# SYSTEMATIC REVIEW, META-ANALYSIS AND WHOLE GENOME SEQUENCE ANALYSIS STUDIES OF AVIAN INFLUENZA VIRUS, INFLUENZA D VIRUS, NEWCASTLE DISEASE VIRUS, AND SEVERE ACUTE RESPIRATORY SYNDROME CORONAVIRUS 2

**By**

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**A Thesis Submitted to the University of Zambia in Fulfilment of the Requirements for the Degree of Doctor of Philosophy in Virology**

**The University of Zambia**
**Lusaka**

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## Declaration

I, **Annie Kalonda,** declare that this thesis is my own work. It is being submitted for the degree of Doctor of Philosophy in Virology at the University of Zambia, Lusaka, Zambia. It has not been submitted either wholly or partially for any degree, diploma or other qualification at this or any other university.

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**Certificate of Approval**

This thesis submitted by Annie Kalonda has been approved as fulfilling the requirements for the award of the degree of Doctor of Philosophy in Virology at the University of Zambia.

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**Abstract**

Avian influenza and Newcastle disease are respiratory and often fatal diseases of birds caused by avian influenza virus (AIV) and Newcastle disease virus (NDV), respectively. They are zoonotic diseases that affect a wide range of avian species and cause substantial economic losses. Moreover, the emergence of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in humans strained the global economy and healthcare systems. Despite disease control efforts, these viruses have continued to circulate. Therefore, the study aimed to evaluate the circulation and genetic characteristics of AIVs, influenza D virus (IDV), NDV and SARS-CoV-2. A total of 2851 and 1150 faecal samples were obtained from wild waterfowl and poultry, respectively, and 198 nasopharyngeal swabs were collected from humans in Zambia. Systematic reviews and meta-analysis were conducted using the Preferred Reporting Items for Systematic Reviews and Meta-Analyses guidelines, and standard methods and next-generation sequencing were used to identify and genetically characterise the viruses. The systematic review revealed an overall prevalence and seroprevalence of 3.0% and 4.1% of AIV in African birds, with H5N1 being the most detected subtype. The meta-analysis demonstrated an estimated pooled prevalence and seroprevalence of IAV in pigs in Africa of 1.6% and 14.9%, respectively, while IDV seroprevalence was at 87.2% in camels, 9.3% in cattle and 2.2% in small ruminants. IAV subtypes H1N1 and H1N1pdm09 were commonly detected in pigs. In Zambian wild waterfowl, 85 (3.0%) low pathogenicity AIVs belonging to various subtypes and 2 (0.07%) avirulent NDVs were isolated. While no AIVs were detected in poultry, 4 (0.3%) virulent NDV were isolated. Moreover, the study reports, for the first time in Africa, the isolation of AIV in glossy ibis and the detection of H2N9, H8N4, and H10N8 AIV subtypes. Phylogenetic analysis of AIVs revealed that all the isolates belonged to the Eurasian lineage, with their closest relatives being viruses from wild and/or domestic birds in Africa, Asia, and Europe, and were grouped into distinct clusters based on the year of isolation. Furthermore, some internal protein genes of the viruses were closely related to H7 low pathogenicity AIVs. Analysis of the NDV strains revealed that the isolates from wild waterfowl belonged to class I viruses. In contrast, those from poultry belonged to class II, genotype VII and were closely related to viruses isolated from the Eastern Province of Zambia in 2015. Genetic analysis of 40 SARS-CoV-2 revealed the circulation of Alpha (B.1.1.7), Beta (B.1.351), Delta (AY.116), and multiple Omicron subvariants. Furthermore, 292 mutations were observed, with the majority being missense mutations. Phylogenetic analysis showed evidence of local transmission and possible multiple introductions of SARS-CoV-2 variants in Zambia from different European and African countries. This study highlights the circulation of low pathogenicity AIVs and NDV in wild waterfowls, NDV in poultry and co-circulations of SARS-CoV-2 variants in the human population in the Southern Province of Zambia. Therefore, the study stresses the need for continuous surveillance and monitoring of AIVs and NDV in wild waterfowl and poultry for a better understanding of the eco-epidemiology and evolutionary dynamics of AIVs in Africa as well as SARS-CoV-2 circulation in Zambia.

## Dedication

I dedicate this to my beloved mother, Mrs Domittila Kapambwe Kalonda, who has been my source of inspiration and continually provides me with moral, spiritual and emotional support. To my late Father, Mr Stephen Njobvu Kalonda, may your soul rest in the Lord's bosom.

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## List of Abbreviations and Acronyms

| | |
|---|---|
| AAvV-1 | *avian avulavirus* 1 |
| ACE-2 | Angiotensin-Converting Enzyme 2 |
| AI | Avian Influenza |
| AIV | Avian Influenza Virus |
| AOAV-1 | *avian orthoavulavirus* 1 |
| APMV-1 | *avian paramyxovirus* 1 |
| CDC | Centers for Disease Control and Prevention |
| CoV | Coronavirus |
| COVID-19 | Coronavirus Disease 2019 |
| BRD | Bovine Respiratory Disease |
| DIVA | Differentiation of Infected from Vaccinated Animals |
| DNA | Deoxyribonucleic acid |
| E | Envelope Protein |
| ECEs | Embryonated Chicken Eggs |
| ELISA | Enzyme Linked Immunosorbent Assay |
| EIV | Equine Influenza Virus |
| FAO | Food and Agriculture Organisation |
| FDA | Food and Drug Administration |
| F | Fusion Protein |
| GICT | Immune Colloidal Gold Technique |
| GISAID | Global Initiative on Sharing All Influenza Data |
| HA | Haemagglutinin |
| HEF | Haemagglutinin-esterase-fusion |
| HI | Haemagglutination Inhibition |
| HN | Haemagglutinin-Neuraminidase |
| HPAIV | High Pathogenicity Avian Influenza Virus |
| HPAI | High Pathogenicity Avian Influenza |
| IAV | Influenza A Virus |
| IBV | Influenza B Virus |
| ICV | Influenza C Virus |
| IDV | Influenza D Virus |
| IFA | Immunofluorescence Antibody Assay |
| IgG | Immunoglobulin G |

| | |
|---|---|
| IgM | Immunoglobulin M |
| L | RNA-dependent RNA Polymerase Protein |
| LAMP | Loop Mediated Isothermal Amplification |
| LBM | Live Bird Market |
| LPAIV | Low Pathogenicity Avian Influenza Virus |
| LPAIV | Low Pathogenicity Avian Influenza |
| M | Matrix Protein |
| MERS-CoV | Middle East respiratory syndrome coronavirus |
| MDCK | Madin–Darby Canine Kidney cells |
| NA | Neuraminidase |
| NABSA | Nucleic Acid Sequence-Based Amplification |
| ND | Newcastle Disease |
| NDV | Newcastle disease virus |
| NI | Neuraminidase Inhibition |
| NP | Nucleoprotein |
| NS | Non-structural Protein |
| NT | Neutralizing Antibodies Test |
| ORF | Open Reading Frame |
| P | Phosphoprotein |
| PA | Polymerase Acid |
| PANGO | Phylogenetic Assignment of Named Global Outbreak Lineages |
| PB1 | Polymerase Basic 1 |
| PB2 | Polymerase Basic 2 |
| PRISMA | Preferred Reporting Items for Systematic Reviews and Meta-Analyses |
| RBCs | Red Blood Cells |
| RNA | Ribonucleic Acid |
| RdRp | RNA-dependent RNA-polymerase |
| S | Spike Glycoprotein |
| SARS | Severe Acute Respiratory Syndrome |
| SARS-CoV | Severe Acute Respiratory Syndrome Coronavirus |
| SARS-CoV-2 | Severe Acute Respiratory Syndrome Coronavirus 2 |
| SPF | Specific Pathogen Free |

| | |
|---|---|
| ST | Swine Testicular cells |
| TMPRSS2 | Type II Transmembrane Serine Protease |
| VLP | Virus-like Particles Vaccines |
| vNDV | virulent Newcastle Disease Virus |
| vRNA | viral Ribonucleic Acid |
| vRNP | viral Ribonucleoprotein |
| VOC | Variants of Concern |
| VOI | Variants of Interest |
| WOAH | World Organisation for Animal Health |
| WHO | World Health Organisation |

# CHAPTER ONE

## 1.0 Introduction

Avian influenza viruses (AIVs) are of global concern, with some subtypes and strains posing a constant threat to the poultry industry (Alqazlan *et al.*, 2022). AIVs are segmented, negative-sense RNA viruses belonging to the *Orthomyxoviridae* family, genus *Alphainfluenzavirus,* species *influenza A virus* (IAV). Wild waterfowl of the orders *Anseriformes* (ducks, geese and swans) and *Charadriiformes* (gulls and waders) are the known major natural reservoirs of AIV and play an essential role in the evolution and spread of these viruses (Medina and García-Sastre, 2011, Webster *et al.*, 1992). The prevalence of AIVs in wild waterfowl differs within and between avian species, age, season and geographical location (Stallknecht and Shane, 1988). During wild waterfowl migration, which may range from short local movements to intercontinental migrations, wild waterfowl can carry AIVs from one area to another and present a formidable risk to susceptible host species along their migratory flyway (Olsen *et al.*, 2006, Webster *et al.*, 1992). Therefore, wild waterfowl are of primary interest in AIV surveillance efforts, especially in places where birds of various geographical origins congregate at high densities, such as at stopover sites within migratory bottlenecks, creating so-called transmission 'hotspots' (Krauss *et al.*, 2010).

AIVs are classified into subtypes based on the antigenic properties of haemagglutinin (HA) and neuraminidase (NA) glycoproteins expressed on the surface of the virus particles. To date, 16 distinct haemagglutinin (HA) (H1–H16) and nine neuraminidase (NA) (N1–N9) which are found in different HA/NA combinations, have been isolated in wild waterfowl across the globe (Fouchier *et al.*, 2005, Röhm *et al.*, 1996, Kawaoka *et al.*, 1990, Hinshaw *et al.*, 1982). Whilst H17, H18, N10, and N11 subtypes have been identified from bats (Tong *et al.*, 2013, Tong *et al.*, 2012), there is currently no evidence that these variants are found in avian species (Spackman, 2020). AIVs are also categorised into two different pathotypes, namely, low pathogenicity avian influenza viruses (LPAIVs) and high pathogenicity avian influenza viruses (HPAIVs) based on the pathogenicity in chickens. LPAIV infections are typically milder or subclinical and restricted to the respiratory and/or

gastrointestinal tract. In contrast, HPAIV infections are associated with multi-organ systemic infection, which can cause severe disease and high mortality in birds (Bergervoet *et al.*, 2019). Outbreaks of HPAIV infection constitute a substantial risk to human health, the poultry industry and the global economy (Walsh *et al.*, 2017) and are capable of mutating into forms that are more transmissible among humans, increasing the risk of a pandemic and high morbidity and mortality event (Zeynalova *et al.*, 2015). Moreover, LPAIVs that circulate in poultry and other animals also pose a threat to public health (Zeynalova *et al.*, 2015). Hence, the identification of AIV hotspots could help develop intervention strategies to block epizootic emergence in domestic poultry before severe losses to poultry stocks accrue or zoonotic transmission to humans ensues.

While AIV cause mild to severe disease in various animal species, IDV has been associated with bovine respiratory disease (BRD) complex which is the most economically significant disease of the beef industry with economic losses being attributed to morbidity, mortality, treatment costs, and reduced carcass value (Collin *et al.*, 2015, Taylor *et al.*, 2010). IDV was recently discovered in swine with respiratory disease in the USA in 2011 (Hause *et al.*, 2013). Since its discovery, serological evidence of IDV has been reported in healthy and symptomatic cattle populations in multiple geographical regions including the USA (Luo *et al.*, 2017, Ferguson *et al.*, 2015, Hause *et al.*, 2014, Hause *et al.*, 2013), Europe (Dane *et al.*, 2019, Chiapponi *et al.*, 2019, Chiapponi *et al.*, 2016, Ducatez *et al.*, 2015), Asia (Zhai *et al.*, 2017, Murakami *et al.*, 2016) and Africa (Salem *et al.*, 2017), suggesting that cattle could be the natural reservoir hosts of this new virus (Hause *et al.*, 2014). Further, serological evidence of IDV has been reported in small ruminants in the USA (Quast *et al.*, 2015). Despite various studies on the virological and serological evidence of IDV, its zoonotic potential and pathogenicity in other hosts, including humans, remain obscure.

Newcastle disease (ND) is another significant disease of poultry. It is highly contagious and is one of the most important viral avian diseases with a considerable threat to the poultry industry worldwide (Bello *et al.*, 2018b). It is considered a differential diagnosis for high pathogenicity avian influenza (HPAI) as it causes clinical symptoms similar to those of HPAIVs, including severe respiratory,

gastrointestinal, and neurological lesions in infected birds (Zeynalova *et al.*, 2015). It is classified as a notifiable disease by the World Organization for Animal Health (WOAH) in its highly pathogenic form (WOAH, 2022). It is caused by virulent strains of *avian orthoavulavirus* 1 (AOAV-1) (formerly designated as *avian avulavirus 1* (AAvV-1)), also known as *avian paramyxovirus 1* (APMV-1) or Newcastle disease virus (NDV, used hereafter), belonging to the genus *Orthoavulavirus* of the family *Paramyxoviridae* in the Order *Mononegavirale* (Amarasinghe *et al.*, 2019, Dimitrov *et al.*, 2019). NDV is an enveloped, single-stranded, negative-sense RNA virus with a non-segmented genome of approximately 15.2kb and has six open reading frames (ORF) which encode six major structural proteins (Locke *et al.*, 2000, Steward *et al.*, 1993). Non-commercial poultry and wild waterfowl seem to be the natural reservoirs for both virulent and avirulent NDV strains (Miller and Koch, 2013, Alexander, 1995).

Based on pathogenicity in chickens, NDV is classified into three major pathotypes; velogenic (highly virulent), mesogenic (moderately virulent) and lentogenic (avirulent or low virulent). Additionally, NDV strains have been classified into two distinct classes based on the phylogenetic analysis of the full-length fusion (F) gene, namely class I (genotype I) and class II (genotypes I–XXI), all within a single serotype (Dimitrov *et al.*, 2019). Class II viruses have been known to have caused at least five ND panzootics, and viruses from genotype VII are responsible for the 5$^{th}$ panzootic (Miller *et al.*, 2015), which originated in South-East Asia, with the earliest known outbreaks beginning around 1985. Genotypes V, VI, and VII of virulent viruses are the predominant genotypes circulating worldwide (Miller *et al.*, 2010). Genotype VII is particularly important as it has been associated with many of the most recent outbreaks in Asia, Africa, and the Middle East (Khan *et al.*, 2010, Kim *et al.*, 2007b, Liu *et al.*, 2003). In Zambia, ND continues to contribute to a high burden of morbidity and mortality among village chickens nationwide, and is considered the leading cause of death in chickens (Stepanovic and Ngulube, 2015 , Musako and Abolnik, 2012, Alders *et al.*, 1994). Recent genetic studies conducted in Zambia revealed the circulation of NDV from genotypes VII.2 and XIII among chickens in the Eastern province (Abolnik *et al.*, 2018, Abolnik *et al.*, 2017). Despite these studies, which were very limited in geographical coverage, there is a paucity of data regarding the circulation of NDV in live bird markets (LBMs) and wild waterfowls

in Zambia. Currently, only one whole genome sequence of genotype XIII exists in GenBank from Zambia.

While AI and ND are zoonotic respiratory diseases of birds, the coronavirus disease 2019 (COVID-19) pandemic caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has resulted in more than 750 million confirmed cases and over six million deaths worldwide as of 29 March 2023 (WHO, 2023). The global fatality of the disease is approximately 1.0% (WHO, 2023), and infected people have a range of clinical outcomes from asymptomatic to severe disease and death (Kim *et al.*, 2022). Despite the concerted efforts to develop therapeutical drugs and effective vaccines for COVID-19, the number of cases continues to rise. Furthermore, several SARS-CoV-2 variants carrying mutations with concerning phenotypic implications on current pandemic management strategies have emerged (Sanyaolu *et al.*, 2021). Of particular significance to the ongoing pandemic are SARS-CoV-2 variants designated variants of concern (VOCs). Several VOCs have been described, including Alpha (B.1.1.7), Beta (B.1.351), Gamma (P.1), Delta (B.1.617.2), and Omicron (B.1.1.529). VOCs are associated with enhanced transmissibility or virulence, reduction in neutralisation by antibodies obtained through natural infection or vaccination, the ability to evade detection, or a decrease in therapeutic or vaccination effectiveness (Aleem *et al.*, 2022, Dinnon *et al.*, 2020).

In Zambia, the first known COVID-19 cases were reported on 18 March 2020 from travellers returning from Europe (Chipimo *et al.*, 2020). Since then, the country has experienced four major COVID-19 pandemic waves characterised by the dominance of a particular SARS-CoV-2 lineage or VOC. Therefore, as the pandemic continues to evolve, it remains crucial to monitor and understand the virus evolution and outbreak dynamics, particularly in strategically positioned regions such as the Southern Province, which is a trade entry point of Zambia for all imports and exports from Southern Africa. However, there is limited data regarding the genomic characteristics of SARS-CoV-2 in Zambia, with only two genomic studies reporting the detection of SARS-CoV-2 belonging to lineage B.1.1. (Simulundu *et al.*, 2021) and the B.1.351 variant (Mwenda *et al.*, 2021).

Therefore, this study was undertaken to sequence the whole genome of AIVs, NDV and SARS-CoV-2 circulating in selected parts of Zambia to improve our understanding of their genetic diversity, phylogenetic relationships and inform disease mitigation strategies.

### 1.1 Statement of the Problem

Outbreaks of avian influenza (AI) and Newcastle disease (ND) can have severe repercussions for animal and public health. They result in losses of unprecedented proportions to the global poultry industry, impacting national economies and international trade of live poultry and poultry products in the affected countries (Ekong *et al.*, 2018, Miller *et al.*, 2010). The losses are usually associated with high morbidity and mortality rates which may approach 100% in severe cases and a reduction in egg production (Awuni *et al.*, 2019, El Houadfi *et al.*, 2016, Miller *et al.*, 2010). Moreover, the rapid and unpredictable evolution of AIVs poses a threat to human health, the poultry industry and the global economy. Despite efforts at disease management that include improved surveillance systems, biotechnology, and modern vaccine development strategies, among others, we still experience significant epidemics in humans, including pandemics such as the 2009 H1N1 (CDC, 2010, Guan *et al.*, 2010, Cao *et al.*, 2009) and the devastating outbreaks in poultry particularly those caused by the Goose/Guangdong (Gs/GD) clade 2.3.4.4 H5Nx across the globe including South Africa, neighbouring Zimbabwe and Botswana (Letsholo *et al.*, 2022, Abolnik *et al.*, 2019, Khomenko *et al.*, 2018). The constant emergence of new AIV strains makes it particularly challenging to predict the behaviour, spread, virulence or potential for human-to-human transmission of AIVs. Moreover, the emergency of IDV causing respiratory illness in livestock including the BRD complex has a major negative economic impact on the beef and dairy industry globally. However, there is limited data on the epidemiology of IDV in Africa. Furthermore, little is known about the NDV strains circulating in wild birds, their evolution and role in epidemiology as well as the role that LBMs may play in the dissemination of the virus including AIV in Zambia.

SARS-CoV-2 has continued to spread globally and poses a threat to human health (Hirotsu and Omata, 2021) despite the concerted efforts to develop therapeutic drugs and vaccines. Of particular concern to the ongoing pandemic are SARS-CoV-2

variants designated variants of concern (VOCs). These variants are associated with enhanced transmissibility or virulence, reduction in neutralisation by antibodies obtained through natural infection or vaccination, the ability to evade detection, or a decrease in therapeutic or vaccination effectiveness (Aleem *et al.*, 2022, Dinnon *et al.*, 2020). Despite studies done in Zambia, which were very limited in scope, there is a paucity of data regarding the circulation of SARS-CoV-2 in the Southern Province of Zambia.

## 1.2 Research Questions

1. What is the prevalence and subtypes of AIV circulating in domestic and wild birds in Africa?
2. What is the prevalence of influenza A and D viruses circulating in non-human mammalian hosts in Africa?
3. What are the AIV subtypes circulating in wild waterfowl and poultry in Zambia?
4. What genotypes of NDV circulate in wild waterfowl and poultry in Zambia?
5. What are the genetic characteristics of AIVs and NDV circulating in wild waterfowl and poultry in selected parts of Zambia?
6. What are the genetic characteristics of SARS-CoV-2 circulating in Southern Province of Zambia?

## 1.3 Objectives

### 1.3.1 General Objective

To evaluate the circulation and genetic characteristics of whole genomes of avian influenza viruses, influenza D virus, Newcastle disease virus and severe acute respiratory syndrome coronavirus 2.

### 1.3.2 Specific Objectives

1. To determine the prevalence, seroprevalence and virus subtypes of avian influenza viruses detected in domestic and wild birds in sub-Saharan Africa.
2. To investigate the prevalence of influenza A virus and influenza D virus in non-human mammalian hosts in Africa.
3. To identify avian influenza viruses and Newcastle disease virus isolated in wild waterfowl and poultry in live bird markets in selected parts of Zambia.

4. To determine the genetic diversity of avian influenza viruses and Newcastle disease virus isolated from wild waterfowl and poultry in selected parts of Zambia.
5. To determine the genetic characteristics of severe acute respiratory syndrome coronavirus detected in humans in Southern Province of Zambia.

### 1.3 Significance of the Study

AIVs are known to constantly evolve and cross-species barriers. The genetic diversity of AIVs is ever-increasing, and every so often, novel subtypes are being discovered (Kuchipudi and Nissly, 2018). Also, the unpredictability of the subtype or strain that may cause the next zoonotic or pandemic influenza threat (Offeddu *et al.*, 2016, Guan *et al.*, 2007) underscores the need to monitor the profile of circulating AIVs as a key element of pandemic preparedness. Moreover, since it is difficult to completely eradicate AIVs, IDVs and NDV from their hosts, animal disease surveillance is fundamental for disease prevention and control (Qiu *et al.*, 2019). It is also an essential tool for obtaining information which can guide prevention, control and eradication strategies, particularly in determining whether existing vaccines and medical interventions will be effective against novel influenza viruses and NDV strains. Furthermore, animal surveillance is crucial in helping to establish the potential for AIVs to infect humans and in identifying novel strains with pandemic potential. Moreover, genomic surveillance of AIV and NDV is crucial in providing insights into the transmission, evolution dynamics, identification of novel variants and virulence determinants. Additionally, SARS-CoV-2 genomic surveillance is cardinal in informing public health mitigation strategies.

Overall, this study is important because it provides novel insights into the circulation of AIV and NDV in live bird markets as well as the circulation of SARS-CoV-2 in the Southern province of Zambia. The utilization of next-generation sequencing further enhances our comprehension of these viruses, which is essential for developing effective control measures.

# CHAPTER TWO

## 2.0 Literature Review

## 2.1 Avian Influenza Viruses

### 2.1.1 Taxonomy and Nomenclature of Avian Influenza Viruses

The nomenclature and taxonomy of the influenza virus are complex and have changed over the years due to a large number of viruses isolated from the late 1960s onwards (Alexander and Brown, 2009). AIV belongs to the *Orthomyxoviridae* family of segmented negative-sense RNA viruses, Genus *Alphainfluenzavirus* (ICTV, 2018). Other viruses within the family *Orthomyxoviridae* include Genus *Betainfluenzavirus, Gammainfluenzavirus, Deltainfluenzavirus, Isavirus, Quaranjavirus* and *Thogotovirus* based on the serological cross-reactivity to the nucleoprotein and matrix protein (Hutchinson, 2018, ICTV, 2018).

The nomenclature system that is in use for influenza virus strains includes the type of influenza (A, B, C or D), host of origin (this is omitted for humans, and if the virus was isolated from inanimate material, this material should be used instead of the host), geographical location (usually a country or state), strain reference number, year of isolation and for type A viruses: the HA and NA subtypes (Alexander and Brown, 2009, WHO, 1980). For example, A/lake water/Alberta/1/77 (H4N6) (Alexander and Brown, 2009) and A/pelican/Zambia/01/06 (H3N6) (Simulundu *et al.*, 2009). Additionally, the WHO, the World Organisation for Animal Health (WOAH), and the FAO (2012) recently established a nomenclature system for classifying H5 HPAIV. This system is based on the phylogenetic analysis of the HA sequences that have evolved from the A/goose/Guangdong/1996 H5N1 virus. This has led to viruses being grouped into clades based on their phylogenetic characteristics and the HA gene sequence homology. The average percentage of pairwise nucleotide distances between clades is higher than 1.5% and is less than 1.5% within clades (WHO/OIE/FAO H5N1 Evolution Working Group, 2012).

### 2.1.2 Avian Influenza Virus Genome

AIV is either spherical or pleomorphic (ranging from spheres to extremely long filaments) with a helical capsid. The spherical virion is 80-120nm in diameter (Hutchinson, 2018, Webster *et al.*, 1992), but the filamentous forms are often more

than 300nm in length. They are enveloped, negative-sense single-stranded RNA viruses with a segmented genome containing eight RNA segments that encode several proteins (Figure 2.1) (Kim *et al.*, 2018, Mostafa *et al.*, 2018, Taubenberger and Kash, 2010). The eight segments of the AIV genome encode at least 10 classical proteins, namely, polymerase basic 1 (PB1), polymerase basic 2 (PB2), polymerase acid (PA), hemagglutinin (HA), nucleoprotein (NP), neuraminidase (NA), matrix 1 (M), matrix 2 (M2), non-structural (NS1) and nuclear export protein (NS2/NEP) (Lamb and Choppin, 1981, Lamb *et al.*, 1978, Inglis *et al.*, 1977, Inglis *et al.*, 1976). In addition, a variable number of accessory proteins that are dependent on the strain have been described PB1-F2, PB1-N40, PA-X, M42, PA-N155, PA-182, NS3 and PB2-S1 (Yamayoshi *et al.*, 2016, Muramoto *et al.*, 2013, Jagger *et al.*, 2012, Selman *et al.*, 2012, Wise *et al.*, 2012, Wise *et al.*, 2011, Wise *et al.*, 2009, Chen *et al.*, 2001, Shih *et al.*, 1998).

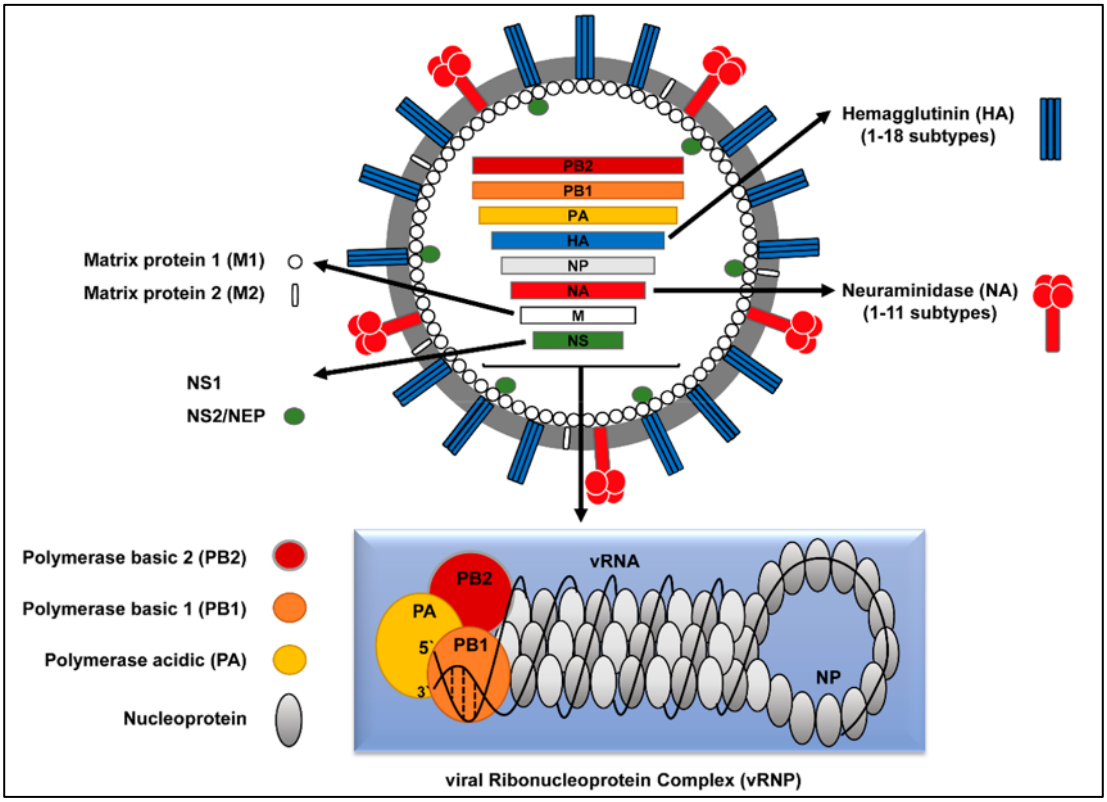


**Figure 2.1.** Diagrammatic representation of Avian Influenza Virus Genome with eight RNA segments and the viral Ribonucleoprotein Complex**.** Adapted from Mostafa *et al.* (2018)

Generally, the AIV particle is made up of the host-derived viral envelope covered with projections of two major viral transmembrane glycoproteins, with HA being the most abundant (~80%), followed by NA (~17%), along with a small number of an

integral tetrameric membrane protein called M2 with ion channel activity (Mostafa *et al.*, 2018, Taubenberger and Kash, 2010). The HA and NA proteins are essential for virus infectivity and classification into subtypes. The HA protein is responsible for virus attachment to the host cell and is the principal target of the humoral immune responses, while the NA protein plays a role in the increase and spread of progeny virions by removing sialic acid from glycoproteins (Kim *et al.*, 2018). The M1 underlies the inner surface of the envelope, and the NEP/NS2 protein is detected in minor amounts and is a consistent component of influenza virions. The main three RNA segments (PB2, PB1 and PA) encode the viral RNA-dependent RNA polymerase (RdRp) responsible for RNA synthesis and replication in the infected cells (Havasi *et al.*, 2022). The viral core comprises the eight viral RNA (vRNA) segments (890–2341 nucleotides) in association with the viral NP and the RdRp subunits (PB2, PB1, and PA) to constitute the biological active replication/transcription units of influenza virus, called viral ribonucleoprotein (vRNP) complexes (Palese and Shaw, 2007).

### 2.1.3 The Replication Cycle of AIVs

Following the entry into a cell, the HA protein on the surface of the virion recognises and binds to cell-surface receptors, containing either α2,3- or α2,6-linked sialic acid residues on the surface of host cells and initiates signaling cascades that facilitate internalisation of the virus via receptor-mediated endocytosis (Meineke *et al.*, 2019). The HA proteins of AIVs preferentially recognise sialic acid linked to galactose by an α2,6 linkage and α2,3-linkage in humans and birds, respectively (Siaα2,3Gal) (Matsuoka *et al.*, 2013). The exact mechanism of endocytosis is unknown, but it has been speculated that the influenza virus can use both clathrin-dependent and clathrin-independent mechanisms to enter the cell (Nuñez and Ross, 2019, Lakadamyali *et al.*, 2004). After binding, the virus is internalised, and the endosome is trafficked and acidified leading to an irreversible conformational change in the HA molecule on the influenza virus and exposes the hydrophobic fusion peptide (Krammer *et al.*, 2018). The fusion peptide inserts into the endosomal membrane that causes the fusion of the viral and endosomal membrane (Nuñez and Ross, 2019). Proton influx through the M2 protein leads to acidification of the interior of the virus particles, thereby separating the viral M1 from viral ribonucleoprotein (vRNP) complexes (Matsuoka *et al.*, 2013). This mechanism allows for the release of vRNP into the cytoplasm and

subsequently into the nucleus, where they serve as templates for genome transcription and replication (Nuñez and Ross, 2019, Matsuoka *et al.*, 2013, Lakadamyali *et al.*, 2004). All this requires the host machinery.

The replication and transcription of AIV genomic RNAs take place in the nucleus and are catalysed by the trimeric viral polymerase complex composed of PB2, PB1, and PA. Replication starts with the synthesis of a positive-sense copy of vRNA, which is then copied to produce large amounts of vRNA (Matsuoka *et al.*, 2013). Influenza vRNA segments do not contain a 5' cap for the RdRp to perform transcription, so the PB1, PB2, and PA components perform 'cap-snatching' of host DNA to complete this process. Cap-containing viral mRNA is released into the cytoplasm to be translated by the host ribosome machinery (Nuñez and Ross, 2019). After transcription, the viral mRNAs are exported to the cytoplasm and translated by cellular ribosomes. After that, the protein components of the vRNP complex are imported into the nucleus, where progeny vRNP complexes are assembled (Eisfeld *et al.*, 2015). The NS2 and M1 help escort the new viral proteins to the host cell membrane, where they assemble and bud newly synthesized virions (Nuñez and Ross, 2019).

### 2.1.4 Evolution and Ecology of Avian Influenza Viruses

#### 2.1.4.1 Antigenic Drift and Shift

AIVs are known to be species jumpers and promiscuous, frequently spilling over from their natural host, infecting new host species, and spread and evolve rapidly (Kuchipudi and Nissly, 2018). This rapid evolution has been attributed to the low fidelity of RdRp and the lack of a proofreading mechanism leading to approximately $1 \times 10^{-3}$ to $8 \times 10^{-3}$ substitution per site annually (Kuchipudi and Nissly, 2018, Taubenberger and Kash, 2010). These evolutionary dynamics of AIVs lie in their complex multi-host ecology, viral structure, and segmented genome (Nelson and Vincent, 2015). Furthermore, this evolution is accomplished by two mechanisms: antigenic drift and antigenic shift.

Antigenic drift involves point mutations over time that may alter antigenic sites such that a host immune system can no longer recognise them (Kuchipudi and Nissly, 2018), thereby offering the viral strains a selective advantage. This is especially

important in influenza A viruses (IAVs) adapted to humans, which are subjected to strong population immunologic pressures (Taubenberger and Kash, 2010). Interestingly, the point mutation rate is lower in AIV compared to that in humans (Mostafa *et al.*, 2018). Also, a slower evolution rate has been observed in AIVs isolated from wild waterfowls compared with those from terrestrial poultry, swine or humans (Mostafa *et al.*, 2018). However, it is not negligible (Olsen *et al.*, 2006). This slow evolution of AIV in their natural host is probably due to the adaptation of AIV to new hosts, while genetic stasis is maintained in its natural reservoir (Mostafa *et al.*, 2018).

Antigenic shift is the exchange of genome segments between two influenza virus subtypes resulting in a variant virus significantly different from both parent viruses (Kuchipudi and Nissly, 2018). It is theoretically believed that antigenic shift or reassortment results from co-infection with two different influenza virus types. However, the proteins of both viruses must be compatible for the reassortment to occur (Kuchipudi and Nissly, 2018). There are supposedly 256 ($2^8$) probable combinations of the eight gene segments from reassortment between two parental viruses (Taubenberger and Kash, 2010). Additionally, reassortment is common and vital in AIV evolution and species barrier crossing (Taubenberger and Kash, 2010, Dugan *et al.*, 2008). Through reassortment, novel AIV with pandemic potential in humans may be created (Urbaniak and Markowska-Daniel, 2014). It is worth mentioning that antigenic shift and drift resulted in five well-documented influenza pandemics since 1900 and in yearly repeated seasonal epidemics, respectively (Mostafa *et al.*, 2018, Urbaniak and Markowska-Daniel, 2014). This makes AIVs important global pathogens which call for consented efforts globally in controlling their spread. Furthermore, surveillance of AIV remains key in mitigating the risk of reassortment and preventing possible pandemics in humans as well as epidemics in poultry and humans.

#### 2.1.4.2 Persistence and Mode of Transmission

The incidence of AIVs in birds exhibits strong seasonal fluctuations and raises questions regarding where the virus is perpetuated between outbreaks. Several factors play a role in this seasonal fluctuation and persistence between epidemics. Some of these factors are based on the characteristics of the aquatic bird reservoir

system, which include subclinical viral infections within these avian hosts, an efficient transmission mechanism involving the aquatic habitats used by these avian species (Webster *et al.*, 1992) and viral stability in water (Faust *et al.*, 2009).

Transmission of AIVs among wild waterfowl populations is believed to occur through an indirect faecal-oral route in which virus-contaminated water facilitates dissemination via surface water (Faust *et al.*, 2009). In birds, LPAIVs preferentially infect cells lining the intestinal tract, replicate within the host and the virus is excreted in high concentrations into the environment in their faeces (Faust et al., 2009, Olsen et al., 2006). In addition to playing an essential role in transmitting AIVs within wild waterfowl populations, the aquatic environment contributes to the long-term maintenance of AIVs within avian populations (Webster *et al.*, 1992). The aquatic environment contributes to the maintenance of AIVs for an extended period because they can remain infectious in lake water for up to four days at 22°C and more than 30 days at 0°C (Olsen *et al.*, 2006). However, the ability of the virus to persist in water depends on water temperature, pH and salinity (Faust *et al.*, 2009). Therefore, it is also important to note that transmission of IAVs is also dependent on the ecology and the virus distribution (Olsen et al., 2006). The persistence of AIVs in aquatic environments supports the theory that AIVs can persist and reappear after a long period.

### 2.1.5 Epidemiology of Avian Influenza Viruses

#### 2.1.5.1 Avian Influenza Viruses in Wild Waterfowls

Genetically and antigenically diverse AIVs are widely distributed in wild avian species worldwide (Taubenberger and Kash, 2010). They are maintained mainly through asymptomatic infections as LPAIVs, most frequently documented in wild waterfowls of the orders *Anseriformes* and *Charadriiformes* (Taubenberger and Kash, 2010). *Anseriformes* include over 170 species of waterfowl, among them the ducks, geese, and swans, while *Charadriiformes* include gulls, terns, and waders. Additionally, *Anseriformes* and *Charadriiformes* are distributed globally, except for the most arid regions of the world (Olsen *et al.*, 2006). Hence, wild birds represent the major natural reservoirs for LPAIVs (Olsen *et al.*, 2006, Webster *et al.*, 1992). At least 105 species of wild birds of 26 different families have been identified as harbouring LPAIVs (Olsen *et al.*, 2006).

AIVs possess antigenically and genetically diverse HA and NA subtypes which are not uniform among wild waterfowl AIV isolates, with some HA subtypes being more common than others (Taubenberger and Kash, 2010). Although most HA subtypes have been isolated from the *Anseriformes,* HA subtypes H13 and H16 have been isolated predominantly from *Charadriiformes*. Furthermore, all combinations of HA 1–16 and NA 1–9 subtypes have been isolated from wild waterfowl (Okazaki *et al.*, 2000, Ito *et al.*, 1995). Moreover, 103 of the possible 144 AIV HA-NA subtype combinations have been found in wild waterfowl (Dugan *et al.,* 2008). Hence, wild waterfowl pose a unique risk for introducing AIVs of all subtypes to poultry kept in free-range or outdoor facilities (Bouwstra *et al.*, 2017).

Maintenance of AIVs in wild waterfowl has been associated with stable host switch events to novel hosts, including poultry, horses, swine, and humans, leading to the emergence of viral lineages transmissible in the new host, though adaptation to poultry species is the most frequent (Taubenberger and Kash, 2010). Stable host switching may involve the acquisition of many mutations, depending on the virus and the species that separates an individual clonally derived AIV strain from the large wild waterfowl AIV gene pool.

The first evidence of AIV infection in wild birds arose in 1961, during which approximately 1,300 common terns (*Sterna hirundo*) died in an outbreak in South Africa (Capua and Alexander, 2006, Becker, 1966). Since then, studies have been conducted across the globe and have documented the presence of LPAIVs and HPAIVs in wild waterfowl (Abolnik *et al.*, 2022, Khomenko *et al.*, 2018, Si *et al.*, 2016, Zhao *et al.*, 2012, Simulundu *et al.*, 2011, Simulundu *et al.*, 2009). The increase in the number of studies has been a result of the introduction of high-throughput molecular diagnostic methods. These studies have provided vital information regarding AIV epidemiology in wild waterfowls. It has been observed that AIV prevalence in wild waterfowl follows a repeatable seasonal pattern, with high incidence in autumn, during migration, followed by a marked drop in winter, and only a few infected birds in the spring (Tolf *et al.*, 2012).

In Europe, surveillance studies conducted in wild waterfowl in the Netherlands (Bergervoet *et al.*, 2019), Sweden (Wallensten *et al.*, 2007), Portugal (Tolf *et al.*,

2012) and Germany (Suss *et al.*, 1994) revealed the circulation of a diversity of LPAIV subtypes with the prevalence ranging from 4–12.5%. In the Netherlands, 55 HA/NA subtype combinations were detected, with the majority being H6N3, H13N8, H13N8, and H3N8, while in Portugal, eight subtypes, including H4N6, H1N1, H3N2, H3N8, H4N6, H6N5, H9N2, H10N7, and H11N3 (Bergervoet *et al.*, 2019, Tolf *et al.*, 2012). Furthermore, 11 HA/NA subtype combinations were detected in Germany, including H1N1, H2N3, H3N3, H3N6, H4N6, H4N7, H6N1, H7N3, H10N4 and H10N8 while 40 HA/NA subtypes were found in Sweden with most prevalent combinations being H4N6, H7N7, and H6N2 (Wallensten *et al.*, 2007, Suss *et al.*, 1994). Moreover, most of the viruses were isolated from mallards (*Anas platyrhynchos*) in the Netherlands, Sweden and Portugal (Bergervoet *et al.*, 2019, Tolf *et al.*, 2012, Wallensten *et al.*, 2007), while most of the isolates were from feral ducks in Germany (Suss *et al.*, 1994). This could have been due to sampling bias, as most of the birds sampled were mallards. Studies in Europe revealed a significant temporal variation in the AIV prevalence, including a pronounced peak in fall and spring and among predominantly young birds compared to adults, indicating that AIV circulates within wetland breeding populations. Phylogenetic analysis of the surface genes of selected AIV subtypes in the Netherlands and Portugal showed that the wild waterfowl viruses were closely related to viruses circulating in local poultry, and the Portuguese AIV were closely related to viral sequences from Central Europe as well as those in the southern parts of Africa, respectively (Bergervoet *et al.*, 2019, Tolf *et al.*, 2012).

In North America, surveillance of wild ducks and shorebirds for 26 and 16 years, respectively, revealed differences in the prevalence of AIVs between these hosts (Krauss *et al.*, 2004). Shorebirds had a high frequency of AIV isolation during their northern migration, while wild ducks had high virus isolation frequencies during their southern migration. Shorebirds manifested a broader range of subtypes suggesting that shorebirds are the leading source of some viruses (such as H5), which are isolated less frequently from wild ducks (Krauss *et al.*, 2004). HA subtypes H1 through H12 occurred in both hosts, H13 occurred only in shorebirds, while H14 and H15 were not detected.

In Asia, studies have detected AIVs from wild waterfowl confirming their importance as the natural reservoir of these pathogens. In China, AIVs have been isolated in wild waterfowl, including the HPAIVs H5N1 initially isolated from geese in the Guangdong Province in 1996, and outbreaks in wild waterfowl at two waterfowl parks in Hong Kong reported in 2002 (Zhao *et al.*, 2012). In spring 2009, one strain of the H5N1 clade 2.3.2 virus was isolated from wild swans in Shanghai, indicating the importance of the wild swan in the ecology of this HPAIV in Eastern China (Zhao *et al.,* 2012). In Japan, Influenza virus surveillance of ducks has been done since the late 1970s (Olsen *et al.*, 2006). As in other studies, AIV prevalence and subtypes varied between years and locations. Surveillance of AIVs in migratory aquatic birds in Eastern Hokkaido in Japan revealed the presence of various subtypes of IAVs, including H3N5, H3N6, H3N8, H4N2, H4N6, H6N5, H6N8, and H11N3 (Bui *et al.,* 2010). Hokkaido is a crucial area of Japan for AIV surveillance because it is located within multiple wild bird flyways (Bui *et al.,* 2010). In Mongolia, the H5N6 HPAIV belonging to subclade 2.3.4.4h was recently isolated from Whooper Swans (*Cygnus cygnus*), and one Swan Goose (*Anser cygnoides*) were found dead at three different locations (Ankhanbaatar *et al.*, 2022). Isolation of HPAIVs and LPAIVs in wild waterfowl indicates these birds' importance in dispersing these viruses.

In sub-Saharan Africa, AIVs have also been isolated in wild birds giving a prevalence rate ranging from 2.5% - 3.5% (Cumming *et al.*, 2011, Gaidet *et al.*, 2007). In Nigeria, an H5N2 LPAIV was isolated in three spur-winged geese (*Plectropterus gambensis*) in the Hadejia–Nguru wetlands (Snoeck *et al.*, 2011). The presence of LPAIV H5N2 in wild birds in the Hadejia–Nguru wetlands, where wild waterfowl and poultry occasionally mix, provides ample opportunity for infection across species boundaries, with the potential risk of generating HPAIVs after extensive circulation in poultry (Snoeck *et al.*, 2011). A study in southern Africa (South Africa, Botswana, Zimbabwe and Mozambique) revealed an overall prevalence of 2.5%. Two viruses (H1N8 and H3N8) were isolated, and additional H5, H6 and H7 strains were identified, and there was no detectable influence of the annual influx of Palearctic migrants (Cumming *et al.*, 2011). A recent study in South Africa demonstrated the presence of the HPAIV clade 2.3.4.4B H5N1 and other

LPAIVs H3N1, H4N2, H5N2, H8Nx and H9N2 in wild waterfowl (Abolnik *et al.*, 2022).

In Zambia, AIV surveillance conducted in 2006 and 2008–2009 resulted in the isolation of 13 viruses of distinct subtypes (H3N6, H3N8, H4N6, H6N2, H9N1 and H11N9) from wild waterfowl (Simulundu *et al.*, 2011, Simulundu *et al.*, 2009). However, compared to surveillance activities in other regions of the world, AIV surveillance in wild birds in Zambia and southern Africa is patchy and limited in geographical coverage (Cumming *et al.*, 2011). Surveillance for AIVs in wild birds should not be underestimated as these viruses have contributed gene segments to virus strains that have caused human influenza pandemics (Taubenberger and Morens, 2009, 2006, Kilbourne, 2006) and have the potential to cause future pandemics. Moreover, from Africa, only a relatively few complete AIV sequences have been deposited in public databases suggesting a gap in the surveillance activities on the continent as a whole. It is also worth noting that some of the viruses isolated from Zambia harboured particular residues predominantly observed in human influenza viruses in their genome and showed higher levels of replication and morbidity in animal experiments (Simulundu *et al.*, 2011). Besides, these isolates had genes related to AIVs isolated from wild and domestic birds in South Africa (Simulundu *et al.*, 2011). Hence, the presence of AIVs in wild waterfowl in Zambia and Southern Africa stresses the need for continuous surveillance of AIVs in the wild, as these viruses are capable of crossing species barriers.

#### 2.1.5.2 Avian Influenza Viruses in Poultry

Domestic birds of the order *Galliformes,* such as turkeys, chickens, and quails, are not a reservoir host of AIVs but are susceptible to infection with wild-bird-derived AIV after adaptation. Once AIVs are adapted to gallinaceous poultry, they rarely circulate in the wild bird AIV viral pool (Taubenberger and Kash, 2010). Poultry may become infected with AIVs through direct contact with infected waterfowl or poultry (Nuñez and Ross, 2019). Infection of poultry with LPAIV can result in little to no disease, whereas infection with HPAIV is associated with systemic infection with high mortality in chickens. This is due to the possession of multiple basic amino acid residues at the HA cleavage site, which are recognised by ubiquitous intracellular proteases such as furin, rendering these viruses capable of causing

systemic infection with fatal outcomes in chickens (Senne *et al.*, 1996). Highly pathogenic avian influenza (HPAI) is restricted to subtypes H5 and H7, though not all H5 or H7 cause HPAI (Aly *et al.*, 2008, Alexander, 2007). Hence, sporadically, strains of poultry-adapted H5 or H7 AIV evolve into HPAI.

HPAI is one of the most important zoonotic infectious diseases of the 21st century, and its emergence constitutes a substantive risk to both human health and the economy (Walsh *et al.*, 2017). It was first identified in 1878 in Italy as a highly lethal, systemic disease of chickens as fowl plague and spread to the rest of Europe (Bertran *et al.*, 2017, Perroncito, 1878) and was later identified in the United States and Africa. The current burden of disease associated with the Gs/Gd-lineage of H5Nx HPAIVs was first documented in China in 1997 when poultry farms were devastated by the emergence of an epizootic (Walsh *et al.*, 2017), though outbreaks had been reported as early as 1996. Since then, the Gs/Gd-lineage of H5Nx HPAIVs virus has spread to other parts of Asia, Europe, the Middle East and West Africa, causing deaths in poultry as well as infections in humans (Fasanmi *et al.*, 2017, Duan *et al.*, 2008, Joannis *et al.*, 2006).

Despite reports of AIV outbreaks among poultry around the globe, poultry farming remains common in most parts of the world. Poultry farming in many countries includes commercial agriculture with a high density of poultry population, free-range rearing (backyards, rooftops) and trading via LBMs. Backyard birds have also become an intermediate transmission host for AIV infection of poultry in the commercial sector and LBMs and human infection (Kim, 2018). Outbreaks and sporadic cases of AIVs have been reported around the world. However, most of the surveillance activities of AIV in poultry have concentrated on the detection of subtypes H5, H7 and/or H9 though other subtypes can be detected if investigated (Naguib *et al.*, 2019). For instance, an H10N7 LPIAV outbreak occurred on a chicken farm in Australia in 2010, leading to transmission from bird to human (Arzey *et al.*, 2012). In southern China, systematic surveillance of minor poultry species, including quail, chukar, guinea fowl, partridges, and pheasants, in live poultry markets from 2000 to 2005 resulted in the isolation of H6N1 and H6N2 influenza viruses (Cheung *et al.*, 2007). This indicated that H6N1 and H6N2 influenza viruses are prevalent year-round in terrestrial minor poultry species and

that the H6 influenza virus is still among the most frequently detected subtypes from domestic ducks (Cheung *et al.*, 2007).

In Africa, the first report of an HPAI outbreak caused by H5N3 was recorded in 1961, during which approximately 1,300 common terns died in South Africa (Becker, 1966). Since then, no reports of HPAI outbreaks were recorded in Africa until 2004 when an outbreak caused by an H5N2 HPAIV was reported in South African ostriches (Olivier, 2006). In 2006, the Gs/Gd-lineage H5N1 HPAIV infection of commercial poultry was first confirmed on the continent on farms in Kaduna state in northern Nigeria (Ducatez *et al.*, 2007b, Ducatez *et al.*, 2006). The virus spread rapidly to North and West African countries and resulted in losses of unprecedented proportions to the poultry industry, impacting national economies and international trade of live poultry and poultry products in the affected countries (Ekong *et al.*, 2018). Moreover, the virus was thought to have been introduced through routes that coincided with the flight pathways of migratory birds (Ducatez *et al.*, 2006), highlighting the importance of migratory birds in AIV dispersal including that of HPAIVs. Since the first outbreak of the Gs/Gd-lineage H5N1 HPAIV infection was reported in 2006 in Africa, two more waves of H5Nx HPAIVs have been confirmed including the H5N1 viruses from clade 2.3.2.1c isolated in 2015 and the 2016 clade 2.3.4.4b H5N8 viruses which emerged in western Africa and spread to southern, central, and eastern Africa (Fusaro *et al.*, 2019, Ekong *et al.*, 2018).

In southern Africa, clade 2.3.4.4b H5N8 viruses reached the Democratic Republic of the Congo (DR Congo), Zimbabwe, and South Africa in 2017 (Abolnik *et al.*, 2019, Twabela *et al.*, 2018). In February 2019, new cases were reported in Namibia (Molini *et al.*, 2020). Furthermore, the HPAI outbreak caused by the H5N1 clade 2.3.4.4b virus was recently reported in Botswana (Letsholo *et al.*, 2022). Remarkably, despite several reports of HPAI outbreaks in neighbouring countries, there has been no report of HPAIVs in Zambia, possibly due to low prevalence of the strain or due to a break in surveillance of AIVs in the country.

Apart from the havoc caused by HPAIVs, H9N2 LPAIV in poultry have gained attention because of the serious repercussions on animal health, public health and the trade of live poultry or poultry products. It also seems widespread in poultry, as

evidenced by its circulation in chickens, quail, and other poultry in Asia and has caused a few cases of influenza in humans (Matrosovich *et al.*, 2001). Since their detection in China in 1992 (Zecchin *et al.*, 2017), H9N2 LPAIVs have been extensively circulating in North African countries since the early 2000s (El Houadfi *et al.*, 2016, Monne *et al.*, 2013). From 2017 to date, H9N2 viruses have been detected in several sub-Saharan African countries: Ghana, Burkina Faso, Uganda, Kenya and Senegal, where a human case was recently reported (Fusade-Boyer *et al.*, 2021, Kariithi *et al.*, 2020, Awuni *et al.*, 2019, Zecchin *et al.*, 2017). Additionally, a surveillance study conducted in Egypt demonstrated the emergence of H9N2, which co-circulated with H5N1 circulating in poultry (Kayali *et al.*, 2014). These strains usually and frequently co-infected the same avian host, which poses a concern for potential reassortment (Kayali *et al.*, 2014). Hence, the co-circulation of HPAI H5N1 and LPAI H9N2 viruses in domestic birds in Egypt signifies a threat to animal and human health. In addition to H9N2 LPAIV, other LPAIVs have been detected in Africa including the H5N9, H6N8, H7N1, H7N7 and H10N1 isolated in ostriches in South Africa (Abolnik *et al.*, 2010). The presence of LPAIV H5N9, H7N1 and H7N7 in South African ostriches is significant in that AIV subtype H5 and H7 can evolve into HPAIV. However, in Zambia, no AIVs have been reported in poultry, and little is known regarding the ecology and genetic characteristics.

#### 2.1.5.3 Avian Influenza Virus in Other Host Species

Interspecies transmission of AIV occur among different animal species via direct or indirect contact, which may result in the introduction of viruses that are new to the recipient species and which have the potential to cause substantial outbreaks (Webster *et al.*, 1995, Webster *et al.*, 1992). Whereas most of these interspecies transmission events may not result in onward transmission and establishment in the new host, sustained influenza outbreaks have been reported in poultry and several mammalian species (Parrish *et al.*, 2015). Of the mammalian hosts, only a limited number are currently recognised as sustaining AIV transmission, and it is not clear what distinguishes these species from those for which influenza has not been reported (Parrish *et al.*, 2015). However, for AIVs to become established and achieve efficient viral replication in other hosts, they must overcome a variety of species barriers (Cauldwell *et al.*, 2014). Such barriers include host innate immune responses, several intracellular factors and recognition of different sialic acid (SA)

receptors, α-2,3 and α-2,6 expressed on host cell surfaces of avian and human respiratory epithelia, respectively (Ayim-Akonor *et al.*, 2020).

The well-known mammalian hosts for which AIVs have established themselves and formed stable lineages include humans, pigs, horses, seals, mink and dogs (Root and Shriner, 2020, Shriner *et al.*, 2012). For example, H1N1 and H3N2 subtypes in humans (Chang and Zaia, 2019, Freidl *et al.*, 2014), H1N1, H1N2, and H3N2 subtypes in swine (Krammer *et al.*, 2018, Lewis *et al.*, 2016) and H3N8 and H7N7 subtypes in horses (Waddell *et al.*, 1963, Sovinova *et al.*, 1958). Dogs emerged as important AIV hosts in the 2000s when the H3N8 equine influenza virus (EIV) and the avian virus-like H3N2 strain introduced from horses and birds, respectively, were detected in the United States of America (USA) and Asia (Li *et al.*, 2010, Crawford *et al.*, 2005). Both of these canine influenza viruses have continuously circulated in the dog population since their emergence, increasing opportunities for human exposure to these zoonotic viruses (Parrish *et al.*, 2015).

The human adapted-AIVs have caused at least four major pandemics (1918 “Spanish flu”, 1957 “Asian flu”, 1968 “Hong Kong flu” and the 2009 “swine flu”) in the human population from the 20th century to date, the worst being the 1918 “Spanish flu” H1N1 pandemic, which recorded nearly 500 million cases and 50 million human deaths globally (Taubenberger and Morens, 2009, Kilbourne, 2006). Apart from the pandemics and seasonal epidemics of influenza, there have been reports of spillovers of AIVs subtypes H5, H6, H7, H9, and H10, particularly, H5N1, H5N6, H6N1, H7N1, H9N2 and H10N8 subtypes, from the natural reservoir to humans (Liu *et al.*, 2016, Shi *et al.*, 2013, Wei *et al.*, 2013, Arzey *et al.*, 2012, Nicholson *et al.*, 2003). For instance, human cases of the H5N1 HPAIVs have been reported in Asia, the Americas and Africa (WHO, 2019, CDC, 2014). Though human H5N1 is believed to be rare, the avian-to-human transmission of influenza H5N1 virus is thought to have occurred in most human cases, and so far, only poultry and wild birds have been implicated in transmission to humans (Zhou *et al.*, 2009). Besides the H5N1 HPAI infections in humans, the H7N9 LPAIV from an avian source emerged in China in February 2013, and by June of the same year, 131 more cases were confirmed (Cowling *et al.*, 2013). It is known to have caused five waves in humans and was reported to have mutated into highly pathogenic variants (Yang *et al.*, 2017). In

Zambia, there have been no reports of HPAI H5N1 and H7N9 in either humans or poultry. However, from the reviewed literature, it is evident that poultry and wild waterfowl pose a threat to human health in terms of AI transmission. Therefore, surveillance and control of AI in poultry is cardinal in preventing the transmission of zoonotic influenza.

Swine play an important role in the ecology and epidemiology of IAVs. They are thought to be a mixing vessel of IAVs and play a significant role in their genetic reassortment. Swine is the only domesticated mammal that is susceptible to all avian and human IAVs, and it is suggested that the swine population may become a reservoir for another lineage of IAV that can be introduced worldwide and pose a significant risk (Urbaniak and Markowska-Daniel, 2014). Influenza in swine was first observed in the United States during the catastrophic 1918 to 1919 human influenza pandemic, and the signs of the disease in pigs were the same as in humans, like nasal discharge, coughing, fever, laboured breathing, and conjunctivitis (Webster *et al.*, 1992). However, it is unclear if IAV infections in swine occurred before the 1918 HINI pandemic (Taubenberger and Kash, 2010). Several studies across the globe have reported the detection of IAVs of various subtypes in swine, with a few causing enzootic infections and many causing only limited outbreaks without continued circulation (Harima *et al.*, 2022, Gomaa *et al.*, 2018, Adeola *et al.*, 2017, Kowalczyk *et al.*, 2012, Adeola *et al.*, 2010, Kowalczyk and Markowska-Daniel, 2010). The presence of these viruses raises concern regarding the potential of influenza virus reassortment in pigs and highlights the need for enhanced global monitoring, control and preventive measures for influenza virus infection in pigs.

Furthermore, AIVs of various subtypes have been documented in wild animals though these reports are mainly limited to captive animals. Examples of these introductions include H5N1 AIV infections in leopards and tigers in Thailand (Amonsin *et al.*, 2006, Keawcharoen *et al.*, 2004) and the H1N1 virus that caused the 2009 pandemic (H1N1pdm09) in cheetahs in California USA (Crossley *et al.*, 2012) and wild boars in Japan (Shimoda *et al.*, 2017). Infections in wild animals are usually thought of as being opportunistic as they usually arise through the consumption of raw meat containing the virus, especially for carnivores; hence limited or no animal-to-animal transmission occurs. However, a study by Thanawongnuwech *et al.* (2005)

in tigers, points to a probable horizontal transmission of AIVs in these animals. Although herbivores might be exempt from diet-driven pathogen transmission, sharing common feeding grounds and water sources with the reservoir host could also lead to potential transmission (Soilemetzidou *et al.*, 2020).

### 2.1.6 Diagnosis of Avian Influenza Virus

Understanding the ecology and epidemiology of AIV and subsequently control lies in the availability of reliable laboratory techniques permitting accurate and sensitive detection of AIVs (Lira *et al.*, 2010, Weinberg *et al.*, 2005). Thus, laboratory diagnosis of AIV has become a cornerstone of the prevention, containment and surveillance of the virus (Petric *et al.*, 2006). Laboratory diagnosis of influenza can be accomplished by the detection of the virus or the host's immune response to the virus. However, the success of the virus diagnosis is essentially dependent on the quality, the conditions for transport and storage of the specimens before it is processed in the laboratory (WHO, 2002). Over the years, various methods have been used for AIV diagnosis, including clinical signs, viral isolation, serological and molecular techniques (Suarez *et al.*, 2007).

#### 2.1.6.1 Clinical Signs

For AIVs presence of clinical disease alone is not diagnostic. Moreover, LPAI may not lead to visible clinical manifestations especially in wild avian species. In addition, for some avian species, disease expression is extremely variable, and clinical disease is a less reliable indicator of infection. Moreover, other respiratory viruses may cause similar nonspecific symptoms, hence, establishing a diagnosis of influenza on the basis of the clinical presentation alone is problematic in both birds and other animal species (Petric *et al.*, 2006). For HPAI infections, clinical manifestations can be a valuable tool for presumptive diagnosis in chickens and turkeys, but the causative agent must be confirmed by specific laboratory methods (Suarez *et al.*, 2007, Swayne and Suarez, 2000). Despite all the drawbacks of clinical diagnosis, it is advantageous as it is helpful in preventing the further spread of the disease by allowing the animal health authorities to initiate quarantines and other control measures until a definitive diagnosis can be made (Suarez *et al.*, 2007).

### 2.1.6.2 Virus Isolation

Virus isolation in specific-pathogen-free (SPF) or specific antibody-negative embryonated chicken eggs (ECEs) is considered a gold-standard technique in AIV diagnosis and remains a highly sensitive and suitable method when used with good-quality specimens (WHO, 2002). Its use is critical in the diagnosis of an index case AI in an outbreak, and it is usually accompanied by subsequent immunologic and/or molecular techniques for virus identification. This method is cardinal as it provides high quantities of live infectious viruses for further antigenic, biological and genetic characterisation and drug susceptibility testing (Suarez *et al.*, 2007, WHO, 2002). It also enables the production of virus stocks for epidemiological monitoring and updating of vaccines, which is a critical requirement for the diagnosis of AI (El Zowalaty *et al.*, 2013). However, virus isolation has several drawbacks that limit its use as a diagnostic test. The technique is time-consuming, labour-intensive, and is a mandatory requirement of a high level of biosecurity facilities (biosafety level 3) if HPAI is suspected (Wang and Taubenberger, 2010).

The other method used in the isolation of AIV is the cell culture which dates back to 1940 when the technique was first introduced (Petric *et al.*, 2006). This technique provides a useful method for the primary isolation of some human or swine influenza isolates that do not grow well in ECEs (Taubenberger and Layne, 2001). Various mammalian and bird cell lines have been used to isolate influenza viruses and most commonly used cells are: primary rhesus monkey kidney and rhesus monkey kidney (LLC MK2) cells, African green monkey kidney cells, mink lung epithelial cell line (Mv1Lu), buffalo green monkey kidney, Madin–Darby canine kidney, green monkey continuous cell line (Vero), human lung embryonating cells (MRC-5), CACO-2 cell lines, CCL-141 (duck embryo) cells, CCL-169 (goose embryonic kidney) cells, duck embryo fibroblast cells and chicken embryo fibroblast cells (Reina *et al.*, 1997, Meguro *et al.*, 1979) However, since AIVs have different hosts and growth properties depending on the strain (Webster, 1997, Shope, 1931) and not all host cells are universally permissive to all AIV subtypes, virus isolation is effective only when the cell culture is sensitive to the inoculated virus (Fouchier *et al.*, 2000). In addition, this method requires the rapid transport of specimens to the laboratory, since delays may lead to inactivation of the virus and hence failure to isolate any infectious agents. Another useful technique for the culture of AIVs is the rapid shell

vial culture, which uses single or mixed cell lines using monolayers of two different cell types in a single vial (Petric *et al.*, 2006). This technique has the advantage of enhancing sensitivity and shortening the time to detection through enhancing the viral infectivity of the cells by centrifugation (time to diagnosis, 24 hrs vs. 13 days), (Petric *et al.*, 2006).

#### 2.1.6.3 Serological Methods

Serological methods for the diagnosis of AI play an essential role in epidemiological strain surveillance and can provide an accurate method for the detection of influenza infection (Taubenberger and Layne, 2001). Serological methods are widely used for trade purposes to show freedom of infection from mainly LPAIVs, although it is of little value for HPAI because most birds die before producing antibodies (Suarez *et al.*, 2007). Therefore, these methods cannot be used for rapid diagnosis even in surviving birds because a considerable delay in diagnosis may allow the virus to continue to spread. Serological methods are based on the detection of the specific AIV antibodies that appear from the second-week post-infection.

The haemagglutination inhibition (HI) and neuraminidase inhibition (NI) assays are the primary methods for determining quantitative antibody titres for AIV. HI is considered the gold standard in the detection of HA-subtype-specific antibodies despite several drawbacks (Zhao *et al.*, 2013, WHO, 2002). Both HI and NI are labour-intensive, time-consuming, and have to be performed for every HA and NA subtype separately (Comin *et al.*, 2013). Alternatives to the gold standard have been developed and are in use, including agar gel precipitation (agar gel immunodiffusion), virus neutralization, complement fixation (CF), enzyme immunoassay, indirect immunofluorescence assay, enzyme-linked immunosorbent assay (ELISA), competitive ELISA also known as inhibition/blocking ELISA, nucleoprotein-based specific indirect ELISA (NP-ELISA) and multiplex serological assay using Luminex xMAP technology (Germeraad *et al.*, 2019, El Zowalaty *et al.*, 2013). As opposed to the gold standard, these methods are quicker and easier to automate. In addition, multiplex serological assay using Luminex xMAP technology is the first assay that is able to subtype all AI antibodies in one single assay, has a higher sensitivity and requires a smaller sample volume (Germeraad *et al.*, 2019).

### 2.1.6.4 Molecular Methods

Molecular detection methods detect viral nucleic acid and share the same goal of amplifying nucleic acid to high levels to allow easy identification of the organism (Suarez *et al.*, 2007). They are generally sensitive and allow timely and specific detection of viral nucleic acids, as compared to culture or antigen detection methods. However, the need for specialized equipment, trained personnel, and the high costs involved hamper their application for both diagnostics in the field and in poor countries (Shojaei *et al.*, 2015). Moreover, for any molecular diagnostic method, the RNA extraction step is a critical step because the quality of the RNA will impact the amplification efficiency (Suarez *et al.*, 2007). Several kinds of molecular methods have been developed for the detection of AI. They include polymerase chain reaction (PCR) based methods (conventional reverse transcription polymerase chain reaction (RT-PCR), real-time PCR (qPCR) and nucleic acid sequence-based methods (DNA microarray, pyrosequencing, nucleic acid sequence-based amplification (NASBA), Loop-mediated isothermal amplification (LAMP) and next generation sequencing (NGS). For RT-PCR based methods, the choice of primer sequences is the most crucial parameter if the sensitivity and specificity of the assay have to be maintained (WHO, 2002).

Conventional RT-PCR, ELISA RT-PCR, and RT-qPCR are similar techniques or utilise the same principle, with the exception of how the PCR products are detected. Therefore, purified viral RNA is reverse transcribed into cDNA by reverse transcriptase, followed by PCR-based amplification with gene-specific primers (Okamatsu *et al.*, 2016, Wang and Taubenberger, 2010). The Uni12 and Uni13 primers have been widely used in AIV diagnosis because, among the eight segments of AIV viral RNA, only nucleotides at the 5′ terminus (Uni13) and 12 nucleotides at the 3′ terminus (Uni12) is fully conserved (Desselberger *et al.*, 1980). Furthermore, primers for the detection of all the AIV HA (H1–H16) and NA (N1–N9) subtypes have been developed, including the universal primers for the full-length amplification of all AIVs (Qiu *et al.*, 2009, Hoffmann *et al.*, 2001). Conventional RT-PCR detects the PCR product by using electrophoresis in an agarose gel to separate the PCR product by size, which allows presumptive identification. Additional confirmation methods can be used to confirm that the PCR product is specific, including using a nucleic acid hybridization ELISA test and Sanger DNA

sequencing. Its main drawback is that the assay is prone to contamination, running the gels is time-consuming and the specificity of the generated PCR amplicon might be altered by nonspecific binding of the primers to other similar sequences on the DNA template. The RT-qPCR method is a rapid and sensitive assay which has eliminated the need for post-PCR screening by gel electrophoresis, allowing the definitive confirmation of the virus within minutes (Dhama, 2013). It uses a fluorescently labelled probe to detect the increase in PCR product while the test is being performed, and the results are reported in real-time. Moreover, it is highly useful in surveillance and monitoring programs.

Nucleic acid sequencing-based methods allow for sequencing and phylogenetic analysis of AIV. NGS is a novel high-throughput sequencing technology that has been developed to investigate any genomic query or clinical activity involving DNA and has enhanced sequencing volume to several billion nucleotides within a very short time and at considerably low cost (Okamatsu *et al.*, 2016). A major advantage of NGS over traditional mutation detection methods is the ability to sequence multiple genes and highlight millions of variants simultaneously. Other advantages include minimal DNA input and faster turnaround time; (Pervez *et al.*, 2022). Thus, NGS can analyse the full-length sequence information of the entire AIV genome at once and can accurately identify subtypes. Furthermore, it allows for the identification of single nucleotide variants, evolutionary analyses and the detection of novel variants (Park *et al.*, 2020, Van Poelvoorde *et al.*, 2020). Moreover, the genotypic information that complete genome sequencing provides could be critical when tracking the origin of outbreaks and forecasting the spread of disease (Van Poelvoorde et al., 2020). NGS is undoubtedly a powerful method, but it has some limitations. One of the biggest challenges is the need for advanced bioinformatics systems, fast data processing, and large data storage capabilities. These requirements can be quite expensive, making NGS a costly undertaking for many organisations (healio.com).

### 2.1.7 Prevention, Control and Treatment of Avian Influenza

Prevention and control of AI are important not only for poultry but also for the prevention of human infections and potential pandemics. Thus, preventative and control measures of AI include measures designed to prevent the introduction of the

virus, reduce the likelihood of infection of birds once the virus is introduced, prevent the movement of the virus from a premise with infected birds to another premise and, if possible, eliminate/eradicate the virus (Swayne *et al.*, 2020). The measures are achieved by five major interrelated components, including improved biosecurity, diagnostics and surveillance, elimination of infected poultry, usually through culling or depopulation, reduction of host susceptibility through vaccines and vaccination and education or public awareness (Swayne *et al.*, 2020, Swayne, 2012).

#### 2.1.7.1 Biosecurity

Biosecurity is very crucial and is the first line of defence in the control of AIV because it is able to greatly reduce the risk of AI. Biosecurity measures involve exclusion measures to keep the virus out of virus-free premises and containment to prevent the virus from spreading once cases occur (Spackman, 2020). Hence, every poultry farm must have a biosecurity plan that includes all potential pathways for entry of AIV and how to reduce the risk of entry. Biosecurity practices include cleaning and disinfection, segregation of susceptible flocks from infected ones, segregation of newly introduced poultry, proper handling of dead carcases and restriction of vehicle entry to areas close to poultry (Swayne *et al.*, 2020). Despite the role proper biosecurity plays in the prevention and control of AIV infection, it has some drawbacks, such as the requirement for strict measures and costly facilities, restricting freedom of poultry workers and animals, and it is unable to alleviate severe situations rapidly (Liu *et al.*, 2020b).

#### 2.1.7.2 Diagnostics and Surveillance

Accurate and rapid diagnosis is key to the control of AI, and the success of control measures are dependent on how fast the index case or cases can be identified, the existing biosecurity measures, and how quickly control strategies are implemented (Swayne *et al.*, 2020). Moreover, early detection and reporting of disease outbreaks allow for a rapid response and putting in place accurate warning systems which in turn is essential to efficiently prevent and control the disease (WOAH, 2023). Surveillance, which can be active or passive, is essential for ongoing evaluation of the success of control strategies as well as decision-making as a prelude to improving control strategies. Passive surveillance is critical for distinguishing LPAI virus as the cause of respiratory disease or drops in egg production from

endemic diseases with similar symptoms, whereas active surveillance is required for detecting infection in species that can be infected subclinically (Swayne *et al.*, 2020). The ultimate goal of surveillance is to notice potential public health threats early so that a proper response can be mounted before a public health crisis ensues.

#### 2.1.7.3 Elimination of Infected Poultry Through Culling

Culling involves the stamping out of all infected poultry flocks and other flocks that have been exposed to the infection through direct or indirect contact; their carcasses and manure are destroyed by burning and/or burial (Swayne *et al.*, 2020). It is often followed by standard cleansing, disinfection and closure of live bird markets. While this strategy is able to reduce the viral spread if well implemented and eliminate the disease with early detection, this strategy alone cannot control the spread of the disease. Moreover, the strategy has several drawbacks that include its unsustainable effects if poultry biosecurity is weak, not applicable for wild waterfowl, requires government and poultry owners to sacrifice greatly, and it's too costly if the virus has already spread (Liu *et al.*, 2020b). Furthermore, this strategy has become uneconomical and not feasible for countries facing endemic infections and limited financial sources for farmers' compensation (Parvin *et al.*, 2020).

#### 2.1.7.4 Vaccines and Vaccination

Vaccines against AI are important tools for protecting the poultry industry and humans since vaccines help increase resistance to infection, prevent illness and death, reduce virus replication and shedding, and transmission to birds and mammals, including humans (Kapczynski *et al.*, 2015, El Zowalaty *et al.*, 2013). They provide protection from both HPAI and LPAI infections. On the other hand, vaccines are very costly, they sometimes lead to silent infections in chickens and may accelerate viral mutation and diversification by complicating virus control measures (Liu *et al.*, 2020b, Parvin *et al.*, 2020). The use of vaccines to control AIV is dependent upon many factors, including the species and age of poultry, pathotype, and HA subtypes of AIV, country freedom or endemic status on AIV, the attitudes of veterinary authorities toward vaccination, and in-country availability of vaccine and logistics for vaccination (Swayne and Sims, 2021). Several AI vaccine technologies have been developed and have demonstrated purity, safety, efficacy and potency in experimental studies (Swayne, 2009). They can be classified into six groups, namely:

(i) inactivated whole AIV adjuvanted, (ii) live-attenuated, (iii) subunit, (vi) vector-based, (v) DNA, and (vi) Virus-like particles (VLP) vaccines (Nurzijah *et al.*, 2022, Swayne *et al.*, 2020).

Inactivated whole AIV adjuvanted vaccines, typically made using LPAI field outbreak strains or sometimes HPAIV or reverse genetic generated AI vaccine strains, followed by chemical inactivation and oil emulsification, are the most commonly used vaccines for the prevention of AI (Dhama, 2013, Swayne, 2009, Swayne, 2006). A number of inactivated AI vaccines are commercially available and include monovalent inactivated vaccines comprising either H5 or H7 strains, bivalent vaccines with H5 and H7 strains, and both monovalent and bivalent vaccines with homologous or heterologous NA (Swayne, 2012). The protection provided by inactivated AI vaccines is dependent on the quantity of antigen in each dose, how well-matched the vaccine is to circulating viruses and an appropriate adjuvant (Nurzijah *et al.*, 2022). Moreover, inactivated AI vaccines confer transient immunity, poor stimulation of cellular immunity, require frequent and multiple doses and requires direct inoculation (de Vries *et al.*, 2018). Live-attenuated vaccines are not recommended for use in birds because they have the potential to revert to virulence and recombination with field viruses (Nurzijah *et al.*, 2022, Dhama, 2013).

Subunit vaccines are effective at inducing humoral and cellular immune responses against specific viral proteins without the risk of handling live viruses during vaccine production or reversion to virulence (Nurzijah *et al.*, 2022). However, subunit vaccines have a more restricted antigenic repertoire, confer low immunogenicity and transient immunity and require direct inoculation. Vector-based vaccines, mainly recombinant, are regarded as “live” vaccines but are severely replication-restricted, leading to an excellent safety profile (de Vries *et al.*, 2018). They induce strong humoral and cellular immune responses and are effective in providing heterologous protective immunity, though multiple doses are often required and interference by vector-specific immunity (Kapczynski *et al.*, 2015). The commonly used vector-based vaccines with AI H5 gene inserts are live recombinant fowl poxvirus (rFPV-AIV-H5), herpesvirus of turkeys (rHVT- AIV-H5), and Newcastle disease vaccines (rNDV-AIV-H5) (Swayne *et al.*, 2020).

DNA vaccines for AI have been in development for 15 to 20 years, and they offer the advantage of being effective at inducing a strong and long-lasting immune response but are poor at inducing antibody responses and require multiple doses (El Zowalaty *et al.*, 2013). The basic principle of DNA vaccination is the induction of an immune response by intramuscular injection or the use of a 'gene gun' of naked DNA (plasmid) encoding the targeted gene into the host cells (Wolff *et al.*, 1990). The other drawbacks of DNA vaccines are cost, problems with delivery into poultry due to their time-consuming application, undue stress to the chickens, low efficiency in birds and its requirement of large amounts of purified plasmids (Lai and Bennett, 1998). Non-replicating, haemagglutinin-based H5 RNA particle, H5 expressed baculovirus, and H5 DNA vaccines have been licensed for use in chickens since 2015, although their usage has been limited (Swayne and Sims, 2021). VLP vaccines are non-infectious nanoparticles made up of assembled viral proteins without viral genetic materials (Yang *et al.*, 2022). This offers many benefits, including the potential for DIVA, the inability to recombination with wild-type AIVs, no need for virus inactivation and the lack of any pre-existing vector immunity in chickens (Alqazlan *et al.*, 2022). In addition, VLP vaccines are safe, contain main epitopes and are capable of inducing both humoral and cell-mediated immune responses. Generally, the effectiveness of vaccines and adjuvants relies on multiple parameters of host health and function, which includes the GIT microbiota (Nurzijah *et al.*, 2022). Hence, maintaining poultry GIT microbiota through the use of probiotics and prebiotics, along with the use of well-characterized dietary supplements, can increase the effectiveness of current poultry AIV vaccines (Alqazlan *et al.*, 2022).

#### 2.1.7.5 Education or Public Awareness Campaigns

Education, training, and public awareness campaigns are important basic measures in the control of AI infections by minimising the spread of the virus (Abdelwhab and Hafez, 2011). Hence, education and training programmes should be organised for all the stakeholders, including poultry industry personnel, veterinarians, para-veterinarians, military and interior ministries, social associations, the media and religious leaders (Dhama, 2013) on appropriate measures to minimize the risk of virus introduction and spread. Mass media platforms should take part in spreading awareness to the general public.

#### 2.1.7.6 Treatment of Avian Influenza Virus

Currently, no practical or specific treatment exists for AI in poultry, although Amantadine has been shown experimentally to be effective in reducing mortality. However, the drug is not approved for food animals. Therefore, symptomatic and antibiotic treatment has been used to reduce the effects of concurrent bacterial infections (Swayne *et al.*, 2020)

## 2.2 Influenza D Virus

### 2.2.1 Taxonomy and Nomenclature of Influenza D Virus

Influenza D viruses belong to the family *Orthomyxoviridae,* genus *Deltainfluenzavirus.* Based on the nucleotide sequences of the Hemagglutinin-Esterase Fusion (HEF) protein that is the primary target of virus-neutralizing antibodies, IDVs can be divided at least into five genetically distinct lineages: D/OK, D/660, D/Yama2016, D/Yama2019 and D/CA2019 (Huang *et al.*, 2021). D/OK lineage is the most frequently reported worldwide, while D/660 lineage is mainly distributed in Europe and North America. Interestingly, both D/Yama2016 and D/Yama2019 lineages seem to be restricted to Japan (Yu *et al.*, 2021, Murakami *et al.*, 2020, Chiapponi *et al.*, 2019, Horimoto *et al.*, 2016). Recently, a novel phylogenetic lineage of IDV with broad antigenicity has been discovered in California, USA, named D/CA2019 (Huang *et al.*, 2021).

### 2.2.2 Influenza D Virus Genome

Influenza D virus is an enveloped single-stranded, negative-sense RNA virus with seven genome segments which encode nine proteins (Wolff and Veit, 2021). The longest three segments encode polymerases PB2, PB1, and P3 which are essential in replication and viral mRNA synthesis. The fourth segment encodes the glycoprotein hemagglutinin-esterase fusion (HEF) which combines the functions of receptor recognition and binding, receptor destroying, and membrane fusion. The fifth segment encodes the NP that associates with the viral genome segments in a double-helical conformation in which two NP strands of opposite polarity are associated with each other. The sixth segment encodes two proteins, the matrix protein M1, a peripheral membrane protein that covers the viral envelope on its inside, and CM2, a short transmembrane protein with proton-channel activity required for virus entry. M1 contributes to the morphology of virus particles (Wolff and Veit, 2021, Ferguson

*et al.*, 2015). Virions are elliptical, spherical with a diameter of 80–120 nm or filamentous with a similar diameter but with lengths in the μm range. IDV is genetically closest to ICV and shares approximately 50% protein sequence identity. Based on the genetic similarities to influenza C viruses, IDV was provisionally designated C/Oklahoma/1334/2011 (C/OK) (Hause *et al.*, 2013). However, there is no cross-reactivity between IDV and human ICV generated in serum. Furthermore, phylogenetic analysis revealed the failure of reassortment between ICV and IDV in field isolates. Owing to the differences in biological, genetic, and antigenic characteristics of the novel virus from those of ICV, it became known as IDV (Sreenivasan *et al.*, 2015, Hause *et al.*, 2014).

### 2.2.3 Replication of Influenza D Virus

The interaction between the virus and the host cell triggers receptor-mediated endocytosis of the virus into the host cell and initiates the infection cycle (Wolff and Veit, 2021, Dou *et al.*, 2018). Upon entry into a host cell, ion channels along the virus surface allow for endosome acidification to facilitate the fusion of viral and endosomal membranes. Membrane fusion enables the release of viral RNA gene segments, packaged as viral ribonucleoprotein complexes (vRNPs), into the host cell cytoplasm. Each vRNP consists of an RNA gene segment neatly wrapped with several copies of influenza NP and bound by the viral RNA-dependent RNA polymerase (RdRp) complex consisting of PB2, PB1, and P3 (Wolff and Veit, 2021, Dou *et al.*, 2018). The vRNPs are transported into the nucleus, where the RdRp complex bound to each RNA segment initiates replication and transcription of viral RNA (vRNA) (Dou *et al.*, 2018). The vRNA that enter the nucleus as negative-sense templates are transcribed by the RdRp complex into “host” positive-sense mRNA that is translated into viral proteins within the cytoplasm using the host’s ribosomal machinery (Wolff and Veit, 2021, Dou *et al.*, 2018). The newly formed vRNPs are transported out of the nucleus with the help of influenza NEP, while appropriate viral proteins are transported to the cell surface. These viral proteins work together to package the newly created proteins and genetic material into budding virions.

### 2.2.4 Transmission of Influenza D Viruses

IDVs are likely to be transmitted similarly to other influenza viruses. They are shed in nasal secretions, and in cattle, they are transmissible in aerosols over short

distances and by direct contact (Spickler, 2021, Ferguson *et al.*, 2016). Transmission through aerosol has been demonstrated through experimental infections of cattle and calves (Salem *et al.*, 2019, Ferguson *et al.*, 2016). The other route of IDV transmission is through contaminated fomites, though this route has not yet been confirmed (Ruiz *et al.*, 2022). However, IDVs have been reported to be more resistant to inactivation by heat or acid when suspended in an unspecified liquid (Yu *et al.*, 2017a), which makes transmission of this virus through fomites more plausible.

### 2.2.5 Epidemiology of Influenza D Virus

IDV was first reported in the USA in swine with respiratory disease in 2011 (Hause *et al.*, 2013) but serological evidence has indicated circulation of the virus among cattle in Nebraska, since 2003 (Luo *et al.*, 2017). Since then, the virus has been detected among sick and healthy cattle across the US, Italy, France, Japan and China (Mekata *et al.*, 2018, Foni *et al.*, 2017, Zhai *et al.*, 2017, Ferguson *et al.*, 2016, Ferguson *et al.*, 2015). IDV infections in other animals have been detected including small ruminants such as sheep and goat, and pigs, wild boar, and camels. Furthermore, the virus has been detected in North America's small ruminant animals such as sheep and goats (Quast *et al.*, 2015). Serological evidence in Canada suggests that 13.5% and 13.3% of sheep and goat farms have been exposed to IDV but not chickens and turkeys (Quast *et al.*, 2015). Besides, the virus is known to have the ability to infect ferrets, a surrogate for human influenza infection, and this suggests that this virus may become a potential threat to human health (Hause *et al.*, 2013).

In the USA, Ferguson *et al.* (2015) recovered 15 isolates of IDV from surveillance of beef cattle in Mississippi. The study also revealed that two distinct virus clades circulated in the same herd. Moreover, serological assessment of neonatal beef cattle showed 94% seropositivity, and serological results of archived sera suggested that IDV has been circulating in the Mississippi cattle populations since at least 2004 (Ferguson *et al.*, 2015). Another study in the USA reported the recovery of six IDVs from cattle and phylogenetic analysis of the isolates revealed two distinct co-circulating lineages represented by D/swine/Oklahoma/1334/2011 (D/OK) and D/bovine/Oklahoma/660/2013 (D/660) (Collin *et al.*, 2015). The presence of co-

circulating lineages suggests IDV is endemic in cattle, and the virus reassorts frequently (Collin *et al.*, 2015). Moreover, a recent study in California identified a novel phylogenetic group of IDV strains in diseased bovines named D/CA2019 which represents the fifth genetic lineage of IDV (Huang *et al.*, 2021). Additionally, specific antibodies against IDV have been reported in humans in the US (White *et al.*, 2016, Hause *et al.*, 2013). Although the seroprevalence of IDV in the general population was reported to be low (1.3%) by Hause *et al.* (2013), a recent study reported a higher seroprevalence of 94–97% among cattle-exposed workers, raising concerns about a possible zoonotic risk (White *et al.*, 2016, Ferguson *et al.*, 2015)

In Europe, IDV was first reported in cattle in France in 2012 (Ducatez *et al.*, 2015) and then, a routine diagnostic investigation of respiratory disease outbreak in swine revealed the presence of IDV among swine in Northern Italy (Chiapponi *et al.*, 2016). Genetic analysis of the isolates revealed the swine and bovine IDV isolates were genetically related (Chiapponi *et al.*, 2016). Other European studies showed a 2.9% IDV viral detection in Italy among swine (Foni *et al.*, 2017) while a 4.5% prevalence was reported in France (Ducatez *et al.*, 2015). Genetic analysis in both studies highlighted that Italian swine IDVs and bovine IDVs in France are closely related to the D/swine/ Oklahoma/1334/2011 cluster (Foni *et al.*, 2017, Ducatez *et al.*, 2015).

Apart from America and Europe, the IDV has also been detected in Asia and Africa. A study in China demonstrated high levels of IDV infection in goats and pigs with over 30% prevalence (Zhai *et al.*, 2017). The high prevalence was attributed to poor biosecurity measures and high-density feeding mode practices in China's animal industry. In Japan, serological evidence suggests an overall positivity rate of 30.5% of IDV in cattle, though the rate varied from 13.5% to 50.0% from region to region within Japan (Horimoto *et al.*, 2016). Furthermore, a molecular epidemiological study for the IDV in sick and healthy cattle in Japan demonstrated that 2.1% were infected. Whole genome analysis revealed a distinct cluster of IDV from those found in other countries (Mekata *et al.*, 2018). Suggesting the virus may have evolved within Japan, giving a possibility of a third lineage of the IDV.

In Africa, serological evidence of IDV has been demonstrated in cattle and small ruminant animals in North and West Africa, particularly Morocco, Togo and Benin (Salem *et al.*, 2017). The results suggest the IDV has at least been circulating in North and West Africa from 2012. In these countries, seroprevalence varied depending on time and place like 23%, 41%, and 42% seroprevalence in Morocco in 2013, 2014, and 2015, respectively; 0% and 21% seroprevalence in Togo in 2009 and 2015, respectively (Salem *et al.*, 2017). Moreover, IDV RNA has been detected in bovines, giraffes, and wildebeest from Namibia and genetic analysis suggests the IDV sequence was distinct from any other sequence for all its seven segments and most likely represents an African-specific genotype within the D/OK lineage (Molini *et al.*, 2022). In Zambia, there is no data regarding the presence of IDV in Zambian livestock. The literature reviewed suggests the presence of IDV in America, Europe and Africa, indicating its wide global geographical distribution. Furthermore, the virus has a wide host range. Hence, continuous surveillance is needed to monitor the evolution and epidemiology of IDV in both ruminant and small ruminant animals.

### 2.2.6 Diagnosis of Influenza D Viruses

Several analytical techniques have been described for the diagnosis of IDV and include serological methods, molecular techniques and virus isolation (Ruiz *et al.*, 2022). The serological tests used include direct detection of virus attachment to sialic acid–linked receptors on RBCs by the hemagglutinin assay (HA), or by detecting virus-specific neutralizing antibodies that inhibit virus binding to RBCs by using hemagglutination inhibition (HI), an agar gel precipitation test (AGP), or the micro-neutralization (MN) assay and ELISA-based virus neutralization assay. HA and HI are the commonly used, however, they labour intensive, require several standardisations and are incapable of differentiang between IDV bovine and swine clades (Hause *et al.*, 2014). Hence, an ELISA test based on two different epitopes HEF and NP and four peptides, and fluorescent focus neutralization assay has been developed and is able to differentiate between IDV bovine and swine clades (Okda *et al.*, 2020). RT-PCR is the most common used molecular diagnostic test for IDV and aims to detect the IDV PB1 gene, one of the most conserved and stable regions within the seven RNA segments of the IDV genome (Okda *et al.*, 2020, Hause *et al.*, 2014). RT-PCR is a highly sensitive assay for virus identification, but has low efficacy in large-scale surveillance, due to early clearance of viral nucleic acids.

Moreover, RT-PCR is also used as part of a multiplex PCR assay for the detection of respiratory viruses in the BRD complex (Spickler, 2021). IDVs can also be propagated in cell cultures. Cell culture is a robust tool to study virus-host interactions and investigate viral protein functions in a controlled environment. Currently available cell culture models for IDVs include Madin-Darby canine kidney cell (MDCK), Madin-Darby bovine kidney (MDBK), African green monkey kidney epithelial cells (Vero) and swine testicular (ST) cells (Spickler, 2021, Uprety *et al.*, 2021).

### 2.2.7 Prevention and Control of Influenza D Viruses

IDV infections are common, even in asymptomatic animals, and there do not appear to be any practical, cost-effective measures to exclude these viruses from herds. However, good animal husbandry is likely to help reduce the effects of these viruses on animal health (Spickler, 2021). Currently, there are no specific vaccines or treatments for IDVs in animals. However, a chemical-inactivated whole virus particle vaccine has been developed and shown to elicit virus-specific immune responses in cattle, providing partial protection against respiratory disease and viral shedding following the homologous IDV challenge (Hause *et al.*, 2017). Additionally, a DNA vaccine expressing the consensus HEF gene of diverse IDV isolates has been successful in protecting guinea pigs from infection by two lineages of IDV: D/OK and D/660 (Wan *et al.*, 2018). While more research is needed to evaluate its protective efficacy against other lineages of IDV in guinea pigs and other animals, these results are encouraging and suggest that a DNA vaccine expressing the consensus HEF has the potential to protect animals from different lineages of IDV infections (Wan *et al.*, 2018). As IDVs will continue to evolve into more diverse lineages, future vaccine research efforts will need to focus on developing a universal vaccine that can effectively protect animals or humans from multiple strains or lineages of IDV. There are promising developments in this area, and with recently described robust reverse genetics systems for IDV (Ishida *et al.*, 2020, Yu *et al.*, 2019), it's possible to achieve rational design of IDV live attenuated vaccine candidates by various mechanisms, representing an alternative platform for developing safe and efficacious vaccines for IDV.

## 2.3 Newcastle Disease Virus

### 2.3.1 Taxonomy and Nomenclature of Newcastle Disease Virus

Newcastle disease virus (NDV), commonly known as Avian paramyxoviruses 1 (APMV-1), were classified by the International Committee on Taxonomy of Viruses as *Avian orthoavulavirus* serotype 1 (formerly *Avian avulavirus 1*) in the new subfamily *Avulavirinae,* family *Paramyxoviridae,* order *Mononegavirale* (Dimitrov *et al.*, 2019, Amarasinghe *et al.*, 2019, ICTV, 2019). Previously, NDV was classified as a member of the genus *Rubulavirus* of the same order, and the new genus *Avulavirus* was created in 2002 (Mayo, 2002a,b).

Over the years, various NDV classification systems have been developed and used for NDV classification based on their genetic information (Cattoli *et al.*, 2010, Snoeck *et al.*, 2009, Aldous *et al.*, 2003, Ballagi-Pordány *et al.*, 1996, Sakaguchi *et al.*, 1989, Toyoda *et al.*, 1989). However, each of these systems was based on different approaches and lacked objectivity for the classification of NDV strains into different genetic groups, which led to inconsistencies in the classification (Dimitrov *et al.*, 2019). To overcome the inconsistencies and challenges posed by these classification systems, a unified and objective NDV classification system was proposed in 2012 based on the complete fusion (F) gene coding sequences and incorporated a number of objective criteria for the classification of NDV (Diel *et al.*, 2012). However, despite the objectivity provided by Diel *et al.* (2012) classification system, the ample circulation and constant evolution of NDV led to the almost simultaneous identification and uncoordinated naming of new genotypes, which necessitated a new classification system. Thus, according to the new and updated NDV classification system with new nomenclature, NDV strains have been classified into two distinct classes based on the phylogenetic analysis of the full-length F gene, namely class I and class II, all within a single serotype (Dimitrov *et al.*, 2019). Class I viruses belong to a single genotype (genotype I) and three sub-genotypes, which mostly consist of avirulent NDV strains, usually from wild birds that occasionally spill over into poultry. In contrast, the more genetically diverse class II viruses are divided into 21 genotypes (I–XXI) with several sub-genotypes, which include both avirulent and virulent isolates from a wide range of domestic and wild birds (Dimitrov *et al.*, 2019). Genotype VII is subdivided into three sub-genotypes, while

genotypes I, V, VI, VII, XII, XIII, XIV, and XVIII are each divided into several sub-genotypes.

Furthermore, NDV strains are classified into three major pathotypes based on the pathogenicity in chickens; velogenic (highly virulent), mesogenic (moderately virulent) and lentogenic (avirulent or low virulent). Furthermore, velogenic strains are classified into viscerotropic and neurotropic velogenic strains and are responsible for severe disease and cause significant mortality in susceptible flocks (Bari *et al.*, 2021, Nagy *et al.*, 2020). According to the World Organisation for Animal Health (WOAH), virulent NDV (vNDV) strains, which include both mesogenic and velogenic strains, must meet one of the following criteria: (i) have an intracerebral pathogenicity index (ICPI) in day-old chicks (*Gallus gallus*) of 0.7 or greater; or (ii) have multiple basic amino acids at the C terminus of the F2 protein and phenylalanine at residue 117, which is the N terminus of the F1 protein (WOAH, 2021). The virulent and avirulent NDV strains have the sequence of $^{112}$R/K–R–Q/K/R–K/ R–R–F$^{117}$ and $^{112}$G/E–K/R–Q–G/E–R–L$^{117}$ in the F protein cleavage site, respectively (WOAH, 2021).

### 2.3.2 Newcastle Disease Virus Genome

NDV is an enveloped, single-stranded, negative-sense RNA virus with a non-segmented genome of approximately 15.2kb that consists of a leader (55 nucleotides) and trailer (114 nucleotides) terminal sequences. However, the genome sizes may vary in different strains, exhibiting genome sizes of 15,186 nucleotides (nt), 15,192 nt, and 15,198 nt, which follow the “rule of six” that is required for the replication of NDV strains (Wu *et al.*, 2015). In terms of the ultra-structure, the NDV particle ranges from 200–300 nm in diameter (Ganar *et al.*, 2014). Its genome has six open reading frames (ORF) which encode six major structural proteins, namely, nucleoprotein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase (HN), and the RNA-dependent RNA polymerase (L) in the order, 3′-NP-P-M-F-HN-L-5′ and two non-structural proteins (V and W) generated by P-gene mRNA editing in virus-infected cells (Figure 2.2) (Locke *et al.*, 2000, Steward *et al.*, 1993).

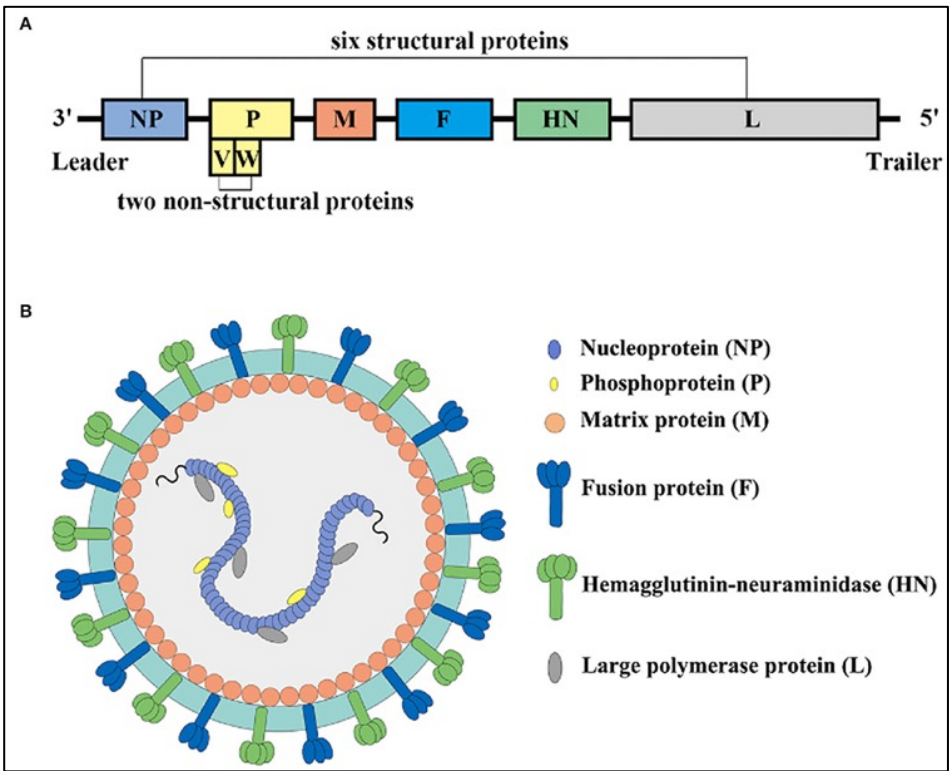


Figure 2.2: Structural features of Newcastle disease virus. A. Arrangement of the genes in the viral genome. B. Structure of the NDV virus particle showing the locations of the viral proteins. Adapted from Mao *et al.* (2022)

The HN, F, and M proteins are tightly linked to the viral envelope, with the HN and F glycoproteins protruding from the viral envelope. The F and HN proteins are crucial in the virulence of the virus (Ren *et al.*, 2019, Liu *et al.*, 2015) and play essential roles in the assembly and development of enveloped viruses and in determining tropism in the host and tissues (Jin *et al.*, 2017). The F protein is significant as a type-I integral membrane protein and is considered the main molecular marker of NDV virulence. In infected cells, NDV-F is synthesized as a precursor F0 without fusion activity, and then it is activated via cleavage by host protease of the Fc into two disulfide-linked subunits F1-F2, which is essential for the progeny virus to become infective. The HN is a multifunctional molecule that recognizes cellular sialic-acid-containing receptors and has neuraminidase (NA) activity to hydrolyze the sialic acid molecules from progeny virus particles, prevent viral self-aggregation, and interact with F to promote fusion and other functions (Connolly *et al.*, 2009, Connaris *et al.*, 2002). The HN also has an effect on the pathogenicity, replication, and biological characteristics of the virus. The M protein is non-glycosylated and is peripherally attached to the inner surface of the viral

envelope, and is involved in the morphogenesis and budding of NDV (Dortmans *et al.*, 2010).

The NP is the main structural protein of NDV nucleocapsid protein and also the most abundant protein present in the viral particles, which encapsidates the RNA genome to protect it from host nucleases. The NP is also important to genome packaging into mature virus particles (Mao *et al.*, 2022). The P and L proteins form a P-L complex and play a vital role in the replication and transcription of NDV. The P-L complex replicates the genome and synthesizes full-length plus-strand antigenomic RNA, which serves as a template for minus-strand genomic RNA (Yu *et al.*, 2017b). The P protein helps stabilize the L protein in the P-L complex and acts as a viral RNA-dependent RNA polymerase (vRdRp) that serves as the viral replicase and transcriptase during the infectious cycle, while the P protein is the cofactor of the polymerase (Zhao *et al.*, 2021, Dortmans *et al.*, 2010). Apart from the structural proteins, the P gene encodes two other non-structural proteins, V and W. The V protein, also known as the antagonist protein, has been suggested to direct host-immune evasion upon NDV infection, whereas the function of the W protein remains unknown (Karsunke *et al.*, 2019, Qiu *et al.*, 2016, Huang *et al.*, 2003).

### 2.3.3 Replication and Pathogenicity of Newcastle Disease Virus

The first step in NDV infection is the binding of the virus to the respiratory epithelial cells of the host through the sialic acid-containing compounds such as gangliosides and N-glycoproteins receptors by its surface glycoproteins F and HN (Dortmans *et al.*, 2010, Connaris *et al.*, 2002). This triggers a conformational change in the F protein, which results in the fusion of the viral envelope and the cellular plasma membrane to allow the entry of the nucleocapsid into the cell cytoplasm (Burman *et al.*, 2020). Furthermore, viral particles are internalised by receptor-mediated endocytosis and sometimes through caveolae-dependent endocytosis (Cantín *et al.*, 2007). Simultaneously, the viral RNA is encapsidated by the NP protein to form an N-RNA template, which is mediated by the P protein and catalysed by the L protein to form an active RNP complex to enter the cytoplasm.

Following viral entry, the M protein dissociates from the RNP complex in the cytoplasm, and the P and L proteins form a polymerase complex that initiates

transcription of the viral RNA (Connaris *et al.*, 2002, Ito *et al.*, 1992). The negative sense RNA genome is transcribed into positive sense mRNA which is then translated into viral proteins (Jadhav *et al.*, 2020). Transcription begins at the extreme 3′ leader sequence and synthesizes the mRNA of individual genes from gene-start to gene-end sequences. The reinitiation of transcription to a downstream gene at the gene-start site is not uniform, which leads to a gradient of mRNA population that decreases according to the distance from the 3′ end of the viral genome (Ganar *et al.*, 2014). The positive-sense RNA is then used as a template for the synthesis of negative-sense genomic RNA. The M protein is required for the assembly of virus particles and the budding process of progeny (Pantua *et al.*, 2006, Takimoto and Portner, 2004). Finally, the HN protein promotes the detachment of the virus from the cell and by removing sialic acid molecules from progeny virions to prevent self-aggregation during budding (Cornax *et al.*, 2013).

NDV infectivity is dependent on the cleavage of F protein precursor F0 and the amino acid sequence of the F protein cleavage site is considered the main determinant of infection. When NDV replicates in an infected cell, viral particles are produced with an inactive precursor fusion glycoprotein, F0 (Aldous and Alexander, 2001). For this protein to be functional and virus particles to be infectious, the precursor has to be cleaved into two peptides, F1 and F2, by host cell proteases leading to varying degrees of virulence (Rott and Klenk, 1988). Avirulent (lentogenic) strains of NDV have fewer basic amino acids in its F protein cleavage site which can only be cleaved by trypsin and trypsin-like proteases found in respiratory and gastrointestinal tracts which leads to restricted host site replication. However, virulent (velogenic/mesogenic) strains of NDV have polybasic amino acids in its F protein cleavage site, which can be cleaved by furin-like proteases found in nearly all cells in the body, allowing for a systemic infection to occur and extensive viral replication (Aldous and Alexander, 2001, Toyoda *et al.*, 1987). It has also been suggested that F and HN proteins in NDV are determinants of NDV virulence in chickens and infected macrophages (Cornax *et al.*, 2013).

NDV has also been reported to naturally or experimentally infect non-avian hosts, including humans, monkeys, rabbits, mink, hamsters and pigs (Shabbir *et al.*, 2021, Kuiken *et al.*, 2018, Zhao *et al.*, 2017, Kuiken *et al.*, 2017, Chen *et al.*, 2013, Samuel

*et al.*, 2011, Charan *et al.*, 1984). Detection of NDV in non-avian hosts, including humans, has raised concerns about its zoonotic potential (Shabbir *et al.*, 2021, Ul-Rahman and Shabbir, 2019, Kuiken *et al.*, 2018). However, information regarding the zoonotic potential of NDV remains limited and it is hard to predict the risk of spillover potential of NDV strains that have been detected in mammalian species (Ul-Rahman *et al.*, 2022).

### 2.3.4 Transmission and Spread of Newcastle Disease Virus

ND is very contagious and is easily spread from one bird to another. It spreads in three ways including, the domestic poultry industry with its vertical integration, intracontinental and intercontinental avian migration, and the pet bird trade (Cross, 1991). However, the most important ways of spread are the movement of domestic poultry, including day-old chicks, hatching eggs, live and dead birds, and poultry offal; the migratory movements of infected wild avian species; the mechanical transfer of virus by rodents; and wind transmission of the virus. The spread of the virus is facilitated by its high resistance to adverse environmental conditions, its wide avian host range, and its ability to persist in poultry carcasses (Cross, 1991).

NDV is primarily transmitted via inhalation or ingestion of virus shed in faeces and respiratory secretions by infected birds for variable lengths of time (Alexander, 2009). The virus is shed during incubation, during the clinical stage, and for a varying but limited period during convalescence. The virus may also be present in eggs laid during clinical disease and in all parts of the carcass during acute vNDV infections. Chickens are readily infected by aerosols and by ingesting contaminated water or food or by the production of small infective particles produced from dried faeces that may be inhaled or impinge on mucous membranes (Alexander, 2009). Furthermore, virus has been found to be present in all parts of the carcass and is able to persist for many months on both chicken skin and bone marrow if kept at refrigerated temperatures (Brown and Bevins, 2017). Infected chickens and other domestic and wild birds may be sources of NDV. Movement of infected birds and transfer of the virus, especially in infective faeces, by the movement of people and contaminated equipment or litter are the main methods of virus spread between poultry flocks. The transmission of ND infection from birds to humans is rare. However, it is believed that transmission results from close contact with infected

birds or materials. No evidence exists to support human-to-human transmission, but the potential for bird-to-human transmission exists (Ul-Rahman *et al.*, 2022)

### 2.3.5 Epidemiology of Newcastle Disease Virus

NDV was first detected in 1926 in Java, Indonesia and Newcastle-upon-Tyne, England, from where it gained its name (Doyle, 1927, Kraneveld, 1926). However, there is evidence of earlier reports of similar disease outbreaks in Central Europe based on literature (Halasz, 1912). NDV is distributed worldwide and is endemic in many developing countries (Ganar *et al.*, 2014). Its continual presence in over 250 avian species including wild and domestic birds presents a constant threat to all poultry industries and other activities that involve the raising or keeping of birds (Miller *et al.*, 2015). Wild aquatic birds seem to be the reservoir of avirulent strains, while poultry is the most likely reservoir of virulent viruses, but both exchange viruses (Snoeck *et al.*, 2013a). The exchange of viruses is believed to occur during the interactions between free-ranging wild birds and poultry at the livestock-wildlife interface (Welch *et al.*, 2019). However, free-living migratory species, such as waterfowls or white storks (*Ciconia ciconia*), may carry virulent NDV strains without obvious contact with poultry (Snoeck *et al.*, 2013a). Furthermore, wild-bird carriers are of great concern regarding the movement and transmission of pathogens, including NDV, to and from the poultry sector (Welch *et al.*, 2019).

Since the first report of ND outbreaks in 1926, four panzootics caused by various NDV genotypes have been documented (Alexander, 2001) and the possibility of another panzootic is imminent (Miller *et al.*, 2015). During the 1920s to 1960s genotypes II–IV were responsible for the first panzootic which began simultaneously in Asia and Europe and spread to the rest of the world (Lomniczi *et al.*, 1998). The second panzootic caused by genotype V began in Europe in the 1960s and became full-fledged within four years probably due to the increased commercialisation of the poultry industry globally, as well as the enhanced international trade of captive cage birds, which were shown to be the reservoirs of vNDV in different parts of the world. Genotype VI isolates were responsible for the third panzootic in the mid-1980s which occurred among the racing pigeons but eventually affected several bird species (Miller and Koch, 2013). Genotype VII viruses are responsible for the fourth and current ongoing panzootic which originated in South-East Asia, with the earliest

known outbreaks beginning in the 1980s (Bello *et al.*, 2018a, Miller *et al.*, 2015). Genotype VII which constitutes the most rapidly evolving strain of the virus has been linked to several economic losses in several countries in South East Asia, the Middle East, Europe, Africa, and America (Miller and Koch, 2013). Genotypes V, VI, VII, VIII and XI emerged after the 1960s and are considered ‘‘late’’ genotypes and only comprise vNDV strains. Currently, genotype VII comprising is the most prevalent genotype circulating worldwide and is associated with many outbreaks among chickens.

In Africa, ND causes devastating losses to the poultry industry, particularly in backyard poultry where the disease is endemic but recurs as frequent epidemic outbreaks with high mortality (Abolnik, 2017, Fisher, 2014, Copland and Alders, 2005). This compromises the livelihoods of poor rural households who depend on poultry as a source of protein and income. Outbreaks of ND in poultry have been reported in North, Central, East, Southern and Western African countries (Twabela *et al.*, 2021, Welch *et al.*, 2019, Molini *et al.*, 2017, Mohamed *et al.*, 2011, Cattoli *et al.*, 2010, Mohamed *et al.*, 2009, Herczeg *et al.*, 1999). A recent systematic review and meta-analysis reported a prevalence and seroprevalence of NDV of 12% and 40.2%, respectively in backyard poultry in Africa (Mngumi et al., 2022). To date, NDV isolates from several African countries have been grouped into genotypes I–VIII, XI, XIII, XIV, XVII, XVIII and XXI (Cattoli, 2019, Dimitrov *et al.*, 2019). Of these, genotypes VI and VII are widely distributed in African countries whereas genotype V is predominant in East Africa, genotypes XIV, XVII, and XVIII are predominant in West and Central Africa, and genotype XI occurs only in Madagascar (Mngumi *et al.*, 2022).

In some North African countries, studies have reported genotypes II and VII of NDV in poultry from LBMs, backyard and commercial farms (Eid *et al.*, 2022, Saad *et al.*, 2017, Ewies *et al.*, 2017, Radwan *et al.*, 2013, Mohamed *et al.*, 2011). In West and Central Africa, circulation of genotypes XIV, XVII, and XVIII have been reported in poultry (Funsho-Sanni *et al.*, 2022, Souley *et al.*, 2021, da Silva *et al.*, 2020, Snoeck *et al.*, 2013b) while genotypes V, VI, VII, and XIII have been reported in recent studies from East Africa (Bari *et al.*, 2021, Kariithi *et al.*, 2021, da Silva *et al.*, 2020, Ogali *et al.*, 2020, Msoffe *et al.*, 2019). Studies conducted in Southern Africa, have

reported the detection of genotypes I, VII, VIII and XIII with genotype VII, sub-genotype VII.2 being the most common (Abolnik *et al.*, 2018, Abolnik *et al.*, 2017, Fringe *et al.*, 2012, Herczeg *et al.*, 1999). Genotype VII, sub-genotype VII.2 (VIIh) was first detected on the African continent in 2011 in vaccinated broiler chickens in Maputo province, Mozambique (Mapaco *et al.*, 2016). Since then, the virus has been detected in several Southern African countries including Namibia (Molini *et al.*, 2017), Botswana (Kgotlele *et al.*, 2020), South Africa, Malawi, Zambia and Zimbabwe (Abolnik *et al.*, 2018). Phylogenetic analysis linked the viruses isolated in Mozambique to viruses from China, South-East Asia and a single isolate from South Africa in 2013 (Mapaco *et al.*, 2016). In addition, the likely source of the introduction to Mozambique in 2011 was thought to be illegal poultry trading or infected waste from ships and not wild migratory birds (Abolnik *et al.*, 2018).

In Zambia, NDV was first detected in 1952 in the Southern Province, where a total of 15 outbreaks were recorded in that year although the pathotypes of the isolates remained unknown (Hussein *et al.*, 1984). By the early 1980s, velogenic NDV strains had been detected in different parts of the country and ND was considered enzootic throughout the country (Hussein *et al.*, 1984). Since then, the government enforced vaccination policy on commercial poultry farms. However, ND continues to contribute to a high burden of morbidity and mortality among village chickens nationwide with a reported seroprevalence of 36.5% and 73.9% in 1994 and 2012, respectively (Musako and Abolnik, 2012, Alders *et al.*, 1994). Moreover, ND is considered the leading cause of death in chickens in Zambia. Recent genetic studies conducted in Zambia revealed the circulation of ND viruses from genotypes VII.2 (VIIh) and XIII among chickens in the Eastern Province (Abolnik *et al.*, 2018, Abolnik *et al.*, 2017). Despite these studies, which were very limited in geographical coverage, there is a paucity of data as regards, the circulation of NDV in LBMs and wild waterfowls.

Apart from the circulation of NDV in domestic birds, various genotypes of NDV have also been detected in wild birds in Africa including avirulent and virulent strains (Abd Elfatah *et al.*, 2021, Wanyana *et al.*, 2018). Genotypes I, VI, and XVIII have been reported in wild birds in West Africa (Snoeck *et al.*, 2013a) whereas genotypes II and VII have been reported in Uganda and Egypt, respectively (Abd

Elfatah *et al.*, 2021, Wanyana *et al.*, 2018, El Naggar *et al.*, 2018). Despite reports of NDV in wild birds in Africa, no NDV strains have been reported from wild birds in Zambia and the extent of the viral burden, the pathotypic and genotypic characteristics remain unknown.

### 2.3.6 Diagnosis of Newcastle Disease Virus

Newcastle disease is generally diagnosed by isolation of NDV in SPF ECEs, (Miller *et al.*, 2010). However, several other diagnostic methods for NDV exist and include clinical diagnosis, virus isolation, serology and molecular methods.

#### 2.3.6.1 Clinical Diagnosis

Clinical diagnosis of NDV is based on the presence of clinical signs and acts as a presumptive diagnosis especially if the disease is known to be present in a given area (Getabalew *et al.*, 2019, Roy, 2012). The clinicopathologic picture of ND gives important clues in making clinical diagnosis. However, clinical signs alone do not present a reliable basis for diagnosis of ND because a number of viral and bacterial diseases may manifest similar clinical features that could be confused with ND (Cross, 1991). Moreover, ND presents with various clinical and pathological signs depending on the form of ND that include velogenic viscerotrophic ND, velogenic neutrotropic ND, mesogenic strain lentogenic ND and asymptomatic enteric ND (WOAH, 2021, Getabalew *et al.*, 2019). Besides, none of the clinical and pathological signs are pathognomonic to NDV.

#### 2.3.6.2 Virus Isolation

Virus isolation is considered as the gold standard method for the definitive diagnosis of ND and is often used for for the validation of other techniques (Bello *et al.*, 2018a). Moreover, virus isolation is important for the prediction and control of epidemics, as well as for vaccines and antiviral drug development (Mao *et al.*, 2022). The preferred method for NDV isolation is by the inoculation of SPF ECEs, or specific antibody negative eggs (WOAH, 2021). However, isolation of NDV can also be performed in n a variety of cell cultures of avian and non-avian origin, such as chicken embryo fibroblasts, DF-1, chicken embryo kidney, chicken embryo liver cells, avian myeloblasts and African green monkey kidney cells (vero cells) (Cattoli *et al.*, 2011). Primary cell cultures of avian origin are all highly permissive to the

virus (Bello *et al.*, 2018a). The choice of samples required for virus isolation is determined by the sites of virus replication and routes of viral shedding which include the cloacal and oropharyngeal swabs, and faecal matter collected in isotonic solution with or without antibiotics. If the birds are already moribund or have recently died, samples should include lungs, kidney, liver, intestine, spleen, and caecal tonsils collected separately or as a pool in addition to cloacal and oropharyngeal swabs (WOAH, 2021). To detect the presence of the virus after inoculation in SPF ECEs, the HA test is performed to determine the HA activity. However, since other viruses such as AIVs and APMVs possess HA activity, HI assay using NDV specific antisera or molecular tests should be performed. Besides, some serological cross-reactivity might occur between NDV and APMV-3 or APMV-7 (Alexander, 2000) and be circumvented by the use of a panel of monoclonal antibodies specific for NDV. The key disadvantages of virus isolation is the need for samples with viable virus, cost and time (Bourgeois and Oaks, 2014).

#### 2.3.6.3 Serological Diagnosis

Serological assays are an important strategy for the detection of NDV and are based on the interaction between antigens and antibodies (Mao *et al.*, 2022). They are used in diagnostic laboratories to assess antibody response following vaccination However, the diagnostic relevance of serology in NDV surveillance is considerably limited due to the almost universal use of vaccines in domestic poultry leading to the inability to differentiate vaccinated from infected animals (DIVA) (WOAH, 2021, Bello *et al.*, 2018a). Hence, there is an urgent need for highly specific and sensitive serological testing methods for the diagnosis of NDV in clinical settings in endemic areas. Currently, several serological assays have been developed for the detection of NDV, among them, HA/HI, ELISA, virus neutralizing test (VNT), Immunofluorescence assay (IFA) and Immune colloidal gold technique (GICT) (Mao *et al.*, 2022).

The sensitive and most inexpensive serological test for NDV is HI which measures the ability of NDV specific antibodies to inhibit the agglutination of RBCs by the NDV particles. It takes advantage of some viruses' ability to hemagglutinate (bind) red blood cells, therefore forming a “lattice” and preventing the red blood cells from clumping (Bourgeois and Oaks, 2014). The assay is used to identify a virus as NDV

and is normally performed using standard amount of NDV (4 or 8 HAU) as HA antigen (WOAH, 2021). This assay has the advantage of being quick and easy to perform, especially if there is a predominant virus subtype that is suspected and also titre gives an indication of the immune status of the bird. However, the HI assay may not detect antibodies in samples that are not cross-reactive to the virus being and the test is difficult to standardise between laboratories (Getabalew *et al.*, 2019, Bourgeois and Oaks, 2014).

One of the most common serological assays currently being used in the detection of NDV is ELISA. It is a qualitative and quantitative detection method that binds soluble antigens or antibodies with solid-phase carriers such as polystyrene and uses the specific recognition and binding effect of antigen and antibody to carry out immune reactions (Mao *et al.*, 2022). Over the years, several ELISA kits based on the whole or part of the virus antigen have been developed for the rapid detection of NDV and are commercially available in the form of sandwich, competitive or indirect ELISA (WOAH, 2021, Makkay *et al.*, 1999). The technique is highly specific, sensitive, and produce results that correlate well with HI test results. However, some limitations make the assay less routinely used compared to the HA/HI tests. Apart from being expensive and unsuitable in the field, these monoclonal antibodies (Mab) based ELISAs may not be able to detect certain strains of NDV that might have some mutation in the single epitope against which the monoclonal antibody was raised (Bello *et al.*, 2018a).

The VNT is another powerful serological test that detects antibodies capable of neutralizing the infectivity of the virus (Bourgeois and Oaks, 2014). The test is the most superior tool for assessing neutralising antibody titre following vaccination (Bello *et al.*, 2018a). The only drawback is that, it is highly laborious and very slow, yielding results only after nearly one week. However, the development of an improved VNT using a recombinant NDV engineered to constitutively express the green fluorescent protein (GFP) or the enhanced GFP (eGFP) giving conclusive results within 24 hours without the need for any additional staining procedure has made it a suitable method to overcome the drawbacks of a conventional VNT (Chumbe *et al.*, 2017). The advantages of the VNT are high sensitivity and specificity, the existence of neutralizing antibodies in the body for a long time, and

the neutralizing antibodies of most viruses are directly related to immunity (Mao *et al.*, 2022). The other serological tests in use are immunofluorescent antibody (IFA) and immune colloidal gold technique (GICT) (Mao *et al.*, 2022).

#### 2.3.6.4 Molecular Based Assays

The cross-reaction between NDV and some APMVs leads to uncertainty in ND serological testing. To overcome this uncertainty several molecular assays capable of quickly identifying NDV and distinguishing it from other closely related pathogens have been developed. These assays include PCR-based, non-PCR-based and sequencing based diagnostic methods.

The most commonly used molecular assay in NDV diagnosis especially in the developing countries is conventional RT-PCR. The test can rapidly and accurately detect viral genome in clinical samples with high sensitivity especially if appropriate samples are taken and is usually designed to simultaneously detect and identify the pathotype of the virus by targeting the F gene portion encompassing the F cleavage site. The shortcomings of this technique are that it is prone to contamination resulting from opening and closing finished reactions and transferring reaction products to new tubes or analytical gels and may be labour intensive. However, to overcome these short comings, several variants of the RT-PCR assay have been designed to decrease the time necessary to perform the assay, minimize manipulations of reactions, and improve sensitivity (Bourgeois and Oaks, 2014) among them RT-qPCR.

RT-qPCR is not only faster and less cumbersome than the conventional RT-PCR, but depending the assay conditions it also provides equal or even greater sensitivity of virus detection than the gold standard virus isolation method (Bello *et al.*, 2018a). The M gene, F gene and L gene-based RT-qPCR assays have been used extensively in many countries for NDV screening and pathotyping directly from clinical samples (Miller *et al.*, 2010, Fuller *et al.*, 2010). The primers and probe for the M-gene assay were designed to detect the highly conserved matrix (M) gene of NDV and, as such, detects most NDV genotypes of class II, regardless of pathotype (Kim *et al.*, 2007a). However, due to the heterogeneous genetic nature of this virus, class I viruses tested often fail to be detected. Due to this failure, a new matrix-polymerase

multiplex RT-qPCR was developed for the detection of a broad range of class I and II NDV isolates (Mia Kim *et al.*, 2008). Another assay capable of detecting class I and II viruses is the L gene-based RT-qPCR, which has a relatively high sensitivity and specificity. The assay targets a region of the polymerase (L) gene because of the high level of genetic conservation it exhibits in different strains of APMV-1 (Fuller *et al.*, 2010). While the M gene-based assay are capable of identifying NDV, the F gene based-qPCR assay used for pathotyping can differentiates the low virulence viruses from the vNDV strains (Aldous *et al.*, 2001). In addition to disease identification and pathotyping, the qPCR assay can also be used in viral load quantification in different organs or virus shedding from the vaccinated animals following challenge with the virulent NDV strain (Niesters, 2001).

The other molecular based technique in the loop mediated isothermal amplification (LAMP). LAMP is a simple, sensitive, and inexpensive diagnostic assay that was developed for the rapid detection of the genetic materials of infectious agents (Bello *et al.*, 2018a). The principle of the assay is to amplify DNA with high specificity, efficiency and rapidity under isothermal conditions by using a DNA polymerase with high displacement strand activity and a set of specifically designed primers to amplify targeted DNA strands (Notomi *et al.*, 2000). Over the years the technique has undergone several advancements in terms of its applications and different forms have been developed based entirely on this technique (Notomi *et al.*, 2015) including conventional LAMP, reverse-transcription LAMP, multiplex LAMP (Wong *et al.*, 2018). LAMP based assay for the detection of NDV directly from clinical samples have been developed and showed that its sensitivity and specificity are similar to those of nested PCR, yet it is simpler and inexpensive (Pham *et al.*, 2005)., Moreover, RT-LAMP has been used in the detection of NDV RNA from cloacal and tracheal swabs obtained from chickens in less than one hour (Kirunda *et al.*, 2012) making the technique a rapid and cheaper alternative especially in developing countries. The other advantage of LAMP is that it can be performed without advanced laboratory equipment (Soroka *et al.*, 2021).

The other molecular diagnostic techniques that are used in NDV detection are microarray hybridisation techniques. Microarrays are usually used in research settings, although their application in clinical diagnostics is becoming more common,

especially for detection of unknown viruses (Bourgeois and Oaks, 2014). Microarrays have the potential to concurrently monitor, detect, and characterise hundreds to thousands of targets without compromising the assay's sensitivity and specificity. Besides, microarray techniques have been able to simultaneously detect NDV and AIVs (Lung *et al.*, 2012, Wang *et al.*, 2008) demonstrating the potential of DNA microarrays in the detection of mixed infection.

#### 2.3.6.5 Sequencing Based Methods

Advances in genetic sequencing that combine biotechnology, molecular biology, and bioinformatics have provided a powerful tool for the rapid detection and characterisation of new viruses in both humans and animals (Bourgeois and Oaks, 2014). Several sequencing methodologies exist for infectious disease diagnostics, and all use PCR for its basic reaction among them is the traditional method of Sanger sequencing (first-generation sequencing) which involves amplification of the DNA sequence of interest using all four deoxynucleotide bases (A, C, T, and G) and one known di-deoxynucleotide (ddA, ddC, ddT, or ddG). It has been widely used for decades and enables the rapid determination of typically a single (F) target gene in 24–36 hours to define virus virulence and still has widespread utility (WOAH, 2021). However, despite many technical improvements, the limitations of Sanger sequencing showed a need for new and improved technologies for sequencing large numbers of human genomes (Metzker, 2010).

One of the most recent sequencing technologies that have revolutionised the diagnosis of infectious diseases is NGS and it is currently the most throughput tool used in the discovery of novel viruses associated with unknown diseases (Deurenberg *et al.*, 2017). NGS is not only important in tracking disease epidemics, but also facilitates the rapid, sensitive, and specific detection and differentiation of mixed infections within a single host (Metzker, 2010). It allows for the sequencing of the whole genome of numerous pathogens in one sequence run (Deurenberg *et al.*, 2017). Moreover, the major advance offered by NGS is the ability to produce an enormous volume of data cheaply, in some cases over one billion short reads per instrument run (Metzker, 2010). Currently, many NGS platforms have emerged and most platforms aimed at viral diagnosis are focusing on improved sequence reads and speed of the assay. With regard to NDV, NGS has been used to simultaneously

characterise the genomic sequences of multiple APMVs and differentiation of virulent from avirulent strains of class II NDV successfully and rapidly (Dimitrov *et al.*, 2017, De Battisti *et al.*, 2013). So far, the disadvantages of the assay are its perceived high cost of a sample run and the possibility of contamination which applies to all molecular techniques (Bello *et al.*, 2018a, Bourgeois and Oaks, 2014).

### 2.3.7 Prevention and Control of Newcastle Disease Virus

Since no treatment exists for ND, prevention and control of the disease are cardinal. The general approach to the control of ND outbreaks lies in the culling of infected birds combined with strict biosecurity and aggressive vaccination protocols (Absalón *et al.*, 2019). Culling infected birds and those in contact with them limits the spread of the disease. However, most developing countries have no strong government-sponsored programs to support eradication through culling and lack compensation schemes for culled birds (Absalón *et al.*, 2019) leaving biosecurity and vaccination as the main alternative in the prevention and control of NDV. Tight biosecurity is the first-line strategy in the control of avian diseases and aims at minimizing the traffic of pathogenic organisms in and out of poultry facilities (Conan *et al.*, 2012). Biosecurity measures include bird-proofing houses, feed and water supplies, minimizing travel on and off the facility, disinfecting vehicles and equipment that enter the farm, and good personal hygiene of the farm staff (Getabalew *et al.*, 2019, Spickler, 2016). However, even with good biosecurity practices, vaccination is still required to optimally protect the birds against economically devastating diseases such as ND.

#### 2.3.7.1 Vaccination

Vaccination against NDV needs to achieve three main goals: i) decrease or eliminate clinical disease; ii) decrease the amount of virulent virus being shed; and iii) increase the infectious dose of the challenge virus (Kapczynski *et al.*, 2013). However, due to the limited practicability of effective tools and skills for assessing virus shedding in the field, the majority of current vaccines and vaccination regimes primarily aim to decrease or eliminate clinical disease (Hu *et al.*, 2022). The strategies for ND vaccination are broadly classified into two, namely the conventional methods and recombinant DNA technology. However, the vast majority of commercially available vaccines are conventionally produced which include both live and inactivated

vaccines (Brown *et al.*, 2021a). Live vaccines are the most commonly used ND vaccines across the world and include lentogenic and mesogenic vaccines (Hu *et al.*, 2022). The commonly used of these vaccines are lentogenic such as the Hitchner-B1, LaSota, Ulster 2C, VG/GA, V4 and C2 (Genotype II) but mesogenic strains such as Komarov and Roakin, which are more pathogenic, may be used primarily in countries where ND is endemic (WOAH, 2021). Among these vaccine strains, LaSota is the most widely used in different countries including Zambia due to its superior immunogenicity. The LaSota strain shows high tropism to the respiratory system and replicates to high levels in chickens (Perozo *et al.*, 2008).

Live ND vaccines present good safety because the vaccine strains present low or no virulence and usually cause few vaccinal reactions in chickens (Hu *et al.*, 2022). Furthermore, live ND vaccines stimulate both mucosal and systemic immune responses similar to those of the natural infection, are suitable for mass application via drinking water or spray making them highly inexpensive and also contribute to overall herd immunity by the vaccine virus from the vaccinated birds spreading to the suboptimally vaccinated ones in the vicinity (de Geus *et al.*, 2012, Rauw *et al.*, 2009, Thornton *et al.*, 1980). Moreover, strains like V4 and I2 are naturally thermostable vaccines (Brown *et al.*, 2021a) with the unique advantages of being used in remote country areas with limited cold chain facilities.

Inactivated vaccines are also extensively used and are the earliest strategy for ND control. They consist of ND virus formulated with an adjuvant which is usually a water and oil emulsion or may also be an aluminium hydroxide adjuvant. Lentogenic NDV strains, including LaSota, Ulster and B1, are usually used as the master seed due to their high virus yield in ECEs (Hu *et al.*, 2022). They are used to boost the immunity of older birds such as layers and breeders that have previously been primed by a live vaccine and are usually administered by an injection of individual birds (Brown *et al.*, 2021a). The safety of inactivated vaccines is good because the viruses cannot replicate and spread among vaccinated chickens (Hu *et al.*, 2022). However, these vaccines are relatively more expensive than live vaccines, they cannot be used in mass vaccination, they are generally poor inducers of the mucosal or cell-mediated immune response, they require a withdrawal period before the immunised birds can be processed for human consumption and each vaccine requires individual

administration by subcutaneous or intramuscular injection (WOAH, 2021, Kapczynski *et al.*, 2013).

Currently, almost all ND vaccines are produced in ECEs, which are a traditional, mature system for poultry vaccine production. However, there are still some shortcomings of the egg-based system and it is necessary to develop alternative platforms for poultry vaccine production (Hu *et al.*, 2022). One of the most promising strategies used is the use of recombinant viral vector vaccines based on recombinant DNA technology (Bello *et al.*, 2018a). The most common vectors used in poultry are the vaccinia virus, fowl pox virus, and herpes virus of turkeys, which due to their large genomes facilitate the insertion of the necessary genetic sequences that code for key protective ND antigens without adversely affecting the viability and efficacy of the vaccine vector itself (Brown *et al.*, 2021a). Currently, only a few recombinant vector vaccines have been authorized in certain countries including recombinant turkey herpes virus (rHVT/ND) expressing the fusion protein of NDV D-26 lentogenic strain (Vectormune® ND), recombinant fowlpox virus expressing ND antigens (Trovac®-NDV, VectorVax® FP-N) and Innovax®-ND among others (Hu *et al.*, 2022, Brown *et al.*, 2021a). Several other vaccines have been produced with the use of recombinant technology including DNA vaccines, NDV Reverse Genetics-Based vaccines, and Virus-Like Particles (VLP) Platforms (Bello *et al.*, 2018a)

## 2.4 Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2)

### 2.4.1 Taxonomy and Nomenclature of SARS-CoV-2

Coronaviruses (CoVs) belong to the order *Nidovirales*, family *Coronaviridae*, subfamily *Coronavirinae* (Coronaviridae Study Group of the International Committee on Taxonomy of Viruses, 2020). The subfamily includes four genera: *Alphacoronavirus*, *Betacoronavirus*, *Gammacoronavirus*, and *Deltacoronavirus* (Figure 2.3), and comprises more than 60 species with several viruses ranging from human to bovine, porcine, canine, feline, murine, leporid, and avian species of CoV (Havasi *et al.*, 2022). SARS-CoV-2 belongs to the genus *Betacoronavirus* (β-CoV), together with SARS-CoV and Middle East respiratory syndrome coronavirus (MERS-CoV) sharing 80% and 50% homology, respectively (Astuti and Ysrafil, 2020, Zhou *et al.*, 2020). Moreover, comparative sequence analysis has demonstrated

that SARS-CoV-2 may have originated from bats as its genome organisation is 96.2% identical to the genome sequence of BatCoV RatG13 (GenBank: MN996532) (Zhou *et al.*, 2020) isolated from horseshoe bats (Cagliani *et al.*, 2020).

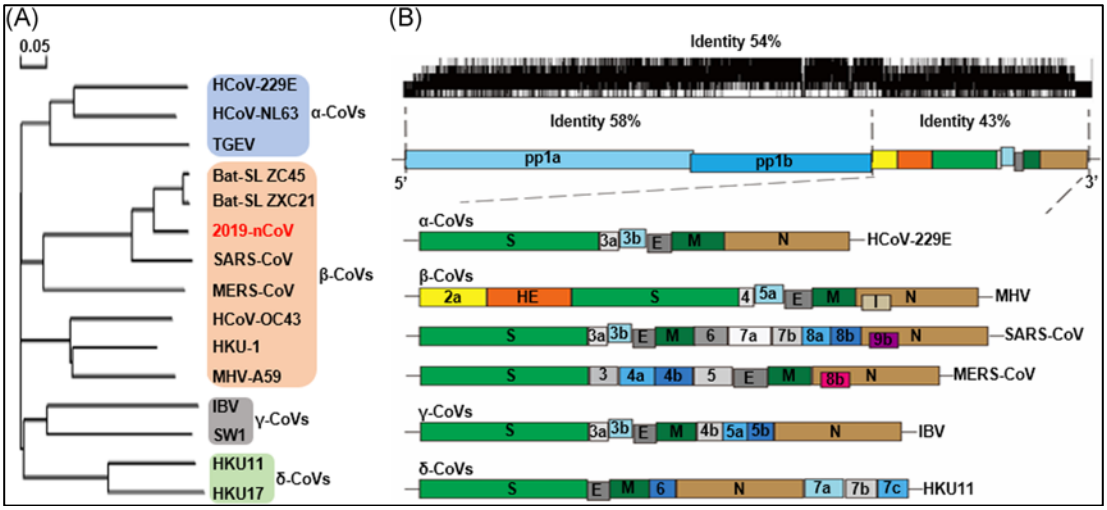


Figure 2.3: The genomic structure and phylogenetic tree of coronaviruses. (A) The phylogenetic tree of representative CoVs, with the new coronavirus 2019-nCoV highlighted in red. (B) The genome structure of four genera of coronaviruses. Adapted from Chen *et al.* (2020). Virus names: HCoV – Human coronavirus; TGEV – Transmissible gastroenteritis coronavirus; Bat-SL – Bat-SARS Like coronavirus; 2019-nCoV – 2019 novel coronavirus (now SARS-CoV-2); SARS-CoV – Severe Acute Respiratory Syndrome Coronavirus; MERS-CoV – Middle East respiratory syndrome Coronavirus; HKU – Human Coronavirus HKU; MHV - Mouse Hepatitis Virus; IBV – Infectious Bronchitis Virus; SW1 – Whale Coronavirus Strain SW1

SAR-CoV-2 has further been classified into lineages, sublineages, groups, or clades based on its genetic diversity. Currently, there are at least four nomenclature systems that have been developed and used to identify and name genetically-distinct SARS-CoV-2 types and lineages including the Global Initiative on Sharing All Influenza Data (GISAID), Nextstrain, Phylogenetic Assignment of Named Global Outbreak Lineages (PANGO) and the 'Greek letter' system proposed by the WHO Virus Evolution Working Group (Konings *et al.*, 2021, WHO, 2021, Alm *et al.*, 2020). Each system has its own scientific approach to classify and name lineages (Konings *et al.*, 2021). While Nextstrain and GISAID clade nomenclatures aim at providing a broad-brush categorisation of globally circulating diversity, PANGO nomenclature is designated to identify the current circulating lineages (Alm *et al.*, 2020). The 'Greek letter' system proposed by the WHO Virus Evolution Working Group is intended for public communication purposes and provides labels only for a small number of variants of concern (VOCs) and variants of interest (VOIs) (WHO, 2021). The WHO SARS-CoV-2 VOCs include Alpha, Beta, Gamma, Delta and Omicron (O'Toole *et*

*al.*, 2022, WHO, 2021). In contrast, the variants Lambda and Mu are classified as VOIs (Flores-Vega *et al.*, 2022).

According to the proposed SARS-CoV-2 lineage nomenclature, two main SARS-CoV-2 lineages (A and B) with multiple sub-lineages have been described (Rambaut *et al.*, 2020). The lineages that begin with the letter A are directly related to the Wuhan/WH04/2020 variant, and the lineages that begin with the letter B are associated with the Wuhan-Hu-1 variant. The new SARS-CoV-2 lineages descending from a lineage A or B are assigned with a numerical value (e.g., lineage A.1 or B.2). With this nomenclature, the variants of concern were designated as B.1.1.7, B.1.351, P.1, B.1.617.2, and B.1.1.529 (Table 2.1) (PANGO, 2022). The GISAID nomenclature classifies the SARS-CoV-2 variants into clades based on the statistical distribution of the viral genomes distance into phylogenetic clusters. Hence, viral variants are classified into eight high-level phylogenetic groups from an early split of S and L, and then by an evolution of L into V and G, and later of G into GH, GR, and GV, and, more recently GR into GRY (Table 2.1) (GISAID, 2021). Nextstrain classifies SARS-CoV-2 into 14 major clades: 19A, 19B, and 20A-20L. The clade name is associated with the year in which a new variant emerges and the new viral variant uses the next letter in the alphabet. Thus, the major clades by year are defined by their emergence and a letter, e.g., 19A, 19B, or 20A (Nextstrain, 2022).

Table 2.1: Classification of SARS-CoV-2 viral variants using the four-classification nomenclature

| **WHO** | **PANGO (Lineage)** | **GISAID (Clade)** | **Nextstrain (Clade)** |
|---|---|---|---|
| Alpha | B.1.1.7 | GRY | 20I (V1) |
| Beta | B.1.351 | GH/501Y.V2 | 20H (V2) |
| Gamma | P.1 | GR/501Y.V3 | 20J (V3) |
| Delta | B.1.617.2 | G/478K.V1 | 21A |
| Epsilon | B.1.427/B.1.429 | GH/452R.V1 | 21C |
| Eta | B.1.525 | G/484K.V3 | 21D |
| Iota | B.1.526 | GH/253G.V1 | 21F |
| Kappa | B.1.617.1 | G/452R.V3 | 21B |
| Lambda | C.37 | GR/452Q.V1 | 21G |
| Mu | B.1.621 | GH | 21H |
| Omicron | B.1.1.529 | BR/484A | 21K |

### 2.4.2 SARS-CoV-2 Genome

SARS-CoV-2 is an enveloped, non-segmented positive-sense, single-stranded RNA virus with a poly-adenylated genome of approximately 30kb (Lu *et al.*, 2020a, Wu *et al.*, 2020). Its diameter is about 65–125 nm, containing single strands of RNA and displaying crown-like spikes on the outer surface (Astuti and Ysrafil, 2020). The SARS-CoV-2 genome contains at least six open-reading frames (ORFs) (Chen *et al.*, 2020). Two-thirds of the SARS-CoV-2 genome is occupied by two large overlapping ORFs, ORF1a and ORF1b which encode 16 non-structural proteins (nsp1-16) (Cagliani *et al.*, 2020, Chen *et al.*, 2020). The other ORFs (ORF3a, ORF6, ORF7b and ORF8) on the one-third of the genome near the 3′- terminus encodes at least four main structural proteins: spike (S), envelope (E), membrane (M), and nucleoprotein (N), as well as a variable number of accessory proteins (Cagliani *et al.*, 2020, Chen *et al.*, 2020, Astuti and Ysrafil, 2020, Yoshimoto, 2020).

The S glycoprotein is a transmembrane protein in the outer portion of the virus and has pivotal roles in viral infection and pathogenesis (Astuti and Ysrafil, 2020, Hofmann *et al.*, 2004). This glycoprotein is cleaved by the host cell furin-like protease into two functional subunits namely S1 and S2. The S1 subunit contains the receptor-binding domain (RBD) which binds directly to the host receptor angiotensin-converting enzyme 2 (ACE2), enabling virus entry into host cells while the S2 subunit mediates virus fusion in host cell membranes (Du *et al.*, 2009). Thus, the S protein defines the infectivity of the virus and its transmissibility in the host cell (Hulswit *et al.*, 2016), and serves as a target for the development of antibodies, entry inhibitors and vaccines (Richter *et al.*, 2021, Tai *et al.*, 2020). The N protein is the structural component of CoV localized in the endoplasmic reticulum-Golgi region that structurally is bound to CoV RNA genome, making up the nucleocapsid (de Haan and Rottier, 2005). It is involved in processes related to the viral genome, the viral replication cycle, and the cellular response of host cells to viral infections (Tai *et al.*, 2020, Schoeman and Fielding, 2019).

The M protein is the most abundant structural protein that defines the shape of the viral envelope. The M protein interacts with other structural viral proteins and plays a central role in coronavirus assembly as it directs envelope formation and provides the matrix to which the nucleocapsid can attach for budding (Neuman *et al.*, 2011).

The binding of M to N protein helps to stabilize nucleocapsids and promotes the completion of viral assembly by stabilizing the N protein-RNA complex, inside the internal virion (Astuti and Ysrafil, 2020). Together, M and E proteins make up the viral envelope and their interaction is sufficient for the production and release of VLPs (Vennema *et al.*, 1996). The E protein is the smallest of the major structural proteins, around 8–12 kDa, and, plays a role in the production and maturation of this virus (Schoeman and Fielding, 2019). During the replication cycle, E is abundantly expressed inside the infected cell, but only a small portion is incorporated into the virion envelope (Venkatagopalan *et al.*, 2015). The majority of the protein is localised at the site of intracellular trafficking, viz. the ER, Golgi, and ERGIC, where it participates in CoV assembly and budding (Venkatagopalan *et al.*, 2015, Nieto-Torres *et al.*, 2011).

### 2.4.3 Replication of SARS-CoV-2

Replication of SARS-CoV-2 begins with attachment and entry of the virus into the host cells. Attachment of the virus to the host cell is initiated by the interactions between the S protein present on the surface of the virus envelope and its receptor the angiotensin-converting enzyme 2 (ACE2) (Malik, 2020) as shown in Figure 2.4. The ACE2 receptors are found in numerous organs, like the heart, lungs, kidneys, and digestive system, enabling the virus to enter target cells (Astuti and Ysrafil, 2020). Moreover, the interaction between the S protein and the ACE receptor is the primary determinant of infecting a host species and also controls viral tissue tropism (Malik, 2020). This attachment occurs in the RBD of the S protein of SARS-CoV-2 which is present at 331 to 524 residues and can bind strongly to human ACE2 and bat ACE2 (Tai *et al.*, 2020). The entry and binding processes are then followed by the fusion of the viral membrane and host cell (Walls *et al.*, 2020).

Following attachment, the SARS-CoV-2 enters the host cell cytosol via acid-dependent proteolytic cleavage and activation of the S protein by type II transmembrane serine protease (TMPRSS2), followed by fusion of the viral and cellular membranes (Malik, 2020, Hoffmann *et al.*, 2020). Once in the host cell, SARS-CoV-2 will subsequently release its genomic material inside the cytoplasm and become translated in the nuclei (Astuti and Ysrafil, 2020) (Figure 2.4). The infecting RNA acts as a messenger RNA (mRNA), which is then translated by host

ribosomes to produce the viral replicative enzymes, which generate new RNA genomes and the mRNAs for the synthesis of the components necessary to assemble the new viral particles (Romano *et al.*, 2020). The genomic RNA acts as the template to directly translate polyprotein 1a/1ab (pp1a/pp1ab), which encodes non-structural proteins (nsps) to form the replicase–transcriptase complex (RTC) in double-membrane vesicles (DMVs). The polyproteins are processed by virally encoded papain-like proteases (PLpro) and a serine-type Mpro (chymotrypsin-like protease (3CLpro) protease that are encoded in nsp3 and nsp5 into 16 nsps.

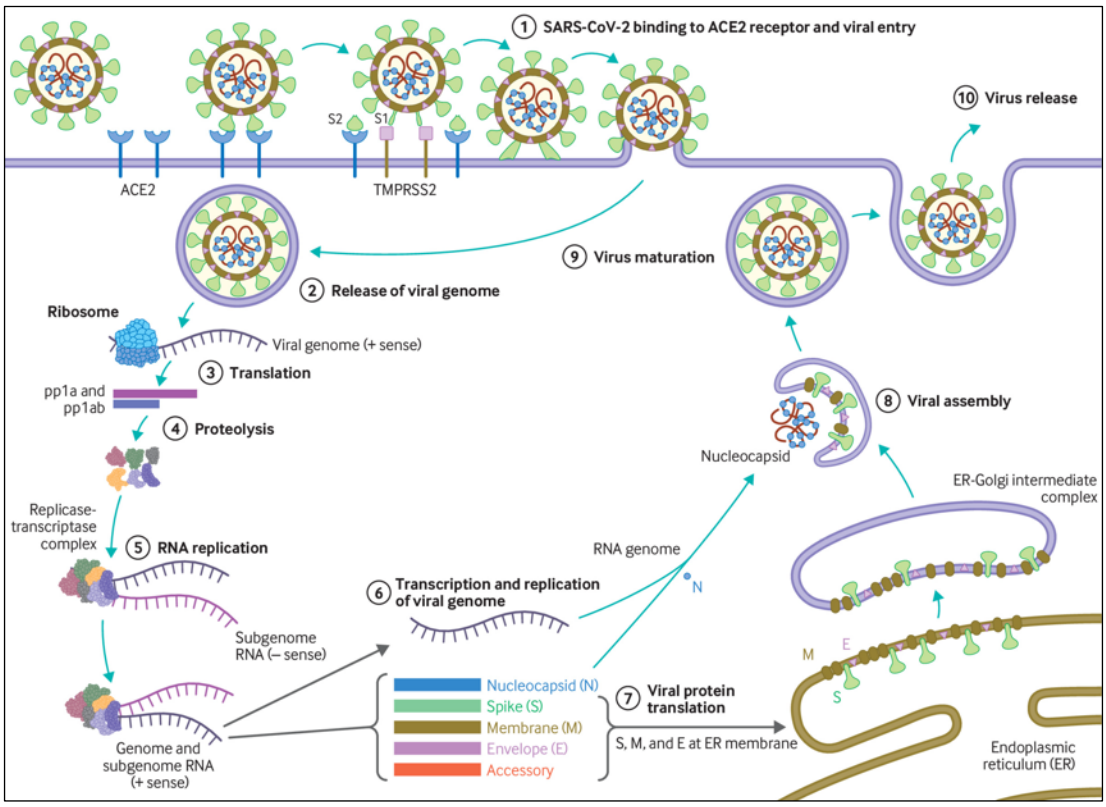


Figure 2.4: SARS-CoV-2 Replication. The virus binds to ACE 2 as the host target cell receptor in synergy with the host's transmembrane serine protease 2 (cell surface protein), which is principally expressed in the airway epithelial cells and vascular endothelial cells. This leads to membrane fusion and releases the viral genome into the host cytoplasm (2). Stages (3-7) show the remaining steps of viral replication, leading to viral assembly, maturation, and virus release. Adapted from Cevik *et al.* (2020)

Many of the nsps assemble into the RTC to create an environment suitable for RNA synthesis and ultimately are responsible for RNA replication and transcription of the sub-genomic RNAs (Fehr and Perlman, 2015). The nsps also contain other enzyme domain functions, including those important for RNA replication, for instance, nsp12 encodes the RdRp domain; nsp13 encodes the RNA helicase domain and RNA 5′-

triphosphatase activity; nsp14 encodes the exoribonuclease (ExoN) involved in replication fidelity and N7-methyltransferase activity among others. Furthermore, the complex transcribes an endogenous genome template of viral entry to negative-sense genes of both the progeny genome and sub-genomic RNA as intermediate products and followed by transcription to positive-sense mRNAs that are mainly mediated by RdRp (Chen *et al.*, 2020).

Following replication and sub-genomic RNA synthesis, the viral structural proteins, S, E, and M are translated and inserted into the endoplasmic reticulum (ER). These proteins move along the secretory pathway into the endoplasmic reticulum–Golgi intermediate compartment (ERGIC). Meanwhile, the previously replicated genome can directly join the N protein to the nucleocapsid form and move into the ERGIC. In this compartment, nucleocapsids will meet with several other structural proteins and small wallet vesicles containing viral structural proteins, forming mature virions to be exported out of the cell through exocytosis (de Haan and Rottier, 2005).

### 2.4.4 Transmission of SARS-CoV-2

SARS-CoV-2 is primarily transmitted through direct or indirect or close contact with infected individuals or their respiratory droplets that are expelled during coughing, speaking, and sneezing, leading to infection of the respiratory tract of an exposed and susceptible person (Leung, 2021). However, virus, host and environmental factors influence whether a successful transmission occurs by governing the infectivity of the respiratory virus. Although SARS-CoV-2 is generally considered to not linger in the air for extended periods of time, experiments have indicated that airborne transmission may be possible for up to 3 hours in certain conditions (van Doremalen *et al.*, 2020). Moreover, fast physical activity can increase the distance these droplets can travel, with simulations suggesting they can reach a maximum of 2 meters. Furthermore, studies have indicated that SARS-CoV-2 can remain infectious for days to weeks on non-porous surfaces such as glass, stainless steel, plastic, and both paper and polymer banknotes (Liu *et al.*, 2021, Riddell *et al.*, 2020, Biryukov *et al.*, 2020, Chin *et al.*, 2020, Kratzel *et al.*, 2020, van Doremalen *et al.*, 2020) thereby making fomites an important mode of transmission. Hence, fomite-mediated transmission occurs when a person touches contaminated surfaces and then subsequently touches their facial area.

SARS-CoV-2 infection is associated with a wide range of clinical presentations, including fever, fatigue, dyspnoea (breathlessness), cough, chest pain, sore throat, and muscle aches (CDC, 2022b, Sheleme *et al.*, 2020). It can also cause lymphopenia (low levels of certain types of white blood cells) and lower respiratory tract infection. Some individuals may experience gastrointestinal issues such as diarrhoea or abdominal pain due to the virus replicating in their intestines (Yang *et al.*, 2020a). Other possible symptoms include rash, changes in taste or smell, headache, and difficulty breathing (Tenforde *et al.*, 2020). In more severe cases, patients may suffer from lung damage, organ failure, shock, and even death (Jin *et al.*, 2020). Research suggests that SARS-CoV-2 affects the body's immune system, causing it to become weaker and creating an environment where other infections are more likely to occur.

### 2.4.5 Epidemiology of SARS-CoV-2

Like AIVs, coronaviruses have crossed the species barrier to cause deadly pneumonia in humans since the beginning of the 21st century. The novel human pathogen SARS-CoV-2 was first identified as the cause of COVID-19 in December 2019 when severe pneumonia-hospitalised cases in Wuhan city, China, were connected through epidemiological links to the Huanan Seafood Market in Wuhan (Telenti *et al.*, 2022). Since then, the virus has resulted caused more than 750 million confirmed cases and claimed over six million lives worldwide as of 29 March 2023 (WHO, 2023). Most of the SARS-CoV-2 infections have been reported in Europe, followed by Western Pacific, the Americas, South-East Asia, Eastern Mediterranean and the least reported cases are in Africa (WHO, 2023). The disease was later declared a pandemic by the world health organization (WHO) in March, 2020.

Since the pandemic began, several SARS-CoV-2 variants carrying mutations with concerning phenotypic implications on current pandemic management strategies emerged (Sanyaolu *et al.*, 2021). The WHO defined such SARS-CoV-2 variant as variants of concern (VOCs) which included Alpha (B.1.1.7), Beta (B.1.351), Gamma (P.1), Delta (B.1.617.2), and Omicron (B.1.1.529). VOCs are associated with enhanced transmissibility or virulence, reduction in neutralization by antibodies obtained through natural infection or vaccination, the ability to evade detection, or a decrease in therapeutic or vaccination effectiveness (Aleem *et al.*, 2022, Dinnon *et al.*, 2020). Furthermore, all the five reported VOCs have mutations in the RBD and

the N-terminal domain (NTD), of which N501Y mutation located on the RBD is common to all variants except the Delta variant (Aleem *et al.*, 2022). The N501Y mutation results in increased binding affinity of the spike (S) protein to angiotensin-converting enzyme (ACE) 2 receptors thereby enhancing the viral attachment and its subsequent entry into the host cells (Ramanathan *et al.*, 2021, Starr *et al.*, 2020). Other genomic changes have been reported, including the extensive deletion in the open reading frame (ORF) 7a, ORF8 (Mazur-Panasiuk *et al.*, 2021, Holland *et al.*, 2020, Addetia *et al.*, 2020), and a deletion in the nsp2 genes (Bal *et al.*, 2020), but these deletions have been associated with mild to moderate clinical symptoms compared to the infection caused by the wildtype SARS-CoV-2 (Mazur-Panasiuk *et al.*, 2021, Young *et al.*, 2020).

The first major variant was observed in September 2020 in the United Kingdom (UK) and was termed VOC 202012/01 (VOC 202012/01) which is now known as Alpha (B.1.1.7). This variant contains point mutations of asparagine to tyrosine in the RBD of the S protein. This N501Y mutation became a growing concern due to the virus being able to adhere to the ACE2 receptor more strongly leading to increased transmissibility, and increased risk of hospitalisation (Volz *et al.*, 2021, Karadag, 2020). The second VOC was the Beta (B.1.351 lineage) variant which was first detected in South Africa in October 2020 and became the most common variant in many African countries (Tegally *et al.*, 2021a). This VOC is characterized by mutations in the S protein, including in the RBD: K417N, E484K, and N501Y (Tegally *et al.*, 2021a, Sanyaolu *et al.*, 2021). In addition, the Beta variant is known to cause severe disease in young and healthy individuals (Tegally *et al.*, 2021a). Similarly, variant Gamma (P.1) was detected in four Brazilians travelling to Japan in January 2021 and was responsible for the resurgence in infections in Manaus, despite high levels of previous infection in the country (Fujino *et al.*, 2021). These variants were then replaced by the highly transmissible Delta (B.1.617.2 lineage) variant which was initially detected in India in December 2020 and spread worldwide among vaccinated as well as unvaccinated individuals (Singanayagam *et al.*, 2022). The Omicron (B.1.1.529) variant, characterised by several mutations in the S protein, including a set of mutations previously observed in other VOCs and novel mutations, was first reported in South Africa on 24 November 2021 (Telenti *et al.*, 2022).

In Africa, despite having a total population of about 1.3 billion, the official reports show a low burden of SARS-CoV-2 infections when compared with other continents. The total number of confirmed cases and fatalities reported in Africa as of 21 March 2023 are 9,509,869 and 175,315, respectively, representing a global burden of 1.2% (WHO, 2023). However, post-mortem and serological studies in some African countries suggest that the true burden of SARS-CoV-2 infections and deaths may be higher than what is officially reported (Gelanew *et al.*, 2022, Chisale *et al.*, 2022, Uyoga *et al.*, 2021, Mwananyanda *et al.*, 2021). Further, a systematic review and meta-analysis on the seroprevalence of SARS-CoV-2 in Africa revealed that over two-thirds of the African population had been infected by SARS-CoV-2 (Lewis *et al.*, 2022). The analysis further revealed that the true number of SARS-CoV-2 infections on the African continent was 97 times higher than the reported confirmed cases and the sharp rise in incidence was attributed to the introduction of the highly transmissible Alpha and Delta variants (Lewis *et al.*, 2022).

The first COVID-19 case in Africa was reported in Egypt on 14 February 2020 (Oluniyi, 2020, Massinga Loembé *et al.*, 2020) followed by Algeria, whose first case was reported on 25 February 2020 (WHO, 2020) and Nigeria on 27 February 2020 (Elimian *et al.*, 2020). Most African countries including Cameroon, Morocco, Senegal, South Africa, Togo, and Tunisia reported their first cases by mid-March 2020 and most of the index cases were imported cases from Europe which by then had become the epicentre of the pandemic (Dufailu *et al.*, 2021, Massinga Loembé *et al.*, 2020). Within three months of Africa's COVID-19 index case, 54 of 55 African Union (AU) Member States (except Western Sahara) had reported over 100,000 cases which included imported and community transmissions (Massinga Loembé *et al.*, 2020). The early phase of the pandemic in Africa was characterized by the predominance of lineage B.1 which was introduced multiple times in African countries (Wilkinson *et al.*, 2021). However, due to a ban on international air travel in most African countries and the world at large in March/April 2020, the number of SARS-CoV-2 importations into Africa decreased and the pandemic entered a phase that was characterized by sustained within-country spread and occasional international viral dissemination between neighbouring countries, presumably via road and rail links between these countries (Wilkinson *et al.*, 2021).

As the pandemic progressed VOCs were also reported in Africa and to date, four VOCs, namely Alpha (B.1.1.7), Beta (B.1.351 lineage), Delta (B.1.617.2 lineage), and Omicron (B.1.1.529) have been detected on the African continent. These variants were associated with the pandemic waves in Africa. Whereas the Beta variant was associated with the second wave of SARS-CoV-2, the Alpha variant did not predominate in many African countries possibly due to a lack of selective advantage over the other VOCs (Hirabara *et al.*, 2022). In addition, the Delta (B.1.617.2 lineage) variant seeded the third wave of the pandemic in 2021 and was introduced in Africa in June 2021. By the end of 2021, the Omicron (B.1.1.529) variant was detected in South Africa on 24 November 2021 and became the dominant driver of the fourth global wave of SARS-CoV-2 (Telenti *et al.*, 2022). To date several sub-lineages of SARS-CoV-2 have been detected in Africa including BA.1, BA.2, BA.1.1, BA.4, BA.5, BA.5.3.1, XBB.2, BQ.1.23 among others (GISAID, 2023)

In Zambia, the first known COVID-19 cases were reported on 18 March 2020 from travellers returning from Europe (Chipimo *et al.*, 2020). Within days, the government implemented restrictions on international travel, school closures, halting of non-essential business, and confinement of people to their homes. Despite these measures, the virus spread to all parts of the country with over 300,000 cases and over 4000 deaths as of 4 February 2023 (ZNPHI, 2023). The course of the pandemic in Zambia can be divided into four major waves: the first wave occurred from July to September 2020 and was mainly driven by B.1.1 and its sub-lineages; the second wave occurred from December 2020 to April 2021 and was dominated by the Beta variant, while the Delta variant dominated the third wave from May to September 2021 (Worldometer). The Omicron variant has dominated the fourth pandemic wave in Zambia, with cases peaking in early January 2022 and then rapidly decreasing to low levels. In the Southern Province, which shares international borders with Botswana, Namibia, and Zimbabwe and is a major tourist destination, SARS-CoV-2 was first detected in May 2020 (ZNPHI, 2023). Hence, As the course of the pandemic continues to evolve, it remains crucial to monitor and understand the virus evolution and outbreak dynamics, particularly in strategically positioned regions such as the Southern Province which is a trade entry point of Zambia for all imports and exports from Southern Africa.

### 2.4.6 Diagnosis of SARS-CoV-2

The proper management of the ongoing COVID-19 pandemic depends on the provision of accurate, accessible, time and cost-effective diagnostic methods. However, this is being hampered by the emergence of novel and evolving variants of SARS-CoV-2 and has necessitated the change in the form of newer and more adaptive diagnostic methods for the detection of infections. On the other hand, developing rapid and sensitive diagnostic technologies is now more challenging due to emerging variants and varying symptoms exhibited in infected individuals (Fernandes *et al.*, 2022). Currently, several commercial SARS-CoV-2 detection technologies exist including technologies that can identify a) specific viral gene regions through nucleic acid amplification techniques (Real-Time Reverse Transcription Polymerase Chain Reaction (RT-qPCR) and isothermal nucleic acid amplification), b) the antibodies produced by the immune system in response to the viral infection (serology/Immunoglobulin M (IgM)/Immunoglobulin G (IgG) tests), and c) the antigen testing by lateral flow assays (Fernandes *et al.*, 2022, Yüce *et al.*, 2021). However, the choice of method depends on the selection of the right test, right sample, and right time as the viral nucleic acid/antigen/antibody detection varies at different time points during the infection (Falzone *et al.*, 2021, Alpdagtas *et al.*, 2020).

#### 2.4.6.1 Molecular Methods

Currently, nucleic acid-based molecular diagnosis via RT-qPCR test is considered the golden standard for the early diagnosis of SARS-CoV-2 infection (Havasi *et al.*, 2022). Moreover, diagnosis based on nucleic acid detection is more sensitive and specific compared to other tests. RT-qPCR assays generally target one or more of the SARS-CoV-2 genes such as ORF1a/b, ORF1b–nsp14, RdRp, envelope (E), spike (S), or nucleocapsid (N) genes (Chan *et al.*, 2020, Alpdagtas *et al.*, 2020). Therefore, viral mutations can potentially alter the accuracy of this method, leading to unpredictable test performances and false negatives. However, to surpass this limitation, the use of specific primers and probes, and multi-target assays is recommended. Moreover, studies are now developing specific primers to enable the rapid detection of VOCs through real-time PCR, thus enabling the differentiation of VOCs from regular SARS-CoV-2 strains (Puvar *et al.*, 2021, Zelyas *et al.*, 2021). Other limitations include its low sensitivity in correctly diagnosing samples with low

viral load, including swabs taken incorrectly or obtained from asymptomatic or paucisymptomatic individuals and it is prone to contaminants and interferers contained in the sample or introduced by the operator capable of inhibiting the reaction (Falzone *et al*., 2021).

LAMP is an isothermal amplification technique which offers a viable alternative to cyclic nucleic acid amplification and the traditional RT-PCR method. LAMP has been developed as a fast, accurate, reliable, and cheaper technique to amplify the target region at a single reaction temperature instead of the thermal cycle required in RT-PCR (Notomi *et al.*, 2000). The advantage of the LAMP method to RT-PCR is that the amount of DNA produced is much higher, and a positive test result can be viewed visually without the need for an additional analysis step. In addition, RT-LAMP has proven to be is highly sensitive and specific in targeting the ORF1ab gene of SARS-CoV-2 (Yang *et al.*, 2020b). Other molecular methods used to detect and characterise SARS-CoV-2 include clustered regularly interspaced short palindromic repeats (CRISPR)-based analysis, microarray assays and NGS (Alpdagtas *et al.*, 2020).

#### 2.4.6.2 Antibody-Based Techniques

Antibody-based techniques can be a useful tool for detecting current and past exposure to SARS-CoV-2. However, these assays are not ideal for the early detection of infection due to the delay in producing an immune response, typically taking up to two weeks from initial infection; a time-point at which viral nucleic acid and antigen levels begin to decline (Falzone *et al.*, 2021). Therefore, it is important to recognize that antibody-based techniques alone cannot provide an early warning of infection; rather, they should be used in conjunction with other diagnostic tests to help ensure patient safety. Various techniques such as immunofluorescence, immunochromatographic, chemiluminescence and ELISA are used for the detection of antibodies generated specific to the SARS-CoV-2 viral antigen (Di Domenico *et al.*, 2021). However, the most common antibody tests are based on lateral flow type assays (LFA) and ELISA (Yüce *et al.*, 2021). Most of the commercially available kits target the antibodies generated against the viral S and N proteins and other easy-to-use kits are based on measuring the ratio between the IgM and IgG in the blood (Edouard *et al.*, 2021). Furthermore, humoral immune responses to SARS-CoV-2

can also be detected using simple blotting systems through which the reactivity of human IgGs against five key SARS-CoV-2 viral antigens is detected (Edouard *et al.*, 2021).

#### 2.4.6.3 Antigen-Based Techniques

Antigen-based techniques are reliable and cost-effective methods for detecting SARS-CoV-2 infections. Unlike PCR-based techniques, tests such as immunofluorescent assays, immunochromatographic assays, chemiluminescent immunoassays, and ELISA, can detect the presence of two main antigens produced by the virus: the S and N proteins or the virus directly without thermal amplification steps (Yüce *et al.*, 2021). However, their effectiveness may vary according to factors such as disease stage and viral load; these should be taken into account when utilizing antigen-based techniques to achieve optimal results (Fernandes *et al.*, 2022). Moreover, clinical specimens such as nasopharyngeal swabs, nasal swabs, and saliva can all be used with antigen-based kits for detection purposes (Alpdagtas *et al.*, 2020). Ultimately, antigen-based methods provide a reliable means of recognizing SARS-CoV-2 infection through unique antigens that cannot otherwise be identified by other common testing strategies.

### 2.4.7 Treatment, Prevention and Control of SAR-CoV-2

#### 2.4.7.1 Treatment of SARS-CoV-2

The treatment of SARS-CoV-2, the causative agent of COVID-19, is an evolving science. Currently, two primary avenues of care have been established; antiviral medications and immune modifiers. Among the antivirals, Remdesivir has gained attention for its efficacy in containing the viral load and was also the first drug approved by the Food and Drug Administration (FDA) for the treatment of patients with pneumonia during oxygen shortages. This drug is a broad-spectrum adenosine nucleotide analogue and phosphoramidite pro-drug which targets a wide range of viruses including coronaviruses. It primarily works by terminating RNA synthesis and inhibits SARS-CoV-2 genome replication in respiratory-associated epithelial cells (de Wit *et al.*, 2020, Li *et al.*, 2020). Additionally, remdesivir triphosphate the active form of Remdesivir resembles the RNA of the virus itself, making it a potent drug for combatting SARS-CoV-2. In addition, studies have demonstrated that a combination of remdesivir and baricitinib is particularly efficacious at reducing

recovery times among patients hospitalized with COVID-19 pneumonia. It appears that baricitinib's antiviral and anti-inflammatory properties stem from its ability to inhibit clathrin-mediated endocytosis, thus controlling cytokine levels. The efficacy of such treatments is certainly encouraging; however, further investigation into novel treatments for SARS-CoV-2 will be paramount for curbing its spread.

Other antivirals currently repurposed for COVID-19 therapy include neuraminidase inhibitors, protease inhibitors and virus fusion inhibitors (Chung *et al.*, 2021). Oseltamivir a neuraminidase inhibitor that has proved successful in the treatment of influenza A and B require neuraminidase for virus release to host cells (Chung *et al.*, 2021). However, SARS-CoV-2 does not express neuraminidase and this has necessitated the use of combination therapy involving protease inhibitors to improve the efficacy against this novel CoV (Rosa and Santos, 2020). Such strategies demonstrate an important shift from single-drug treatments commonly used in the past towards integrated therapies targeting multiple points of viral replication. Lopinavir and ritonavir, two protease inhibitors approved for treating HIV-1 infection (Croxtall and Perry, 2010), have been co-formulated and used in combination therapy with PEG-IFN-α, ribavirin, chloroquine or hydroxychloroquine to combat COVID-19 (Hung *et al.*, 2020, Hashem *et al.*, 2020). Although these treatments may provide some relief from symptoms, their efficacy in treating SARS-CoV-2 is as yet unproven, making them far from ideal solutions to this pandemic (Mitja *et al.*, 2021, Ma and Wang, 2021). The other antiviral arbidol, also known as umifenovir a protease inhibitor is capable of preventing virus-cell membrane fusion and the subsequent joining of the viral membrane to endosomes after they have undergone endocytosis (Monteil *et al.*, 2020). Additionally, studies have found that when used in tandem with protease inhibitors, umifenovir can interfere with Vero E6 cell binding processes for SARS-CoV-2, thus serving as an effective treatment for COVID-19 (Wang *et al.*, 2020).

Tocilizumab and Sarilumab, have recently been utilized as immunotherapies for their potential to treat the cytokine storm caused by critical COVID-19 infections (Soin *et al.*, 2021). These treatments work by blocking downstream signals from interleukin-6 receptors (Brown *et al.*, 2021b) and ultimately prevent the release of proinflammatory cytokines (Zhang *et al.*, 2020) which can be extremely detrimental

in cases of SARS-CoV-2. infection. The other immune modifiers are Casirivimab with Imdevimab which forms a unique monoclonal antibody cocktail named REGEN- COVTM that binds non-competitively to the SARS- CoV-2 spike protein, thus being beneficial in targeting the novel mutant SARS-CoV-2 variants and lowering chances of their immune escape (Bergman *et al.*, 2021).

#### 2.4.7.2 Prevention and Control of SAR-CoV-2

SARS-CoV-2 has had a devastating impact on the world, and it's important to understand how to prevent its spread. To prevent infection from SARS-CoV-2, the CDC recommends physical distancing by staying at least 6 feet away from others, especially those who are suspected or confirmed to have the virus, community use of well-fitting masks (e.g., barrier face coverings, procedure/surgical masks), adequate ventilation, and avoidance of crowded indoor spaces (CDC, 2021). Furthermore, the practice of good hygiene by covering your mouth and nose when coughing or sneezing and hand washing is important in the prevention and control of SARS-CoV-2 infections. These methods reduce transmission both from inhalation of the virus and deposition of the virus on exposed mucous membranes (CDC, 2021).

Vaccines are also available to help prevent SARS-CoV-2 infection. Vaccines are a crucial preventative measure for minimizing the risk of COVID-19, as well as any potential variants that could arise (CDC, 2022a). While there is an initial financial cost to produce and distribute such vaccines, their usage can result in substantial cost savings; both in terms of lives lost due to infectious diseases and medical costs associated with treating those affected. Moreover, vaccines are safe and often more palatable than other conventional treatments and vaccinating against SARS-CoV-2 may be our most pragmatic solution for combating this current pandemic (CDC, 2022a). COVID-19 vaccines include protein subunits, mRNA, live attenuated and whole inactivated, viral vectors, VLP and cell-based vaccines, all sharing the same goal of stimulating the immune system against SARS-CoV-2 with the generation of memory cells (Chung *et al.*, 2021).

Protein subunit vaccines are based on microorganisms' fragments and only include the pathogens' antigenic components that are required to elicit effective immune responses rather than complete viral particles (Heidary *et al.*, 2022). The S protein of

SARS-CoV-2 has been shown, to be an ideal target for vaccine development on multiple platforms due to its high antigenicity and potency to induce robust immune responses. Epitopes of the S, M, N and E proteins of SARS-CoV-2 have been screened to identify the immune response for enhanced antibody production and T-cell responses against SARS-CoV-2 (Shang *et al.*, 2020, Ahmed *et al.*, 2020). Moreover, alternative subunit protein vaccines using RBD, S1, and S2 domains have been explored (Liu *et al.*, 2020a). The S2 protein subunit vaccines primed receptor binding and entry with antiviral effects while the vaccine targeting the S1 protein and RBD prevented the entry to host cells thereby controlling viral infection (Quinlan *et al.*, 2020). Protein subunits have limited immunogenicity and require adjuvants to produce neutralizing antibodies. NVX-CoV2373 (Novavax) is an example of a protein subunit SARS-CoV-2 vaccine consisting of nanoparticles containing baculovirus-expressed full-length SARS-CoV-2 S protein produced in insect cells and Matrix-M1, a saponin-based adjuvant (Golob *et al.*, 2021). The vaccine is capable of inducing a predominant CD4+ T cell response characterized by high production of IFN-γ, IL-2, and TNF-α. Furthermore, the clinical trials from the UK indicate that the efficacy of NVX-CoV2373 is estimated at 89.7% among different subgroups including participants with comorbidities, with no hospitalization or deaths reported in vaccinated individuals (Heath *et al.*, 2021).

The mRNA-based vaccines contain the mRNA molecules that encode protein antigens (Fathizadeh *et al.*, 2021). This technology is based on the principle that mRNA is an intermediate messenger that can be easily delivered into host cells and translated into an antigen of interest that will trigger a protective antigen-specific immune response in the human body. Shortly after the onset of the COVID-19 pandemic, two mRNA vaccines, namely, BNT162b2 (Pfizer-BioNTech) and mRNA-1273 (Moderna biotechnologies Inc.) were approved by the FDA for emergency use as a prevention against SARS-CoV-2 infection (Teo, 2022). Both vaccines, BNT162b2 and mRNA-1273, carry a nucleoside-modified mRNA encoding the full-length SARS-CoV-2 spike protein (S) stabilized in the prefusion conformation and formulated in lipid nanoparticles (LNPs). These LNPs lipid nanoparticles protect the vaccine against degradation, facilitate endosomal escape and can target the required cell type through ligands on its surface (Shang *et al.*, 2020). The advantage of this approach is that the SARS-CoV-2 S protein is produced by the vaccine recipients'

cells, such that the target antigens can be processed for presentation via class I and II MHC from the transfected cells and professional antigen-presenting cells, respectively (Golob *et al.*, 2021). Moreover, mRNA vaccine production is rapid and can be manufactured on larger scales at relatively lower costs, mRNA vaccines are considered safe since they do not contain the full pathogen and do not carry the viral DNA material that might be associated with genotoxic concerns (Teo, 2022, Fernandes *et al.*, 2022). While other vaccines can only be used in adults and older children, the FDA approved the use of mRNA-1273 and BNT162b2 COVID-19 vaccines in children aged 6 months–5 years and in children aged 6 months–4 years, respectively (Fleming-Dutra *et al.*, 2022). However, the major drawback with mRNA vaccines is the stability of the formulation since they require strict temperature control for shipment and storage to avoid the degradation of the mRNA and the induced activation of the immune system would potentially lead to side effects associated with enhanced inflammatory processes (Fernandes *et al.*, 2022).

Live attenuated and whole inactivated vaccines rely on the use of the whole pathogen in a weakened or inactivated state through chemical or physical alterations, and have resulted in many clinically successful vaccinations. Though this approach has historically proven effective in many vaccinations, there is always a concern that mutations could lead to virulence reversal and reactivation in immunocompromised individuals, a risk which should not be overlooked. Another problem with this approach is the selection of the appropriate viral strain (Fathizadeh *et al.*, 2021). Several candidate SARS-CoV-2 vaccines employ this well-established method in which reference strains of the targeted virus are grown and then inactivated formalin, formaldehyde, β-propiolactone and UV alone or a combination of these methods (Golob *et al.*, 2021). Sinopharm and Sinovac are among the manufacturers farthest along in the development of this type of vaccine, which has been evaluated by phase 3 trials that have attained international authorizations for use (Creech *et al.*, 2021). Moreover, both Sinovac and Sinopharm vaccines have been approved for emergency use authorisation in China and also in some other countries (Golob *et al.*, 2021).

Viral vector vaccines employ replication-deficient viruses engineered to express the genetic sequence of the antigen of interest in host cells (Creech *et al.*, 2021). SARS-CoV-2 viral vector vaccines are usually based on the adenovirus type 5 (Ad5) and

most of these vaccines express the S protein or RBD subunit of SARS-CoV-2 (Zhu *et al.*, 2020). The approach uses the host cellular machinery for transcription of the SARS-CoV-2 S protein gene to mRNA and then translation to the SARS-CoV-2 S protein (Golob *et al.*, 2021). However, a major challenge to this approach is the potential presence of pre-existing cellular immunity or neutralising antibodies against the viral vector (Xiang *et al.*, 2002). The two commonly used SARS-CoV-2 viral vector vaccines are AZD1222 (AstraZeneca/University of Oxford), and Ad26.COV2.S (Janssen), the Gam-COVID-Vac. The AZD1222 is a recombinant adenovirus-based SARS-CoV-2 vaccine constructed from the replication-deficient simian chimpanzee adenovirus vector (ChAdOx2) expressing the full-length SARS-COV-2 S glycoprotein. The AZD1222 vaccine has been approved by the WHO and has been used in several countries (Tracker, 2022) including Zambia. However, many European countries suspended the use of the AZD1222 vaccine due to reports linking it to episodes of thrombocytopenia, bleeding, and arterial and venous thromboses occurring within days to weeks after vaccination. Despite the reported side effects which call for more investigations, the AZD1222 vaccine is safe and induces high cellular and humoral immune responses (Fernandes *et al.*, 2022, Chung *et al.*, 2021).

The other vaccine technologies include the VLP and cell-based vaccines. Virus-like particles (VLPs) are produced by recombinant expression of structural proteins. VLP technology enables the creation of an artificial SARS-CoV-2 particle that lacks the viral genome, rendering them non-infectious but structurally identical to the virus. These particles can fuse with host cells via ACE2 receptors, allowing for priming by TMPRSS2 without directly engaging B cell receptors responsible for antibody production. This makes VLPs highly stable, scalable for production, cheaper and ultimately safe (Ortega-Berlanga and Pniewski, 2022). Cell-based therapy has also been employed to create vaccines for SARS-CoV-2, specifically, researchers have developed a "synthetic mini-gene" which expresses the four viral proteins S, M, E, N, and polymerase protease of the virus in an artificial APC (NCT04276896). Furthermore, DCs modified to express viral antigens were also generated with the purpose of stimulating cytotoxic T cells ex vivo (NCT04299724). In addition, Aivita Biomedical Inc.'s AV-COVID-19 is being tested via phase I clinical trials and utilizes autologous DCs containing SARS-CoV-2 antigens (NCT04386252).

Unfortunately, these types of vaccinations are limited by their low productivity and expensive costs (Chung *et al.*, 2021).

# CHAPTER THREE

## 3.0 Materials and Methods

### 3.1 Study Design

The research was preceded by systematic reviews and a meta-analysis of the distribution of AIVs collected from birds in sub-Saharan Africa, and IAVs and IDV collected from non-human mammalian hosts in Africa, respectively. In addition, descriptive cross-sectional studies were conducted on AIV, NDV and SARS-CoV-2 in 2015 and 2020–2022 in selected parts of Zambia.

#### 3.1.1 Study Sites

The Systematic review focussing on the distribution of AIV in birds was done on studies conducted in the sub-Saharan Africa while a systematic review and meta-analysis of IAV and IDV in non-human mammalian hosts was conducted on articles from Africa. Genetic studies of AIV, NDV and SARS-CoV-2 were conducted in the Copperbelt, Lusaka and Southern provinces of Zambia from 2015 and 2020 to 2022 (Figure 3.1). For wild waterfowl, samples were collected from Lochinvar National Park (LNP) located in southern Zambia (Figure 3.1A). The LNP (15°51'S 27°13'E) is home to over 420 bird species and more than 30,000 endemic Kafue Lechwe (*Kobus leche kafuensis*) (Zambia Tourism, 2022). It is an important site for AIV surveillance as it receives migratory birds and various AIV subtypes have been previously detected in faecal samples of birds found in this park (Simulundu *et al.*, 2011, Simulundu *et al.*, 2009). Poultry samples were collected from live bird markets (LBMs) in various towns of the Copperbelt, Lusaka and Southern Provinces of Zambia (Figure 3.1A). The sampling sites were purposively chosen based on their high population density of poultry and availability of LBMs. Traders in the LBMs acquire their bird stocks from various commercial and backyard poultry farms across the country for restocking (especially indigenous (village/traditional) chicken keepers), and home and hospitality businesses for slaughter. Poultry in LBMs sampled were kept in wire-mesh cages throughout without separation of cages among different bird species (Figure 3.2). Human samples for SARS-CoV-2 were collected between December 2020 and April 2022 from eight districts in the Southern Province as shown in Figure 3.1B. The Southern Province was specifically

chosen for the SARS-CoV-2 because of its strategic location as a tourist capital and border province.

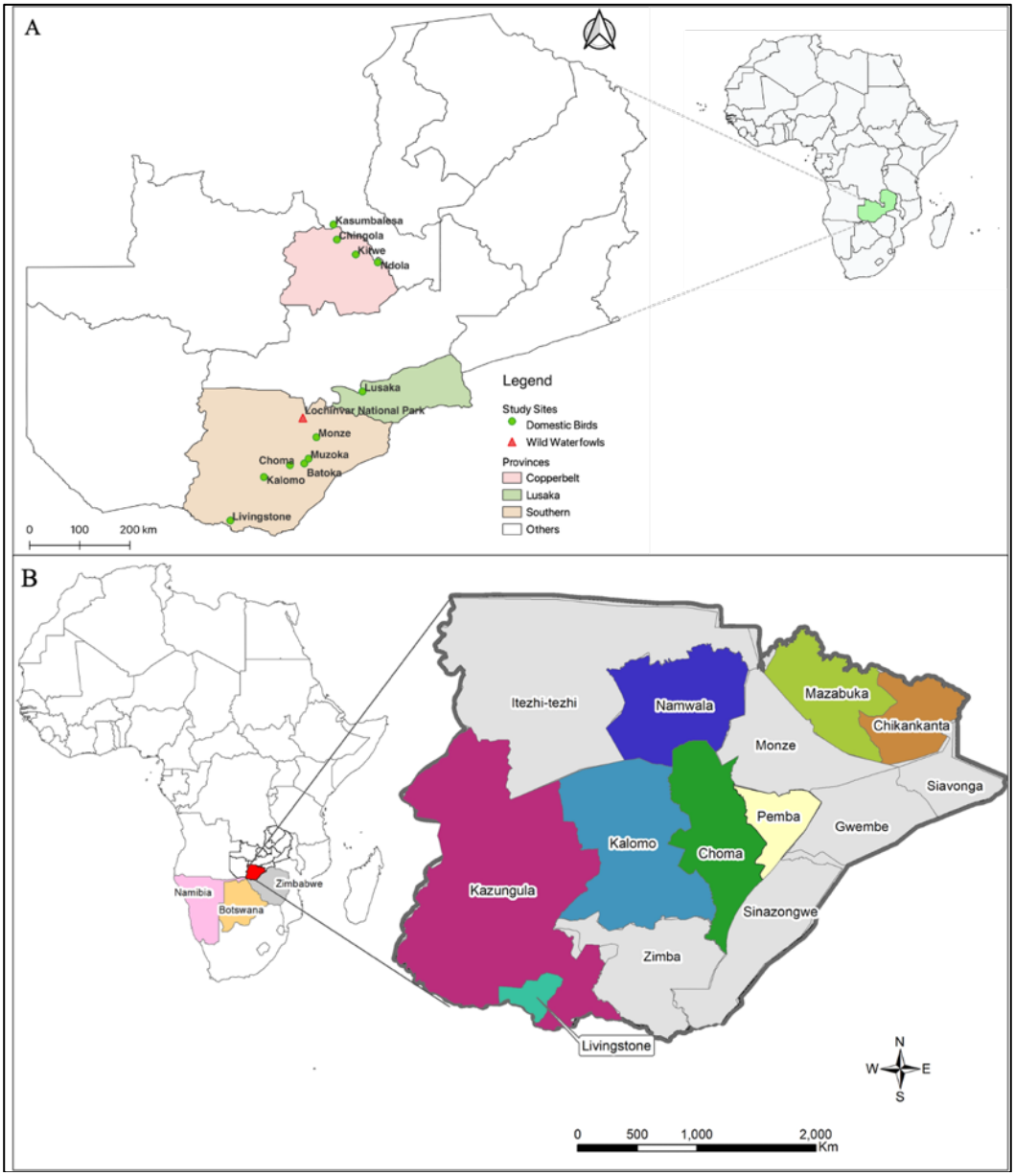


**Figure 3.1:** Maps showing the study sites. A. The locator map showing Zambia with neighbouring countries that share the border with Southern Province. The insert map showing Zambia with study sites namely Copperbelt, Lusaka and Southern provinces. B. The locator map depicts Zambia with neighbouring countries that share the border with Southern Province. The insert map shows the Southern Province of Zambia with the study sites namely Chikankata, Choma, Kalomo, Kazungula, Livingstone, Mazabuka, Namwala and Pemba districts. The maps were generated using Quantum Geographic Information System (QGIS) version 3.10 (http://www.qgis.org).

Figure 3.2. Poultry in wire mesh cages at one of the live bird markets.

### 3.1.2 Sample Size and Sample Collection

#### 3.1.2.1 Systematic Review and Meta-analysis

To investigate the distribution of AIVs in domestic and wild birds, a systematic review was conducted in sub-Saharan Africa to identify all publications reporting the detection of AIVs in birds between 2000 and 2019. Additionally, to investigate the distribution of IAV and IDV in non-human mammalian hosts, a systematic review and meta-analysis was conducted to search for literature reporting IAV and IDV from 2000 to 2020.

#### 3.1.2.2 Avian Influenza Virus and Newcastle Disease Virus

For AIV and NDV, 2,851 fresh faecal samples were conveniently collected from various wild waterfowl whereas 1,150 feacal samples were collected from poultry.

For wild waterfowl, sample collection was carried out once every month from January–February and May–December in 2015, September and October in 2020, and October–December in 2021. Approximately 200 samples were collected each month except for some months in the rainy season (March–April 2015 and January–April 2020–2021) when the wetland was inaccessible due to extreme flooding. The sampling strategy was to collect samples from sites where waterfowl were physically seen congregating for easier morphological identification of the birds by the trained ornithologist from the Department of National Parks and Wildlife and to collect well-separated fresh faecal samples. A sample was collected in a sterile airtight plastic container from each fresh faecal material in the field and placed on ice packs. Poultry samples were collected in 2020 and 2021, and sampling was done once in each LBM in the selected provinces of Zambia. However, sampling was not done in some dry months of 2020 and 2021 for both wild waterfowl and poultry due to public health restrictions on movement during the coronavirus disease 2019 (COVID-19) pandemic. Samples were kept at 4°C and transported to the University of Zambia, School of Veterinary Medicine laboratories for further analysis within 24 hours of being collected or stored at -80ºC.

#### 3.1.2.3 SARS-CoV-2

For SARS-CoV-2, samples were collected by the Ministry of Health through the Zambia National Public Health Institute under the coordination of the Zambia Genomic Sequencing Consortium. Therefore, a convenient sample of 198 nasal pharyngeal swabs representing the number of samples received from the Ministry of Health for sequencing was used. The samples were collected through routine surveillance (i.e., point of entry screening and routine screening for influenza-like illnesses) and targeted surveillance of cluster outbreaks. Samples were collected between December 2020 and April 2022. Upon receipt, all samples were retested to determine the cycle threshold (Ct) value of each sample. Samples that had a Ct value of ≤30 and were submitted with the relevant metadata were included to undergo WGS. Samples that did not meet the inclusion criteria and those that could not be amplified or had poor genomic coverage were excluded from further analysis.

## 3.2 Determination of Prevalence, Seroprevalence and Virus Subtypes of AIV in Birds in sub-Saharan Africa

### 3.2.1 Literature Search

Using the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) protocol 2010 guidelines (Appendix A Checklist) (Moher *et al.*, 2010), a systematic literature search was conducted to identify all publications reporting the detection of AIVs in birds in sub-Saharan Africa between 2000 and 2019. Three electronic databases namely PubMed, SpringerLink electronic journals and African Journals Online (AJOL) were searched using the medical subject headings (MeSH) keywords and Boolean connectors. The following keywords were used: "influenza in birds," "influenza," "birds," "avian," "avian influenza," "avian influenza virus," "sub-Saharan Africa," "countries in sub-Saharan Africa," "epidemiology," "prevalence" and "subtype." Furthermore, the search was restricted to original articles, titles, abstracts and keywords published in English, which reported on AIVs using serological and/or molecular methods. The last search was conducted on 17th January 2020. All references located in the searches were entered into Endnote, a web-based reference manager. Furthermore, a database was built that included the references of all selected publications, as well as the title, author, year of publication, country or countries where the study was conducted and language of publication. The articles were selected using a two-stage approach. During the first stage, the publications were selected based on their titles and abstracts while the full text of articles selected in the first stage was assessed for eligibility in the second stage. At this point, the articles that did not meet the inclusion criteria were excluded.

### 3.2.2 Inclusion and Exclusion Criteria

All study designs were included in this review except experimental studies as these do not represent natural infections. Additionally, studies published between 2000 and 2019, serological and molecular studies on AIV in birds in sub-Saharan Africa were investigated. Moreover, publications containing data on the positive diagnostic test result, data on incidence, prevalence and distribution of AIV in any naturally infected birds were included.

For the exclusion criteria, editorials, comments/letter to the editor, congress or conference abstracts, review articles, perspectives, personal opinions, theoretical

models, pathogenesis models, animal models, case reports in humans, or reports in non-avian species and studies reported in languages other than English were excluded. Moreover, studies with the following characteristics were excluded: the diagnostic test not specified, sample source not described, publications reporting data published elsewhere other than sub-Saharan Africa, outbreak reports without laboratory-based confirmation, reporting a zero incidence/prevalence in any diagnostic test, studies with data overlapping with another included study and publications exclusively on the experimental infection. For prevalence and seasonality of AIV analysis, all studies without sampling time, sample size, prevalence or rate were excluded. Additionally, studies with a sample size of less than five were excluded.

### 3.2.3 Data Extraction

A database on reference information regarding the author's name, title and year of publication was recorded in the data extraction file. Furthermore, from the included publications, data were extracted on country or countries of study including region, years of sample collection, avian species, the purpose of study, number of samples analysed, type of samples collected, the diagnostic method(s) used, number of positives and pathogenicity of the AIV subtypes (LPAI or HPAI).

### 3.2.4 Assessment of Quality and Risk Bias of the Included Studies

To assess the quality and risk of bias of the included studies, the McMaster Critical Review Form—Qualitative Studies (version 2.0) (Letts *et al.*, 2007) and McMaster Critical Review Form—Quantitative Studies (Law *et al.*, 1998) were used.

## 3.3 Investigation of the Prevalence and Seroprevalence of IAV and IDV in Non-Human Mammalian Hosts in Africa

### 3.3.1 Literature Search Strategy

The systematic review and meta-analysis were conducted according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (Appendix B Checklist) (Moher *et al.*, 2009). A systematic literature search was conducted to identify all publications reporting IAV and IDV in non-avian species in Africa from the year 2000-2020. Three databases, PubMed, Google Scholar and Wiley Online Library were searched using the following 13 search terms: "animal

influenza viruses, influenza A virus, influenza D virus, influenza viruses, influenza A virus in animals, influenza D virus in animals, influenza in animals, influenza in swine, influenza A virus in pigs, influenza A virus in livestock, prevalence of influenza A virus in livestock, influenza outbreaks in livestock, epidemiology of influenza A virus in livestock and Africa". The Boolean terms "AND", "OR" and "NOT" were also used. Additional articles were identified by reviewing the reference list of the primary articles. All references located in the searches were imported into Endnote Version 8, a web-based reference manager, and a database for all relevant articles was generated.

### 3.3.2 Study Selection

All studies identified in the search were assessed, duplicates removed and checked for eligibility. The studies were initially selected based on the relevance of their titles and abstracts regarding the prevalence and circulation of IAV and IDV in non-avian species in Africa. Thereafter, full-texts of the remaining articles were screened and those that did not meet the inclusion criteria were excluded.

### 3.3.3 Inclusion and Exclusion Criteria

The review included all study types on IAV and IDV in non-human mammalian species with the exception of (i) experimental studies, (ii) studies on the development of new diagnostic methods and (iii) vaccine development. Only studies written in English and published between 2000 and 2020 were included in this review and meta-analysis. Studies excluded from the review included those not published in Africa, editorials, conference proceedings, review articles, animal experiments, theoretical models, and studies in human and avian species. Studies were further excluded if the diagnostic test was not indicated, had overlapping data with another included study and were excluded from the meta-analysis if the sample size was less than five.

### 3.3.4 Data Extraction

We extracted study information regarding the author's name, title and year of publication. Additional information extracted included country/region, study type, animal species, sample type, diagnostic method, sample size, number of positive samples, IAV sub- type, strain, vaccination status (important for swine and equids),

and premises (indicating where the sample was collected such as farm and slaughterhouse, etc.).

### 3.3.5 Assessment of Quality and Risk of Bias

We assessed the quality and risk of bias of included studies using a quality assessment checklist (Appendix C Checklist) (Chidumayo, 2018, Abdelzaher *et al.*, 2010). The checklist included ten questions that had a 'yes' or 'no' answer. A point was scored if the response was 'yes' and zero for 'no'. Overall study quality was categorised as 'high' (scores ≥ 8 points), 'moderate' (scores 5 to 7 points) or 'low' (scores < 5 points). Funnel and doi plots were used to assess publication bias using the LFK index. Based on the LFK index no asymmetry was defined as LFK index values within ±1, minor asymmetry as values exceeding ±1 but within ±2, while major asymmetry as values exceeding ± 2 (Barendregt and Doi, 2016).

## 3.4 Detection of Avian Influenza Viruses and Newcastle Disease Virus

### 3.4.1 Virus Isolation and Identification

Faecal samples were processed according to the previously described protocol (Simulundu *et al.*, 2011, WHO, 2002). Briefly, faecal samples were suspended in phosphate-buffered saline (PBS) (pH 7.4) supplemented with antimicrobials to prepare a 20% homogenate. The antimicrobial supplements included penicillin G (2 x $10^6$ U/litre), streptomycin (200 mg/litre), gentamycin (250 mg/litre), and Nystatin (0.5 x $10^6$ U/litre) (Meiji Seika Pharma Co., Ltd, Tokyo, Japan). The faecal homogenates were centrifuged at 2000 x g for 10 min at 4°C. Then, 0.5 ml of the supernatant was inoculated into the allantoic cavity of 9- to 11-day-old embryonated chicken eggs (two eggs per homogenate sample) (Ross Breeders Zambia Ltd). The eggs were incubated at 37°C for 48 hours and then chilled overnight at 4°C. The allantoic fluids (AFs) collected from the eggs were screened by a haemagglutination (HA) test with 0.5% (v/v) chicken red blood cells (RBCs), a stock of NDV strain as a positive control and PBS as a negative control. The test involved a two-fold serial dilution of the virus suspended in PBS and a 0.5% suspension of chicken RBCs added and incubated at room temperature for 30 minutes. The sample was interpreted as HA positive when tilting the microtiter plate showed complete RBCs agglutination with no RBCs streaming in comparison to the negative control. The AF samples that did not show HA activity were passaged to a second egg inoculation followed by an

HA test. Samples that were HA-negative on the second passage were considered negative.

Thereafter, haemagglutination-inhibition (HI) and neuraminidase-inhibition (NI) assays were used to determine the HA and NA subtypes by using a panel of hyperimmune antisera against H1 to H16 and NDV, and N1 to N9 subtypes, respectively, according to the standard protocol (WHO, 2002). The HI assay was conducted as follows: Two-fold serial dilution of 25µl of reference antisera in PBS was made in U-bottom microtitre plates after which 25µl of predetermined 4HA units of the HA positive allantoic fluids were added and allowed to incubate at room temperature for 30–60 minutes to facilitate antigen-antibody reaction. Thereafter, 50µl of 0.5% chicken RBC suspension was added into each well and allowed to stand at room temperature for 40 minutes. The HI titre was thereafter determined by observing teardrop streaming of the microtitre plate when tilted and HI titre ≥ 1:16 was considered positive, while the negative one showed complete HA without tear streaming of RBCs (WHO, 2002). Positive and negative controls were run simultaneously with the test samples to validate the test.

The neuraminidase inhibition (NI) assay was conducted by diluting the AIV antiserum stock tenfold (1/10) and then making a twofold dilution (1/20) using PBS. Following this, 100µl of N1-N9 antisera were added to nine separate tubes, with tube 10 containing a negative antiserum, while 100µl of PBS was added to a positive control and 200µl to a blank. After that, 100µl of the diluted HI-positive virus was added to all the tubes except the blank and incubated at room temperature for 20 minutes. Thereafter, 300µl of substrate Fetuin was added to all the tubes and incubated at 37ºC for 12-24 hours. The following day, 200µl of 0.025M of Sodium Periodate was added to each tube and incubated at 37ºC for 30 minutes. Finally, 2% Sodium Arsenate was added to all the tubes, and then 2ml Thiobarbituric acid was added to each of the tubes, mixed thoroughly, and placed in a boiling water bath until the pink colour developed in the virus control tubes. Then, the NI results was determined by checking the colour change in the tubes. The liquid in the two control tubes was pink, while that in the blank was clear. Hence, the tube containing the homologous N subtype antiserum was clear, showing inhibition and the rest of the tubes were pink. This indicated the neuraminidase subtype of the isolate.

### 3.5 Molecular Characterisation of AIVs and NDV

#### 3.5.1 RNA Extraction and Whole Genome Sequencing

Viral RNA was extracted from the virus containing AF using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The extracted RNA was subjected to next-generation sequencing (NGS) using the Illumina MiSeq System (Illumina, San Diego, CA, USA). Libraries were prepared using KAPA RNA Hyper Prep Kit (Illumina, Inc., San Diego, CA, USA) and KAPA Dual-Indexed Adapter Kit (Roche, Basel, Switzerland). Libraries were then purified with Agencourt®AMPure®XP beads (Beckman Coulter, Brea, CA, USA). The library quantity and quality were verified using Agilent High Sensitivity DNA Kit on an Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Santa Clara, CA, USA). For sequencing, the pooled libraries were diluted to a final concentration of 10 pM, followed by the addition of a 3% PhiX control library (Illumina, San Diego, CA, USA). The prepared libraries were sequenced on a MiSeq by using MiSeq Reagent kit v3 (600 cycles) (Illumina, San Diego, CA, USA) with 2 × 300 bp paired-end read length. Sequence reads were mapped to reference sequences representing IAV subtypes H1 to H16, N1 to N9 and the six internal gene segments (GenBank accession numbers: MT090424, MT090343, MF146097, GQ404728, LC349399, MT406833, JX069105, CY076976, FJ183474, KT777885, MN049535, CY041260, MF694247, AB569521, MH071484, LC339733, KT777929, KT777932, KT777839, AB569497, MT406955, AB569553, MN049537, MN208007, MT126633, MT566219, MW188636, MW333882, MN588198, MW188640, MF694125, AB569484–AB569486, AB569488, AB569490, MW188635, MF146131, MF147767, MF694210, MK414709, KT777895, KT777897, KX979438, KY415917, KY765299) and APMV (GenBank accession numbers: KR074405, KR074404, KR815908, MH105247, MW927492, MW927497, MZ666228), and the consensus sequences were rebuilt until all mismatches were solved using the CLC Genomic Workbench, version 22.0 (CLC bio, Aarhus, Denmark). The nucleotide sequences obtained in this study were submitted to GenBank under accession numbers OQ120633 to OQ120872.

#### 3.5.2 Phylogenetic and Molecular Analysis

Nucleotide similarity searches were performed on the National Center for Biotechnology Information (NCBI) website with the Basic Local Alignment Search

Tool (BLAST) (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and GISAID EpiFlu. For phylogenetic analysis, reference sequences were obtained from the NCBI database and included viruses that showed high sequence identity to our isolates, representative viruses from Africa and viruses previously isolated in Zambia. Additionally, for NDV, a dataset was prepared using the pilot datasets of the full-length NDV F gene sequences proposed by Dimitrov *et al.* (2019) in which the Zambian isolates from the current study were inserted. Multiple sequence alignment was performed using the Multiple Alignment with Fast Fourier Transformation (MAFFT) (https: https://mafft.cbrc.jp/alignment/software/) (Accessed on 1st December 2022) according to default parameters (Katoh *et al.*, 2017) and the aligned sequences were manually edited and trimmed using Geneious Prime® v2022.2.2. Maximum likelihood trees of all the eight full gene segments (PB2, PB1, PA, HA, NP, NA, M, NS) of all the sequenced AIV isolates and the full-length NDV F gene were generated using Molecular Evolutionary Genetics Analysis version X (MEGA X) (Stecher *et al.*, 2020, Kumar *et al.*, 2018) applying the Tamura-Nei model (Tamura and Nei, 1993) and 1,000 bootstrap replicates. For the H2 AIV isolates, the partial HA genes were used to allow for the inclusion of two sequences from Africa (Réunion island). The HA and F genes cleavage site of all the Zambian isolates of AIV and NDV, respectively were assessed using GENETYX Version 12.0 (Genetyx Co., Tokyo, Japan). Furthermore, the percent similarities of the Zambian, and other AIV and NDV isolates were assessed using BLAST.

### 3.6 Detection of SARS-CoV-2

#### 3.6.1 RNA Extraction and Virus Genome Amplification

Viral RNA was extracted from nasopharyngeal swabs using the commercial kits (QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) and MagMax kit (Thermo Fisher Scientific, USA) in accordance with the manufacturer's guidelines. Amplification of the SARS-CoV-2 genome was done using the Centre for Disease Control and Prevention (CDC) SARS-CoV-2 qRT-PCR assay (Lu *et al.*, 2020b).

### 3.7 Molecular Characterisation of SARS-CoV-2

#### 3.7.1 Next Generation Sequencing

Next generation sequencing (NGS) was performed using the Oxford Nanopore technologies and Illumina NextSeq platforms. For Oxford Nanopore, cDNA

synthesis reaction was performed on 36 samples (based on cycle threshold values <30) using SuperScript IV Reverse Transcriptase kit (Invitrogen, USA), following the manufacturer's protocol. Library preparation was done using the ARTIC protocol V.3 (Quick, 2020, Tyson *et al.*, 2020). Whole-genome sequencing was done using custom-designed primers (Simulundu *et al.*, 2021). The PCR products were cleaned using AMPure XP beads (Beckman Coulter) and DNA quantification was done using a Qubit fluorometer (Thermo Fisher Scientific). End-repair on the amplified samples was done using NEBNext Ultra II End Repair Module (New England BioLabs). Native barcode expansion kits 1-12 and 13-24 were used in combination with Ligation Sequencing Kit (SQK-LSK109) (Oxford Nanopore Technologies). Subsequently, genomic sequencing was done using the MinION 1MkB (Oxford Nanopore Technologies). The RAMPART (v1.0.6) software package was used to monitor sequencing performance in real-time, with runs proceeding until a minimum of approximately 200-fold coverage was achieved across all amplicons. At this point, the run was terminated and the resulting reads were basecalled using Guppy (4.0.14). Consensus sequence, generation was done using the ARTIC bioinformatics pipeline (https://artic.network/ncov-2019/ncov2019-bioinformatics-sop.html) (accessed on 7 October 2021).

For Illumina NextSeq, V.3 primers pools designed by ARTIC Network were used (https://github.com/joshquick/artic-ncov2019/blob/master/primer_schemes/nCoV-2019/V3/nCoV-2019.tsv (accessed on 7 October 2021)). Sequencing libraries for 37 samples were prepared using the Illumina COVIDSeq kit on the automated Hamilton robotic instrument, ABI 7500 fast, and the Quant Studio thermo-cyclers. After successful library clean-up and pooling, pooled samples were quantified and normalized using a Qubit dsDNA HS Assay kit by diluting from a starting concentration of 4 nM to a final loading concentration of 1 nM. Thereafter, 25 µL was loaded on the Illumina NextSeq 2000 instrument through a cartridge loaded with a flow cell for SARS-CoV-2 genomic sequencing. A customized version of the DRAGEN DNA pipeline was used to perform Kmer-based detection of SARS-CoV-2. The Nextseq 2000 then aligned the reads to a reference genome, calls variants, and generates consensus genome sequences. The NextSeq 2000 optionally performs lineage/clade analysis using Pangolin and NextClade.

### 3.7.2 Genome Annotation and Phylogenetic Analysis of SARS-CoV-2

Whole-genome sequences were annotated using the reference genome of hCoV-19/Wuhan/Hu-1/2019|EPI_ISL_402125 (GISAID, 2020). A dataset of 176 whole genomes was created, which included 36 generated from this study and 140 retrieved from the GISAID database. Audacity*Instant* was used to retrieve SARS-CoV-2 whole-genome sequences from GISAID that were most similar to the sequences generated in this study. We also included reference sequences of VOCs detected in Southern Africa and other parts of the world. Reference sequences with stretches of more than 10% 'NNNN' were excluded from the analysis. Multiple sequence alignment of the sequences was performed using the FFT-NS-2 algorithm available in the multiple sequence alignment programme (MAFFT), but otherwise using default settings (https://mafft.cbrc.jp/alignment/server/index.html) (Katoh *et al.*, 2017). The alignment was inspected in Geneious Prime v2022.0.1 (https://www.geneious.com) and gaps were trimmed. The maximum likelihood (ML) tree was constructed using the PhyML Online server (www.atgc-montpellier.fr/phyml/) (Guindon *et al.*, 2005) using the smart model selection (SMS) (Lefort *et al.*, 2017) and the Bayesian Information Criterion. Branch support was estimated through the SH-like approximate likelihood ratio test (SH-aLRT). The ML tree was then rooted using TempEst v1.5.3 (Rambaut *et al.*, 2016), which estimated the best-fitting root of this phylogeny using the heuristic residual mean squared function, aimed at minimizing the variance of root-to-tip distances. The resultant ML tree file was edited using Interactive Tree of Life (iTOL) v5, an online tool for phylogenetic tree display and annotation (Letunic and Bork, 2021).

PANGO lineage identification was performed using Pangolin v3.1.16 (https://pangolin.cog-uk.io/) (accessed on 8 August 2022). Identification of single nucleotide polymorphisms (SNPs) was performed using the coronapp web application http://giorgilab.unibo.it/coronannotator/ (accessed on 8 August 2022). SNPs were identified based on the number of high confidence base calls (consensus sequence variations of the assembly) that do not agree with the reference bases for the genome position of interest. These variations were then exported to a vcf file and visualized in Microsoft Excel. The genomes generated in this study have been deposited in the GISAID EpiCoV (https://www.epicov.org/epi3/frontend#1cfb94).

### 4.8 Statistical Analysis

Descriptive statistics were used to describe the overall search results, characteristics of included studies, distribution of distribution of AIVs, IAVs and IDV in birds and non-human mammalian hosts, respectively using MS Office Excel® 2016. The Kruskal–Wallis test and Wilcoxon signed-rank sum test were used to determine associations between seasonality, regions and prevalence of AIV in birds. A Pearson correlation was carried out to determine the association between time in years and number of papers published between 2000 and 2019. For the meta-analysis, MetaXL version 3.1 (https://epigear.com accessed on 26 July 2021) a tool for meta-analysis in Microsoft Excel was used to pool prevalence and seroprevalences from each study (Barendregt *et al.*, 2013). Seroprevalence was defined as the presence of antibodies against IVs by any serological test while prevalence was defined as the isolation or detection of IVs by culture or reverse-transcriptase polymerase chain reaction. The quality effects model was used to calculate the pooled prevalences and their 95% confidence intervals (CI). The $I^2$ was used to assess study heterogeneity and $I^2$ values of 25%, 50% and 75% were considered as having a low, moderate and high degree of heterogeneity, respectively (Higgins *et al.*, 2003). We divided studies into subgroups based on the geographical regions (African Islands, Central, East, Southern and West Africa) and animal hosts to investigate the potential sources of heterogeneity.

### 4.9 Ethical Consideration

For the AIV and NDV study, permission to conduct the study in the Lochinvar National Park and LBMs was obtained from the Ministry of Tourism and Arts, and the Ministry of Livestock and Fisheries in Zambia, respectively. All protocols for the study were approved by the University of Zambia Biomedical Research Ethics Committee (Reference Number: 616–2019) and the National Health Research Authority. For the SARS-CoV-2 study, ethical approval to conduct the study was obtained from ERES Converge (Ref. No. 2020-Aug-008). Additionally, for human samples waiver of consent was obtained and the study used de-identified nasopharyngeal swabs obtained for routine clinical diagnosis. De-identification of samples was done to ensure that the samples could not be linked to any individual.

# CHAPTER FOUR

## 4.0 Results

## 4.1 Determination of the Prevalence and Seroprevalence of AIVs in Birds in sub-Saharan Africa

### 4.1.1 Search Results, Study Selection and Characteristics of the Studies

During the literature search, PubMed yielded 1313 records, SpringerLink electronic journals had 293 and AJOL showed 50 records, giving a total of 1656 research records. Of the 1656 research records, 678 (40.9%) were duplicates and were discarded. Using the set inclusion and exclusion criteria, we screened the remaining 978 records following a flow chart (Figure 4.1). During the screening, 870/978 (89.0%) articles were excluded based on their titles and abstracts, while 108 articles were retained. The 108 full-text articles were further screened for eligibility and 40/108 (37.0%) were excluded, while a total of 68/108 (63.0%) articles were deemed eligible for inclusion in this systematic review.

The reviewed articles were published between 2000 and 2019, from 22 sub-Saharan African countries. One article (1.5%) was published between 2000 and 2004, 16 (23.5%) between 2005 and 2009, 23 (33.8%) between 2010 and 2014 and 28 (41.2%) between 2015 and 2019. A Pearson correlation was computed to assess the relationship between years of publication and the numbers of articles published. There was a positive correlation between the two variables ($r = 0.81$, $n = 20$, $p < 0.0001$). This shows that there was a fairly strong positive and significant increase in the number of published articles between the years 2000 to 2019. A review of selected publications yielded a total of 83 published records on the presence of avian influenza in sub-Saharan Africa. Most articles (26.8%) were for studies done in Nigeria followed by those in South Africa (17.1%). Of the articles included in this review, 39 (57.4%) were surveillance studies, while 29 (42.6%) were a combination of either case studies or case reports. Additionally, the studies used either molecular, serological or both molecular and serological techniques to detect AIVs in different species of birds. Specifically, 29 (42.6%) studies employed molecular methods, 12 (17.6%) applied serological methods and 27 (39.7%) used both molecular and serological methods.

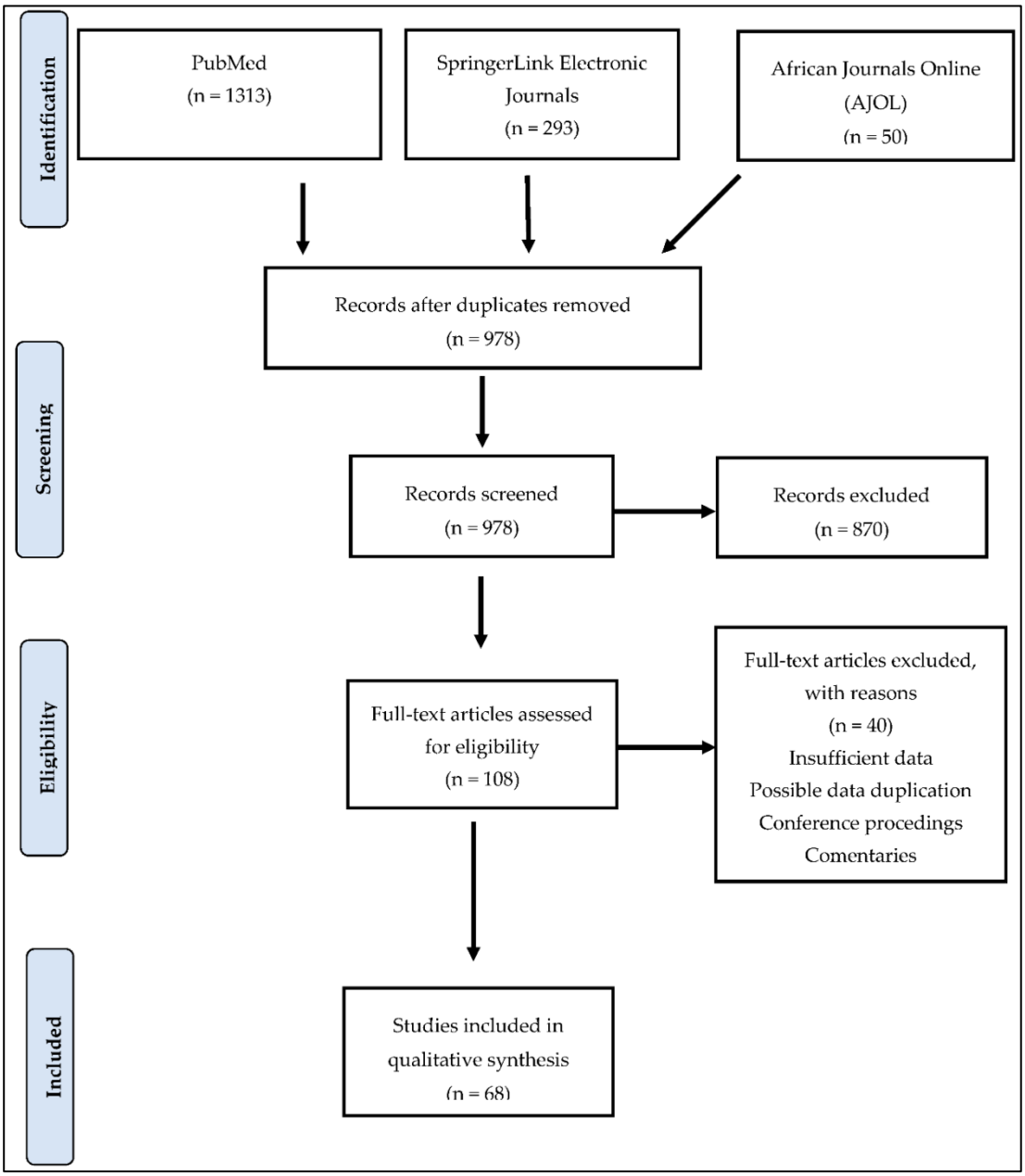


**Figure 4.1.** PRISMA Flow chart of the literature search, screening, assessing eligibility and article selection.

### 4.1.2 The Prevalence and Seasonality of AIV in Birds in Sub-Saharan Africa

The overall prevalence (determined based on virus isolation and genome detection) and seroprevalence of AIVs in avian species in sub-Saharan Africa was 3.0% (Table 4.1) and 4.1% (Table 4.2), respectively. During the analysis, sub-Saharan Africa was divided into regions namely, Central, East, Southern and West Africa for easier analysis and based on their unique seasonality. The prevalence varied between regions and ranged from 1.1–7.1% (Table 4.1) while seroprevalence ranged from

2.2–4.1% (Table 4.2). The majority of the serosurveys were conducted in poultry and focused on the detection of H5 and H7 antibodies.

**Table 4.1.** Prevalence of AIV in sub-Saharan Africa.

| Study Region | No. of Studies | No. Samples | No. Positive | Prevalence (%) |
|---|---|---|---|---|
| Central Africa [1] | 4 | 4868 | 344 | 7.1 |
| East Africa [2] | 6 | 13,875 | 146 | 1.1 |
| West Africa [3] | 19 | 38,203 | 1269 | 3.3 |
| Southern Africa [4] | 12 | 12,518 | 349 | 2.8 |
| **Total** | 41 | 69,464 | 2108 | **Overall prevalence: 3.0** |

Study regions: [1] Central African Republic, Congo Brazzaville, Cameroon and Democratic Republic of Congo, [2] Kenya, Uganda, [3] Benin, Burkina Faso, Ivory Coast, Gabon, Ghana, Mali, Niger, Nigeria, Togo, [4] Botswana, Namibia, South Africa, Zambia, Zimbabwe.

**Table 4.2.** Seroprevalence of AIV in sub-Saharan Africa.

| Study Regions | No. of Studies | No. of Samples | No. Samples | Seroprevalence (%) |
|---|---|---|---|---|
| Central Africa [1] | 0 | - | - | - |
| East Africa [2] | 2 | 3517 | 77 | 2.2 |
| West Africa [3] | 11 | 16,669 | 875 | 5.2 |
| Southern Africa [2] | 4 | 235,084 | 9605 | 4.1 |
| **Total** | 18 | 255,270 | 10,557 | **Overall seroprevalence: 4.1** |

Study regions: [1] Central African Republic, Congo Brazzaville, Cameroon and Democratic Republic of Congo, [2] Kenya, Uganda, [3] Benin, Burkina Faso, Ivory Coast, Gabon, Ghana, Mali, Niger, Nigeria, Togo, [4] Botswana, Namibia, South Africa, Zambia, Zimbabwe.

The Kruskal-Wallis Test of independent samples and the Wilcoxon Signed Rank Sum Test showed that there was no significant difference in prevalence AIV in birds across regions $\chi^2(3) = 5.237$, $p = 0.1553$ and seasons $T = 820$, $z = -1.244$, $p = 0.2136$ respectively. However, highest detection rates of AIVs were observed during the dry season (6.7%) (that is May to October in Central Africa (Table 4.3). The lowest prevalence was obtained in the wet season (0.4%) November to April in Central Africa (Table 4.3).

**Table 4.3.** Prevalence of AIV According to Regions and Seasons

| Study Region | No. of Studies | No. of Samples | Dry Season (%) | Wet Season (%) |
|---|---|---|---|---|
| Central Africa | 4 | 4868 | 325 (6.7%) | 19 (0.4%) |
| East Africa | 5 | 9556 | 71 (0.7%) | 46 (0.5%) |
| West Africa | 18 | 11,680 | 319 (2.7%) | 158 (1.4%) |
| Southern Africa | 11 | 6009 | 165 (2.7%) | 48 (0.7%) |
| **Total (Overall Prevalence%)** | 38 | 32,113 | 880 (2.7%) | 271 (0.8%) |

Note: In sub-Saharan Africa, seasons differ according to the regions: Central/Southern Africa—Dry season (May–October) and Wet season (November–April); East Africa—Dry season (January–March/June–October) and Wet season (April–June/November–December); West Africa—Dry season (January–March/June–October) and Wet season (April–June/November–December).

### 4.1.3 Distribution of the AIV Subtypes and Avian Species

As depicted in Table 4.4, the included studies reported a diverse range of AIV subtypes in different avian species in the past 20 years. According to the analysed articles, 52/68 (76.5%) studies specified the AIV subtypes, while 16/68 (23.5%) did not. During the period under review, nine different HA subtypes and six NA subtypes were found in 19 different subtype combinations (Table 4.4). The 19 AIV subtypes detected were: H1N2, H1N8, H3N6, H3N8, H4N2, H4N6, H4N8, H5N1, H5N2, H5N8, H6N2, H6N8, H7N1, H7N7, H9N1, H9N2, H10N7, H10N9 and H11N9. Southern Africa recorded a wider range of AIV subtype combinations than any other region in sub-Saharan Africa (Table 4.4). Of the 5073 viruses detected, the H5 (78.5%) subtype was the most common, followed by H9 (2.5%), H6 (1.4%), H7 (1.1%) and the rest falling below 1.0%. The H5Nx and H7Nx were either characterized as LPAI viruses or were not characterised at all (Supplementary Table S2). Overall, the most detected subtype combinations were H5N1, followed by H9N2, H5N2, H5N8 and H6N2. The majority of H5N1 and H5N8 subtypes were HPAI viruses and were commonly detected in domestic poultry especially chicken (*Gallus gallus*) and domestic ducks (*Anas platyrhynchos domestica*).

**Table 4.4** AIV Subtype Detection in sub-Saharan Africa according to the included studies.

| Study Region | Central Africa | East Africa | Southern Africa | West Africa |
|---|---|---|---|---|
| **HA Subtypes Detected** | H5 | H4, H5, H9 | H1, H3, H4, H5, H6, H7, H9, H10, H11 | H3, H5, H7, H9 |
| **Most Prevalent HA Subtypes** | H5 | H5 | H5 | H5 |
| **HA Subtypes not Detected** | H1–H4, H6–H16 | H1–H3, H6–H8, H10–H16 | H2, H8, H12–H16 | H1–H2, H4, H6, H8, H10–H16 |
| **Prevalent NA Subtypes** | N1, N8 | N2, N6, N8 | N1, N2, N6, N7, N8, N9 | N1, N2, N7, N8 |
| **NA Subtypes not Detected** | N2, N3, N4, N5, N6, N7, N9 | N1, N3, N4, N5, N7, N9 | N3, N4, N5 | N3, N4, N5, N6, N9 |
| **Prevalent Subtype Combinations** | H5N1, H5N8, H5Nx | H4N6, H5N8, H9N2, H5Nx | H1N2, H1N8, H3N6, H3N8, H4N2, H4N6, H4N8, H5N1, H5N2, H5N8, H6N2, H6N8, H9N2, H10N7, H10N9, H11N9, H5Nx, H6Nx, H7Nx | H3N8, H5N1, H5N2, H7N7, H9N2, H5Nx, H7Nx |
| **Most Prevalent Subtype Combinations** | H5N1 | H5N8 | H5N2, H5N8, H6N2 | H5N1, H9N2 |

Note: Nx = Unknown NA subtype.

Overall, the 52 studies that specified the AIV subtypes recorded both HPAI or LPAI viruses among domestic and wild birds. Majority of the studies that reported HPAIVs were conducted between 2006–2008 and 2017–2018. Furthermore, the majority of studies that reported HPAIV were conducted in Nigeria 11 (21.2%), followed by Burkina Faso and South Africa 4 (7.6%) each, Cameroon 3 (5.8%) and 2 (3.8%) from Cote d'Ivoire. The Democratic Republic of Congo (DRC), Ghana, Niger, Namibia, Togo and Uganda recorded 1 (1.9%) HPAIV studies each. The rest of the studies 22 (42.3%) recorded either LPAIVs or did not specify the pathogenicity of the detected subtype. Zambia and Zimbabwe did not report any HPAIVs according to the 68 studies included in this review. All the HPAIVs from Cameroon, DRC, Ghana, Niger and Togo were only detected in domestic birds while those from Burkina Faso, Cote d'Ivoire, Nigeria and South Africa were detected in both domestic and wild birds. H5N1, H5N2 and H5N8 HPAI viruses were detected mostly in domestic poultry. Further, H5N2 HPAI virus was more common among ostriches (*Struthio camelus australis*) than any other bird species included in this study. Notably, H5N8 HPAIV was detected in African penguins (*Spheniscus demersus*) in Namibia leading to the most severe mortality on record for this species in Namibia with more than 350 penguins dead (Molini *et al.*, 2020). Furthermore, the H5N1 HPAI virus was detected in Burkina Faso from hooded vultures (*Necrosyrtes monachus*), which exhibited dyspnea and neurological signs signs (Ducatez *et al.*, 2007b).

## 4.2 Investigation of IAV and IDV in Non-Human Mammalian Hosts in Africa

### 4.2.1 Search Results, Study Selection and Characteristics of the Studies

A total of 8785 articles and records were identified, of which 49 (45 articles and four records from OIE-WAHIS) were included in this study (Figure 4.2a and Appendix D). Of the 49 articles/records included in the systematic review, 29 were included in the meta-analysis. Further, some articles reported datasets from more than one country in Africa. Overall, the included articles/records reported data from 19 African countries as shown in Figure 4.2b. The highest number of articles was for studies conducted in West Africa (24/49; 49.0%) with the majority of articles being from Nigeria while the least of articles were for studies conducted in Southern Africa and the African Islands which recorded one study each (1/49; 2.0%). Furthermore,

the majority of the articles/records were published between 2011 and 2020 (40) while only nine articles/records were published between 2000 and 2010.

According to the included studies, more studies/records (28 (45.2%)) were conducted in pigs than in any other animal species included in this study Appendix E. Further, most studies collected serum or both serum and nasal swabs (18 (36.7%)) and used multiple methods (serological or virological methods) (22 (44.9%)) for the identification of IVs Appendix E. Only four (8.2%) articles that reported on IDVs in Africa were included in this study. Furthermore, most studies did not report whether the animals had influenza-like illness (ILI), or on their vaccination status Appendix E.

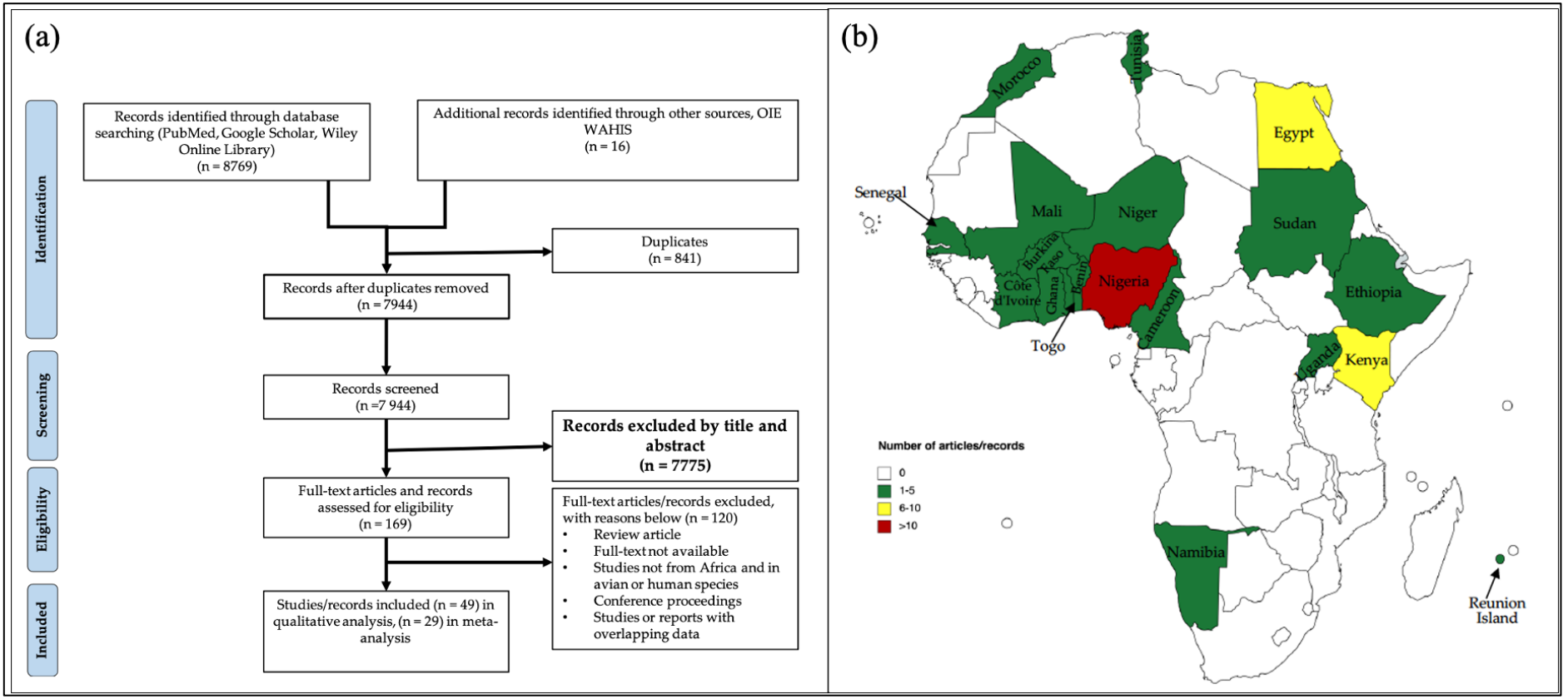


**Figure 4.2.** PRISMA flow diagram and Map of Africa. (a) PRISMA flow diagram of the selection process used to determine eligible studies; (b) Map of Africa showing the distribution of the number of articles/records (n=49) included in the review. Some articles reported data from several countries. The map was created online at https://mapchart.net/ (accessed on 30 November 2021).

### 4.2.2 Assessment of Quality and Risk of Publication Bias of Selected Studies

According to our quality assessment criteria, of the 29 publications included in the meta-analysis, five publications were of high quality, 13 were of moderate quality and 11 were of low quality. Publication bias in studies was measured and detected using the funnel and doi plots. Overall, the funnel and doi plots (Appendix F and G) showed minor and major asymmetry with the LFK index of 3.52 and 1.48 for prevalence and seroprevalence of IAV in pigs, respectively, and 1.10 for seroprevalence of IDV in non-human mammalian species, demonstrating a potential risk of publication bias among the selected papers.

### 4.2.3 Distribution of Influenza A and D Viruses and Detection of Antibodies in Non-Human Mammalian Hosts in Africa

The distribution of IAV and IDV in non-human mammalian hosts is depicted in Tables 4.5 and 4.6. West Africa had the highest number of countries (n = 9) with studies or reports on IAV in non-human mammalian species followed by North Africa (n = 4), East Africa (n = 3), African Island (n = 1), Central Africa (n = 1) and Southern Africa (n = 1). All the regions (African islands, Central, East, North, Southern and West Africa) included in this review reported at least virological or serological evidence of IAV in non-human mammalian species (Table 4.5).

The majority of the studies included in this review provided virological and/or serological evidence of the circulation of H1N1pdm09 in pigs in Africa (Table 4.5). The countries reporting H1N1pdm09 in pigs included Cameroon, Egypt, Ghana, Kenya, Nigeria, Togo and the Reunion Island. Apart from H1N1pdm09, classical H1N1, H3N2, H1 and H3 viruses were also reported in pigs in Burkina Faso, Kenya, Egypt, Uganda, Ghana and Nigeria (Table 4.5). The populations of pigs in various studies included piglets, weaners, growers, finishers, sows and boars. Additionally, pigs were either sampled from farms or slaughterhouses, though some articles did not indicate the sources of the pigs sampled. Further virological and serological evidence of H5N1 highly pathogenic avian influenza viruses (HPAIVs) were reported in Egypt and Nigeria, as well as H5N2 and H9N2 viruses in Egypt (Table 4.5).

Additionally, virological and/or serological evidence of exposure to IAVs was also reported in cats, dogs, rats, olive baboons (*Papio anubis*), equids, bats, spotted hyena

(*Crocuta crocuta*), black rhinos (*Diceros bicornis*), wildebeest (*Connochaetes taurinus*) and caracals (*Caracal caracal*) (Table 4.5). IAV-specific antibodies for H1 and H1N1 were detected in cats and dogs in Kenya (Munyua *et al.*, 2018), H3N8 and other unidentified subtypes in hunting, pet and village dogs in Nigeria (Daodu *et al.*, 2019, Oluwayelu *et al.*, 2014), while antibodies against H5N1 were detected in cats, dogs and rats in Egypt (El-Sayed *et al.*, 2013) (Table 4.5). Further, influenza A viral RNA was detected in dogs in Kenya (Munyua *et al.*, 2018). Equine influenza virus (EIV) subtype H7N7 (EIV-1) and/or, H3N8 (EIV-2), and their respective antibodies were reported in horses, donkeys and mules in Egypt (WOAH, 2008, Abd El-Rahim and Hussein, 2004). Moreover, antibodies specific for EIV were detected in donkeys, horses and mules in Morocco (Boukharta *et al.*, 2012), Sudan (WOAH, 2019b), Tunisia (Family - Orthomyxoviridae, 2012), Mali (WOAH, 2019a) and in camels in Kenya (Kimber *et al.*, 2002). Apart from EIVs, H5N1 HPAIV and antibodies against this virus were detected in donkeys in Egypt (Abdel-Moneim *et al.*, 2010) (Table 4.5). Furthermore, specific antibodies against H3, H5, H8, H9 and H12 viruses were also detected in wild mammals such as bats in Ghana (Freidl *et al.*, 2015) and IAV A/bat/Egypt/381OP/2017 was detected from Egyptian fruit bats in Egypt (Kandeil *et al.*, 2019). Moreover, a study conducted in Namibia demonstrated the exposure of various wildlife animals such as lions, black rhinos, spotted hyena, wildebeest, caracal, honey badgers and black-backed jackal to various IAVs (Soilemetzidou *et al.*, 2020) (Table 4.5).

**Table 4.5.** Distribution of influenza A virus and detection of antibodies in non-human mammalian hosts in Africa.

| Region | Country | Influenza A Virus | Influenza A Virus Antibodies | Host Species |
|---|---|---|---|---|
| **African Island** | Reunion Island | H1N1pdm09 | H1N1, H1, H3 | Pigs |
| **Central Africa** | Cameroon | H1N1pdm09 | H1N1pdm09, H1N2, H3N2 | Pigs |
| **East Africa** | Ethiopia | ND [1] | None [2] | Horses |
| | Kenya | H1N1pdm09, IAV | H1N1, H3N2, IAV | Pigs |
| | | | ND [1] | Olive baboons |
| | | H1N1, H3N2 | IAV [3] | Cats |
| | | None [2] | H1N1 | Dogs |
| | | IAV [3] | EIV [4] | Camels |
| | | ND [1] | | |
| | Uganda | IAV [3] | IAV [3], H1 | Pigs |
| **North Africa** | Egypt | H3N8, H7N7, H5N1 | H3N8, H7N7, H5N1 | Equids |
| | | H1N1pdm09, H3N2, H5N1, H9N2 | H1N1, H1N1pdm09, H5N1, H5N2, H5, H9 | Pigs |
| | | | H5N1 | Cats, Dogs, Rats |
| | | ND [1] | None [2] | Buffaloes, Cattle, Goats, Sheep |
| | | ND [1] | ND [1] | Bats |
| | | H9N2-like virus | | |
| | Morocco | ND [1] | H3N8, H7N8 | Equids [5] |
| | Sudan | ND [1] | EIV [4] | Equids [5] |
| | Tunisia | ND [1] | EIV [4] | Horses |
| **Southern Africa** | Namibia | ND [1] | H1, H5 | Black Rhino |
| | | ND [1] | H4, H11 | Wildebeest |
| | | ND [1] | H1, H3, H5, H7, H8, H9, H11, H13, H14, H16 | Caracals |
| | | ND [1] | H7 | Honey Badger |
| | | ND [1] | H1 | Lion |
| **West Africa** | Benin | None [2] | ND [1] | Pigs |
| | Burkina Faso | None [2] | H1N1, H1N1pdm09 | Pigs |
| | Côte d'Ivoire | None [2] | None [2] | Pigs |
| | Ghana | H1N1pdm09 | H1N1pdm09, H3N2 | Pigs |
| | | ND [1] | H3, H5, H8, H9, H12 | Bats |
| | Mali | ND [1] | H3N8 | Donkeys |
| | Niger | H3N8 | ND [1] | Donkeys, Horses |
| | Nigeria | H1N1, H3N2, H5N1, H1, H3, H5 | H1N1, H1N1pdm09, H3N2, H5N1, H3, H7, IAV [3] | Pigs |
| | | H3N8 | ND [1] | Donkeys, Horses |
| | | None [2] | IAV [3], H3N8 | Dogs |
| | Senegal | H3N8 | ND [1] | Donkeys, Horses |
| | Togo | H1N1pdm09 | ND [1] | Pigs |

[1] ND–Not Done; [2] None–Investigated but not detected; [3] IAV–Influenza A Virus (IAV–matrix gene detected but not subtyped; IAV antibodies–used multispecies ELISA kit); [4] EIV–Equine influenza virus (subtype not specified); [5] Equid-Horses, donkeys, mule.

Exposure to IDV was reported in East Africa, North Africa and West Africa. IDV antibodies were detected in cattle from Benin (Salem *et al.*, 2017), Morocco (Saegerman *et al.*, 2020) and Togo (Fusade-Boyer *et al.*, 2020, Salem *et al.*, 2017), in dromedary camels from Kenya (Salem *et al.*, 2017) and Ethiopia (Murakami *et al.*, 2019), and in small ruminants from Ethiopia and Togo (Table 4.6). Further, the review of the literature suggests that IDV has been circulating in Africa since 2012 as evidenced by the antibodies detected in Morocco (Salem *et al.*, 2017).

**Table 4.6.** Distribution of influenza D virus and their antibodies in non-human mammalian species in Africa.

| Region | Country | Influenza D Virus | Influenza D Virus Antibodies | Host Species |
|---|---|---|---|---|
| East Africa | Ethiopia | ND [1] | IDV | Camels, Goats |
| | Kenya | ND [1] | IDV | Camels |
| North Africa | Morocco | ND [1] | IDV | Cattle |
| West Africa | Benin | ND [1] | IDV | Cattle |
| | | ND [1] | None [2] | Sheep, Goat |
| | Togo | None [2] | IDV | Cattle, Small ruminants |
| | | ND [1] | IDV | Cattle, Goats, Sheep |
| | | None [2] | None [2] | Pigs |

[1] ND–Not done; [2] Not detected.

### 4.2.4 Pooled Prevalence, Seroprevalence and Heterogeneity of IAVs in Pigs in Africa

The estimated pooled prevalence of IAV in pigs in Africa was 1.6% (95% CI: 0–5%), $I^2$ = 98%, $p < 0.0001$ as shown in Table 4.7 and Figure 4.3a. African Islands and North Africa had the highest prevalence of 13.2% (95%: 10–16%) and 10.4% (0–100%), respectively, while the lowest prevalence of 0.3% (95% CI: 0–1%) was observed in East Africa. Furthermore, the pooled prevalence of IAV in pigs varied across studies ranging from 0–63% (Figure 4.3a).

**Table 4.7.** Estimated pooled prevalence and seroprevalence of IAV in pigs in Africa.

| IAV | Regions | Sample Size | No. Positive | Pooled Prevalence/ Seroprevalence (%) | 95% CI [1] | $I^2$ (%) |
|---|---|---|---|---|---|---|
| Overall Prevalence | Africa | 10,703 | 370 | 1.6% | 0–55 | 98 |
| | African Islands | 474 | 62 | 13.2 | 10–16 | - |
| | Central Africa | 104 | 2 | 2.4 | 0–6 | - |
| | East Africa | 5196 | 23 | 0.3 | 0–1 | 91 |
| | North Africa | 433 | 122 | 10.4 | 0–100 | 100 |
| | West Africa | 4496 | 161 | 2.2 | 0–5 | 98 |
| Overall Seroprevalence | Africa | 10,870 | 2095 | 14.9 | 5–28 | 99 |
| | African Islands | 1203 | 399 | 33.2 | 31–36 | - |
| | Central Africa | 98 | 27 | 27.8 | 19–37 | - |
| | East Africa | 5098 | 680 | 12.6 | 7–13 | 96 |
| | North Africa | 585 | 226 | 25.8 | 0–100 | 100 |
| | West Africa | 3886 | 763 | 14.9 | 0–41 | 99 |

[1] CI–Confidence Interval.

The estimated pooled seroprevalence of IAV in Africa was 14.9% (95% CI: 5–28%), $I^2 = 99\%$, $p < 0.001$ among pigs (Table 4.7 and Figure 4.3b). The highest prevalence was recorded in African Islands with 33.2% (95% CI: 31–36%), followed by Central Africa with 27.8% (95% CI: 19–37%), North Africa with 25.8% (95% CI: 0–100%), West Africa with 14.9% (95% CI: 0–41%) and the least was 12.6% (95% CI: 7–18%) for East Africa (Table 4.7 and Figure 4.3b). Further, there was a variation in the pooled seroprevalence of IAV in pigs among individual studies ranging from 0–94% as shown in Figure 4.3.

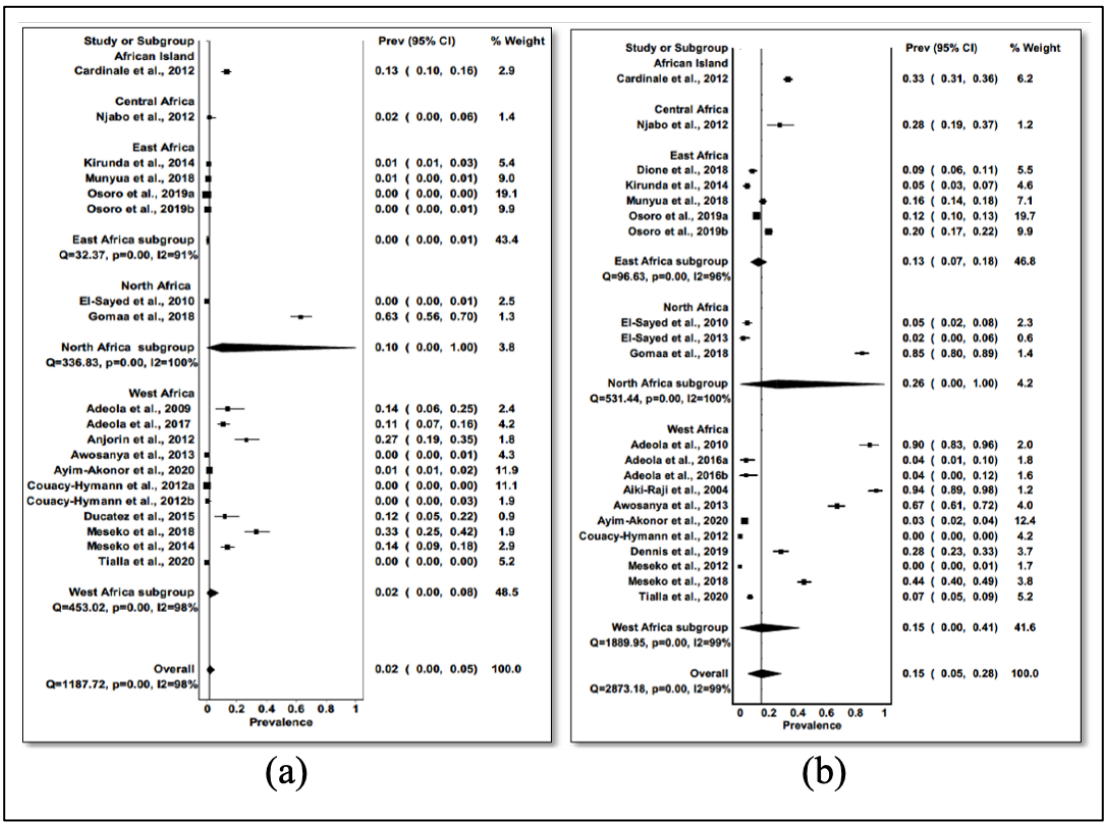


**Figure 4.3.** Forest plot of the prevalence and seroprevalence estimates IAV. (a) Forest plot of the prevalence estimates of IAV in pigs in Africa by region; (b) Forest plot of the seroprevalence estimates of IAV in pigs in Africa by region.

### 4.2.5 Pooled Seroprevalence and Heterogeneity of IDV in Non-Human Mammalian Hosts

Of the 29 studies included in the meta-analysis, only four were on IDV. The overall seroprevalence of IDV in non-human mammalian species was 9.9% (95% CI: 0–28%), $I^2$ = 99%, $p < 0.001$ as shown in Table 4.8. The seroprevalence of IDV was highest in camels with 87.2% (95% CI: 24–100%) and lowest in pigs with 0.0% (95% CI: 0–2%) (Table 4.8 and Figure 4.4).

**Table 4.8.** Estimated Pooled seroprevalence of IDV in non-human mammalian species in Africa.

| Subgroup | Sample Size | No. Positive | Pooled Seroprevalence (%) | 95% CI [1] | $I^2$ (%) |
|---|---|---|---|---|---|
| Overall seroprevalence | 3992 | 536 | 9.9 | 0–28 | 99 |
| Cattle | 2260 | 190 | 9.3 | 0–23 | 99 |
| Small Ruminants | 1321 | 35 | 2.2 | 0–4 | 73 |
| Pigs | 80 | 0 | 0.0 | 0–2 | - |
| Camels | 331 | 311 | 87.2 | 24–100 | 98 |

[1] CI–Confidence Interval.

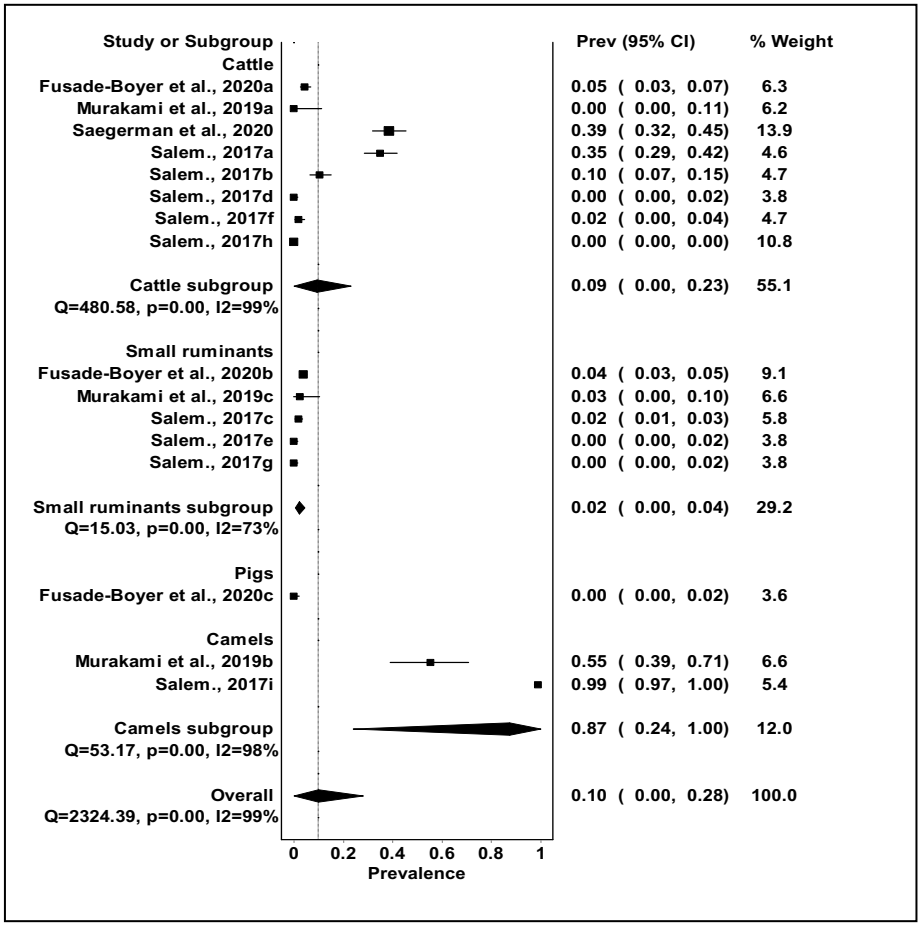

Figure 4.4. Forest Plot of the Seroprevalence of IDV according to subgroups

## 4.3 Detection and Identification of AIV and NDV from Wild Waterfowls and Poultry Faecal Samples

Of the 2,851 faecal samples collected from wild waterfowl in the LNP, 85 samples were obtained from ducks, geese and ibises and two samples from an ibis and duck were positive for AIV and NDV, respectively (Table 4.9). The positivity rate of AIV was 3.0%, whereas that of NDV was 0.07% by virus isolation and HI, as shown in Table 4.9. For poultry samples collected from LBMs, 4(0.3%) of 1150 samples were positive for NDV, while all the samples were negative for AIV by virus isolation and HI. All four NDV strains from poultry were isolated from chickens (2 broilers and 2 village chicken (free range) during the dry season in Lusaka province (Table 4.9) at one LBM. In wild waterfowl, the AIV positivity rate differed across bird species, seasons and sampling years. Among the bird species, the positivity rate was highest in ibis birds (23.8%), followed by ducks (0.8%) and geese (0.2%). No virus was detected in great white pelicans and white egrets. High positivity rates were observed in the wet season (5.7%) and the 2015 (4.1%) sampling period (Table 4.9). Based on

the HI and NI assay results, 10 AIV HA/NA subtypes combinations were detected in wild waterfowl namely, H2N9, H3N8, H4N6, H8N9, H10N7, H10N8, H10N9, H11N8, H11N9, H13N6 and three HA subtypes, H1, H3 and H8 with NA subtype not determined.

Of the 85 AIV positive samples, 50 isolates representing all the subtypes determined by HI/NI assays in this study were subjected to NGS for subtype confirmation and genetic characterisation. Additionally, six NDV samples were also subjected NGS for whole genome sequencing and genetic characterisation. We obtained good sequences for 30 and six AIV and NDV samples, respectively, by NGS. Through NGS, eight HA/NA subtypes of AIV (i.e., H1N8, H2N9, H8N4, H10N6, H10N8, H10N9, H11N6 and H11N9) were detected. All the H1N8, H2N9, H8N4, H10N6, H10N9 and H11N9 isolates were detected in 2015, with the majority being samples collected in November. All the two H8N4 and four H11N6 isolates were detected in samples collected in September 2020 and November 2021, respectively. The most prevalent subtypes in the present study were H10N8 (20%; 6/30), followed by H11N9 (16.7%; 5/30). The most prevalent HA subtype was H10 (40%; 12/30) which occurred in combination with three NA (N6, N8, N9) subtypes.

**Table 4.9.** Number of samples collected and positivity rates over the surveillance period 2015, 2020 and 2021

| Characteristics | Variables | Number of Samples Collected | No. of AIV Positive Samples (%) | No. of NDV Positive Samples (%) |
|---|---|---|---|---|
| Wild Waterfowls | **Sampling Sites** | | | |
| | Southern Province – Lochinvar[1] | 2851 | 85 (3.0) | 2 (0.07) |
| | **Bird Species** | | | |
| | Ducks | 1214 | 10 (0.8) | 1 (0.08) |
| | Geese | 1227 | 3 (0.2) | 0 (0) |
| | Ibises | 302 | 72 (23.8) | 1 (0.3) |
| | Pelicans | 98 | 0 (0) | 0 (0) |
| | White Egrets | 10 | 0 (0) | 0 (0) |
| | **Season [2]** | | | |
| | Dry | 1539 | 7 (0.5) | 0 (0) |
| | Wet | 1312 | 78 (5.9) | 2 (0.07) |
| | **Sampling Year** | | | |
| | 2015 | 1921 | 79 (4.1) | 1 (0.05) |
| | 2020 | 242 | 2 (0.2) | 0 (0) |
| | 2021 | 688 | 4 (0.6) | 1 (0.1) |
| Poultry/Domestic Birds | **Sampling Sites** | | | |
| | Copperbelt Province | 402 | 0 (0) | 0 (0) |
| | Lusaka Province | 410 | 0 (0) | 4 (1.0) |
| | Southern Province | 338 | 0 (0) | 0 (0) |
| | **Bird Species** | | | |
| | Chickens | 1077 | 0 (0) | 4 (0.4) |
| | Other Poultry [3] | 73 | 0 (0) | 0 (0) |
| | **Season [2]** | | | |
| | Dry | 576 | 0 (0) | 4 (0.7) |
| | Wet | 574 | 0 (0) | 0 (0) |
| | **Year of Collection** | | | |
| | 2020 | 812 | 0 (0) | 4 (0.5) |
| | 2021 | 338 | 0 (0) | 0 (0) |

[1] Lochinvar National Park; [2] Season: Dry season: May–October, Wet season: November–April; [3] Other poultry – Include ducks, doves, geese, guinea fowls, ostriches, quails and turkeys

## 4.4 Genetic Diversity of AIVs

### 4.4.1 Genomic Characteristics of AIV

The consensus genome sequence length of all the segments of the 30 Zambian AIV isolates are shown in Table 4.10, with the length sizes ranging from 2309–2336 nucleotides (nt) for PB2, 1716–1759 nt for HA and 1426–1454 nt for NA. Isolates A/ibis/Zambia/1625/2015(H2N9) and A/ibis/Zambia/1765/2015(H2N9) had partial sequence lengths for the M and NS genes while A/ibis/Zambia/1786/2015(H11N9) had an NS partial sequence (Table 4.10). Further, the nucleotide similarities of all the eight segments of AIV isolates (PB2, PB1, PA, HA, NP, NA, M, NS) were assessed using BLAST (Table 4.11). The HA genes of the H1N8, H10N8, H10N9 and H11N9 isolates showed the highest nucleotide sequence similarity (94.4–95.5%) to viruses isolated from ostriches, Pekin ducks and red-billed teals from South Africa while the HA genes of the H2N9 and H10N6 isolates shared 95.1% and 94.9% nucleotide sequence identity with A/tufted duck/Georgia/1/2012 (H2N3) and A/duck/Mongolia/371/2010 (H10N8), respectively. The HA genes of the H8N4 and H11N6 isolates showed 97.2–98.9% nucleotide sequence similarity to H8N4 and H11N3 viruses isolated from ducks in Bangladesh in 2019. Analysis of NA gene segments of all the isolates indicated that they shared 96.1–98.8% nucleotide sequence identity with viruses isolated in wild waterfowl from Asia, Africa and Europe (Table 4.11).

The nucleotide sequence analysis further revealed that the PB2 genes of the H1N8, H2N9, H10N6, H10N8, H10N9 and H11N9 isolates were highly similar (97.9–98.6%) to A/tufted duck/Georgia/1/2012 (H2N3) while those of the H8N4 and H11N6 isolates were similar (97.2–97.4%) to A/pintail/Egypt/MB-D-384C/2015 (H3N6). The PB1 genes of the H1N8 isolates showed high nucleotide similarity (98.2%) to A/shelduck/South Africa/DLH/2012 (H7N8). The PA genes of the HIN8, H2N9, H10N8 and H10N9 isolates showed 98.2–98.5% nucleotide similarity to an H7N7 virus, A/ostrich/South Africa/KRB/2013 (H7N7) while the NP genes of the H10N8, H10N9 and H11N9 isolates showed a high nucleotide identity of 98.5–98.7% with an H7N8 virus isolated in Egypt (Table 4.11).

**Table 4.10.** Consensus nucleotide sequence lengths of the full-length or nearly full-length AIV Genomes obtained by next-generation sequencing (NGS)

| Virus Name | PB2 | PB1 | PA | HA | NP | NA | M | NS |
|---|---|---|---|---|---|---|---|---|
| A/duck/Zambia/28/2020(H8N4) | 2331 | 2323 | 2216 | 1718 | 1553 | 1426 | 1008 | 879 |
| A/duck/Zambia/29/2020(H8N4) | 2329 | 2325 | 2219 | 1717 | 1552 | 1447 | 1012 | 876 |
| A/duck/Zambia/264/2021(H11N6) | 2332 | 2323 | 2216 | 1735 | 1556 | 1454 | 1016 | 883 |
| A/duck/Zambia/268/2021(H11N6) | 2330 | 2323 | 2216 | 1735 | 1549 | 1447 | 1008 | 875 |
| A/duck/Zambia/360/2021(H11N6) | 2327 | 2323 | 2215 | 1735 | 1552 | 1452 | 1013 | 872 |
| A/duck/Zambia/390/2021(H11N6) | 2330 | 2329 | 2222 | 1745 | 1555 | 1453 | 1015 | 879 |
| A/duck/Zambia/514/2015(H2N9) | 2328 | 2320 | 2221 | 1759 | 1553 | 1443 | 1014 | 878 |
| A/ibis/Zambia/1032/2015(H10N6) | 2330 | 2329 | 2222 | 1723 | 1552 | 1449 | 1014 | 876 |
| A/ibis/Zambia/1033/2015(H10N6) | 2332 | 2321 | 2222 | 1717 | 1552 | 1449 | 1015 | 879 |
| A/ibis/Zambia/1608/2015(H10N8) | 2330 | 2319 | 2222 | 1716 | 1562 | 1447 | 1016 | 879 |
| A/ibis/Zambia/1609/2015(H10N9) | 2336 | 2320 | 2224 | 1717 | 1559 | 1446 | 1020 | 879 |
| A/ibis/Zambia/1610/2015(H10N8) | 2323 | 2328 | 2219 | 1710 | 1555 | 1444 | 1009 | 877 |
| A/ibis/Zambia/1625/2015(H2N9) | 2320 | 2185 | 2140 | 1752 | 1547 | 1438 | 990 [1] | 768 [1] |
| A/ibis/Zambia/1626/2015(H10N8) | 2331 | 2321 | 2223 | 1716 | 1554 | 1449 | 1016 | 883 |
| A/ibis/Zambia/1642/2015(H1N8) | 2309 | 2299 | 2221 | 1743 | 1551 | 1447 | 1013 | 873 |
| A/ibis/Zambia/1687/2015(H10N6) | 2336 | 2325 | 2228 | 1719 | 1556 | 1449 | 1016 | 881 |
| A/ibis/Zambia/1709/2015(H10N8) | 2335 | 2321 | 2228 | 1717 | 1553 | 1450 | 1016 | 879 |
| A/ibis/Zambia/1740/2015(H11N9) | 2328 | 2319 | 2223 | 1742 | 1552 | 1447 | 1014 | 877 |
| A/ibis/Zambia/1741/2015(H11N9) | 2332 | 2319 | 2222 | 1743 | 1553 | 1447 | 1016 | 879 |
| A/ibis/Zambia/1745/2015(H11N9) | 2336 | 2320 | 2227 | 1743 | 1555 | 1451 | 1017 | 880 |
| A/ibis/Zambia/1750/2015(H10N8) | 2330 | 2319 | 2222 | 1717 | 1550 | 1449 | 1020 | 878 |
| A/ibis/Zambia/1765/2015(H2N9) | 2324 | 2319 | 2218 | 1757 | 1544 | 1444 | 964 [1] | 840 [1] |
| A/ibis/Zambia/1766/2015(H10N8) | 2333 | 2321 | 2224 | 1718 | 1556 | 1448 | 1019 | 880 |
| A/ibis/Zambia/1767/2015(H10N9) | 2332 | 2321 | 2226 | 1717 | 1557 | 1448 | 1017 | 882 |
| A/ibis/Zambia/1773/2015(H1N8) | 2329 | 2327 | 2219 | 1743 | 1553 | 1448 | 1013 | 875 |
| A/ibis/Zambia/1774/2015(H11N9) | 2331 | 2320 | 2226 | 1743 | 1558 | 1443 | 1015 | 883 |
| A/ibis/Zambia/1786/2015(H11N9) | 2333 | 2322 | 2229 | 1745 | 1555 | 1433 | 1017 | 600 [1] |
| A/ibis/Zambia/1787/2015(H1N8) | 2341 | 2299 | 2223 | 1743 | 1554 | 1451 | 1016 | 880 |
| A/ibis/Zambia/1788/2015(H1N8) | 2335 | 2299 | 2225 | 1743 | 1555 | 1450 | 1016 | 880 |
| A/ibis/Zambia/1794/2015(H10N9) | 2334 | 2320 | 2232 | 1718 | 1559 | 1433 | 1017 | 882 |

Note: Length = nucleotides (nt); [1] Partial sequence lengths

**Table 4.11.** AIVs with the highest nucleotide sequence similarity to viruses in the current study

| Representative Virus Subtype | Gene Segment | Highest Homology Influenza A Virus | GenBank Accession # | % Homology |
|---|---|---|---|---|
| H1N8 | PB2 | A/tufted duck/Georgia/1/2012 (H2N3) | MF147767.1 | 97.9 |
| | PB1 | A/shelduck/South Africa/DLH/2012 (H7N8) | KT777839.1 | 98.2 |
| | PA | A/ostrich/South Africa/KRB/2013 (H7N7) | KT777875.1 | 98.2 |
| | HA | A/ostrich/South Africa/AI2887/2011 (H1N2) | JX069105.1 | 95.5 |
| | NP | A/mallard duck/Netherlands/18/2012 (H4N2) | MF146131.1 | 97.8 |
| | NA | A/duck/Zambia/04/2008 (H3N8) | AB569497.1 | 96.5 |
| | M | A/Mallard/Netherlands/31/2014 (H4N3) | MK414709.1 | 98.3 |
| | NS | A/mallard/Netherlands/25/2013 (mixed) | MK192309.1 | 99.0 |
| H2N9 | PB2 | A/tufted duck/Georgia/1/2012 (H2N3) | MF147767.1 | 98.5 |
| | PB1 | A/yellow-billed duck/South Africa/STR0963/2013 (H4N8) | KT777923.1 | 97.7 |
| | PA | A/ostrich/South Africa/KRB/2013 (H7N7) | KT777875.1 | 98.5 |
| | HA | A/tufted duck/Georgia/1/2012 (H2N3) | MF146097.1 | 95.1 |
| | NP | A/mallard duck/Netherlands/15/2011 (H6N8) | KX979542.1 | 98.5 |
| | NA | A/duck/Bangladesh/8987/2010 (H10N9) | MH071484.1 | 96.7 |
| | M | A/Anas Platyrhynchos/Belgium/108116/2019 (H5N6) | MT406810.1 | 98.2 |
| | NS | A/ostrich/South Africa/MKT/2012 (H7N1) | KT777895.1 | 99.4 |
| H8N4 | PB2 | A/pintail/Egypt/MB-D-384C/2015 (H3N6) | MN208007.1 | 97.4 |
| | PB1 | A/garganey/North Kazakhstan/45/2018 (H3N8) | MT126633.1 | 98.4 |
| | PA | A/duck/Mongolia/451/2018 (H4N1) | MW188636.1 | 97.7 |
| | HA | A/duck/Bangladesh/37509/2019 (H8N4) | MT090424.1 | 98.0 |
| | NP | A/duck/Moscow/4952/2013 (H5N3) | MN588198.1 | 97.6 |
| | NA | A/common teal/Shanghai/JDS120613/2018 (H10N4) | MN049535.1 | 96.2 |
| | M | A/duck/Mongolia/451/2018 (H4N1) | MW188640.1 | 98.9 |
| | NS | A/mallard duck/Netherlands/41/2015 (H5N1) | MF694125.1 | 99.1 |
| H10N6 | PB2 | A/tufted duck/Georgia/1/2012 (H2N3) | MF147767.1 | 97.9 |
| | PB1 | A/yellow-billed duck/South Africa/STR0963/2013 (H4N8) | KT777923.1 | 97.7 |
| | PA | A/cape shoveler/South Africa/STR0982/2013 (H4N8) | KT777929.1 | 97.8 |
| | HA | A/Pekin duck/South Africa/AI1642/2009 (H10N7) | GQ404728.2 | 95.0 |
| | NP | A/mallard duck/Netherlands/18/2012 (H4N2) | MF146131.1 | 97.8 |
| | NA | A/duck/Hokkaido/10/2015 (H3N6) | LC339733.1 | 97.0 |
| | M | A/Mallard/Netherlands/31/2014 (H4N3) | MK414709.1 | 98.3 |
| | NS | A/duck/Bangladesh/821/2009 (H10N7) | MH071464.1 | 99.0 |
| H10N8/H10N9 | PB2 | A/tufted duck/Georgia/1/2012 (H2N3) | MF147767.1 | 98.5 |
| | PB1 | A/yellow-billed duck/South Africa/STR0963/2013 (H4N8) | KT777923.1 | 97.3 |
| | PA | A/ostrich/South Africa/KRB/2013 (H7N7) | KT777875.1 | 98.3 |
| | HA | A/Pekin duck/South Africa/AI1642/2009 (H10N7) | GQ404728.2 | 94.8 |
| | NP | A/teal/Egypt/MB-D-487OP/2016(H7N3) | MN208011.1 | 98.5 |
| | NA | A/cape shoveler/South Africa/STR0982/2013 (H4N8) | KT777932.1 | 96.1 |
| | M | A/teal/Egypt/MB-D-698OP/2016 (H7N3) | MN207981.1 | 99.5 |
| | NS | A/duck/Bangladesh/821/2009 (H10N7) | MH071464.1 | 98.8 |
| H11N6 | PB2 | A/pintail/Egypt/MB-D-384C/2015 (H3N6) | MN208007.1 | 97.2 |
| | PB1 | A/duck/Mongolia/451/2018 (H4N1) | MW188635.1 | 97.8 |
| | PA | A/duck/Mongolia/451/2018 (H4N1) | MW188636.1 | 97.5 |
| | HA | A/duck/Bangladesh/38285/2019 (H11N3) | MT090343.1 | 97.2 |
| | NP | A/mallard duck/Netherlands/35/2015 (H4N6) | MF694210.1 | 97.5 |
| | NA | A/domestic duck/Georgia/1/2016 (H4N6) | MF694247.1 | 97.4 |
| | M | A/duck/Mongolia/451/2018 (H4N1) | MW188640.1 | 98.9 |

| | | | | |
|---|---|---|---|---|
| | NS | A/ostrich/South Africa/MKT/2012 (H7N1) | KT777895.1 | 97.9 |
| H11N9 | PB2 | A/tufted duck/Georgia/1/2012 (H2N3) | MF147767.1 | 98.3 |
| | PB1 | A/yellow-billed duck/South Africa/STR0963/2013 (H4N8) | KT777923.1 | 97.5 |
| | PA | A/cape shoveler/South Africa/STR0982/2013 (H4N8) | KT777929.1 | 97.8 |
| | HA | A/red-billed teal/South Africa/KZN002/2012 (H11N2) | KT777885.1 | 97.4 |
| | NP | A/teal/Egypt/MB-D-487OP/2016 (H7N3) | MN208011.1 | 98.7 |
| | NA | A/Anas platyrhynchos/Belgium/195_7/2018 (H11N9) | MT406955.1 | 98.6 |
| | M | A/Anas Platyrhynchos/Belgium/10811_6/2019(H5N6) | MT406810.1 | 98.5 |
| | NS | A/mallard duck/Netherlands/43/2011 (H7N1) | KX979531.1 | 97.3 |

### 4.4.2 Phylogenetic Analysis of the Viral Surface Glycoprotein Genes

Phylogenetic analysis of the HA and NA gene sequences revealed that the viruses isolated in the current study belonged to the Eurasian virus lineage (Figures 4.5–4.9). Analysis of the tree topology of the HA gene indicated that the H1N8, H2N9, H8N4, H10N6, H10N8 and H10N9 isolates grouped together and formed distinguishable clusters in their respective trees (Figure 4.5–4.7). The H11N6 viruses isolated in 2021 clustered distinctly from the H11N9 viruses isolated in 2015. A/ostrich/South Africa/AI2887/2011 (H1N2) formed a precursor-like relationship to the H1N8 isolates characterised in this study (Figure 4.5a). Interestingly, the HA genes of the H2N9 isolates formed a separate and distinct "African lineage-like" cluster and were not closely related to the H2Nx viruses detected in Réunion island (Figure 4.5b). The analysis further indicated that the HA genes of the H8N4 isolates were closely related to the H8N4 viruses isolated in Bangladesh in 2019 (Figure 4.5c). Similar to the H1 gene tree, the virus A/pekin duck/South Africa/AI1642/2009 (H10N7) had a precursor-like relationship to the HA gene of all the H10 isolates characterised in this study (Figure 4.6). The H11 HA phylogeny revealed that the H11N6 and H11N9 isolates formed distinct clusters and were distantly related to H11N9 viruses previously isolated in Zambia in 2009 (Figure 4.7).

Phylogenetic analysis of the NA gene revealed that the H8N4 isolates of the current study formed a separate cluster and were closely related to viruses isolated from wild waterfowl in Asia (Figure 4.8a). The N6 NA genes of the H10N6 and H11N6 viruses isolated in 2015 and 2021 formed distinct clusters based on the year of isolation and were distantly related to the NA genes of the H3N6 and H4N6 viruses isolated in

Zambia previously (Figure 4.8b). Analysis of the NA genes of the H1N8 and H10N8 isolates indicated that all the isolates grouped together and were closely related to viruses isolated from wild waterfowl in South Africa (A/Cape shoveler/South Africa/STR0982/2013 (H4N8)); A/yellow-billed duck/South Africa/STR0963/2013 (H4N8)) and Zambia (A/duck/Zambia/04/2008 (H3N8)) (Figure 4.9a). Except for A/duck/Zambia/514/2015 (H2N9) which clustered separately, the N9 NA gene sequences of the other N9 viruses isolated in the current study were closely related to each other and belonged to a clade which included viruses from Belgium, South Korea and Egypt (Figure 4.9b).

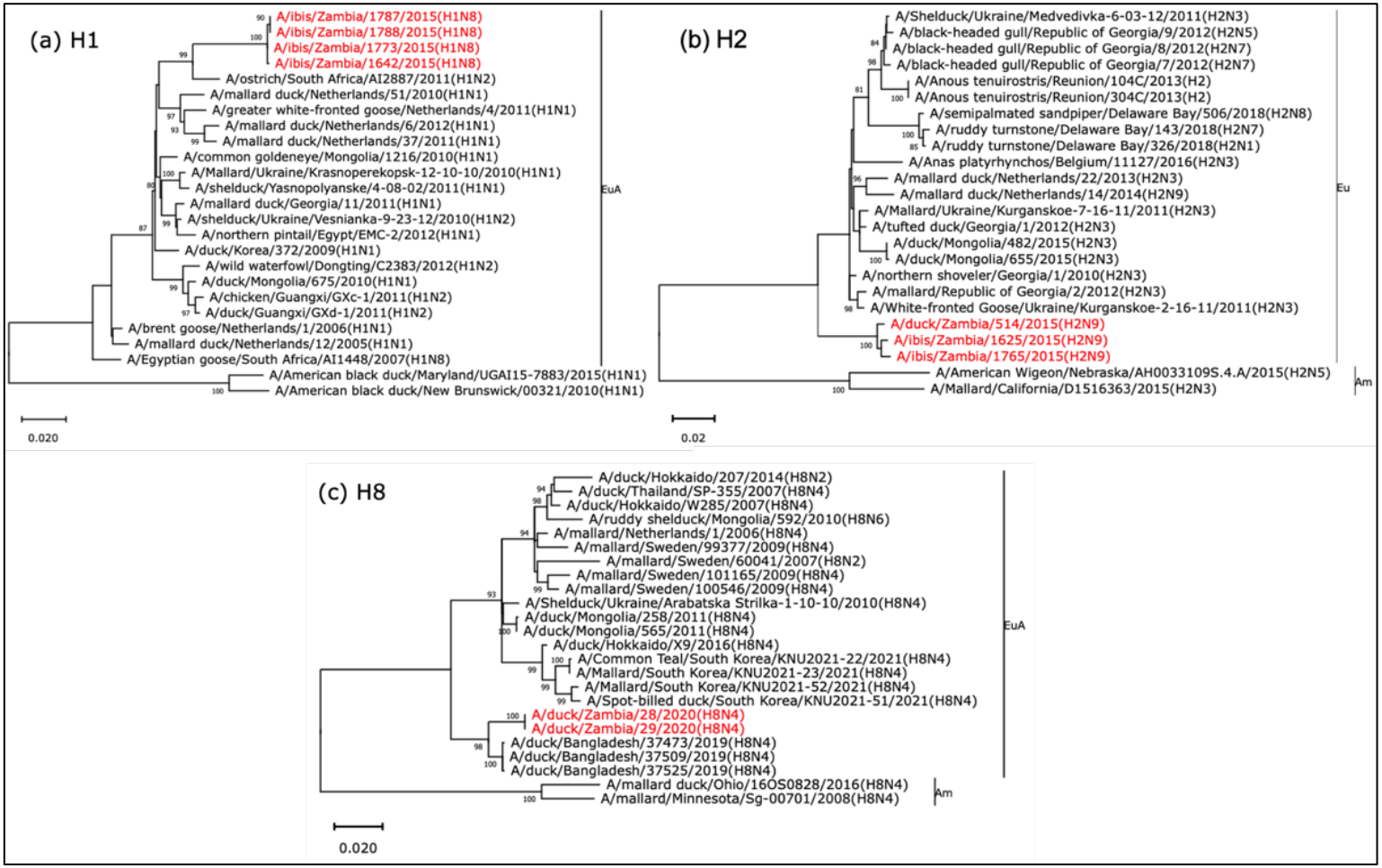


**Figure 4.5.** Phylogenetic analysis of the H1, H2 and H8 genes of AIVs: (a) Phylogenetic tree of H1 genes based on 1702 nucleotides; (b) Phylogenetic tree of H2 genes based on 1695 nucleotides; (c) Phylogenetic tree of H1 genes based on 1712 nucleotides. The viruses isolated in this study are in red text. Numbers at branch nodes are bootstrap values ≥70%. Am – American lineage; EuA – Eurasian lineage. Bar, number of substitutions per site.

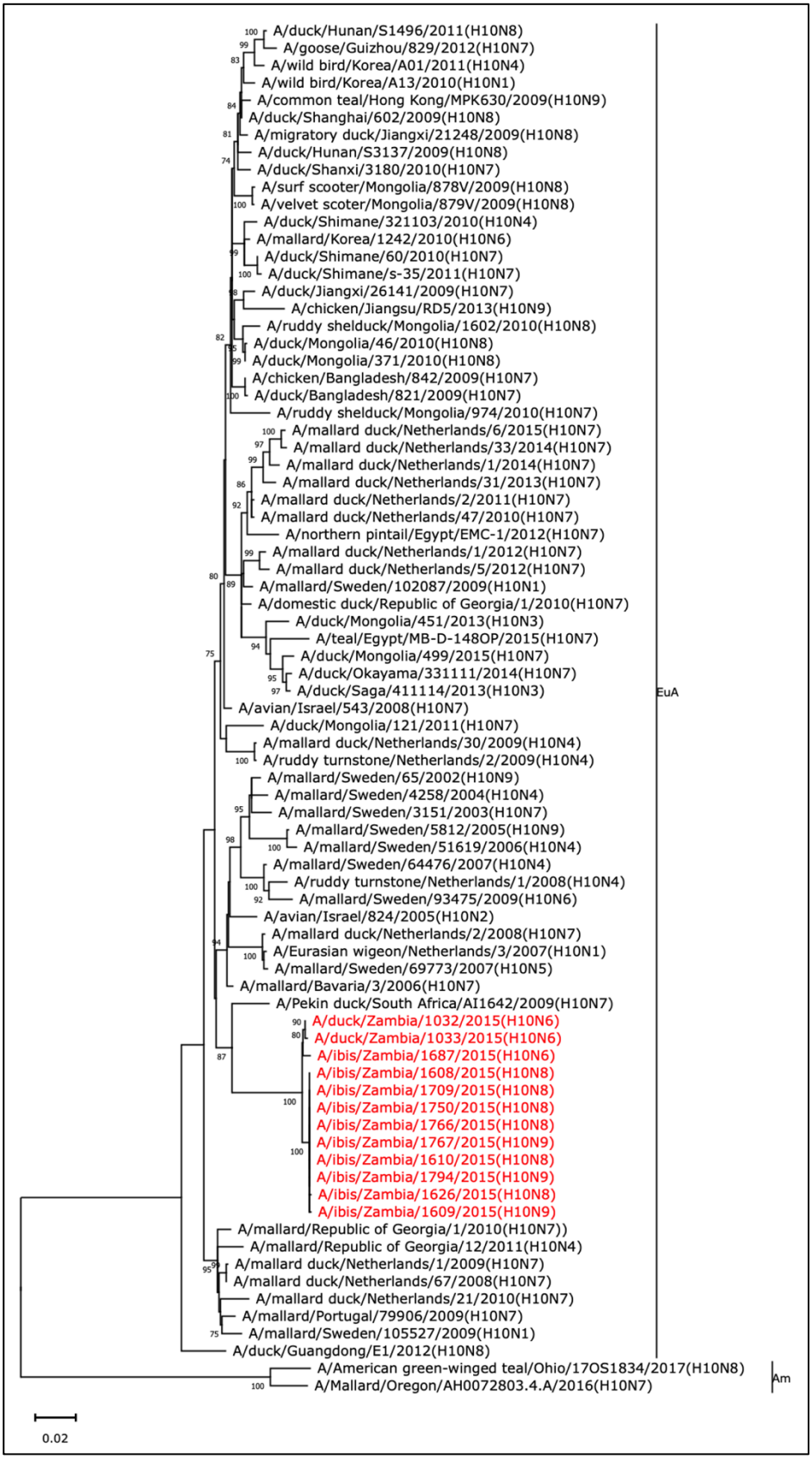


**Figure 4.6.** Phylogenetic analysis of the H10 gene based on 1686 nucleotides. The viruses isolated in this study are in red text. Numbers at branch nodes are bootstrap values ≥70%. Am – American lineage; EuA – Eurasian lineage. Bar, number of substitutions per site.

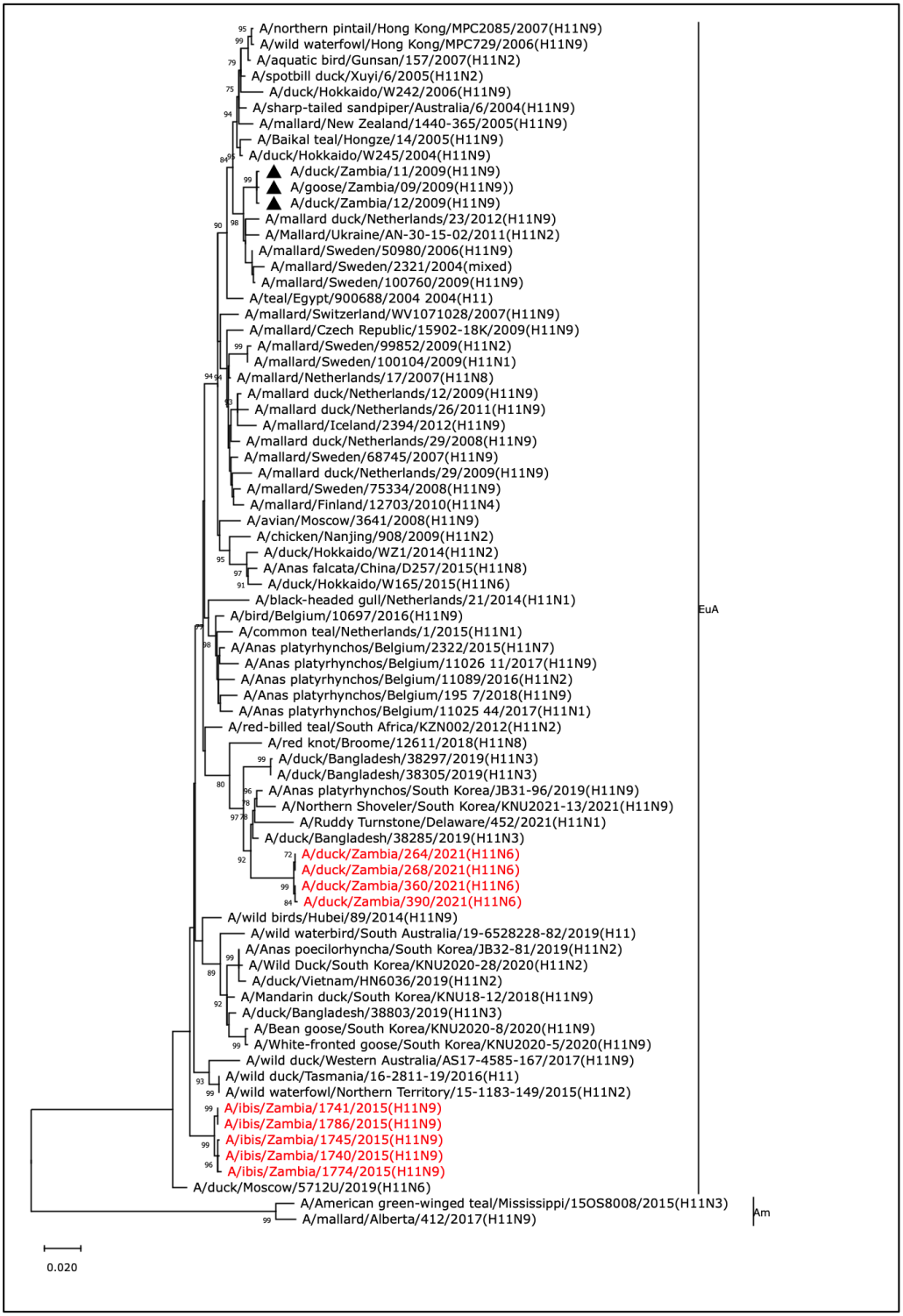


**Figure 4.7.** Phylogenetic analysis of the of H11 gene based on 1707 nucleotides. The viruses isolated in this study are in the red text while those previously isolated in Zambia are shown with a black triangle. Numbers at branch nodes are bootstrap values ≥70%. Am – American lineage; EuA – Eurasian lineage. Bar, number of substitutions per site.

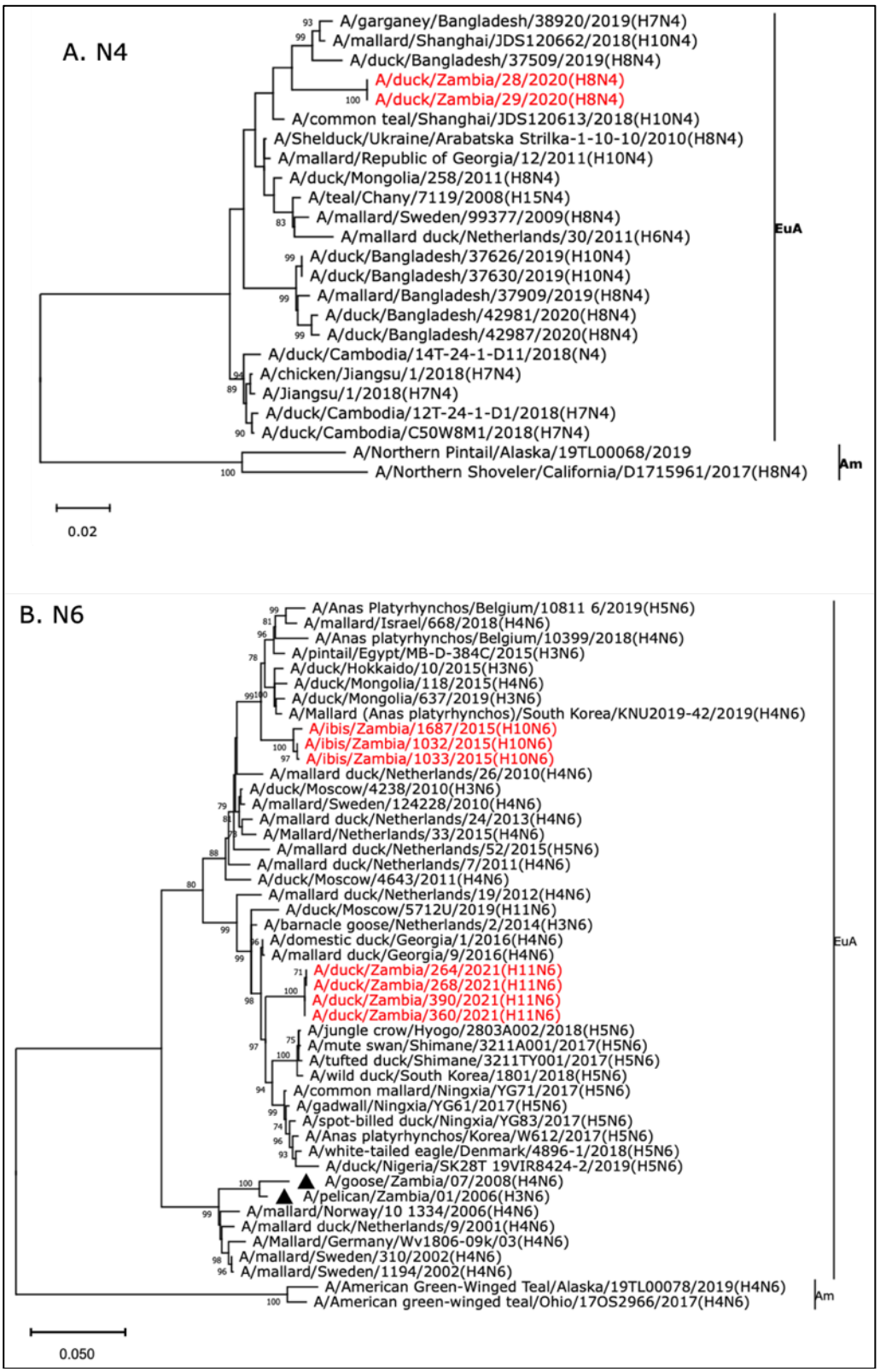


**Figure 4.8.** Phylogenetic analysis of the N4 and N6 NA genes: (a) Phylogenetic tree of N4 based on 1405 nucleotides; (b) Phylogenetic tree of N6 based on 1413 nucleotides. The viruses isolated in this study are in the red text while those previously isolated in Zambia are shown with a black triangle. Numbers at branch nodes are bootstrap values ≥70%. Am – American lineage; EuA – Eurasian lineage. Bar, number of substitutions per site.

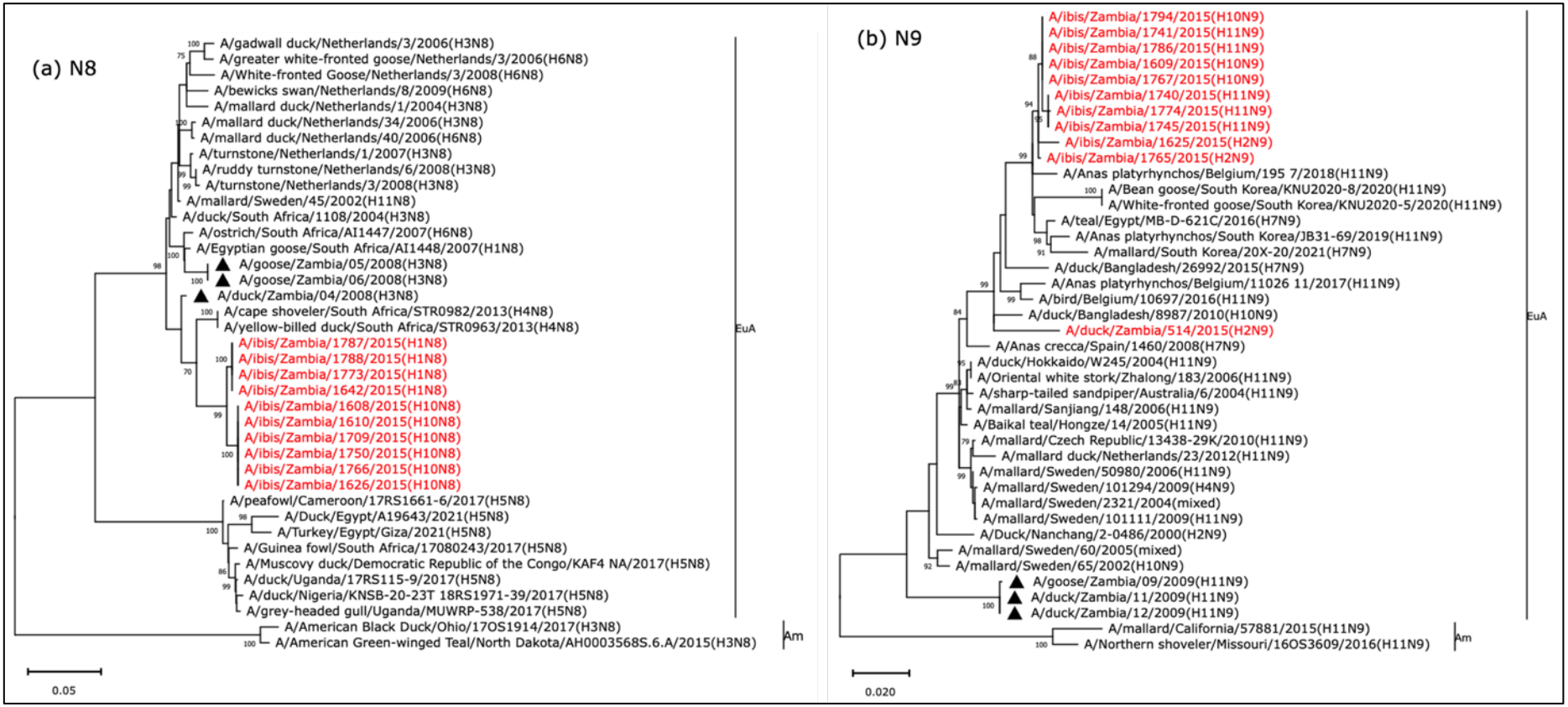


**Figure 4.9.** Phylogenetic analysis of the N8 and N9 NA genes. (a) Phylogenetic tree of N8 based on 1413 nucleotides; (b) Phylogenetic tree of N9 based on 1413 nucleotides. The viruses isolated in this study are in the red text while those previously isolated in Zambia are shown with a black triangle. Numbers at branch nodes are bootstrap values ≥70%. Am – American lineage; EuA – Eurasian lineage. Bar, number of substitutions per site.

### 4.4.3 Phylogenetic Analysis of the Viral Internal Protein Genes

In general, the tree topologies of all internal protein genes revealed that the viruses isolated in the present study clustered among the Eurasian virus lineage (Figures 4.10–4.12, Appendix H). The analysis also revealed that the viruses isolated in 2015 formed separate clusters from viruses isolated in 2020 and 2021 and were distinct from those previously analysed in 2008-2009 except for the M and NS gene segments whose sequences were clustered with some of the Zambian viral sequences characterised previously (Figures 4.10–4.12, Appendix H). Some internal protein genes characterised in this study were closely related to H7 LPAIVs.

The PB1 genes of the viruses isolated in 2015 were closely related to A/yellow-billed duck/South Africa/STR0963/2013 (H4N8) and A/cape shoveler/South Africa/STR0982/2013 (H4N8) except for PB1 genes of the H1N8 isolates which had a close relationship to an H7N8 LPAIV isolate, A/shelduck/South Africa/DLH/2012 (H7N8), as shown in Figure 4.10. Additionally, the PB1 genes of the H8N4 isolates were closely related to that of viruses isolated in Japan and Slovakia while those of the H11N6 isolates were closely related to viruses isolated in Moscow (A/duck/Moscow/5712U/2019 (H11N6)) and Mongolia (A/duck/Mongolia/451/2018 (H4N1)) (Figure 4.10).

The NP gene phylogeny revealed that the viruses isolated in 2020–2021 formed a single cluster related to viruses isolated in Belgium, Netherlands, Georgia, Russia and Egypt (Figure 4.11). In contrast, viruses isolated in 2015 formed two clusters, with the majority of the sequences belonging to a clade which included H7N3 viruses detected from wild birds in Egypt in 2016 (Figure 4.11).

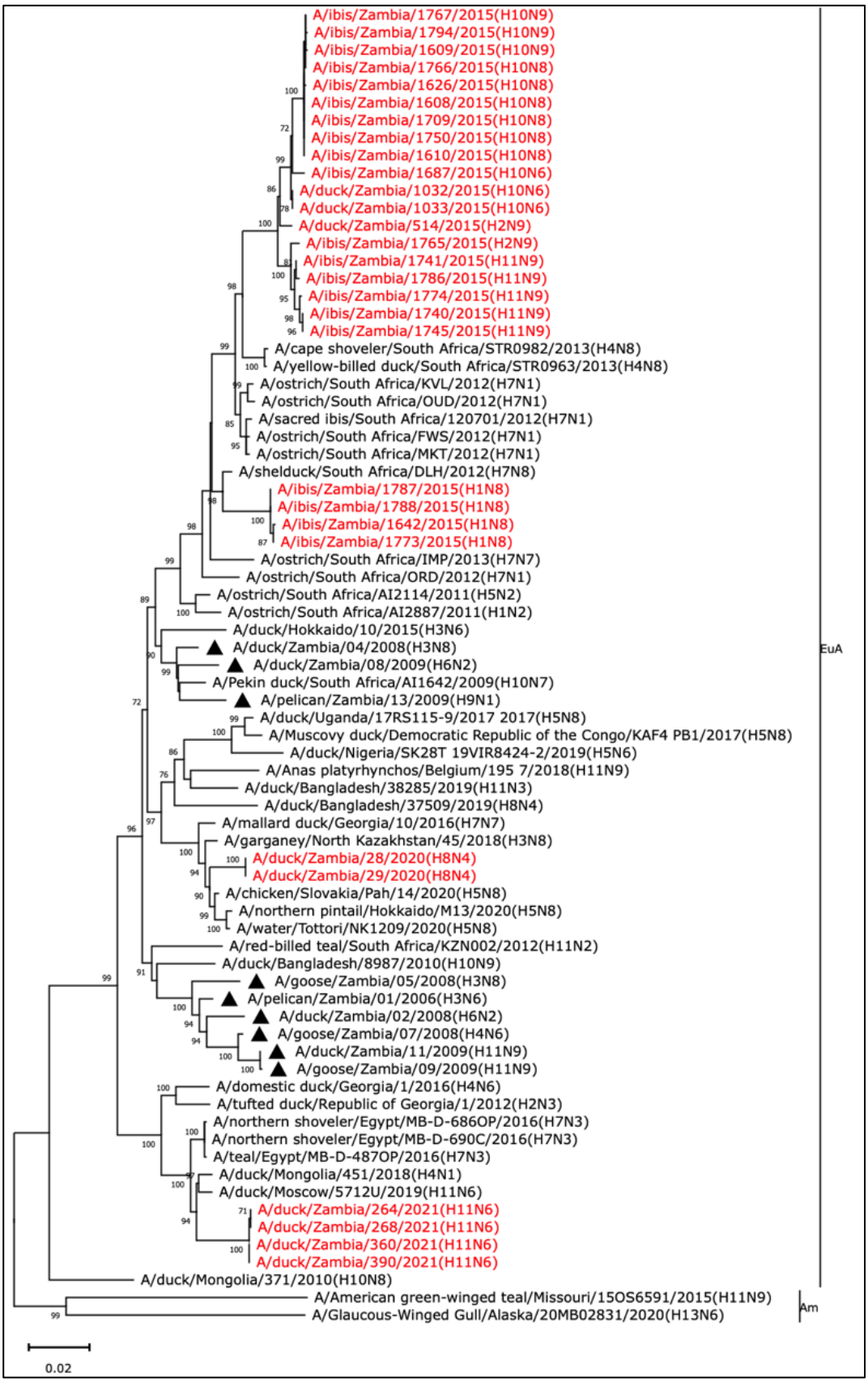


**Figure 4.10.** Phylogenetic analysis of the PB1 genes based on 2279 nucleotides. The viruses isolated in this study are in the red text while those previously isolated in Zambia are shown with a black triangle. Numbers at branch nodes are bootstrap values ≥70%. Am – American lineage; EuA – Eurasian lineage. Bar, number of substitutions per site.

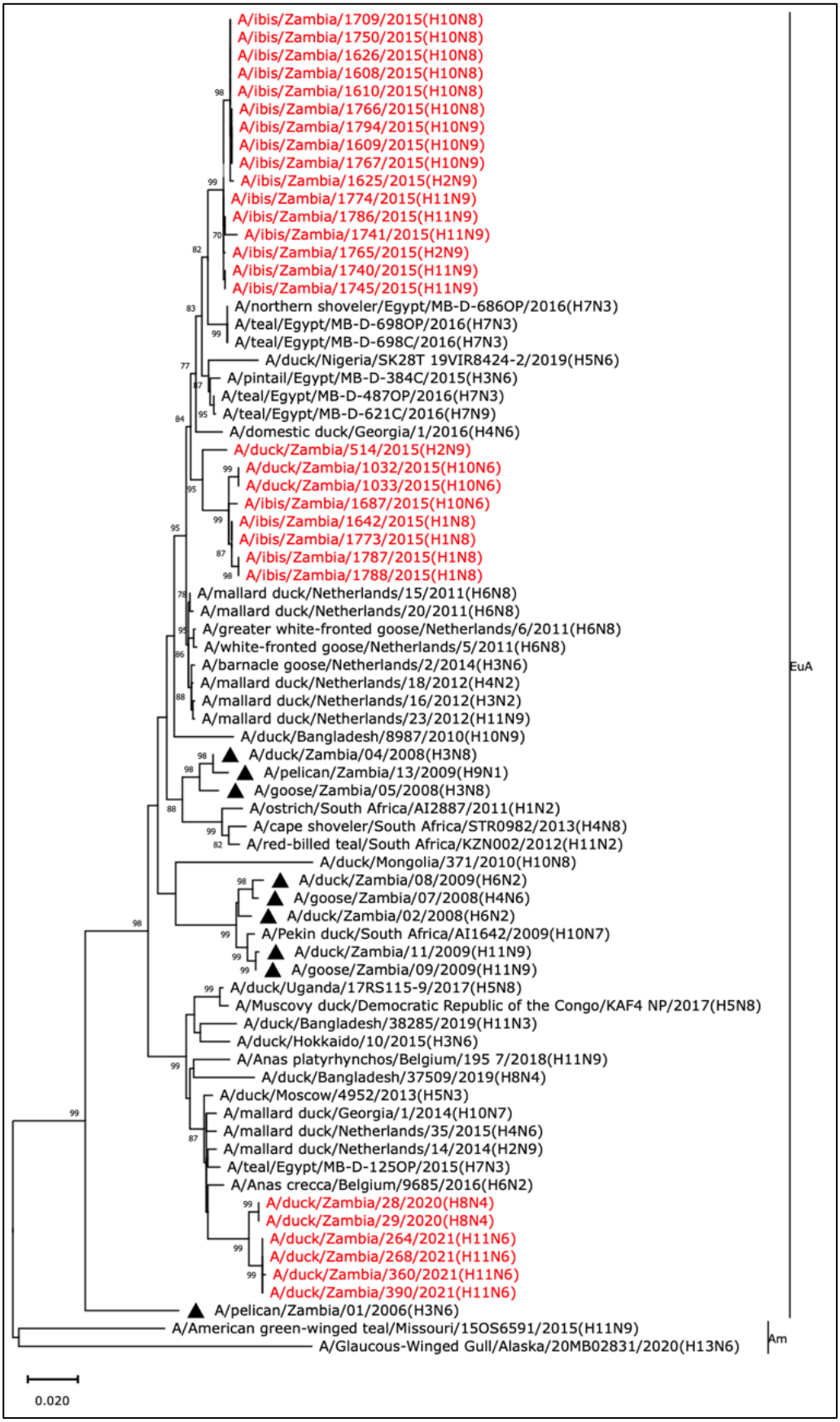


**Figure 4.11.** Phylogenetic analysis of the NP genes based on 1506 nucleotides**.** The viruses isolated in this study are in the red text while those previously isolated in Zambia are shown with a black triangle. Numbers at branch nodes are bootstrap values ≥70%. Am – American lineage; EuA – Eurasian lineage. Bar, number of substitutions per site.

Phylogenetic analysis of the NS gene demonstrated that the NS genes of the isolates characterised in the current study grouped into two separate alleles; Alleles A and B with the majority of the viruses being grouped into Allele A (Figure 4.12). The H11N6 isolates in allele B formed a distinct cluster and clustered closely with viruses isolated in Zambia in 2006 and 2008 and those isolated in Europe and Asia. In addition, the Zambian allele B sequences clustered close to H7N1 South African viruses detected in ostriches in 2012 (Figure 4.12). Within allele A, all the NS gene sequences of the H1 and H10 isolates were closely related to A/duck/Bangladesh/8987/2010 (H10N9) (Figure 4.12). In contrast, the H8N4 isolates belonged to a cluster of isolates detected from wild birds mainly in the Netherlands and included H5N1, H5N6 and H7N5 LPAIVs, though none of these was closely related to our isolates (Figure 4.12).

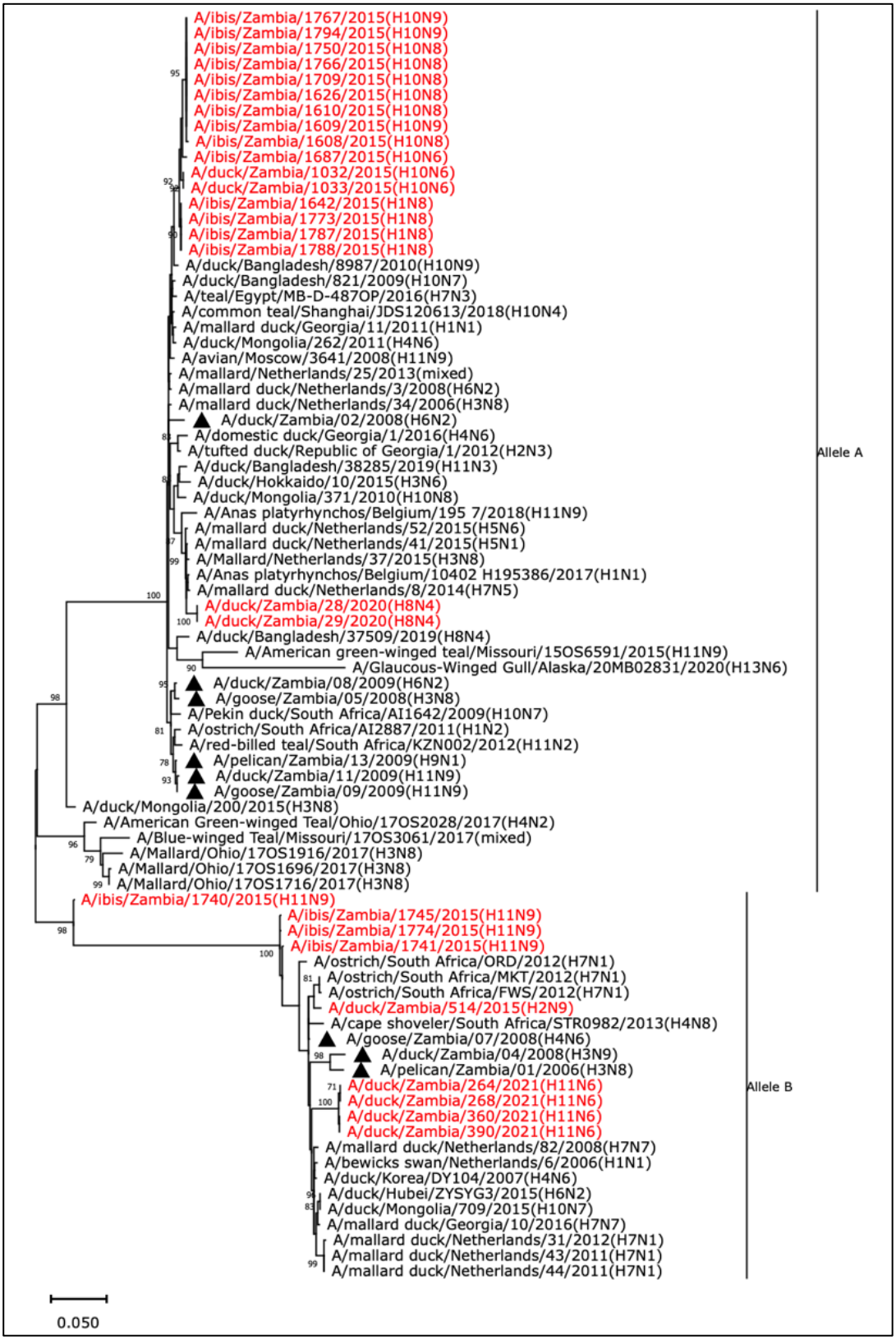


**Figure 4.12.** Phylogenetic analysis of the AIV NS genes based on 852 nucleotides. The viruses isolated in this study are in the red text while those previously isolated in Zambia are shown with a black triangle. Numbers at branch nodes are bootstrap values ≥70%. Bar, number of substitutions per site.

### 4.4.4 Genetic Analysis of the HA Cleavage Site

Genetic analysis of the HA gene segment indicated that the isolates with the same HA subtypes shared similar motifs at the cleavage site of the HA protein as follows: H1N8 (PSIQSR/GLF), H2N9 (PQIESR/GLF), H8N4 (PSIEPK/GLF), H10N6/H10N8/H10N9 (PEVMQGR/GLF) and H11N6/H11N9 (PAIASR/GLF). The sequences for all the isolates were typical for LPAIVs as none of the viruses had multiple basic amino acids at the HA cleavage site.

## 4.5 Genetic Diversity of Newcastle Disease Virus

### 4.5.1 Genomic Characteristics of Newcastle Disease Virus

Sequence analysis showed that out of six Zambian NDV isolates, four had either 9–14 sequences missing at either the 5' or 3' ends giving a consensus genomic sequence lengths ranging from 15,178–15,183 nt. The other two isolates, NDV/duck/Zambia/708/2021 and NDV/Chicken/Zambia/48/2020 had sequence lengths of 15,166 and 15,122 nt respectively, due to missing sequences at the 5' or 3' ends. The similarities of the full-length F gene sequence between the Zambian NDV isolates and other strains were assessed using BLAST search. For strains isolated from poultry, the identity similarity with the top ten hit strains was 98.4–97.4% and mostly contained strains from Southern Africa. For NDV/duck/Zambia/708/2021 and NDV/ibis/Zambia/1636/2015, the identity similarity to the top ten hits was 98.9–98.3% and 98.9–98.4%, respectively.

### 4.5.2 Phylogenetic Analysis of Newcastle Disease Virus

Phylogenetic analysis of the four NDV strains from poultry using the full-length F gene revealed that all the strains clustered together and were closely related to NDV strains detected in Eastern Province of Zambia in 2015. These viruses belonged to class II viruses, genotype VII, sub-genotype VII.2 (Figure 4.13). Furthermore, the analysis also indicated that the NDV strains isolated from wild birds clustered with strains from Eurasia among class I viruses, genotype 1 (Figure 4.13).

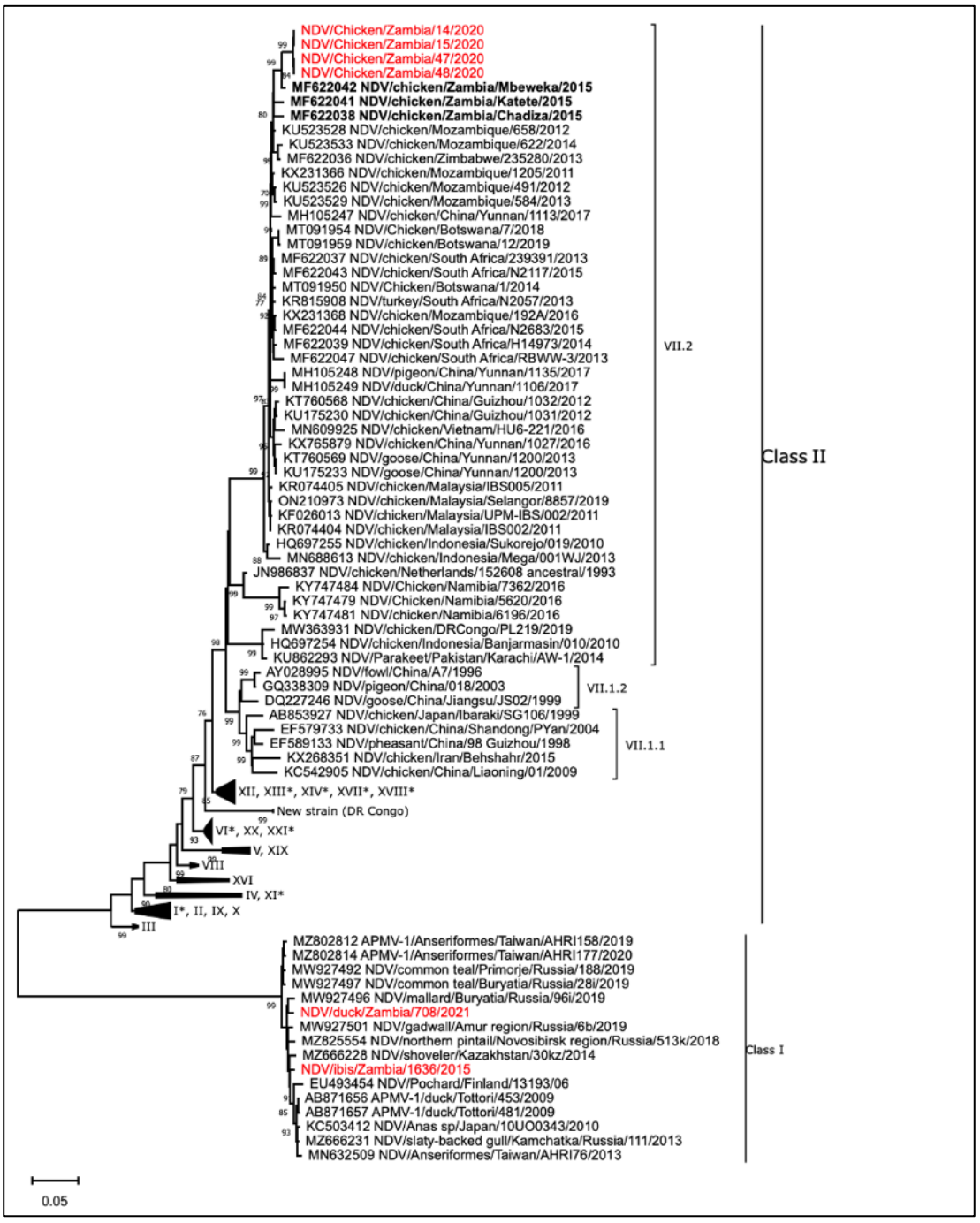


**Figure 4.13.** Phylogenetic tree of class I and II NDV based on the full-length nucleotide sequence of the fusion gene. Genotypes identified in Africa are shown with an asterisk. The sequences from this study are shown in red text while those previously isolated in Zambia are in bold. The roman numerals on the right represent the genotypic classification of the viruses using the current nomenclature by Dimitrov *et al.,* 2019.

### 4.5.3 Genetic Analysis of the F Gene Cleavage Site

The genome lengths of the isolates described in this study followed the 'rule-of-six', which is considered an essential feature for efficient replication of paramyxoviruses (Kolakofsky *et al.*, 2005), and they all had the genome organization 3′-N-P-M-F-HN-L-5′, as has been observed for all known NDV isolates. Analysis of the amino acid

(aa) proteolytic cleavage site of the F gene revealed that the $F_0$ precursor cleavage site of the four Zambian isolates from poultry contained multiple basic amino acid residues at the C-terminus of the $F_2$ protein and a phenylalanine (F) residue at the N-terminus of the $F_1$ protein ($^{112}$RRQKR|F$^{117}$) which is a characteristic feature of mesogenic and velogenic strains of NDV while the two isolates from wild birds possessed a combination motif $^{112}$ERQER|L$^{117}$ of the lentogenic strains of NDV as shown in Table 4.12.

**Table 4.12.** Characteristics of Zambian Newcastle disease virus isolates analysed in this study

| Virus Strain | Date of Isolation | Genotype | F Protein Cleavage Site |
|---|---|---|---|
| NDV/chicken/Zambia/14/2020 | October 2020 | VII.2 | $^{112}$RRQKR\|F$^{117}$ |
| NDV/chicken/Zambia/15/2020 | October 2020 | VII.2 | $^{112}$RRQKR\|F$^{117}$ |
| NDV/chicken/Zambia/47/2020 | October 2020 | VII.2 | $^{112}$RRQKR\|F$^{117}$ |
| NDV/chicken/Zambia/48/2020 | October 2020 | VII.2 | $^{112}$RRQKR\|F$^{117}$ |
| NDV/ibis/Zambia/1636/2015 | November 2015 | 1 | $^{112}$ERQER\|L$^{117}$ |
| NDV/duck/Zambia/708/2021 | December 2021 | 1 | $^{112}$ERQER\|L$^{117}$ |

## 4.6 Detection of SARS-CoV-2

### 4.6.1 Characteristics of Patients with COVID-19 from the Southern Province of Zambia

A total of 198 samples were received for WGS from districts in the Southern Province, 74 were negative for SARS-CoV-2, 51 had Ct values > 30, and 33 had a low genome coverage after sequencing. Only 40 samples were successfully sequenced and yielded whole genome consensus lengths ranging from 29,606–29,815nt with an average genome coverage of ≥98.7%. Demographic data were analyzed for all the 198 samples and the majority of the samples (104/198; 52.5%) were from females as shown in Table 4.13. The mean age of the participants was 28 (range: 0–82). The data set for gender and age were not available for one and five samples, respectively (Table 4.13).

**Table 4.13.** Characteristics of the genotyped samples infected with SARS-CoV-2.

| Parameters | Sample Distribution n (%), Overall, n = 198 |
|---|---|
| **Age Group** | |
| 0–14 Years | 7 (3.5) |
| 15–50 Years | 171 (86.4) |
| >50 Years | 15 (7.6) |
| Unknown | 5 (2.5) |
| **Gender** | |
| Female | 104 (52.5) |
| Male | 93 (47.0) |
| Unknown | 1 (0.5) |

### 4.6.2 SARS-CoV-2 Lineage Assignment and Distribution in Southern Province

SARS-CoV-2 lineage assignment using the PANGOLIN application (https://pangolin.cog-uk.io/ (accessed on 8 August 2022), revealed that the 40 genomes detected in this study were distributed into seven lineages, namely AY.116 (Delta), B.1.1.7 (Alpha), B.1.351 (Beta), and Omicron (BA.1, BA.1.1, BA.1.14, and BA.2) (Figure 4.14a). The largest number of the sequences (*n* = 17, 42.5%) belonged to lineage BA.1/GRA (Figure 4.14a). All lineage AY.116 sequences came from Choma District, whereas the six B.1.351 lineage was detected in Choma (*n* = 2), Namwala (*n* = 2), Kalomo (*n* = 1), and Mazabuka (*n* = 1) districts (Figure 4.14b; Appendix I). The Alpha variant (B.1.1.7) viruses were found in Namwala, Pemba, and Chikankata districts (Figure 4.14b; Appendix I). Of the 27 Omicron variants, 11 (40.7%) were from Livingstone, 9 (33.3%) from Chikankata, 4 (14.8%) from Kazungula, 2 (7.4%) from Choma and 1 (3.7%) from Namwala. Most lineage BA.1 viruses were detected in Chikankata and Livingstone districts where 7/27 (25.9%) viruses of this lineage were found in each district. Lineage BA.1.1 was detected in Chikankata and Kazungula districts whereas B.1.14 was only detected in Livingstone (Appendix I). Three of the five BA.2 lineage viruses were detected in Livingstone whereas the other two were detected in Choma and Kazungula districts as shown in Figure 4.14b and Appendix I.

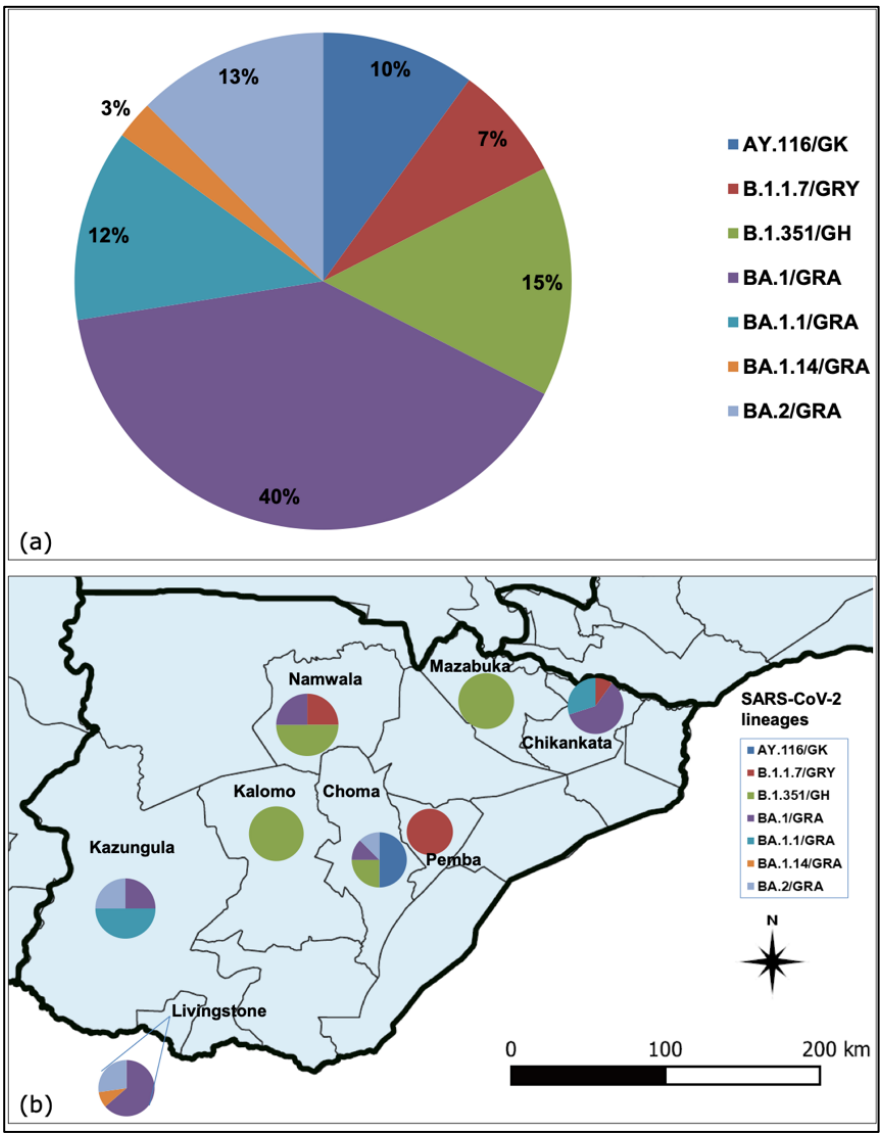


**Figure 4.14.** Distribution of SARS-CoV-2 lineage in the Southern Province of Zambia. Panel (**A**) pie chart showing SARS-CoV-2 lineages detected in the Southern Province; panel (**B**) proportionate distribution of SARS-CoV-2 lineages in the eight districts of the Southern Province. The map was generated using Quantum Geographic Information System (QGIS) version 3.10 (http://www.qgis.org (accessed on 8 August 2022).

## 4.7 Molecular Characterisation of SARS-CoV-2

### 4.7.1 Phylogenetic Analysis of SARS-CoV-2

Phylogenetic analysis revealed that the sequences separated into four clades namely Delta, Beta, Alpha, and Omicron (Figure 4.15). In the Delta clade four Southern Province sequences (Zambia/SP250/2021|EPI_ISL_6761088, Zambia/SP253/2021|EPI_ISL_6762977, Zambia/SP251/2021|EPI_ISL_6761106, and Zambia/SP252/2021|EPI_ISL_6761100), separated into two groups of which two formed a distinct cluster with a Zambian isolate whereas the other two clustered with sequences from Angola, Eswatini, and Zambia (Figure 4.15). Six sequences

analysed in this study belonged to the Beta clade and they separated into four distinct clusters. Two of the sequences (Zambia/SP30/2021|EPI_ISL_6760973 and Zambia/SP87/2021|EPI_ISL_6764745) analysed in this study clustered with isolates from Zambia, Zimbabwe, England, and the Democratic Republic of Congo (DRC), and another set of two formed a distinct cluster with sequences from Zambia. The last two sequences (Zambia/SP11/2021|EPI_ISL_6760905 and Zambia/SP10/2021|EPI_ISL_6760707) belonged to separate clusters with the former sequence grouping with Zambian sequences whereas the latter was closely related to sequences obtained in Malawi, Eswatini, and Botswana (Figure 4.15). In the Alpha clade, three Southern Province sequences, namely Zambia/SP32/2021|EPI_ISL_6761015, Zambia/SP37/2020|EPI_ISL_6761027, and Zambia/SP172/2021|EPI_ISL_6761052 formed a distinguishable cluster with sequences from England and Zambia (Figure 4.15). The Omicron clade was separated into two clusters (Figure 4.15). The majority (22/27; 81.5%) of the Zambian sequences in this clade belonged to the BA.1 sub-lineage cluster whereas the rest (5/27; 18.5%) were of the BA.2 lineage. Phylogenetic analysis further showed that the Omicron sequences from this study were mainly closely related to sequences from European and African countries (Figure 4.15).

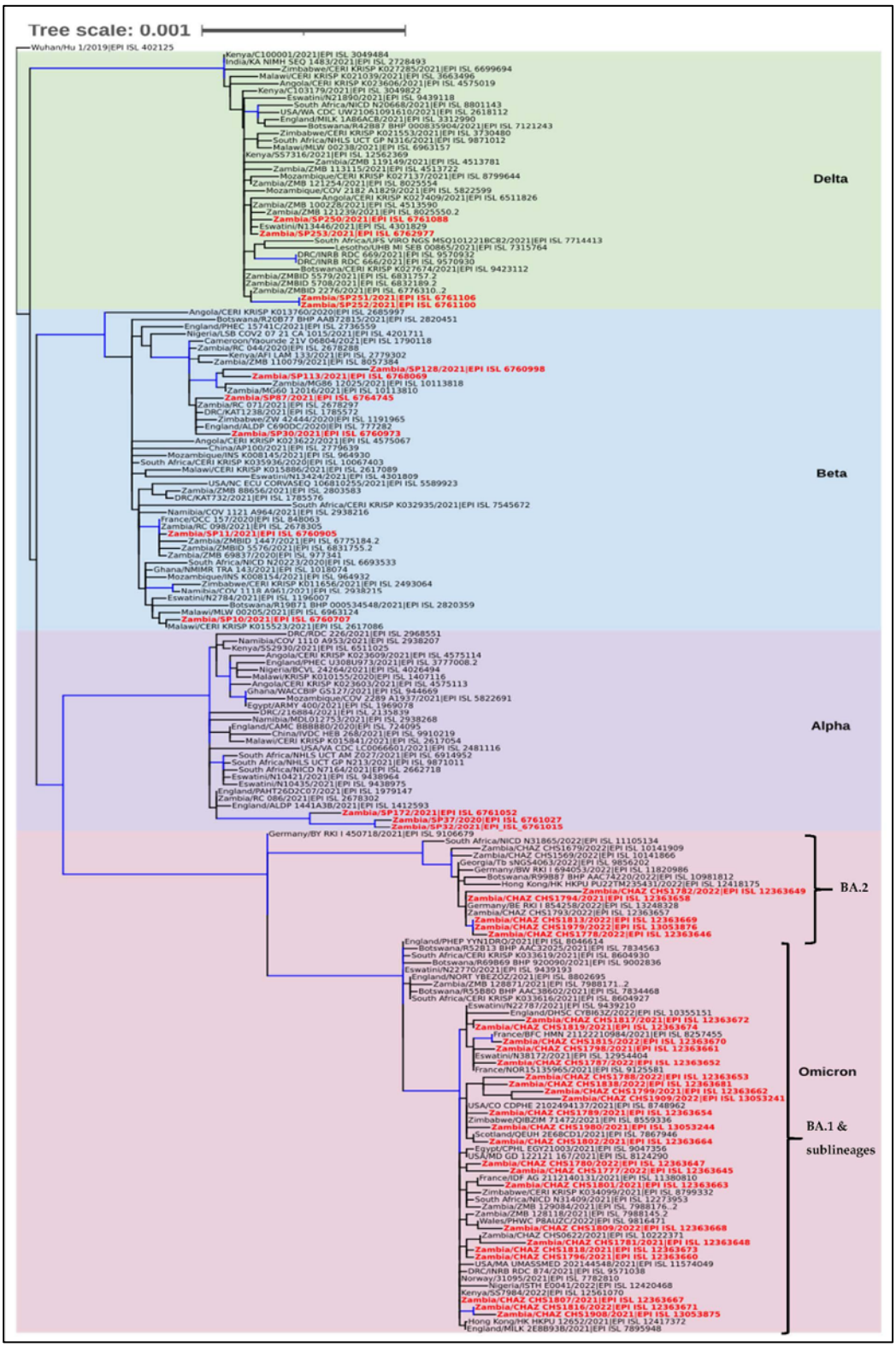


**Figure 4.15.** Phylogenetic analysis of SARS-CoV-2 genomes from Zambia and other countries. The genomes generated in this study are indicated in red whereas the shaded areas indicate the clades of variants of concern. Each sequence was named with the country name first followed by the isolate name and then the GISAID accession number. The tree branches highlighted in blue indicate tree branches that had a strong maximum likelihood ratio greater than 0.9, whereas the tree scale represents the nucleotide substitutions per site.

### 4.7.2. Molecular Analysis

A total of 292 different mutations were detected from the 40 genomes studied when compared to the Wuhan/Hu 1/2019|EPI_ISL_402125 reference sequence (Table 4.14). Most (96.2%) mutations were detected in the coding regions of the genomes. Of the mutations detected in the coding region, 64.8% (182/281) were missense mutations, 23.5% (66/281) were synonymous mutations, 8.2% (23/281) were deletions, 3.2% (9/281) insertions, and one was a stop codon (0.4%), gained with a single nucleotide polymorphism (SNP) on the ORF8 (Tables 4.14 and Appendix I). Deletions and insertion included in-frame and out-of-frame mutations. When gene mutations were stratified according to the VOCs, the Alpha variant had a total of 53 different mutations of which 31 (58.5%) were missense mutations and 8 (15.1%) synonymous mutations. The number of mutations in the Alpha variant genomes ranged between 41 and 45 with (EPI_ISL_6761027) having the most mutations. The Beta variant had a total of 68 different mutations with 44 (64.7%) missense mutations and 15 (22.1%) synonymous mutations. The mutations in the Beta variant genomes ranged between 26 and 45 with one sequence (EPI_ISL_6760998) having the most mutations. Further, sequences of the Delta variant had a total of 50 mutations with 37 (74%) missense mutations and 7 (14%) synonymous mutations. The Delta variant mutations ranged between 39 and 44 with two sequences (EPI_ISL_6761106; EPI_ISL_6761100) having the most mutations. Sequences of the Omicron variant had the highest number of mutations; 149 different mutations with 90 (60.4%) missense and 39 (26.2%) synonymous mutations, with the genomes having a mutation range between 48 and 67 with three sequences (EPI_ISL_12363648; EPI_ISL_12363649; EPI_ISL_12363661) having the most mutations. Deletions, insertions, stop-codons, and upstream/downstream gene variants had a frequency below 18% in all the VOCs.

When the number of mutations per gene was counted only once, the S protein was the most mutated gene with 82 mutations whereas the second most mutated gene was the NSP3 protein with 42 mutations (Appendix I). Of the 82 mutations in the S protein, 65/82 were mis- sense mutations, 3/82 synonymous mutations, 10/82 deletions, and 4 insertions as shown in Table 4.14. Among all the SNPs, the most common change was C > T followed by A > G and G > A. Further, a large deletion

of 26 nucleotides was observed on position 29734 of the 3′UTR of the four sequences (EPI_ISL_12363646, EPI_ISL_12363658, EPI_ISL_12363649 and EPI_ISL_12363669).

The most common mutation was the D614G substitution on the S protein and P314L substitution on the NSP12b (RdRp) protein which occurred in all the sequences studied and 67.5% (27/40) showed other amino acid substitution in the S protein including T95I, G339D, S373P, S375F, H655Y, N679K, N764K, D796K, Q954H, and D1146D (Appendix I). The second most common amino acid change (39/40; 97.5%) was the F106F substitution on the NSP3 followed by the K417N (31/40; 77.5%) substitution on the S protein T492I substitution on the NSP4, followed by P681H (30/40; 75%), and (29/40; 72.5%) N501Y substitutions on the S protein. In addition to these mutations, several substitutions, deletions, and insertions in other genomic areas were also present (Appendix I).

Comparison of mutations on the S protein of the SARS-CoV-2 variants in this study with the wildtype (Wuhan-Hu-1) SARS-CoV-2 revealed that the Omicron variant had the highest number of mutations in this protein compared to the other VOCs in this study. The Omicron variant had 58 amino acid (AA) mutations which included six deletions and four insertions (Table 4.15). Of the 60 AA mutations in the Omicron variant, 22 were found to be in the RBD of the S protein including G339D, R346K, Y369Y, S371L, S371F, S373P, S375F, T376A, D405N, R408S, K417N, N440K, G446S, T470A, S477N, T478K, E484A, Q493R, G496S, Q498R, N501Y, and Y505H (Tables 4.14–4.15). The other AA variations in the RBD included N501Y in Alpha variants, S325P, I326K, V327A, K417N, E484K, and N501Y in the Beta variant and L452R and T478K (Table 4.15).

**Table 4.14.** Distribution of mutations along different genomic regions of SARS-CoV-2 sequences detected in Southern Province.

| Genome Segment | Missense Mutation | Synonymous Mutation | Deletion | Insertion | Others | Total Mutation |
|---|---|---|---|---|---|---|
| **Coding Region** | | | | | | |
| ORF1ab | 74 | 48 | 9 | 3 | 0 | 134 |
| Spike | 65 | 3 | 10 | 4 | 0 | 82 |
| ORF3a | 5 | 4 | 0 | 0 | 0 | 9 |
| Envelope | 5 | 0 | 0 | 0 | 0 | 5 |
| Membrane | 5 | 2 | 0 | 0 | 0 | 7 |
| ORF6 | 2 | 2 | 0 | 0 | 0 | 4 |
| ORF7a | 2 | 0 | 0 | 0 | 0 | 2 |
| ORF7b | 4 | 1 | 2 | 2 | 0 | 9 |
| ORF8 | 4 | 3 | 1 | 0 | 1[1] | 9 |
| Nucleocapsid | 16 | 3 | 1 | 0 | 0 | 20 |
| **Non-coding Region** [2] | | | | | | |
| 5′UTR | 0 | 0 | 0 | 0 | 4 | 4 |
| 3′UTR | 0 | 0 | 0 | 0 | 7 | 7 |
| Total | 182 | 66 | 23 | 9 | 12 | 292 |

[1] Stop codon in the ORF8; [2] all the mutations in the non-coding region are extragenic.

**Table 4.15.** Spike protein mutations in different SARS-CoV-2 variants compared to the wild-type (Wuhan-Hu-1).

| SARS-CoV-2 Variants | Spike Mutations [1] |
|---|---|
| Wuhan-Hu-1 (wild-type) | - |
| Alpha (B.1.1.7) | ΔH69, ΔY145, **N501Y**, A570D, D614G, P681H, T716I, T874I, S982A, D1118H |
| Beta (B.1.351) | L18F, D80A, D215G, ΔL242, T307P, N317F, **S325P, I326K, V327A, K417N, E484K, N501Y**, D614G, A701V, A1087S |
| Delta (AY.116) | T19R, T95I, G142D, ΔE156, **L452R, T478K,** D614G, P681R, D950N |
| Omicron (BA.1, BA.1.1, BA.1.14, BA.2) | T19I, ΔL24, ΔA67, ΔA67, ΔI68, T95I, ΔG142, G142D, V193L, Y200C, insI210, ΔN211, N211K, L212C, V213G, insS214, insV213, insR214, insV213, insR214, R214R, A243S, L244S, **G339D, R346K, Y369Y, S371L, S371F, S373P, S375F, T376A, D405N, R408S, K417N, N440K, G446S, T470A, S477N, T478K, E484A, Q493R, G496S, Q498R, N501Y, Y505H,** T547K, D614G, H655Y, N679K, P681H, N764K, D796Y, N856K, Q954H, N969K, L981F, V1104L, D1127G, D1146D, V1264L |

[1] Receptor-binding domain (residues 319–541) is marked as bold in all the variants. Δ Represents deletion, ins represent insertion.

# CHAPTER FIVE

## 5.0 Discussion

### 5.1 Distribution of Avian Influenza Viruses in Birds in sub-Saharan Africa

The systematic review aimed at investigating the prevalence, spatiotemporal distribution, and virus subtypes of AIVs detected in domestic and wild birds over the two-decade period (2000–2019) in sub-Saharan Africa. Many articles were retrieved from the databases, but only 68 studies from 22 different countries in sub-Saharan Africa met the inclusion criteria. The findings showed a significant increase in the number of studies published between 2000 and 2019. This increase could be attributed to the increase in the number of AIV outbreaks recorded within the region with Nigeria recording 1205 suspected outbreaks (Fasina *et al.*, 2009) between 2006 and 2007. Additionally, there has been an increase in surveillance studies regionally and in different countries (Fuller *et al.*, 2015, Coker *et al.*, 2014, Cappelle *et al.*, 2012, Abolnik *et al.*, 2012, Cumming *et al.*, 2011, Simulundu *et al.*, 2011, Couacy-Hymann *et al.*, 2009, Simulundu *et al.*, 2009), improved laboratory diagnostic capacity and generally enhanced surveillance systems aimed at preventing HPAI outbreaks.

The AIV prevalence generally varied from region to region with the highest prevalence being reported in Central Africa (7.1%) and the lowest in East Africa (1.1%) (Table 1). Also, the findings indicate that AIVs were detected throughout the year in sub-Saharan Africa with higher prevalence during the dry season. Whilst it is generally understood that the prevalence of AIV infection tends to increase during the period when Eurasian migratory water birds overwinter in sub-Saharan Africa and decrease after they migrate back to Eurasia (Gaidet *et al.*, 2012), this study revealed a higher prevalence in the dry season when Eurasian migratory birds are absent or rare. It is possible that the limited water bodies in the dry season may allow increased interaction of waterfowl by congregating at particular sites, which provides opportunities for AIV transmission as well as detection during surveillance activities. However, despite the observed variations in prevalence and seasonality of AIV infection, the difference was not statistically significant. Further, the finding indicates that AIVs are perpetuated in migratory water birds originating from Eurasia as well as in indigenous African species that remain in the continent throughout the year.

Wild migratory aquatic birds are known to be the natural reservoir of AIVs globally (Olsen *et al.*, 2006, Webster *et al.*, 1992). Therefore, it is not surprising that different wild birds in sub-Saharan Africa harbour AIVs. The findings also highlight the impact of AIVs on migratory and non-migratory local birds. Analysis of HA subtype diversity revealed that H5 was the most predominant HA subtype detected in the review followed by H9, H6 and H7. The predominance of these subtypes could be attributed to the fact that most of the AIV surveillance activities have concentrated on the detection of subtypes H5, H7 and/or H9 (Chen *et al.*, 2019, Naguib *et al.*, 2019) and that they cause devastation, particularly HPAI, that is difficult to miss. The H5 and H7 viruses were detected in both wild and domestic birds implying possible transmission from wild birds to domestic birds.

The HA subtype diversity in this study has similarities and differences to that found in studies in China (Chen *et al.*, 2019), Netherlands (Bergervoet *et al.*, 2019), North America (Diskin *et al.*, 2020, Krauss *et al.*, 2004), Germany (Suss *et al.*, 1994) and Northern Europe (Wallensten *et al.*, 2007) though a number of these studies were done in wild aquatic birds. For example, the findings of this study were comparable to the studies in China (Chen *et al.*, 2019) and that in the Netherlands (Bergervoet *et al.*, 2019), which did not detect H8 and H12–H16. Furthermore, this study only detected six NA subtypes and 19 HA/NA combinations, while studies in Germany (Suss *et al.*, 1994), Northern Europe (Wallensten *et al.*, 2007) and North America (Diskin *et al.*, 2020, Krauss *et al.*, 2004) detected 40 or more subtype combinations. The higher subtype diversity in these studies could be because the surveillance was focused on wild waterfowl, which are expected to harbor a large pool of various AIV subtypes, which could also be true for Southern Africa, which had the highest subtype diversity in sub-Saharan Africa. The most prevalent NA subtype was N1, followed by N2 and N8, while N3–N5 and H2, H8 and H12–H16 were never detected, which may be due to limited surveillance efforts in wild birds or that there may be avian species-specific niches of certain HA and NA subtypes in the studied region.

Also, the findings of this systematic review revealed the presence of LPAI and HPAI viruses in both wild and domestic birds. However, the presence of HPAI viruses was more common among domestic birds, with the highest detection rate being in

chickens and ducks. The dominant subtype was H5N1, which circulated in both wild and domestic birds. The results further demonstrated that West African countries were the most affected by the H5N1 HPAI viruses with Nigeria (Fasina *et al.*, 2009, Ducatez *et al.*, 2007a), Niger (Tassoni *et al.*, 2016), Ghana (Tassoni *et al.*, 2016), Burkina Faso (Ducatez *et al.*, 2007a, Ducatez *et al.*, 2007b), Cote d'Ivoire (Couacy-Hymann *et al.*, 2009) and Togo (Fusade-Boyer *et al.*, 2019) recording one or more outbreaks during the evaluated period. This may be because West Africa is a major wintering area for the migratory waterbirds (*Anseriformes* and *Charadriiformes*) which are the natural reservoirs of AIVs (Olsen *et al.*, 2006). Moreover, the H5N1 HPAI viruses have persisted within the West African ecosystem since their introduction in Nigeria in 2006 (Fasanmi *et al.*, 2017, Joannis *et al.*, 2006). The persistence and transmission of H5N1 HPAI viruses in West Africa have been attributed to the illegal movement of infected poultry and products, multispecies livestock farming and poor biosecurity compliance levels in live bird markets (LBMs) (Fasanmi *et al.*, 2017, Fasanmi *et al.*, 2016). Furthermore, an HPAI outbreak caused by H5N1 viruses has been reported in Cameroon, a central African country that shares borders with Nigeria, which suggests transboundary transmission due to porous borders, leading to illegal trade in livestock, especially birds, between these countries (Njouom *et al.*, 2008).

Apart from the H5N1 HPAI viruses, the study also revealed the presence of other HPAI virus subtypes namely H5N8 and H5N2 in different avian species in sub-Saharan Africa. The first case of H5N8 HPAI infection in Africa was reported around the same time in Egypt and Nigeria and later spread to other neighboring countries (Khomenko *et al.*, 2018). This outbreak spread to Uganda, South Africa, Zimbabwe and DRC (Twabela *et al.*, 2020, Abolnik *et al.*, 2019, Khomenko *et al.*, 2018, Twabela *et al.*, 2018, Ndumu *et al.*, 2018). Additionally, HPAI outbreaks caused by H5N2 viruses have been reported in farmed ostriches in South Africa. These outbreaks caused a devastating impact on the ostrich industry of South Africa, which account for at least 65% of global ostrich production (Abolnik *et al.*, 2013). The presence and spread of H5N8 and H5N2 HPAI viruses have been attributed to migratory waterfowl due to the long-distance seasonal movements along their migration routes and also the other sedentary birds that have been implicated in facilitating the intracontinental dissemination of the virus (Twabela *et al.*, 2020). In

fact, H5N2 HPAI viruses have been detected in apparently healthy wild waterfowl in Nigeria (Gaidet *et al.*, 2008). Although HPAI viruses have been detected in many countries in sub-Saharan Africa, our results did not report any HPAI viruses in Zambia and Zimbabwe. Further, other countries such as Mali, Central Africa Republic, Congo-Brazzaville, Gabon and Madagascar did not specify the subtypes and pathogenicity of the viruses detected. However, the presence of HPAI viruses highlights the importance of continued and better epidemiological monitoring systems to allow their timely detection and institution of mitigatory measures.

A large diversity of LPAI virus subtypes was detected in the review with H9N2 being the most predominant followed by H6N2 and H3N8. The H9N2 and H6N2 LPAI viruses were exclusively detected in domestic birds, in which they caused asymptomatic or symptomatic infections. Symptomatic infections caused by LPAI viruses include severe clinical signs in poultry, such as respiratory distress, intestinal signs and a drop in egg production (El Houadfi *et al.*, 2016). These observations are consistent with previous studies in Iraq (Khamas, 2008). Moreover, H9N2 AIV infection is known to be endemic among poultry in Eurasia (Lindh *et al.*, 2014, Monne *et al.*, 2013), and its circulation has been reported in North Africa, Europe and Asia among others (Youk *et al.*, 2020, Ali *et al.*, 2018, Zhu *et al.*, 2018, El Houadfi *et al.*, 2016, Lindh *et al.*, 2014, Monne *et al.*, 2013). H9N2 viruses are also known to circulate between wild birds and poultry sold at LBMs. LBMs are known to be reservoirs, amplifiers and sources of AIVs (Offeddu *et al.*, 2016). Furthermore, the transmission of H9N2 viruses from poultry to humans has been reported (Peiris *et al.*, 1999).

While H4N6 and H7N7 were the most prevalent AIV subtypes detected in Northern Europe and Germany (Wallensten *et al.*, 2007, Suss *et al.*, 1994), these subtypes were among the least detected in this review. H3N6 and H9N1 were the least and the only AIV subtypes detected in the great white pelican (*Pelecanus onocrotalus*) in Zambia (Simulundu *et al.*, 2011, Simulundu *et al.*, 2009). Further, the presence of AIVs in wild waterfowl such as white-winged black terns (*Chlidonias leucopterus*), Egyptian geese (*Alopochen aegyptiacus*), yellow-billed duck (*Anas undulata*), shelduck (*Tadorna cana*) among others is important as these birds are known to be the primary reservoir of AIVs. Although our data seem to suggest an increase in the

incidence of AIV infection in migratory waterfowl and domestic birds, this review also reports the detection of H5N1 HPAI viruses in African wild birds, hooded vultures (*Necrosyrtes monachus)* in Burkina Faso (Ducatez *et al.*, 2007b). The detection of AIVs in wild migratory birds and minor bird reservoirs highlights the important role they play in the maintenance and transmission of these viruses. AIVs with H5Nx, H7Nx and other subtypes (not fully identified) were detected in Afro-tropical waterfowl and swallows in Zimbabwe throughout the year, and the detection rate was higher when Palearctic birds were present, suggesting a year-round persistence of LPAI viruses in Afro-tropical waterfowl and other wild birds (Caron *et al.*, 2011).

**5.2 Distribution of IAV and IDV in Non-Human Mammalian Hosts in Africa**

The main objective of the systematic review and meta-analysis was to investigate the prevalence and circulation of IAVs and IDVs in non-human mammalian hosts in Africa. This review included all studies found in the searched databases which reported data on prevalence, seroprevalence, virus isolation and genome detection rates of influenza A and D viruses in non-human mammalian hosts in Africa between 2000 and 2020. A total of 8785 articles were retrieved from the databases and other sources of which 169 full-texts were screened and 49 were selected and included in this review.

The findings showed that the majority of the studies were conducted in West Africa, predominantly from Nigeria. Additionally, the findings demonstrated an increase in the number of studies conducted after 2011. This increase in the number of studies could be attributed to the heightened interest in IAV in non-human mammalian species, especially swine, after the 2009 H1N1 pandemic. It is also possible that rigorous sampling and reporting of surveillance activities in non-human mammalian species were absent before the pandemic and more surveillance effort was concentrated on the emergence of H5N1 HPAIV as evidenced by numerous studies conducted in avian species (Kalonda *et al.*, 2020). Furthermore, the discovery of the novel IDV virus in swine in the USA and bat influenza in South America in 2011 could also have contributed to the increased number of studies of IVs in non-human mammalian species after 2011. Moreover, more studies were reported in pigs than any other animal species included in this review and meta-analysis.

The review showed that the predominant IAVs circulating in pigs in Africa from 2000 to 2020 were H1N1 and H1N1pdm09 followed by H3N2 viruses. RNA and antibodies of the H1N1pdm09 virus were the most frequently detected among studies included in this review, suggesting that reverse zoonosis could be a common occurrence in Africa. The H1 subtypes were detected in five regions of Africa namely African Islands (Cardinale *et al.*, 2012), Central Africa (Snoeck *et al.*, 2015, Larison *et al.*, 2014, Njabo *et al.*, 2012), East Africa (Osoro *et al.*, 2019, Munyua *et al.*, 2018, Kirunda *et al.*, 2014), North Africa (Gomaa *et al.*, 2018) and West Africa (Meseko *et al.*, 2018, Adeola *et al.*, 2017, Snoeck *et al.*, 2015, Meseko *et al.*, 2014, Anjorin *et al.*, 2012, Adeola *et al.*, 2010, Adeola *et al.*, 2009, Aiki-Raji *et al.*, 2004), but not Southern Africa, where no reports of studies in pigs were included in this review. While H3 was detected in Central Africa (Larison *et al.*, 2014), North Africa (Gomaa *et al.*, 2018), and West Africa (Gomaa *et al.*, 2018, Adeola *et al.*, 2017, 2016, Snoeck *et al.*, 2015, El-Sayed *et al.*, 2013, Adeola *et al.*, 2010, El-Sayed *et al.*, 2010, Adeola *et al.*, 2009), it was not detected in African Island, East and Southern Africa. Our findings are in line with those of studies in China (Liu *et al.*, 2011) and Korea (Jung *et al.*, 2007, Jung and Song, 2007) which detected H1 and H3 subtypes as being predominant in pigs. Furthermore, the findings of the review and meta-analysis are in agreement with the general notion that H1N1, H1N2 and H3N1 IAVs are endemic in pigs throughout the world (Krammer *et al.*, 2018, Lewis *et al.*, 2016).

The findings also revealed the circulation of other non-pig-adapted IAV subtypes in apparently healthy pigs including the H5N1 HPAIV clade 2.3.2.1c reported in Nigeria (Meseko *et al.*, 2018), H5N1 clade 2.2.1.2, H5N1 and H5N2 viruses in Egypt (Gomaa *et al.*, 2018, Kirunda *et al.*, 2014, El-Sayed *et al.*, 2013). The detection of viral RNA in apparently healthy pigs in Nigeria is a public health concern as it shows the silent circulation of a potentially zoonotic HPAIV in a country with a large population of pigs reared under intensive and free-range husbandry systems (Meseko *et al.*, 2018). The other subtype detected in pigs was the H9N2 low pathogenic avian influenza virus reported in Egypt (Gomaa *et al.*, 2018). Similar observations of H5N1 and H9N2 circulation in pigs have been reported in China (Liu *et al.*, 2011). The exposure of pigs to avian influenza viruses (AIVs) has been attributed to the increased occurrence of AIV outbreaks in poultry in the two regions (North and West

Africa) as well as pigs feeding on dead poultry carcasses or droppings of wild birds, which typically share their food (Meseko *et al.*, 2018, Abdel-Moneim *et al.*, 2010). Moreover, the co-circulation of pig adapted IAVs, non-pig-adapted IAVs and AIVs in pigs in Africa raise concern, as this may result in co-infections and possibly the generation of new reassortant viruses with pandemic potential as pigs are recognized to be "mixing vessel" of pandemic influenza virus strains (Meseko *et al.*, 2018).

The results of the meta-analysis showed an estimated pooled prevalence of 1.6% (95% CI: 0–5%) of IAV in pigs in Africa. This finding is comparable to a study in Cambodia which reported a prevalence of 1.5% of IAV in pigs (Osbjer *et al.*, 2017) but lower than the 11.7–15.7% and 19.67% reported in Guatemala (Gonzalez-Reiche *et al.*, 2017) and Mexico (Maya-Badillo *et al.*, 2020), respectively. Further, the meta-analysis demonstrated an estimated pooled seroprevalence of 14.9% of IAV in pigs in Africa. The findings are relatively similar to other studies in Britain and Wales (Mastin *et al.*, 2011), Cambodia (Rith *et al.*, 2013), and Malaysia (Suriya *et al.*, 2008), which reported an overall seroprevalence of 12%–14.9%. In contrast, higher seroprevalences of 30 to ≥ 50% in Belgium, Germany, Italy and Spain (Van Reeth *et al.*, 2008, Maldonado *et al.*, 2006), 46.1% in Korea (Pascua *et al.*, 2008), 37.7% in Taiwan (Shieh *et al.*, 2008) and 22.8% in the USA (Choi *et al.*, 2002) have been reported in pigs. The differences observed in prevalence and seroprevalence of IAV in pigs could be attributed to the region where the studies were conducted, the status of the animals (healthy or diseased), age of the animals, type of sample, sample sizes and diagnostic tests used.

The findings also demonstrated the presence or circulation of equine influenza virus (EIV) in camels in Kenya (Kimber *et al.*, 2002), and horses, donkeys and mules in Egypt (Abdel-Moneim *et al.*, 2010, WOAH, 2008, Abd El-Rahim and Hussein, 2004), Mali (WOAH, 2019a), Niger (Diallo *et al.*, 2020), Nigeria (Shittu *et al.*, 2020, Meseko *et al.*, 2016) Senegal (Diallo *et al.*, 2020), Sudan (WOAH, 2019b) and Tunisia (WOAH, 2005). The present review reported the detection of H3N8 and H7N7 antibodies and viral RNA of EIV in horses, donkeys and mules. These two subtypes of IAV have been associated with influenza virus disease in horses (Waddell *et al.*, 1963, Sovinova *et al.*, 1958). Despite the idea that H7N7 may be extinct, our review reported serological evidence of this subtype in Egypt (Abd El-

Rahim and Hussein, 2004) and Nigeria (Boukharta *et al.*, 2012). Further, the horses, donkeys and mules in these two studies were not vaccinated, indicating natural exposure of these equids to EIVs. Therefore, this finding may suggest the possible silent or undetected circulation of H7N7 EIV in African equids. In addition, H5N1 HPAIV clade 2.2, sub-clade 2.2.1 was detected from donkeys showing influenza-like illness in Egypt (Abdel-Moneim *et al.*, 2010) suggesting active infection. Exposure of Egyptian horses and donkeys to H5N1 AIV suggests the susceptibility of equids to this virus and raises concern regarding the role of equids in the spread of the H5N1 virus to other animal species (Abdel-Moneim *et al.*, 2010). Transboundary movement of donkeys, horses and mules has been implicated in EIV infections in West Africa. It has been suggested that herders often use donkeys to transport goods and once infected these animals can carry pathogens between regions and countries due to porous borders (Diallo *et al.*, 2020).

Serological evidence has shown that dogs could be infected with human influenza viruses, and different subtypes of IAVs even coexist in dogs (Horimoto *et al.*, 2014, Ramírez-Martínez *et al.*, 2013). The present review demonstrated serological evidence of H1N1, H3N8 and H5N1 IAV in dogs and cats from Nigeria, Kenya and Egypt (Daodu *et al.*, 2019, Munyua *et al.*, 2018, Oluwayelu *et al.*, 2014, El-Sayed *et al.*, 2013). These results suggest that IAV could be circulating in household dogs and cats in Africa. Furthermore, pet dogs and cats share the same environment with backyard poultry and are in close contact with their owners, therefore increasing the opportunities for human exposure to these viruses. Therefore, continued surveillance of IAVs in dogs and cats is cardinal to determine the risk posed by canine-derived IAVs to public health.

This review further demonstrated the exposure of African wildlife to IAVs including lions, black rhino, spotted hyena, wildebeest, caracal, black-backed jackal, olive baboons, rats and bats (Soilemetzidou *et al.*, 2020, Bunuma *et al.*, 2018, Freidl *et al.*, 2015, El-Sayed *et al.*, 2013, Cardinale *et al.*, 2012). The detection of IAV antibodies or antigens in wild mammals correlates with a study in Thailand and China that reported the detection of H5N1 HPAIV in leopards and tigers (Hu *et al.*, 2016, Keawcharoen *et al.*, 2004) though the present review did not determine whether the strains identified serologically represent low- or highly pathogenic IAV strains. The

exposure of wild mammals to IAVs could be attributed to the consumption of contaminated meat in carnivores or contaminated water or feeding grounds for herbivores (Soilemetzidou *et al.*, 2020). For example, captive carnivores, including tigers, leopards, dogs, cats, and raccoons, have been observed with influenza symptoms after consumption of contaminated meat (Qi *et al.*, 2009, Rimmelzwaan *et al.*, 2006, Klingeborn *et al.*, 1985).

Bats are reservoir hosts of many zoonotic viruses, such as the severe acute respiratory syndrome (SARS) coronaviruses, Middle East respiratory syndrome (MERS) coronavirus, Nipah and Hendra viruses among others, which can cause severe disease and significant mortality in humans (Han *et al.*, 2015, Ge *et al.*, 2013). In contrast to known bat influenza viruses (H17N10 and H18N11), this review found a report of a novel H9N2-like virus (A/bat/Egypt/381OP/2017) which was detected in oral and faecal swab samples collected from Egyptian fruit bats in a densely populated agricultural area in Egypt (Kandeil *et al.*, 2019). We also found studies reporting serological evidence of IAV subtype H3, H5, H8, H9 and H12 in straw-coloured fruit bats in Ghana (Freidl *et al.*, 2015). The H9N2-like virus is thought to be transmitted through the faecal-oral route which suggests opportunities for human exposure to this kind of virus through bat faeces and saliva on contaminated fruits (Yang *et al.*, 2021, Kandeil *et al.*, 2019). The virological and serological detection of IAV in wild mammals highlights the risk that IAVs pose to many mammals, including humans, as their transmission dynamics and host ranges are unclear.

Studies around the globe have reported the circulation of IDV in either healthy or sick cattle, small ruminants and swine from China, France and the USA (Family - Orthomyxoviridae, 2012). This review and meta-analysis demonstrated the presence of IDV specific antibodies in cattle from Benin, Morocco and Togo (Fusade-Boyer *et al.*, 2020, Saegerman *et al.*, 2020, Salem *et al.*, 2017), camels from Ethiopia and Kenya (Murakami *et al.*, 2019, Salem *et al.*, 2017) and small ruminants from Ethiopia and Togo (Fusade-Boyer *et al.*, 2020, Murakami *et al.*, 2019, Salem *et al.*, 2017). However, no viral RNA of IDV was detected, possibly due to the absence of active infection in the animals during the period of sampling, the limited number of samples collected in each study, and the limited number of studies conducted in Africa. The estimated pooled seroprevalence of IDV varied widely among different

host species ranging from 0.0% (95% CI: 1–2%) in pigs to 87.2% in camels (95% CI: 24–100%) with an overall seroprevalence of 10% (0–28%). It was intriguing that the highest seroprevalence of IDV was observed in dromedary camels, especially since cattle are considered the reservoir host of the virus. This suggests that these animals could be susceptible to IDV infection and are worthy of monitoring to better understand their role in the epidemiology of IDV. The seroprevalence observed in cattle, small ruminants and pigs in Africa was lower than that reported in the USA, France and Japan (Oliva *et al.*, 2019, Ferguson *et al.*, 2018, Luo *et al.*, 2017, Horimoto *et al.*, 2016, Ferguson *et al.*, 2015). While studies in other parts of the world have reported IDV in pigs (Ferguson *et al.*, 2018, Chiapponi *et al.*, 2016), the findings of this review reported a zero seroprevalence rate. This could be attributed to the small sample size of the included study which was the only study investigating IDV in pigs in this review and meta-analysis. This calls for more IDV studies to be conducted in Africa to ascertain the true picture of IDV circulation in pigs. Furthermore, the serological data of IDV in cattle, camels and small ruminants is likely to reflect natural infection as there is no IDV vaccination in place (Fusade-Boyer *et al.*, 2020).

### 5.3 Avian Influenza Viruses in Wild Waterfowl and Poultry

This study provides evidence of AIV circulation in the wild waterfowl in LNP whereas no AIV was detected in poultry from various LBMs in Zambia. The positivity rate of AIV in wild waterfowl was 3.0% during the three years of sampling with the highest positivity rate being detected in 2015 (4.1%). The positivity rate of AIVs in wild waterfowl in this study was consistent with the finding of the previous review which found a prevalence of 3% in birds in Africa (Kalonda *et al.*, 2020). While bird species in the order *Anseriformes* and *Charadriiformes* are known to be the main natural reservoir of AIV, the highest positivity rate in the current study was detected in glossy ibis (*Plegadis falcinellus*), order *Pelecaniformes*. This is the first study to report AIVs in glossy ibis in Africa. AIV has also been detected in African sacred ibis (*Threskiornis aethiopicus*) in South Africa (Abolnik *et al.*, 2016) and the first AIV detected in Zambia was from a Great white pelican (Simulundu *et al.*, 2009), which is also from the order Pelecaniformes. These data suggest that birds of the order *Pelecaniformes* may be frequently infected with AIVs and may play an important role in the eco-epidemiology of these viruses. Moreover, experimental

infection of adult ibis has revealed their susceptibility to and capability of shedding multiple AIV subtypes (Bahnson *et al.*, 2020).

In this study, the isolation rate of AIVs was higher in the wet season compared to the dry season. This could be attributed to the presence of migratory waterfowl which are known to be natural reservoirs of AIVs as most of the migratory birds begin to arrive in LNP from November to April which coincides with the wet season in Zambia. Further, the findings corroborate the general understanding that the prevalence of AIVs tends to increase during the period when Eurasian migratory water birds overwinter in sub-Saharan Africa and decrease after they migrate back to Eurasia (Gaidet *et al.*, 2012). However, these findings do not agree with the previous review report that found a higher prevalence in the dry season in sub-Saharan Africa (Kalonda *et al.*, 2020). Higher prevalence in dry seasons could be attributed to limited water bodies that may allow increased interaction of waterfowl by congregating at particular sites, which provides opportunities for AIV transmission as well as detection during surveillance activities (Kalonda *et al.*, 2020). The difference in the findings could be because sampling was not done in some months of 2020–2021 due to restrictions of movements brought about by the COVID-19 pandemic. Despite a higher positivity rate of AIV in the wet season, the isolation of AIVs in the dry season in this study may denote that AIV transmission by wild birds may be possible at any time of the year.

In the current study, eight LPAIV subtypes were detected. Although there was a disparity in the HA/NA subtypes obtained using HI/NI assays and NGS, the detected subtypes included H1N8, H2N9, H8N4, H10N6, H10N8, H10N9, H11N6 and H11N9. The disparity could be due to possible cross-reactivity of some AIV subtypes (Zhao *et al.*, 2013, WHO, 2002), a pitfall which was resolved by NGS. Along with previous studies (Simulundu *et al.*, 2011, Simulundu *et al.*, 2009), the total number of HA and NA subtypes that have been identified in Zambia are nine (H1–H4, H6, H8–H11) and six NA (N1, N2, N4, N6, N8, N9), respectively. These findings demonstrated a considerably high HA and NA diversity of AIVs circulating among wild waterfowl in Zambia. Previous studies conducted in Zambia have also reported the isolation of LPAIVs from ducks, geese and pelicans (Simulundu *et al.*, 2011, Simulundu *et al.*, 2009) within LNP, indicating the continuous circulation of

these viruses among migratory and indigenous bird species in the park. The findings confirm the idea that wild waterfowl are important in the maintenance and introduction of a wide range of viruses into the Zambian environment.

Similar studies in Africa have also reported LPAIVs in different avian species including wild and domestic birds (Kariithi *et al.*, 2020, Kayed *et al.*, 2019, Kirunda *et al.*, 2014, Abolnik *et al.*, 2010, Abolnik *et al.*, 2007). However, to the best of our knowledge, no H2N9, H8N4 and H10N8 viruses have been reported in Africa and we did not find any sequences of these subtypes on the continent in GenBank or GISAID (Global Initiative on Sharing All Influenza Data). This suggests that this is the first time that these HA/NA combinations are being reported in Zambia and Africa as a whole. For some subtypes that are being reported in this study such as H10N6, H10N9 and H11N6, though they have been reported previously (Kayed *et al.*, 2019, Soliman *et al.*, 2012, Amin *et al.*, 1980), sequence data is not available and therefore this study adds to the genetic resource of AIV detected in Africa. Hence, continuous surveillance and monitoring of wild waterfowl for AIVs should be supported to facilitate the creation of a library of isolates circulating in Africa that can be used for diagnosis and control strategies such as vaccine development in the event that any of these viruses causes an outbreak in poultry or other mammals including humans.

Phylogenetic analysis of all eight gene segments of AIVs revealed that the viruses isolated in the current study clustered with the viruses of the Eurasian lineage. However, it was noted that the HA genes of H2N9 viruses clustered separately from the major Eurasian clade, which may suggest possible independent evolution of these genes and raises the temptation to speculate on the possible existence of an African lineage of AIVs. The analysis further revealed that AIVs characterised in this study and those previously isolated in Zambia grouped into distinct clusters according to the period of isolation signifying that these viruses were introduced in the Zambian environment independently at different times. Most of the genes were closely related to AIVs isolated from wild and domestic birds in Bangladesh, Belgium, Egypt, Georgia, Mongolia, the Netherlands and South Africa. The close phylogenetic clustering of sequences analysed in this study with those of Eurasian isolates, along with the observation that most sequences characterised herein were distantly related

to those previously isolated in Zambia may suggest that these viruses were introduced in the country by Palearctic migratory birds. Additionally, the viral internal protein genes of some viruses in the current study were closely related to notifiable H7 LPAIVs, indicating possible gene exchange with viruses with the potential to mutate into HPAIVs. Remarkably, only the PB2 and NS genes of the H8N4 and H11N6 viruses isolated in 2020 and 2021 were closely related to AIVs isolated in Africa. Therefore, this might indicate a gap in the surveillance of AIVs in Africa or that these viruses were recently introduced into the African ecosystem. Furthermore, our phylogenetic analysis showed that some of the AIV genes studied were closely related to those identified in poultry, confirming the understanding that the wild waterfowl population act as a source of AIV infection for domestic birds. The findings highlight the need for continuous surveillance of AIVs in both wild and domestic birds to monitor the introduction of viruses of veterinary and public health significance.

### 5.4 Newcastle Disease Virus in Wild Waterfowl and Poultry

This study, reports for the first time the isolation of NDV from domestic and wild birds in Zambia from a LBM and LNP, respectively. Previous studies have reported the isolation of NDV from backyard poultry in the Eastern Province of Zambia (Abolnik *et al.*, 2018, Abolnik *et al.*, 2017). In addition, most work on NDV in many parts of the world has focused on poultry, where the occurrence of both virulent and avirulent strains of class II has been reported with limited studies in wild birds (de Almeida *et al.*, 2013, Miller *et al.*, 2010).

The present study demonstrated the circulation of NDV strains in broiler and village chickens sold at a LBM in Lusaka and wild birds in the LNP, indicating a positivity rate of 0.3% and 0.07%, respectively. The positivity rate of NDV recorded in chickens at LBMs in the current study was lower than 4.36–91.67% reported in Ghana and Tanzania (da Silva *et al.*, 2020), 65.9% in Egypt (Eid *et al.*, 2022), 54% in Nigeria (Funsho-Sanni *et al.*, 2022) and 45% in Libya (Gedara *et al.*, 2020). Moreover, the positivity rate of NDV in wild birds was also lower compared to 2–10% reported in other African countries (Abd Elfatah *et al.*, 2021, Wanyana *et al.*, 2018, El Naggar *et al.*, 2018, de Almeida *et al.*, 2013). The observed difference in the positivity rates could be attributed to the differences in the health status of the

birds sampled, the type of samples collected, and the methods of detection used. For example, other studies collected organ samples, cloacal or oropharyngeal swabs from sick or both sick and apparently health birds and used RT-PCR for detection (Funsho-Sanni *et al.*, 2022, Gedara *et al.*, 2020) which may increase the rate of detection. The low isolation rate of NDV in poultry could also be that the sampled poultry had been vaccinated against NDV. However, information on vaccination status was not collected during sampling. In addition, NDV from poultry were all isolated in the summer months of the dry season. The findings are in agreement with studies done by Gedara *et al.* (2020) and Munmun *et al.* (2016) who reported high prevalence of NDV in summer. Moreover, it was previously reported that ND epidemics usually occur at times of climatic stress, leading to seasonal occurrence (Awan *et al.*, 1994). The detection of NDV in wild birds is cardinal since wild birds are known to be natural reservoirs of NDV of low virulence, which can spill over into poultry (Bansal *et al.*, 2022, Rahman *et al.*, 2018).

Despite, the low positivity rate, isolation of NDV from poultry sold at LBMs is cardinal because LBMs may likely act as foci of uninterrupted replication, sustained maintenance, and dissemination of NDV variants. Furthermore, LBMs are places where birds tend to mix in unhygienic and crowded cages usually with no biosecurity measures making the transmission of any avian disease possible. Interestingly all positive samples were detected from one LBM in Lusaka district (Lusaka province) commonly known as Soweto Market from apparently health birds. Soweto LBM is the largest market located in Lusaka the capital city of Zambia and birds sold at this market originate from various parts of the country. Moreover, the market serves as the chicken source to many urban markets within the country and to farmers intending to rear chickens especially backyard poultry famers. Therefore, LBMs such as the in Lusaka province can easily foster the transmission as NDV is primarily transmitted via aerosols or ingestion of virus shed in faeces and respiratory secretions originating from infected birds (Brown and Bevins, 2017).

Phylogenetic analysis showed that NDV isolates detected in chickens belonged to class II, genotype VII, sub-genotype VII.2 previously classified as sub-genotype VIIh (Dimitrov *et al.*, 2019, Diel *et al.*, 2012). Viruses from genotype VII are prevalent worldwide and have been responsible for the most recent ND panzootic.

Detection of genotype VII, sub-genotype VII.2 (VIIh) isolates have been previously reported in Botswana, Malawi, Mozambique, South Africa, Zambia and Zimbabwe (Kgotlele *et al.*, 2020, Abolnik *et al.*, 2018, Mapaco *et al.*, 2016). The circulation of this endemic strain of NDV in Southern Africa could be attributed to the uncontrolled cross-border trading of live birds (Abolnik *et al.*, 2018). Moreover, the isolates were closely related to Zambian NDVs and those detected in neighbouring countries in Southern Africa. The close relationship between the 2015 Zambian NDV isolates and those from the current study may indicate that this NDV variant may be endemic in the country. Further, the detection of class II, genotype VII viruses emphasise the need for continued molecular surveillance of NDV to monitor the evolution and distribution of endemic strains in Zambia and the African continent.

The findings further demonstrated that the two NDV isolates from wild waterfowl belonged to class I, genotype 1 strains and were closely related to NDV strains from Europe and Asia. The close relationship of our isolates with those obtained from wild birds in Eurasia points to wild bird migration as a conduit for the introduction of these viruses. Therefore, genotype 1 NDV strains from the current study could have been introduced into the wild bird population in Zambia by Eurasian migratory birds as the isolation period corresponds to the season when migratory birds are resident in the Zambian wetlands. In Africa, studies have reported both NDV class I and II in wild waterfowl. For instance, Snoeck *et al.* (2013a) reported NDV class I (avirulent) and class II, genotype XVIII (virulent) strains in wild birds in Nigeria and Côte d'Ivoire, respectively. Other strains reported in wild birds in Africa include, avirulent class II, genotype II and virulent genotype VII NDV strains (Abd Elfatah *et al.*, 2021, Wanyana *et al.*, 2018, El Naggar *et al.*, 2018). Thus, molecular surveillance in world birds is critical in the control of NDV as previous studies suggest that wild birds may not only act as reservoirs of low virulence strains but may also play a critical role in the epidemiology of different variants of NDV strains persisting in Africa, including virulent strains responsible for poultry outbreaks (de Leeuw *et al.*, 2005, Aldous *et al.*, 2003).

The F protein is an important determinant of NDV pathogenicity. This protein in virulent strains is characterised by the presence of polybasic amino acid residues and phenylalanine at position 117 of the F gene, while avirulent strains are characterised

by the presence of only two single amino acids at the fusion cleavage site (WOAH, 2021, Courtney *et al.*, 2013). In the present study, the four strains of NDV isolated from chicken were classified as virulent based on the aa sequence motif $^{112}$RRQKR|F$^{117}$ at the F protein cleavage site whereas the two NDV strains from wild birds were classified as avirulent as they possessed aa sequence motif $^{112}$ERQER|L$^{117}$ typical of avirulent strains. Moreover, several studies have also reported the isolation of virulent strains of NDV in chickens (Liu *et al.*, 2022, Twabela *et al.*, 2021, Tran *et al.*, 2020) as well as avirulent strains in wild birds (Liu *et al.*, 2022, Wanyana *et al.*, 2018, Jindal *et al.*, 2010). While this study reported avirulent strains of NDV in wild birds, virulent class II strains responsible for many outbreaks have also been isolated in wild birds (Welch *et al.*, 2019, Miller *et al.*, 2010) indicating the importance of these birds in the dissemination of the virus.

### 5.5 Severe Acute Respiratory Syndrome Coronavirus 2

In this study, from the 198 samples obtained for genomic sequencing in eight districts of the Southern Province of Zambia, 40 SARS-CoV-2 whole genomes were successfully sequenced and analysed. Our dataset revealed that there were more cases of COVID-19 observed in females compared to males. However, other studies have recorded a higher disease burden in males than females (Taboada *et al.*, 2021, Yadav *et al.*, 2021, Jin *et al.*, 2020). The mean age of patients was 28, with a minimum and maximum age of 0 and 82 years, respectively. However, it cannot be ruled out that the small number of samples analysed in this study may have impacted the observed gender distribution and the mean age of COVID-19 patients.

Furthermore, lineage assignment revealed that BA.1 was the most prevalent lineage among our sequences, followed by B.1.351. This could be explained by the fact that most of the successfully sequenced samples were collected during the Omicron wave. The B.1.351 predominated in the second wave, AY.116 in the third wave, and BA.1 in the fourth wave. The findings corroborate those of other authors who reported the predominance of Beta (B.1.351), Delta (B.1.617.2), and Omicron BA.1 variants in the second, third, and fourth waves of the pandemic in Africa, respectively (Viana *et al.*, 2022, Wilkinson *et al.*, 2021, Tegally *et al.*, 2021a, Tegally *et al.*, 2021b). Moreover, the detection of AY.116 and B.1.351 coincided with a rapid increase in the number of confirmed cases and deaths in Zambia

(Mwenda *et al.*, 2021, Salyer *et al.*, 2021). Despite the small sample size of this study, SARS-CoV-2 lineages were detected in different districts of the Southern Province. Most of the Omicron variants were detected in Chikankata and Livingstone districts, with the latter having more subvariants. It is plausible that Livingstone, being a border town, a tourist capital, and a major transportation link to Zambia's neighbouring countries, could be at increased risk of introducing novel VOCs. Except for the Alpha variant, all the other VOCs detected in this study were found in Choma District. This could be explained by the fact that Macha Research Trust, where sequencing was conducted, is located in the Choma District. Thus, the institution was more likely to receive samples throughout the different phases of the COVID-19 waves.

Phylogenetic analysis revealed that the 40 SARS-CoV-2 genomes generated in this study belonged to four SARS-CoV-2 VOCs: Alpha, Beta, Delta, and Omicron variants. These VOCs have presented a formidable public health challenge during the COVID-19 pandemic because of their increased viral transmissibility and disease severity (Plante *et al.*, 2021). Additionally, the early detection of some of the VOCs in Africa highlights the importance of coordinated molecular surveillance systems in all parts of the world, and the role Africa has played in enabling the early detection and characterisation of new lineages and informing the global pandemic response. The close phylogenetic relatedness of sequences generated in this study with those from European and African countries supports the idea of possible multiple introductions of the virus from different regions. Phylogenetic analysis further revealed that some sequences from this study clustered together and among other Zambian sequences, which may signify the local circulation of these viruses. Notably, sequences obtained in this study that grouped within the Alpha variant clade were phylogenetically distinguishable and were detected in three different districts, which may suggest independent introductions, mainly from Europe, as these sequences were closely related to isolates from England. This introduction could be attributed to the relaxation of flight restrictions at the time these samples were collected. The Zambian Alpha cluster also displayed a longer branch length than the other sequences in this clade, indicating the continued evolution as the virus circulated.

Interestingly, the Alpha variant has not been associated with any COVID-19 wave in Zambia. This observation may suggest that the Alpha variant has no selective advantage over the other VOCs, such as the Beta and Delta variants (Hirabara *et al.*, 2022). Although some Beta and Delta variants were closely related to isolates from Europe and Zambia, others showed a close relationship to isolates from Eswatini, DRC, Malawi, and Zimbabwe, suggesting that public health measures implemented by the authorities may have been compromised by porous borders and thus permitting the variants to spread within the region. Phylogenetic analysis also revealed that Omicron variants separated into two major clusters, BA.1 and BA.2, signifying the continued evolution of this VOC. The BA.4 and BA.5 subvariants, which have been associated with driving current waves of infection in South Africa (Khan *et al.*, 2022, Tegally *et al.*, 2022) were not detected in this study.

The S glycoprotein of SARS-CoV-2 plays a pivotal role in viral infection and pathogenesis because of its role in host cell receptor recognition, viral attachment, and entry (Duan *et al.*, 2020, Tai *et al.*, 2020, Du *et al.*, 2016, Lu *et al.*, 2014, Du *et al.*, 2009). The present study demonstrated the presence of the D614G mutation in the S protein in all 40 genomes. Similar findings have been reported in many countries, including Turkey (Sahin *et al.*, 2021), Oman (Al-Mahruqi *et al.*, 2021), Egypt (Zekri *et al.*, 2021), and the Comoros Islands (Agoti *et al.*, 2021). In addition to the D614G mutation in the S glycoprotein (23403A > G), a P314L mutation (14408C > T) in the NSP12/RdRp was detected in all the sequences analysed. This finding agrees with previous research, which reported a high co-occurrence of these mutations around the globe (Obeid *et al.*, 2021, Flores-Alanis *et al.*, 2021, Rahman *et al.*, 2021). The D614G mutation is associated with a high viral load, infectivity, and transmissibility (Plante *et al.*, 2021). In contrast, mutations in the RdRp protein results in a dysfunctional enzyme that generates errors during RNA synthesis, increasing the chances of mutations occurring (Rahman *et al.*, 2021, Obeid *et al.*, 2021, Eskier *et al.*, 2020). It is also suggested that the co-occurrence of the D614G and NSP12_P314L mutations may enhance viral entry and replication, respectively (Haddad *et al.*, 2021). Therefore, the S protein mutations and their effects on virulence should be closely monitored and evaluated, as this protein is the main target for vaccine development (Korber *et al.*, 2020).

Alpha, Beta, and Omicron variants share the N501Y mutation, located in the S protein's receptor-binding domain (RBD). It is known to confer an increased binding affinity of the RBD for the ACE2 receptor, raising the viral transmission rate (Gómez *et al.*, 2021). This mutation was detected in all Alpha, Beta, and in 74.1% (20/27) Omicron variants of our sequences. Furthermore, the K417N and E484K mutations in the S protein, common to all Beta variants (Tegally *et al.*, 2021a) were also detected in our sequences. Other mutations detected in this present study included the Q27 stop in the ORF8 in all three Alpha variants. This mutation has been observed in the Alpha (B.1.1.7) variant and is known to truncate the ORF8 protein or make it inactive, allowing the accumulation of additional mutations in other regions (Gómez *et al.*, 2021). Further, eight mutations, namely D614G, D950N, F157Δ, L452R, P681R, R158Δ, T19R, and T478K, were detected in the S protein in the four sequences of the Delta variant identified in this study. These mutations are identical to those detected in the Indian Delta variants (B.1.617.2) (Zhan *et al.*, 2022, Augusto *et al.*, 2022). Deletions, insertions, frameshift variants, and up/downstream variants were much rarer. This observation is also in line with the finding of Malune and colleagues whose study reported less than 10% of these mutations (Malune *et al.*, 2022).

Sequences of the Omicron variant obtained in this study were highly mutated, having 149 mutations across the 27 sequences examined. The findings are consistent with Saxena and colleagues who detected more mutations in Omicron variants than the Delta variant (Saxena *et al.*, 2022). When the S protein mutations of the VOCs in this study were compared to the hCoV-19/Wuhan/Hu-1/2019|EPI_ISL_402125, Omicron was highly mutated with 58 mutations and 22 amino acid mutations in the RBD. These mutations are crucial as they are thought to increase the overall risk of reinfection and partial resistance to existing vaccines (Geddes, 2021). In addition to mutations in the S protein, several substitutions and deletions in other genomic regions are also present in all the SARS-CoV-2 variants in this study. Moreover, some mutations may increase the pathogenicity of SARS-CoV-2 and may also impact diagnostic assays as well as the effectiveness of antivirals and vaccines. Therefore, monitoring of mutations and characterisation of their roles in virulence-related conditions in SARS-CoV-2 is very vital in the control and prevention of the spread of the virus.

# CHAPTER SIX

## 6.0 Conclusions and Recommendations

### 6.1 Conclusions

1. The systematic review of AIVs in sub-Saharan Africa has provided an insight into the ecology and epidemiological trends of AIVs in birds over a twenty-year period (2000–2019). A considerable diversity of AIV subtypes in sub-Saharan Africa was found, with some subtypes being detected frequently in both wild and domestic birds. Furthermore, the occurrence of AIV in wildfowl and domestic birds in both the wet and dry seasons, with viruses being detected in both Eurasian migratory and indigenous African wild birds may suggest a year-round perpetuation of AIVs in Afrotropical ecosystems, with seasonal variation.
2. The review and meta-analysis revealed the circulation of IAVs and IDVs in non-human mammalian hosts in Africa with estimated pooled prevalence and seroprevalence of 1.6% and 14.9% in pigs, respectively, while the seroprevalence of IDV was estimated to be 9.9%. Pig and non-pig adapted IAVs are currently circulating in Africa with H1N1 and H1N1pdm09 predominating. Therefore, the circulation of these viruses in birds and non-human mammalian hosts underscores the need for continued IAV and IDV surveillance in different animal species. This is to better understand the eco-epidemiology of these viruses, along with improved biosecurity on poultry farms, enhanced extension services and engagement of various disciplines under a “One Health” approach in tackling influenza viruses could assist in mitigating the potential threats that these viruses may pose to public health, wildlife and the livestock industry.
3. The study further provides evidence of circulation of LPAIV and avirulent NDV in wild waterfowl in Zambia. Moreover, it reports for the first time the isolation of AIVs in glossy ibis in Africa. While no AIV was detected in poultry in LBMs, virulent strains of NDV strains were detected in a LBM in Lusaka Province of Zambia.
4. Phylogenetic analysis revealed that the AIVs isolated in this study clustered with isolates of the Eurasian lineage. The viruses isolated in 2015 formed separate clusters from those isolated in 2020–2021 suggesting independent introductions of AIVs in wild waterfowl in Zambia. Furthermore, some internal protein genes clustered with H7 LPAIVs isolated from South Africa and Egypt. Phylogenetic

analysis revealed that the LBM NDV strains belonged to genotype II and clustered mainly with viruses of the same genotype from Southern Africa and were closely related to a Zambian strain detected in 2015, signifying local circulation and transmission of the virus in Zambia. In addition, wild bird NDV strains belonged to avirulent class I, genotype 1 viruses and were closely related to viruses isolated in Europe and Asia, suggesting the possible introduction of these viruses into the Zambian ecosystem by Eurasian migratory birds.

5. For SARS-CoV-2, the findings highlighted the circulation of four VOCs in the Southern Province of Zambia namely Alpha (B.1.1.7), Beta (B.1.351), Delta (AY.116), and Omicron (BA.1, BA.1.1, BA.1.14 and BA.2). Phylogenetic analysis revealed that the genomes were closely related to genomes from Europe and Southern Africa indicating intra- and intercontinental introductions of the virus to the country. Additionally, some sequences that clustered with Zambian sequences may signify local transmission of the virus. The Omicron variant exhibited the highest number of amino acid substitutions in the S glycoprotein as compared to the other three variants in this study. Moreover, SARS-CoV-2 with the D614G and P314L mutation was the major circulating virus in Southern Province, Zambia. Our findings stress the need for continued monitoring of SARS-CoV-2 circulation in Zambia, especially in strategically positioned regions such as the Southern Province which could be at increased risk of introduction of novel VOCs. This analysis further represents the first genomic study in the Southern Province of Zambia and highlights the importance of the Zambia Genomic Sequencing Consortium in the expansion of SARS-CoV-2 genomic surveillance in understanding the spread of the virus at national and community levels.
6. Therefore, this study emphasizes the importance of genomic surveillance of AIVs and NDV in wild waterfowl and poultry as well as SARS-CoV-2 in order to understand their genetic characteristics, including evolutionary relationships to inform control strategies of veterinary and public health of these global pathogens.

### 6.2 Recommendations

The following recommendations are made based on the findings of this study.

1. The findings show evidence of AIV and NDV circulation in wild waterfowl in the LNP in Zambia. Therefore, the researcher recommends strengthening genomic surveillance systems of AIV and NDV in wild waterfowl to enable investigation of the evolutionary and transmission dynamics of these viruses. Moreover, surveillance activities are crucial for the early detection of changing AIV dynamics in reservoir populations which may provide inputs to support public health and pandemic preparedness.
2. Following the isolation AIVs in wild waterfowl in the LNP which is one of the wetlands and home to several migratory and indigenous birds, the researcher recommends extending surveillance activities to other wetlands that are home to indigenous and migratory waterfowl in Zambia to completely understand the epidemiology and seasonality of AIVs in reservoir populations in Zambia. It is also emphasized that surveillance is of global interest because this type of information in one country is important for other countries.
3. Circulation of NDV in LBMs. Following this finding there is a need to establish government-sponsored active surveillance systems, enforce biosecurity measures at the LBMs, optimize vaccination regimes, and foster general awareness of poultry viral infections amongst rural farmers including LBM marketeers.
4. Circulation of SARS-CoV-2 VOCs in Southern province. Based on this finding, it is important to strengthen genomic surveillance of SARS-CoV-2 in border towns such as Livingstone to monitor the spread of the virus and the introduction of novel variants.
5. Strengthening the capacity for sample storage and courier in rural areas should be prioritised by the Zambian Ministry of Health and other stake orders to improve SARS-CoV-2 genomic surveillance.

### 6.3 Limitations of the Study

The potential limitations of the reviews and meta-analysis include language restriction due to papers published only in English and the distribution of IAV subtypes and IDV might not be limited to the present findings since these studies included only publications with original data, well-elaborated methodological approach and laboratory-confirmed cases of IAV and IDV. For the meta-analysis the limitations included, large heterogeneity and publication bias observed across studies, sub-regions and host species and many studies were conducted in a limited

number of African countries, with West Africa being overrepresented. Reasons for this discrepancy are unclear but may reflect limited technical and financial capacity, underreporting, with few articles being published in journals accessible online, and animal influenza not being a research priority for some regions of the continent. Therefore, more studies on IAVs and IDVs in non-human mammalian species need to be conducted in Africa to identify the annual and seasonal patterns in prevalence and seroprevalence as well as to monitor the evolution and circulation of these viruses, thus assisting in preparing for potentially emerging influenza viruses of animal origin in humans.

The study was intended to determine the genetic characteristics of whole genomes of AIV, NDV and SARS-CoV-2 in selected parts of Zambia. However, the study was not without limitations. Wild bird and poultry sampling was not carried out in all the months of the dry season in the 2020 and 2021 sampling periods due to the emergence of the COVID-19 pandemic and the associated public health measures that involved movement restrictions. In addition, no sampling was conducted during the 2016 to 2019 period which leaves a considerable gap in our understanding of AIV and NDV ecology and epidemiology in the country and region at large. Furthermore, we did not use molecular tools in wild waterfowl species identification but identified them morphologically. The inclusion of an ornithologist from the Department of National Parks and Wildlife alleviated this shortcoming. In the SARS-CoV-2 study, most of the samples could not be successfully sequenced because they had a Ct > 30, whereas others had poor genomic coverage leading to low number of sequences obtained. This could have been due poor sample quality potentially caused by poor storage and transportation conditions (i.e., failure to maintain a good cold chain), as some of the samples came from far-lying rural districts.

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# APPENDICES

## Appendix A: PRISMA 2009 Checklist-Avian Influenza Viruses Detected in sub-Sahara Africa: A Systematic Review

### PRISMA 2009 Checklist. Avian Influenza Viruses Detected in Birds in sub-Saharan Africa: A Systematic Review

Annie Kalonda[1,2,3], Ngonda Saasa[2], Panji Nkhoma[1], Masahiro Kajihara[4], Hirofumi Sawa[3,4], Ayato Takada[3,4] and Edgar Simulundu[2,5,6]

| Section/topic | # | Checklist item | Reported on page # |
|---|---|---|---|
| **TITLE** | | | |
| Title | 1 | Identify the report as a systematic review, meta-analysis, or both. | 1 |
| **ABSTRACT** | | | |
| Structured summary | 2 | Provide a structured summary including, as applicable: background; objectives; data sources; study eligibility criteria, participants, and interventions; study appraisal and synthesis methods; results; limitations; conclusions and implications of key findings; systematic review registration number. | 1 |
| **INTRODUCTION** | | | |
| Rationale | 3 | Describe the rationale for the review in the context of what is already known. | 2 - 3 |
| Objectives | 4 | Provide an explicit statement of questions being addressed with reference to participants, interventions, comparisons, outcomes, and study design (PICOS). | 2 - 3 |
| **METHODS** | | | |
| Protocol and registration | 5 | Indicate if a review protocol exists, if and where it can be accessed (e.g., Web address), and, if available, provide registration information including registration number. | N/A |
| Eligibility criteria | 6 | Specify study characteristics (e.g., PICOS, length of follow-up) and report characteristics (e.g., years considered, language, publication status) used as criteria for eligibility, giving rationale. | 3 |
| Information sources | 7 | Describe all information sources (e.g., databases with dates of coverage, contact with study authors to identify additional studies) in the search and date last searched. | 3 |
| Search | 8 | Present full electronic search strategy for at least one database, including any limits used, such that it could be repeated. | 3 |
| Study selection | 9 | State the process for selecting studies (i.e., screening, eligibility, included in systematic review, and, if applicable, included in the meta-analysis). | 3 |
| Data collection process | 10 | Describe method of data extraction from reports (e.g., piloted forms, independently, in duplicate) and any processes for obtaining and confirming data from investigators. | 3 - 4 |
| Data items | 11 | List and define all variables for which data were sought (e.g., PICOS, funding sources) and any assumptions and simplifications made. | 3 |
| Risk of bias in individual studies | 12 | Describe methods used for assessing risk of bias of individual studies (including specification of whether this was done at the study or outcome level), and how this information is to be used in any data synthesis. | 4 |
| Summary measures | 13 | State the principal summary measures (e.g., risk ratio, difference in means). | 3 |
| Synthesis of results | 14 | Describe the methods of handling data and combining results of studies, if done, including measures of consistency (e.g., $I^2$) for each meta-analysis. | N/A |



| Section/topic | # | Checklist item | Reported on page # |
|---|---|---|---|
| Risk of bias across studies | 15 | Specify any assessment of risk of bias that may affect the cumulative evidence (e.g., publication bias, selective reporting within studies). | 3 |
| Additional analyses | 16 | Describe methods of additional analyses (e.g., sensitivity or subgroup analyses, meta-regression), if done, indicating which were pre-specified. | 4 |
| **RESULTS** | | | |
| Study selection | 17 | Give numbers of studies screened, assessed for eligibility, and included in the review, with reasons for exclusions at each stage, ideally with a flow diagram. | 4 - 5 |
| Study characteristics | 18 | For each study, present characteristics for which data were extracted (e.g., study size, PICOS, follow-up period) and provide the citations. | 8 - 14 |
| Risk of bias within studies | 19 | Present data on risk of bias of each study and, if available, any outcome level assessment (see item 12). | NA |
| Results of individual studies | 20 | For all outcomes considered (benefits or harms), present, for each study: (a) simple summary data for each intervention group (b) effect estimates and confidence intervals, ideally with a forest plot. | 8 - 14 |
| Synthesis of results | 21 | Present results of each meta-analysis done, including confidence intervals and measures of consistency. | N/A |
| Risk of bias across studies | 22 | Present results of any assessment of risk of bias across studies (see Item 15). | N/A |
| Additional analysis | 23 | Give results of additional analyses, if done (e.g., sensitivity or subgroup analyses, meta-regression [see Item 16]). | 6 - 16 |
| **DISCUSSION** | | | |
| Summary of evidence | 24 | Summarize the main findings including the strength of evidence for each main outcome; consider their relevance to key groups (e.g., healthcare providers, users, and policy makers). | 16 - 18 |
| Limitations | 25 | Discuss limitations at study and outcome level (e.g., risk of bias), and at review-level (e.g., incomplete retrieval of identified research, reporting bias). | 18 |
| Conclusions | 26 | Provide a general interpretation of the results in the context of other evidence, and implications for future research. | 18 |
| **FUNDING** | | | |
| Funding | 27 | Describe sources of funding for the systematic review and other support (e.g., supply of data); role of funders for the systematic review. | 19 |

*From:* Moher D, Liberati A, Tetzlaff J, Altman DG, The PRISMA Group (2009). Preferred Reporting Items for Systematic Reviews and Meta-Analyses: The PRISMA Statement. PLoS Med 6(7): e1000097. doi:10.1371/journal.pmed1000097

For more information, visit: www.prisma-statement.org.

## Appendix B: PRISMA 2009 Checklist: Influenza A and D Viruses in Non-avian Hosts in Africa: A Systematic Review and Meta-analysis

### PRISMA 2009 Checklist S1: Influenza A and D Viruses in Non-avian Hosts in Africa: A Systematic Review and Meta-analysis

Annie Kalonda [1,2,3], Marvin Phonera [2,3,4], Ngonda Saasa[2] and Edgar Simulundu [2,5]

| Section/topic | # | Checklist item | Reported on page # |
|---|---|---|---|
| **TITLE** | | | |
| Title | 1 | Identify the report as a systematic review, meta-analysis, or both. | 1 |
| **ABSTRACT** | | | |
| Structured summary | 2 | Provide a structured summary including, as applicable: background; objectives; data sources; study eligibility criteria, participants, and interventions; study appraisal and synthesis methods; results; limitations; conclusions and implications of key findings; systematic review registration number. | 1 |
| **INTRODUCTION** | | | |
| Rationale | 3 | Describe the rationale for the review in the context of what is already known. | 1-3 |
| Objectives | 4 | Provide an explicit statement of questions being addressed with reference to participants, interventions, comparisons, outcomes, and study design (PICOS). | 3 |
| **METHODS** | | | |
| Protocol and registration | 5 | Indicate if a review protocol exists, if and where it can be accessed (e.g., Web address), and, if available, provide registration information including registration number. | NA |
| Eligibility criteria | 6 | Specify study characteristics (e.g., PICOS, length of follow-up) and report characteristics (e.g., years considered, language, publication status) used as criteria for eligibility, giving rationale. | 3-4 |
| Information sources | 7 | Describe all information sources (e.g., databases with dates of coverage, contact with study authors to identify additional studies) in the search and date last searched. | 3-4 |
| Search | 8 | Present full electronic search strategy for at least one database, including any limits used, such that it could be repeated. | 3 |
| Study selection | 9 | State the process for selecting studies (i.e., screening, eligibility, included in systematic review, and, if applicable, included in the meta-analysis). | 4 |
| Data collection process | 10 | Describe method of data extraction from reports (e.g., piloted forms, independently, in duplicate) and any processes for obtaining and confirming data from investigators. | 4 |
| Data items | 11 | List and define all variables for which data were sought (e.g., PICOS, funding sources) and any assumptions and simplifications made. | 4 |
| Risk of bias in individual studies | 12 | Describe methods used for assessing risk of bias of individual studies (including specification of whether this was done at the study or outcome level), and how this information is to be used in any data synthesis. | 4-5, 16 |
| Summary measures | 13 | State the principal summary measures (e.g., risk ratio, difference in means). | 5 |
| Synthesis of results | 14 | Describe the methods of handling data and combining results of studies, if done, including measures of consistency (e.g., $I^2$) for each meta-analysis. | 4-5 |



| Section/topic | # | Checklist item | Reported on page # |
|---|---|---|---|
| Risk of bias across studies | 15 | Specify any assessment of risk of bias that may affect the cumulative evidence (e.g., publication bias, selective reporting within studies). | 4-5, 16 |
| Additional analyses | 16 | Describe methods of additional analyses (e.g., sensitivity or subgroup analyses, meta-regression), if done, indicating which were pre-specified. | 5 |
| **RESULTS** | | | |
| Study selection | 17 | Give numbers of studies screened, assessed for eligibility, and included in the review, with reasons for exclusions at each stage, ideally with a flow diagram. | 5-6 |
| Study characteristics | 18 | For each study, present characteristics for which data were extracted (e.g., study size, PICOS, follow-up period) and provide the citations. | 8-10 |
| Risk of bias within studies | 19 | Present data on risk of bias of each study and, if available, any outcome level assessment (see item 12). | 8, 16 |
| Results of individual studies | 20 | For all outcomes considered (benefits or harms), present, for each study: (a) simple summary data for each intervention group (b) effect estimates and confidence intervals, ideally with a forest plot. | 11-13 |
| Synthesis of results | 21 | Present results of each meta-analysis done, including confidence intervals and measures of consistency. | 10-13 |
| Risk of bias across studies | 22 | Present results of any assessment of risk of bias across studies (see Item 15). | 8, 16 |
| Additional analysis | 23 | Give results of additional analyses, if done (e.g., sensitivity or subgroup analyses, meta-regression [see Item 16]). | 11-13 |
| **DISCUSSION** | | | |
| Summary of evidence | 24 | Summarize the main findings including the strength of evidence for each main outcome; consider their relevance to key groups (e.g., healthcare providers, users, and policy makers). | 17 |
| Limitations | 25 | Discuss limitations at study and outcome level (e.g., risk of bias), and at review-level (e.g., incomplete retrieval of identified research, reporting bias). | 16-17 |
| Conclusions | 26 | Provide a general interpretation of the results in the context of other evidence, and implications for future research. | 13-17 |
| **FUNDING** | | | |
| Funding | 27 | Describe sources of funding for the systematic review and other support (e.g., supply of data); role of funders for the systematic review. | 17 |

*From:* Moher D, Liberati A, Tetzlaff J, Altman DG, The PRISMA Group (2009). Preferred Reporting Items for Systematic Reviews and Meta-Analyses: The PRISMA Statement. PLoS Med 6(7): e1000097.

**Appendix C: Quality Assessment Checklist**

The following items were examined and given a score based on a simple scale system (1 for "yes", 0 for "no").

1. Was the research objective clearly stated?
2. Was the sampling area clearly described with reference to the location, climate and level of development (rural, peri-urban or urban)?
3. Was the period of the study stated?
4. Was the target sample a close representation of the general population?
5. Was some form of random selection used to select the samples?
6. Was a minimum sample size calculated?
7. Were the sample processing and diagnostic method clearly described?
8. Were the subjects categorised by sex?
9. Were the subjects categorised by ownership and movement restriction and were the categories clearly defined?
10. Were the subjects categorised by age and were the age categories clearly defined?

The quality index score for each study was calculated by dividing the study quality score by 10.

## Appendix D: Studies Included in the Systematic Review and Meta-analysis

Table S1: Studies included in the systematic review and meta-analysis

| No. | Title of Study | Studies Included Meta-analysis (✓) |
|---|---|---|
| 1 | Abd El-Rahim, I. H. & Hussein, M. 2004. An epizootic of equine influenza in Upper Egypt in 2000. *Rev Sci Tech,* 23**,** 921-30 DOI: 10.20506/rst.23.3.1539. | |
| 2 | Abdel-Moneim, A. S., Abdel-Ghany, A. E. & Shany, S. A. 2010. Isolation and characterization of highly pathogenic avian influenza virus subtype H5N1 from donkeys. *J Biomed Sci,* 17**,** 25 DOI: 10.1186/1423-0127-17-25. | |
| 3 | Adeola, O.A.; Adeniji, J.A.; Olugasa, B.O. Isolation of influenza A viruses from pigs in Ibadan, Nigeria. *Vet Ital*. **2009**, *45*, 383-390. | ✓ |
| 4 | Adeola, O. A., Adeniji, J. A. & Olugasa, B. O. 2010. Detection of haemagglutination-inhibiting antibodies against human H1 and H3 strains of influenza A viruses in pigs in Ibadan, Nigeria. *Zoonoses Public Health,* 57**,** e89-94 DOI: 10.1111/j.1863-2378.2009.01268.x. | ✓ |
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## Appendix E: Characteristics of the Included Studies

Table S2: Characteristics of the included studies

| Characteristics | Studies n (%) |
|---|---|
| **Animal Species** [1] | |
| Pigs | 28 (62.2) |
| Cattle | 4 (8.9) |
| Small ruminants – Goat and Sheep | 4 (8.9) |
| Camels | 3 (6.7) |
| Equids (Donkeys, Horses, Mule) | 8 (17.8) |
| Domestic Canine – Cats and Dogs | 4 (8.9) |
| Rats | 1 (2.2) |
| Olive baboons | 1 (2.2) |
| Zebra | 1 (2.2) |
| Buffaloes | 1 (2.2) |
| Bats | 2 (4.4) |
| Herbivores – African elephant, Springbok, Black Rhinos and Wildebeest | 1 (2.2) |
| Carnivores – Black rhino, Brown hyena, Spotted hyena, Bat-eared fox, Lion, Leopard, Cheetah, Caracal and Black-backed Jackal | 1 (2.2) |
| **Type of Samples** | |
| Nasal Swabs | 11 (24.4) |
| Serum | 17 (37.8) |
| Mixed [2] | 17 (37.8) |
| **Method of Testing** | |
| Haemagglutinin Inhibition (HI) | 6 (13.3) |
| ELISA | 9 (20) |
| Virus Neutralisation Assay | 1 (2.2) |
| Protein Microarray | 1 (2.2) |
| RT-PCR | 5 (11.1) |
| Culture | 1 (2.2) |
| Mixed [3] | 22 (48.9) |
| **Influenza Viruses** | |
| Influenza A virus | 37 (82.2) |
| Influenza D virus | 4 (8.9) |
| ND [4] | 4 (8.9) |
| **Healthy Status** | |
| Asymptomatic (Apparently healthy) | 14 (31.1) |
| Symptomatic | 5 (11.1) |
| Mixed [5] | 2 (4.4) |
| Not reported | 24 (53.3) |
| **Vaccination Status** | |
| Vaccinated | 1 (2.2) |
| Not Vaccinated | 14 (31.1) |
| Not Reported | 30 (66.7) |
| **Types of Study** | |
| Cross-sectional | 23 (51.1) |
| Longitudinal | 2 (4.4) |
| Surveillance | 20 (44.4) |

[1] Some studies reported more than one animal species, [2] Mixed means two types of samples were collected i.e., nasal swab and serum, [3] Two or more methods were used, [4] ND – Not Detected, [5] Asymptomatic and symptomatic population.

## Appendix F: Funnel Plots Used to Examine Publication Bias

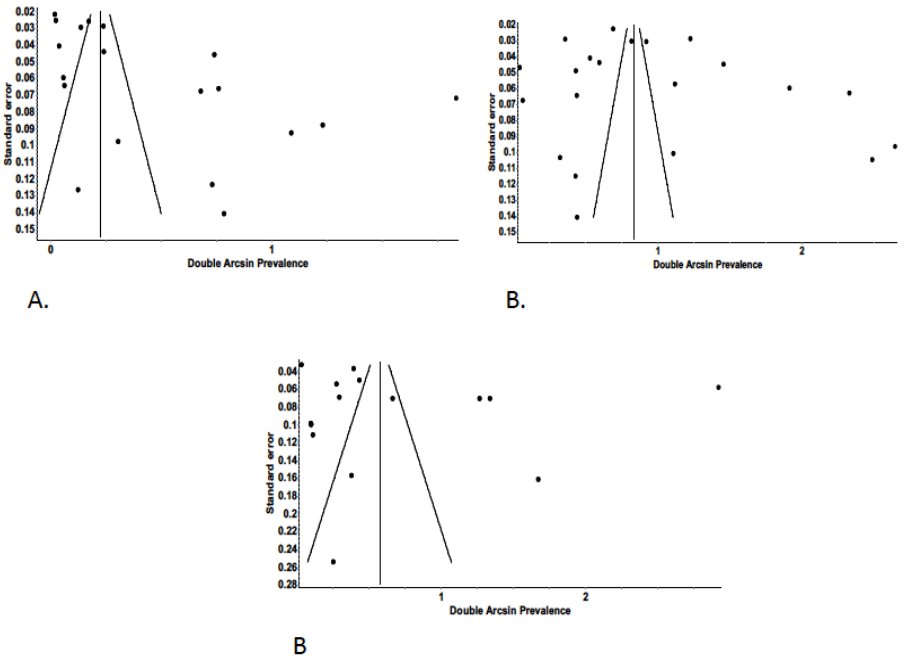


Figure S1: Funnel plots used to examine publication bias. A: Publication bias in studies related to IAV in pigs in Africa; B: Publication bias in studies related to seroprevalence of IAV in pigs in Africa; C: Publication bias related to seroprevalence of IDV in non-avian species in Africa.

## Appendix G: Doi Plots

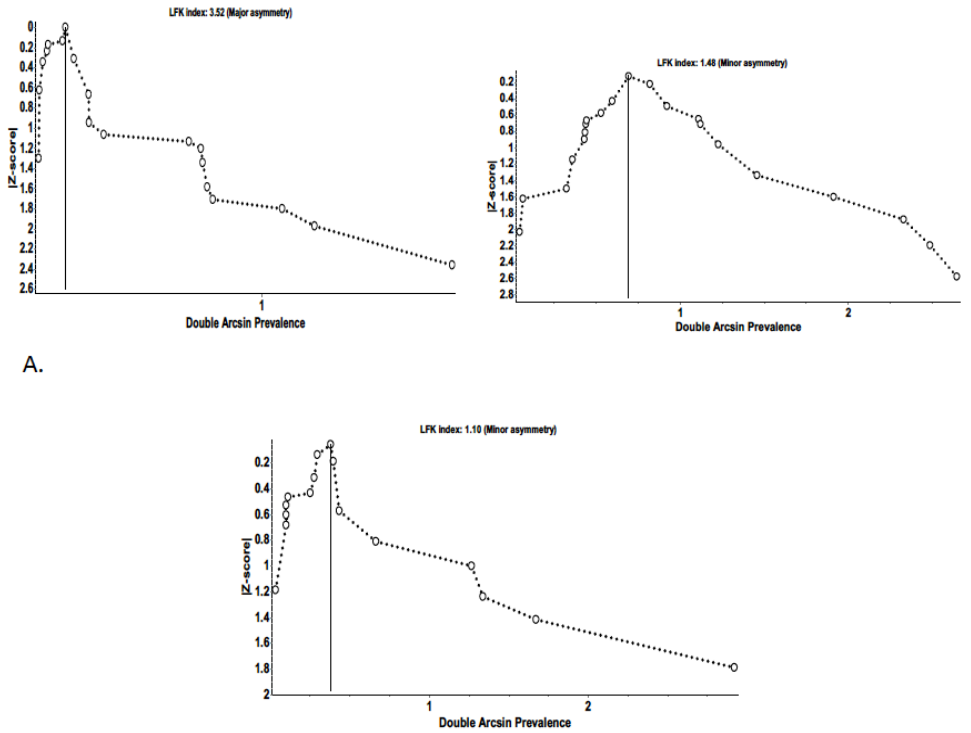


Figure S2: Doi plots with pseudo 95% confidence limits used to examine publication bias. A: Publication bias in studies related to IAV in pigs in Africa; B: Publication bias in studies related to seroprevalence of IAV in pigs in Africa; C: Publication bias related to seroprevalence of IDV in non-avian species in Africa.

## Appendix H: Phylogenetic Trees of Internal Genes of AIVs

PB2 Gene

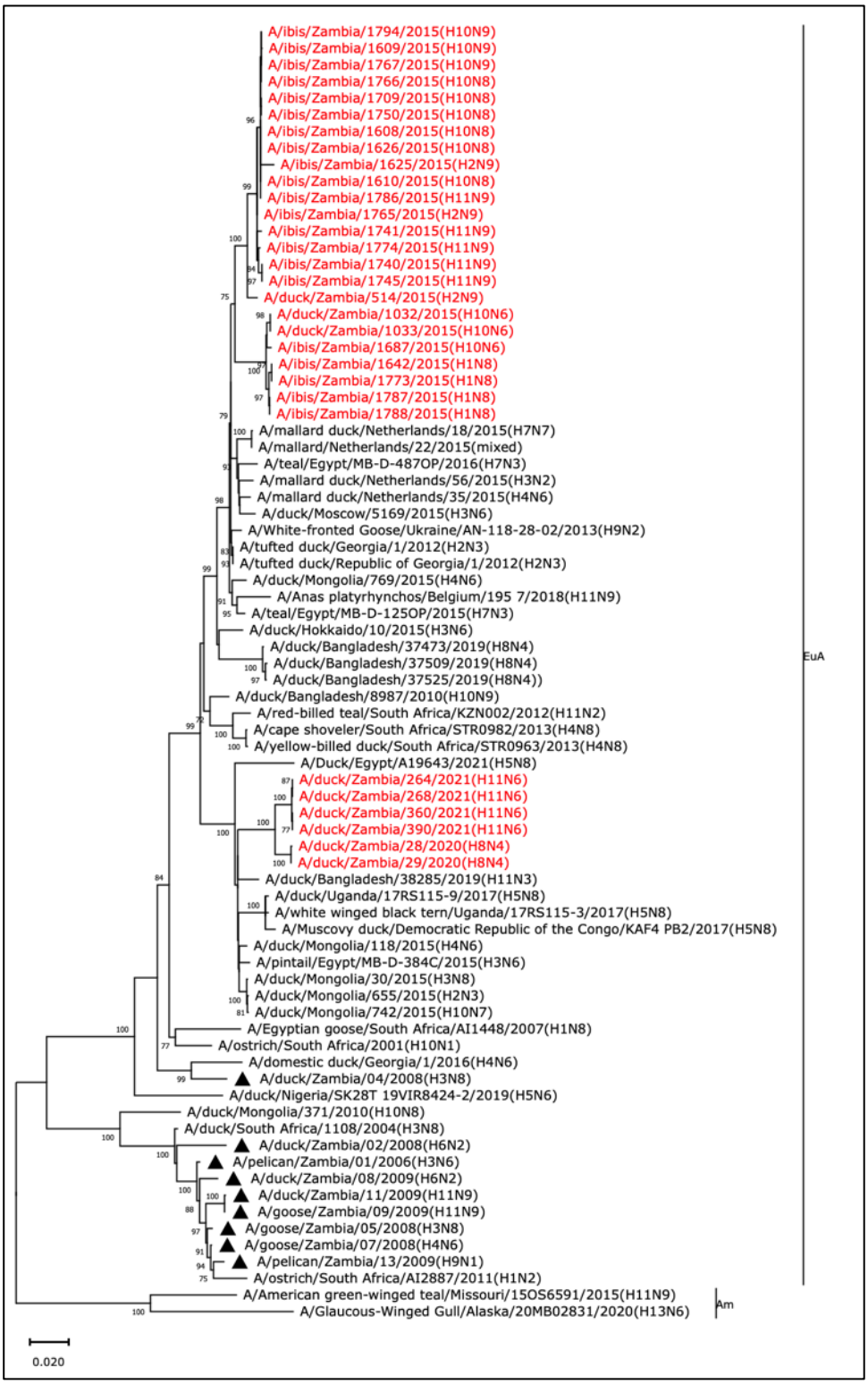


**Figure 1.** Phylogenetic analysis of the AIV PB2 genes based on 2280 nucleotides. The viruses isolated in this study are in red text while those previously isolated in Zambia are shown with a black triangle. Numbers at branch nodes are bootstrap values ≥70%. Am – American lineage; EuA – Eurasian lineage. Bar, number of substitutions per site.

PA Gene


**Figure 2.** Phylogenetic analysis of the AIV PA genes based on 2118 nucleotides. The viruses isolated in this study are in red text while those previously isolated in Zambia are shown with a black triangle. Numbers at branch nodes are bootstrap values ≥70%. Am – American lineage; EuA – Eurasian lineage. Bar, number of substitutions per site.

M Gene

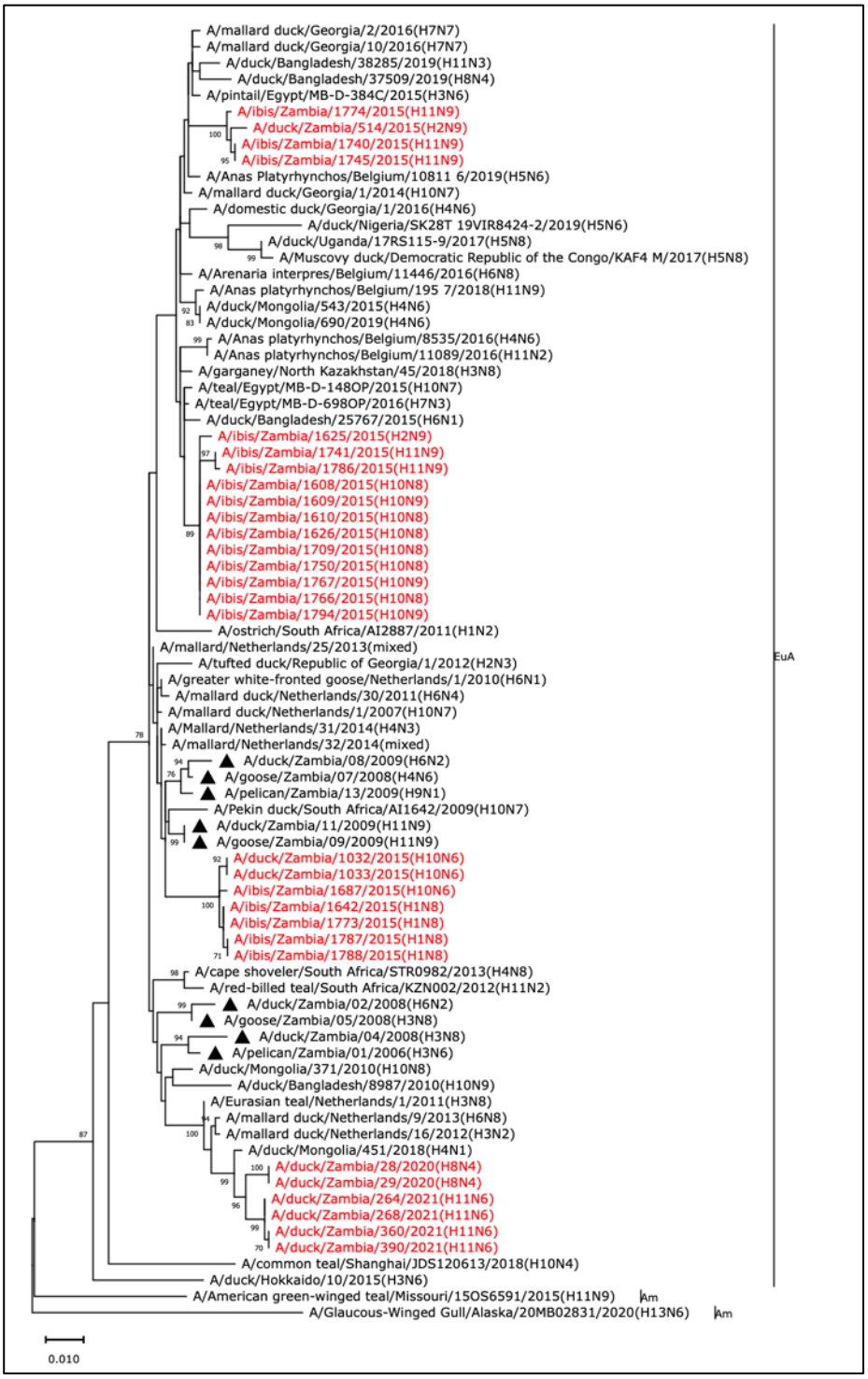


**Figure 3.** Phylogenetic analysis of the AIV M genes based on 989 nucleotides. The viruses isolated in this study are in red text while those previously isolated in Zambia are shown with a black triangle. Numbers at branch nodes are bootstrap values ≥70%. Am – American lineage; EuA – Eurasian lineage. Bar, number of substitutions per site.

## Appendix I: Characteristics of SARS-CoV-2 Genomes, Nucleic Acid and Amino Acid Mutations in SARS-CoV-2 Genomes Obtained from the Southern Province of Zambia

**Table 1:** Characteristics of 36 SARS-CoV-2 whole-genomes from the Southern Province of Zambia

| Patient ID. | Sex | Age | $C_T$ Value | Virus name (GISAID Accession ID) | Collection Date | District | Lineage | Covid-19 Wave |
|---|---|---|---|---|---|---|---|---|
| 1 | Female | 17 | 20.3 | hCoV-19/Zambia/SP113/2021\|EPI_ISL_6768069 | 2 February 2021 | Choma | B.1.351 | Second |
| 2 | Male | 19 | 18 | hCoV-19/Zambia/SP87/2021\|EPI_ISL_6764745 | 15 February 2021 | Namwala | B.1.351 | Second |
| 3 | Male | 38 | 18.4 | hCoV-19/Zambia/SP10/2021\|EPI_ISL_6760707 | 28 January 2021 | Mazabuka | B.1.351 | Second |
| 4 | Male | 16 | 22.3 | hCoV-19/Zambia/SP11/2021\|EPI_ISL_6760905 | 3 February 2021 | Kalomo | B.1.351 | Second |
| 5 | Male | 34 | 16.7 | hCoV-19/Zambia/SP30/2021\|EPI_ISL_6760973 | 5 February 2021 | Namwala | B.1.351 | Second |
| 6 | Female | 17 | 16.8 | hCoV-19/Zambia/SP128/2021\|EPI_ISL_6760998 | 18 February 2021 | Choma | B.1.351 | Second |
| 7 | Male | 23 | 18.2 | hCoV-19/Zambia/SP32/2021\|EPI_ISL_6761015 | 15 February 2021 | Namwala | B.1.1.7 | Second |
| 8 | Male | 57 | 19.2 | hCoV-19/Zambia/SP37/2020\|EPI_ISL_6761027 | 1 December 2020 | Pemba | B.1.1.7 | Second |
| 9 | Female | 44 | 26.4 | hCoV-19/Zambia/SP172/2021\|EPI_ISL_6761052 | 2 September 2021 | Chikankata | B.1.1.7 | Third |
| 10 | Female | 23 | 20.1 | hCoV-19/Zambia/SP250/2021\|EPI_ISL_6761088 | 26 June 2021 | Choma | AY.116 | Third |
| 11 | Female | 5 | 28.7 | hCoV-19/Zambia/SP252/2021\|EPI_ISL_6761100 | 19 August 2021 | Choma | AY.116 | Third |
| 12 | Male | - | 19.5 | hCoV-19/Zambia/SP253/2021\|EPI_ISL_6762977 | 17 June 2021 | Choma | AY.116 | Third |
| 13 | Male | 44 | 29.2 | hCoV-19/Zambia/SP251/2021\|EPI_ISL_6761106 | 8 September 2021 | Choma | AY.116 | Third |
| 14 | Unknown | 0 | 14.3 | hCoV-19/Zambia/CHAZ-CHS1777/2022\|EPI_ISL_12363645 | Unknown | Chikankata | BA.1 | Fourth |
| 15 | Male | 20 | 20.5 | hCoV-19/Zambia/CHAZ-CHS1778/2022\|EPI_ISL_12363646 | 28 February 2022 | Livingstone | BA.2 | Fourth |
| 16 | Male | 0 | 23 | hCoV-19/Zambia/CHAZ-CHS1780/2022\|EPI_ISL_12363647 | Unknown | Chikankata | BA.1 | Fourth |
| 17 | Male | 46 | 24.4 | hCoV-19/Zambia/CHAZ-CHS1781/2021\|EPI_ISL_12363648 | 21 December 2021 | Livingstone | BA.1 | Fourth |
| 18 | Female | 33 | 26.3 | hCoV-19/Zambia/CHAZ-CHS1782/2022\|EPI_ISL_12363649 | 6 April 2022 | Choma | BA.2 | Fourth |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 19 | Male | 32 | 17.9 | hCoV-19/Zambia/CHAZ-CHS1787/2022\|EPI_ISL_12363652 | 5 January 2022 | Chikankata | BA.1 | Fourth |
| 20 | Female | 0 | 28.9 | hCoV-19/Zambia/CHAZ-CHS1788/2022\|EPI_ISL_12363653 | Unknown | Chikankata | BA.1 | Fourth |
| 21 | Male | 17 | 15 | hCoV-19/Zambia/CHAZ-CHS1789/2021\|EPI_ISL_12363654 | 12 December 2021 | Livingstone | BA.1 | Fourth |
| 22 | Female | 30 | 28.9 | hCoV-19/Zambia/CHAZ-CHS1794/2021\|EPI_ISL_12363658 | 14 December 2021 | Kazungula | BA.2 | Fourth |
| 23 | Female | 52 | 17.4 | hCoV-19/Zambia/CHAZ-CHS1796/2021\|EPI_ISL_12363660 | 7 December 2021 | Livingstone | BA.1 | Fourth |
| 24 | Female | 23 | 22 | hCoV-19/Zambia/CHAZ-CHS1798/2021\|EPI_ISL_12363661 | 30 December 2021 | Chikankata | BA.1.1 | Fourth |
| 25 | Male | 48 | 11 | hCoV-19/Zambia/CHAZ-CHS1799/2021\|EPI_ISL_12363662 | 28 December 2021 | Chikankata | BA.1 | Fourth |
| 26 | Female | 23 | 28.8 | hCoV-19/Zambia/CHAZ-CHS1801/2021\|EPI_ISL_12363663 | 13 December 2021 | Livingstone | BA.1.14 | Fourth |
| 27 | Male | 0 | 28.8 | hCoV-19/Zambia/CHAZ-CHS1802/2021\|EPI_ISL_12363664 | 13 December 2021 | Livingstone | BA.1 | Fourth |
| 28 | Male | - | 20.5 | hCoV-19/Zambia/CHAZ-CHS1807/2021\|EPI_ISL_12363667 | 14 December 2021 | Kazungula | BA.1 | Fourth |
| 29 | Male | 40 | 17.9 | hCoV-19/Zambia/CHAZ-CHS1809/2022\|EPI_ISL_12363668 | 20 January 2022 | Livingstone | BA.1 | Fourth |
| 30 | Male | 19 | 25.4 | hCoV-19/Zambia/CHAZ-CHS1813/2022\|EPI_ISL_12363669 | 28 February 2022 | Livingstone | BA.2 | Fourth |
| 31 | Male | 49 | 17.3 | hCoV-19/Zambia/CHAZ-CHS1815/2022\|EPI_ISL_12363670 | 5 January 2022 | Chikankata | BA.1.1 | Fourth |
| 32 | Female | 0 | 24 | hCoV-19/Zambia/CHAZ-CHS1816/2022\|EPI_ISL_12363671 | Unknown | Chikankata | BA.1 | Fourth |
| 33 | Male | 45 | 23.7 | hCoV-19/Zambia/CHAZ- | 14 December 2021 | Kazungula | BA.1.1 | Fourth |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | CHS1817/2021\|EPI_ISL_12363672 | | | | |
| 34 | Male | 39 | 15.2 | hCoV-19/Zambia/CHAZ-CHS1818/2021\|EPI_ISL_12363673 | 14 December 2021 | Livingstone | BA.1 | Fourth |
| 35 | Male | 39 | 28.6 | hCoV-19/Zambia/CHAZ-CHS1819/2021\|EPI_ISL_12363674 | 14 December 2021 | Kazungula | BA.1.1 | Fourth |
| 36 | Male | 25 | - | hCoV-19/Zambia/CHAZ-CHS1838/2022\|EPI_ISL_12363681 | Unknown | Namwala | BA.1 | Fourth |
| 37 | Male | 48 | - | hCoV-19/Zambia/CHAZ-CHS1908/2021\|EPI_ISL_13053875 | 28 December 2021 | Choma | BA.1 | Fourth |
| 38 | Female | - | - | >hCoV-19/Zambia/CHAZ-CHS1909/2022\|EPI_ISL_13053241 | Unknown | Chikankata | BA.1 | Fourth |
| 39 | Male | 19 | - | >hCoV-19/Zambia/CHAZ-CHS1979/2022\|EPI_ISL_13053876 | 28 February 2022 | Livingstone | BA.2 | Fourth |
| 40 | Male | 46 | - | hCoV-19/Zambia/CHAZ-CHS1980/2021\|EPI_ISL_13053244 | 21 December 2021 | Livingstone | BA.1 | Fourth |

**Table 2:** Nucleic acid and amino acid mutations observed in the 36 SARS-CoV-2 genomes obtained from the Southern Province of Zambia

| No. | Position | Genome Region | Number of Mutations | Reference Nucleotide | Sample Nucleotide | AA Change | Frequency | Mutation Type |
|---|---|---|---|---|---|---|---|---|
| 1 | 174 | **5'UTR** | 4 | G | T | 174 | 6 | extragenic |
| 2 | 210 | | | G | T | 210 | 4 | extragenic |
| 3 | 222 | | | C | T | 222 | 1 | extragenic |
| 4 | 241 | | | C | T | 241 | 31 | extragenic |
| 5 | 343 | **NSP1** | 3 | . | C | V26 | 2 | Insertion |
| 6 | 478 | | | C | T | I71I | 1 | Synonymous |
| 7 | 670 | | | **T** | **G** | **S135R** | 5 | **Missense** |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 8 | 913 | **NSP2** | 5 | C | T | S36S | 3 | Synonymous |
| 9 | 926 | | | **T** | **C** | **F41L** | 2 | **Missense** |
| 10 | 940 | | | G | A | K45K | 1 | Synonymous |
| 11 | 1059 | | | **C** | **T** | **T85I** | 6 | **Missense** |
| 12 | 2692 | | | A | T | T629T | 2 | Synonymous |
| 13 | 2790 | **NSP3** | 42 | **C** | **T** | **T24I** | 5 | **Missense** |
| 14 | 2832 | | | **A** | **G** | **K38R** | 20 | **Missense** |
| 15 | 2903 | | | **A** | **G** | **I62V** | 1 | **Missense** |
| 16 | 3037 | | | C | T | F106F | 39 | Synonymous |
| 17 | 3177 | | | **C** | **T** | **P153L** | 3 | **Missense** |
| 18 | 3267 | | | **C** | **T** | **T183I** | 3 | **Missense** |
| 19 | 4150 | | | T | C | V477V | 2 | Synonymous |
| 20 | 4181 | | | **G** | **T** | **A488S** | 4 | **Missense** |
| 21 | 4184 | | | **G** | **A** | **G489S** | 5 | **Missense** |
| 22 | 4255 | | | G | T | P512P | 2 | Synonymous |
| 23 | 4276 | | | C | T | Y519Y | 2 | Synonymous |
| 24 | 4321 | | | C | T | A534A | 5 | Synonymous |
| 25 | 4540 | | | C | T | Y607Y | 2 | Synonymous |
| 26 | 4576 | | | A | T | T619T | 2 | Synonymous |
| 27 | 4579 | | | T | A | L620L | 3 | Synonymous |
| 28 | 4655 | | | **C** | **T** | **R646W** | 1 | **Missense** |
| 29 | 5161 | | | **T** | **A** | **F814L** | 1 | **Missense** |
| 30 | 5230 | | | **G** | **T** | **K837N** | 6 | **Missense** |
| 31 | 5260 | | | T | A | T847T | 1 | Synonymous |
| 32 | 5386 | | | T | G | A889A | 22 | Synonymous |
| 33 | 5388 | | | **C** | **A** | **A890D** | 3 | **Missense** |
| 34 | 5866 | | | C | T | F1049F | 1 | Synonymous |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 35 | 5953 | | | G | A | L1078L | 2 | Synonymous |
| 36 | 5986 | | | C | T | F1089F | 3 | Synonymous |
| 37 | 6106 | | | T | C | Y1129Y | 1 | Synonymous |
| 38 | 6402 | | | **C** | **T** | **P1228L** | 4 | **Missense** |
| 39 | 6513 | | | GTT | . | S1265 | 21 | Deletion |
| 40 | 6568 | | | C | T | D1283D | 1 | Synonymous |
| 41 | 6954 | | | **T** | **C** | **I1412T** | 3 | **Missense** |
| 42 | 6968 | | | C | T | L1416L | 1 | Synonymous |
| 43 | 7069 | | | A | G | E1450E | 1 | Synonymous |
| 44 | 7071 | | | **G** | **T** | **G1451V** | 1 | **Missense** |
| 45 | 7086 | | | **C** | **T** | **T1456I** | 2 | **Missense** |
| 46 | 7124 | | | **C** | **T** | **P1469S** | 4 | **Missense** |
| 47 | 7252 | | | **G** | **C** | **L1511F** | 1 | **Missense** |
| 48 | 7292 | | | T | C | G1524G | 1 | Synonymous |
| 49 | 7787 | | | **G** | **C** | **A1690P** | 1 | **Missense** |
| 50 | 7923 | | | **C** | **T** | **S1735F** | 1 | **Missense** |
| 51 | 8035 | | | **G** | **T** | **M1772I** | 2 | **Missense** |
| 52 | 8290 | | | C | T | L1857L | 1 | Synonymous |
| 53 | 8393 | | | **G** | **A** | **A1892T** | 22 | **Missense** |
| 54 | 8499 | | | **C** | **T** | **A1927V** | 2 | **Missense** |
| 55 | 8723 | **NSP4** | 9 | **A** | **C** | **I57L** | 1 | **Missense** |
| 56 | 8986 | | | C | T | D144D | 4 | Synonymous |
| 57 | 9053 | | | **G** | **T** | **V167L** | 4 | **Missense** |
| 58 | 9344 | | | **C** | **T** | **L264F** | 5 | **Missense** |
| 59 | 9424 | | | A | G | V290V | 3 | Synonymous |
| 60 | 9474 | | | **C** | **T** | **A307V** | 1 | **Missense** |
| 61 | 9534 | | | **C** | **T** | **T327I** | 5 | **Missense** |

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|---|---|---|---|---|---|---|---|---|
| 62 | 9866 | | | **C** | **T** | **L438F** | 5 | **Missense** |
| 63 | 10029 | | | **C** | **T** | **T492I** | 31 | **Missense** |
| 64 | 10181 | **NSP5** | 7 | **A** | **G** | **I43V** | 1 | **Missense** |
| 65 | 10198 | | | C | T | D48D | 2 | Synonymous |
| 66 | 10323 | | | **A** | **G** | **K90R** | 6 | **Missense** |
| 67 | 10447 | | | G | A | R131R | 5 | Synonymous |
| 68 | 10449 | | | **C** | **A** | **P132H** | 27 | **Missense** |
| 69 | 10691 | | | **A** | **G** | **I213V** | 4 | **Missense** |
| 70 | 10892 | | | **A** | **G** | **T280A** | 1 | **Missense** |
| 71 | 11083 | **NSP6** | 9 | **G** | **T** | **L37F** | 1 | **Missense** |
| 72 | 11109 | | | **C** | **T** | **A46V** | 1 | **Missense** |
| 73 | 11201 | | | **A** | **G** | **T77A** | 4 | **Missense** |
| 74 | 11286 | | | TGTCTGGTT | . | L105 | 22 | Deletion |
| 75 | 11288 | | | TCTGGTTTT | . | S106 | 14 | Deletion |
| 76 | 11332 | | | A | G | V120V | 4 | Synonymous |
| 77 | 11537 | | | **A** | **G** | **I189V** | 20 | **Missense** |
| 78 | 11653 | | | C | T | L227L | 2 | Synonymous |
| 79 | 11713 | | | T | C | S247S | 1 | Synonymous |
| 80 | 11941 | **NSP7** | 1 | C | T | V33V | 1 | Synonymous |
| 81 | 12406 | **NSP8** | 4 | C | T | N105N | 1 | Synonymous |
| 82 | 12452 | | | **C** | **T** | **P121S** | 2 | **Missense** |
| 83 | 12473 | | | C | T | K127K | 1 | Synonymous |
| 84 | 12557 | | | **A** | **C** | **I156L** | 2 | **Missense** |
| 85 | 12786 | **NSP9** | 2 | **C** | **T** | **T34I** | 1 | **Missense** |
| 86 | 12880 | | | C | T | I65I | 5 | Synonymous |
| 87 | 13195 | **NSP10** | 2 | T | C | V57V | 22 | Synonymous |
| 88 | 13339 | | | **T** | **A** | **N105K** | 2 | **Missense** |

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|---|---|---|---|---|---|---|---|---|
| 89 | 13592 | **NSP12b** | 11 | **C** | **T** | **T42I** | 2 | **Missense** |
| 90 | 13756 | | | **A** | **G** | **I97V** | 1 | **Missense** |
| 91 | 14408 | | | **C** | **T** | **P314L** | 40 | **Missense** |
| 92 | 14676 | | | C | T | P403P | 3 | Synonymous |
| 93 | 15240 | | | C | T | N591N | 22 | Synonymous |
| 94 | 15279 | | | C | T | H604H | 3 | Synonymous |
| 95 | 15451 | | | **G** | **A** | **G662S** | 4 | **Missense** |
| 96 | 15714 | | | C | T | L749L | 5 | Synonymous |
| 97 | 15939 | | | T | C | D824D | 1 | Synonymous |
| 98 | 15960 | | | C | T | A831A | 1 | Synonymous |
| 99 | 16176 | | | T | C | T903T | 3 | Synonymous |
| 100 | 16406 | **NSP13** | 6 | **T** | **G** | **V57G** | 2 | **Missense** |
| 101 | 16466 | | | **C** | **T** | **P77L** | 4 | **Missense** |
| 102 | 16636 | | | **G** | **T** | **A134S** | 1 | **Missense** |
| 103 | 17280 | | | G | T | V348V | 1 | Synonymous |
| 104 | 17410 | | | **C** | **T** | **R392C** | 5 | **Missense** |
| 105 | 17999 | | | **C** | **T** | **T588I** | 4 | **Missense** |
| 106 | 18163 | **NSP14** | 2 | **A** | **G** | **I42V** | 27 | **Missense** |
| 107 | 19220 | | | **C** | **T** | **A394V** | 4 | **Missense** |
| 108 | 19684 | **NSP15** | 8 | **G** | **T** | **V22L** | 1 | **Missense** |
| 109 | 19803 | | | C | T | R61R | 4 | Synonymous |
| 110 | 19884 | | | C | T | Y88Y | 1 | Synonymous |
| 111 | 19955 | | | **C** | **T** | **T112I** | 5 | **Missense** |
| 112 | 19999 | | | **G** | **T** | **V127F** | 1 | **Missense** |
| 113 | 20055 | | | A | G | E145E | 5 | Synonymous |
| 114 | 20094 | | | A | G | K158K | 2 | Synonymous |
| 115 | 20132 | | | **C** | **T** | **A171V** | 2 | **Missense** |

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|---|---|---|---|---|---|---|---|---|
| 116 | 20741 | **NSP16** | 23 | **A** | **G** | **Q28R** | 1 | **Missense** |
| 117 | 21082 | | | **GA** | **CT** | **E142L** | 2 | **Missense** |
| 118 | 21110 | | | **C** | **T** | **T151I** | 4 | **Missense** |
| 119 | 21512 | | | **A** | **C** | **N285T** | 4 | **Missense** |
| 120 | 21517 | | | **AG** | **CA** | **R287Q** | 4 | **Missense** |
| 121 | 21520 | | | **G** | **C** | **V288L** | 4 | **Missense** |
| 122 | 21523 | | | **GTT** | **TCG** | **V289S** | 4 | **Missense** |
| 123 | 21528 | | | T | C | I290I | 4 | Synonymous |
| 124 | 21532 | | | **A** | **T** | **S292C** | 4 | **Missense** |
| 125 | 21534 | | | . | A | S292 | 2 | Insertion |
| 126 | 21535 | | | G | . | S292 | 1 | Deletion |
| 127 | 21536 | | | . | TC | D293 | 1 | Insertion |
| 128 | 21537 | | | **T** | **A** | **D293E** | 1 | **Missense** |
| 129 | 21537 | | | T | . | D293 | 1 | Deletion |
| 130 | 21539 | | | **T** | **A** | **V294D** | 2 | **Missense** |
| 131 | 21542 | | | T | . | L295 | 1 | Deletion |
| 132 | 21543 | | | **TGTT** | **AACG** | **V296T** | 1 | **Missense** |
| 133 | 21543 | | | TGTTA | . | L295 | 1 | Deletion |
| 134 | 21547 | | | **AA** | **CT** | **N297L** | 1 | **Missense** |
| 135 | 21547 | | | AA | . | V296 | 1 | Deletion |
| 136 | 21549 | | | **C** | **G** | **N297K** | 1 | **Missense** |
| 137 | 21550 | | | A | . | N297 | 1 | Deletion |
| 138 | 21551 | | | **A** | **T** | **N298I** | 1 | **Missense** |
| 139 | 21614 | **Spike** | 82 | **C** | **T** | **L18F** | 1 | **Missense** |
| 140 | 21618 | | | **C** | **G** | **T19R** | 9 | **Missense** |
| 141 | 21618 | | | **C** | **T** | **T19I** | 5 | **Missense** |
| 142 | 21633 | | | TACCCCCTG | . | L24 | 5 | Deletion |

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|---|---|---|---|---|---|---|
| 143 | 21762 | C | . | A67 | 22 | Deletion |
| 144 | 21764 | A | . | A67 | 22 | Deletion |
| 145 | 21765 | TACATG | . | H69 | 3 | Deletion |
| 146 | 21767 | CATG | . | I68 | 22 | Deletion |
| 147 | 21801 | **A** | **C** | **D80A** | 6 | **Missense** |
| 148 | 21846 | **C** | **T** | **T95I** | 26 | **Missense** |
| 149 | 21987 | **G** | **A** | **G142D** | 9 | **Missense** |
| 150 | 21987 | GTGTTTATT | . | G142 | 22 | Deletion |
| 151 | 21993 | ATT | . | Y145 | 3 | Deletion |
| 152 | 22029 | AGTTCA | . | E156 | 4 | Deletion |
| 153 | 22139 | **G** | **C** | **V193L** | 1 | **Missense** |
| 154 | 22161 | **A** | **G** | **Y200C** | 1 | **Missense** |
| 155 | 22193 | . | T | I210 | 16 | Insertion |
| 156 | 22194 | ATT | . | N211 | 2 | Deletion |
| 157 | 22195 | **T** | **G** | **N211K** | 16 | **Missense** |
| 158 | 22197 | **TA** | **GC** | **L212C** | 16 | **Missense** |
| 159 | 22200 | **T** | **G** | **V213G** | 5 | **Missense** |
| 160 | 22201 | . | AGC | S214 | 16 | Insertion |
| 161 | 22202 | . | A | V213 | 1 | Insertion |
| 162 | 22203 | . | A | R214 | 1 | Insertion |
| 163 | 22204 | T | A | R214R | 16 | Synonymous |
| 164 | 22206 | **A** | **G** | **D215G** | 6 | **Missense** |
| 165 | 22286 | CTTGCTTTA | . | L242 | 4 | Deletion |
| 166 | 22289 | **G** | **T** | **A243S** | 1 | **Missense** |
| 167 | 22293 | **T** | **C** | **L244S** | 1 | **Missense** |
| 168 | 22481 | **A** | **C** | **T307P** | 1 | **Missense** |
| 169 | 22511 | **AA** | **TT** | **N317F** | 1 | **Missense** |

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|---|---|---|---|---|---|---|
| 170 | 22535 | **T** | **C** | **S325P** | 1 | **Missense** |
| 171 | 22539 | **TT** | **AA** | **I326K** | 1 | **Missense** |
| 172 | 22542 | **T** | **C** | **V327A** | 1 | **Missense** |
| 173 | 22578 | **G** | **A** | **G339D** | 27 | **Missense** |
| 174 | 22599 | **G** | **A** | **R346K** | 5 | **Missense** |
| 175 | 22669 | T | C | Y369Y | 1 | Synonymous |
| 176 | 22673 | **TC** | **CT** | **S371L** | 22 | **Missense** |
| 177 | 22674 | **C** | **T** | **S371F** | 5 | **Missense** |
| 178 | 22679 | **T** | **C** | **S373P** | 27 | **Missense** |
| 179 | 22686 | **C** | **T** | **S375F** | 27 | **Missense** |
| 180 | 22688 | **A** | **G** | **T376A** | 5 | **Missense** |
| 181 | 22775 | **G** | **A** | **D405N** | 5 | **Missense** |
| 182 | 22786 | **A** | **C** | **R408S** | 5 | **Missense** |
| 183 | 22813 | **G** | **T** | **K417N** | 31 | **Missense** |
| 184 | 22882 | **T** | **G** | **N440K** | 7 | **Missense** |
| 185 | 22898 | **G** | **A** | **G446S** | 16 | **Missense** |
| 186 | 22917 | **T** | **G** | **L452R** | 4 | **Missense** |
| 187 | 22969 | **AA** | **GG** | **T470A** | 1 | **Missense** |
| 188 | 22992 | **G** | **A** | **S477N** | 21 | **Missense** |
| 189 | 22995 | **C** | **A** | **T478K** | 24 | **Missense** |
| 190 | 23012 | **G** | **A** | **E484K** | 6 | **Missense** |
| 191 | 23013 | **A** | **C** | **E484A** | 20 | **Missense** |
| 192 | 23040 | **A** | **G** | **Q493R** | 20 | **Missense** |
| 193 | 23048 | **G** | **A** | **G496S** | 15 | **Missense** |
| 194 | 23055 | **A** | **G** | **Q498R** | 20 | **Missense** |
| 195 | 23063 | **A** | **T** | **N501Y** | 29 | **Missense** |
| 196 | 23075 | **T** | **C** | **Y505H** | 19 | **Missense** |

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|---|---|---|---|---|---|---|---|---|
| 197 | 23202 | | | **C** | **A** | **T547K** | 22 | **Missense** |
| 198 | 23271 | | | **C** | **A** | **A570D** | 3 | **Missense** |
| 199 | 23403 | | | **A** | **G** | **D614G** | 40 | **Missense** |
| 200 | 23525 | | | **C** | **T** | **H655Y** | 27 | **Missense** |
| 201 | 23599 | | | **T** | **G** | **N679K** | 27 | **Missense** |
| 202 | 23604 | | | **C** | **G** | **P681R** | 29 | **Missense** |
| 203 | 23604 | | | **C** | **A** | **P681H** | 5 | **Missense** |
| 204 | 23664 | | | **C** | **T** | **A701V** | 6 | **Missense** |
| 205 | 23709 | | | **C** | **T** | **T716I** | 3 | **Missense** |
| 206 | 23854 | | | **C** | **A** | **N764K** | 27 | **Missense** |
| 207 | 23948 | | | **G** | **T** | **D796Y** | 27 | **Missense** |
| 208 | 24130 | | | **C** | **A** | **N856K** | 22 | **Missense** |
| 209 | 24183 | | | **C** | **T** | **T874I** | 1 | **Missense** |
| 210 | 24410 | | | **G** | **A** | **D950N** | 4 | **Missense** |
| 211 | 24424 | | | **A** | **T** | **Q954H** | 27 | **Missense** |
| 212 | 24469 | | | **T** | **A** | **N969K** | 26 | **Missense** |
| 213 | 24503 | | | **C** | **T** | **L981F** | 21 | **Missense** |
| 214 | 24506 | | | **T** | **G** | **S982A** | 3 | **Missense** |
| 215 | 24821 | | | **G** | **T** | **A1087S** | 1 | **Missense** |
| 216 | 24872 | | | **G** | **T** | **V1104L** | 2 | **Missense** |
| 217 | 24914 | | | **G** | **C** | **D1118H** | 3 | **Missense** |
| 218 | 24942 | | | **A** | **G** | **D1127G** | 1 | **Missense** |
| 219 | 25000 | | | C | T | D1146D | 27 | Synonymous |
| 220 | 25352 | | | **G** | **T** | **V1264L** | 3 | **Missense** |
| 221 | 25469 | **ORF3a** | 9 | **C** | **T** | **S26L** | 4 | **Missense** |
| 222 | 25546 | | | **C** | **T** | **L52F** | 4 | **Missense** |
| 223 | 25563 | | | **G** | **T** | **Q57H** | 6 | **Missense** |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 224 | 25584 | | | C | T | T64T | 27 | Synonymous |
| 225 | 25587 | | | C | T | L65L | 5 | Synonymous |
| 226 | 25665 | | | C | T | Y91Y | 1 | Synonymous |
| 227 | 25904 | | | **C** | **T** | **S171L** | 6 | **Missense** |
| 228 | 26058 | | | C | T | D222D | 1 | Synonymous |
| 229 | 26060 | | | **C** | **T** | **T223I** | 5 | **Missense** |
| 230 | 26270 | **Envelope** | 5 | **C** | **T** | **T9I** | 27 | **Missense** |
| 231 | 26296 | | | **C** | **A** | **L18I** | 1 | **Missense** |
| 232 | 26392 | | | **A** | **T** | **S50C** | 1 | **Missense** |
| 233 | 26438 | | | **T** | **C** | **L65P** | 2 | **Missense** |
| 234 | 26456 | | | **C** | **T** | **P71L** | 6 | **Missense** |
| 235 | 26530 | **Membrane** | 7 | **A** | **G** | **D3G** | 19 | **Missense** |
| 236 | 26577 | | | **C** | **G** | **Q19E** | 25 | **Missense** |
| 237 | 26709 | | | **G** | **A** | **A63T** | 24 | **Missense** |
| 238 | 26767 | | | **T** | **C** | **I82T** | 4 | **Missense** |
| 239 | 26858 | | | C | T | F112F | 4 | Synonymous |
| 240 | 26997 | | | **T** | **G** | **C159G** | 1 | **Missense** |
| 241 | 27059 | | | C | T | Y179Y | 4 | Synonymous |
| 242 | 27193 | **3′UTR** | 1 | T | C | 27193 | 1 | extragenic |
| 243 | 27209 | **ORF6** | 4 | **A** | **G** | **H3R** | 1 | **Missense** |
| 244 | 27259 | | | A | C | M19M | 27 | Synonymous |
| 245 | 27382 | | | **GAT** | **CTC** | **D61L** | 5 | **Missense** |
| 246 | 27384 | | | T | C | D61D | 1 | Synonymous |
| 247 | 27638 | **ORF7a** | 2 | **T** | **C** | **V82A** | 4 | **Missense** |
| 248 | 27752 | | | **C** | **T** | **T120I** | 4 | **Missense** |
| 249 | 27807 | **ORF7b** | 9 | C | T | L17L | 20 | Synonymous |
| 250 | 27873 | | | . | G | E39 | 1 | Insertion |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 251 | 27874 | | | **C** | **A** | **T40N** | 1 | **Missense** |
| 252 | 27874 | | | **C** | **T** | **T40I** | 4 | **Missense** |
| 253 | 27875 | | | . | C | T40 | 1 | Insertion |
| 254 | 27879 | | | C | . | C41 | 1 | Deletion |
| 255 | 27880 | | | **A** | **T** | **H42L** | 1 | **Missense** |
| 256 | 27882 | | | G | . | H42 | 1 | Deletion |
| 257 | 27883 | | | **C** | **T** | **A43V** | 1 | **Missense** |
| 258 | 27921 | **ORF8** | 9 | **A** | **T** | **I10F** | 1 | **Missense** |
| 259 | 27972 | | | **C** | **T** | **Q27*** | 3 | SNP_stop |
| 260 | 27998 | | | C | T | D35D | 1 | Synonymous |
| 261 | 28048 | | | **G** | **T** | **R52I** | 3 | **Missense** |
| 262 | 28079 | | | G | C | V62V | 1 | Synonymous |
| 263 | 28111 | | | **A** | **G** | **Y73C** | 3 | **Missense** |
| 264 | 28248 | | | **GATTTC** | **.** | **D119** | 4 | Deletion |
| 265 | 28253 | | | C | T | F120F | 1 | Synonymous |
| 266 | 28253 | | | **CA** | **TC** | **I121L** | 5 | **Missense** |
| 267 | 28271 | **3′UTR** | 2 | A | T | 28271 | 27 | extragenic |
| 268 | 28273 | | | A | . | 28273 | 7 | extragenic |
| 269 | 28280 | **Nucleocapsid** | 20 | **GAT** | **CTA** | **D3L** | 3 | **Missense** |
| 270 | 28310 | | | **C** | **T** | **P13S** | 1 | **Missense** |
| 271 | 28311 | | | **C** | **T** | **P13L** | 26 | **Missense** |
| 272 | 28362 | | | **GAGAACGCA** | **.** | **E31** | 26 | Deletion |
| 273 | 28461 | | | **A** | **G** | **D63G** | 4 | **Missense** |
| 274 | 28881 | | | **G** | **T** | **R203M** | 2 | **Missense** |
| 275 | 28881 | | | **GG** | **TT** | **R203I** | 2 | **Missense** |
| 276 | 28881 | | | **GGG** | **AAC** | **RG203KR** | 30 | **Missense** |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 277 | 28887 | | | **C** | **T** | **T205I** | 6 | **Missense** |
| 278 | 28916 | | | **G** | **T** | **G215C** | 4 | **Missense** |
| 279 | 28977 | | | **C** | **T** | **S235F** | 3 | **Missense** |
| 280 | 28985 | | | **G** | **T** | **G238C** | 1 | **Missense** |
| 281 | 29014 | | | T | C | T247T | 1 | Synonymous |
| 282 | 29095 | | | C | T | F274F | 1 | Synonymous |
| 283 | 29144 | | | C | T | E290E | 1 | Synonymous |
| 284 | 29192 | | | **T** | **C** | **F307L** | 2 | **Missense** |
| 285 | 29304 | | | **C** | **T** | **P344L** | 5 | **Missense** |
| 286 | 29402 | | | **G** | **T** | **D377Y** | 4 | **Missense** |
| 287 | 29433 | | | **A** | **G** | **K387R** | 2 | **Missense** |
| 288 | 29510 | | | **A** | **C** | **S413R** | 5 | **Missense** |
| 289 | 29545 | **3′UTR** | 4 | C | T | 29545 | 2 | extragenic |
| 290 | 29734 | | | GAGGCCAC GCGGAGTAC GATCGAGTG | . | 29734 | 4 | extragenic |
| 291 | 29736 | | | G | T | 29736 | 1 | extragenic |
| 292 | 29742 | | | G | T | 29742 | 4 | extragenic |

**Appendix J: Reprint of Published Material**

**Kalonda, A**., Saasa, N., Kajihara, M., Nao, N., Moonga, L., Ndebe, J., Mori-Kajihara, A., Mukubesa, A. N., Samutela, M., Munjita, S., Sakoda, Y., Sawa, H., Takada, A. & Simulundu, E. 2023. Surveillance and Phylogenetic Characterisation of Avian Influenza Viruses Isolated from Wild Waterfowl in Zambia in 2015, 2020, and 2021. Transboundary and Emerging Diseases, 2023, 4606850, DOI: 10.1155/2023/4606850.

Katowa, B., **Kalonda, A**., Mubemba, B., Matoba, J., Shempela, D. M., Sikalima, J., Kabungo, B., Changula, K., Chitanga, S., Kasonde, M., Kapona, O., Kapata, N., Musonda, K., Monze, M., Tembo, J., Bates, M., Zumla, A., Sutcliffe, C. G., Kajihara, M., Yamagishi, J., Takada, A., Sawa, H., Chilengi, R., Mukonka, V., Muleya, W. & Simulundu, E. 2022. Genomic Surveillance of SARS-CoV-2 in the Southern Province of Zambia: Detection and Characterization of Alpha, Beta, Delta, and Omicron Variants of Concern. *Viruses,* 14, DOI: 10.3390/v14091865.

**Kalonda, A**., Phonera, M., Saasa, N., Kajihara, M., Sutcliffe, C. G., Sawa, H., Takada, A. & Simulundu, E. 2021. Influenza A and D Viruses in Non-Human Mammalian Hosts in Africa: A Systematic Review and Meta-Analysis. Viruses, 13, DOI: 10.3390/v13122411.

**Kalonda, A**., Saasa, N., Nkhoma, P., Kajihara, M., Sawa, H., Takada, A. & Simulundu, E. 2020. Avian Influenza Viruses Detected in Birds in Sub-Saharan Africa: A Systematic Review. *Viruses,* 12, DOI: 10.3390/v12090993.

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*Research Article*

# Surveillance and Phylogenetic Characterisation of Avian Influenza Viruses Isolated from Wild Waterfowl in Zambia in 2015, 2020, and 2021

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In recent years, the southern African region has experienced repeated incursions of highly pathogenic avian influenza viruses (HPAIVs), with wild migratory birds being implicated in the spread. To understand the profile of avian influenza viruses (AIVs) circulating in Zambia, we surveyed wild waterfowl for AIVs and phylogenetically characterised the isolates detected in 2015, 2020, and 2021. A total of 2,851 faecal samples of wild waterfowl were collected from Lochinvar National Park in the Southern Province of Zambia. During the study period, 85 (3.0%) low pathogenicity AIVs belonging to various subtypes were isolated, with H2N9, H8N4, and H10N8 being reported for the first time in avian species in Africa. The majority of the isolates were detected from glossy ibis (order *Pelecaniformes*) making it the first report of AIV from these birds in Zambia. Phylogenetic analysis of all eight gene segments of the 30 full genomes obtained in this study revealed that all the isolates belonged to the Eurasian lineage with their closest relatives being viruses isolated from wild and/or domestic birds in Bangladesh, Belgium, Egypt, Georgia, Mongolia, the Netherlands, and South Africa. Additionally, the Zambian viruses were

grouped into distinct clusters based on the year of isolation. While no notifiable AIVs of the H5 or H7 subtypes were detected in wild birds in Zambia, viral internal protein genes of some viruses were closely related to H7 low pathogenicity AIVs. This study shows that periodically, a considerable diversity of AIV subtypes are introduced into the Zambian ecosystem by wild migratory waterfowl. The findings highlight the importance of continuous surveillance and monitoring of AIVs in wild waterfowl, including birds traditionally not considered to be major AIV reservoirs, for a better understanding of the eco-epidemiology and evolutionary dynamics of AIVs in Africa.

## 1. Introduction

Avian influenza viruses (AIVs) are of global concern, with some subtypes and strains posing a constant threat to the poultry industry [1]. They are segmented, negative-sense RNA viruses belonging to the *Orthomyxoviridae* family, genus *Alpha influenza virus*. Wild waterfowl are the known natural reservoir of AIVs and play an important role in the evolution and spread of these viruses [2, 3]. Wild waterfowl of the orders *Anseriformes* and *Charadriiformes* are thought to be the most common reservoirs of diverse AIV subtypes, including 16 distinct haemagglutinin (HA) (H1–H16) and nine neuraminidase (NA) (N1–N9) [4]. Wild waterfowl can carry AIVs from one area to another and present a formidable risk to susceptible host species along their migratory flyway [5]. Therefore, wild waterfowl are of primary interest in AIV surveillance efforts, especially in places where birds of various geographical origins congregate at high densities, such as at stopover sites within migratory bottlenecks, creating so-called transmission "hotspots" [6].

AIVs exist in two forms: low and high pathogenicity viruses based on their pathotypes in chickens. Low pathogenicity AIVs (LPAIVs) circulating widely in waterfowl generally cause asymptomatic infections in these birds and are primarily shed in faeces [5]. In addition, LPAIVs are known to cause mild to no symptoms in chickens due to their restricted replication in the respiratory and/or intestinal tracts [7]. By contrast, high pathogenicity avian influenza viruses (HPAIVs) cause systemic infections and high mortality of up to 100% [8]. Particularly, the Gs/Gd-lineage H5N1 HPAIV that was first reported in Hong Kong in 1997 and H5Nx HPAIVs whose HA have the same origin as the H5N1 virus have caused a large number of outbreaks in poultry and wild birds across the globe [8, 9]. So far, HPAIVs have been restricted to subtypes H5 and H7, although not all viruses of these subtypes cause high pathogenicity avian influenza (HPAI) [4, 9, 10]. However, H5 and H7 LPAIVs may evolve spontaneously into HPAIVs, especially upon introduction into birds of the order *Galliformes* [5, 11–14].

In Africa, the first report of HPAI outbreak was recorded in 1961 during which approximately 1,300 common terns died in South Africa [15, 16]. Since then, no reports of HPAI outbreaks were recorded in Africa until 2004 when an outbreak caused by an H5N2 HPAIV was reported in South African ostriches [17]. In 2006, the Gs/Gd-lineage H5N1 HPAIV infection of commercial poultry was first confirmed on the continent on farms in Kaduna state in northern Nigeria [18, 19]. The virus spread rapidly to various African countries and resulted in losses of unprecedented proportions to the poultry industry, impacting national economies and international trade of live poultry and poultry products in the affected countries [20]. Moreover, the virus was thought to have been introduced through routes that coincided with the flight pathways of migratory birds [19], highlighting the importance of migratory birds in AIV dispersal including that of HPAIVs. Since the first outbreak of the Gs/Gd-lineage H5N1 HPAIV infection was reported in 2006 in Africa, two more waves of H5Nx HPAIVs have been confirmed including the H5N1 viruses from clade 2.3.2.1c isolated in 2015 and the 2016 clade 2.3.4.4b H5N8 viruses which emerged in western Africa and spread to southern, central, and eastern Africa [20, 21]. In southern Africa, clade 2.3.4.4b H5N8 viruses reached the Democratic Republic of the Congo (DR Congo), Zimbabwe, and South Africa in 2017 [22, 23]. In February 2019, new cases were reported in Namibia [24]. Furthermore, the HPAI outbreak caused by the H5N1 clade 2.3.4.4b virus was recently reported in Botswana [25]. Remarkably, despite several reports of HPAI outbreaks in neighbouring countries, there has been no report of HPAIVs in Zambia, possibly due to nondominance of the strain or due to a break in surveillance of AIVs in the country.

Apart from the havoc caused by HPAIVs, H9N2 LPAIV in poultry has gained attention because of the serious repercussions on animal health, public health, and the trade of live poultry or poultry products. Since their detection in China in 1992 [26], H9N2 LPAIVs have been extensively circulating in North African countries since the early 2000s [27, 28]. From 2017 to date, H9N2 viruses have been detected in several sub-Saharan African countries: Ghana, Burkina Faso, Uganda, Kenya, and Senegal, where a human case was recently reported [26, 29–31]. Surveillance studies have also been conducted in wild waterfowl in Africa which led to the detection of diverse subtypes of LPAIVs [32–35]. Our AIV surveillance in Zambia in 2006 and 2008–2009 resulted in the isolation of 13 viruses of distinct subtypes (H3N6, H3N8, H4N6, H6N2, H9N1, and H11N9) from wild waterfowl [36, 37]. However, compared to surveillance activities in other regions of the world, AIV surveillance in wild birds in Africa, especially southern Africa, is patchy and limited in geographical coverage [38]. Nevertheless, surveillance for AIVs in wild birds should not be underestimated as these viruses have contributed some gene segments to virus strains that have caused human influenza pandemics [39–41] and have the potential to cause future pandemics. Moreover, from Africa, only a few complete AIV sequences have been deposited in public databases suggesting a gap in the surveillance activities on the continent as a whole. Here, we phylogenetically characterised AIVs

obtained from wild waterfowl in Zambia to better understand the profile of circulating viruses in these hosts.

## 2. Materials and Methods

*2.1. Study Area and Sample Collection.* A total of 2,851 fresh faecal samples from various wild waterfowl were collected in 2015, 2020, and 2021 from Lochinvar National Park (LNP) in the Southern Province of Zambia. The LNP (15°51′S 27°13′E) is home to over 420 bird species and more than 30,000 endemic Kafue lechwe (*Kobus leche kafuensis*) (Kafue lechwe are a species of antelopes found in the Kafue flats in Zambia) [42]. The LNP is important for continued AIV surveillance as it receives migratory birds, and various AIV subtypes have been previously detected in faecal samples of birds found in this park [36, 37]. Sample collection was carried out once every month from January–February and May–December in 2015, September and October in 2020, and October–December in 2021. Approximately 200 samples were collected each month except for some months in the rainy season (March–April 2015 and January–April 2020–2021) when the wetland was inaccessible due to extreme flooding. Furthermore, sampling was not carried out in the other dry months of 2020 and 2021 due to public health restrictions on movement during the coronavirus disease 2019 (COVID-19) pandemic. Our sampling strategy was to collect samples from sites where waterfowl were physically seen congregating for easier morphological identification of the birds by the trained ornithologist from the Department of National Parks and Wildlife and collecting well-separated fresh faecal samples. A sample was collected from each fresh faecal material in the field. Samples were kept at 4°C and transported to the University of Zambia, School of Veterinary Medicine laboratories for further analysis within 24 hours of being collected.

*2.2. Virus Isolation and Subtyping.* Faecal samples were processed according to the previously described protocol [36, 43]. Briefly, faecal samples were suspended in phosphate-buffered saline (PBS) (pH 7.4) supplemented with antimicrobials to prepare a 20% homogenate. The antimicrobial supplements included penicillin G ($2 \times 10^6$ U/litre), streptomycin (200 mg/litre), gentamycin (250 mg/litre), and nystatin ($0.5 \times 10^6$ U/litre) (Meiji Seika Pharma Co., Ltd, Tokyo, Japan). The faecal homogenates were centrifuged at 2000 ×g for 10 min at 4°C. Then, 0.5 ml of the supernatant was inoculated into the allantoic cavity of 9 to 11-day-old embryonated chicken eggs (two eggs per homogenate sample). The eggs were incubated at 37°C for 48 hours and then chilled overnight at 4°C. The amino-allantoic fluids (AAFs) collected from the eggs were screened by a haemagglutination (HA) test with 0.5% chicken red blood cells. The AAF samples that did not show HA activity were passaged to a second egg inoculation followed by an HA test. Samples that were HA-negative on the second passage were considered negative. Haemagglutination-inhibition (HI) and neuraminidase-inhibition (NI) assays were used to determine the HA and NA subtypes by using a panel of hyperimmune antisera against H1 to H16 and N1 to N9 subtypes, respectively, according to the standard protocol [43].

*2.3. RNA Extraction and Whole Genome Sequencing.* RNA was extracted from the AAF using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The extracted RNA was subjected to next-generation sequencing (NGS) using the Illumina MiSeq System (Illumina, San Diego, CA, USA). Libraries were prepared using KAPA RNA Hyper Prep Kit (Illumina, Inc., San Diego, CA, USA) and KAPA Dual-Indexed Adapter Kit (Roche, Basel, Switzerland). Libraries were then purified with Agencourt®AMPure®XP beads (Beckman Coulter, Brea, CA, USA). The library quantity and quality were verified using Agilent High Sensitivity DNA Kit on an Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Santa Clara, CA, USA). For sequencing, the pooled libraries were diluted to a final concentration of 10 pM, followed by the addition of a 3% PhiX control library (Illumina, San Diego, CA, USA). The prepared libraries were sequenced on a MiSeq by using MiSeq Reagent kit v3 (600 cycles) (Illumina, San Diego, CA, USA) with 2 × 300 bp paired-end read length. Sequence reads were mapped to reference sequences of AIV gene segments, and the consensus sequences were rebuilt until all mismatches were solved using the CLC Genomic Workbench, version 22.0 (CLC bio, Aarhus, Denmark). The nucleotide sequences obtained in this study were submitted to GenBank under accession numbers OQ120633 to OQ120872.

*2.4. Phylogenetic and Molecular Analysis.* Nucleotide similarity searches were performed on the National Centre for Biotechnology Information (NCBI) website with the Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov/Blast.cgi). For phylogenetic analysis, reference sequences were obtained from the NCBI database and included viruses that showed high sequence identity to our isolates, representative viruses from Africa, and viruses previously isolated in Zambia. Multiple sequence alignment was performed using the Multiple Alignment with Fast Fourier Transformation (MAFFT) (https: https://mafft.cbrc.jp/alignment/software/) (Accessed on 1st December 2022) according to default parameters [44], and the aligned sequences were manually edited and trimmed using Geneious Prime® v2022.2.2. Maximum likelihood trees of all the eight full gene segments (PB2, PB1, PA, HA, NP, NA, M, and NS) of all the sequenced isolates with the exception of the HA gene for H2 isolates were generated using Molecular Evolutionary Genetics Analysis version X (MEGA X) [45, 46] applying the Tamura-Nei model [47] and 1,000 replicates of bootstrap. For the H2 isolates, the partial HA genes were used to allow for the inclusion of two sequences from Africa (Réunion Island). The software, GENETYX Version 12.0 (Genetyx Co., Tokyo, Japan), was used to assess the HA cleavage site of all the Zambian isolates.

## 3. Results

*3.1. Surveillance of AIVs in Wild Waterfowl.* In 2015, 2020, and 2021, a total of 2,851 faecal samples of wild waterfowl were collected from LNP in the Southern Province of Zambia. A total of 85 samples obtained from ducks, geese, and ibises were positive for AIV with a positivity rate of 3.0% by virus isolation as shown in Table 1. The positivity rate differed among bird species, seasons, and sampling years. Among the bird species, the positivity rate was highest in ibis birds (23.8%), followed by ducks (0.8%), and geese (0.2%). No virus was detected in great white pelicans and white egrets. High positivity rates were observed in the wet season (5.7%) and the 2015 (4.1%) sampling period (Table 1).

Based on the HI and NI assay results, 10 HA/NA subtype combinations were detected, namely, H2N9, H3N8, H4N6, H8N9, H10N7, H10N8, H10N9, H11N8, H11N9, and H13N6 and three HA subtypes, H1, H3, and H8 with NA subtypes not determined. Of the 85 positive samples, 50 representing all the subtypes determined by HI/NI assays in this study were subjected to NGS for subtype confirmation and genetic characterisation. We obtained good sequences for 30 samples by NGS. Through NGS, eight HA/NA subtypes (i.e., H1N8, H2N9, H8N4, H10N6, H10N8, H10N9, H11N6, and H11N9) were detected. All the H1N8, H2N9, H8N4, H10N6, H10N9, and H11N9 isolates were detected in 2015 with the majority being samples collected in November. All the two H8N4 and four H11N6 isolates were detected in samples collected in September 2020 and November 2021, respectively. The most prevalent subtypes in the present study were H10N8 (20%; 6/30), followed by H11N9 (16.7%; 5/30). The most prevalent HA subtype was H10 (40%; 12/30) which occurred in combination with three NA (N6, N8, and N9) subtypes.

*3.2. Nucleotide Sequence Analysis of the Isolates.* To further characterise the 30 isolates that were sequenced in this study, the nucleotide similarities of the full-length sequences of all eight segments of AIVs (PB2, PB1, PA, HA, NP, NA, M, and NS) were assessed using BLAST (Table 2). The HA genes of the H1N8, H10N8, H10N9, and H11N9 isolates showed the highest nucleotide sequence similarity (94.4–95.5%) to viruses isolated from ostriches, Pekin ducks, and red-billed teals from South Africa, while the HA genes of the H2N9 and H10N6 isolates shared 95.1% and 94.9% nucleotide sequence identity with A/tufted duck/Georgia/1/2012 (H2N3) and A/duck/Mongolia/371/2010 (H10N8), respectively. The HA genes of the H8N4 and H11N6 isolates showed 97.2–98.9% nucleotide sequence similarity to H8N4 and H11N3 viruses isolated from ducks in Bangladesh in 2019. Analysis of NA gene segments of all isolates indicated that they shared 96.1–98.8% nucleotide sequence identity with viruses isolated in wild waterfowl from Asia, Africa, and Europe (Table 2).

The nucleotide sequence analysis further revealed that the PB2 genes of the H1N8, H2N9, H10N6, H10N8, H10N9, and H11N9 isolates were highly similar (97.9–98.6%) to A/tufted duck/Georgia/1/2012 (H2N3), while those of the H8N4 and H11N6 isolates were similar (97.2–97.4%) to A/pintail/Egypt/MB-D-384C/2015 (H3N6). The PB1 genes of the H1N8 isolates showed high nucleotide similarity (98.2%) to A/shelduck/South Africa/DLH/2012 (H7N8). The PA genes of the HIN8, H2N9, H10N8, and H10N9 isolates showed 98.2–98.5% nucleotide similarity to an H7N7 virus, A/ostrich/South Africa/KRB/2013 (H7N7), while the NP genes of the H10N8, H10N9, and H11N9 isolates showed a high nucleotide identity of 98.5–98.7% with an H7N8 virus isolated in Egypt (Table 2).

*3.3. Phylogenetic Analysis of the Viral Surface Glycoprotein Genes.* Phylogenetic analysis of the HA and NA gene sequences revealed that the viruses isolated in the current study belonged to the Eurasian virus lineage (Figures 1–4). Analysis of the tree topology of the HA gene indicated that the H1N8, H2N9, H8N4, H10N6, H10N8, and H10N9 isolates grouped together and formed distinguishable clusters in their respective trees (Figures 1 and 2). The H11N6 viruses isolated in 2021 clustered distinctly from the H11N9 viruses isolated in 2015. A/ostrich/South Africa/AI2887/2011 (H1N2) formed a precursor-like relationship to the H1N8 isolates characterised in this study (Figure 1(a)). Interestingly, the HA genes of the H2N9 isolates formed a separate and distinct "African lineage-like" cluster and were not closely related to the H2Nx viruses detected in Réunion Island (Figure 1(b)). The analysis further indicated that the HA genes of the H8N4 isolates were closely related to the H8N4 viruses isolated in Bangladesh in 2019 (Figure 1(c)). Similar to the H1 gene tree, the virus A/pekin duck/South Africa/AI1642/2009 (H10N7) had a precursor-like relationship to the HA gene of all the H10 isolates characterised in this study (Figure 2(a)). The H11 HA phylogeny revealed that the H11N6 and H11N9 isolates formed distinct clusters and were distantly related to H11N9 viruses previously isolated in Zambia in 2009 (Figure 2(b)).

Phylogenetic analysis of the NA gene revealed that the H8N4 isolates of the current study formed a separate cluster and were closely related to viruses isolated from wild waterfowl in Asia (Figure 3(a)). The N6 NA genes of the H10N6 and H11N6 viruses isolated in 2015 and 2021 formed distinct clusters based on the year of isolation and were distantly related to the NA genes of the H3N6 and H4N6 viruses isolated in Zambia previously (Figure 3(b)). Analysis of the NA genes of the H1N8 and H10N8 isolates indicated that all the isolates grouped together and were closely related to viruses isolated from wild waterfowl in South Africa (A/cape shoveler/South Africa/STR0982/2013 (H4N8); A/yellow-billed duck/South Africa/STR0963/2013 (H4N8)) and Zambia (A/duck/Zambia/04/2008 (H3N8)) (Figure 4(a)). Except for A/duck/Zambia/514/2015 (H2N9) which clustered separately, the N9 NA gene sequences of the other N9 viruses isolated in the current study were closely related to each other and belonged to a clade that included viruses from Belgium, South Korea, and Egypt (Figure 4(b)).

*3.4. Phylogenetic Analysis of the Viral Internal Protein Genes.* In general, the tree topologies of all internal protein genes revealed that the viruses isolated in the present study clustered among the Eurasian virus lineage (Figures 5–7,

Table 1: Number of samples collected and positivity rates over the surveillance period 2015, 2020, and 2021.

| Variable | No. of samples collected | No. of AIV positive samples (%) | AIV subtypes detected |
|---|---|---|---|
| Sample Type | | | |
| Faecal | 2851 | 85 (3.0) | H1N8, H2N9, H8N4, H10N6, H10N8, H10N9, H11N6, and H11N9 |
| Bird species | | | |
| Ducks | 1214 | 10 (0.8) | H2N9, H8N4, H10N6, and H11N6 |
| Geese | 1227 | 3 (0.2) | H10N8 and H11N9 |
| Ibises | 302 | 72 (23.8) | H1N8, H2N9, H10N6, H10N8, and H11N9 |
| Pelicans | 98 | 0 (0) | — |
| White egrets | 10 | 0 (0) | — |
| Season | | | |
| Dry† | 1539 | 7 (0.5) | H2N9, H8N4, H10N6, and H11N9 |
| Wet ‡ | 1312 | 78 (5.9) | H1N8, H2N9, H10N6, H10N8, H10N9, H11N6, and H11N9 |
| Sampling year | | | |
| 2015 | 1921 | 79 (4.1) | H1N8, H2N9, H10N6, H10N8, H10N9, and H11N9 |
| 2020 | 242 | 2 (0.8) | H8N4 |
| 2021 | 688 | 4 (0.6) | H11N6 |

†Dry season: May–October; ‡wet season: November–April.

Supplemental Figures S1–S3). The analysis also revealed that the viruses isolated in 2015 formed separate clusters from viruses isolated in 2020 and 2021 and were distinct from those previously analysed in 2008–2009, except for the M and NS gene segments, whose sequences were clustered with some of the Zambian viral sequences characterised previously (Figures 5–7, Supplemental Figures S1–S3). Some internal protein genes characterised in this study were closely related to H7 LPAIVs.

The PB1 genes of the viruses isolated in 2015 were closely related to A/yellow-billed duck/South Africa/STR0963/2013 (H4N8) and A/cape shoveler/South Africa/STR0982/2013 (H4N8) except for PB1 genes of the H1N8 isolates which had a close relationship to an H7N8 LPAIV isolate, A/shelduck/South Africa/DLH/2012 (H7N8), as shown in Figure 5. Additionally, the PB1 genes of the H8N4 isolates were closely related to that of viruses isolated in Japan and Slovakia, while those of the H11N6 isolates were closely related to viruses isolated in Moscow (A/duck/Moscow/5712U/2019 (H11N6)) and Mongolia (A/duck/Mongolia/451/2018 (H4N1)) (Figure 5).

The NP gene phylogeny revealed that the viruses isolated in 2020–2021 formed a single cluster related to viruses isolated in Belgium, Netherlands, Georgia, Russia, and Egypt (Figure 6). In contrast, viruses isolated in 2015 formed two clusters, with the majority of the sequences belonging to a clade that included H7N3 viruses detected from wild birds in Egypt in 2016 (Figure 6).

Phylogenetic analysis of the NS gene demonstrated that the NS genes of the isolates characterised in the current study grouped into two separate alleles, alleles A and B, with the majority of the viruses being grouped into allele A (Figure 7). The H11N6 isolates in allele B formed a distinct cluster and clustered closely with viruses isolated in Zambia in 2006 and 2008 and those isolated in Europe and Asia. In addition, the Zambian allele B sequences clustered close to H7N1 South African viruses detected in ostriches in 2012 (Figure 7). Within allele A, all the NS gene sequences of the H1 and H10 isolates were closely related to A/duck/Bangladesh/8987/2010 (H10N9) (Figure 7). In contrast, the H8N4 isolates belonged to a cluster of isolates detected from wild birds mainly in the Netherlands and included H5N1, H5N6, and H7N5 LPAIVs, though none of these was closely related to our isolates (Figure 7).

*3.5. Genetic Analysis of the HA Cleavage Site.* Genetic analysis of the HA gene segment indicated that the isolates with the same HA subtypes shared similar motifs at the cleavage site of the HA protein as follows: H1N8 (PSIQSR/GLF), H2N9 (PQIESR/GLF), H8N4 (PSIEPK/GLF), H10N6/H10N8/H10N9 (PEVMQGR/GLF), and H11N6/H11N9 (PAIASR/GLF). The sequences for all the isolates were typical for LPAIVs as none of the viruses had multiple basic amino acids at the HA cleavage site.

## 4. Discussion

In this study, we found evidence of AIV circulation in the wild waterfowl found in LNP in Zambia with a positivity rate of 3.0% during the three years of sampling with the highest positivity rate being detected in 2015 (4.1%). The positivity rate of AIVs in wild waterfowl in this study was consistent with the finding of the previous review which found a prevalence of 3% in birds in Africa [48]. While bird species in the order *Anseriformes* and *Charadriiformes* are known to be the main natural reservoir of AIV, the highest positivity rate in the current study was detected in glossy ibis (*Plegadis falcinellus*), order *Pelecaniformes*. This is the first study to report AIVs in glossy ibis in Zambia. AIV has also been detected in African sacred ibis (*Threskiornis aethiopicus*) in South Africa [49], and the first AIV detected in Zambia was from a great white pelican [37] which is also from the order *Pelecaniformes*. These data suggest that birds of the order *Pelecaniformes* may be frequently infected with AIVs and may play an important role in the eco-epidemiology of these viruses. Moreover, experimental infection of adult ibis has revealed their susceptibility to and capability of shedding multiple AIV subtypes [50].

Table 2: Influenza viruses with the highest nucleotide sequence similarity to viruses in the current study.

| Representative virus subtype | Gene segment | Highest homology influenza A virus | GenBank accession # | % homology |
|---|---|---|---|---|
| H1N8 | PB2 | A/tufted duck/Georgia/1/2012 (H2N3) | MF147767.1 | 97.9 |
| | PB1 | A/shelduck/South Africa/DLH/2012 (H7N8) | KT777839.1 | 98.2 |
| | PA | A/ostrich/South Africa/KRB/2013 (H7N7) | KT777875.1 | 98.2 |
| | HA | A/ostrich/South Africa/AI2887/2011 (H1N2) | JX069105.1 | 95.5 |
| | NP | A/mallard duck/Netherlands/18/2012 (H4N2) | MF146131.1 | 97.8 |
| | NA | A/duck/Zambia/04/2008 (H3N8) | AB569497.1 | 96.5 |
| | M | A/Mallard/Netherlands/31/2014 (H4N3) | MK414709.1 | 98.3 |
| | NS | A/mallard/Netherlands/25/2013 (mixed) | MK192309.1 | 99.0 |
| H2N9 | PB2 | A/tufted duck/Georgia/1/2012 (H2N3) | MF147767.1 | 98.5 |
| | PB1 | A/yellow-billed duck/South Africa/STR0963/2013 (H4N8) | KT777923.1 | 97.7 |
| | PA | A/ostrich/South Africa/KRB/2013 (H7N7) | KT777875.1 | 98.5 |
| | HA | A/tufted duck/Georgia/1/2012 (H2N3) | MF146097.1 | 95.1 |
| | NP | A/mallard duck/Netherlands/15/2011 (H6N8) | KX979542.1 | 98.5 |
| | NA | A/duck/Bangladesh/8987/2010 (H10N9) | MH071484.1 | 96.7 |
| | M | A/*Anas platyrhynchos*/Belgium/108116/2019 (H5N6) | MT406810.1 | 98.2 |
| | NS | A/ostrich/South Africa/MKT/2012 (H7N1) | KT777895.1 | 99.4 |
| H8N4 | PB2 | A/pintail/Egypt/MB-D-384C/2015 (H3N6) | MN208007.1 | 97.4 |
| | PB1 | A/garganey/North Kazakhstan/45/2018 (H3N8) | MT126633.1 | 98.4 |
| | PA | A/duck/Mongolia/451/2018 (H4N1) | MW188636.1 | 97.7 |
| | HA | A/duck/Bangladesh/37509/2019 (H8N4) | MT090424.1 | 98.0 |
| | NP | A/duck/Moscow/4952/2013 (H5N3) | MN588198.1 | 97.6 |
| | NA | A/common teal/Shanghai/JDS120613/2018 (H10N4) | MN049535.1 | 96.2 |
| | M | A/duck/Mongolia/451/2018 (H4N1) | MW188640.1 | 98.9 |
| | NS | A/mallard duck/Netherlands/41/2015 (H5N1) | MF694125.1 | 99.1 |

Table 2: Continued.

| Representative virus subtype | Gene segment | Highest homology influenza A virus | GenBank accession # | % homology |
|---|---|---|---|---|
| H10N6 | PB2 | A/tufted duck/Georgia/1/2012 (H2N3) | MF147767.1 | 97.9 |
| | PB1 | A/yellow-billed duck/South Africa/STR0963/2013 (H4N8) | KT777923.1 | 97.7 |
| | PA | A/cape shoveler/South Africa/STR0982/2013 (H4N8) | KT777929.1 | 97.8 |
| | HA | A/Pekin duck/South Africa/A11642/2009 (H10N7) | GQ404728.2 | 95.0 |
| | NP | A/mallard duck/Netherlands/18/2012 (H4N2) | MF146131.1 | 97.8 |
| | NA | A/duck/Hokkaido/10/2015 (H3N6) | LC339733.1 | 97.0 |
| | M | A/Mallard/Netherlands/31/2014 (H4N3) | MK414709.1 | 98.3 |
| | NS | A/duck/Bangladesh/821/2009 (H10N7) | MH071464.1 | 99.0 |
| H10N8/H10N9 | PB2 | A/tufted duck/Georgia/1/2012 (H2N3) | MF147767.1 | 98.5 |
| | PB1 | A/yellow-billed duck/South Africa/STR0963/2013 (H4N8) | KT777923.1 | 97.3 |
| | PA | A/ostrich/South Africa/KRB/2013 (H7N7) | KT777875.1 | 98.3 |
| | HA | A/Pekin duck/South Africa/A11642/2009 (H10N7) | GQ404728.2 | 94.8 |
| | NP | A/teal/Egypt/MB-D-487OP/2016(H7N3) | MN208011.1 | 98.5 |
| | NA | A/cape shoveler/South Africa/STR0982/2013 (H4N8) | KT777932.1 | 96.1 |
| | M | A/teal/Egypt/MB-D-698OP/2016 (H7N3) | MN207981.1 | 99.5 |
| | NS | A/duck/Bangladesh/821/2009 (H10N7) | MH071464.1 | 98.8 |
| H11N6 | PB2 | A/pintail/Egypt/MB-D-384C/2015 (H3N6) | MN208007.1 | 97.2 |
| | PB1 | A/duck/Mongolia/451/2018 (H4N1) | MW188635.1 | 97.8 |
| | PA | A/duck/Mongolia/451/2018 (H4N1) | MW188636.1 | 97.5 |
| | HA | A/duck/Bangladesh/38285/2019 (H11N3) | MT090343.1 | 97.2 |
| | NP | A/mallard duck/Netherlands/35/2015 (H4N6) | MF694210.1 | 97.5 |
| | NA | A/domestic duck/Georgia/1/2016 (H4N6) | MF694247.1 | 97.4 |
| | M | A/duck/Mongolia/451/2018 (H4N1) | MW188640.1 | 98.9 |
| | NS | A/ostrich/South Africa/MKT/2012 (H7N1) | KT777895.1 | 97.9 |
| H11N9 | PB2 | A/tufted duck/Georgia/1/2012 (H2N3) | MF147767.1 | 98.3 |
| | PB1 | A/yellow-billed duck/South Africa/STR0963/2013 (H4N8) | KT777923.1 | 97.5 |
| | PA | A/cape shoveler/South Africa/STR0982/2013 (H4N8) | KT777929.1 | 97.8 |
| | HA | A/red-billed teal/South Africa/KZN002/2012 (H11N2) | KT777885.1 | 97.4 |
| | NP | A/teal/Egypt/MB-D-487OP/2016 (H7N3) | MN208011.1 | 98.7 |
| | NA | A/Anas platyrhynchos/Belgium/195_7/2018 (H11N9) | MT406955.1 | 98.6 |
| | M | A/*Anas platyrhynchos*/Belgium/10811_6/2019(H5N6) | MT406810.1 | 98.5 |
| | NS | A/mallard duck/Netherlands/43/2011 (H7N1) | KX979531.1 | 97.3 |

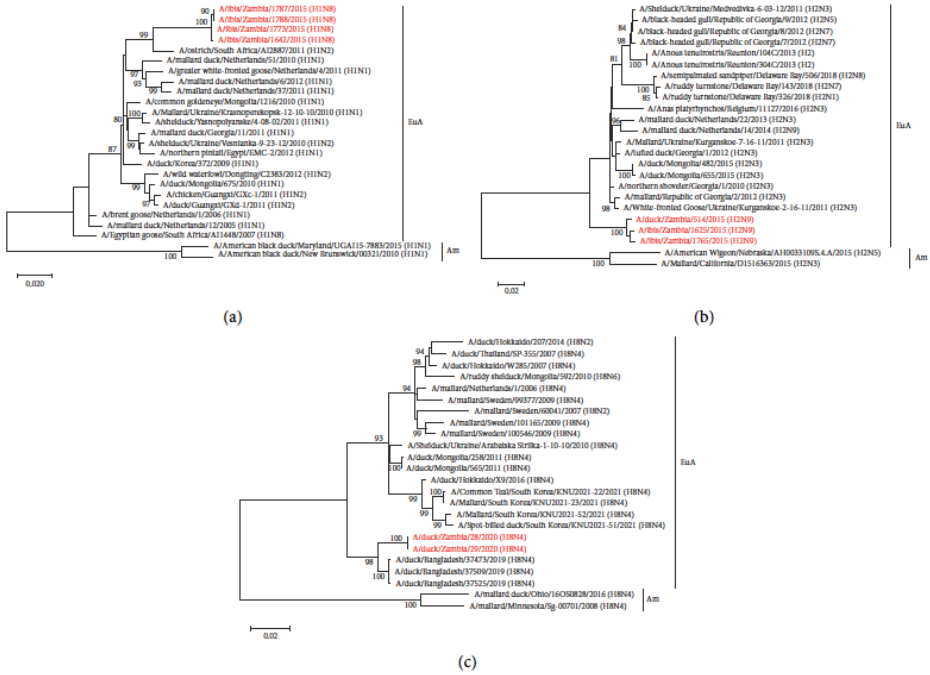


FIGURE 1: Phylogenetic analysis of the H1, H2, and H8 genes of AIVs: (a) phylogenetic tree of H1 genes based on 1702 nucleotides; (b) phylogenetic tree of H2 genes based on 1695 nucleotides; (c) phylogenetic tree of H1 genes based on 1712 nucleotides. The viruses isolated in this study are in red text. Numbers at branch nodes are bootstrap values ≥ 70%. Am–American lineage; EuA–Eurasian lineage. Bar, number of substitutions per site.

In this study, we found that the isolation rate of AIVs was higher in the wet season compared to the dry season. This could be attributed to the presence of migratory waterfowl which are known to be natural reservoirs of AIVs as most of the migratory birds begin to arrive in LNP from November to April, which coincides with the wet season in Zambia. Further, our findings corroborate the general understanding that the prevalence of AIVs tends to increase during the period when Eurasian migratory water birds overwinter in sub-Saharan Africa and decrease after they migrate back to Eurasia [51]. However, our findings do not agree with the previous review report that found a higher prevalence in the dry season in sub-Saharan Africa [48]. Higher prevalence in dry seasons could be attributed to limited water bodies that may allow increased interaction of waterfowl by congregating at particular sites, which provides opportunities for AIV transmission as well as detection during surveillance activities [48]. The difference in the findings could be because sampling was not carried out in some months of 2020–2021 due to restrictions of movements brought about by the COVID-19 pandemic. Despite a higher positivity rate of AIV in the wet season, the isolation of AIVs in the dry season in this study may denote that AIV transmission by wild birds may be possible at any time of the year.

In the current study, we detected eight LPAIV subtypes. Although there was a disparity in the HA/NA subtypes obtained using HI/NI assays and NGS, the detected subtypes included H1N8, H2N9, H8N4, H10N6, H10N8, H10N9, H11N6, and H11N9. The disparity could be due to possible cross-reactivity of some AIV subtypes [43, 52], a pitfall that was resolved by NGS.

Along with previous studies [36, 37], the total number of HA and NA subtypes that have been identified in Zambia are nine (H1–H4, H6, and H8–H11) and six NA (N1, N2, N4, N6, N8, and N9), respectively. These findings demonstrated a considerably high HA and NA diversity of AIVs circulating among wild waterfowl in Zambia. Previous studies conducted in Zambia have also reported the isolation of LPAIVs from ducks, geese, and pelicans [36, 37] within LNP, indicating the continuous circulation of these viruses among migratory and indigenous bird species in the park. The findings confirm the idea that wild waterfowl are important in the maintenance and introduction of a wide range of viruses into the Zambian environment. Similar studies in Africa have also reported LPAIVs in different avian species including wild and domestic birds [30, 32, 34, 53, 54]. However, to the best of our knowledge, no H2N9, H8N4, and H10N8 viruses have been reported in Africa, and we did not find any sequences of these subtypes on the

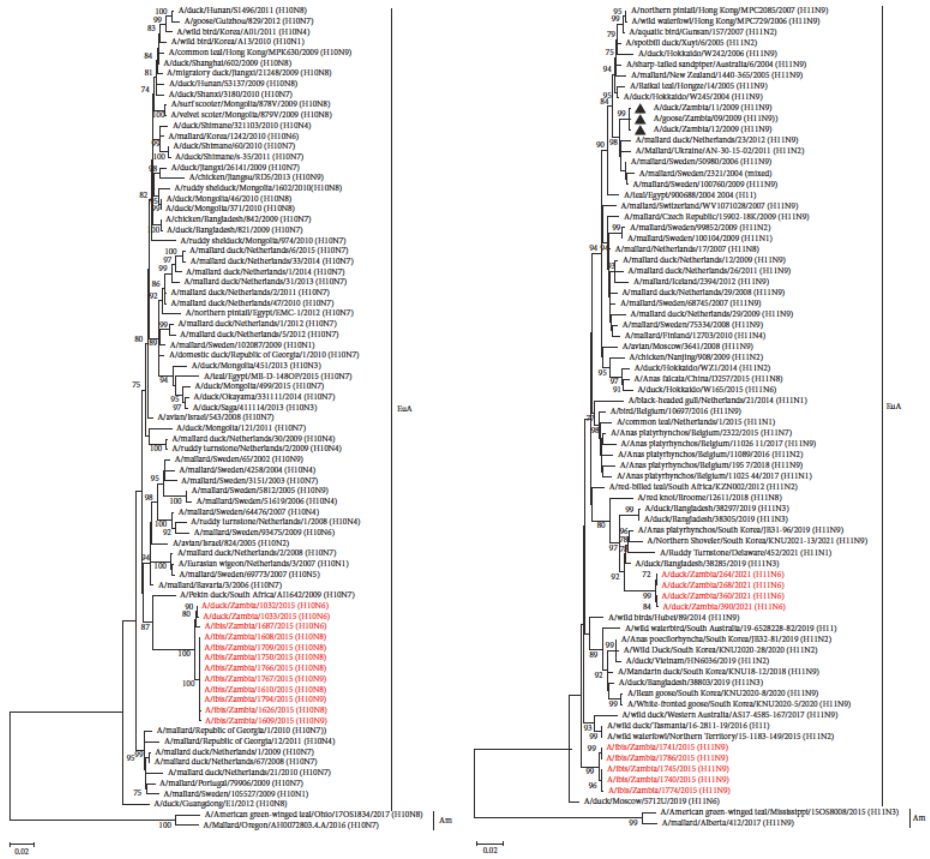


FIGURE 2: Phylogenetic analysis of the H10 and H11 HA genes of AIVs: (a) phylogenetic tree of H10 gene based on 1686 nucleotides; (b) phylogenetic tree of H11 gene based on 1707 nucleotides. The viruses isolated in this study are in red text, while those previously isolated in Zambia are shown with a black triangle. Numbers at branch nodes are bootstrap values ≥ 70%. Am–American lineage; EuA–Eurasian lineage. Bar, number of substitutions per site.

continent in GenBank or GISAID (i.e., the Global initiative on sharing all influenza data). This suggests that this is the first time that these HA/NA combinations are being reported in Zambia and Africa as a whole. For some subtypes that we are reporting now such as H10N6, H10N9, and H11N6, though they have been reported previously [32, 55, 56], sequence data are not available, and therefore, this study adds to the genetic resource of AIV detected in Africa. Hence, continuous surveillance and monitoring of wild waterfowl for AIVs should be supported to facilitate the creation of a library of isolates circulating in Africa that can be used for diagnosis and control strategies such as vaccine development in the event that any of these viruses causes an outbreak in poultry or other mammals including humans.

Phylogenetic analysis of all eight gene segments of AIVs revealed that the viruses isolated in the current study clustered with the viruses of the Eurasian lineage. However, it was noted that the HA genes of H2N9 viruses clustered separately from the major Eurasian clade, which may suggest possible independent evolution of these genes and raises the temptation to speculate on the possible existence of an African lineage of AIVs. The analysis further revealed that AIVs characterised in this study and those previously isolated in Zambia grouped into distinct clusters according to the period of isolation, signifying that these viruses were introduced in the Zambian environment independently at different times. Most of the genes were closely related to AIVs isolated from wild and domestic birds in Bangladesh, Belgium, Egypt, Georgia, Mongolia, the Netherlands, and South Africa. The close phylogenetic clustering of sequences analysed in this study with those of Eurasian isolates, along with the observation that most sequences characterised herein were distantly related to those previously isolated in

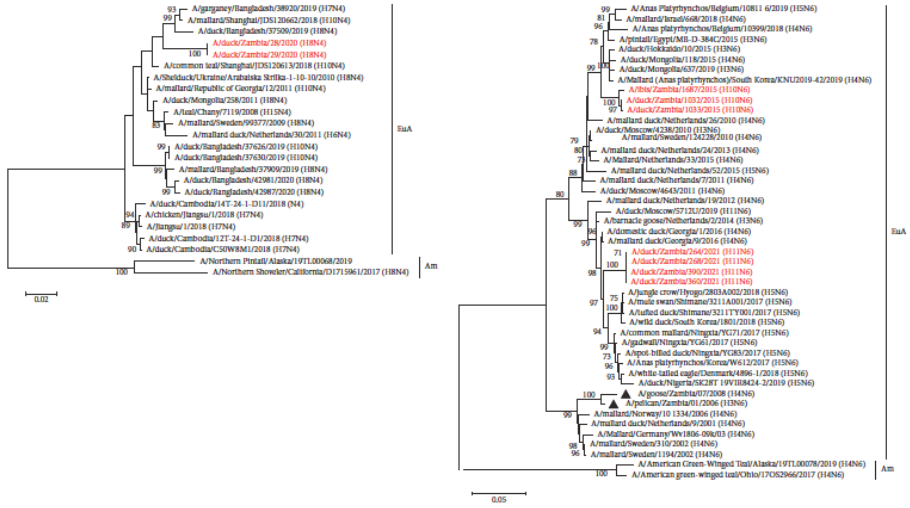


FIGURE 3: Phylogenetic analysis of the N4 and N6 NA genes: (a) phylogenetic tree of N4 based on 1405 nucleotides; (b) phylogenetic tree of N6 based on 1413 nucleotides. The viruses isolated in this study are in red text, while those previously isolated in Zambia are shown with a black triangle. Numbers at branch nodes are bootstrap values ≥ 70%. Am–American lineage; EuA–Eurasian lineage. Bar, number of substitutions per site.

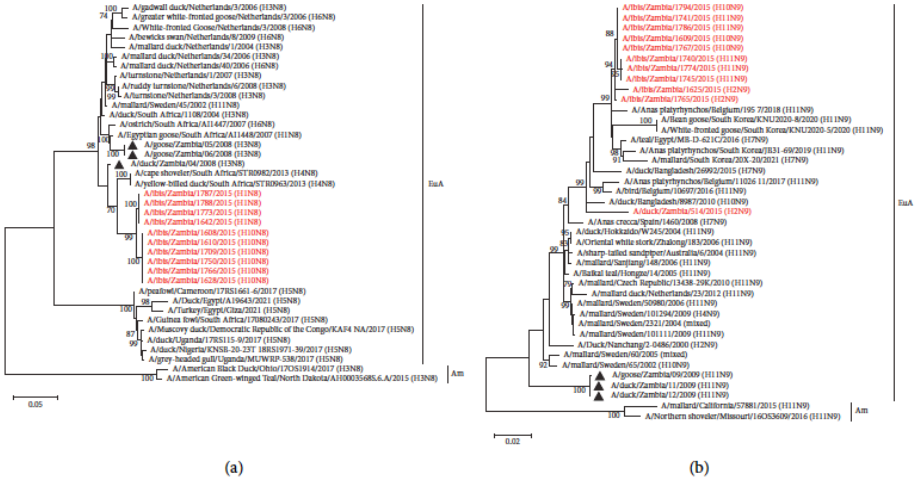


FIGURE 4: Phylogenetic analysis of the N8 and N9 NA genes. (a) Phylogenetic tree of N8 based on 1413 nucleotides; (b) phylogenetic tree of N9 based on 1413 nucleotides. The viruses isolated in this study are in red text, while those previously isolated in Zambia are shown with a black triangle. Numbers at branch nodes are bootstrap values ≥ 70%. Am–American lineage; EuA–Eurasian lineage. Bar, number of substitutions per site.

Zambia, may suggest that these viruses were introduced in the country by Palearctic migratory birds. Additionally, the viral internal protein genes of some viruses in the current study were closely related to notifiable H7 LPAIVs, indicating possible gene exchange with viruses with the potential to mutate into HPAIVs. Remarkably, only the PB2 and NS genes of the H8N4 and H11N6 viruses isolated in 2020 and 2021 were closely related to AIVs isolated in Africa. Therefore, this might indicate a gap in the surveillance of AIVs in Africa or that these viruses were


FIGURE 5: Phylogenetic analysis of the PB1 genes based on 2279 nucleotides. The viruses isolated in this study are in red text, while those previously isolated in Zambia are shown with a black triangle. Numbers at branch nodes are bootstrap values ≥ 70%. Am–American lineage; EuA–Eurasian lineage. Bar, number of substitutions per site.

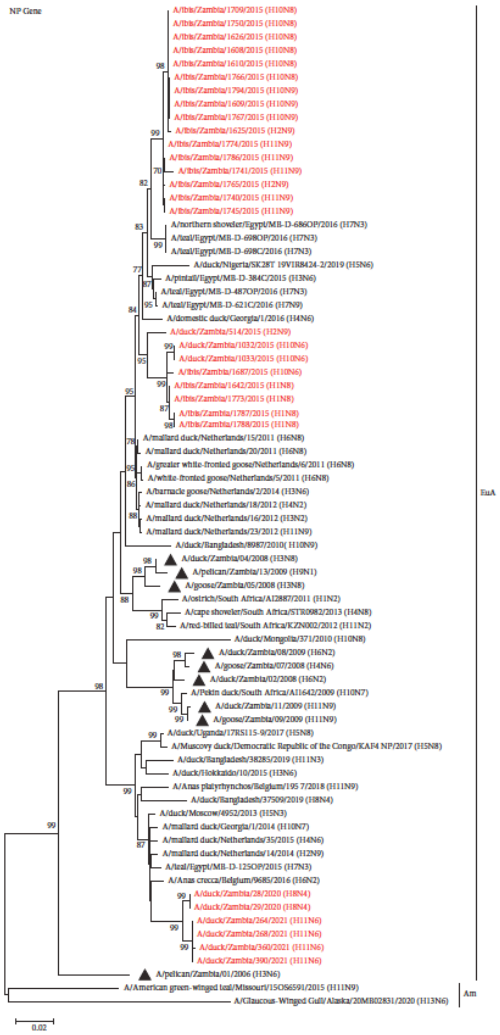


FIGURE 6: Phylogenetic analysis of the NP genes based on 1506 nucleotides. The viruses isolated in this study are in red text, while those previously isolated in Zambia are shown with a black triangle. Numbers at branch nodes are bootstrap values ≥ 70%. Am–American lineage; EuA–Eurasian lineage. Bar, number of substitutions per site.

recently introduced into the African ecosystem. Furthermore, our phylogenetic analysis showed that some of the AIV genes studied were closely related to those identified in poultry, confirming the understanding that the wild waterfowl population act as a source of AIV infection for domestic birds. The findings highlight the need for continuous surveillance of AIVs in both wild and domestic birds to monitor the introduction of viruses of veterinary and public health significance.

This study was not without limitations. We did not carry out sampling in all the months of the dry season in the 2020 and 2021 sampling periods due to the emergence of the COVID-19 pandemic and the associated public health measures that involved movement restrictions. In addition, no sampling was carried out during the 2016 to 2019 period which leaves a considerable gap in our understanding of AIV ecology and epidemiology in the country and region at large. Furthermore, we did not use

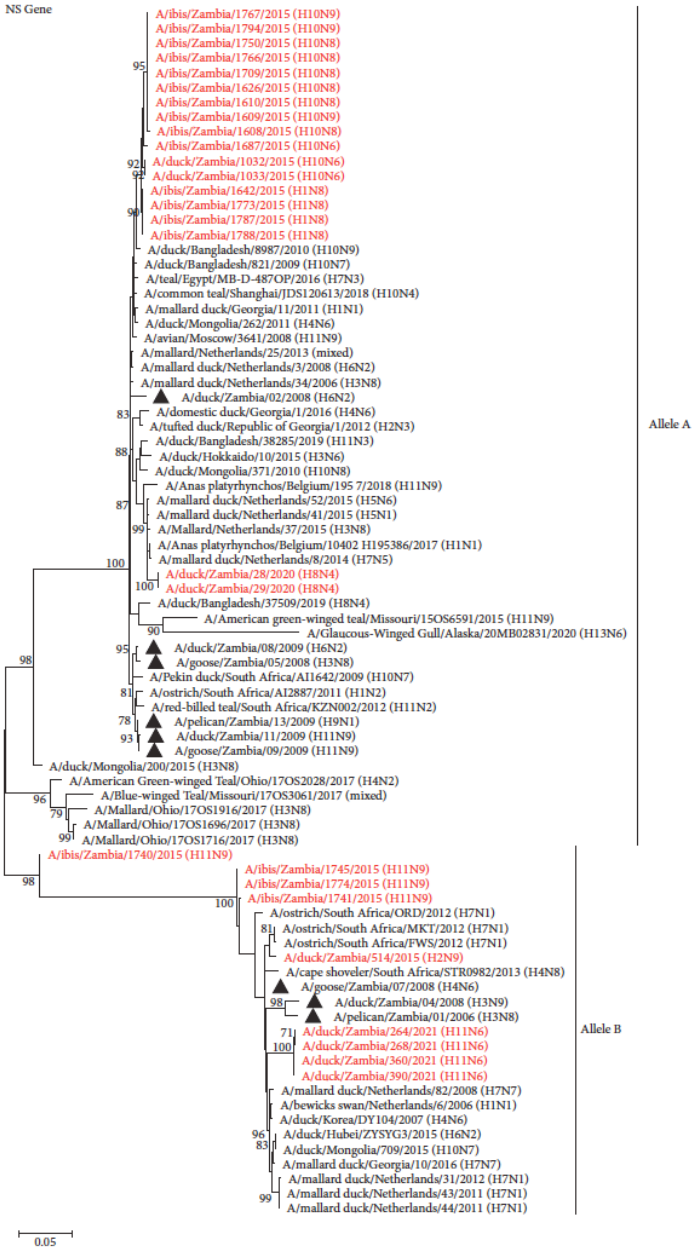


FIGURE 7: Phylogenetic analysis of the AIV NS genes based on 852 nucleotides. The viruses isolated in this study are in red text, while those previously isolated in Zambia are shown with a black triangle. Numbers at branch nodes are bootstrap values ≥ 70%. Bar, number of substitutions per site.

molecular tools in wild waterfowl species identification but identified them morphologically. The inclusion of an ornithologist from the Department of National Parks and Wildlife alleviated this shortcoming.

## 5. Conclusion

Our study revealed the active circulation of multiple LPAIV subtypes in wild waterfowl in Zambia and reports for the first time the isolation of AIVs in glossy ibis in Africa. Phylogenetic analysis revealed that the AIVs isolated in this study clustered with isolates of the Eurasian lineage. The viruses isolated in 2015 formed separate clusters from those isolated in 2020–2021 suggesting independent introductions of AIVs in wild waterfowl in Zambia. Further, some internal protein genes clustered with H7 LPAIVs isolated from South Africa and Egypt. This study emphasises the importance of continuous surveillance and monitoring of AIVs in wild waterfowl including birds traditionally not considered to be major reservoirs of AIVs in order to be better prepared to protect animal and public health from zoonotic influenza.

## Data Availability

All data generated in this study are included within the article along with their supplementary files.

## Ethical Approval

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to, and the appropriate ethical review committee approval has been received. Permission to conduct the study in the Lochinvar National Park was obtained from the Ministry of Tourism and Arts in Zambia, and all protocols for the study were approved by the University of Zambia Biomedical Research Ethics Committee (Reference Number: 616–2019).

## Conflicts of Interest

The authors declare that they have no conflicts of interest.

## Authors' Contributions

Conceptualisation was performed by Annie Kalonda and Edgar Simulundu; methodology was prepared by Annie Kalonda, Ngonda Saasa, Masahiro Kajihara, Naganori Nao, Ladislav Moonga, Joseph Ndebe, Akina Mori-Kajihara, Andrew Nalishuwa Mukubesa, Yoshihiro Sakoda, and Edgar Simulundu; data analysis was carried out by Annie Kalonda, Mulemba Samutela, Samuel Munjita, and Edgar Simulundu; investigation was carried out by Annie Kalonda, Masahiro Kajihara, Nao Naganori, Ladslav Moonga, Joseph Ndebe, Akina Mori-Kajihara, Andrew N. Mukubesa, and Edgar Simulundu; resources were collected by Ayato Takada, Hirofumi Sawa, Yoshihiro Sakoda, Ngonda Saasa, and Edgar Simulundu; Annie Kalonda and Edgar Simulundu wrote the original draft; all the authors reviewed and edited the draft; supervision was performed by Ngonda Saasa, Ayato Takada, and Edgar Simulundu; project administration was performed by Ayato Takada, Masahiro Kajihara, and Edgar Simulundu; funding acquisition was carried out by Ayato Takada, Hirofumi Sawa, and Edgar Simulundu. All the authors have read and agreed to the published version of the manuscript.

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## Supplementary Materials

Figure S1. Phylogenetic analysis of the AIV PB2 genes based on 2280 nucleotides. The viruses isolated in this study are in red text, while those previously isolated in Zambia are shown with a black triangle. Numbers at branch nodes are bootstrap values ≥ 70%. Am–American lineage; EuA–Eurasian lineage. Bar, number of substitutions per site. Figure S2. Phylogenetic analysis of the AIV PA genes based on 2118 nucleotides. The viruses isolated in this study are in red text, while those previously isolated in Zambia are shown with a black triangle. Numbers at branch nodes are bootstrap values ≥ 70%. Am–American lineage; EuA–Eurasian lineage. Bar, number of substitutions per site. Figure S3. Phylogenetic analysis of the AIV M genes based on 989 nucleotides. The viruses isolated in this study are in red text, while those previously isolated in Zambia are shown with a black triangle. Numbers at branch nodes are bootstrap values ≥ 70%. Am–American lineage; EuA–Eurasian lineage. Bar, number of substitutions per site. (*Supplementary Materials*)

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# Genomic Surveillance of SARS-CoV-2 in the Southern Province of Zambia: Detection and Characterization of Alpha, Beta, Delta, and Omicron Variants of Concern

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**Abstract:** Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) variants of concern (VOCs) have significantly impacted the global epidemiology of the pandemic. From December 2020 to April 2022, we conducted genomic surveillance of SARS-CoV-2 in the Southern Province of Zambia, a region that shares international borders with Botswana, Namibia, and Zimbabwe and is a major tourist destination. Genetic analysis of 40 SARS-CoV-2 whole genomes revealed the circulation of Alpha (B.1.1.7), Beta (B.1.351), Delta (AY.116), and multiple Omicron subvariants with the BA.1 subvariant being predominant. Whereas Beta, Delta, and Omicron variants were associated with the second, third, and fourth pandemic waves, respectively, the Alpha variant was not associated with any wave in the country. Phylogenetic analysis showed evidence of local transmission and possible multiple introductions of SARS-CoV-2 VOCs in Zambia from different European and African countries. Across the 40 genomes analysed, a total of 292 mutations were observed, including 182 missense mutations, 66 synonymous mutations, 23 deletions, 9 insertions, 1 stop codon, and 11 mutations in the non-coding region. This study stresses the need for the continued monitoring of SARS-CoV-2 circulation in Zambia, particularly in strategically positioned regions such as the Southern Province which could be at increased risk of introduction of novel VOCs.

**Keywords:** SARS-CoV-2; COVID-19; variants of concern; spike mutations; whole-genome sequencing; Zambia

## 1. Introduction

As of 6 July 2022, the ongoing coronavirus disease 2019 (COVID-19) pandemic has caused over 548,990,094 confirmed cases including 6,341,637 deaths [1]. In Africa, despite having a total population of about 1.3 billion, the official reports show a low burden of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infections when compared with other continents. The total number of confirmed cases and fatalities reported in Africa were 9,138,803 and 173,674, respectively, representing a global burden of 1.7% [1]. However, post-mortem and serological studies in some African countries suggest that the true burden of SARS-CoV-2 infections and deaths may be higher than what is officially reported [2–5]. Further, a recent systematic review by the World Health Organisation (WHO) on the seroprevalence of SARS-CoV-2 in Africa revealed that over two-thirds of the African population had been infected by SARS-CoV-2 [6]. The analysis further revealed that the true number of SARS-CoV-2 infections on the African continent was 97 times higher than the reported confirmed cases and the sharp rise in incidence was attributed to the introduction of the highly transmissible Alpha and Delta variants [6,7].

The first COVID-19 case in Africa was reported in Egypt on 14 February 2020 [8,9] followed by Algeria, with its first case being reported on 25 February 2020 [10] and Nigeria on 27 February 2020 [11]. Most African countries including Cameroon, Morocco, Senegal, South Africa, Togo, and Tunisia reported their first cases by mid-March 2020 [8,12] and most of the index cases were imported cases from Europe which by then had become the epicentre of the pandemic [8,12]. Within three months of Africa's COVID-19 index case, 54 of 55 African Union (AU) Member States (except Western Sahara) had reported over 100,000 cases which included imported and community transmissions [8]. The early phase of the pandemic in Africa was characterized by the predominance of lineage B.1 which was introduced multiple times in African countries [13]. However, due to a ban on international air travel in most African countries and the world at large in March/April 2020, the number of SARS-CoV-2 importations into Africa decreased and the pandemic entered a phase that was characterized by sustained low levels of within-country spread and occasional international viral dissemination between neighbouring countries, presumably via road and rail links between these countries [13].

As the pandemic progressed, several SARS-CoV-2 variants carrying mutations with concerning phenotypic implications on current pandemic management strategies emerged [14]. Of particular significance to the ongoing pandemic are SARS-CoV-2 variants designated

variants of concern (VOCs). Several VOCs have been described including Alpha (B.1.1.7), Beta (B.1.351), Gamma (P.1), Delta (B.1.617.2), and Omicron (B.1.1.529). VOCs are associated with enhanced transmissibility or virulence, reduction in neutralization by antibodies obtained through natural infection or vaccination, the ability to evade detection, or a decrease in therapeutic or vaccination effectiveness [15,16]. Further, all the five reported VOCs have mutations in the receptor-binding domain (RBD) and the N-terminal domain (NTD), of which N501Y mutation located on the RBD is common to all variants except the Delta variant [16]. The N501Y mutation results in increased binding affinity of the spike (S) protein to angiotensin-converting enzyme (ACE) 2 receptors thereby enhancing the viral attachment and its subsequent entry into the host cells [17,18]. Other genomic changes have been reported, including the extensive deletion in the open reading frame (ORF) 7a, ORF8 [19–21], and a deletion in the nsp2 genes [22], but these deletions have been associated with mild to moderate clinical symptoms compared to the infection caused by the wildtype SARS-CoV-2 [21,23].

To date, four VOCs, namely Alpha, Beta, Delta, and Omicron have been detected on the African continent. The first VOC, designated Alpha (B.1.1.7), was detected in September 2020 in the United Kingdom (UK) and was introduced into Africa between November 2020 and February 2021 with evidence of local transmission in Nigeria and Ghana [13]. This variant is characterised by nine mutations in the S protein, increased transmissibility, and increased risk of hospitalisation [24,25]. The second VOC was the Beta (B.1.351 lineage) variant which was first detected in South Africa in October 2020 and became the most common variant in many African countries [26]. This VOC is characterized by mutations in the S protein, including in the RBD—K417N, E484K, and N501Y [14,26]. In addition, the Beta variant is known to cause severe disease in young and healthy individuals [26]. Whereas the Beta variant was associated with the second wave of SARS-CoV-2 in Africa, the Alpha variant did not predominate in many African countries possibly due to a lack of selective advantage over the other VOCs [27]. These variants were replaced by the highly transmissible Delta (B.1.617.2 lineage) variant which was initially detected in India in December 2020 and spread worldwide among vaccinated as well as unvaccinated individuals [28]. This variant seeded the third wave of the pandemic in 2021 and was introduced in Africa in June 2021. The Omicron variant, characterised by several mutations in the S protein, including a set of mutations previously observed in other VOCs and novel mutations, was first reported in South Africa on 24 November 2021 and became the dominant driver of the fourth global wave of SARS-CoV-2 [29].

In Zambia, the first known COVID-19 cases were reported on 18 March 2020 from travellers returning from Europe [30]. Within days, the government implemented restrictions on international travel, school closures, halting of non-essential business, and confinement of people to their homes. Despite these measures, the virus spread to all parts of the country with over 300,000 cases and over 4000 deaths as of 6 July 2022 [31]. The course of the pandemic in Zambia can be divided into four major waves: the first wave occurred from July to September 2020 and was mainly driven by B.1.1 and its sub-lineages; the second wave occurred from December 2020 to April 2021 and was dominated by the Beta variant, while the Delta variant dominated the third wave from May to September 2021 [32,33]. The Omicron variant has dominated the fourth pandemic wave in Zambia, with cases peaking in early January 2022 and then rapidly decreasing to low levels. In the Southern Province, which shares international borders with Botswana, Namibia, and Zimbabwe and is a major tourist destination, SARS-CoV-2 was first detected in May 2020 [31]. As the course of the pandemic continues to evolve, it remains crucial to monitor and understand the virus evolution and outbreak dynamics, particularly in strategically positioned regions such as the Southern Province which is a trade entry point of Zambia for all imports and exports from Southern Africa. However, there is limited data regarding the molecular epidemiology of SARS-CoV-2 in Zambia, with only two genomic studies reporting the detection of SARS-CoV-2 belonging to lineage B.1.1. [34] and the B.1.351 variant [35]. Moreover, to our best knowledge, no reports have described the genetic characteristics of SARS-CoV-2 VOCs

circulating in the Southern Province. Therefore, this study used whole-genome sequencing (WGS) and phylogenetic analyses to describe the genetic characteristics of SARS-CoV-2 in the Southern Province of Zambia.

## 2. Materials and Methods

### *2.1. Study Site and Sample Collection*

The study samples were collected between December 2020 and April 2022 from eight districts in the Southern Province (Figure 1). Sample collection was conducted through the Zambia National Public Health Institute under the coordination of the Zambia Genomic Sequencing Consortium. The samples were collected through routine surveillance (i.e., point of entry screening and routine screening for influenza-like illnesses) and targeted surveillance of cluster outbreaks. A total of 198 samples were collected from different parts of the Southern Province and were brought to Macha Research Trust (MRT) for WGS. WGS was conducted in collaboration with the Churches Health Association of Zambia (CHAZ) with 161 samples collected between December 2020 and November 2021 being transported to MRT, while 37 samples collected from December 2021 to April 2022 were transported to CHAZ Complex laboratory for sequencing. Upon receipt, all samples were retested to determine the cycle threshold (Ct) value of each sample. Samples that had a Ct value of $\leq$30 and were submitted with the relevant metadata were included to undergo WGS. Samples that did not meet the inclusion criteria and those that could not be amplified or had poor genomic coverage were excluded from further analysis.

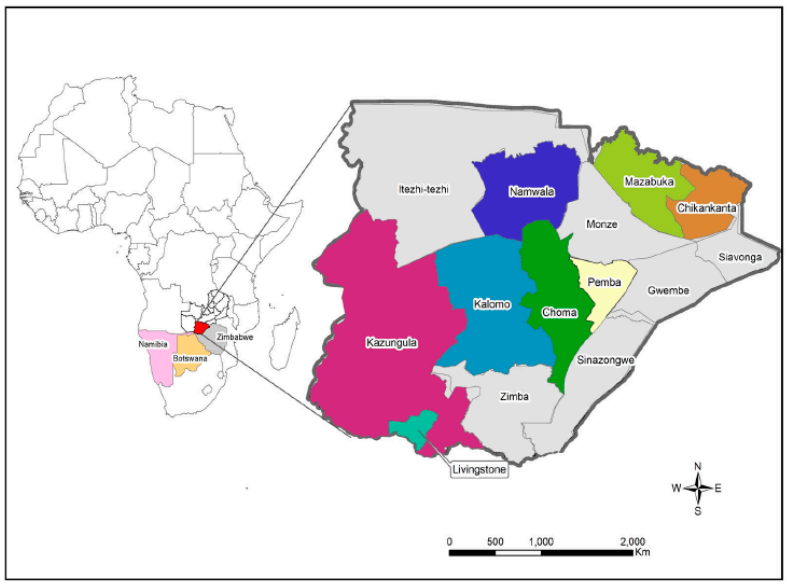


**Figure 1.** Map showing the location of study sites in Southern Province. The locator map depicts Zambia with neighbouring countries that share the border with Southern Province. The insert map shows the Southern Province of Zambia with the study sites namely Chikankata, Choma, Kalomo, Kazungula, Livingstone, Mazabuka, Namwala and Pemba districts. The maps were generated using Quantum Geographic Information System (QGIS) version 3.10 (http://www.qgis.org (accessed on 8 August 2022).

### *2.2. RNA Extraction and Virus Genome Amplification*

Viral RNA was extracted from nasopharyngeal swabs using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) or the MagMax kit (Thermo Fisher Scientific, Waltham,

MA, USA) on an automated KingFisher Flex platform (Thermo Fisher Scientific, USA) according to manufacturer specifications and protocols. Amplification of the SARS-CoV-2 genome in preparation for WGS was conducted using the Centre for Disease Control and Prevention (CDC) SARS-CoV-2 qRT-PCR assay [36].

### *2.3. Next-Generation Sequencing*

Whole-genome sequencing was performed using the Oxford Nanopore technologies and Illumina NextSeq platforms. For Oxford Nanopore, a cDNA synthesis reaction was performed on 36 samples (based on cycle threshold values < 30) using SuperScript IV Reverse Transcriptase kit (Invitrogen, Waltham, MA, USA), following the manufacturer's instructions. Library preparation was conducted using the ARTIC protocol version 3 [37,38]. Whole-genome sequencing was conducted using custom-designed primers (Tables S3 and S4) [34]. The PCR products were cleaned using AMPure XP beads (Beckman Coulter, Brea, CA, USA) and DNA quantification was conducted using a Qubit fluorometer (Thermo Fisher Scientific). End-repair on the amplified samples was conducted using NEBNext Ultra II End Repair Module (New England BioLabs, Ipswich, MA, USA). Native barcode expansion kits 1–12 and 13–24 was used in combination with Ligation Sequencing Kit (SQK-LSK109) (Oxford Nanopore Technologies). Subsequently, genomic sequencing was conducted using the MinION 1MkB (Oxford Nanopore Technologies, Oxford, UK). The RAMPART (v1.0.6) software package was used to monitor sequencing performance in real-time, with runs proceeding until a minimum of approximately 200-fold coverage was achieved across all amplicons. At this point, the run was terminated and the resulting reads were basecalled using Guppy (4.0.14). Consensus sequence generation was conducted using the ARTIC bioinformatics pipeline (https://artic.network/ncov-2019/ncov2019-bioinformatics-sop.html (accessed on 7 October 2021).

For Illumina NextSeq, V.3 primers pools designed by ARTIC Network were used (https://github.com/joshquick/artic-ncov2019/blob/master/primer_schemes/nCoV-2019/V3/nCoV-2019.tsv (accessed on 7 October 2021)). Sequencing libraries for 37 samples were prepared using the Illumina COVIDSeq kit on the automated Hamilton robotic instrument, ABI 7500 fast, and the Quant Studio thermo-cyclers. After successful library clean-up and pooling, pooled samples were quantified and normalized using a Qubit dsDNA HS Assay kit by diluting from a starting concentration of 4 nM to a final loading concentration of 1 nM. Thereafter, 25 µL was loaded on the Illumina NextSeq 2000 instrument through a cartridge loaded with a flow cell for SARS-CoV-2 genomic sequencing. A customized version of the DRAGEN DNA pipeline was used to perform Kmer-based detection of SARS-CoV-2. The Nextseq 2000 then aligned the reads to a reference genome, calls variants, and generates consensus genome sequences. The NextSeq 2000 optionally performs lineage/clade analysis using Pangolin and NextClade.

### *2.4. Genome Annotation and Phylogenetic Analysis*

Whole-genome sequences were annotated using the reference genome of hCoV-19/Wuhan/Hu-1/2019 | EPI_ISL_402125 [33]. A dataset of 180 whole genomes was created, which included 40 generated from this study and 140 retrieved from the GISAID database. Audacity *Instant* was used to retrieve SARS-CoV-2 whole-genome sequences from GISAID that were most similar to the sequences generated in this study. We also included reference sequences of VOCs detected in Southern Africa and other parts of the world, targeting those isolated within the same period and belonging to the same lineage as those characterized in this study. Reference sequences with stretches of more than 10% 'NNNN' were excluded from the analysis. Multiple sequence alignment of the sequences was performed using the FFT-NS-2 algorithm available in the multiple sequence alignment programme (MAFFT), but otherwise using default settings (https://mafft.cbrc.jp/alignment/server/index.html (accessed on 9 August 2022) [39]. The alignment was inspected in Geneious Prime v2022.0.1 (https://www.geneious.com (accessed on 9 August 2022) and gaps were trimmed. Follow-

ing alignment, a maximum likelihood (ML) phylogenetic tree was constructed using the PhyML Online server (www.atgc-montpellier.fr/phyml/ (accessed on 9 August 2022) [40] using the smart model selection (SMS) [41] and the Bayesian Information Criterion. Branch support was estimated through the SH-like approximate likelihood ratio test (SH-aLRT). The ML tree was then rooted using TempEst v1.5.3 [42], which estimated the best-fitting root of this phylogeny using the heuristic residual mean squared function, aimed at minimizing the variance of root-to-tip distances. The resultant ML tree file was edited using Interactive Tree of Life (iTOL) v5, an online tool for phylogenetic tree display and annotation [43].

PANGO lineage identification was performed using Pangolin v3.1.16 (https://pangolin.cog-uk.io/ (accessed on 8 August 2022)). Identification of single nucleotide polymorphisms (SNPs) was performed using the coronapp web application http://giorgilab.unibo.it/coronannotator/ (accessed on 8 August 2022). SNPs were identified based on the number of high confidence base calls (consensus sequence variations of the assembly) that do not agree with the reference bases for the genome position of interest. These variations were then exported to a vcf file and visualized in Microsoft Excel. The GISAID accession IDs of the genomes generated in this study can be found in Table S1.

## 3. Results

### *3.1. Characteristics of Patients with COVID-19 from the Southern Province of Zambia*

A total of 198 samples were received for WGS from districts in the Southern Province, 74 were negative for SARS-CoV-2, 51 had Ct values > 30, and 33 had a low genome coverage. Only 40 samples were successfully sequenced, 13 samples at MRT and 27 at CHAZ Complex laboratory.

Demographic data were analyzed for all the 198 samples and the majority of the samples (104/198; 52.5%) were from females as shown in Table 1. The mean age of the participants was 28 (range: 0–82). The data set for gender and age were not available for one and five samples, respectively (Table 1).

**Table 1.** Characteristics of the genotyped samples infected with SARS-CoV-2.

| Parameters | Sample Distribution *n* (%), Overall, *n* = 198 |
|---|---|
| **Age Group** | |
| 0–14 Years | 7 (3.5) |
| 15–50 Years | 171 (86.4) |
| >50 Years | 15 (7.6) |
| Unknown | 5 (2.5) |
| **Gender** | |
| Female | 104 (52.5) |
| Male | 93 (47.0) |
| Unknown | 1 (0.5) |

### *3.2. SARS-CoV-2 Lineage Assignment and Distribution in Southern Province*

SARS-CoV-2 lineage assignment using the PANGOLIN application (https://pangolin.cog-uk.io/ (accessed on 8 August 2022), showed that the 40 genomes detected in this study were distributed into seven lineages, namely AY.116 (Delta), B.1.1.7 (Alpha), B.1.351 (Beta), and Omicron (BA.1, BA.1.1, BA.1.14, and BA.2) (Figure 2A). The largest number of the sequences ($n$ = 17, 42.5%) belonged to lineage BA.1/GRA (Figure 2A). All lineage AY.116 sequences came from Choma District, whereas the six B.1.351 lineage was detected in Choma ($n$ = 2), Namwala ($n$ = 2), Kalomo ($n$ = 1), and Mazabuka ($n$ = 1) districts (Figure 2B; Table S1). The Alpha variant (B.1.1.7) viruses were found in Namwala, Pemba, and Chikankata districts (Figure 2B; Table S1). Of the 27 Omicron variants, 11 (40.7%) were from Livingstone, 9 (33.3%) from Chikankata, 4 (14.8%) from Kazungula, 2 (7.4%) from Choma and 1 (3.7%) from Namwala. Most lineage BA.1 viruses were detected in Chikankata and Livingstone districts where 7/27 (25.9%) viruses of this lineage were found in each district. Lineage BA.1.1 was detected in Chikankata and Kazungula districts

whereas B.1.14 was only detected in Livingstone (Table S1). Three of the five BA.2 lineage viruses were detected in Livingstone whereas the other two were detected in Choma and Kazungula districts as shown in Figure 2B and Table S1.

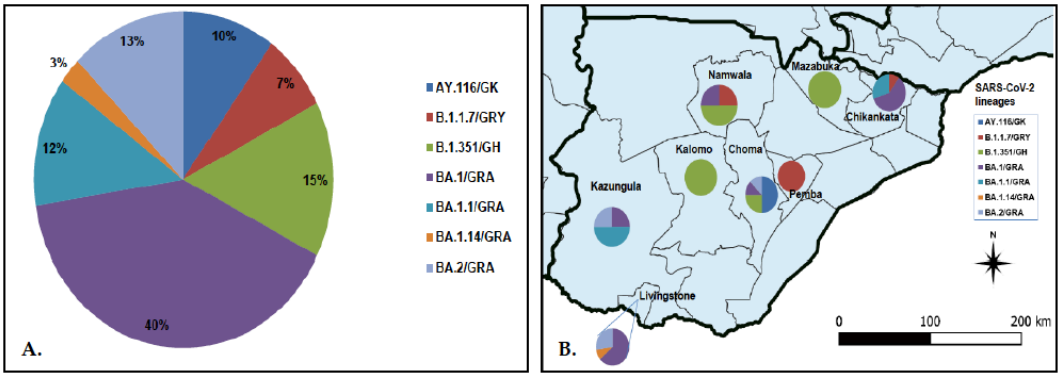


**Figure 2.** Distribution of SARS-CoV-2 lineage in the Southern Province of Zambia. Panel (**A**) pie chart showing SARS-CoV-2 lineages detected in the Southern Province; panel (**B**) proportionate distribution of SARS-CoV-2 lineages in the eight districts of the Southern Province. The map was generated using Quantum Geographic Information System (QGIS) version 3.10 (http://www.qgis.org (accessed on 8 August 2022)).

### *3.3. Phylogenetic Analysis*

Phylogenetic analysis revealed that the sequences separated into four clades namely Delta, Beta, Alpha, and Omicron (Figure 3). In the Delta clade four Southern Province sequences (Zambia/SP250/2021 | EPI ISL 6761088, Zambia/SP253/2021 | EPI ISL 6762977, Zambia/SP251/2021 | EPI ISL 6761106, and Zambia/SP252/2021 | EPI ISL 6761100), separated into two groups of which two formed a distinct cluster with a Zambian isolate whereas the other two clustered with sequences from Angola, Eswatini, and Zambia (Figure 3). Six sequences analysed in this study belonged to the Beta clade and they separated into four distinct clusters. Two of the sequences (Zambia/SP30/2021 | EPI ISL 6760973 and Zambia/SP87/2021 | EPI ISL 6764745) analysed in this study clustered with isolates from Zambia, Zimbabwe, England, and the Democratic Republic of Congo (DRC), and another set of two formed a distinct cluster with sequences from Zambia. The last two sequences (Zambia/SP11/2021 | EPI_ISL_6760905 and Zambia/SP10/2021 | EPI_ISL_6760707) belonged to separate clusters with the former sequence grouping with Zambian sequences whereas the latter was closely related to sequences obtained in Malawi, Eswatini, and Botswana (Figure 3). In the Alpha clade, three Southern Province sequences, namely Zambia/SP32/2021 | EPI_ISL_6761015, Zambia/SP37/2020 | EPI_ISL_6761027, and Zambia/SP172/2021 | EPI_ISL_6761052 formed a distinguishable cluster with sequences from England and Zambia (Figure 3). The Omicron clade was separated into two clusters (Figure 3). The majority (22/27; 81.5%) of the Zambian sequences in this clade belonged to the BA.1 sub-lineage cluster whereas the rest (5/27; 18.5%) were of the BA.2 lineage. Phylogenetic analysis further showed that the Omicron sequences from this study were mainly closely related to sequences from European and African countries (Figure 3).

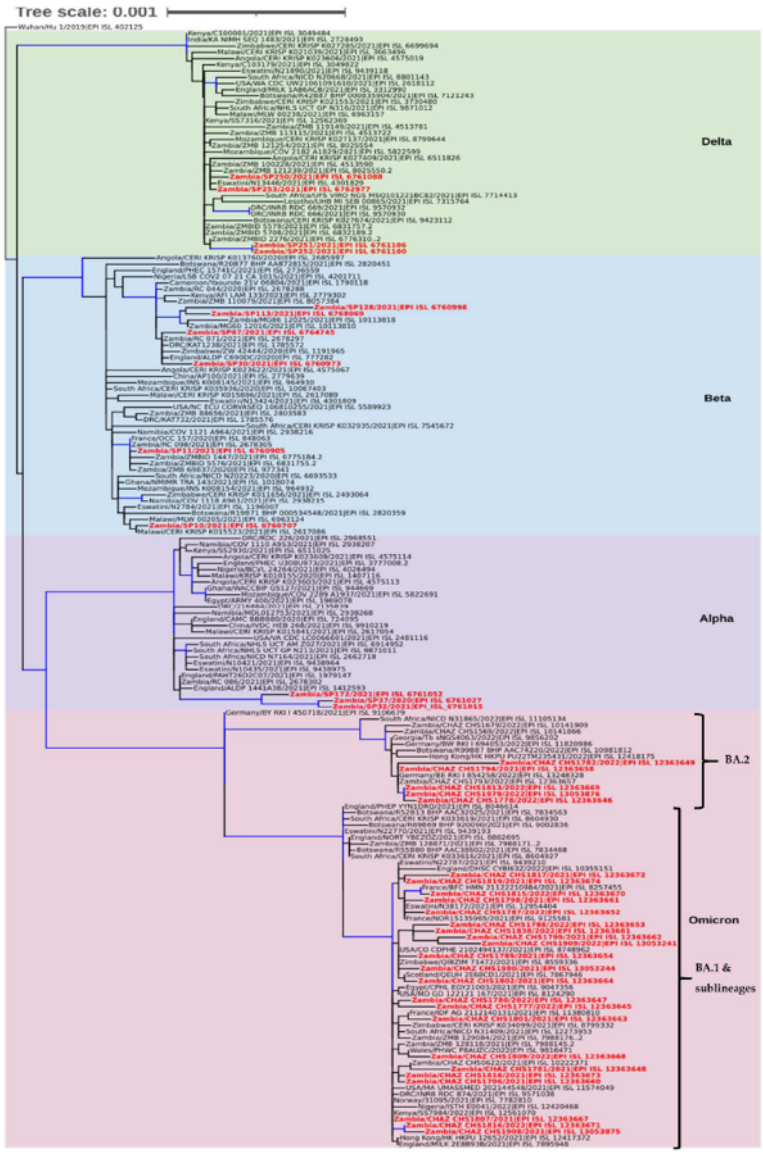


**Figure 3.** Phylogenetic analysis of SARS-CoV-2 genomes from Zambia and other countries. The genomes generated in this study are indicated in red whereas the shaded areas indicate the clades of variants of concern. Each sequence was named with the country name first followed by the isolate name and then the GISAID accession number. The tree branches highlighted in blue indicate tree branches that had a strong maximum likelihood ratio greater than 0.9, whereas the tree scale represents the nucleotide substitutions per site.

### *3.4. Molecular Analysis*

A total of 292 different mutations were detected from the 40 genomes studied when compared to the Wuhan/Hu 1/2019 | EPI ISL 402125 reference sequence (Table 2). Most (96.2%) mutations were detected in the coding regions of the genomes. Of the mutations detected in the coding region, 64.8% (182/281) were missense mutations, 23.5% (66/281) were synonymous mutations, 8.2% (23/281) were deletions, 3.2% (9/281) insertions, and one was a stop codon (0.4%), gained with a single nucleotide polymorphism (SNP) on the ORF8 (Tables 2 and S2). Deletions and insertion included in-frame and out-of-frame mutations. When gene mutations were stratified according to the VOCs, the Alpha variant had a total of 53 different mutations of which 31 (58.5%) were missense mutations and 8 (15.1%) synonymous mutations. The number of mutations in the Alpha variant genomes ranged between 41 and 45 with (EPI_ISL_6761027) having the most mutations. The Beta variant had a total of 68 different mutations with 44 (64.7%) missense mutations and 15 (22.1%) synonymous mutations. The mutations in the Beta variant genomes ranged between 26 and 45 with one sequence (EPI_ISL_6760998) having the most mutations. Further, sequences of the Delta variant had a total of 50 mutations with 37 (74%) missense mutations and 7 (14%) synonymous mutations. The Delta variant mutations ranged between 39 and 44 with two sequences (EPI_ISL_6761106; EPI_ISL_6761100) having the most mutations. Sequences of the Omicron variant had the highest number of mutations; 149 different mutations with 90 (60.4%) missense and 39 (26.2%) synonymous mutations, with the genomes having a mutation range between 48 and 67 with three sequences (EPI_ISL_12363648; EPI_ISL_12363649; EPI_ISL_12363661) having the most mutations. Deletions, insertions, stop-codons, and upstream/downstream gene variants had a frequency below 18% in all the VOCs.

**Table 2.** Distribution of mutations along different genomic regions of SARS-CoV-2 sequences detected in Southern Province.

| Genome Segment | Missense Mutation | Synonymous Mutation | Deletion | Insertion | Others | Total Mutation |
|---|---|---|---|---|---|---|
| **Coding Region** | | | | | | |
| ORF1ab | 74 | 48 | 9 | 3 | 0 | 134 |
| Spike | 65 | 3 | 10 | 4 | 0 | 82 |
| ORF3a | 5 | 4 | 0 | 0 | 0 | 9 |
| Envelope | 5 | 0 | 0 | 0 | 0 | 5 |
| Membrane | 5 | 2 | 0 | 0 | 0 | 7 |
| ORF6 | 2 | 2 | 0 | 0 | 0 | 4 |
| ORF7a | 2 | 0 | 0 | 0 | 0 | 2 |
| ORF7b | 4 | 1 | 2 | 2 | 0 | 9 |
| ORF8 | 4 | 3 | 1 | 0 | 1 [1] | 9 |
| Nucleocapsid | 16 | 3 | 1 | 0 | 0 | 20 |
| **Non-coding Region** [2] | | | | | | |
| 5′UTR | 0 | 0 | 0 | 0 | 4 | 4 |
| 3′UTR | 0 | 0 | 0 | 0 | 7 | 7 |
| Total | 182 | 66 | 23 | 9 | 12 | 292 |

[1] Stop codon in the ORF8; [2] all the mutations in the non-coding region are extragenic.

When the number of mutations per gene was counted only once, the S protein was the most mutated gene with 82 mutations whereas the second mutated gene was the NSP3 protein with 42 mutations (Table S2). Of the 82 mutations in the S protein, 65/82 were missense mutations, 3/82 synonymous mutations, 10/82 deletions, and 4 insertions as shown in Table 2. Among all the SNPs, the most common change was C > T followed by A > G and G > A. Further, a large deletion of 26 nucleotides was observed on position 29734 of the 3′UTR of the four sequences (EPI_ISL_12363646, EPI_ISL_12363658, EPI_ISL_12363649 and EPI_ISL_12363669).

The most common mutation was the D614G substitution on the S protein and P314L substitution on the NSP12b (RdRp) protein which occurred in all the sequences studied and

67.5% (27/40) showed other amino acid substitution in the S protein including T95I, G339D, S373P, S375F, H655Y, N679K, N764K, D796K, Q954H, and D1146D (Table S2). The second most common amino acid change (39/40; 97.5%) was the F106F substitution on the NSP3 followed by the K417N (31/40; 77.5%) substitution on the S protein T492I substitution on the NSP4, followed by P681H (30/40; 75%), and (29/40; 72.5%) N501Y substitutions on the S protein. In addition to these mutations, several substitutions, deletions, and insertions in other genomic areas were also present (Table S2).

Comparison of mutations on the S protein of the SARS-CoV-2 variants in this study with the wildtype (Wuhan-Hu-1) SARS-CoV-2 revealed that the Omicron variant had the highest number of mutations in this protein compared to the other VOCs in this study. The Omicron variant had 58 amino acid (AA) mutations which included six deletions and four insertions (Table 3). Of the 60 AA mutations in the Omicron variant, 22 were found to be in the RBD of the S protein including G339D, R346K, Y369Y, S371L, S371F, S373P, S375F, T376A, D405N, R408S, K417N, N440K, G446S, T470A, S477N, T478K, E484A, Q493R, G496S, Q498R, N501Y, and Y505H (Tables 2 and 3). The other AA variations in the RBD included N501Y in Alpha variants, S325P, I326K, V327A, K417N, E484K, and N501Y in the Beta variant and L452R and T478K (Table 3).

**Table 3.** Spike protein mutations in different SARS-CoV-2 variants compared to the wild-type (Wuhan-Hu-1).

| SARS-CoV-2 Variants | Spike Mutations [1] |
|---|---|
| Wuhan-Hu-1 (wild-type) | - |
| Alpha (B.1.1.7) | ΔH69, ΔY145, **N501Y**, A570D, D614G, P681H, T716I, T874I, S982A, D1118H |
| Beta (B.1.351) | L18F, D80A, D215G, ΔL242, T307P, N317F, **S325P, I326K, V327A, K417N, E484K, N501Y**, D614G, A701V, A1087S |
| Delta (AY.116) | T19R, T95I, G142D, ΔE156, **L452R, T478K**, D614G, P681R, D950N |
| Omicron (BA.1, BA.1.1, BA.1.14, BA.2) | T19I, ΔL24, ΔA67, ΔA67, ΔI68, T95I, ΔG142, G142D, V193L, Y200C, insI210, ΔN211, N211K, L212C, V213G, insS214, insV213, insR214, insV213, insR214, R214R, A243S, L244S, **G339D, R346K, Y369Y, S371L, S371F, S373P, S375F, T376A, D405N, R408S, K417N, N440K, G446S, T470A, S477N, T478K, E484A, Q493R, G496S, Q498R, N501Y, Y505H**, T547K, D614G, H655Y, N679K, P681H, N764K, D796Y, N856K, Q954H, N969K, L981F, V1104L, D1127G, D1146D, V1264L |

[1] Receptor-binding domain (residues 319–541) is marked as bold in all the variants. Δ Represents deletion, ins represent insertion.

## 4. Discussion

In this study, from the 198 samples that were obtained for genomic sequencing in eight districts of the Southern Province of Zambia, 40 SARS-CoV-2 whole genomes were successfully sequenced and analysed. Our dataset revealed that there were more cases of COVID-19 observed in females compared to males. However, other studies have recorded a higher disease burden in males compared to females [44–46]. The mean age of patients was 28 with a minimum and maximum age of 0 and 82 years, respectively. However, it cannot be ruled out that the small number of samples analysed in this study may have had an impact on the observed gender distribution and the mean age of COVID-19 patients. Furthermore, lineage assignment revealed that BA.1 was the most prevalent lineage among our sequences followed by B.1.351. This could be explained by the fact that most of the successfully sequenced samples were collected during the Omicron wave. The B.1.351 predominated in the second wave, AY.116 in the third wave, and BA.1 in the fourth wave. The findings corroborate those of other authors who reported the predominance of Beta (B.1.351), Delta (B.1.617.2), and Omicron BA.1 variants in the second, third, and fourth waves of the pandemic in Africa, respectively [13,26,47,48]. Moreover, the detection of AY.116 and B.1.351 coincided with a rapid increase in the number of confirmed cases and deaths in Zambia [35,49]. Despite the small sample size of this study, SARS-CoV-2 lineages were detected in different districts of the Southern Province. The majority of the Omicron

variants were detected in Chikankata and Livingstone districts, with the latter having more subvariants. It is plausible that Livingstone, being a border town, a tourist capital, and a major transportation link to Zambia's neighbouring countries, the area could be at increased risk of the introduction of novel VOCs. Except for the Alpha variant, all the other VOCs detected in this study were found in Choma District. This could be explained by the fact that Macha Research Trust where sequencing was conducted is located in the Choma District and thus the institution was more likely to receive samples throughout the different phases of the COVID-19 waves.

Phylogenetic analysis revealed that the 40 SARS-CoV-2 genomes generated in this study belonged to four SARS-CoV-2 VOCs namely Alpha, Beta, Delta, and Omicron variants. These VOCs have presented a formidable public health challenge during the COVID-19 pandemic because of their increased viral transmissibility and disease severity [50]. Additionally, the early detection of some of the VOCs in Africa highlights the importance of coordinated molecular surveillance systems in all parts of the world and the role Africa has played in enabling the early detection and characterization of new lineages and informing the global pandemic response. The close phylogenetic relatedness of sequences generated in this study with those from European and African countries supports the idea of possible multiple introductions of the virus from different regions. Phylogenetic analysis further revealed that some sequences from this study clustered together and among other Zambian sequences which may signify the local circulation of these viruses. Notably, sequences obtained in this study that grouped within the Alpha variant clade were phylogenetically distinguishable and were detected in three different districts, which may suggest independent introductions, particularly from Europe, as these sequences were closely related to isolates from England. This introduction could be attributed to the relaxation of flight restrictions at the time these samples were collected. The Zambian Alpha cluster also displayed a longer branch length compared to the other sequences in this clade indicating the continued evolution as the virus circulated. Interestingly, the Alpha variant has not been associated with any COVID-19 wave in Zambia. This observation may suggest that the Alpha variant has no selective advantage over the other VOCs such as the Beta and Delta variants [27]. Although some Beta and Delta variants were closely related to isolates from Europe and Zambia, others showed a close relationship to isolates from Eswatini, DRC, Malawi, and Zimbabwe, suggesting that public health measures implemented by the authorities may have been compromised by porous borders and thus permitting the variants to spread within the region. Phylogenetic analysis also revealed that Omicron variants separated into two major clusters, BA.1 and BA.2, signifying the continued evolution of this VOC. The BA.4 and BA.5 subvariants which have been associated with driving current waves of infection in South Africa [51,52] were not detected in this study.

The S glycoprotein of SARS-CoV-2 plays a pivotal role in viral infection and pathogenesis because of its role in host cell receptor recognition, viral attachment, and entry [53–57]. The present study demonstrated the presence of the D614G mutation in the S protein in all 40 genomes. Similar findings have been reported in many countries including Turkey [58], Oman [59], Egypt [60], and the Comoros Island [61]. In addition to the D614G mutation in the S glycoprotein (23403A > G), a P314L mutation (14408C > T) in the NSP12/RdRp was detected in all the sequences analysed. This finding agrees with previous research which reported a high co-occurrence of these mutations around the globe [62–64]. The D614G mutation is associated with a high viral load, infectivity, and transmissibility [50] whereas mutations in the RdRp protein results in a dysfunctional enzyme that generates errors during RNA synthesis, increasing the chances of mutations occurring [62,64,65]. It is also suggested that the co-occurrence of the D614G and NSP12_P314L mutations may enhance viral entry and replication, respectively [66]. Therefore, the S protein mutations and their effects on virulence should be closely monitored and evaluated, as this protein is the main target for vaccine development [67].

Alpha, Beta, and Omicron variants share the N501Y mutation, located in the receptor-binding domain (RBD) of the S protein. It is known to confer an increased binding affinity of the RBD for the ACE2 receptor, raising the viral transmission rate [68]. This mutation was detected in all Alpha, Beta, and 20 Omicron variants of our sequences. Furthermore, the K417N and E484K mutations in the S protein, common to all Beta variants [26] were also detected in our sequences. Other mutations in this present study included the Q27 stop in the ORF8 in all three Alpha variants. This mutation has been observed in the Alpha (B.1.1.7) variant and is known to truncate the ORF8 protein or make it inactive, allowing the accumulation of additional mutations in other regions [68]. Further, eight mutations namely D614G, D950N, F157Δ, L452R, P681R, R158Δ, T19R, and T478K were detected in the S protein in the four sequences of the Delta variant identified in this study. These mutations are identical to those detected in the Indian Delta variants (B.1.617.2) [69]. Deletions, insertions, frameshift variants, and up/downstream variants were much rarer. This observation is also in line with the finding of Malune et al., whose study reported less than 10% of these mutations [70].

Sequences of the Omicron variant obtained in this study were highly mutated, having 149 mutations across the 27 sequences examined. The findings are consistent with the findings of Saxena et al., who detected more mutations in Omicron variants than the Delta variant [71]. When the S protein mutations of the VOCs in this study were compared to the hCoV-19/Wuhan/Hu-1/2019 | EPI_ISL_402125, Omicron was highly mutated with 58 mutations and 22 amino acid mutations in the RBD. These mutations are crucial as they are thought to increase the overall risk of reinfection and partial resistance to existing vaccines [72]. In addition to mutations in the S protein, several substitutions and deletions in other genomic regions are also present in all the SARS-CoV-2 variants in this study. Moreover, mutations have an adverse impact on the pathogenicity of SARS-CoV-2 and the development of diagnostic assays, antivirals, and vaccines. Therefore, monitoring of mutations and characterization of their roles in virulence-related conditions in SARS-CoV-2 is very vital in the control and prevention of the spread of the virus.

The limitations of the study are that most of the samples could not be successfully sequenced because they had a Ct > 30, whereas others had poor genomic coverage. We believe poor sample quality was the main reason for the considerably low number of sequences obtained in this study which may have been due to poor storage and transportation conditions (i.e., failure to maintain a good cold chain), as some of the samples came from far-lying rural districts. For improved SARS-CoV-2 genomic surveillance, strengthening the capacity for sample storage and courier in rural areas should be prioritized by the Zambian Ministry of Health.

## 5. Conclusions

The findings highlighted the circulation of four VOCs in the Southern Province of Zambia namely Alpha (B.1.1.7), Beta (B.1.351), Delta (AY.116), and Omicron (BA.1, BA.1.1, BA.1.14 and BA.2). Phylogenetic analysis revealed that our genomes were closely related to genomes from Europe and Southern Africa indicating intra- and intercontinental introductions of the virus to the country. Additionally, some sequences that clustered with Zambian sequences may signify local transmission of the virus. The Omicron variant exhibited the highest number of amino acid substitutions in the S glycoprotein as compared to the other three variants in this study. Moreover, SARS-CoV-2 with the D614G and P314L mutation was the major circulating virus in Southern Province, Zambia. Our findings stress the need for continued monitoring of SARS-CoV-2 circulation in Zambia, especially in strategically positioned regions such as the Southern Province which could be at increased risk of introduction of novel VOCs. This analysis further represents the first genomic study in the Southern Province of Zambia and highlights the importance of the Zambia Genomic Sequencing Consortium in the expansion of SARS-CoV-2 genomic surveillance in understanding the spread of the virus at national and community levels. It has further contributed to the decentralization of sequencing facilities encompassing

among them public, private, and academic public health laboratories which have led to the rapid dissemination of sequences into the public domain.

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/v14091865/s1, Table S1: Characteristics of 40 SARS-CoV-2 whole-genomes from the Southern Province of Zambia; Table S2: Nucleic acid and amino acid mutations observed in the 40 SARS-CoV-2 genomes obtained from the Southern Province of Zambia; Table S3: SARS-CoV-2 primer sequences; Table S4: SARS-CoV-2 genome map.

**Author Contributions:** Conceptualization, B.K. (Ben Katowa), A.K., B.M., W.M. and E.S.; methodology, B.K. (Ben Katowa), A.K., B.M., J.M., W.M. and E.S.; software, B.M.; formal analysis, B.K. (Ben Katowa), A.K. and B.M.; investigation, B.K. (Ben Katowa), A.K., B.M., W.M., and E.S.; resources, M.K. (Masahiro Kajihara), J.Y., A.T., H.S., R.C., V.M. and E.S.; writing—original draft preparation, B.K. (Ben Katowa) and A.K.; writing—review and editing, B.K. (Ben Katowa), A.K., B.M., J.M., D.M.S., J.S., B.K. (Boniface Kabungo), K.C., S.C., M.K. (Mpanga Kasonde), O.K., N.K., K.M., M.M., J.T., M.B., A.Z., C.G.S., M.K. (Masahiro Kajihara), J.Y., A.T., H.S., R.C., W.M. and E.S; supervision, W.M. and E.S.; project administration, M.K. (Masahiro Kajihara), J.Y., A.T., H.S., R.C., V.M. and E.S.; funding acquisition, A.T., H.S., R.C., V.M., M.B., A.Z. and E.S. All authors have read and agreed to the published version of the manuscript.

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**Data Availability Statement:** The sequences have been deposited in the GISAID EpiCoV (https://www.gisaid.org/ (accessed on 8 August 2022) under accession numbers: EPI_ISL_6768069; EPI_ISL_6764745; EPI_ISL_6760707; EPI_ISL_6760905; EPI_ISL_6760973; EPI_ISL_6760998; EPI_ISL_6761015; EPI_ISL_6761027; EPI_ISL_6761052; EPI_ISL_6761088; EPI_ISL_6761100; EPI_ISL_6762977; EPI_ISL_6761106; EPI_ISL_12363645; EPI_ISL_12363646; EPI_ISL_12363647; EPI_ISL_12363648; EPI_ISL_12363649; EPI_ISL_12363652; EPI_ISL_12363653; EPI_ISL_12363654; EPI_ISL_12363658; EPI_ISL_12363660; EPI_ISL_12363661; EPI_ISL_12363662; EPI_ISL_12363663; EPI_ISL_12363664; EPI_ISL_12363667; EPI_ISL_12363668; EPI_ISL_12363669; EPI_ISL_12363670; EPI_ISL_12363671; EPI_ISL_12363672; EPI_ISL_12363673; EPI_ISL_12363674; EPI_ISL_12363681; EPI_ISL_13053241; EPI_ISL_13053244; EPI_ISL_13053875 and EPI_ISL_13053876.

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*Review*

# Influenza A and D Viruses in Non-Human Mammalian Hosts in Africa: A Systematic Review and Meta-Analysis

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**Abstract:** We conducted a systematic review and meta-analysis to investigate the prevalence and current knowledge of influenza A virus (IAV) and influenza D virus (IDV) in non-human mammalian hosts in Africa. PubMed, Google Scholar, Wiley Online Library and World Organisation for Animal Health (OIE-WAHIS) were searched for studies on IAV and IDV from 2000 to 2020. Pooled prevalence and seroprevalences were estimated using the quality effects meta-analysis model. The estimated pooled prevalence and seroprevalence of IAV in pigs in Africa was 1.6% (95% CI: 0–5%) and 14.9% (95% CI: 5–28%), respectively. The seroprevalence of IDV was 87.2% (95% CI: 24–100%) in camels, 9.3% (95% CI: 0–24%) in cattle, 2.2% (95% CI: 0–4%) in small ruminants and 0.0% (95% CI: 0–2%) in pigs. In pigs, H1N1 and H1N1pdm09 IAVs were commonly detected. Notably, the highly pathogenic H5N1 virus was also detected in pigs. Other subtypes detected serologically and/or virologically included H3N8 and H7N7 in equids, H1N1, and H3N8 and H5N1 in dogs and cats. Furthermore, various wildlife animals were exposed to different IAV subtypes. For prudent mitigation of influenza epizootics and possible human infections, influenza surveillance efforts in Africa should not neglect non-human mammalian hosts. The impact of IAV and IDV in non-human mammalian hosts in Africa deserves further investigation.

**Keywords:** animal influenza; influenza A virus; influenza D virus; Africa; prevalence; seroprevalence



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## 1. Introduction

Influenza viruses (IVs) are enveloped, single-stranded RNA viruses with segmented genomes containing 7–8 gene segments. They belong to the family *Orthomyxoviridae* and consist of four genera: *Alphainfluenzavirus* (Species: *Influenza A virus* (IAV)), *Betainfluenzavirus* (Species: *Influenza B virus* (IBV)), *Gammainfluenzavirus* (Species: *Influenza C virus* (ICV)) and *Deltainfluenzavirus* (Species: *Influenza D virus* (IDV)) that are classified according to antigenic variations of their nucleoprotein (NP) and matrix 1 (M1) proteins [1–4]. The

four influenza virus genera differ in host range and pathogenicity and are likely to have diverged evolutionarily at least several thousand years ago [5]. Among these genera, IAVs are the most virulent and are known to cause severe disease. Further, only IAVs pose a significant risk of zoonotic transmission, host switching, and the generation of pandemic IAVs [5].

Wild waterfowl among the orders Anseriformes and Charadriiformes are considered to be the natural reservoirs for IAVs [6]. IAVs are classified into subtypes based on their antigenic and genetic diversity of two surface glycoproteins, haemagglutinin (HA) and neuraminidase (NA). To date 16 HA (H1–H16) and 9 NA (N1–N9) subtypes of IAVs have been detected and circulate in wild waterfowl and poultry [7]. In addition, IAV-like viruses, H17N10 and H18N11, were recently detected in bats from Guatemala and Peru, respectively [8,9]. Some IAV subtypes have crossed species barriers, establishing stable lineages in a wide variety of animals [10,11], for example H1N1 and H3N2 subtypes in humans [12,13], H1N1, H1N2, and H3N2 subtypes in swine [14,15] and H3N8 and H7N7 subtypes in horses [16,17].

Interspecies transmission of IAVs is common among different animal species via direct or indirect contact which may result in the introduction of viruses that are new to the recipient species and which have the potential to cause substantial outbreaks [6]. Whereas most of these interspecies transmission events may not result in onward transmission and establishment in the new host, sustained influenza outbreaks have been reported in poultry and several mammalian species [18]. Of the mammalian hosts, only a limited number are currently recognised as sustaining IAV transmission, and it is not clear what distinguishes these species from those for which influenza has not been reported [18]. However, for IAVs to become established and achieve efficient viral replication in other hosts, they must overcome a variety of species barriers [19]. Such barriers include host innate immune responses, several intracellular factors and recognition of different sialic acid (SA) receptors, α-2,3 and α-2,6 expressed on host cell surfaces of avian and human respiratory epithelia, respectively [20]. The well-known mammalian hosts for which IAVs have established themselves include humans, pigs, horses, seals, mink and dogs. Dogs emerged as important IAV hosts in the 2000s when the H3N8 equine influenza virus (EIV) and the avian virus-like H3N2 strain introduced from horses and birds, respectively, were detected in the United States of America (USA) and Asia [21,22]. Both of these canine influenza viruses have continuously circulated in the dog population since their emergence, increasing opportunities for human exposure to these zoonotic viruses [18].

Apart from reports of IAVs in domestic animals, IAVs of various subtypes have been documented in wild animals though these reports are mainly limited to captive animals. Examples of these introductions include H5N1 IAV infections in leopards and tigers in Thailand [23,24], and the H1N1 virus that caused the 2009 pandemic (H1N1pdm09) in cheetahs in California USA [25] and wild boars in Japan [26]. Infections in wild animals are usually thought of as being opportunistic as they usually arise through the consumption of raw meat containing the virus especially for carnivores, hence limited or no animal to animal transmission occurs. However, a study by Thanawongnuwech et al. [27] in tigers points to a probable horizontal transmission of IAVs in these animals. Although herbivores might be exempt from diet-driven pathogen transmission, sharing common feeding grounds and water sources with the reservoir host could also lead to potential transmission [28].

While IAVs cause mild to severe disease in various animal species, IDV has been associated with bovine respiratory disease complex which is the most economically significant disease of the beef industry with economic losses being attributed to morbidity, mortality, treatment costs, and reduced carcass value [29,30]. IDV was recently discovered in swine with respiratory disease in the USA in 2011 [31]. Since its discovery, serological evidence of IDV has been reported in healthy and symptomatic cattle populations in multiple geographical regions including the USA [1,31–33], Europe [34–37], Asia [38,39] and Africa [40], suggesting that cattle could be the natural reservoir hosts of this new virus [1]. Further,

serological evidence of IDV has been reported in small ruminants in the USA [41]. Despite various studies on the virological and serological evidence of IDV, its zoonotic potential and pathogenicity in other hosts including humans remain obscure.

Despite the increasing knowledge of the dynamics of IVs in different avian and non-human mammalian species around the globe, current data in Africa are limited, characterised by patchy surveillance studies, thereby limiting a more comprehensive understanding of the prevalence and circulation of these viruses in non-human mammalian species. Furthermore, the potential public health risk of these viruses arising from the close relationship between non-human mammalian species (pigs, dogs, cats and horses among others) and their owners underscores the need to study their prevalence and circulation in these animals. Therefore, we conducted a systematic review and meta-analysis to investigate the prevalence and current knowledge of IAV and IDV in non-human mammalian hosts in Africa.

## 2. Materials and Methods

### *2.1. Literature Search Strategy*

The systematic review and meta-analysis were conducted according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (Checklist S1) [42], Figure 1. Three databases, PubMed, Google Scholar and Wiley Online Library were searched using terms related to IAV and IDV in non-human mammalian hosts (Protocol S1). In addition, the World Organisation for Animal Health–World Animal Health Information System (OIE–WAHIS) platform was also used as a data source as it provides direct up-to-date information on animal health situations worldwide. All references located in the searches were imported into Endnote Version 8, a web-based reference manager, and a database for all relevant articles was generated.

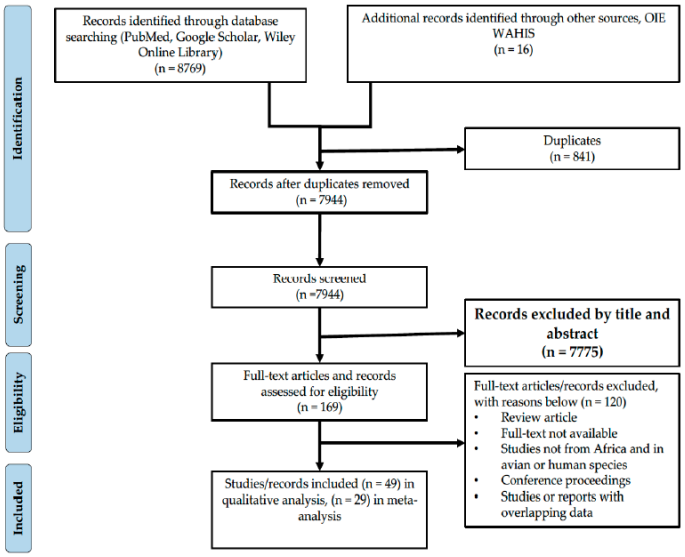


**Figure 1.** PRISMA flow diagram of the selection process used to determine eligible studies.

### *2.2. Study Selection*

All studies identified in the search were assessed, and duplicates removed and checked for eligibility. The studies were initially selected based on the relevance of their titles and abstracts regarding the prevalence and circulation of IAV and IDV in non-human

mammalian hosts in Africa. Thereafter, full texts of the remaining articles were screened and those that did not meet the inclusion criteria were excluded.

### *2.3. Inclusion and Exclusion Criteria*

The review included all study types on IAV and IDV in non-human mammalian species with the exception of (i) experimental studies, (ii) studies on the development of new diagnostic methods and (iii) vaccine development. Only studies written in English and published between 2000 and 2020 were included in this review and meta-analysis.

Studies excluded from the review included those not published in Africa, those published before 2000 or after 2020, editorials, conference proceedings, review articles, animal experiments, theoretical models, and studies in human and avian species. Studies were further excluded if the diagnostic test was not indicated, had overlapping data with another included study and were excluded from the meta-analysis if the sample size was less than five.

### *2.4. Data Extraction*

We extracted study information regarding the author's name, title and year of publication. Additional information extracted included country/region, study type, animal species, sample type, diagnostic method, sample size, number of positive samples, IAV subtype, strain, vaccination status (important for swine and equids), and premises (indicating where the sample was collected such as farm and slaughterhouse, etc.).

### *2.5. Assessment of Quality and Risk of Bias*

We assessed the quality and risk of bias of included studies using a quality assessment checklist (Checklist S2) [43,44]. The checklist included ten questions that had a 'yes' or 'no' answer. A point was scored if the response was 'yes' and zero for 'no'. Overall study quality was categorised as 'high' (scores $\geq$ 8 points), 'moderate' (scores 5 to 7 points) or 'low' (scores < 5 points). Funnel and doi plots were used to assess publication bias using the LFK index. Based on the LFK index no asymmetry was defined as LFK index values within $\pm$1, minor asymmetry as values exceeding $\pm$1 but within $\pm$2, while major asymmetry as values exceeding $\pm$ 2 [45].

### *2.6. Statistical Analysis*

Descriptive statistics were used to describe the overall search results, characteristics of included studies and distribution of IAVs and IDV in non-human mammalian hosts using MS Office Excel® 2016. For the meta-analysis, MetaXL version 3.1 (https://epigear.com accessed on 26 July 2021) a tool for meta-analysis in Microsoft Excel was used to pool prevalence and seroprevalences from each study [46]. Seroprevalence was defined as the presence of antibodies against IVs by any serological test while prevalence was defined as the isolation or detection of IVs by culture or reverse-transcriptase polymerase chain reaction. The quality effects model was used to calculate the pooled prevalences and their 95% confidence intervals (CI). The $I^2$ was used to assess study heterogeneity and $I^2$ values of 25%, 50% and 75% were considered as having a low, moderate and high degree of heterogeneity, respectively [47]. We divided studies into subgroups based on the geographical regions (African Islands, Central, East, Southern and West Africa) and animal hosts to investigate the potential sources of heterogeneity.

## 3. Results

### *3.1. Search Results, Study Selection and Characteristics of the Included Studies*

A total of 8785 articles and records were identified, of which 49 (45 articles and four records from OIE-WAHIS) were included in this study (Figure 1 and Table S1). Of the 49 articles/records included in the systematic review, 29 were included in the meta-analysis. Further, some articles reported datasets from more than one country in Africa. Overall the included articles/records reported data from 19 African countries as shown in Figure 2.

The highest number of articles was for studies conducted in West Africa (24/49; 49.0%) with the majority of articles being from Nigeria while the least of articles were for studies conducted in Southern Africa and the African Islands which recorded one study each (1/49; 2.0%). Furthermore, the majority of the articles/records were published between 2011–2020 (40) while only nine articles/records were published between 2000–2010 (Figure 3).

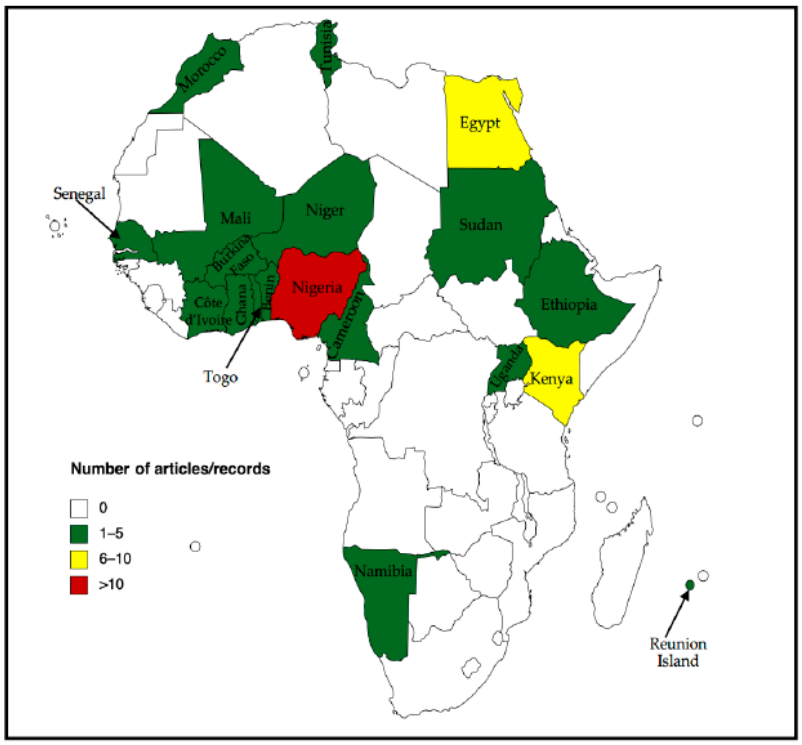


**Figure 2.** Map of Africa showing the distribution of the number of articles/records (n = 49) included in the review. Some articles reported data from several countries. The map was created online at https://mapchart.net/ (accessed on 30 November 2021).

According to the included studies, more studies/records (28 (45.2%)) were conducted in pigs than in any other animal species included in this study (Table S2). Further, most studies collected serum or both serum and nasal swabs (18 (36.7%)) and used multiple methods (serological or virological methods) (22 (44.9%)) for the identification of IVs (Table S2). Only four (8.2%) articles that reported on IDVs in Africa were included in this study. Furthermore, most studies did not report whether the animals had an influenza-like illness (ILI), or on their vaccination status (Table S2).

### *3.2. Assessment of Quality and Risk of Publication Bias of Selected Studies*

According to our quality assessment criteria, of the 29 publications included in the meta-analysis, five publications were of high quality, 13 were of moderate quality and 11 were of low quality. Publication bias in studies was measured and detected using the funnel and doi plots. Overall, the funnel and doi plots (Figures S1 and S2) showed minor and major asymmetry with the LFK index of 3.52 and 1.48 for prevalence and seroprevalence of IAV in pigs, respectively, and 1.10 for seroprevalence of IDV in non-human mammalian species, demonstrating a potential risk of publication bias among the selected papers.

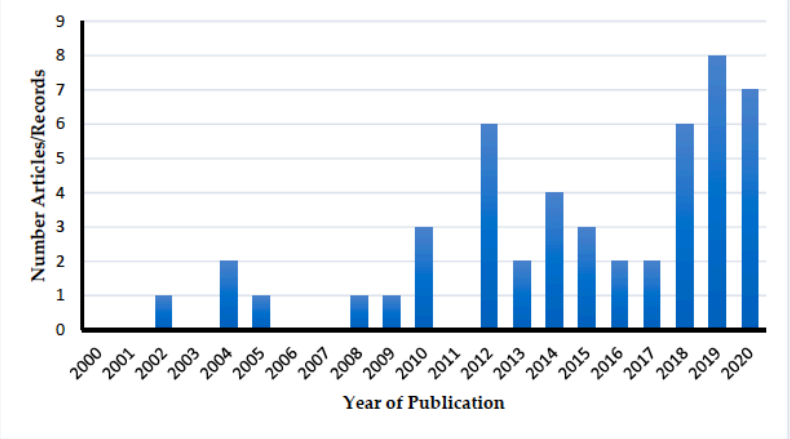


**Figure 3.** Number of publications included in the present study from 2000 to 2020.

### *3.3. Distribution of Influenza A and D Viruses and Detection of Antibodies in Non-Human Mammalian Hosts in Africa*

The distribution of IAV and IDV in non-human mammalian hosts is depicted in Tables 1 and 2. West Africa had the highest number of countries (n = 9) with studies or reports on IAV in non-human mammalian species followed by North Africa (n = 4), East Africa (n = 3), African Island (n = 1), Central Africa (n = 1) and Southern Africa (n = 1). All the regions (African islands, Central, East, North, Southern and West Africa) included in this review reported at least virological or serological evidence of IAV in non-human mammalian species (Table 1).

The majority of the studies included in this review provided virological and/or serological evidence of the circulation of H1N1pdm09 in pigs in Africa (Table 1). The countries reporting H1N1pdm09 in pigs included Cameroon, Egypt, Ghana, Kenya, Nigeria, Togo and the Reunion Island. Apart from H1N1pdm09, classical H1N1, H3N2, H1 and H3 viruses were also reported in pigs in Burkina Faso, Kenya, Egypt, Uganda, Ghana and Nigeria (Table 1). The populations of pigs in various studies included piglets, weaners, growers, finishers, sows and boars. Additionally, pigs were either sampled from farms or slaughterhouses, though some articles did not indicate the sources of the pigs sampled. Further virological and serological evidence of H5N1 highly pathogenic avian influenza viruses (HPAIVs) were reported in Egypt and Nigeria, as well as H5N2 and H9N2 viruses in Egypt (Table 1).

Additionally, virological and/or serological evidence of exposure to IAVs was also reported in cats, dogs, rats, olive baboons (*Papio anubis*), equids, bats, spotted hyena (*Crocuta crocuta*), black rhinos (*Diceros bicornis*), wildebeest (*Connochaetes taurinus*) and caracals (*Caracal caracal*) (Table 1). IAV-specific antibodies for H1 and H1N1 were detected in cats and dogs in Kenya [48], H3N8 and other unidentified subtypes in hunting, pet and village dogs in Nigeria [49,50], while antibodies against H5N1 were detected in cats, dogs and rats in Egypt [51] (Table 1). Further, influenza A viral RNA was detected in dogs in Kenya [48]. Equine influenza virus (EIV) subtype H7N7 (EIV-1) and/or, H3N8 (EIV-2), and their respective antibodies were reported in horses, donkeys and mules in Egypt [52,53]. Moreover, antibodies specific for EIV were detected in donkeys, horses and mules in Morocco [54], Sudan [55], Tunisia [56], Mali [57] and in camels in Kenya [58]. Apart from EIVs, H5N1 HPAIV and antibodies against this virus were detected in donkeys in Egypt [59] (Table 1). Furthermore, specific antibodies against H3, H5, H8, H9 and H12 viruses were also detected in wild mammals such as bats in Ghana [60] and IAV

A/bat/Egypt/381OP/2017 was detected from Egyptian fruit bats in Egypt [61]. Moreover, a study conducted in Namibia demonstrated the exposure of various wildlife animals such as lions, black rhinos, spotted hyena, wildebeest, caracal, honey badgers and black-backed jackal to various IAVs [28] (Table 1).

**Table 1.** Distribution of influenza A virus and detection of antibodies in non-human mammalian hosts in Africa.

| Region | Country | Influenza A Virus | Influenza A Virus Antibodies | Host Species | Reference |
|---|---|---|---|---|---|
| **African Island** | Reunion Island | H1N1pdm09 | H1N1, H1, H3 | Pigs | [62] |
| **Central Africa** | Cameroon | H1N1pdm09 | H1N1pdm09, H1N2, H3N2 | Pigs | [63–65] |
| **East Africa** | Ethiopia | ND [1] | None [2] | Horses | [66] |
| | Kenya | H1N1pdm09, IAV | H1N1, H3N2, IAV | Pigs | [48,67,68] |
| | | H1N1, H3N2 | ND [1] | Olive baboons | [69] |
| | | None [2] | IAV [3] | Cats | [48] |
| | | IAV [3] | H1N1 | Dogs | [48] |
| | | ND [1] | EIV [4] | Camels | [58] |
| | Uganda | IAV [3] | IAV [3], H1 | Pigs | [70,71] |
| **North Africa** | Egypt | H3N8, H7N7, H5N1 | H3N8, H7N7, H5N1 | Equids | [52,53,59] |
| | | H1N1pdm09, H3N2, | H1N1, H1N1pdm09, H5N1, | Pigs | [51,72,73] |
| | | H5N1, H9N2 | H5N2, H5, H9 | | |
| | | ND [1] | H5N1 | Cats, Dogs, Rats | [51] |
| | | ND [1] | None [2] | Buffaloes, Cattle, | [51] |
| | | | | Goats, Sheep | |
| | | H9N2-like virus | ND [1] | Bats | [61] |
| | Morocco | ND [1] | H3N8, H7N8 | Equids [5] | [54] |
| | Sudan | ND [1] | EIV [4] | Equids [5] | [55] |
| | Tunisia | ND [1] | EIV [4] | Horses | [56] |
| **Southern Africa** | Namibia | ND [1] | H1, H5 | Black Rhino | [28] |
| | | ND [1] | H4, H11 | Wildebeest | [28] |
| | | ND [1] | H1, H3, H5, H7, H8, H9, H11, | Caracals | [28] |
| | | | H13, H14, H16 | | |
| | | ND [1] | H7 | Honey Badger | [28] |
| | | ND [1] | H1 | Lion | [28] |
| **West Africa** | Benin | None [2] | ND [1] | Pigs | [74] |
| | Burkina Faso | None [2] | H1N1, H1N1pdm09 | Pigs | [75] |
| | Côte d'Ivoire | None [2] | None [2] | Pigs | [74] |
| | Ghana | H1N1pdm09 | H1N1pdm09, H3N2 | Pigs | [20,76] |
| | | ND [1] | H3, H5, H8, H9, H12 | Bats | [60] |
| | Mali | ND [1] | H3N8 | Donkeys | [57] |
| | Niger | H3N8 | ND [1] | Donkeys, Horses | [77] |
| | Nigeria | H1N1, H3N2, H5N1, | H1N1, H1N1pdm09, H3N2, | Pigs | [65,76,78–87] |
| | | H1, H3, H5 | H5N1, H3, H7, IAV [3] | | |
| | | H3N8 | ND [1] | Donkeys, Horses | [88] |
| | | None [2] | IAV [3], H3N8 | Dogs | [49,50] |
| | Senegal | H3N8 | ND [1] | Donkeys, Horses | [77] |
| | Togo | H1N1pdm09 | ND [1] | Pigs | [89] |

[1] ND–Not Done; [2] None–Investigated but not detected; [3] IAV–Influenza A Virus (IAV–matrix gene detected but not subtyped; IAV antibodies–used multispecies ELISA kit); [4] EIV–Equine influenza virus (subtype not specified); [5] Equid-Horses, donkeys, mule.

**Table 2.** Distribution of influenza D virus and their antibodies in non-human mammalian species in Africa.

| Region | Country | Influenza D Virus | Influenza D Virus Antibodies | Host Species | Reference |
|---|---|---|---|---|---|
| **East Africa** | Ethiopia | ND [1] | IDV | Camels, Goats | [40] |
| | Kenya | ND [1] | IDV | Camels | [40] |
| **North Africa** | Morocco | ND [1] | IDV | Cattle | [40,90] |
| **West Africa** | Benin | ND [1] | IDV | Cattle | [40] |
| | | ND [1] | None [2] | Sheep, Goat | [40] |
| | Togo | None [2] | IDV | Cattle, Smail ruminants | [91] |
| | | ND [1] | IDV | Cattle, Goats, Sheep | [40] |
| | | None [2] | None [2] | Pigs | [91] |

[1] ND–Not done; [2] Not detected.

Exposure to IDV was reported in East Africa, North Africa and West Africa. IDV antibodies were detected in cattle from Benin [40], Morocco [90] and Togo [40,91], in dromedary camels from Kenya [40] and Ethiopia [92], and in small ruminants from Ethiopia and Togo (Table 2). Further, the review of the literature suggests that IDV has been circulating in Africa since 2012 as evidenced by the antibodies detected in Morocco [40].

### *3.4. Pooled Prevalence, Seroprevalence and Heterogeneity of IAVs in Pigs in Africa*

The estimated pooled prevalence of IAV in pigs in Africa was 1.6% (95% CI: 0–5%), $I^2$ = 98%, $p < 0.0001$ as shown in Table 3 and Figure 4A. African Islands and North Africa had the highest prevalence of 13.2% (95%: 10–16%) and 10.4% (0–100%), respectively, while the lowest prevalence of 0.3% (95% CI: 0–1%) was observed in East Africa. Furthermore, the pooled prevalence of IAV in pigs varied across studies ranging from 0–63% (Figure 4A).

**Table 3.** Estimated pooled prevalence and seroprevalence of IAV in pigs in Africa.

| IAV | Regions | Sample Size | No. Positive | Pooled Prevalence/Seroprevalence (%) | 95% CI [1] | $I^2$ (%) |
|---|---|---|---|---|---|---|
| Overall Prevalence | Africa | 10,703 | 370 | 1.6% | 0–55 | 98 |
| | African Islands | 474 | 62 | 13.2 | 10–16 | - |
| | Central Africa | 104 | 2 | 2.4 | 0–6 | - |
| | East Africa | 5196 | 23 | 0.3 | 0–1 | 91 |
| | North Africa | 433 | 122 | 10.4 | 0–100 | 100 |
| | West Africa | 4496 | 161 | 2.2 | 0–5 | 98 |
| Overall Sero-prevalence | Africa | 10,870 | 2095 | 14.9 | 5–28 | 99 |
| | African Islands | 1203 | 399 | 33.2 | 31–36 | - |
| | Central Africa | 98 | 27 | 27.8 | 19–37 | - |
| | East Africa | 5098 | 680 | 12.6 | 7–13 | 96 |
| | North Africa | 585 | 226 | 25.8 | 0–100 | 100 |
| | West Africa | 3886 | 763 | 14.9 | 0–41 | 99 |

[1] CI–Confidence Interval.

The estimated pooled seroprevalence of IAV in Africa was 14.9% (95% CI: 5–28%), $I^2$ = 99%, $p < 0.001$ among pigs (Table 3 and Figure 4B). The highest prevalence was recorded in African Islands with 33.2% (95% CI: 31–36%), followed by Central Africa with 27.8% (95% CI: 19–37%), North Africa with 25.8% (95% CI: 0–100%), West Africa with 14.9% (95% CI: 0–41%) and the least was 12.6% (95% CI: 7–18%) for East Africa (Table 3 and Figure 4B). Further, there was a variation in the pooled seroprevalence of IAV in pigs among individual studies ranging from 0–94% as shown in Figure 4.

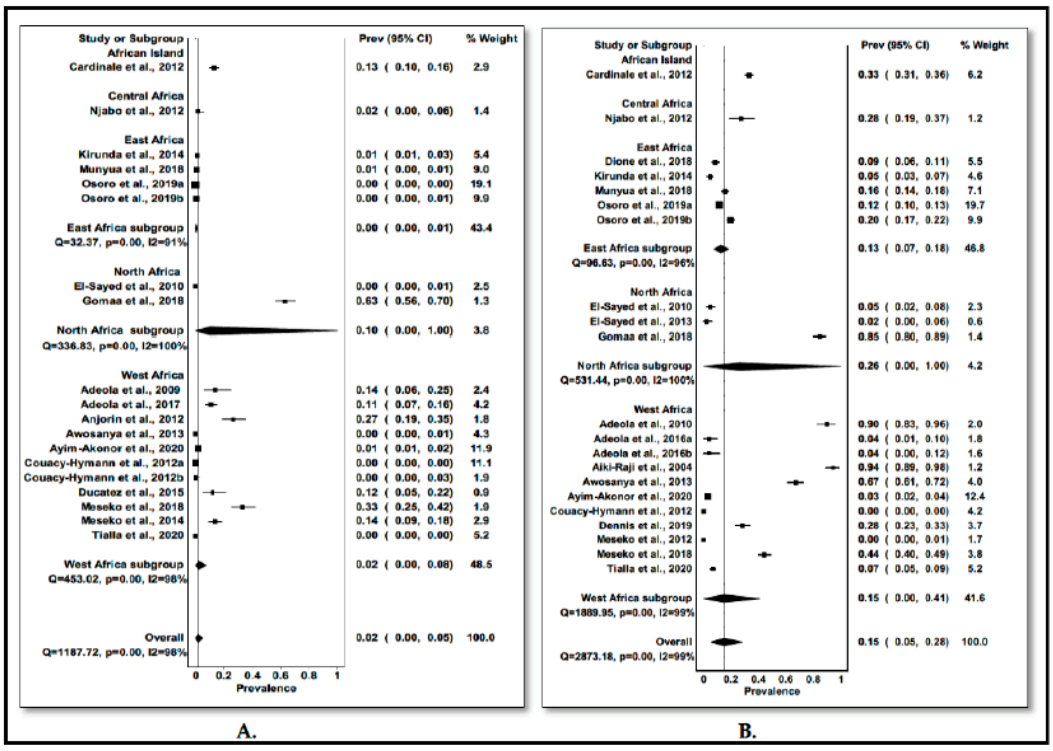


**Figure 4.** Forest plot of the prevalence and seroprevalence estimates IAV. (**A**). Forest plot of the prevalence estimates of IAV in pigs in Africa by region; (**B**). Forest plot of the seroprevalence estimates of IAV in pigs in Africa by region.

### *3.5. Pooled Seroprevalence and Heterogeneity of IDV in Non-Human Mammalian Hosts*

Of the 29 studies included in the meta-analysis, only four were on IDV. The overall seroprevalence of IDV in non-human mammalian species was 9.9% (95% CI: 0–28%), $I^2 = 99\%$, $p < 0.001$ as shown in Table 4. The seroprevalence of IDV was highest in camels with 87.2% (95% CI: 24–100%) and lowest in pigs with 0.0% (95% CI: 0–2%) (Table 4 and Figure 5).

**Table 4.** Estimated Pooled seroprevalence of IDV in non-human mammalian species in Africa.

| Subgroup | Sample Size | No. Positive | Pooled Seroprevalence (%) | 95% CI [1] | $I^2$ (%) |
|---|---|---|---|---|---|
| **Overall seroprevalence** | 3992 | 536 | 9.9 | 0–28 | 99 |
| **Cattle** | 2260 | 190 | 9.3 | 0–23 | 99 |
| **Small Ruminants** | 1321 | 35 | 2.2 | 0–4 | 73 |
| **Pigs** | 80 | 0 | 0.0 | 0–2 | - |
| **Camels** | 331 | 311 | 87.2 | 24–100 | 98 |

[1] CI–Confidence Interval.

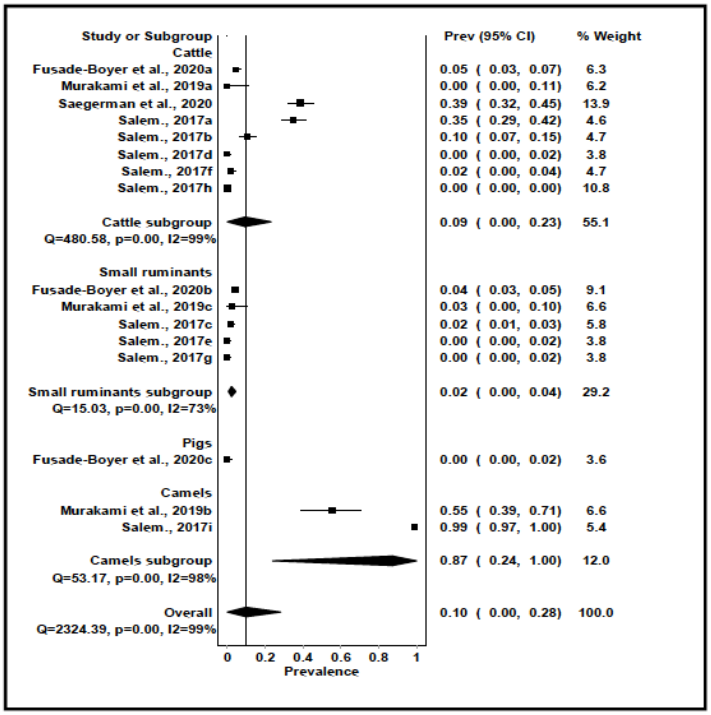


**Figure 5.** Forest Plot of the Seroprevalence of IDV according to subgroups.

## 4. Discussion

The main objective of this systematic review and meta-analysis was to investigate the prevalence and circulation of IAVs and IDVs in non-human mammalian hosts in Africa. This review included all studies found in the searched databases which reported data on prevalence, seroprevalence, virus isolation and genome detection rates of influenza A and D viruses in non-human mammalian hosts in Africa between 2000 and 2020. A total of 8785 articles were retrieved from the databases and other sources of which 169 full-texts were screened and 49 were selected and included in this review.

The results of this review and meta-analysis showed that the majority of studies were conducted in West Africa, predominantly from Nigeria. Additionally, our review demonstrated an increase in the number of studies performed after 2011. This increase in the number of studies could be attributed to the heightened interest in IAV in non-human mammalian species, especially swine, after the 2009 H1N1 pandemic. It is also possible that rigorous sampling and reporting of surveillance activities in non-human mammalian species were absent before the pandemic and more surveillance effort was concentrated on the emergence of H5N1 HPAIV as evidenced by numerous studies conducted in avian species [93]. Furthermore, the discovery of the novel IDV virus in swine in the USA and bat influenza in South America in 2011 could also have contributed to the increased number of studies of IVs in non-human mammalian species after 2011. Moreover, more studies were reported in pigs than any other animal species included in this review and meta-analysis.

The present review showed that the predominant IAVs circulating in pigs in Africa from 2000 to 2020 were H1N1 and H1N1pdm09 followed by H3N2 viruses. RNA and antibodies of the H1N1pdm09 virus were the most frequently detected among studies included in this review, suggesting that reverse zoonosis could be a common occurrence

in Africa. The H1 subtypes were detected in five regions of Africa namely African Islands [62], Central Africa [63–65], East Africa [48,68,71], North Africa [73] and West Africa [65,78,79,82,84–87], but not Southern Africa, where no reports of studies in pigs were included in this review. While H3 was detected in Central Africa [63], North Africa [73], and West Africa [65,76,78,84,85], it was not detected in African Island, East and Southern Africa. Our findings are in line with those of studies in China [94] and Korea [95,96] which detected H1 and H3 subtypes as being predominant in pigs. Furthermore, our review is in agreement with the general notion that H1N1, H1N2 and H3N1 IAVs are endemic in pigs throughout the world [14,15]. The findings also revealed the circulation of other non-pig-adapted IAV subtypes in apparently healthy pigs including the H5N1 HPAIV clade 2.3.2.1c reported in Nigeria [82], H5N1 clade 2.2.1.2, H5N1 and H5N2 viruses in Egypt [51,72,73]. The detection of viral RNA in apparently healthy pigs in Nigeria is a public health concern as it shows the silent circulation of a potentially zoonotic HPAIV in a country with a large population of pigs reared under intensive and free-range husbandry systems [82]. The other subtype detected in pigs was the H9N2 low pathogenic avian influenza virus reported in Egypt [73]. Similar observations of H5N1 and H9N2 circulation in pigs have been reported in China [94]. The exposure of pigs to avian influenza viruses (AIVs) has been attributed to the increased occurrence of AIV outbreaks in poultry in the two regions (North and West Africa) as well as pigs feeding on dead poultry carcasses or droppings of wild birds, which typically share their food [59,82]. Moreover, the co-circulation of pig adapted IAVs, non-pig-adapted IAVs and AIVs in pigs in Africa raise concern, as this may result in co-infections and possibly the generation of new reassortant viruses with pandemic potential as pigs are recognized to be "mixing vessel" of pandemic influenza virus strains [82].

The results of the meta-analysis showed an estimated pooled prevalence of 1.6% (95% CI: 0–5%) of IAV in pigs in Africa. This finding is comparable to a study in Cambodia which reported a prevalence of 1.5% of IAV in pigs [97] but lower than the 11.7–15.7% and 19.67% reported in Guatemala [98] and Mexico [99], respectively. Further, the meta-analysis demonstrated an estimated pooled seroprevalence of 14.9% of IAV in pigs in Africa. The findings are relatively similar to other studies in Britain and Wales [100], Cambodia [101], and Malaysia [102], which reported an overall seroprevalence of 12–14.9%. In contrast, higher seroprevalences of 30 to ≥50% in Belgium, Germany, Italy and Spain [103,104], 46.1% in Korea [105], 37.7% in Taiwan [106] and 22.8% in the USA [107] have been reported in pigs. The differences observed in prevalence and seroprevalence of IAV in pigs could be attributed to the region where the studies were conducted, the status of the animals (healthy or diseased), age of the animals, type of sample, sample sizes and diagnostic tests used.

The findings also demonstrated the presence or circulation of EIV in camels in Kenya [58], and horses, donkeys and mules in Egypt [52,53,59], Mali [57], Niger [77], Nigeria [83,88] Senegal [77], Sudan [55] and Tunisia [56]. The present review reported the detection of H3N8 and H7N7 antibodies and viral RNA of EIV in horses, donkeys and mules. These two subtypes of IAV have been associated with influenza virus disease in horses [16,17]. Despite the idea that H7N7 may be extinct, our review reported serological evidence of this subtype in Egypt [52] and Nigeria [54]. Further, the horses, donkeys and mules in these two studies were not vaccinated, indicating natural exposure of these equids to EIVs. Therefore, this finding may suggest the possible silent or undetected circulation of H7N7 EIV in African equids. In addition, H5N1 HPAIV clade 2.2, sub-clade 2.2.1 was detected from donkeys showing influenza-like illness in Egypt [59] suggesting active infection. Exposure of Egyptian horses and donkeys to H5N1 AIV suggests the susceptibility of equids to this virus and raises concern regarding the role of equids in the spread of the H5N1 virus to other animal species [59]. Transboundary movement of donkeys, horses and mules has been implicated in EIV infections in West Africa. It has been suggested that

herders often use donkeys to transport goods and once infected these animals can carry pathogens between regions and countries due to porous borders [77].

Serological evidence has shown that dogs could be infected with human influenza viruses, and different subtypes of IVs even coexist in dogs [108,109]. The present review demonstrated serological evidence of H1N1, H3N8 and H5N1 IAV in dogs and cats from Nigeria, Kenya and Egypt [48–51]. These results suggest that IAV could be circulating in household dogs and cats in Africa. Furthermore, pet dogs and cats share the same environment with backyard poultry and are in close contact with their owners, therefore increasing the opportunities for human exposure to these viruses. Therefore, continued surveillance of IAVs in dogs and cats is cardinal to determine the risk posed by canine-derived IAVs to public health.

This review further demonstrated the exposure of African wildlife to IAVs including lions, black rhino, spotted hyena, wildebeest, caracal, black-backed jackal, olive baboons, rats and bats [28,51,60,61,69]. The detection of IAV antibodies or antigens in wild mammals correlates with a study in Thailand and China that reported the detection of H5N1 HPAIV in leopards and tigers [23,110] though the present review did not determine whether the strains identified serologically represent low- or highly pathogenic IAV strains. The exposure of wild mammals to IAVs could be attributed to the consumption of contaminated meat in carnivores or contaminated water or feeding grounds for herbivores [28]. For example, captive carnivores, including tigers, leopards, dogs, cats, and raccoons, have been observed with influenza symptoms after consumption of contaminated meat [111–113].

Bats are reservoir hosts of many zoonotic viruses, such as the severe acute respiratory syndrome (SARS) coronaviruses, Middle East respiratory syndrome coronavirus (MERS), Nipah and Hendra viruses among others, which can cause severe disease and significant mortality in humans [114,115]. In contrast to known bat influenza viruses (H17N10 and H18N11), this review found a report of a novel H9N2-like virus (A/bat/Egypt/381OP/2017) which was detected in oral and faecal swab samples collected from Egyptian fruit bats in a densely populated agricultural area in Egypt [61]. We also found studies reporting serological evidence of IAV subtype H3, H5, H8, H9 and H12 in straw-coloured fruit bats in Ghana [60]. The H9N2-like virus is thought to be transmitted through the faecal-oral route which suggests opportunities for human exposure to this kind of virus through bat faeces and saliva on contaminated fruits [61,116]. The virological and serological detection of IAV in wild mammals highlights the risk that IAVs pose to many mammals, including humans, as their transmission dynamics and host ranges are unclear.

Studies around the globe have reported the circulation of IDV in either healthy or sick cattle, small ruminants and swine from China, France and the USA [30,32,34,117,118]. This review and meta-analysis demonstrated the presence of IDV specific antibodies in cattle from Benin, Morocco and Togo [40,90,91], camels from Ethiopia and Kenya [40,92] and small ruminants from Ethiopia and Togo [40,91,92]. However, no viral RNA of IDV was detected, possibly due to the absence of active infection in the animals during the period of sampling, the limited number of samples collected in each study, and the limited number of studies conducted in Africa. The estimated pooled seroprevalence of IDV varied widely among different host species ranging from 0.0% (95% CI: 1–2%) in pigs to 87.2% in camels (95% CI: 24–100%) with an overall seroprevalence of 10% (0–28%). With cattle being considered to be the reservoir host of IDV, it was intriguing that the highest seroprevalence was observed in dromedary camels. This suggests that these animals could be susceptible to IDV infection and are worthy of monitoring to better understand their role in the epidemiology of IDV. The seroprevalence observed in cattle, small ruminants and pigs in Africa was lower than that reported in the USA, France and Japan [32,33,117,119,120]. While studies in other parts of the world have reported IDV in pigs [36,117], the findings of this review reported a zero seroprevalence rate. This could be attributed to the small sample size of the included study which was the only study investigating IDV in pigs in this review and meta-analysis. This calls for more IDV studies to be conducted in Africa to ascertain the true picture of IDV circulation in pigs. Furthermore, the serological data of

IDV in cattle, camels and small ruminants is likely to reflect natural infection as there is no IDV vaccination in place [91].

The potential limitations of this review include language restriction due to papers published only in English, the large heterogeneity and publication bias observed across studies, sub-regions and host species. Studies were conducted in a limited number of African countries, with West Africa being overrepresented. Reasons for this discrepancy are unclear but may reflect limited technical and financial capacity, underreporting, with few articles being published in journals accessible online, and animal influenza not being a research priority for some regions of the continent. Therefore, more studies on IAVs and IDVs in non-human mammalian species need to be conducted in Africa to identify the annual and seasonal patterns in prevalence and seroprevalence as well as to monitor the evolution and circulation of these viruses, thus assisting in preparing for potentially emerging influenza viruses of animal origin in humans.

## 5. Conclusions

This review and meta-analysis found that IAVs and IDVs are currently circulating in non-human mammalian hosts in Africa with an estimated pooled prevalence and seroprevalence of 1.6% and 14.9% in pigs, respectively, while the seroprevalence of IDV was estimated to be 9.9%. Pig and non-pig adapted IAVs are currently circulating in Africa with H1N1 and H1N1pdm09 predominating. Furthermore, virological and/or serological evidence of H3N8 and H7N7 in equids, H1N1, H3N8 and H5N1 in dogs and cats were reported. Therefore, the circulation of these viruses in non-human mammalian hosts underscores the need for continued IAV surveillance in different animal species to evaluate and possibly mitigate potential threats that these viruses may pose to public health, wildlife and the livestock industry. This may help develop new surveillance plans and determine high-risk regions. Further, we recommend more research to be conducted across Africa to ascertain the impact of influenza A and D viruses in non-human mammalian hosts in Africa.

**Supplementary Materials:** The following are available online at https://www.mdpi.com/article/10.3390/v13122411/s1, Checklist S1: PRISMA checklist, Protocol S1: Detailed literature search strategy, Checklist S2: Quality assessment checklist, Table S1: Studies included in the systematic review and meta-analysis, Table S2: Characteristics of the included studies, Figure S1: Funnel plots used to assess publication bias, Figure S2: Doi plots with a pseudo 95% confidence used to assess publication bias.

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viruses

MDPI

Review

# Avian Influenza Viruses Detected in Birds in Sub-Saharan Africa: A Systematic Review

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**Abstract:** In the recent past, sub-Saharan Africa has not escaped the devastating effects of avian influenza virus (AIV) in poultry and wild birds. This systematic review describes the prevalence, spatiotemporal distribution, and virus subtypes detected in domestic and wild birds for the past two decades (2000–2019). We collected data from three electronic databases, PubMed, SpringerLink electronic journals and African Journals Online, using the Preferred Reporting Items for Systematic reviews and Meta-Analyses protocol. A total of 1656 articles were reviewed, from which 68 were selected. An overall prevalence of 3.0% AIV in birds was observed. The prevalence varied between regions and ranged from 1.1% to 7.1%. The Kruskal–Wallis and Wilcoxon signed-rank sum test showed no significant difference in the prevalence of AIV across regions, $\chi^2(3) = 5.237$, $p = 0.1553$ and seasons, $T = 820$, $z = -1.244$, $p = 0.2136$. Nineteen hemagglutinin/neuraminidase subtype combinations were detected during the reviewed period, with southern Africa recording more diverse AIV subtypes than other regions. The most detected subtype was H5N1, followed by H9N2, H5N2, H5N8 and H6N2. Whilst these predominant subtypes were mostly detected in domestic poultry, H1N6, H3N6, H4N6, H4N8, H9N1 and H11N9 were exclusively detected in wild birds. Meanwhile, H5N1, H5N2 and H5N8 were detected in both wild and domestic birds suggesting circulation of these subtypes among wild and domestic birds. Our findings provide critical information on the eco-epidemiology of AIVs that can be used to improve surveillance strategies for the prevention and control of avian influenza in sub-Saharan Africa.

**Keywords:** *Orthomyxoviridae*; avian influenza; avian influenza virus; subtype; ecology; epidemiology; poultry; wild waterfowl; sub-Saharan Africa

## 1. Introduction

Avian influenza is an acute and highly infectious viral disease caused by influenza A viruses (IAVs) of the genus *Alphainfluenzavirus*, family *Orthomyxoviridae*. Avian influenza viruses (AIVs) are important zoonotic pathogens that may cause high morbidity and mortality resulting in substantial economic losses to the poultry industry [1–3]. Migratory avian species within the orders Anseriformes

(ducks, geese and swans) and Charadriiformes (gulls, terns and shorebirds) are known to be the natural reservoirs of IAVs [4,5]. While IAVs are principally found in wild aquatic birds, they infect other mammalian species such as humans, horses, pigs, cats, dogs, seals and whales among others [6,7]. Moreover, an IAV-like virus has recently been discovered in bats [8,9], suggesting a possible new natural reservoir host.

IAVs are enveloped, single-stranded, negative-sense RNA viruses with a segmented genome. Eight segments of the IAV genome encode up to 18 proteins: polymerase basic 1 (PB1), polymerase basic 2 (PB2), polymerase acid (PA), hemagglutinin (HA), nucleoprotein (NP), neuraminidase (NA), matrix 1 (M), matrix 2 (M2), nonstructural (NS1), nuclear export protein (NS2/NEP), PB1-F2, PB1-N40, PA-X, M42, PA-N155, PA-182, NS3 and PB2-S1 [10–24]. The HA and NA proteins are surface glycoproteins, essential for virus infectivity, and are used in the classification of IAVs into subtypes. Currently, 18 HA and 11 NA subtypes of IAV have been identified, of which 16 HA (H1–H16) and 9 NA (N1–N9) are maintained up to now in avian species [25,26], while 2 HA (H17–H18) and 2 NA (N10–N11) were found in bats [9]. However, some IAV strains have been known to be maintained in mammalian hosts, for example, H1N1 and H3N2 circulate seasonally in humans [27]. Additionally, the HA and NA proteins have the highest evolutionary rates of all influenza virus proteins, which contributes to the genetic and antigenic diversity of these viruses. The genetic diversity is mainly due to two mechanisms, antigenic drift and shift, which involve the accumulation of point mutations over time and the exchange of genome segments between two or more influenza viruses (i.e., genetic reassortment), respectively [28]. These two mechanisms may lead to the emergence of novel strains of IAVs with zoonotic and pandemic potential, which may pose a challenge for control [29].

AIVs can be classified based on their pathogenicity or virulence in chickens as either highly pathogenic avian influenza (HPAI) or low pathogenic avian influenza (LPAI) viruses. The HPAI viruses often become highly pathogenic through the acquisition of multiple basic amino acid residues at the HA cleavage site and are restricted to two subtypes H5 and H7 [30]. However, not all H5 or H7 viruses have the capacity to become HPAI viruses [31,32]. Outbreaks of HPAI viruses constitute a substantial risk to human health, the poultry industry and the global economy [33]. Since the first detection of H5N1 HPAI viruses in Asia, the viruses have spread throughout the world leading to multiple outbreaks affecting millions of birds and considerable human infections [34–36]. Moreover, in March 2013, H7N9 LPAI virus emerged in eastern China and caused high morbidity and mortality in humans with an overall case fatality ratio of approximately 37% [37,38]. Furthermore, IAVs have caused at least four major pandemics (1918 "Spanish flu", 1957 "Asian flu", 1968 "Hong Kong flu" and the 2009 "swine flu") in the human population from the 20th century to date, with the worst being the 1918 "Spanish flu" H1N1 pandemic, which recorded nearly 500 million cases and 50 million human deaths globally [39,40]. Although LPAI viruses have typically been known to cause inapparent infections in poultry, some subtypes have caused severe clinical signs in poultry, such as the H9N2 LPAI virus, which has been reported to cause respiratory disease, a reduction in egg production and mortality in birds [41,42].

The importance of poultry farming in sub-Saharan Africa cannot be overemphasized as it is one of the most rapidly growing sectors and an important source of protein and income for several rural households in the region [43]. In Africa, poultry numbers have been estimated to be approximately 1.1 billion [44,45]. Despite the growth of poultry production in the region, the birds are usually reared under poor biosecurity measures, which provide the ideal setting for zoonotic transmission of AIVs [46]. It is also worthy to note that sub-Saharan Africa is a seasonal shelter for a large number of migratory aquatic birds that make their seasonal movements between the temperate zone and the tropics [47], with approximately 5.4 million ducks that gather during the northern winter [48]. These birds congregate and mix with the indigenous water birds in their overwintering sites, which provides opportunities for dissemination and transmission of AIVs between different populations and continents [48].

Despite several studies documenting the presence and impact of AIVs in sub-Saharan Africa [48–54], there is a lack of consolidated data on the eco-epidemiology of these viruses in birds in the region. Therefore, in this review, we aimed at systematically integrating data from different studies within the region to provide the prevalence, spatiotemporal distribution and virus subtypes detected in domestic and wild birds from January 2000 to December 2019.

## 2. Materials and Methods

### *2.1. Literature Search and Data Collection*

To systematically review the literature, we followed the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) protocol 2010 guidelines (S1 Checklist) [55]. Inclusion and exclusion criteria were defined in terms of the relevance of the articles to achieve the study objectives.

A systematic literature search was conducted to identify all publications reporting the detection of avian influenza virus in birds in sub-Saharan Africa between 2000 and 2019. Three electronic databases namely PubMed, SpringerLink electronic journals and African Journals Online (AJOL) were searched using the medical subject headings (MeSH) keywords and Boolean connectors. The following keywords were used: "influenza in birds," "influenza," "birds," "avian," "avian influenza," "avian influenza virus," "sub-Saharan Africa," "countries in sub-Saharan Africa," "epidemiology," "prevalence" and "subtype." Furthermore, the search was restricted to original articles, titles, abstracts and keywords published in English, which reported on AIVs using serological and/or molecular methods. The last search was conducted on 17th January 2020. All references located in the searches were entered into Endnote, a web-based reference manager. Furthermore, a database was built that included the references of all selected publications, as well as the title, author, year of publication, country or countries where the study was conducted and language of publication. The articles were selected using a two-stage approach. During the first stage, the publications were selected based on their titles and abstracts. During the second stage, the full text of articles selected in the first stage was assessed for eligibility. At this point, the articles that did not meet the inclusion criteria were excluded.

### *2.2. Inclusion Criteria*

All study designs were included in this review except experimental studies as these do not represent natural infections. Additionally, studies published between 2000 and 2019, serological, molecular and both serological and molecular studies on AIV in birds in sub-Saharan Africa were investigated. All AIV subtypes and avian species from which the virus was detected were included in this review. Additionally, publications containing data on the positive diagnostic test result, data on incidence, prevalence and distribution of avian influenza in any naturally infected birds were included.

### *2.3. Exclusion Criteria*

Studies published before 2000 and after 31st December 2019, editorials, comments/letter to the editor, congress or conference abstracts, review articles, perspectives, personal opinions, theoretical models, pathogenesis models, animal models, case reports in humans, or reports in non-avian species and studies reported in languages other than English were not included. Moreover, studies with the following characteristics were excluded: the diagnostic test not specified, sample source not described, publications reporting data published elsewhere other than sub-Saharan Africa, outbreak reports without laboratory-based confirmation, reporting a zero incidence/prevalence in any diagnostic test, studies with data overlapping with another included study and publications exclusively on the experimental infection. For prevalence and seasonality of AIV analysis, all studies without sampling time, sample size, prevalence or rate were excluded. Additionally, studies with a sample size of less than five were excluded.

### *2.4. Data Extraction*

A database on reference information regarding the author's name, title and year of publication was recorded in the data extraction file. Furthermore, from the included publications, data were extracted on country or countries of study including region, years of sample collection, avian species, the purpose of study, number of samples analyzed, type of samples collected, the diagnostic method(s) used, number of positives and pathogenicity of the AIV subtypes (LPAI or HPAI).

### *2.5. Assessment of Quality and Risk Bias of the Included Studies*

To assess the quality and risk of bias of the included studies, the McMaster Critical Review Form—Qualitative Studies (version 2.0) [56] and McMaster Critical Review Form—Quantitative Studies [57] were used.

### *2.6. Statistical Analysis*

Data were entered in Microsoft Office Excel 2018 and analyzed using Python 3.7 for Mac. Prevalence and distributions of AIV were reported using descriptive statistics in the form of frequencies, percentages and presented as tables and graphs. Since data were not normally distributed, non-parametric tests such as the Kruskal–Wallis test and Wilcoxon signed-rank sum test were used to determine associations between seasonality, regions and prevalence of AIV. A Pearson correlation was carried out to determine the association between time in years and number of papers published between 2000 and 2019.

## 3. Results

### *3.1. Search Results and Study Selection*

During the literature search, PubMed yielded 1313 records, SpringerLink electronic journals had 293 and AJOL showed 50 records, giving a total of 1656 research records. Of the 1656 research records, 678 (40.9%) were duplicates and were discarded. Using the set inclusion and exclusion criteria, we screened the remaining 978 records following a flow chart (Figure 1). During the screening, 870/978 (89.0%) articles were excluded based on their titles and abstracts, while 108 articles were retained. The 108 full-text articles were further screened for eligibility and 40/108 (37.0%) were excluded, while a total of 68/108 (63.0%) articles were deemed eligible for inclusion in this systematic review. Table S1 is provided as a Supplemental Material of all included studies.

### *3.2. Characteristics of the Included Studies*

The reviewed articles were published between 2000 and 2019, from 22 sub-Saharan African countries. One article (1.5%) was published between 2000 and 2004, 16 (23.5%) between 2005 and 2009, 23 (33.8%) between 2010 and 2014 and 28 (41.2%) between 2015 and 2019 (Figure 2).

A Pearson correlation was computed to assess the relationship between years of publication and the numbers of articles published. There was a positive correlation between the two variables ($r = 0.81$, $n = 20$, $p < 0.0001$). This shows that there was a fairly strong positive and significant increase in the number of published articles between the years 2000 to 2019 (Figure 3).

A review of selected publications yielded a total of 83 published records on the presence of avian influenza in sub-Saharan Africa. Most articles (26.8%) were for studies done in Nigeria followed by those in South Africa (17.1%) (Figure 4).

Of the articles included in this review, 39 (57.4%) were surveillance studies, while 29 (42.6%) were a combination of either case studies or case reports. Additionally, the studies used either molecular, serological or both molecular and serological techniques to detect AIVs in different species of birds. Specifically, 29 (42.6%) studies employed molecular methods, 12 (17.6%) applied serological methods and 27 (39.7%) used both molecular and serological methods.

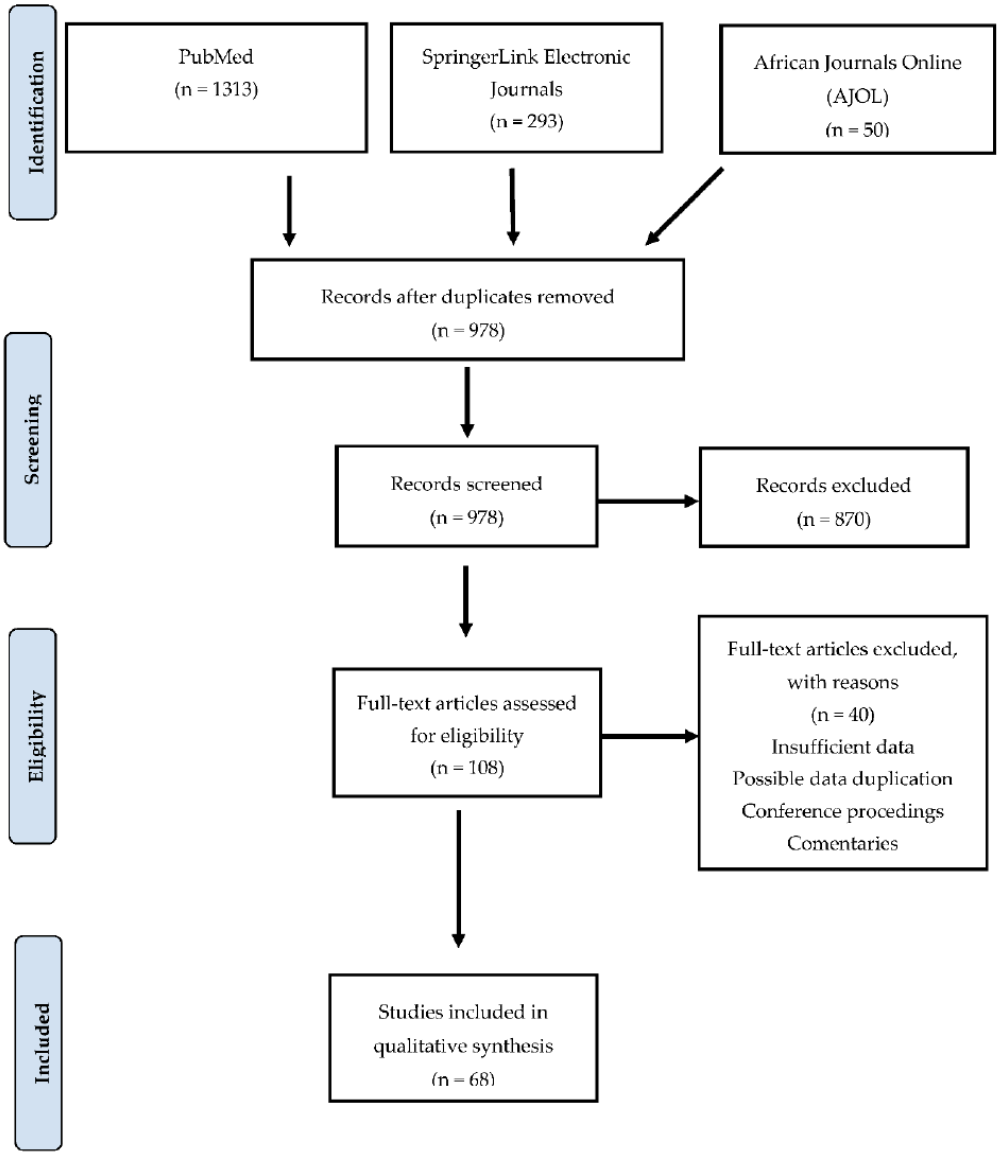


**Figure 1.** Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) flow chart of the literature search, screening, assessing eligibility and article selection.

### *3.3. The Prevalence and Seasonality of AIV in Birds in Sub-Saharan Africa*

In many studies, neither prevalence nor sample size was included. Of the 68 studies included in this review, 55 studies with either prevalence values or sample sizes were found. The overall prevalence (determined based on virus isolation and genome detection) and seroprevalence of AIVs and in avian species in sub-Saharan Africa was 3.0% (Table 1) and 4.1% (Table 2), respectively. During the analysis, we divided sub-Saharan Africa into regions namely, Central, East, Southern and West Africa for easier analysis and due to their unique seasons of the year. The prevalence varied between regions and ranged from 1.1% to 7.1% (Table 1), while seroprevalence ranged from 2.2% to 4.1% (Table 2). The majority of the serosurveys were conducted in poultry studies and focused on the detection of H5 and H7 antibodies. We carried out the Kruskal–Wallis test of independent samples and the Wilcoxon signed rank sum test to determine whether the prevalence distribution of AIV was different across

seasons and regions in sub-Saharan Africa. Our results showed that there was no significant difference in prevalence across regions $\chi^2(3) = 5.237$, $p = 0.1553$ and seasons T = 820, z = −1.244, $p = 0.2136$, respectively. However, the highest detection rates of AIVs were observed during the dry season (6.7%) (that is May to October in Central Africa) (Table 3). The lowest prevalence was obtained in the wet season (0.4%) (November to April in Central Africa) (Table 3).

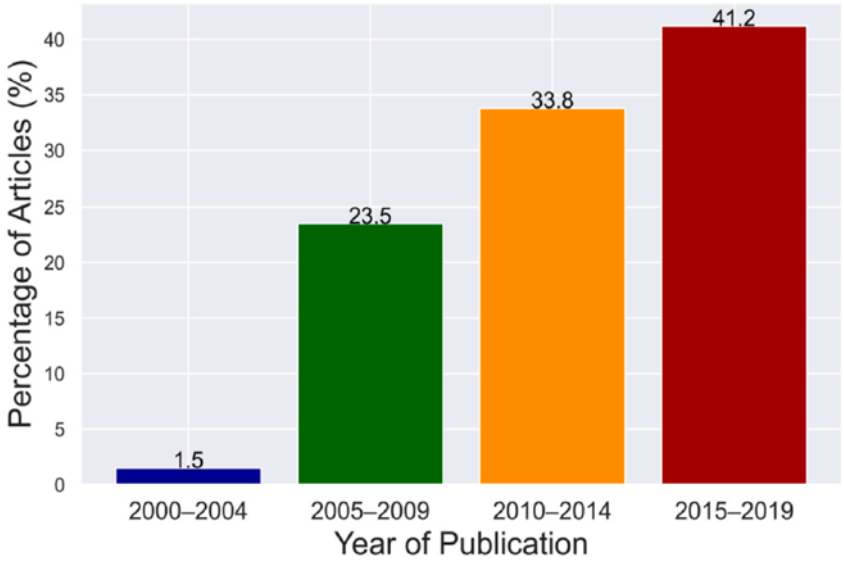


**Figure 2.** Number of selected articles per quarter from 2000 to 2019.

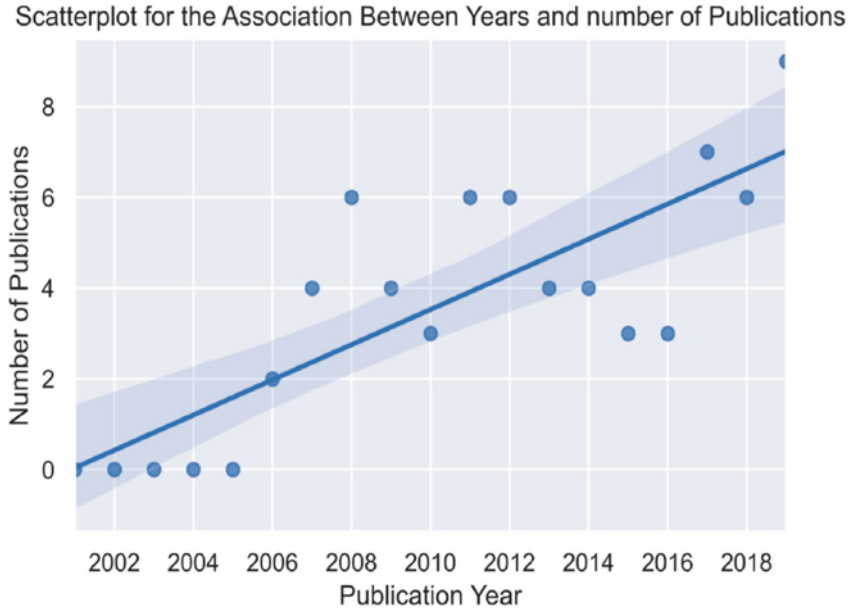


**Figure 3.** Scatter plot showing the correlation between years and number of publications.

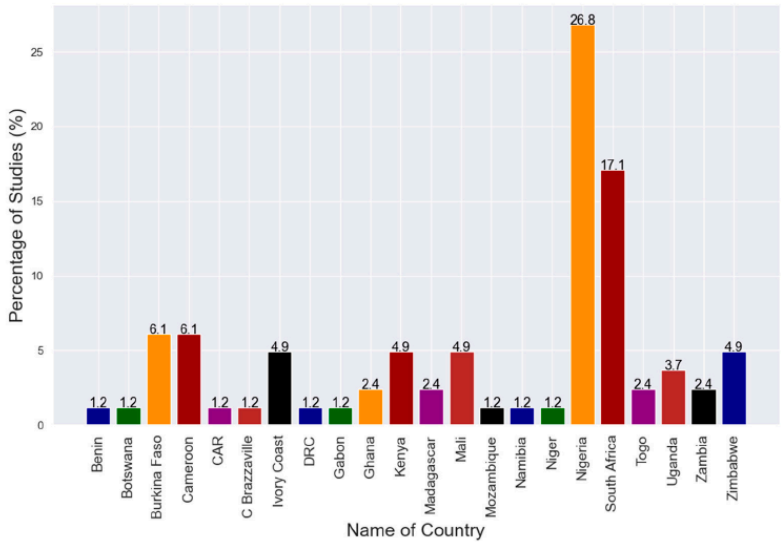


**Figure 4.** Reviewed articles according to countries.

**Table 1.** Prevalence of avian influenza virus (AIV) in sub-Saharan Africa.

| Study Region | No. of Studies | No. Samples | No. Positive | Prevalence (%) |
|---|---|---|---|---|
| Central Africa [a] | 4 | 4868 | 344 | 7.1 |
| East Africa [b] | 6 | 13,875 | 146 | 1.1 |
| West Africa [c] | 19 | 38,203 | 1269 | 3.3 |
| Southern Africa [d] | 12 | 12,518 | 349 | 2.8 |
| Total | 41 | 69,464 | 2108 | Overall prevalence: 3.0 |

Study regions: [a] Central African Republic, Congo Brazzaville, Cameroon and Democratic Republic of Congo, [b] Kenya, Uganda, [c] Benin, Burkina Faso, Ivory Coast, Gabon, Ghana, Mali, Niger, Nigeria, Togo, [d] Botswana, Namibia, South Africa, Zambia, Zimbabwe.

**Table 2.** Seroprevalence of AIV in sub-Saharan Africa.

| Study Regions | No. of Studies | No. of Samples | No. Samples | Seroprevalence (%) |
|---|---|---|---|---|
| Central Africa [a] | 0 | - | - | - |
| East Africa [b] | 2 | 3517 | 77 | 2.2 |
| West Africa [c] | 11 | 16,669 | 875 | 5.2 |
| Southern Africa [d] | 4 | 235,084 | 9605 | 4.1 |
| Total | 18 | 255,270 | 10,557 | Overall seroprevalence: 4.1 |

Study regions: [a] Central African Republic, Congo Brazzaville, Cameroon and Democratic Republic of Congo, [b] Kenya, Uganda, [c] Benin, Burkina Faso, Ivory Coast, Gabon, Ghana, Mali, Niger, Nigeria, Togo, [d] Botswana, Namibia, South Africa, Zambia, Zimbabwe.

**Table 3.** Prevalence of AIV according to regions and seasons [a].

| Study Region | No. of Studies | No. of Samples | Dry Season (%) | Wet Season (%) | Reference |
|---|---|---|---|---|---|
| **Central Africa** | 4 | 4868 | 325 (6.7%) | 19 (0.4%) | [53,58–60] |
| **East Africa** | 5 | 9556 | 71 (0.7%) | 46 (0.5%) | [45,61–64] |
| **West Africa** | 18 | 11,680 | 319 (2.7%) | 158 (1.4%) | [41,65–81] |
| **Southern Africa** | 11 | 6009 | 165 (2.7%) | 48 (0.7%) | [49,51,82–90] |
| **Total (Overall Prevalence%)** | 38 | 32,113 | 880 (2.7%) | 271 (0.8%) | |

[a] In sub-Saharan Africa, seasons differ according to the regions: Central/Southern Africa—dry season (May–October) and wet season (November–April); East Africa—dry season (January–March/June–October) and wet season (April–June/November–December); West Africa—dry season (January–March/June–October) and wet season (April–June/November–December).

### *3.4. Distribution of the AIV Subtypes and Avian Species*

As depicted in Table 4 and Supplementary Table S2, the included studies reported a diverse range of AIV subtypes in different avian species in the past 20 years. According to the analyzed articles, 52/68 (76.5%) of studies specified the AIV subtypes, while 16/68 (23.5%) did not. During the period under review, nine different HA subtypes and six NA subtypes were found in 19 different subtype combinations (Table 4). The 19 AIV subtypes detected were as follows: H1N2, H1N8, H3N6, H3N8, H4N2, H4N6, H4N8, H5N1, H5N2, H5N8, H6N2, H6N8, H7N1, H7N7, H9N1, H9N2, H10N7, H10N9 and H11N9. Southern Africa recorded a wider range of AIV subtype combinations than any other region in sub-Saharan Africa (Table 4). The NA subtypes were not determined for three HA subtypes H5, H6 and H7. Of the 5073 viruses detected, the H5 (78.5%) subtype was the most common, followed by H9 (2.5%), H6 (1.4%), H7 (1.1%) and the rest falling below 1.0%. The H5Nx and H7Nx were either characterized as LPAI viruses or were not characterized at all (Supplementary Table S2). Overall, the most detected subtype combinations were H5N1, followed by H9N2, H5N2, H5N8 and H6N2. The majority of H5N1 and H5N8 subtypes were HPAI viruses and were commonly detected in domestic poultry especially chicken (*Gallus gallus*) and domestic ducks (*Anas platyrhynchos domestica*) (Supplementary Table S2).

**Table 4.** AIV subtype detection in sub-Saharan Africa according to the included studies.

| Study Region | Central Africa | East Africa | Southern Africa | West Africa |
|---|---|---|---|---|
| HA Subtypes Detected | H5 | H4, H5, H9 | H1, H3, H4, H5, H6, H7, H9, H10, H11 | H3, H5, H7, H9 |
| Most Prevalent HA Subtypes | H5 | H5 | H5 | H5 |
| HA Subtypes not Detected | H1–H4, H6–H16 | H1–H3, H6–H8, H10–H16 | H2, H8, H12–H16 | H1–H2, H4, H6, H8, H10–H16 |
| Prevalent NA Subtypes | N1, N8 | N2, N6, N8 | N1, N2, N6, N7, N8, N9 | N1, N2, N7, N8 |
| NA Subtypes not Detected | N2, N3, N4, N5, N6, N7, N9 | N1, N3, N4, N5, N7, N9 | N3, N4, N5 | N3, N4, N5, N6, N9 |
| Prevalent Subtype Combinations | H5N1, H5N8, H5Nx | H4N6, H5N8, H9N2, H5Nx | H1N2, H1N8, H3N6, H3N8, H4N2, H4N6, H4N8, H5N1, H5N2, H5N8, H6N2, H6N8, H9N2, H10N7, H10N9, H11N9, H5Nx, H6Nx, H7Nx | H3N8, H5N1, H5N2, H7N7, H9N2, H5Nx, H7Nx |
| Most Prevalent Subtype Combinations | H5N1 | H5N8 | H5N2, H5N8, H6N2 | H5N1, H9N2 |

Note: HA = hemagglutinin; NA = neuraminidase; Nx = unknown NA subtype.

Overall, the 52 studies that specified the AIV subtypes recorded both HPAI or LPAI viruses among domestic and wild birds (Supplementary Table S2). Majority of the studies that reported HPAIVs were conducted between 2006–2008 and 2017–2018. Furthermore, the majority of studies that reported HPAIVs were conducted in Nigeria 11 (21.2%), followed by Burkina Faso and South Africa 4 (7.6%) each, Cameroon 3 (5.8%) and 2 (3.8%) from Cote d'Ivoire. The Democratic Republic of Congo (DRC), Ghana, Niger, Namibia, Togo and Uganda recorded 1 (1.9%) HPAIV study each. The rest of the studies 22 (42.3%) recorded either LPAIVs or did not specify the pathogenicity of the detected subtype. Zambia and Zimbabwe did not report any HPAIVs according to the 68 studies included in this review (Figure 5). As depicted in Supplementary Table S2, all the HPAIVs from Cameroon, DRC, Ghana, Niger and Togo were only detected in domestic birds, while those from Burkina Faso, Cote d'Ivoire, Nigeria and South Africa were detected in both domestic and wild birds. H5N1, H5N2 and H5N8 HPAI viruses were detected mostly in domestic poultry. Further, the H5N2 HPAI virus was more common among ostriches (*Struthio camelus australis*) than any other bird species included in this study. Notably, the H5N8 HPAI virus was detected in African penguins (*Spheniscus demersus*) in Namibia leading to the most severe mortality on record for this species in Namibia, with more than 350

penguins dead [89]. Furthermore, the H5N1 HPAI virus was detected in Burkina Faso from hooded vultures (*Necrosyrtes monachus*) that exhibited dyspnea and neurological signs [69].

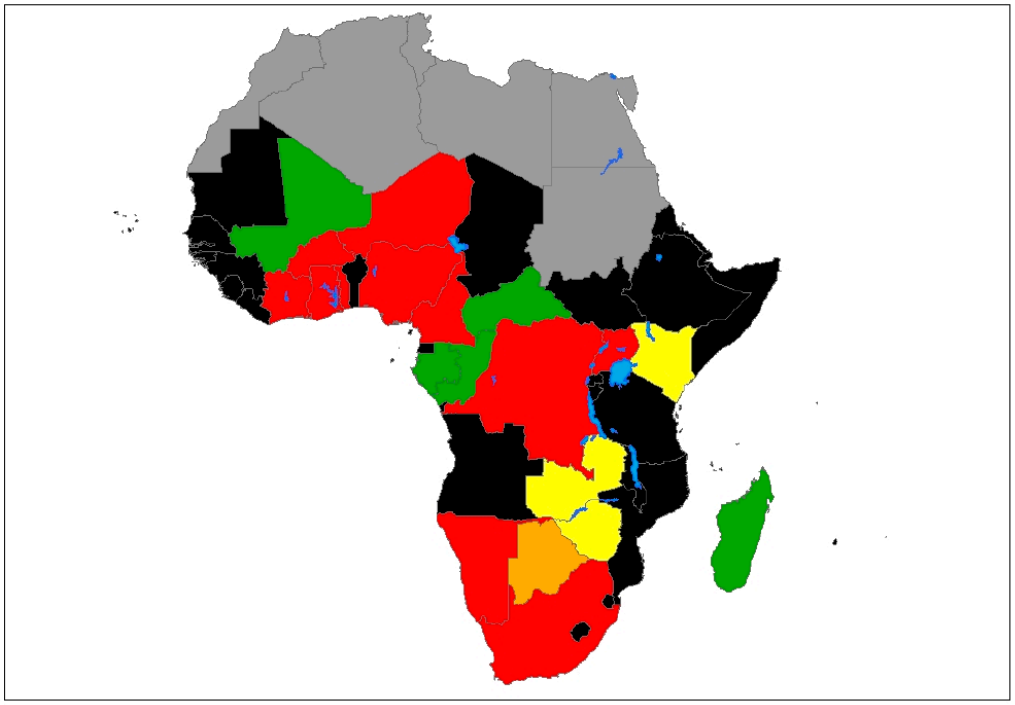

**Figure 5.** Geographical distribution of highly pathogenic avian influenza (HPAI) and low pathogenic avian influenza (LPAI) subtypes in sub-Saharan Africa. Color codes: red denotes countries reporting either HPAI only or both HPAI and LPAI; orange denotes countries reporting H5 or H7, but whose pathogenicity was not determined; yellow denotes countries reporting LPAI; green denotes countries reporting AIV whose subtypes and pathogenicity were not determined; black denotes countries in sub-Saharan Africa with no reports of AIV in birds in the study period; gray denotes North African countries not included in the study; blue denotes major water bodies.

H9N2, H3N8, H5N2, H7N7 and H6N2 LPAI viruses were also detected in domestic poultry. For example, H9N2 LPAI virus detected in Burkina Faso [91] and Ghana [41] and H6N2 LPAI virus in South Africa [92,93] were from symptomatic poultry with signs of respiratory distress, decreased egg production and increased mortality, while in Kenya [64] and Nigeria [78,94] viruses with subtypes H9N2, H7N7, H5N2 and H3N8 were detected in asymptomatic birds. Moreover, mixed infection of H3N8 and H5N2 LPAI viruses was detected in apparently health turkeys (*Meleagris* Spp.) in Nigeria. Furthermore, H1N6, H3N6, H4N6, H4N8, H9N1 and H11N9 LPAI viruses were exclusively detected in wild birds.

### *3.5. Distribution of AIVs among Different Avian Species*

The distribution of AIVs among different avian species according to the articles included in this review is depicted in Table 5. The majority of the studies were conducted in domestic birds with the highest number of studies (35) being in chicken (*Gallus gallus*) and (20) in domestic ducks (*Anas platyrhynchos domestica*) in 12 and 10 different countries, respectively (Table 5). Notably, studies in ostriches were reported only in South Africa [83,86,95–99]. In wild birds, most studies (6) were in Egyptian geese (*Alopochen aegyptiacus*) done in Kenya, South Africa and Zambia followed by 4 studies in unspecified wild birds. For the rest of the free-flying and aquatic birds, 1 to 3 studies were conducted in various sub-Saharan African countries as shown in Table 5.

**Table 5.** Avian host range of AIV according to the 68 publications included in the review.

| Host | Number of Publications * | Number of Countries | Names of Countries |
|---|---|---|---|
| **Domestic Birds** | | | |
| Chicken | 35 | 12 | Burkina Faso, Cameroon, Cote d'Ivoire, Ghana, Kenya, Madagascar, Mali, Nigeria, South Africa, Togo, Uganda |
| Domestic duck | 20 | 10 | Cameroon, Cote d'Ivoire, DRC, Ghana, Kenya, Madagascar, Mali, Nigeria, South Africa, Uganda |
| Ostrich | 8 | 1 | South Africa |
| Turkey | 7 | 7 | Cameroon, Cote d'Ivoire, Kenya, Mali, Nigeria, South Africa, Uganda |
| Domestic guinea fowl | 7 | 5 | Burkina Faso, Cameroon, Mali, Nigeria, South Africa |
| Pigeon | 5 | 3 | Cameroon, Nigeria, South Africa |
| Domestic geese | 4 | 4 | Cameroon, Kenya, Madagascar, South Africa |
| Poultry [a] | 3 | 3 | Nigeria |
| Indian peafowl | 2 | 1 | Cameroon |
| **Wild Birds** | | | |
| Egyptian goose | 6 | 3 | Kenya, South Africa, Zambia |
| Wild species [a] | 4 | 3 | Nigeria, Africa, South Africa |
| White-faced whistling duck | 3 | 3 | Kenya, Nigeria, Mali |
| Yellow-billed duck | 3 | 2 | Kenya, South Africa |
| Hooded vulture | 3 | 1 | Burkina Faso |
| Red-billed quelea | 2 | 1 | Mali, Zimbabwe |
| Red-billed teal | 2 | 1 | South Africa |
| Great white pelican | 2 | 1 | Zambia |
| Spur-winged goose | 2 | 1 | Nigeria |
| Duck | 2 | 2 | Zambia, Zimbabwe |
| Cattle egret | 2 | 2 | Nigeria, Zimbabwe |
| Barn swallow | 2 | 1 | Zimbabwe |
| African sacred ibis | 2 | 1 | South Africa |
| Turtle dove | 2 | 1 | Cote d'Ivoire |
| Cape teal | 2 | 2 | Kenya, South Africa |
| African penguin [b] | 1 | 1 | Namibia |
| Sparrowhawk | 1 | 1 | Cote d'Ivoire |
| Dove | 1 | 1 | Cote d'Ivoire |
| Crow | 1 | 1 | Cote d'Ivoire |
| Weaver | 1 | 1 | Cote d'Ivoire |

**Table 5.** *Cont.*

| Host | Number of Publications * | Number of Countries | Names of Countries |
|---|---|---|---|
| Hottentot teal | 1 | 1 | Kenya |
| Red-knobbed coot | 1 | 1 | Kenya |
| Garganey | 1 | 1 | Mali |
| Ruff | 1 | 1 | Mali |
| Northern pintail | 1 | 1 | Mali |
| Purple swamphen | 1 | 1 | Mali |
| Common moorhen | 1 | 1 | Mali |
| Comb duck | 1 | 1 | Mali |
| Gull-billed tern | 1 | 1 | Mali |
| Spotted redshank | 1 | 1 | Mali |
| Speckled pigeon | 1 | 1 | Nigeria |
| Canada goose | 1 | 1 | Nigeria |
| Gray crown crane | 1 | 1 | Nigeria |
| African gray parrot | 1 | 1 | Nigeria |
| Cape shoveler | 1 | 1 | South Africa |
| Swift tern | 1 | 1 | South Africa |
| White-winged black tern | 1 | 1 | South Africa |
| Hadada ibis | 1 | 1 | South Africa |
| Shelduck | 1 | 1 | South Africa |
| Brown-throated martin | 1 | 1 | Kenya |

* Multiple publications reported on multiple animal species; [a] poultry and wild species not clearly specified; [b] sea bird; wild birds include free-flying wild birds and aquatic waterfowl.

## 4. Discussion

This systematic review is aimed at investigating the prevalence, spatiotemporal distribution, and virus subtypes of AIVs detected in domestic and wild birds over a two-decade period (2000–2019) in sub-Saharan Africa. We retrieved many study articles from the databases, but only 68 studies from 22 different countries in sub-Saharan Africa met our inclusion criteria. Our findings showed a significant increase in the number of studies published between 2000 and 2019. This increase could be attributed to the increase in the number of AIV outbreaks recorded within the region, with Nigeria recording 1205 suspected outbreaks [100] between 2006 and 2007. Additionally, there has been an increase in surveillance studies [49,51,66,67,85,98,101–104] regionally and in different countries, improved laboratory diagnostic capacity and generally enhanced surveillance systems aimed at preventing HPAI outbreaks.

The AIV prevalence generally varied from region to region with the highest prevalence being reported in Central Africa (7.1%) and the lowest in East Africa (1.1%) (Table 1). Moreover, our findings indicate that AIVs were detected throughout the year in sub-Saharan Africa with higher prevalence during the dry season. Whilst it is generally understood that the prevalence of AIV infection tends to increase during the period when Eurasian migratory water birds overwinter in sub-Saharan Africa and decrease after they migrate back to Eurasia [105], this study revealed a higher prevalence in the dry season when Eurasian migratory birds are absent or rare. It is possible that the limited water bodies in the dry season may allow increased interaction of waterfowl by congregating at particular sites, which provides opportunities for AIV transmission as well as detection during surveillance activities. However, despite the observed variations in prevalence and seasonality of AIV infection, the difference was not statistically significant. Further, the finding indicates that AIVs are perpetuated in migratory water birds originating from Eurasia as well as in indigenous African species that remain in the continent throughout the year.

Wild migratory aquatic birds are known to be the natural reservoir of AIVs globally [4,5]. Therefore, it is not surprising that different wild birds in sub-Saharan Africa harbor AIVs according to the findings of this review. Our findings also highlight the impact of AIVs on migratory and non-migratory local birds. Analysis of HA subtype diversity revealed that H5 was the most predominant HA subtype detected in this review followed by H9, H6 and H7. The predominance of these subtypes could be attributed to the fact that most of the AIV surveillance activities have concentrated on the detection of subtypes H5, H7 and/or H9 [106,107] and that they cause dramatic devastation, particularly HPAI that is difficult to miss. The H5 and H7 viruses were detected in both wild and domestic birds implying possible transmission from wild birds to domestic birds or vice versa.

The HA subtype diversity in our study has similarities and differences to that found in studies in China [107], Netherlands [108], North America [109,110], Germany [111] and Northern Europe [112] though a number of these studies were done in wild aquatic birds. For example, our study was comparable to the study in China [107] and that in the Netherlands [108], which did not detect H8, H12–H16. Furthermore, our study only detected six NA subtypes and 19 HA/NA combinations, while studies in Germany [111], Northern Europe [112] and North America [109,110] detected 40 or more subtype combinations. This higher subtype diversity in these studies could be because the surveillance was focused on wild waterfowl, which are expected to harbor a large pool of various AIV subtypes, which could also be true for Southern Africa, which had the highest subtype diversity in sub-Saharan Africa. The most prevalent NA subtype was N1, followed by N2 and N8, while N3–N5 and H2, H8 and H12–H16 were never detected, which may be due to limited surveillance efforts in wild birds or that there may be avian species-specific niches of certain HA and NA subtypes in the studied region.

Furthermore, the findings of this study revealed the presence of LPAI and HPAI viruses in both wild and domestic birds. However, the presence of HPAI viruses was more common among domestic birds, with the highest detection rate being in chickens and ducks. The dominant subtype was H5N1, which circulated in both wild and domestic birds. Our results further demonstrated that West African

countries were the most hit by the H5N1 HPAI viruses with at least Nigeria [100,113], Niger [81], Ghana [81], Burkina Faso [69,113], Cote d'Ivoire [68] and Togo [71] recording one or more outbreaks during the evaluated period. This may be because West Africa is a major wintering area for migratory water birds (Anseriformes and Charadriiformes), which are the natural reservoirs of AIVs [5]. Moreover, the H5N1 HPAI viruses have persisted within the West African ecosystem since their introduction in Nigeria in 2006 [35,114]. The persistence and transmission of H5N1 HPAI viruses in West Africa have been attributed to the illegal movement of infected poultry and products, multispecies livestock farming and poor biosecurity compliance levels in live bird markets (LBMs) [35,115]. Furthermore, an HPAI outbreak caused by H5N1 viruses has been reported in Cameroon, a Central African country that shares borders with Nigeria, which suggests transboundary transmission due to porous borders, leading to illegal trade in livestock, especially birds, between these countries [58].

Apart from the H5N1 HPAI viruses, the study also revealed the presence of other HPAI virus subtypes namely H5N8 and H5N2 in different avian species in sub-Saharan Africa. The first case of H5N8 HPAI infection in Africa was reported around the same time in Egypt and Nigeria and later spread to other neighboring countries [52]. This outbreak spread to Uganda, South Africa, Zimbabwe and DRC [52,53,63,116,117]. Additionally, HPAI outbreaks caused by H5N2 viruses have been reported in farmed ostriches in South Africa. These outbreaks caused a devastating impact on the ostrich industry of South Africa, which account for at least 65% of global ostrich production [118]. The presence and spread of H5N8 and H5N2 HPAI viruses have been attributed to migratory waterfowl due to the long-distance seasonal movements along their migration routes and also the other sedentary birds that have been implicated in facilitating the intracontinental dissemination of the virus [117]. In fact, H5N2 HPAI viruses have been detected in apparently healthy wild waterfowl in Nigeria [72]. Although HPAI viruses have been detected in many countries in sub-Saharan Africa, our results did not report any HPAI viruses in Zambia and Zimbabwe. Further, other countries such as Mali, Central Africa Republic, Congo-Brazzaville, Gabon and Madagascar did not specify the subtypes and pathogenicity of the viruses detected. However, the presence of HPAI viruses highlights the importance of continued and better epidemiological monitoring systems to allow their timely detection for mitigatory measures.

A large diversity of LPAI virus subtypes was detected in this review with H9N2 being the most predominant followed by H6N2 and H3N8. The H9N2 and H6N2 LPAI viruses were exclusively detected in domestic birds, in which they caused asymptomatic or symptomatic infections. Symptomatic infections caused by LPAI viruses include severe clinical signs in poultry, such as respiratory distress, intestinal signs and a drop in egg production [42]. These observations are consistent with previous studies in Iraq [119]. Moreover, H9N2 AIV infection is known to be endemic among poultry in Eurasia [120,121], and its circulation has been reported in North Africa, Europe and Asia among others [42,120–124]. H9N2 viruses are also known to circulate between wild birds and poultry sold at LBMs. LBMs are known to be reservoirs, amplifiers and sources of AIVs [46]. Furthermore, the transmission of H9N2 viruses from poultry to humans has been reported [125].

While H4N6 and H7N7 were the most prevalent AIV subtypes detected in Northern Europe and Germany [111,112], these subtypes were among the least detected in this review. H3N6 and H9N1 were the least and the only AIV subtypes detected in the great white pelican (*Pelecanus onocrotalus*) in Zambia [49,51]. Further, the presence of AIVs in wild waterfowl such as white-winged black terns (*Chlidonias leucopterus*), Egyptian geese (*Alopochen aegyptiacus*), yellow-billed duck (*Anas undulata*), shelduck (*Tadoma cana*) among others is important as these birds are known to be the primary reservoir of AIVs. Although our data seem to suggest an increase in the incidence of AIV infection in migratory waterfowl and domestic birds, this review also reports the detection of H5N1 HPAI viruses in African wild birds, hooded vultures (*Necrosyrtes monachus)* in Burkina Faso [69]. The detection of AIVs in wild migratory birds and minor bird reservoirs highlights the important role they play in the maintenance and transmission of these viruses. AIVs with H5Nx, H7Nx and other subtypes (not fully identified) were detected in Afro-tropical waterfowl and swallows in Zimbabwe throughout the year, and the

detection rate was higher when Palearctic birds were present, suggesting the yearly persistence of LPAI viruses in Afro-tropical waterfowl and other wild birds [126].

Although the benefits of systematic reviews are enormous, they are also not short of the challenges and limitations that come with aggregating data. For example, the distribution of AIV subtypes and avian species might not be limited to the present findings since our study included only publications with original data, well-elaborated methodological approach and laboratory-confirmed cases of AIVs. Furthermore, we only searched three recognized electronic databases and only included articles written in English, making it possible to leave out studies or publications that may be relevant to this review. Avian influenza subtypes depicted in this study may not be the true reflection of the subtype diversity in sub-Saharan Africa as some studies did not perform subtype identification due to insufficient samples and lack of laboratory capacity for influenza diagnostics.

## 5. Conclusions

Our review of AIVs in sub-Saharan Africa has provided an insight into the ecology and epidemiological trends of AIVs in birds over a twenty-year period (2000–2019). We found a considerable diversity of AIV subtypes in sub-Saharan Africa, with some subtypes being detected frequently in both wild and domestic birds. Furthermore, AIV was detected in wildfowl and domestic birds in both the wet and dry seasons, with viruses being detected in both Eurasian migratory and indigenous African wild birds. These results suggest a year-round perpetuation of AIVs in Afrotropical ecosystems, with seasonal variation. Continued surveillance, especially in wild birds, to better understand the eco-epidemiology of IAVs, along with improved biosecurity on poultry farms, enhanced extension services and engagement of various disciplines under a "One Health" approach in tackling avian influenza could assist in mitigating the impact of AIV in sub-Saharan Africa.

**Supplementary Materials:** The following are available online at http://www.mdpi.com/1999-4915/12/9/993/s1, S1 Checklist-PRISMA checklist. Table S1. References of all included studies. Table S2. Countries with reported AIV of diverse subtypes and avian species.

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## Appendix K: Ethical Approval Letters and Study Permits

**UNIVERSITY OF ZAMBIA**
**BIOMEDICAL RESEARCH ETHICS COMMITTEE**

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14th April 2020.

**Your REF. No. 616-2019.**

Ms. Annie Kalonda,
University of Zambia,
School of Veterinary Medicine,
P.O Box 32379,
**Lusaka.**

Dear Ms. Kalonda,

**RE: "ISOLATION AND CHARACTERIZATION OF INFLUENZA A AND D VIRUSES ISOLATED FROM WILD WATERFOWL, BATS, CATTLE AND POULTRY IN SELECTED PARTS OF ZAMBIA" (REF. NO. 616-2019)**

The above-mentioned research proposal was presented to the Biomedical Research Ethics Committee on 14th April, 2020. The proposal is **approved**. The approval is based on the following documents that were submitted for review:

**a) Study proposal**
**b) Participant Consent Form**

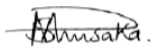
**APPROVAL NUMBER : REF. 616-2019**

**This number should be used on all correspondence, consent forms and documents as appropriate.**

- **APPROVAL DATE : 14th April 2020**
- **TYPE OF APPROVAL : Expedited**
- **EXPIRATION DATE OF APPROVAL : 13th April 2021**
  After this date, this project may only continue upon renewal. For purposes of renewal, a progress report on a standard form obtainable from the UNZABREC Offices should be submitted one month before the expiration date for continuing review.
- **SERIOUS ADVERSE EVENT REPORTING**: All SAEs and any other serious challenges/problems having to do with participant welfare, participant safety and study integrity must be reported to UNZABREC within 3 working days using standard forms obtainable from UNZABREC.
- **MODIFICATIONS**: Prior UNZABREC approval using standard forms obtainable from the UNZABREC Offices is required before implementing any changes in the Protocol (including changes in the consent documents).
- **TERMINATION OF STUDY**: On termination of a study, a report has to be submitted to the UNZABREC using standard forms obtainable from the UNZABREC Offices.

- **NHRA:** You are advised to obtain final study clearance and approval to conduct research in Zambia from the National Health Research Authority (NHRA) before commencing the research project.
- **QUESTIONS**: Please contact the UNZABREC on Telephone No.256067 or by e-mail on unzarec@unza.zm.
- **OTHER**: Please be reminded to send in copies of your research findings/results for our records. You're also required to submit electronic copies of your publications in peer-reviewed journals that may emanate from this study. Use the online portal: unza.rhinno.net for further submissions.

Yours sincerely,



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21st December 2021

**Your REF. No. 616-2019**

Ms. Annie Kalonda,
University of Zambia,
School of Veterinary Medicine,
P.O Box 32379,
**Lusaka.**

Dear Ms. Kalonda,

**RE: "ISOLATION AND CHARACTERIZATION OF INFLUENZA A AND D VIRUSES** ISOLATED FROM WILD WATERFOWL, BATS, CATTLE AND CHICKEN IN **SELECTED PARTS OF ZAMBIA" (REF. NO. 616-2019)**

We acknowledge receipt of your request for Annual Continuing Review for the aforementioned protocol and enclosed progress report therewith.

Renewal was granted for the period 13th April 2021 to 20th December 2022.

Please be reminded to submit progress report and annual reviews timely.

Yours sincerely,

Munsaka.

Sody Mweetwa Munsaka, BSc., MSc., PhD
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**NATIONAL HEALTH RESEARCH AUTHORITY**
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**Ref No:……** **Date: 1st June, 2020**

The Principal Investigator
Ms. Annie Kalonda,
University of Zambia,
School of Veterinary Medicine,
P.O Box 32379,
**Lusaka.**

Dear Ms. Kalonda,

## Re: Request for Authority to Conduct Research

The National Health Research Ethics Board (NHREB) is in receipt of your request for authority to conduct research titled **"Isolation and Characterisation of Influenza A and D Viruses Isolated from Wild Waterfowl, Bats, Cattle and Poultry in Selected Parts of Zambia"**

I wish to inform you that following submission of your request to the Board, its review of the same and in view of the ethical clearance, this study has been **approved** on condition that:

1. **A Material Transfer Agreement is obtained and cleared by the National Health Research Ethics Board should there be any need for samples to be sent outside the country for analysis.**
2. The relevant Provincial and District Medical Officers where the study is being conducted are fully appraised;
3. Progress updates are provided to NHRA quarterly from the date of commencement of the study;
4. The final study report is cleared by the NHRA before any publication or dissemination within or outside the country;
5. After clearance for publication or dissemination by the NHRA, the final study report is shared with all relevant Provincial and District Directors of Health where the study was being conducted, and all key respondents.

Yours sincerely,

Prof. Patrick Musonda
Chairperson
**National Health Research Ethics Board**

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All correspondences should be addressed to the Director/CEO National Health Research Authority

Tel: +260 211 223 930
Fax: +260 211 223 930
Telex: ZA 45510
Email: psmta@mta.gov.zm

REPUBLIC OF ZAMBIA

# MINISTRY OF TOURISM AND ARTS

OFFICE OF THE PERMANENT SECRETARY

*In reply please quote*

No.....

KWACHA HOUSE
CAIRO ROAD
P.O.BOX 30575
10101 LUSAKA

**TJ/NPW/8/27/1**

21st February 2020

Annie Kalonda
PhD Student (Microbiology)
Department of Disease Control
School of Veterinary Medicine
University of Zambia
**Zambia**

Dear Madam,

**AUTHORITY TO UNDERTAKE RESEARCH ON INFLUENZA VIRUS IN WILD WATERFOWL AND BATS IN ZAMBIA**

Reference is made to your letter received on 13th February 2020 concerning the above subject matter

I am pleased to inform you that you have been granted permission to conduct research on the theme ***"isolation and characterization of influenza A and D viruses isolated from wild waterfowls, bats, cattle and poultry in selected parts of Zambia"***. Your sample area will include Lochinvar National Park, Liuwa Plains National Park, Kasanka National Park, Lukanga Swamps, Bangweulu Wetlands and Leopards Hill Caves. Your permit only allows you to collect faecal samples from wild waterfowls and bats. The research permit is valid from 19th February 2020 to 31st December 2020. Be advised that issuance of the research permit is subject to payment of all prescribed research fees.

This permit is granted to the following people:

| NAME | ID | NATIONALITY | FEES |
|---|---|---|---|
| Application Review Fee | | | ZMK 500.10 |
| Annie Kalonda | 225965/31/1 | Zambian | ZMK 667.60 |
| Dr. Edgar Simulundu | 191720/71/1 | Zambian | ZMK 667.60 |

This permit is granted on the following conditions:

1. You shall report to DNPW offices in each protected area before you collect data for your research
2. You shall conduct your research under the supervision of the Area Ecologist for each protected area you sample at your own cost
3. You shall adhere to all rules and regulations when in the National Parks and Game Management Area.
4. You shall submit a copy of the research results and report to DNPW before publication.
5. You shall be escorted into the park by a wildlife police officer at all times and at your own cost.
6. No unmanned Aerial Vehicles shall be used in this study.
7. The researcher shall not obtain or attempt to obtain patent coverage on the samples (unmodified derivatives or progeny) without prior written consent of DNPW.
8. The researcher shall promptly notify DNPW in writing of any invention and discoveries arising out of the use of the samples. DNPW and the Researcher shall jointly decide on protection and commercialization of such inventions and discoveries and take into account DNPW contributions including its provision of the samples.
9. The samples shall not be sold, distributed or otherwise made available to any other third party or stored at any other facility for any purpose.
10. The permit is subject to any other written laws of Zambia

Kindly, note that you may be requested to make an oral presentation to DNPW of your research findings and its conservation and management implications.

Yours faithfully,

Chuma Simukonda, DSc.
**ACTING DIRECTOR- NATIONAL PARKS AND WILDLIFE**
**For/PERMANENT SECRETARY-MINISTRY OF TOURISM AND ARTS**

cc: Acting Assistant Director Research and Veterinary Services

All correspondence should be addressed to
The Director Veterinary Services
Tel: 0211-252608

In reply please quote
No.....

REPUBLIC OF ZAMBIA

# MINISTRY OF FISHERIES AND LIVESTOCK

DEPARTMENT OF VETERINARY SERVICES
MULUNGUSHI HOUSE
P.O. Box 50060
RIDGEWAY 15100
LUSAKA - ZAMBIA

20th March 2020

Assistant Dean

Post Graduate Studies

School of Veterinary Medicine

University of Zambia

**Lusaka**

Ref: **Request for authorisation to conduct a study entitled "Isolation and Characterisation of Influenza A and D Viruses currently circulating in bats, wild waterfowls, cattle and poultry in selected parts of Zambia" – Annie Kalonda.**

Following your request dated 12th February 202 to authorise Annie Kalonda to conduct a study entitled **"Isolation and Characterisation of Influenza A and D Viruses currently circulating in bats, wild waterfowls, cattle and poultry in selected parts of Zambia"** under the University of Zambia, the Department referred the concept note submitted to scrutiny by the Ethics Committee of the Ministry.

The Ministry is of the view that the concept note was concisely written and meets most of the expectations of the Ministry. The Department will therefore conditionally grant authorisation for the study to go ahead as long as the following are met;

1) A full proposal needs to be availed to the Department to assess how the study will make the study subjects lose weight without subjecting them to inhuman conditions
2) Elaboration on how the 'Biological Characteristics' of the viruses as per specific objective 4 will be determined
3) There should be a 6-monthly feedback to the Department on the findings of the study and any harmful exposure of animals or humans due to this study and the remedial measures implemented should be reported immediately to the Department.
4) If some tests will be done out of Zambia, a Material Transfer Agreement (MTA) will need to be drawn and approved by the Department between the University and the other party outside the country.

Further to the above requirements, the Department advises the study organisers to ensure further permission is sought from the Department of National Parks and Wildlife for the Bats/waterfowls that will be used in the study.

Yours Sincerely,

*Dr. Swithine Kabilika*

*Director*

**DEPARTMENT OF VETERINARY SERVICES**