicken eggs containing samples C, D, and E revealed embryo death with areas of necrosis and haemorrhages in the embryonic fluid and yolk sac (Plate 4.7). However, embryonic fluid and yolk sac membrane of the eggs showed no viral activity when evaluated with hemagglutination assay (HA) (Table 4.15) and RT-PCR (Plate 4.8).

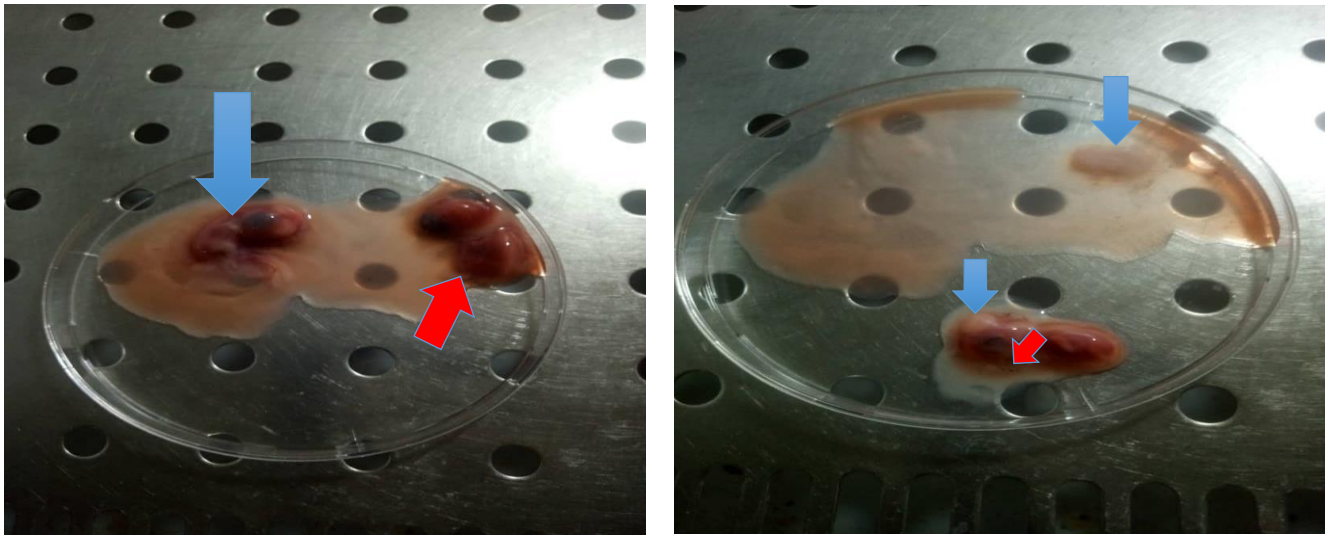
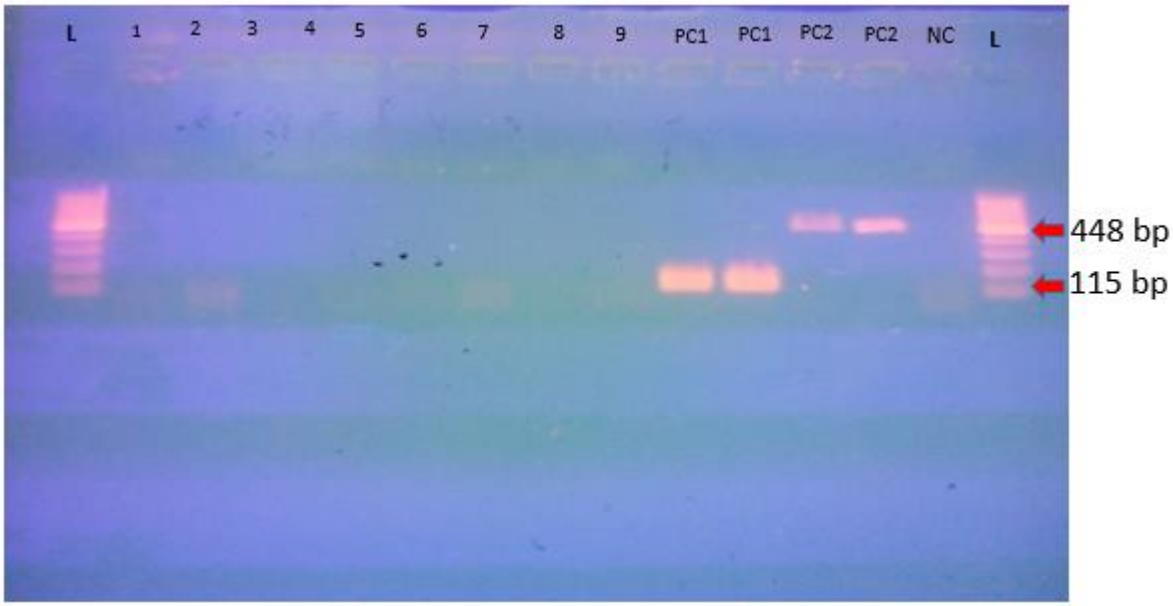


Plate 4.7: Harvested embryonic fluid and yolk sac after four passages

*There was cell death with areas of necrosis shown with the blue arrows. Haemorrhages (red arrow) in the embryonic fluid and the embryo

Table 4.15: Haemagglutination assay test result of the embryonic fluid and yolk sac of the Specific antibody negative-chicken embryonated eggs

Test	Results	Interpretation
Hemagglutination assay test	Negative	Absence of viral particles



PC: Positive control

NC: Negative control

L: Ladder

Plate 4.8: Gel image of the PCR result produced using the G-gene and N-gene specific primer on embryonic fluid and membrane of the SAN-Embryonated Eggs

4.5. Isolation and Identification of Bacteria Associated with Avian Metapneumovirus Infection in the Three States in Nigeria

The microbial analysis on samples collected in the flocks with respiratory disease revealed bacteria presence as presented in Figure 4.7 with their respective occurence. *Escherichia coli* had the highest prevalence of 52.38% (22 out of 42), *Aeromonas hydrophilia* 11.91% (5/42), *Pseudomonas aeruginosa* 7.14% (3/42), *Citrobacter freudi* was 4.76% (3/42), *Citrobacter youngae* 4.76% (2/42), *Procteus mirabilis* 4.76% (2/42), *Vibrio cholera* 4.76% (2/42), and *Procteus vulgaris* 2.38% (1/42), *Klebsiella pneumonia* 2.38 % (1/42) and *Vibrio mimicus* 2.38% (1/42).

Considering the five flocks that were positive for aMPV, *Escherichia coli* had the highest occurrence of 60% (3/5) while *Pseudomonas aeruginosa* was 20% (1/5) and *Klebsiella pneumonia* 20% (1/5) (Figure 4.8). Plate 4.9 depicted E. *coli* growth on MacConkey and Eosin Methylene Blue (EMB) agars while Plate 4.10 shows *Pseudomonas aeruginosa* growth on trypticase soy agar.

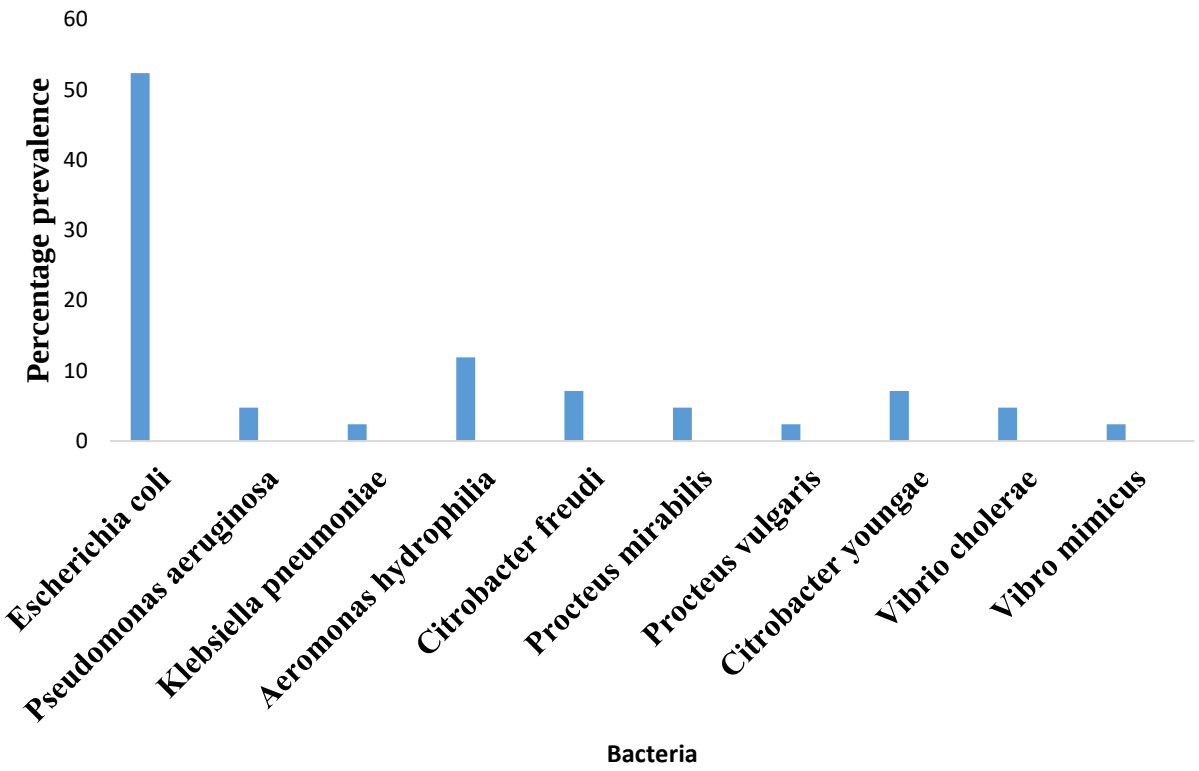


Figure 4.7: Bacteria isolated from cases of respiratory diseases presented at Veterinary Teaching Hospitals and Veterinary Clinics during harmattan in the three climatic zones

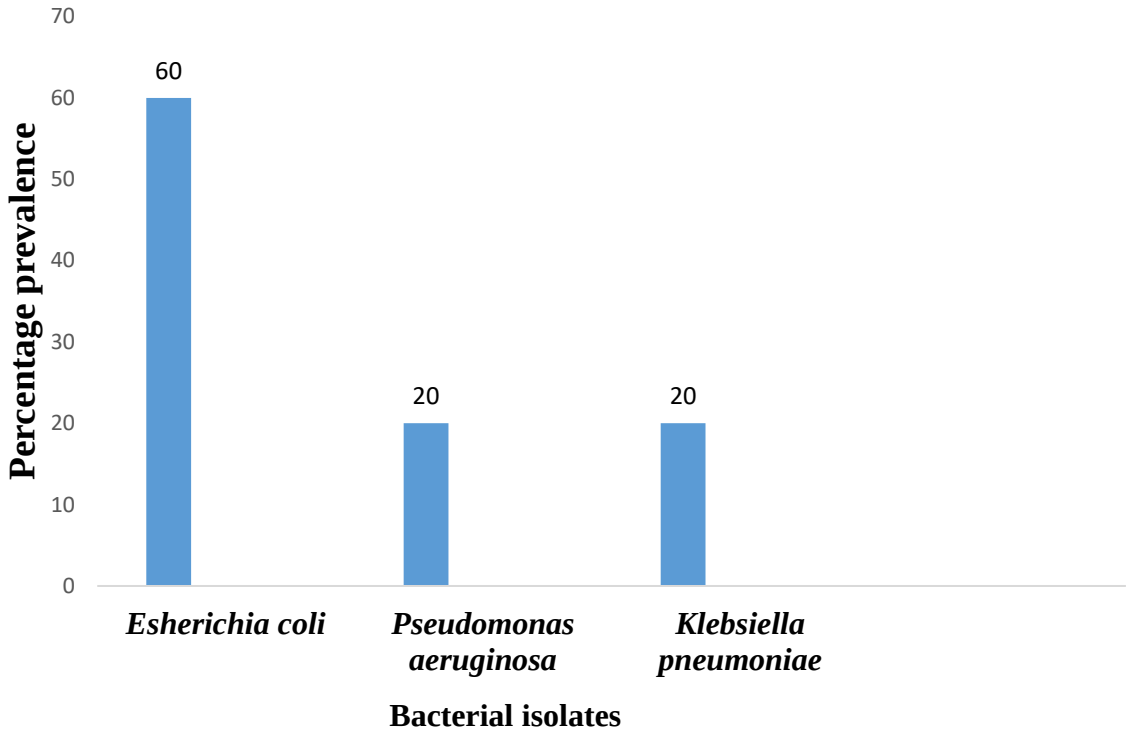


Figure 4.8: Bacteria isolated from flocks of chickens that were positive for Avian metapneumovirus infection

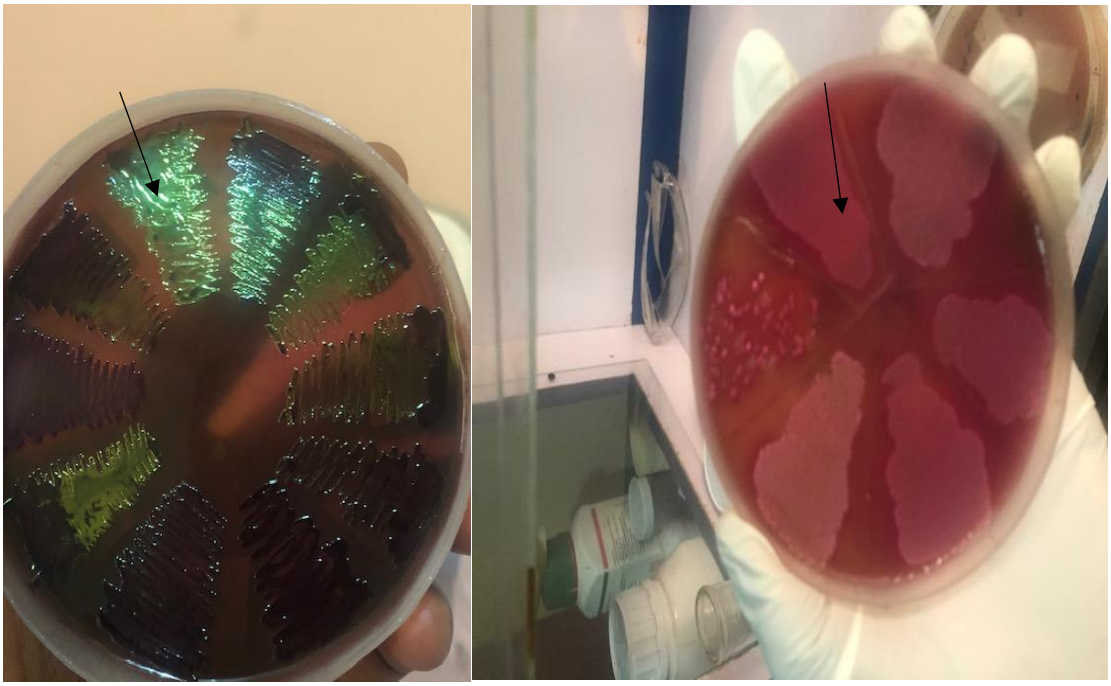


Plate 4.9: Growth of *Escherichia coli* isolated from avian metapneumovirus positive cases is indicated with black arrow on MacConkey (Right) and Eosin Methylene Blue agar (Left)

Plate 4.10: Growth of *Pseudomonas aeruginosa* isolated from avian metapneumovirus positive cases is indicated with black arrow on Trypticase soy

CHAPTER FIVE

DISCUSSION

Positive serological results in unvaccinated flocks are conclusive indication that the birds were exposed to the infectious agent under review (Xu *et al.,* 2021). As observed in the present study and generally in Nigeria, vaccination against aMPV has not been incorporated into the routine vaccination in commercial poultry farming.

5.1 Seroprevalence of Avian Metapneumovirus in Commercial Chickens from Three Climatic Zones in Nigeria

The existence of antibodies to aMPV was considered evidence of spontaneous infection. The findings of an overall seroprevalence of 59.79% for avian metapneumovirus in the three climatic zones evaluated in Nigeria are in consistent with earlier reports on the disease. In Southwestern Nigeria, Owoade *et al*. (2006) reported a prevalence of 40%. This implies that aMPV is widespread in Nigeria. Similarly, a seroprevalence of 56.4% for aMPV was reported in Poland, (Minta *et al*., 1995), 34.02% in India (Eswaran *et al*., 2014), 48% in both Iran (Rahimi, 2011) and Jordan (Gharaibeh and Agharaibeh, 2007) and 53.29% in Bangladesh (Ali *et al*., 2019). In addition, a seasonal pattern of aMPV infection was observed in this study with a higher seroprevalence rate 68.3% recorded during the dry season with mean antibody titre of 2990.9 ± 231.6 when compared with the wet season having 51.3% with mean antibody titre of 572.9 ± 64.1. This indicates that aMPV circulated more in the study areas during the dry season, when the transmission of respiratory disease by aerosol is believed to be more frequent in poultry flocks. In Nigeria, Olarenwaju *et al*. (2015) and Ali *et al*. (2019) previously found that infectious bronchitis, Newcastle disease, and avian metapneumovirus are more prevalent during the dry season (November to March) than during the rainy season (June to September). It has also

been proposed that wind velocity and the amount of dust in the air may influence the transmission of airborne diseases, with cold harmattan potentially worsening the outcome (Abdu *et al*., 1992; Sa'idu *et al*., 2006). In Bangladesh, the winter season (December to March) also saw a greater seroprevalence of respiratory illnesses according to Ali *et al*. (2019). Birds of prey have also been identified as aMPV carriers in addition to aerosol transmission, hence the seasonality of aMPV epidemic may be related to the seasonal migratory pattern of these wild species (Shin *et al.,* 2000). Wild birds such as geese, ducks and swans migrate to escape harsh winters in places such as Europe to a more favorable climate in Nigeria, which is located in a tropical humid region with abundant wetlands, providing an ideal environment for the life of wild bird (Mesenko *et al*., 2018). Most respiratory viruses spread because of the migration of these birds and their interaction with resident birds and poultry (Kaplan and Webby, 2013). For example, in 2009, hunters caught a migratory raptor from Finland with the ring tag number 258211 in a wetland environment in Taraba State, and pathological and virological examination revealed that it was positive for Newcastle disease, which is related to avian metapneumovirus in both the virus family and clinical manifestations (Suarez, 2020).

A comparison of the three climatic zones, depicted by the three states under study during dry season showed that Plateau State had the highest seroprevalence of 100%, while Sokoto State had 56.2% and Oyo State had 48.8% with mean antibody titers of 4757.9 ± 223.5, 2800.9 ± 313.1 and 1414.0 ± 158.1, respectively. This indicates that the weather in Plateau State is conducive to the growth and spread of avian metapneumovirus, presumably as a result of the dry harmattan wind and the generally low temperatures associated with its near-temperate climatic zone (Al-Ankari *et al.,* 2001; Sai'du *et al.,* 2006). The temperature of Plateau State (13 ^{o}C-22 ^{o}C) due to high altitude of 1,829 metres above sea level is close to winter temperature obtainable in temperate regions (Brousse *et al*., 2019).

It is important to remember that seasonality and occurrence of many infectious diseases are controlled by climate changes (Tiruneh and Tegene, 2018) such that they can abruptly alter or drift their usual trends of occurrence over time (Ricardo-Izurieta and Clem, 2008; Shima *et al*., 2015a).

During the wet season, Sokoto State recorded the highest prevalence of 65%, followed by Plateau State with 52.5% and Oyo State with 36.2% with mean antibody titers of 670.7 ± 74.9, 548.8 ± 61.4, and 499.4 ± 55.8 respectively. Additionally, during the rainy season, Plateau State's average monthly temperature (18–22^{o} C) is lower than that of Oyo States (24–25^{o} C) and Sokoto State (26–28^{o} C) (Climate-Data.org, 2020) Nonetheless, Sokoto State has the lowest monthly average humidity (53-68%) compared to the States of the Plateau (60-75%) and Oyo (85-90%). This could be the cause of increased seroprevalence of aMPV in Sokoto State during the wet season. Olarenwaju *et al*. (2015) have reported by that low humidity and higher temperature play a key role in the spread of airborne diseases.

5.2 Detection and Molecular Characterisation of Avian Metapneumovirus in Three Climatic Zones in Nigeria

All 5 samples that were positive for aMPV 11.91% (5/42) were detected in Plateau State, which lies in the near temperate climatic zone. It can be inferred from this study that the weather in Plateau State during the harmattan period supports the proliferation of aMPV due to its extreme coldness which could be as low as 4^{o}C. The study lends credence to the results of the sero-prevalence aspect of this study.

The aMPV isolates that were sequenced showed 96.4-97.3% similarity to European isolates, especially the Hungary isolate with accession number MN729604.1 (Goraichuk *et al.*, 2020), Attachment protein (G) mRNA complete Cds strain (2119) from Turkey rhinotracheitis with accession number AB548428.1 in Japan (Sugiyama *et al.,* 2010) and the Russian isolate with accession number JN651915.1 (Giovanardi, 2014) present in the GenBank, all of which were identified as Subtype B. This subtype has shown a larger tissue diffusion pattern, higher pathogenicity, and longer tenacity than Subtype A (Franzo *et al*., 2020). Al-Shekaili *et al*. (2015) had also reported its occurrence in backyard flocks in Oman, laying chicken flocks in Brazil (Chacon *et al*., 2011), Italian broiler farms (Tucciarone *et al.,* 2018b), turkey farm in Iran (Mayahi *et al*., 2017) and in Greece (Tucciarone *et al.,* 2017; Andreopoulou *et al*., 2019). According to *Franzo et al*. (2020), this subtype B is the predominant subgroup in Europe. However, a previous investigation by Owoade *et al*. (2008) revealed that both subtypes A and B were present in asymptomatic flocks of commercial chicken in the southwest region of Nigeria.

The incursion of aMPV subtype B into the Nigerian poultry industry could be related to the practice of importation of chickens from Europea and Asia (Meseko *et al*., 2018; FAO, 2019). In their investigation, Owoade *et al*. (2008) similarly came to the conclusion that aMPV subtype B might have originated from wild and feral birds, which have been blamed for the spread of aMPV.

Considering the sequence of aMPV subtype B-G attachment protein in this study, substitution A8S present at position 8 is consistent with the other subtypes A and B previously reported in Nigeria (Owoade *et al*., 2008). This isolate has a continuous presence of serine residue at position 8, which is only found in Nigerian aMPV viruses. This shows that an unnamed serine 8 viral reservoir served as the direct parent of all Nigerian isolates discovered thus far. Arginine and tyrosine are also present at positions 183 and 224, respectively, in the current isolate and the previously identified Nigerian isolate B (Owoade *et al*., 2008) which shows similarity between the strains. Other non-synonymous mutations are T12I, G223E and A238V substitutions at positions 12, 223 and 238, respectively, which occurred only in the isolate detected in this study have not been previously reported. According to Cecchinato *et al*. (2010) the G- attachment glycoprotein is the most variable of the metapneumovirus proteins and therefore prone to a lot of mutations in its amino acids. The unique substitutions detected in the aMPV in this study could pose challenge to the development of vaccine against aMPV infections as have been observed in other RNA viruses affecting the respiratory system of birds such as, avian influenza and infectious bronchitis viruses. This must therefore be considered in the development of vaccines against aMPV in Nigeria.

As previously reported by Bayon Auboyer (1999) in the identification of turkey rhinotracheitis virus (aMPV) in swabs, the findings of this investigation show that the RT-PCR technique can find aMPV in swab samples without the requirement for prior proliferation of this virus.

5.3 Clinical presentation and Epidemiological Factors Associated with Avian metapneumovirus Infections in Nigeria

aMPV was only found in the conjunctiva and turbinate taking into account the clinical manifestations and epidemiological aspects linked to the epidemic of respiratory disease in this study. This supports the previous assertions made by Umar *et al.* (2016) and Franzo *et al*. (2020) that it is an upper respiratory tract disease and supports the

finding of Hosseini and Ghalyanchi-Langeroudi (2012) that turbinates and conjunctiva are the most suitable sources of aMPV in chickens. Similarly, to this, Catelli *et al*. (1998); Cook (2000) and Gharaibeh and Sharmoun, (2012) confirmed that the turbinate contained the highest viral load.

The cases of respiratory disease outbreaks that were presented in the VTHs and Clinics during this study covered two basic chicken types (Table 4.2.3); however, the five isolates of aMPV were from only the layer flocks. Chacón *et al*. (2007) detected and isolated aMPV from laying hens alone in Brazil. Similarly in Mexico, Rivera-Benitez *et al.* (2014) detected aMPV from five pooled samples from pullets. In contrast, Tucciarone *et al*. (2018b) detected avian metapneumovirus mainly in broilers when compared to other breeds of chickens in Italy.

The results showed that chickens of all age groups were affected, although, higher rate (40%) of occurrence was recorded in those above 31 weeks of age. Jones (2010) had earlier inferred that aMPV infects all ages of layers and could be detected at the onset of egg production with higher prevalence around the peak of egg production when its effects will be shown on both respiratory and reproductory performances. Similarly, other researchers, (Rivera-Bernitez *et al*., 2014; Nguyen *et al*., 2021) reported incidences of aMPV in all ages of chicken. It is worthy of note that, all flocks associated with aMPV infection in this study, practice intensive management system which aids disease transmission via the oral route. Poultry farms in North Central Nigeria are mostly operated on the intensive management system (Pousga *et al*., 2018).

The positive chicken flocks in this study showed mortality rates that ranged from 2% to 50%. This is in agreement with the report of Falchieri (2016) that avian metapneumovirus causes mortality of 2-50% in affected flocks. However, Bao *et al*. (2020) affirmed that uncomplicated cases have low mortality of 2–5% and 100% morbidity However, mortality rates for infections with associated microbial infections could reach 25%.

The clinical signs presented were by flocks that were positive for aMPV were acute and severe respiratory signs and included rales and coughing (80%), sneezing (80%), dyspnea (40%), swollen infraorbital sinus (60%), anorexia (60%), mucoid nasal discharges (60%), stunted growth (40%), torticollis (60%) and purulent ocular discharge (40%). This is similar to the observations of Umar *et al*. (2016) who explained that swollen head syndrome (i.e., aMPV infection in chicken) is characterized by coughing, sneezing, trachea rales, sneezing, nasal and ocular

discharges. In a recent research, Umar *et al*. (2019) explained that aMPV infection does not only show respiratory signs, it also causes head swelling resulting in opisthotonos, disorientation and torticollis, which are all examples of neurological disorder. Similarly, Gough *et al*. (2016) affirmed that torticollis and opisthotonos are major clinical signs of aMPV.

In this investigation, flocks that had been exposed to aMPV showed decreased egg production and the development of shell less eggs. This conclusion agrees with Hassan and Abdul-careem (2020) who classified aMPV as one of the viruses causing decreased egg production and the creation of shell-less eggs in chickens and turkeys. Similarly, to this, an aMPV strain (PLE8T1) derived from swollen head syndrome has been used in an experimental investigation to demonstrate a decrease in egg production in hens (Sugiyama *et al.,* 2006). Furthermore, aMPV infection in hens had been linked to poor egg quality (Wei *et al*., 2013; Ali *et al*., 2019). The gross findings in cases that were positive for aMPV in this study i.e., consolidated lungs with frothy exudates, haemorrhagic tracheitis and sometimes mucoid exudate and caseous plugs in trachea and cloudy air sacs are similar to those reported by Homayounfar *et al*. (2015). In addition to these, swelling of the infraorbital sinus, watery to mucoid exudate in the upper respiratory system, oedema and conjunctivitis have also been reported by Ali *et al*. (2019).

Other postmortem findings from the aMPV positive cases included haemorrhages in proventricular mucosa and colorectal mucosae, mucoid caecal content, eroded periventricular mucosa with area of suffusion, haemorrhages in the ileocecal junction, and congested and/or atrophic spleen and urinary system (20%) with nephritis, congested kidneys and distended ureters. According to Aung (2008), aMPV infection affects other organs like the Harderian gland, kidney, spleen, cecal tonsils and bursa of Fabricius, apart from the respiratory system. In as much as these lesions could be an extension of aMPV infection of the respiratory tract, they could also be indications of other diseases whose investigation is beyond the scope of this study. The reproductive system showed mishapen, congested, pedunculated, and occasionally regressed ovarian follicles, thin-shelled eggs in the oviduct. Previous studies by Villarreal *et al*. (2007) and Choi *et al.* (2010) described folded shell membrane, prolapsed oviducts, and egg yolk peritonitis in breeders with aMPV infection.

Since aMPV has been shown to suppress the immune system of chickens, favouring secondary bacterial infections, clinical signs and gross lesions showing involvement

beyond the upper respiratory system as well as mortality rates as high as 50% in aMPV positive cases reported in this study may be related to complications with other secondary pathogens (Legnardi *et al*., 2021; Smialek *et al*., 2021a). In many investigations, efforts have been made to identify the immunosuppression processes caused by the virus. The bovine respiratory syncytial virus and the human respiratory syncytial virus are two paramyxoviruses that also cause T cells to exhibit mitogenic inhibition (Woldehiwet and Sharma, 1992). Concanavalin A, a plant mitogen that can stimulate the Mouse T-cell subset, has been demonstrated to stimulate T cells, but a European strain of aMPV has also been shown to inhibit the proliferative response, demonstrating the immunosuppressive character of the virus (Chary *et al*., 2002b). Recently, Kaboudi and Lachheb, (2021) have shown a reduced weight of the thymus in birds infected with aMPV.

This study has demonstrated that many farmers utilize antibiotics to lessen the severity of clinical disease and mortality in the absence of systematic vaccination against illnesses brought on by aMPV infection. This study showed that the most commonly used antibiotics in the outbreaks were tylosin (71.23%), doxycline (66.7%) and enrofloxacin (59.5%) as reported by respondent farmers. These antibiotics were used indiscriminately without an attempt at bacterial isolation or sensitivity test for antibiotics. Several authors have worked extensively on the use of antibiotics among poultry farmers and have established that many are multi-drug users (Adelowo *et al*., 2009; Awogbemi *et al*., 2018).

Indiscriminate use of antibiotics as observed in this study is a major contributory factor to antimicrobial resistance and should be discouraged. An essential tool in this framework is efficient vaccinations, which serve to reduce or avoid animal susceptibility to illnesses and their effects (Rodrigues *et al*., 2020). The records from the flocks that tested positive for aMPV revealed that all bird had received vaccinations against poultry pox, infectious bursal disease, infectious bronchitis, Newcastle disease, and egg drop syndrome but not for aMPV.

5.4 Isolation and Identification of avian metapneumovirus

In this study, although cell death was detected after 48 hours in each serial passage in the embryonated eggs, no viral activity was observed from the embryonic fluid and yolk sac. The absence of positive result in the embryonated eggs could be due to the live virus in the field samples being inactivated. Shin *et al*. (2000) reported that unless

the samples were collected at the right time, there is always extreme difficulty in aMPV isolation, since most conventional methods used have proved unsuccessful due to poor replication of the virus in chicken embryos rather than turkey embryos. However, SPF chicken and turkey embryonated eggs are not readily available in Nigeria. Similarly, Nagy *et al*. (2018) stated that aMPV isolation is extremely complicated because the virus is evident in tissues and excretions for a very brief duration after infection, and because establishing the emergence of infection is difficult, the time to sample collection in a field outbreak will also be difficult.

Ongor *et al.* (2010) questioned the suitability of Vero cells and chicken embryo fibroblast for aMPV isolation because he found no positive results after four blind passages of RT-PCR positive samples on Vero cells and chicken embryo fibroblast, which are the best methods for viral isolation. Also, Chacón *et al*. (2007) detected that aMPV nucleic acid was not detected after the cytopathic effect was observed in the third passage in embryonated eggs in samples collected in Brazil. In contrast to these, however, Kwon *et al*. (2010) reported isolation of aMPV from samples from Vero cells that were positive by real- time PCR. Although unsuccessful, this study is the first reported attempt to isolate aMPV from field samples in Nigeria. According to Gough (2003), isolating aMPV in embryonating eggs is a slow, costly, and labour-intensive process that necessitates multiple subsequent cell culture passages for classification. In order to successfully isolate aMPV in Nigeria and produce vaccines, proper timing of sample collection post-infection is required.

5.5 Isolation and Identification of Bacteria Associated with Avian Metapneumovirus Infection in the Three States in Nigeria

It could be inferred from the microbial analysis of post-mortem samples collected from cases of respiratory diseases presented at the VTHs and Clinics that, *E. coli,* was the most prevalent (52.38%) bacteria in this study, followed by *Aeromonas. hydrophilia (A. hydrophilia)* being 11.91% and each of *Pseudomonas. aeruginosa (P. aeruginosa)* and *Citrobacter. Freudi (C. freudi)* being 7.14%. *E. coli* is the most frequently identified secondary bacteria organism in respiratory disease, according to Kahn (2010), and it can survive for a long time in harmattan. *A. hydrophilia and P. aeruginosa* are prevalent avian infections in the upper respiratory tract, which begins when waste and feed are contaminated as a result of poor environmental/managemental conditions (Kebede, 2010; Sirdar *et al*., 2012). Kebede

(2010) further observed in his study that *P. aeruginosa* is an opportunistic organism that produces respiratory disease in susceptible birds and also causes low egg production in layer birds, although it is not so commonly reported. Infections can occur through skin wounds, adulterated vaccines and antibiotic solutions, or injection needles. The disease can be systemic, affecting multiple organs and tissues, or localized, affecting only the infraorbital sinus or air sacs (Wei *et al*., 2013), causing swelling of the head, wattles, sinuses and joints in poultry birds as seen in the present study.

In samples from MPV positive flocks, it was also observed that *E. coli* was the predominant (60%) opportunistic bacteria followed by *P. aeruginosa* (20%) and *K. pneumonia* processes (20%). This investigation indicates that *E. coli*, *P. aeruginosa* and *K. pneumonia* were the secondary bacterial isolates associated with aMPV infection in this study. Other researchers have reported that aMPV infection is usually aggravated by the existence of *E. coli* and other bacteria organisms (Naqi *et al.,* 2001; Matthijs *et al.,* 2003; Landman and Feberwee, 2004; Jirjis *et al.,* 2009; Giovarnardi *et al*., 2014; Seifi and Boroomand, 2015). This study also corroborates the study of Abdelmoez *et al*. (2019) who also isolated *E. coli* and *P. aeruginosa* from aMPV infected broiler chickens in Egypt. However, other researchers reported other secondary bacteria. Gough (2003) and Marien (2005), for example, reported that aMPV is associated with secondary agents like *Mycoplasma spp*, *Bordetella avium*, and *Ornitobacterium rhinotracheale*. Furthermore, Ongor *et al*. (2015) isolated *Mycoplasma species* alone in the trachea of turkeys infected with aMPV in a flock in California.

Clinical indications are worsened when aMPV and *E. coli* co-infect, showing that the two organisms have harmonious effects on clinical pictures in domesticated birds. (Kwon *et al.,* 2010).

CHAPTER SIX

SUMMARY, CONCLUSION AND RECOMMENDATIONS

6.1 Summary

The high seroprevalence of aMPV recorded in commercial chickens in this investigation despite the absence of vaccination against infections shows that commercial chickens are constantly vulnerable to the virus in Nigeria. Seroprevalence was higher in the dry season than in the wet season, and Plateau State with its near-temperate climatic zone had the highest seroprevalence in the dry season. During the wet season, seroprevalence was highest in Sokoto State due to its low humidity compared to Plateau and Oyo States. The geographical/climatic distribution of infection shows that low ambient temperature and relative humidity are necessary factors in the spread of aMPV and seasonal variation. Bacterial co-infection with aMPV cause morbidity, mortality and low egg production in commercial layers thereby inflicting immense economic impact to the poultry industry in Nigeria.

Avian metapneumovirus Serotype B was detected in Plateau State only, in 5-layer flocks of chickens on intensive management system. The clinical signs and postmortem lesions observed in the aMPV infected flocks were as previously recorded for other respiratory diseases and the virus was detected only in the tissues of the turbinate and conjunctiva. Secondary bacterial organisms isolated from aMPV positive cases were *E coli, P. aeruginosa and K. pneumoniae* and farmers were found to use antibiotics indiscriminately without attempt at bacterial isolation and antibiotic sensitivity test.

The aMPV detected in this study is 96-97% related to the European and Asian strains of subtype B aMPV. The sequence of aMPV subtype B-G attachment protein has various mutations in its amino acid with substitution A8S present at position 8 showing consistency with the other subtypes A and B previously reported in Nigeria. Also, Arginine and Tyrosine present at positions 183 and 224, respectively, in the present isolate is as in the previously detected isolate B from Nigeria. Unique to this isolate are

substituted at positions T12I, G223E and A238V which must be considered in the development of vaccines against aMPV in Nigeria.

6.2 Conclusion

An overall high seroprevalence (59.79%) of Avian metapneumovirus (aMPV) infection was obtained in the study locations with Plateau State having the highest seroprevalence (100%) during dry season. There was a seasonal pattern of aMPV occurrence, with higher seroprevalence in the dry season than in the wet season.

All aMPV-positive samples were found only in Plateau State, which has a near-temperate climate. This study found avian metapneumovirus subtype-B with new mutations in its G-gene, genetically identical to some European and Asian strains, in chickens with respiratory disease.

Infection with avian metapneumovirus occurred more frequently in layers with no discernible age distribution. Only the turbinates and conjunctivae were found to be virally infected, implying that these are the best tissues for disease diagnosis. aMPV growth was not supported by embryonated chicken eggs. *E. coli, P. aeruginosa* and *K. pneumoniae* occurred concurrently with aMPV infection in this study.

6.3 Recommendations

Since aMPV is known to exhibit genetic diversity and resists most disinfection procedures, eradication is often difficult. In addition to the institution of good management practices on farms, the following recommendations are hereby proposed to control aMPV infections.

a. Further attempts should be made at isolating the virus using cell lines such chicken embryo fibroblast as previously reported (Coswig *et al*., 2010).
b. Continuous surveillance for early detection of aMPV to prevent outbreak of associated respiratory diseases in poultry and to recognise circulating strains at a time.
c. Bacterial organisms associated with outbreaks of diseases caused by aMPV (i.e., Turkey rhinotracheitis and Swollen head syndrome) must be isolated and antibiotic sensitivity test conducted for effective control.
d. In poultry with respiratory and reproductive diseases, avian metapneumovirus infection should be considered as a differential diagnosis.

e. Awareness of the existence of aMPV infection in the Nigerian poultry industry and its possible impact must be created among poultry veterinarians and farmers to mitigate potential losses.

6.4. Contributions to knowledge

This research has contributed substantially to clinical and scientific knowledge as follows:

1. This is the first study to establish the seroprevalence of avian metapneumovirus in apparently healthy chickens in Nigeria that involves three different climatic zones i.e., the cold, temperate-like climate of Plateau State, the rainforest of Oyo State and the semi-arid climate of Sokoto State.
2. The possible role of season as an influence in the transmission of aMPV infection was demonstrated.
3. This research is the first confirmation of aMPV in chickens with respiratory disease in Nigeria.
4. Avian metapneumovirus has a predilection for the turbinate and conjunctiva.
5. The disease was more common in layers without a specific age predisposition in Nigeria.
6. The aMPV currently circulating in Nigeria is the subtype B strain with Accession number MZ408311which is genetically related to the European and Asian strains
7. Documented the unique non-synonymous substitutions seen at positions T12I, G223E, and A238V in the G-gene of aMPV subtype B.
8. *Escherichia coli*, *Pseudomonas aeruginosa* and *Klebsiella pneumonia* co-infection with aMPV in commercial layers.

6.5. Further Research

Few aMPV sequences from Nigeria are available in the GenBank as such, more researches are needed to fully explain epidemiological trends of circulating strains. It is also important to develop suitable methods of aMPV isolation with concurrent sequencing of the virus. This will probably give new insights into virus-host-environment interactions. Furthermore, it is important to analyse the aMPV genome of human and animal (including birds) origin to assess relatedness and transmission between species.

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APPENDICES

Appendix I: Ethical approval for the research

UNIVERSITY OF IBADAN
ANIMAL CARE AND USE RESEARCH ETHICS COMMITTEE
(UI-ACUREC)

E.mail: animaluseresearch@gmail.com/animaluseresearch@yahoo.com

Our Ref: ..... 08176917269 Date: 4-6-2018

Assigned number: UI-ACUREC/17/0091

NOTICE OF FULL APPROVAL AFTER FULL COMMITTEE REVIEW

Re: Clinico-Pathological and Molecular Characterization of Avian Pneumovirus Strains from Different Climatic Regions in Nigeria

Name of Principal Investigator: Bakre Adetolase Azizat

Address of Principal Investigator: Department of Veterinary Medicine, Faculty of Veterinary Medicine, University of Ibadan, Ibadan

Date of receipt of valid application: 26/9/2017

Date of meeting when final determination on ethical approval was made: **16/5/2018**

This is to inform you that the research described in the submitted protocol, have been reviewed and *given full approval by the* UI-ACUREC.

This approval dates from **16/5/2018 to 15/5/2019.** If there is delay in starting the research, please inform U1-ACUREC so that the dates of approval can be adjusted accordingly.
Note that no activity related to this research may be conducted outside of these dates. It is expected that you submit your annual report as well as an annual request for the project renewal to the UI-ACUREC at least four weeks before the expiration of this approval in order to avoid disruption of your research.

The National Code for Health Research Ethics requires you to comply with all institutional guidelines, rules and regulations and with the tenet of the Code including ensuring that all adverse events are reported promptly to the UI-ACUREC. *No changes are permitted in the research without prior approval by the UI-ACUREC except in circumstances outlined in the code.* The UI-ACUREC *reserves the right to conduct compliance visit to your research site without previous notification*

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.....
Prof. S.I.B. Cadmus
Chairman, UI-ACUREC

Chairman: Professor S. I. B. Cadmus (DVM, Ph.D)
Department of Veterinary Public Health and Preventive Medicine, Faculty of Veterinary Medicine, University of Ibadan, Nigeria

APPENDIX II: DISTRIBUTION OF SERUM SAMPLES COLLECTED FROM PLATEAU, OYO AND SOKOTO STATE DURING WET SEASON.

PLATEAU STATE

Plateau north senatorial district

Jos North local government

Sample A: 30 weeks

Sample B: 40 weeks

Sample C: 50 weeks

Plateau south senatorial district

Langtang north

Sample D: 26 weeks

Sample E: 30 weeks

Plateau central senatorial district

Mangu local government

Sample F: 30 weeks

Sample G: 32 weeks

Sample H: 7 weeks broilers

SAMPLE	S/P RATIO	ELISA ANTIBODY	IMMUNE STATUS
F1	0.141	270.3	NEG
F2	0.305	630.9	POS
F3	0.500	1071.5	POS
F4	0.140	269.2	NEG
F5	1.182	288.4	NEG
F6	0.278	575.4	NEG
F7	0.126	251.2	NEG
F8	0.432	683.9	POS
F9	0.298	610.9	POS
F10	0.308	635.3	POS
A1	0.433	912.0	POS
A2	0.138	263.0	NEG
A3	0.330	683.9	POS
A4	0.138	263.0	NEG
A5	0.330	683.9	POS
A6	0.450	954.9	POS
A7	0.147	281.3	NEG
A8	0.135	258.2	NEG
A9	0.143	269.2	NEG
A10	0.147	281.8	NEG
B1	0.145	281.4	NEG
B2	0.351	724.4	POS
B3	0.175	338.8	NEG
B4	0.442	933.3	POS
B5	0.450	955	POS
B6	0.145	281.8	NEG
B7	0.138	263.0	NEG
B8	1.361	338.8	NEG
B9	3.114	933.3	POS
B10	3.152	955	POS
C1	1.167	281.8	NEG
C2	1.122	263.0	NEG
C3	1.516	398.1	POS
C4	1.565	407.4	POS
C5	3.278	1023.3	POS
C6	2.314	660.7	POS
C7	1.397	354.8	NEG
C8	2.136	602.6	POS
C9	1.352	338.8	NEG
C10	2.247	645.7	POS
D1	1.085	247.7	POS
D2	1.135	269.2	NEG
D3	1.154	602.6	POS
D4	1.352	338.8	NEG
D5	2.247	645.7	NEG
D6	1.094	251.2	NEG
D7	1.132	263.0	NEG
D8	1.112	257.04	NEG

D9	1.298	239.9	NEG
D10	1.102	258.2	NEG
E1	1.152	269.15	NEG
E2	1.177	281.8	NEG
E3	3.139	912.0	POS
E4	1.168	279.8	NEG
E5	2.509	724.4	POS
E6	1.076	258.2	NEG
E7	1.102	248.2	NEG
E8	3.139	954.9	POS
E9	1.178	281.3	NEG
E10	2.225	635.3	POS
G1	3.039	912.0	POS
G2	2.038	562.3	POS
G3	1.122	263.0	NEG
G4	2.353	691.8	POS
G5	1.120	263.0	NEG
G6	3.170	954.9	POS
G7	1.144	275.4	NEG
G8	1.178	281.8	NEG
G9	3.139	954.9	POS
G10	3.203	977.2	POS
H1	2.509	724	POS
H2	3.039	912.0	POS
H3	3.139	954.9	POS
H4	1.178	281.8	NEG
H5	2.038	562.3	NEG
H6	2.247	645.7	POS
H7	3.278	1023.3	POS
H8	3.114	933.3	POS
H9	3.152	955	POS
H10	2.313	660.7	POS

SOKOTO STATE

Sokoto East Senatorial District

Rabah Local Government

Sample I: 25 weeks Layers

Sample J: 30 weeks Layer

Sample K: 35 weeks Layer

Sokoto South Senatorial District

Boringa Local Government

Sample L: 20 weeks Layer

Sample M: 6 weeks Broiler

Sample N: 25 weeks Layers

Sokoto North Senatorial District

Wamakko Local Government

Sample O: 20 weeks Layers

Sample P: 30 weeks Layers

Sample Q: 6 weeks Broiler

SAMPLE	S/P RATIO	ELISA ANTIBODY TITRE	IMMUNE STATUS
I1	0.366	765.6	POS
I2	0.4551	954.9	POS
I3	0.584	1,288.3	POS
I4	1.820	794.3	POS
I5	0.327	677.6	POS
I6	0.139	269.0	NEG
I7	0.012	18.45	NEG
I8	0.239	591.6	POS
I9	0.359	749.9	POS
I10	0.223	446.6	POS
J1	0.161	312.6	NEG
J2	0.078	142.2	NEG
J3	0.314	648.6	POS
J4	0.181	354.81	NEG
J5	0.272	553.4	POS
J6	0.127	234.0	NEG
J7	0.221	441.6	POS
J8	0.163	316.0	NEG
J9	0.271	550.8	POS
J10	0.162	315.0	NEG
K1	0.267	543.3	POS
K2	0.434	920.0	POS
K3	0.251	502.3	POS
K4	0.021	33.65	NEG
K5	0.205	407.4	POS
K6	0.074	134.0	NEG
K7	0.212	421.7	POS
K8	0.145	279.3	NEG
K9	0.290	594.3	POS
K10	0.178	348.0	NEG
L1	0.027	43.75	NEG
L2	0.470	1,006.9	POS
L3	0.062	110.0	NEG
L4	0.470	1,006.93	POS
L5	0.142	272.9	NEG
L6	0.088	161.8	NEG
L7	0.127	241.6	NEG
L8	0.523	1129.8	POS
L9	0.153	295.8	NEG
L10	0.069	124.2	NEG
M1	0.099	184.1	NEG
M2	0.250	505.8	POS
M3	0.542	1,174.9	POS
M4	0.157	304.8	NEG
M5	0.003	3.192	NEG
M6	0.166	323.6	NEG

M7	0.044	76.0	NEG
M8	0.538	1,166.0	POS
M9	0.653	1,438.8	POS
M10	0.654	1,442.1	POS
N1	0.687	1,520.5	POS
N2	0.288	588.8	POS
N3	0.439	933.3	POS
N4	0.182	357.3	NEG
N5	0.317	654.6	POS
N6	0.298	612.4	POS
N7	0.592	1,291.2	POS
N8	0.544	1,180.3	POS
N9	0.669	1,475.7	POS
N10	0.044	76.03	NEG
O1	0.279	586.1	POS
O2	0.592	1,291.2	POS
O3	0.544	1,180.3	POS
O4	0.669	1,475.7	POS
O5	0.044	76.03	NEG
O6	0.434	928.4	POS
O7	0.581	1,267.7	POS
O8	0.542	1,096.5	POS
O9	0.503	1,083.9	POS
O10	0.199	394.5	POS
Q1	0.544	1,180.3	POS
Q2	0.669	1,475.7	POS
Q3	0.279	586.1	POS
Q4	0.556	1,207.8	POS
Q5	0.439	933.3	POS
Q6	0.182	357.3	NEG
Q7	0.317	654.6	POS
Q8	0.298	612.4	POS
Q9	0.687	1, 520.5	POS
Q10	0.653	1,438.8	POS

OYO STATE

Oyo South Senatorial District

Ibarapa Central Local Government

Farm R: 30 weeks Layer birds

Farm S: 45 weeks Layer birds

Farm T: 23 weeks Layer bird

Oyo Central Senatorial District

Egbeda Local Government

Farm U: 20 weeks Layer bird

Farm V: 30 weeks layer bird

Farm W: 30 weeks layer bird

Oyo North Senatorial District

Iseyin Local Government

Farm X: 30 weeks Layer bird

Farm Y: 20 weeks Layer bird

SAMPLE	S/P RATION	ELISA ANTIBODY TITRE	IMMUNE STATUS
R1	0.0572	100.0	NEG
R2	0.0510	89.50	NEG
R3	0.0405	69.18	NEG
R4	-0.046	78.80	NEG
R5	0.058	102.3	NEG
R6	0.055	97.05	NEG
R7	0.227	169.8	NEG
R8	0.046	79.80	NEG
R9	0.0085	12.70	NEG
R10	0.333	691.0	POS
S1	0.0465	80.70	NEG
S2	0.297	616.0	POS
S3	0.061	109.6	NEG
S4	0.0431	74.5	NEG
S5	-0.0363	61.65	NEG
S6	0.0546	96.60	NEG
S7	0.048	83.60	NEG
S8	-0.0513	89.90	NEG
S9	0.138	263.3	NEG
S10	0.140	54.95	NEG
T1	0.286	588.8	POS
T2	0.138	265.2	NEG
T3	0.293	602.6	POS
T4	0.141	271.02	NEG
T5	0.439	933.25	POS
T6	0.141	271.02	NEG
T7	0.164	316.2	NEG
T8	0.290	588.8	POS
T9	0.142	272.9	NEG
T10	0.122	229.1	NEG
U1	0.149	288.4	NEG
U2	0.461	984.0	POS
U3	0.154	295.1	NEG
U4	0.308	630.95	POS
U5	0.036	61.09	NEG
U6	0.029	47.09	NEG
U7	0.135	257.0	NEG
U8	0.460	9772	NEG
U9	0.289	588.8	POS
U10	0.294	630.9	POS
V1	0.460	977.2	POS
V2	0.1443	275.4	NEG
V3	0.300	630.9	POS
V4	0.140	269.2	NEG
V5	0.140	269.2	NEG

V6	0.289	630.9	POS
V7	0.443	933.3	POS
V8	0.134	257.0	NEG
V9	0.289	588.8	POS
V10	0.134	257.0	NEG
W1	0.140	269.2	NEG
W2	0.138	264.2	NEG
W3	0.286	588.8	POS
W4	0.293	602.6	POS
W5	0.141	271.02	NEG
W6	0.439	933.3	POS
W7	0.164	316.2	NEG
W8	0.142	272.9	NEG
W9	0.122	229.1	NEG
W10	0.149	288.4	NEG
X1	0.154	295.1	NEG
X2	0.308	631.0	POS
X3	0.036	61.09	NEG
X4	0.029	47.90	NEG
X5	0.460	977.2	POS
X6	0.289	588.8	POS
X7	0.294	630.9	POS
X8	0.460	977.2	POS
X9	0.144	275.4	NEG
X10	0.300	630.9	POS
Y1	0.140	269.2	NEG
Y2	0.289	630.9	POS
Y3	0.140	269.2	NEG
Y4	0.443	933.3	POS
Y5	0.134	257.0	NEG
Y6	0.141	270.3	NEG
Y7	0.289	630.9	POS
Y8	0.300	630.9	POS
Y9	0.308	631.0	POS
Y10	0.142	272.9	NEG

Appendix III: DISTRIBUTION OF SERUM SAMPLES COLLECTED FROM PLATEAU, OYO AND SOKOTO STATE DURING DRY SEASON

PLATEAU STATE

Jos North Local government

Sample A: 64 weeks

Sample B: 51 weeks

Sample C: 68 weeks

Plateau south

Langtang North

Sample D: 14 weeks

Sample E: 12 weeks

Plateau central

Mangu Local Government

Sample F: 45 weeks

Sample G: 25 weeks

Sample H: 6 weeks broiler

SAMPLE	S/P RATIO	ELISA ANTIBODY TITRE	IMMUNE STATUS
F1	0.662	1,462.2	POS
F2	1.188	2,760.6	POS
F3	1.153	2,673.0	POS
F4	2.189	5,370.3	POS
F5	2.145	5,260.2	POS
F6	2.532	6,123.5	POS
F7	2.100	5,140.4	POS
F8	1.776	2,301.5	POS
F9	1.061	2,454.7	POS
F10	2.560	6,382.6	POS
A1	2.455	6,095.4	POS
A2	2.951	7,447.3	POS
A3	2.927	7,379.0	POS
A4	2.551	6,353.3	POS
A5	1.415	3,341.95	POS
A6	2.086	5,105.1	POS
A7	2.399	5,861.4	POS
A8	2.127	5,942.9	POS
A9	1.175	2,728.9	POS
A10	2.259	5,571.9	POS
B1	3.138	7,961.6	POS
B2	2.808	7.063.2	POS
B3	2.596	6,486.3	POS
B4	2.511	6,251.7	POS
B5	2.633	6,576.6	POS
B6	2.586	6,441.7	POS
B7	2.430	6,025.6	POS
B8	3.098	7,852.4	POS
B9	2.626	6,561.5	POS
B10	2.833	7,128.5	POS
C1	2.611	6,561.3	POS
C2	1.831	4,425.9	POS
C3	2.633	6,531.3	POS
C4	2.573	6,412.1	POS
C5	2.455	6,095.4	POS
C6	3.018	7,638.4	POS
C7	2.927	7,379.0	POS
C8	2.440	6,053.4	POS
C9	2.210	5,432.0	POS
C10	2.277	5,623.4	POS
D1	1.970	4,797.3	POS
D2	2.401	5,942.9	POS
D3	1.838	4,466.8	POS
D4	2.218	5,457.6	POS
D5	0.230	461.31	POS

D6	1.330	3,162.4	POS
D7	2.165	5,321.1	POS
D8	1.716	4,130.5	POS
D9	1.643	3,981.1	POS
D10	2.560	6,382.6	POS
E1	2.053	5,011.9	POS
E2	0.789	1,766.0	POS
E3	0.258	523.6	POS
E4	1.367	3,221.1	POS
E5	2.535	6,309.6	POS
E6	2.400	5,942.9	POS
E7	2.501	6,223.0	POS
E8	2.040	4,977.4	POS
E9	2.316	5,714.8	POS
E10	2.635	6,591.7	POS
G1	0.661	1,458.8	POS
G2	1.046	2,437.8	POS
G3	1.121	2,754.2	POS
G4	2.358	5,834.4	POS
G5	1.786	4,305.3	POS
G6	1.994	4,864.1	POS
G7	1.462	3,459.4	POS
G8	1.414	3,341.9	POS
G9	0.400	851.1	POS
G10	0.728	1,621.8	POS
H1	1.074	2,471.7	POS
H2	1.708	4,102.0	POS
H3	2.512	6,251.7	POS
H4	2.400	5,942.9	POS
H5	2.272	5,597.6	POS
H6	1.688	3,357.4	POS
H7	0.711	1,577.6	POS
H8	0.562	1,221.8	POS
H9	0.592	1,294.2	POS
H10	0.572	1,244.5	POS

SOKOTO STATE

Sokoto east Senatorial District

Rabah Local Government

Sample I: 25 weeks layers

Sample J: 30 weeks layer

Sample K: 35 weeks layer

Sokoto South Senatorial District

Boringa Local Government

Sample L: 20 weeks layer

Sample M: 6 weeks broiler

Sample N: 25 weeks layers

Sokoto North Senatorial District

Wamakko Local Government

Sample O: 20 weeks layers

Sample P: 30 weeks layers

Sample Q: 6 weeks broiler

SAMPLE	S/P RATIO	ELISA ANTIBODY TITRE	IMMUNE STATUS
I1	0.366	765.6	POS
I2	0.4551	954.9	POS
I3	0.584	1,288.3	POS
I4	1.820	794.3	POS
I5	0.327	677.6	POS
I6	0.139	269.0	NEG
I7	0.012	18.45	NEG
I8	0.239	591.6	POS
I9	0.359	749.9	POS
I10	0.223	446.6	POS
J1	0.161	312.6	NEG
J2	0.078	142.2	NEG
J3	0.314	648.6	POS
J4	0.181	354.81	NEG
J5	0.272	553.4	POS
J6	0.127	234.0	NEG
J7	0.221	441.6	POS
J8	0.163	316.0	NEG
J9	0.271	550.8	POS
J10	0.162	315.0	NEG
K1	0.267	543.3	POS
K2	0.434	920.0	POS
K3	0.251	502.3	POS
K4	0.021	33.65	NEG
K5	0.205	407.4	POS
K6	0.074	134.0	NEG
K7	0.212	421.7	POS
K8	0.145	279.3	NEG
K9	0.290	594.3	POS
K10	0.178	348.0	NEG
L1	0.027	43.75	NEG
L2	0.470	1,006.9	POS
L3	0.062	110.0	NEG
L4	0.470	1,006.93	POS
L5	0.142	272.9	NEG
L6	0.088	161.8	NEG
L7	0.127	241.6	NEG
L8	0.523	1129.8	POS
L9	0.153	295.8	NEG
L10	0.069	124.2	NEG
M1	0.099	184.1	NEG
M2	0.250	505.8	POS
M3	0.542	1,174.9	POS
M4	0.157	304.8	NEG
M5	0.003	3.192	NEG
M6	0.166	323.6	NEG
M7	0.044	76.0	NEG

M8	0.538	1,166.0	POS
M9	0.653	1,438.8	POS
M10	0.654	1,442.1	POS
N1	0.687	1,520.5	POS
N2	0.288	588.8	POS
N3	0.439	933.3	POS
N4	0.182	357.3	NEG
N5	0.317	654.6	POS
N6	0.298	612.4	POS
N7	0.592	1,291.2	POS
N8	0.544	1,180.3	POS
N9	0.669	1,475.7	POS
N10	0.044	76.03	NEG
O1	0.279	586.1	POS
O2	0.592	1,291.2	POS
O3	0.544	1,180.3	POS
O4	0.669	1,475.7	POS
O5	0.044	76.03	NEG
O6	0.434	928.4	POS
O7	0.581	1,267.7	POS
O8	0.542	1,096.5	POS
O9	0.503	1,083.9	POS
O10	0.199	394.5	POS
Q1	0.544	1,180.3	POS
Q2	0.669	1,475.7	POS
Q3	0.279	586.1	POS
Q4	0.556	1,207.8	POS
Q5	0.439	933.3	POS
Q6	0.182	357.3	NEG
Q7	0.317	654.6	POS
Q8	0.298	612.4	POS
Q9	0.687	1, 520.5	POS
Q10	0.653	1,438.8	POS

OYO STATE

Oyo South Senatorial District

Ibarapa Central Local Government

Farm R: 34 weeks commercial layer birds

Farm S: 70 weeks broiler breeder birds

Farm T: 40 weeks commercial layer birds

Oyo Central Senatorial District

Egbeda Local Government

Farm U: 20 weeks commercial layer birds

Farm V: 30 weeks commercial layer birds

Farm W: 35 weeks commercial layer birds

Oyo North Local Senatorial district

Iseyin Local Government

Farm x: 32 weeks commercial layer birds

Farm y: 15 weeks commercial layer birds

SAMPLE	O.D TITRE	S/P RATIO	ELISA ANTIBODY TITRE	IMMUNE STATUS
R1	1.048	0.924	2,089.3	POS
R2	2.090	1.966	4,786.3	POS
R3	2.084	1.960	4,786.3	POS
R4	2.063	1.940	4,677.4	POS
R5	1.080	1.870	4,570.9	POS
R6	1.068	0.944	2,137.9	POS
R7	1.104	0.980	2,238.7	POS
R8	1.091	0.970	2,238.7	POS
R9	1.056	0.932	2,118.4	POS
R10	1.137	1.014	2,238.7	POS
S1	1.103	0.979	2,238.7	POS
S2	2.128	2.000	4,265.8	POS
S3	1.058	0.934	2,123.2	POS
S4	1.072	0.948	2,162.7	POS
S5	1.104	0.980	2,238.7	POS
S6	1.480	1.356	3,191.5	POS
S7	1.840	1.716	4,130.5	POS
S8	1.084	0.960	2,192.8	POS
S9	1.850	1.726	4,149.5	POS
S10	1.740	1.616	3,863.7	POS
T1	0.078	0.020	32.4	NEG
T2	0.083	0.030	49.2	NEG
T3	0.109	0.078	141.9	NEG
T4	0.076	0.017	26.3	NEG
T5	0.091	0.044	76.6	NEG
T6	0.107	0.040	66.1	NEG
T7	0.138	0.014	21.9	NEG
T8	0.196	0.072	130.01	NEG
T9	0.082	0.024	39.08	NEG
T10	0.177	0.203	402.7	NEG
U1	0.111	0.081	147.9	NEG
U2	0.094	0.050	87.1	NEG
U3	0.044	-0.0769	-144.5	NEG
U4	0.109	0.074	134.9	NEG
U5	0.122	0.102	190.6	NEG
U6	0.131	0.118	223.9	NEG
U7	0.082	0.024	39.08	NEG
U8	0.044	-0.0769	-144.5	NEG
U9	0.075	0.017	26.3	NEG
U10	0.100	0.051	68.2	NEG
V1	1.730	1.603	3,801.9	POS
V2	1.860	1.736	4,168.7	POS
V3	1.600	1.480	3,467.4	POS
V4	1.730	1.606	3,801.9	POS
V5	1.550	1.430	3,388.4	POS
V6	1.830	1.706	4,102.0	POS
V7	1.720	1.596	3,801.9	POS

V8	1.600	1.476	3,467.4	POS
V9	1.770	1.650	3,981.0	POS
V10	2.450	2.330	5,754.4	POS
W1	0.450	0.326	660.7	POS
W2	0.600	0.476	1000.0	POS
W4	0.500	0.330	661.8	POS
W5	0.200	0.076	138.0	NEG
W6	0.320	0.196	380.0	POS
W7	0.400	0.276	562.3	POS
W8	0.080	0.024	39.26	NEG
W9	0.820	0.696	1513.6	POS
W10	0.079	0.024	138.0	NEG
X1	0.050	-0.017	27.00	NEG
X2	0.067	0.00	0.00	NEG
X3	0.080	0.024	39.26	NEG
X4	0.090	0.042	72.4	NEG
X5	0.030	-0.094	-173.8	NEG
X6	0.010	-0.114	-213	NEG
X7	0.050	-0.074	-132.7	NEG
X8	0.100	0.061	109.6	NEG
X9	0.120	0.098	182.0	NEG
X10	0.150	0.026	43.7	NEG
Y1	0.080	0.024	39.26	NEG
Y2	0.050	-0.017	27.00	NEG
Y3	0.820	0.700	1515.6	POS
Y4	0.109	0.078	141.9	NEG
Y5	0.450	0.326	660.7	POS
Y6	0.100	0.061	109.6	NEG
Y7	0.030	-0.094	-173.8	NEG
Y8	0.122	0.102	190.6	NEG
Y9	0.094	0.050	87.1	NEG
Y10	0.044	-0.080	-145.0	NEG

Note: POS-POSITIVE

NEG-NEGATIVE

APPENDIX IV: CROSS-SECTIONAL SURVEY USING QUESTIONNAIRE ON THE PREVALENCE OF RESPIRATORY DISEASE

Sample ID: …….

Date: …… Name of Farm…….
Farm Address & Phone No. ……
……
……
Age of flock……. Type of Flock
……
Flock size ……. Mortality Onset ……
Per day……... Last one week…….
Vaccination record: Disease Date
……. ……
……. ……
……. ……
Antibiotics administered since onset:
…… ……...
Pattern of Antibiotic usage ……
Clinical
Signs……
……
……
Postmortem
Findings……..…
……
……
Tentative
Diagnosis……

Administered by:

Name……. Signature…….

APPENDIX V: SET OF AVIAN METAPNEUMOVIRUS (AMPV) PRIMERS USED IN GENETIC ANALYSIS.

AFRICAN BIOSCIENCES LTD
Head Office: Poatson House, 4th Floor, 142 Oba Akran Avenue, Ikeja, Lagos
Operations Office& Lab: JaaGee House, 88, Km 6 Ibadan – Ife Expy, Ibadan
0800-BIOTECH (Toll-Free)
info@africanbio.com
www.africanbio.com

Dr. Bakre,

Dept. of Veterinary Medicine,

University of Ibadan.

08130846021.

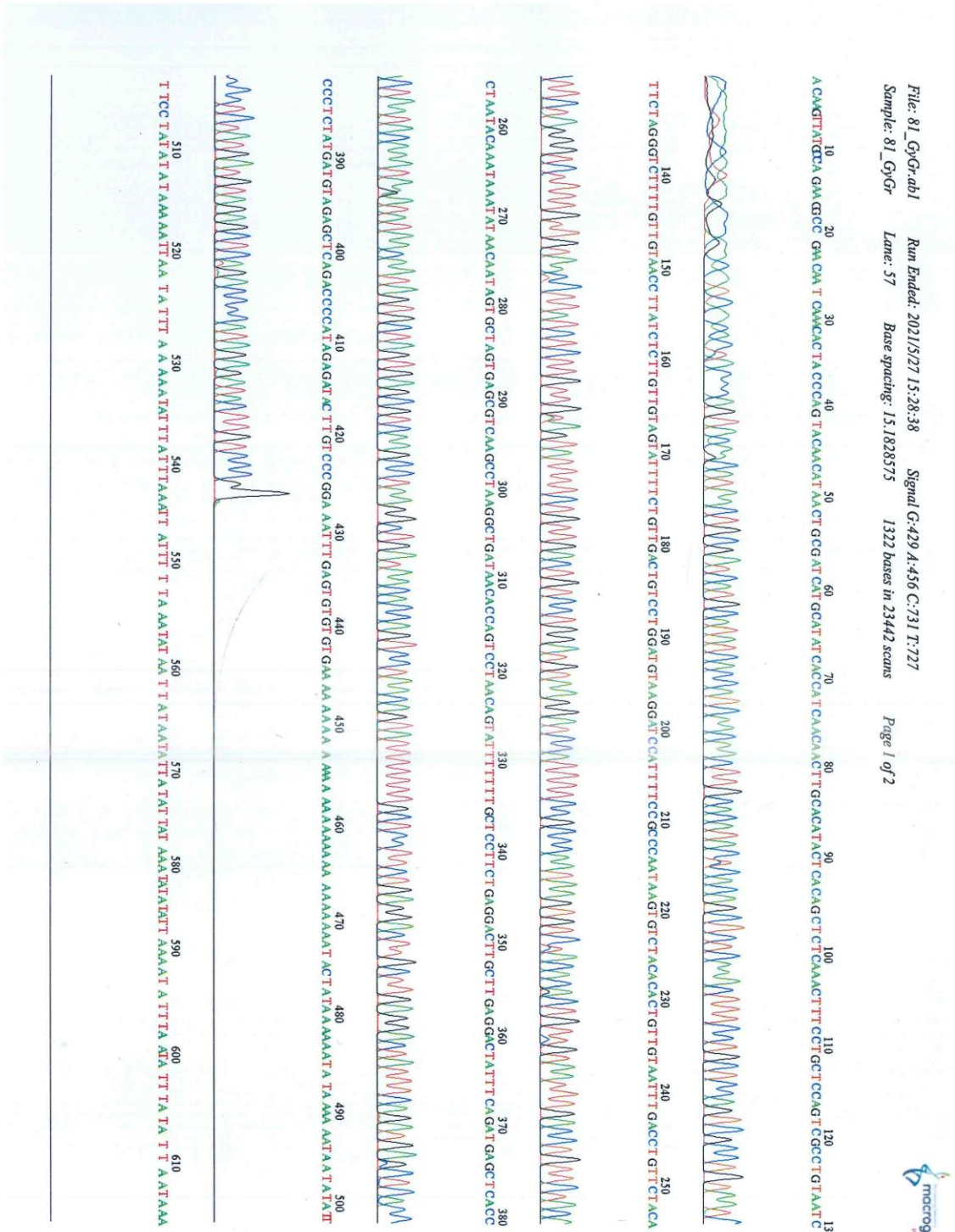
Synthesis Report

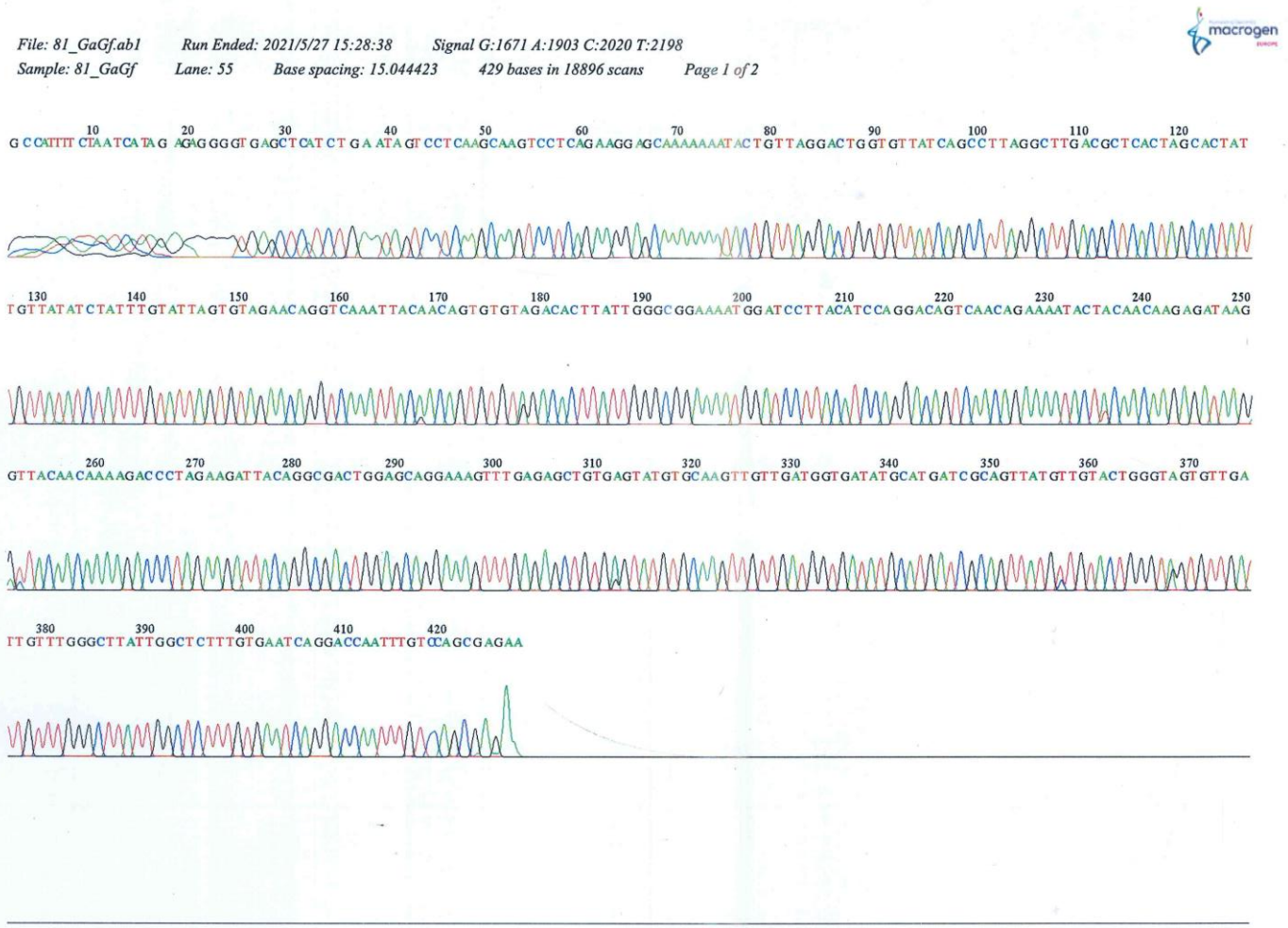
Name	Sequence	Mol. Wt.	nmoles	Tm	GC Content
Nd-F	5'AGCAGGATGGAGAGCCTCTTTG 3'	6816.4g/mol	22.8	60.0^{0}C	54.5%
Nx-R	5` CAT GGC CCA ACA TTA TGT T 3`	5763.8g/mol	26.0	54.0^{0}C	42.1%
GaG-F	5` CCG GGA CAA GTA TCT CTA TGG 3'	6447.2g/mol	25.3	57.8^{0}C	52.4%
GyG-R	5` TCT CGC TGA CAA ATT GGT CCT GA 3'	7015.6g/mol	20.1	58.4^{0}C	47.8%
C1M-F	5` GAT GAC TAC AGC AAA CTA GAG 3	6473.2g/mol	24.5	53.9^{0}C	42.9%
C2M-R	5` CTT CAG GAC ATA TCT CGT AC 3'	6053.0g/mol	25.1	58.0^{0}C	45.0%
G+50-F	5` GCG ATG CCC AGT TAA TGA A 3'	5837g/mol	25.8	56.0^{0}C	47.4%
G-1005-R	5` CCC CTT ACA AAC ACT GTT C 3	5668g/mol	26.3	56.0^{0}C	47.4%

Recommendations for Handling and Storage

- Lyophilised oligo pellets may become displaced from the bottom of the tube during shipment. Briefly centrifuge each tube before opening to prevent loss of pellet.
- Prepare Stock solution of oligos (100µM =100pmole per µl) with sterile buffered solution such as TE (10mM Tris, pH 7.5 to 8.0, 1mM EDTA). If sterile distilled water is used, make sure the pH is above 7.0 since acidic solutions favour oligo depurination and subsequent loss of activity.

APPENDIX VI: SEQUENCE OF ATTACHMENT PROTEIN (G-GENE) OF AVIAN METAPNEUMOVIRUS IN COMMERCIAL LAYER CHICKEN IN PLATEAU STATE (NEAR TEMPERATE ZONE)




File: 81_GaGf.ab1 Run Ended: 2021/5/27 15:28:38 Signal G:1671 A:1903 C:2020 T:2198
Sample: 81_GaGf Lane: 55 Base spacing: 15.044423 429 bases in 18896 scans Page 1 of 2
macrogen
10 20 30 40 50 60 70 80 90 100 110 120
G CCATTTT CTAATCATA G AGAG GGGT GAGCTCAT CT G A ATA GT CCT CAAGCAAGTCCT CAG AAGG AGCAAAAAAAT ACT GTT AGGACT GGT GTTAT CAGCCTT AGGCTTGACGCT CACTAGCACTAT
130 140 150 160 170 180 190 200 210 220 230 240 250
TGTTATATCTATTTGTATTAGTGTAGAACAGGTCAAATTACAACAGTGTGTAGACACTTATTGGGCGGAAAATGGATCCTTACATCCAGGACAGTCAACAGAAAATACTACAACAAGAGATAAG
260 270 280 290 300 310 320 330 340 350 360 370
GTTACAACAAAAGACCCTAGAAGATTACAGGCGACTGGAGCAGGAAAGTTTGAGAGCTGTGAGTATGTGCAAGTTGTTGATGGTGATATGCATGATCGCAGTTATGTTGTACTGGGTAGTGTTGA
380 390 400 410 420
TTGTTTGGGCTTATTGGCTCTTTGTGAATCAGGACCAATTTGTCCAGCGAGAA

APPENDIX VII: AVIAN METAPNEUMOVIRUS ISOLATE NGA_PLATEAU/2021/03 G-GENE, PARTIAL CDS

LOCUS Seq1 450 bp mRNA linear VRL 11-JUN-2021
DEFINITION isolate=NGA_Plateau/2021/03.
ACCESSION
VERSION
KEYWORDS.
SOURCE Avian metapneumovirus
ORGANISM Avian metapneumovirus
Viruses; Riboviria; Orthornavirae; Negarnaviricota;
Haploviricotina; Monjiviricetes; Mononegavirales; Pneumoviridae;
Metapneumovirus.
REFERENCE 1 (bases 1 to 450)
AUTHORS Bakre, A.A., Oladele,O.A., Meseko,C.A., Shittu,I.A., Inuwa,B. and Oluwayelu,D.O.
TITLE Detection and molecular characterization of avian metapneumovirus in commercial chicken in climatic zones in Nigeria
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 450)
AUTHORS Bakre,A.A., Oladele,O.A., Meseko,C.A., Shittu,I.A., Inuwa,B. and Oluwayelu,D.O.
TITLE Direct Submission
JOURNAL Submitted (11-JUN-2021) Veterinary Medicine, University of Ibadan, Abadina Road, UI, Ibadan, Oyo 200005, Nigeria
COMMENT Bankit Comment: Local ID: Seq1.
Bankit Comment: BankIt2471295.

##Assembly-Data-START##
Sequencing Technology: Sanger dideoxy sequencing
##Assembly-Data-END##
FEATURES Location/Qualifiers
Source 1.450
/organism="Avian metapneumovirus"

/isolate="NGA_Plateau 2021/03"
/isolation_source="Secretions"
/host="Chicken"
/bio_material="Swab"
/db_xref="taxon:38525"
/tissue_type="Turbinate"
/country="Nigeria"
/collection_date="10-Mar-2021"
/subtype="B"
/breed="Layers"
gene <1.>450

APPENDIX VIII: AVIAN METAPNEUMOVIRUS ISOLATE NGA_PLATEAU/2021/03 G-GENE, PARTIAL CDS

gene="G gene

GenBank MZ408311

Inbox

gb-admin@ncbi.nlm.nih.gov Jun 17, 2021, 12:03 PM

to me, tholyng

Dear GenBank Submitter:

Thank you for your direct submission of sequence data to GenBank. We have provided a GenBank accession number for your nucleotide sequence:

BankIt2473006 Avian MZ408311

The GenBank accession number should appear in any publication that reports or discusses these data, as it gives the community a unique label with which they may retrieve your data from our on-line servers. You may prepare and submit your manuscript before your accession is released in GenBank.

Submissions are not automatically deposited into GenBank after being accessioned. Each sequence record is individually examined and processed by the GenBank annotation staff to ensure that it is free of errors or problems.

You have not requested a specific release date for your sequence data. Therefore, your record(s) will be released to the public database once they are processed. If this is not what you intended, please contact us as soon as possible with the correct release date.

Since the flatfile record is a display format only and is not an editable

format of the data, do not make changes directly to a flatfile. For complete information about different methods to update a sequence record, see: https://www.ncbi.nlm.nih.gov/Genbank/update.html

Any inquiries about your submission should be sent to gb-admin@ncbi.nlm.nih.gov

For more information about the submission process or the available submission tools, please contact GenBank User Support at info@ncbi.nlm.nih.gov.

APPENDIX IX: BLAST RESULT SIMILARITIES BETWEEN THE ISOLATED AMPV-G-GENE AND OTHER STRAINS IN THE GENBANK

Turkey rhinotracheitis virus (strain 2119) attachment protein (G) mRNA, complete cds

Sequence ID: L34031.1

Length: 1260, Number of Matches: 1

Score: 718 bits (795), Expect: 0.0

Identities: 434/450(96%), Gaps: 1/450(0%), Strand: Plus/Plus

CDS: Putative 1 1 M G S E L Y I I E G V S S S E

Query 1
GGGACAAGTATCTCTATGGGGTCTGAGCTCTACATCATAGAGGGGGTGAGCTCA
TCTGAA 60

|||||||||||| |||||||| ||||||||||||||||||||||||||||||||

Sbjct 1
GGGACAAGTATCCAGATGGGGTCAGAGCTCTACATCATAGAGGGGGTGAGCTCA
TCTGAA 60

CDS:attachment prote 1 M G S E L Y I I E G V S S S E

CDS: Putative 1 16 I V L K Q V L R R S K K I L L G L V L S

Query 61
ATAGTCCTCAAGCAAGTCCTCAGAAGGAGCaaaaaaaTACTGTTAGGACTGGTGTTA
TCA 120

|||||||||||||||||||||||||| ||||||||||||||||||||||||

Sbjct 61
ATAGTCCTCAAGCAAGTCCTCAGAAGGAGCCAAAAAATACTGTTAGGACTGGTG
TTATCA 120

CDS:attachment prote 16 I V L K Q V L R R S Q K I L L G L V L S

CDS: Putative 1 36 A L G L T L T S T I V I X I C I S V E Q

Query 121
GCCTTAGGCTTGACGCTCACTAGCACTATTGTTATATYTATTTGTATTAGTGTAGA
ACAG 180

|||||||||||||||||||||||||||||||||||| ||||||||||||||||||||

Sbjct 121
GCCTTAGGCTTGACGCTCACTAGCACTATTGTTATATCTATTTGTATTAGTGTAGA
ACAG 180

CDS: attachment protein 36 A L G L T L T S T I V I S I C I S V E Q

CDS: Putative 1 56 V K L Q Q C V D T Y W A E N G S L H P G

Query 181
GTCAAATTACAACAGTGTGTAGACACTTATTGGGCGGAAAATGGATCCTTACATC
CAGGA 240

|||||||||| |||||||| ||||||||||||||||||||||||||||||||||||

Sbjct 181
GTCAAATTACGACAGTGTGTGGACACTTATTGGGCGGAAAATGGATCCTTACATC
CAGGA 240

CDS: attachment prote 56 V K L R Q C V D T Y W A E N G S L H P G

CDS : Putative 1 76 Q S T E N T T T R D K V T T K D P R R L

Query 241
CAGTCAACAGAAAATACTACAACAAGAGATAAGGTTACAACAAAAGACCCTAG
AAGATTA 300

|||||||||||||||| ||||||||||||| ||||||||||||||||||||||

Sbjct 241
CAGTCAACAGAAAATACTTCAACAAGAGATAAGACTACAACAAAAGACCCTAGA
AGATTA 300

CDS:attachment prote 76 Q S T E N T S T R D K T T T K D P R R L

CDS: Putative 1 96 Q A T G A G K F E S C E Y V Q V V D G D

Query 301
CAGGCGACTGGAGCAGGAAAGTTTGAGAGCTGTGAGTATGTGCAAGTTGTTGAT
GGTGAT 360

||||||||||||||||||||||||||||||||| ||||||||||||||||||||||

Sbjct 301
CAGGCGACTGGAGCAGGAAAGTTTGAGAGCTGTGGGTATGTGCAAGTTGTTGAT
GGTGAT 360

CDS:attachment prote 96 Q A T G A G K F E S C G Y V Q V V D G D

CDS: Putative 1 116 M H D R S Y V V L G S V D C L G L L A L

Query 361
ATGCATGATCGCAGTTATGTTGTACTGGGTAGTGTTGATTGTTTGGGCTTATTGGC
TCTT 420

|||||||||||||||| ||||||||| ||||||||||||||||||||||||||

Sbjct 361
ATGCATGATCGCAGTTATGCTGTACTGGGTGGTGTTGATTGTTTGGGCTTATTGGC
TCTT 420

CDS: attachment protein 116 M H D R S Y A V L G G V D C L G L L A L

CDS: Putative 1 136 C E S G P I C P A R

Query 421 TGTGAATCAGGACCAATTTGTCCAGCGAGA 450

|||||||||||||||||| ||| ||||

Sbjct 421 TGTGAATCAGGACCAATTTGT-CAGGGAGA 449

CDS: attachment protein 136 C E S G P I C Q G D

Avian metapneumovirus isolate Hungary/657/4, complete genome

Sequence ID: MN729604.1

Length: 13508, Number of Matches: 1

Score: 713 bits (790), Expect: 0.0

Identities: 434/450(96%), Gaps: 1/450(0%), Strand: Plus/Plus

CDS: Putative 1 1 M G S E L Y I I E G V S S S E

Query 1
GGGACAAGTATCTCTATGGGGTCTGAGCTCTACATCATAGAGGGGGTGAGCTCA
TCTGAA 60

Sbjct 5999
GGGACAAGTATCCAGATGGGGTCAGAGCTCTACATCATAGAGGGGGTGAGCTCA
TCTGAA 6058

CDS:attachment glyco 1 M G S E L Y I I E G V S S S E

CDS: Putative 1 16 I V L K Q V L R R S K K I L L G L V L S

Query 61
ATAGTCCTCAAGCAAGTCCTCAGAAGGAGCaaaaaaaTACTGTTAGGACTGGTGTTA
TCA 120

Sbjct 6059
ATAGTCCTCAAGCAAGTCCTCAGAAGGAGCAAAAAAAATACTGTTAGGACTGGTG
TTATCA 6118

CDS:attachment glyco 16 I V L K Q V L R R S K K I L L G L V L S

CDS: Putative 1 36 A L G L T L T S T I V I X I C I S V E Q

Query 121
GCCTTAGGCTTGACGCTCACTAGCACTATTGTTATATYTATTTGTATTAGTGTAGA
ACAG 180

Sbjct 6119
GCCTTAGGCTTGACGCTCACTAGCACTATTGTTATATCTATTTGTATTAGTGTAGA
ACAG 6178

CDS:attachment glyco 36 A L G L T L T S T I V I S I C I S V E Q

CDS: Putative 1 56 V K L Q Q C V D T Y W A E N G S L H P G

Query 181
GTCAAATTACAACAGTGTGTAGACACTTATTGGGCGGAAAATGGATCCTTACATC

CAGGA 240

|||||||||| |||||||| ||||||||||||||||||||||||||||||||||||||||

Sbjct 6179
GTCAAATTACGACAGTGTGTGGACACTTATTGGGCGGAAAATGGATCCTTACATC
CAGGA 6238

CDS:attachment glyco 56 V K L R Q C V D T Y W A E N G S L H P G

CDS: Putative 1 76 Q S T E N T T T R D K V T T K D P R R L

Query 241
CAGTCAACAGAAAATACTACAACAAGAGATAAGGTTACAACAAAAGACCCTAG
AAGATTA 300

|||||||||||||||| |||||||| |||| ||||||||||||||||||||||

Sbjct 6239
CAGTCAACAGAAAATACTTCAACAAGAGGTAAGACTACAACAAAAGACCCTAGA
AGATTA 6298

CDS:attachment glyco 76 Q S T E N T S T R G K T T T K D P R R L

CDS: Putative 1 96 Q A T G A G K F E S C E Y V Q V V D G D

Query 301
CAGGCGACTGGAGCAGGAAAGTTTGAGAGCTGTGAGTATGTGCAAGTTGTTGAT
GGTGAT 360

|||||||||||||||||||||||||||||||| ||||||||||||||||||||||

Sbjct 6299
CAGGCGACTGGAGCAGGAAAGTTTGAGAGCTGTGGGTATGTGCAAGTTGTTGAT
GGTGAT 6358

CDS:attachment glyco 96 Q A T G A G K F E S C G Y V Q V V D G D

CDS: Putative 1 116 M H D R S Y V V L G S V D C L G L L A L

Query 361
ATGCATGATCGCAGTTATGTTGTACTGGGTAGTGTTGATTGTTTGGGCTTATTGGC
TCTT 420

|||||||||||||||| ||||||||| ||||||||||||||||||||||||||||

Sbjct 6359
ATGCATGATCGCAGTTATGCTGTACTGGGTGGTGTTGATTGTTTGGGCTTATTGGC

GCAGTTATGCTGTACTGGGTGGTGTTGATTGTTTGGGCTTATTGGCTCTT 6418

CDS: attachment glyco 116 M H D R S Y A V L G G V D C L G L L A L

CDS: Putative 1 136 C E S G P I C P A R

Query 421 TGTGAATCAGGACCAATTTGTCCAGCGAGA 450

||||||||||||||||||| ||| |||||

Sbjct 6419 TGTGAATCAGGACCAATTTGT-CAGGGAGA 6447

CDS:attachment glyco 136 C E S G P I C Q G D

APPENDIX X: ARRAY OF BACTERIA ISOLATED DURING DRY SEASON IN PLATEAU, OYO AND SOKOTO STATE

23rd March, 2021.

TO: **DR. BAKRE,**
University of Ibadan,
Ibadan, Oyo State.

S/N	SAMPLE	CATALASE	OXIDASE	GRAM	SHAPE	MOTILITY	GROWTH ON BLOOD AGER	GROWTH ON MACCONKEY	METHYL - RED	VOGES -PROSKEUR	INDOLE	STARCH HYDROLYSIS	UREASE HYDROLYSIS	GAS FORMATION	GLUCOSE	LACTOSE	MALTOSE	SUCROSE	CITRATE	H_2S	HEMOLYSIS	MANNITOL	ORGANISM
1	OY 1	+	-	-	ROD	+	+	+	+	-	+	-	-	-	+	+	-	+	+	+	+	+	*Citrobacter Freundii*
2	OY 2	+	-	-	ROD	+	+	+	-	+	+	-	+	+	+	+	-	-	+	+	+	-	*Proteus Mirabilis*
3	OY 3	+	-	-	ROD	+	+	+	-	+	+	-	+	+	+	+	-	-	+	+	+	-	*Proteus Mirabilis*
4	OY 4	+	-	-	ROD	+	+	+	+	-	+	-	-	+	+	+	+	+	-	-	+	-	*Escherichia Coli*
5	OY 5	+	-	-	ROD	+	+	+	+	-	+	-	+	-	+	+	+	+	-	+	+	-	*Proteus Vulgaris*
6	OY 6	+	+	-	ROD	+	+	+	+	-	+	-	-	+	+	+	+	+	+	+	+	+	*Aeromenas Hydrophila*
7	OY 7	+	-	-	ROD	+	+	+	+	-	+	-	-	+	+	+	+	+	-	-	+	-	*Escherichia Coli*
8	OY 8	+	-	-	ROD	+	+	+	+	-	+	-	-	+	+	+	+	+	-	-	+	-	*Escherichia Coli*
9	OY 9	+	-	-	ROD	+	+	+	+	-	+	-	-	+	+	+	+	+	-	-	+	-	*Escherichia*

IBDL/0321/223

...working for animal health and production

																								Coli
10	OY 10	+	-	-	ROD	+	+	+	+	-	+	-	-	+	+	+	+	+	-	-	+	-		*Escherichia Coli*
11	OY 11	+	-	-	ROD	+	+	+	+	-	+	-	-	+	+	+	+	+	-	-	+	-		*Escherichia Coli*
12	OY 12	+	-	-	ROD	+	+	+	+	-	+	-	-	+	+	+	+	+	-	-	+	-		*Escherichia Coli*
13	OY 13	+	-	-	ROD	+	+	+	+	-	+	-	-	-	+	+	-	+	+	+	+	+		*Citrobacter Freundii*
14	OY 14	+	-	-	ROD	+	+	+	+	-	+	-	-	+	+	+	+	+	-	-	+	-		*Escherichia Coli*
15	S 1	+	-	+	ROD	+	+	+	+	-	+	+	-	+	+	+	-	+	+	-	+	+		*Aeromenas Hydrophila*
16	S 2	+	-	-	ROD	+	+	+	+	-	+	-	-	+	+	+	+	+	-	-	+	-		*Escherichia Coli*
17	S 3	+	-	-	ROD	+	+	+	+	-	+	-	-	+	+	+	+	+	-	-	+	-		*Escherichia Coli*
18	S 4	+	+	-	ROD	+	+	+	+	-	+	-	+	-	+	+	+	-	+	-		+		*Vibrio Mimicus*
19	S 5	+	-	-	ROD	+	+	+	+	-	+	-	-	+	+	+	+	+	-	-	+	-		*Escherichia Coli*
20	S 6	+	-	-	ROD	+	+	+	+	-	+	-	-	+	+	+	+	+	-	-	+	-		*Escherichia Coli*
21	S 7	+	-	-	ROD	+	+	+	+	-	+	-	-	+	+	+	+	+	-	-	+	-		*Escherichia Coli*
22	S 8	+	+	-	ROD	+	+	+	+	-	+	-	-	+	+	+	+	+	+	+	+	+		*Aeromenas Hydrophila*
23	S 9	+	-	-	ROD	+	+	+	+	-	+	-	-	+	+	+	+	+	-	-	+	-		*Escherichia*

ANIMAL CARE®

ANIMAL CARE TECHNICAL SERVICES DIVISION
(DISEASE DIAGNOSIS/CONTROL AND FEED ANALYSIS LABORATORY)
Plot 17, Block 1, 19th Avenue, Oluyole, Ibadan, Oyo State

																							Coli
24	S 10	+	+	-	ROD	+	+	+	-	-	+	-	-	+	+	-	+	+	+	-	+	+	*Vibrio Cholerae*
25	S 11	+	+	-	ROD	+	+	+	-	-	+	-	-	+	+	-	+	+	+	-	+	+	*Vibrio Cholerae*
26	S 12	+	+	-	ROD	+	+	+	+	-	+	-	-	+	+	+	+	+	+	+	+	+	*Aeromenas Hydrophila*
27	S 13	+	+	-	ROD	+	+	+	+	-	+	-	-	+	+	+	+	+	+	+	+	+	*Aeromenas Hydrophila*
28	S 14	+	-	-	ROD	+	+	+	+	-	+	-	-	+	+	+	+	+	-	-	+	-	*Escherichia Coli*
29	P 1	+	-	-	ROD	+	+	+	+	-	+	+	+	-	+	+	-	-	+	+	+	-	*Citrobacter Youngae*
30	P 2	+	-	-	ROD	+	+	+	+	-	+	+	+	-	+	+	-	-	+	+	+	-	*Citrobacter Youngae*
31	P 3	+	+	-	ROD	+	+	+	-	-	-	-	-	-	-	-	-	+	-	-	+		*Pseudomonas Aeruginosa*
32	P 4	+	+	-	ROD	+	+	+	-	-	-	-	-	-	-	-	-	+	-	-	+		*Pseudomonas Aeruginosa*
33	P 5	+	-	-	ROD	+	+	+	+	-	+	-	-	+	+	+	+	+	-	-	+	-	*Escherichia Coli*
34	P 6	+	-	-	ROD	-	+	+	-	+	-	-	+	+	+	+	+	+	+	-	+		*Klebsiella Pneumoniae*
35	P 7	+	-	-	ROD	+	+	+	+	-	+	-	-	+	+	+	+	+	-	-	+	-	*Escherichia Coli*
36	P 8	+	-	-	ROD	+	+	+	+	-	+	-	-	+	+	+	+	+	-	-	+	-	*Escherichia Coli*
37	P 9	+	-	-	ROD	+	+	+	+	-	+	-	-	+	+	+	+	+	-	-	+	-	*Escherichia*

																						Coli
	38	P 10	+	-	-	ROD	+	+	+	+	-	+	-	-	+	+	+	+	+	-	-	+	-	*Escherichia Coli*
P11	39	P 12	+	-	-	ROD	+	+	+	+	-	+	-	-	-	+	+	-	+	+	+	+	+	*Citrobacter Freundii*
P12	40	P 13	+	-	-	ROD	+	+	+	+	-	+	-	-	+	+	+	+	+	-	-	+	-	*Escherichia Coli*
P13	41	P 14	+	-	-	ROD	+	+	+	+	-	+	-	-	+	+	+	+	+	-	-	+	-	*Escherichia Coli*
P4	42	P 15	+	+	-	ROD	+	+	+	-	-	-	-	-	-	-	-	-	+	-	-	-	+	*Pseudomonas Aeruginosa*

KEY:

+ **Posistive**
- **Negative**

Thank you for your patronage.
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