ISOLATION AND MOLECULAR CHARACTERISATION OF PANDEMIC A/H1N1 INFLUENZA VIRUS FROM PIGS IN AN INTENSIVE PIGGERY COMPLEX IN LAGOS, NIGERIA

MESEKO, CLEMENT ADEBAJO

ISOLATION AND MOLECULAR CHARACTERISATION OF PANDEMIC A/H1N1 INFLUENZA VIRUS FROM PIGS IN AN INTENSIVE PIGGERY COMPLEX IN LAGOS, NIGERIA

BY

MESEKO, CLEMENT ADEBAJO
Matriculation Number: 124412
DVM (Zaria), M.Sc. Virology (Ibadan)

A Thesis in the Department of Virology
Submitted to the Faculty of Basic Medical Science, College of Medicine in partial fulfillment for the degree of

DOCTOR OF PHILOSOPHY

of the

UNIVERSITY OF IBADAN

OCTOBER, 2014

ABSTRACT

Novel pandemic A/H1N1 influenza virus containing gene segments from pig, bird, and human influenza viruses was first detected in Mexico in 2009. Emergence of the virus further underscores the importance of animals in the epidemiology of influenza viruses. It is also known that intensification of livestock farming increases the potential for circulation of influenza virus at the human-animal interface but there is dearth of data on the situation in Nigeria. This study was therefore designed to characterise and determine the phylogeny of pandemic A/H1N1 influenza virus circulating in an intensive piggery complex in Lagos, Nigeria.

Sentinel surveillance was carried out in a large intensive piggery at Oke Aro, Lagos State with monthly sampling for two years (2010-2012). Two hundred and twenty seven and 40 nasal swabs was respectively collected from pigs and pig handlers presenting with influenza-like illness, were analysed by Real-Time Reverse Transcriptase Polymerase Chain Reaction (RRT-PCR). Virus isolation from samples that were confirmed positive by RRT-PCR was carried out in 8-10 days old embryonated chicken eggs. Isolates were identified by RRT-PCR and haemagglutination inhibition using subtype specific primers and homologous reference antisera respectively. The virus was also visualized with an electron microscope following negative stain with 2% phosphotungstic acid. Cycle sequencing of the isolates was carried out and basic local alignment search tool was used to compare gene sequences obtained with others in the Genbank and global initiative for sharing all influenza data. Nucleotide sequence alignment and construction of phylogenetic trees of full gene segments were done using MEGA5 software and the Neighbour-Joining method with 1,000 bootstrap replicates. Data were analysed using descriptive statistics at P=0.05

Thirty one (13.7%) of the 227 specimens from pigs analysed were positive for influenza A matrix gene by RRT-PCR while none of the 40 specimens from pig handlers was positive for influenza A virus. Twenty nine (12.8%) isolates were

obtained from the samples out of which 18 (7.9%) were subtyped as pandemic A/H1N1 influenza virus. The haemagglutinin and neuraminidase genes of representative isolate A/swine/Nigeria/12VIR4047/2011 with Genbank accession numbers JX442481 and JX442482 showed 99% homology with pandemic A/H1N1 influenza virus earlier isolated from human in San Diego, Cameroon, Ghana and Nigeria. However there were 13 nucleotides and 3 amino acid substitutions in the haemagglutinin gene compared with similar viruses and the prototype A/California/07/2009 especially aspartate to arginine mutation at antigenic binding site 240 position (H1-numbering) on the haemagglutinin gene. All the internal genes clustered with global 2009 pandemic influenza A/H1N1 virus on the phylogenetic tree but were more related to human isolates.

This study showed for the first time, isolation and some molecular properties of pandemic A/H1N1 influenza virus circulating in Nigeria. The observed mutations have implications on the transmissibility and pathogenicity of the virus in different hosts. Surveillance and control measures incorporating human and veterinary components are recommended since uninterrupted circulation of the virus in pigs may lead to reassortment in the animal host and emergence of novel subtypes with more severe consequences to human and animal populations.

Word count: 496

Keywords: Pandemic A/H1N1, Influenza virus, Molecular, Characterisation, Piggery in Nigeria.

ACKNOWLEDGEMENTS

In April 2009, I was walking down the tarmac of Dakar International Airport along side my mentor and supervisor, Professor D.O. Olaleye as we just finished attending the NIH sponsored influenza morbidity and mortality conference and he inquired if I heard the news of the "swine influenza" outbreak in Mexico. We shared views on the story that dominated the airwaves including CNN the previous night. Our conclusion was that there is a need to direct searchlight on millions of pigs in Nigeria. There was the motivation to embark on this study.

I therefore sincerely appreciate Professor Olaleye who spares nothing including his personal resources and private time to discuss, impart knowledge and solve problems. To accomplish these herculean tasks he is ably assisted by indefatigable Dr. Georgina N. Odaibo and to her also I give credit for this work. In carrying out my laboratory analysis, God sent helpers to me in the persons of Mr. M. Ibeh, Mr. L. Uche, Drs. S. Bakarey, B. Onoja, A. Faneye, O. Olayinka and O. Adewumi. I am grateful for their assistance.

All the various laboratory and sectional Staff of the Department of Virology including WHO-NIC, APIN-HIV, WHO-Polio, and MEPIN were helpful. I thank Dr. J.A. Adeniji who though I did not directly work with would occasionally inquire on the progress of my research. I have all the postgraduate students of virology to thank for the opportunity I had to interact and learn from them. I wish to single out Mr. Olumide Odurinde who was appointed research assistant without pay. We both saw the thick and thin of Lagos highway, we were kicked by pigs, harassed by policemen, threatened by touts and beaten by rain in the course of the field investigation, but we survived all to the glory of God.

The past and present Executive Directors of National Veterinary Research Institute - NVRI (Dr. Mrs. L.H. Lombin and Dr. M.S. Ahmed) are highly appreciated for initiating and sustaining the pursuit of postgraduate training among Staff of the Institute. The Director of Research, Dr. D. Shamaki is a man of many virtues. Dr T.M. Joannis does encourage hard work and productivity. Dr. P.A. Okewole is fatherly and Dr C.I. Nwosuh sacrifices convenience to ensure young researchers make progress in their career, we cannot forget. The period Professor D.F. Adene spent in NVRI on

sabbatical was scientifically stimulating. To all my colleagues and collaborators in NVRI, I say thank you for your challenge and encouragement.

The Lagos State Director of Veterinary Services, Dr. G. Adams and his lieutenants, Drs Okopido and Allison were very helpful. I wish also to acknowledge the resilience of Mr. Lanloye, the Veterinary Department Staff who was detailed to assist me in breaking some of the iron cast resistance of some Oke-Aro farmers. I appreciate the co-operation of all the pig farmers especially Mr. Olumide Babasola (M.D., Latosa Farms), Mrs Beatrice Jemirin, Ayo, Dare and Dr. Boye etc. I thank the executive council of the various co-operative societies and unions of pig farmers for their cooperation in the course of carrying out this study.

Renowned Drs Capua and Cattoli are warmly appreciated for opening the research gate of IZSVe to me. I thank all the Staff of IZSVe for their friendship and opportunities given me to learn and work beyond my anticipated schedules. I treasure all the experience in and out of the laboratories; in Padova, in Sienna and Florence, in Venice by the waters, In Verona at the opera, mountain expedition in Recoaro Terme and at the little dolomites, by the Alps. I have you all to thank for calming my nerves after every successive strong earthquakes and making my stay in Italy a combination of business with pleasure.

The two miracles the Lord performed in the course of this study are, first by painstaking and consistent inquiry, I found a virus that was unique in its own right, Secondly I needed fund to carry out in depth analysis of this virus and it was provided by the Robert S. McNammara (RSM)-World Bank Fellowship Award, 2011.

I sincerely appreciate and acknowledge the ideals of the RSM fellowship programme as it continues to assist scholars from all over the world to fulfill their dreams. The fellowship period abroad was actually a stepping stone that is opening further doors of research opportunities and awards to me. I shall continue to mention this as I progress in life and career.

My greatest assets are my family members. God has placed them strategically in my life to be succor and support; Col & Mrs Francis Olaniran, Pastor and Deaconess Godwin Meseko, Mr and Ms Richard Meseko, Pharm & Mrs Alexander Meseko, Arc

& Mrs King-Alfred Meseko, Barrister & Barrister Mrs Christian Meseko, Prince & Mrs Pius Meseko, Mr & Mrs Meshack Ijagbemi and Prince Jones Meseko. I salute my nephews/niece: Ademola, Adedoyin, Martins and Seyi. I appreciate the hospitality of my siblings in Lagos where I had several options. In Alfred's house, Omobolade was so attached and would want me to come always. Pius became my field assistance especially on weekends. I remember how Alex struggled to ensure I had sufficient ice blocks to keep my samples cool.

Special appreciation to my wife and daughter Taiwo and Temitayo for their endurance during many days that I stayed away from home in pursuit of the 'Golden Fleece'. May you all see the goodness of the Lord.

CERTIFICATION

We certify that this work was carried out by Dr. Clement Adebajo Meseko at the WHO National Influenza Center (NIC) in the Department of Virology, College of Medicine, University of Ibadan, Ibadan Nigeria & FAO/OIE Reference Center for Influenza and Newcastle Disease and WHO Collaborating Center for diseases at human-animal interface, Istituto Zooprofillatico Sperimentale delle Venezia (IZSVe) degli Universita di Padova, Italy.

.....	.....
Supervisor	Co-supervisor
DO Olaleye, DVM, Ph.D (Ib.)	**Georgina N. Odaibo, Ph.D (Ib.)**
Professor and Consultant Virologist	Reader
Department of Virology	Department of Virology
Faculty of Basic Medical Science	Faculty of Basic Medical Science
College of Medicine	College of Medicine,
University of Ibadan, Ibadan, Nigeria	University of Ibadan, Ibadan, Nigeria

DEDICATION

My father (HIS ROYAL HIGHNESS OBA P.A. MESEKO ALASORIN III of Okebukun)

encouraged me to start masters' degree that was completed in his last days on earth and as was typical of him, he inquired, "When are you starting your PhD". It was also his philosophy that what matter in life is *not how fast and far, but how well*.

To this man of vision and the driver of my ambition, I dedicate this doctoral thesis.

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ACRONYMS

AI - Avian Influenza

ASF - African Swine Fever

BLAST - Basic Local Alignment Search Tool

CE - Chicken Embryo

CFO - Confine Feeding Operation

CI - Confidence Interval

CDC - Center for Diseases Control

CVO - Chief Veterinary Officer

CPE - Cytopathic Effect

dNTPs - deoxynucleotide triphosphates

DVS - Director of Veterinary Services

D-MEM - Dulbecco- Minimum Essential Medium

EC - Extract control

EDTA - Ethylene diamine tetra acetic acid

FAO - Food and Agriculture Organization

FDL - Federal Livestock Department

FRET - Fluorescent Energy Transfer

FBS - Fetal Bovine Serum

GIS - Geographic Information System

GISAID - Global Initiative for Sharing of All Influenza Data

HA - Haemagglutination

HAI - Human-Animal Interface

HI - Haemagglutination Inhibition

HPAI - Highly Pathogenic Avian Influenza

IZSVe - Istituto Zooprofillatioc Sperimentale delle Venezie

LBM - Live Bird Market

NIC - National Influenza Center

NTC - Negative Test Control

NVRI - National Veterinary Research Institute

PTC - Positive Test Control

RBC - Red Blood Cell

LPAI - Low Pathogenic Avian Influenza

NMA - Nigeria Medical Association

NVMA - Nigeria Veterinary Medical Association

MEGA - Molecular Evolution Gene Analysis

MDCK - Marbin Darbin Canine Kidney

MoA - Ministry of Agriculture

MoH - Ministry of Health

NA - Neuraminidase

NDV - Newcastle Disease Virus

EU - European Union

EM - Electron Microscope

OIE - Office International des Epizooties (World Organization for Animal Health)

RRT- PCR Real time Reverse Transcriptase Polymerase Chain Reaction

RDE - Receptor Destroying Enzyme

PSGA - Penicillin Streptomycin Gentamycin Amphotericin

SAN - Specific Antibody Negative

SPF - Specific Pathogen Free

STAR - Sequential Transfer and Aliquoting Robotic

SIV - Swine Influenza Virus

USDA - United State Department of Agriculture

WHO - World Health Organization

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND

Exceptionally high morbidity and mortality rate in chickens caused by influenza virus was observed in Italy in 1878 and was described as fowl plague (Perroncito, 1878). That was one of the earliest and most quoted records of influenza virus infection (OIE, 2009). About half a century later, another highly pathogenic influenza pandemic was reported in human population where over 50 million people were said to have died. The mortality rate was more than was caused by the first world war (Webster, *et al*., 1992; Johnson and Mueller, 2002; Taubenberger and Morens, 2006). About the same period and almost simultaneously, influenza virus was diagnosed in pigs with epidemiologic evidence indicating the spread of the causative virus from humans to swine. The virus remained in pigs relatively unchanged until detected in 1930 by Shope (1931) at the Animal Pathology Department of Rockefeller Institute of Medical Research, New Jersey USA.

The work by Shope was the first isolation of influenza virus from mammals (OIE, 2009; CDC, 2010). Retrospective serological and molecular studies later identified these viruses as human H1N1 and swine H1N1 respectively (Taubenberger and Morens, 2006). Many years later there were recurrent pandemic of influenza in 1957 and 1968 (Dowdle, 1999). By 1976, isolates of virus from military recruits at Fort Dix, New Jersey, who had influenza like illness, were sent to the Center for Disease Control and Prevention in Atlanta. Most of the isolates were identified as A/Victoria/75(H3N2), the contemporary epidemic strain. Two of the isolates were initially not typeable but were later identified as a strain of swine influenza known as A/New Jersey/76(Hsw/N1/swH1N1) (CDC, 1997).

This virus was similar to the virus of the 1918 pandemic and was described as “swine flu” for its relatedness to earlier swine flu isolates. Though no direct contact of the patients with swine was established as at that time, there were strong evidence suggesting an introduction and ressortment of human and swine influenza (Top and Russel, 1977; CDC, 2010). Emerging strains of influenza virus has since then continued to ravage animal and human populations, each time taking steps ahead in evolution, pathogenicity and

transmissibility than science and medicine could readily understand (Webster, *et al*., 1993).

Swine influenza is an important zoonotic and contagious disease of pigs occurring worldwide and is typically caused by a number of closely related influenza A viruses of the Orthomyxoviridae family (Kawaoka, *et al*., 2005). The influenza A viruses are noted for their ability to change antigenic structure and evolve new strains. Each serotype is identified by surface glycoprotein referred to as "H" and "N" that could give rise to subtypes of various combinations (Fouchier, *et al*., 2005; Luke and Subbarao, 2006).

There are four main types of swine influenza A affecting pigs viz: H1N1, H1N2, H3N2 and H3N1 (CDC, 2010). H1N1 is more frequently isolated while novel H3N1 was isolated in coughing pigs in Italy by Moreno *et al.* in 2009 and was described as a reassortant between H3N2 and H1N1. Many other subtypes, strains and clades consequent of genetic reassortment and mutations circulate in different geo-ecological zones that are yet to be fully understood and described.

Pigs serve as reservoir and transmitter of many subtypes of influenza A to other species of animals including human (Taubenberger and Morens, 2006). The maintenance of influenza viruses in pigs and the frequent introduction of novel influenza viruses from other species, contributes to the generation of strains of human influenza virus with pandemic potential. This evolution was observed in the influenza swH1N1 virus that originated in Mexico and caused the 2009 influenza pandemic. It was a product of the combination of swine, avian and human influenza genes (Smith, *et al*., 2009, Garten, *et al*., 2009). The importance of pigs as an arbiter of influenza between man and other species cannot therefore be overemphasized (Hodder *et al*., 1977).

Pigs are especially important in influenza ecology, molecular virology, pathology and epidemiology because it characteristically possess receptors for both avian and human influenza. It is thus described as a “mixing vessel” permitting co-infection of many viruses that subsequently reassort giving rise to novel strains (Webster, *et al*., 1992; WHO, 2005). Thus pigs are known to have a most important role in the epidemiology and evolution of new influenza viruses (Ma, *et al*., 2009).

Outbreaks of swine influenza in human population were recorded in 1974 and 1975 in the United States of America. In both cases, infected persons had close contact with pigs. Abortive swine flu of 1976 at Fort Dix also had its origin in pigs (Sencer and Millar, 2006) and in 1988 a woman previously exposed to swine influenza H1N1, died of pneumonia eight days after infection (CDC, 2010).

In April 2009, the Centre for Disease Control (CDC) confirmed outbreak of 'swine flu' in Mexico. The virus spread to several other countries including America, Canada, and Africa (Scoones, 2010). On the 11th of June 2009, the World Health Organization (WHO) through its Director General declared it as the first pandemic of the 21st century (Butler, 2009) and as at December 2009, the virus had spread to 208 countries infecting over 1 million people out of which 9,596 died (WHO, 2009). Pandemic H1N1 infection was also detected in human in Nigeria, a country that has had history of avian influenza H5N1 in poultry and human (Joannis *et al*., 2006; Breiman, *et al.*, 2007, Dalhatu *et al*., 2012).

Data on influenza virus transmission and circulation at the human-animal interface in Nigeria is scanty. Neither are there genomic data describing molecular properties of the virus that may be in circulation in the country. Therefore, virological surveillance to detect, isolate and characterise both pandemic and classical swine influenza in pigs is important as an early warning of virus evolution and pandemic indicators for public health action. The opportunity to pre-empt pandemic therefore is to embark on a systematic surveillance for influenza in animals. If infection can be detected in pigs and is quickly followed by rapid and safe control, the surveillance system for humans would be better managed and the infection more easy to contain.

Pig husbandry is a major animal agriculture- the country is the largest pork producer in Africa accounting for over 30% total pig population of over 30 million (Mckay, 1963a and b; FAO, 2009) and piggery is a major subsector of the livestock economy contributing substantially to Agriculture gross domestic product (GDP). It contributes significantly to the economic welfare of the rural population. Consumption of pork and pork products also plays an important role in the social and cultural life of most of the communities in the southern and central states of Nigeria (FAO, 1998).

These pigs are either kept intensively in commercial quantities in brick/cement constructed pens or free range and reared in urban, peri-urban and rural areas with a current population estimate of about 10 million post African Swine Fever (ASF) devastation of 1997, competing with cattle and poultry estimated at 75 and 150 million respectively (FLD, 2006). As with other livestock farming in Nigeria, pigs are intimately associated with humans where they are kept with minimal or no biosecurity measures with high chances of emergence and transmission of pathogens (FAO, 1998).

Influenza virus may be spread at the human-animal interface by formites and movement from infected and uninfected pigs in close contacts (Olsen, *et al*., 2006), pig farmers are thus more likely to be infected or infect pigs as have been previously observed (Ma, *et al*., 2008). The incubation period being very short, as little as 12-48 hours and the onset of clinical sign is usually rapid (Lange, *et al*., 2009). Swine influenza in large herds may then become endemic with intermittent episodes of disease and reassortments of the virus, thus causing many different strains to sequentially infect the herd (Smith *et al*., 2009). This creates a scenario of multiplicity of evolving strains and clades from season to season, which is yet to be described.

The diagnosis of swine influenza in farms can be made reliably on clinical grounds because of rapid onset and clinical effects. Few other respiratory diseases affect so many pigs as quickly and symptomatic pigs are readily noticed in the herd by observing cough, sneezing and severe respiratory distress. Blood samples taken at the time of onset of disease from affected sows and repeated 2-3 weeks later showing rising levels of total antibody to the specific virus can also be diagnostic and seroconversion can be as high as 100% (Li *et al*., 2003). However, virus isolation from nasal and throat swabs in egg or tissue culture and identified by molecular or serological subtyping is often the best approach to confirm the diagnosis of swine influenza (Smith, *et al*., 2009).

Further molecular characterization and full or partial genome sequencing would elucidate antigenic drift or shift of the various isolates and provide insight into the molecular changes that may promote evolution of pandemic strain and its relatedness to circulating or seasonal human influenza, or such mutations that could cause antiviral resistance and a degree of variation from vaccine strains and immune evasion (Ito, *et al*., 2000).

The need to understand these mutations and frequent reassortment is the thrust of the global influenza control programme of the World Health Organization, which relies on a network of National influenza centers/collaborating laboratories and researchers to gather epidemiological data on influenza in the course of surveillance (WHO, 2009). Field isolates are then characterised to determine serotypes and antigenic properties. Information generated could then be used to plan selection of vaccine strains, antiviral development and pandemic preparedness.

The global animal-human disease interface in the environment is complex and dynamic, with potential for zoonotic transmission of known and unknown pathogens, reassortants, variants, novel and emerging infectious agents. Animal reservoirs and humans, both with the potential for global movement and distribution in time and space face these shared infectious challenges. If humans can be infected by influenza from pigs, can human also donate virus to pigs (Tim and Tomy, 2010).

While the novel swH1N1 that was first detected in Mexico is assumed to be of animal origin, it spread rapidly within the human population because of their susceptibility. It was not detected earlier in animal population because of gaps in surveillance for animal influenza. Measures that may be implemented in both outbreak areas and outbreak free areas include vigilance and early detection by adopting effective surveillance and monitoring in both human and animal population.

1.2 RESEARCH JUSTIFICATION

There have been four influenza pandemics between 1918-2009. Since 2003, events in the Veterinary and Public health sectors in terms of interaction between human and animals in a shared environment continued to favour emergence of influenza and other zoonotic diseases. Avian influenza H5N1 was first detected in poultry in 1997 in Hong Kong and by 2003 the host range of avian influenza expanded to include, wild and migratory birds. Other affected animals include dogs, cats, tigers and pigs. Though animal influenza viruses are not natural pathogens of humans and are not easily transmitted but a pandemic can be initiated when the virus:

- evolves a new strain to which human are susceptible

- the new virus replicates and cause disease in humans
- the new virus is transmitted efficiently from human to human

The first and second criteria have been observed in nature by influenza H5N1. While scientists were still contemplating when and how the newly emerged H5N1 would evolve to fulfill the third criteria, a novel strain of influenza virus H1N1pdm emerged in 2009 spreading overwhelmingly, prompting WHO to declare it as the first pandemic of the 21st century.

H1N1pdm virus with genetic materials from avian, human, and swine subtypes caught scientists unaware and unprepared. This is because while attention was on human and avian influenza, the virus was evolving in pigs, and while attention was on Asia; the virus emerged in Latin America. Not enough surveillance among pigs was undertaken as at the period the virus emerged. This lack of data is also revealed in the Genbank which as at April 2010 had a total of 4001 full genome sequences of influenza virus isolated from humans, 2590 from bird and only 325 (8%) from swine (Capua and Cattoli, 2010).

The probability that influenza strain more deadly than H5N1, H1N1pdm with genetic composition for efficient adaptation and pathology in human would emerge is high. One of the more likely hosts is the pig being a "mixing vessel" and the most important species in influenza epidemiology (Ma, *et al*., 2008; Brown, 2008). In recognition of this important factor in the global influenza control, WHO public health research agenda expert forum that was convened in Geneva in November 2009 identified surveillance in animals, particularly pig as one of the major research prerequisite necessary for reducing risk of emergence of pandemic strain, early warning or the mitigation of pandemics (WHO, 2009).

Investigating how influenza viruses circulate in certain species such as pigs, avian, dogs, and horses is vital in pandemic preparedness and mitigation (Capua and Cattoli, 2010). Thus in the periods between pandemics, surveillance in animals that are major reservoir host of influenza viruses is the first of the six influenza pandemic phases described by WHO (2008).

Experts estimate impact of future pandemic at over 1 billion cases, 7.4 million deaths, greater than 200 billion USD losses in the economy and unimaginable socio-political disruptions due to global panic (World Bank, 2006, WHO, 2010). Unlike other global, transboundary or zoonotic diseases, there is no hiding from influenza virus when it is unleashed on mankind as it pollutes the environment, cripples life, economy and the society (Scoones, 2010).

1.3 PROBLEM STATEMENT

The WHO global influenza programme seeks to gather information on subtypes of influenza virus in circulation so as to be able to plan effective control measures. This is even more important in the veterinary sector in Nigeria and by extension Public health where there is a dearth of information on circulating strains of influenza virus in swine population. Targeted point surveillance of pigs in swine producing areas in farms and slaughter slabs of cases that meet the definition of swine influenza or respiratory infections is vital for public health and welfare. An ecological melting point with sufficient interspecies exposures as exemplified by about 160 million people and 10 million pigs in close proximity could contribute to influenza circulation and evolution. This could also provide specimens for laboratory tests, detection and research on circulating swine influenza virus in Nigeria.

Research in pig rearing countries to study changes in the influenza virus so as to gather data for planning intervention is critical. Evidence based capacity development for rapid response to pandemic strains of Influenza viruses could subsequently be put in place to promote sustainable improvement in human-animal health and welfare in the context of one world, onehealth.

1.4 AIMS AND OBJECTIVES

This study is therefore aimed at monitoring circulating influenza virus at the human-animal interface in swine population in Nigeria within the global influenza surveillance framework. This is expected to generate data and scientific basis for subsequent research,

intervention and control while developing capacity for rapid response to pandemic outbreaks.

To achieve this, the study is thus designed to accomplish the following specific objectives:

(1) Detect, isolate and identify influenza viruses circulating at the human-animal interface in intensive pig farm complex in Lagos

(2) Characterise the swine influenza isolates using molecular techniques

(3) Phylogenetic analysis of obtained swine influenza virus and selected sequences from GenBank and GISAID

(4) Development and production of antigen from isolated virus for diagnostic capacity

CHAPTER TWO

LITERATURE REVIEW

2.1 HISTORICAL BACKGROUND

Romans named the virus that caused massive mortalities in chicken as influenza, attributing it to the influence of the star gods as early as 1375 AD. It was probably earlier described but not named by Hippocrates in 412 AD (Hoehling 1961). Edoardo Perroncito first documented fowl plague in 1878 and the "avian" virus was recognized as the precursor of all other subtypes of influenza A virus responsible for epidemics and pandemics in animal and human populations till date (Gorman *et al*., 1991; Slomka *et al*. 2010)

Influenza virus, in the year of the great pandemic (1918) also described as the mother of all pandemics (Webster *et al*., 1992) ravaged human population killing about 50 to 100 million people. That was more deaths than recorded for World War 1, which also took its toll on mankind as at the time of the pandemic (Johnson and Mueller, 2002; Taubenberger and Morens, 2006). In the period between 1918 and 1930, beside human fatalities due to influenza, herds of pigs were also dying of a respiratory disease that closely resemble the clinical syndrome affecting man (Zimmer and Burke 2009) and because of the similarity in clinical signs and pathological features, it was thought that the human influenza adapted to swine and studies on the etiological agent was rigorously pursued by scientists.

In 1931 Robert Shope, working in the animal pathology laboratory of Rockefeller Institute of Medical Research Princeton, New Jersey, successfully transmitted the infectious agent of swine influenza earlier described as hog flu from sick pigs by filtering virus containing secretion to healthy animals in what was recorded as the first isolation of influenza virus (Shope, 1931; Shope, 1934; Brown, *et al*., 2000). That work gave impetus to research by Smith and colleagues who also succeeded in using ferret model to isolate and demonstrate the transmissibility of human influenza three years after (Smith, *et al*. 1933). Virological and Immunological experiments by Shope and other scientists suggested that classical swine influenza has been in circulation in the human population for a long time and had originated from 1918 pandemic strain (Gorman *et al*., 1991;Slomka *et al*. (2010).

Studies since then have shown the circulations of Influenza A viruses in pigs in many parts of the world (Slomka *et al*. (2010). A/(H1N1) was confirmed to be in circulation in North American pigs as early as 1930 and was also isolated in Italy in 1976 when pigs were shipped there from United States hence introducing the classical influenza (H1N1) to Europe and spreading it in the pig population (Nardelli, *et al*., 1978). In 1970, humanlike viruses of the H3N2 subtype were first isolated from pigs in Taiwan, prevalence in North American pigs was however low (Knipe and Howley, 2007). Serologic surveillance studies suggested that human H1N1 viruses transmit easily to pigs and have led to occasional isolation of human virus in swine through exposure to humans involved in pig breeding. The A/Hong Kong/68 (H3N2) influenza virus that has not been isolated from man for several years was also isolated from pigs in China.

Influenza virus A/Victoria/3/75-like that was circulating in man in the 70s was also isolated from pigs. (Hai *et al*., 2007). Both the A/Hong Kong/68 and the A/Victoria/75-like viruses transmitted readily from pig to pig in experimental studies (Webster *et al.*, 1977). In 1976, isolates that were initially not typeable as compared to A/Victoria/75 were later identified as A/New Jersey/76(Hsw/N1/swH1N1) of swine origin (CDC, 1997). Since 1979 till date, three subtypes, avian-like H1N1, reassortant H1N2 and H3N2 viruses, co-circulates in European swine.

Before 1998, classical H1N1 viruses were the exclusive cause of swine influenza in North America. However, after 1998 triple-reassortant H1N1, H1N2 and H3N1, viruses with genes of human, swine and avian virus began to emerge in pigs. A new triple reassortant swine influenza virus - with genes from avian, pigs and humans was also identified in the North America swine population in 1998 (Olsen, 2002).

Twenty two swine influenza viruses (SIVs) H1N2 subtype isolated from 1999 to 2001, from 9 States of the United States were genetically screened. The HA genes of 12 H1N2 isolates showed that 8 of them had a close phylogenetic relationship with the Indiana isolate of H1N2 (A/Swine/Indiana/9K035/99), while 4 isolates were closely related to classical SIV H1N1. (Choi *et al*., 2002). Studies in 1976/1977, 1988/1989 and 2000/2006 demonstrated that pigs in America and Europe were commonly infected with classical swine H1N1 viruses, whereas human H3 and avian influenza virus H5, H7 and H9

infections were very rare. In recent years however, human H3, avian H1 and H5 HPAI viruses have been isolated from pigs in Europe, Asia and Africa (Olsen *et al*., 2000; Maldonado *et al.*, 2006; Van *et al*., 2008; Bálint *et al*., 2009; El-Sayed, *et al*.,2010; Nidom *et al*., 2010).

Influenza A viruses circulating in pigs are distinct in the genetic characteristics and genetic sources. (Van *et al*., 2008; Qi *et al*., 2009). The three predominant subtypes of influenza viruses viz H1N1, H1N2, and H3N2 are prevalent in pig populations worldwide, also included are classical swine H1N1, avian-like H1N1, human-like H3N2, reassortant H3N2 and various genotype H1N2 viruses. In Europe, North America and China and Italy, these subtypes of swine influenza viruses, H1N1, H1N2, and H3N2 have also been isolated.

In 2006, a novel swine influenza virus subtype (H3N1) was isolated from pigs in Italy (Moreno *et al.*, 2009). Several influenza A viral subtypes were also isolated from pigs during severe outbreak of respiratory disease in Korea and china during 2005 and 2006. They include a classical swine H1N1 subtype, two swine-human-avian triple-recombinant H1N2 subtypes, and a swine-human-avian triple-recombinant H3N2 subtype. Influenza A viruses of subtypes H1N1 and H3N2 have also been widely reported in pigs associated with clinical disease. These mainly include classical swine H1N1, avian-like H1N1 and human-like or avian-like H3N2 viruses from 2005 to 2006 (Hai *et al*., 2007; Lee *et al*., 2008).

Unlike Europe, America and even Asia, there is obviously a paucity of information in literature on circulating swine influenza viruses in Africa. Even though only about 325 genomic sequences of swine influenza is available worldwide in the Genbank as at April 2010 (Capua and Cattoli, 2010). Data on the genomic characteristic of SIVs and its reassortant in Africa is equally important especially after H5N1 and H1N1 widely circulated in some parts of the continent in recent time.

The 2009 swine flu H1N1 pandemic was confirmed by CDC to be caused by influenza A virus genetically similar to swine virus that has not been previously identified in the United States or elsewhere. Preliminary research showed that this could be transmitted to

pigs through contact with human with demonstrable clinical signs, virus shedding and pathology though less able to cause mortalities (OIE, 2010). Swine influenza viruses (SIVs) are also known to infect human and poultry (Lipkind *et al*., 1984; Rota *et al*, 1989; Wright *et al*., 1992; Wood *et al*., 1997; Suarez *et al*., 2002; Gregory, *et al*., 2003). While human influenza A viruses are also known to transmit to pigs (Yu *et al*., 2009). Circulation of H5N1 and H1N1 that have been witnessed recently could become established in pigs, which could then serve as a source of further ressortment/evolution and precursor of future Zoonoses or pandemic strain (Van Reeth, 2007; Slomka *et al*. 2010).

2.2. THE INFLUENZA VIRUS

Influenza viruses are widely distributed in nature affecting humans, animals and are shed in the environment, thus a part and parcel of the ecosystem (Kaplan 1980; Webster, 2000). The natural reservoirs are the avian species especially waterfowls where they persist without causing apparent infection (Alexander, 2000). Continuous circulations in these hosts and antigenic drift and shift result in changes that increase host range and pathogenicity. Resulting influenza viruses are therefore responsible for fowl plague, high or low pathogenic influenza and epidemics/pandemics of influenza in human and animals (Alexander and Brown, 2000).

The virus influenza was the second after Foot and mouth disease (FMD) virus to be recognized as ultrafilterable by Centanib and Savonuzzi (1901). It was difficult for virologist to identify the virus until the use of embryonated egg in 1934 (Burnet and Ferry, 1934). The haemagglutination property of the virus was later to be used in its detection, identification and serological studies (Lush, 1934, Katz *et al*., 2011). Avian influenza virus and Newcastle disease virus share similar clinico-pathological features but avian, swine and human influenza were later grouped together when researchers showed that influenza virus shares some internal genes with the mammalian virus which the Newcastle disease virus does not have in common (Shafer, 1955; Andrew and Worthington 1959; Andrew, 1962). It has also become apparent that all known subtypes of influenza A viruses are found among wild waterfowls that also serve as primary reservoir to other animals and human influenza viruses (Hinshaw and Webster, 1982)

2.2.1 CLASSIFICATION

The virus is grouped in the family Orthomyxoviridae that is defined by viruses that have a negative-sense, single-stranded, and segmented RNA genome (Katz *et al.*, 2011). The definition of negative-sense RNA viruses came from work by David Baltimore (1971) that showed that the genome of the virus is complementary to the mRNA, which is defined as positive. Though influenza has similar physical, biological and clinico-pathological similarity with Newcastle Disease Virus (NDV) as described earlier the later is classified in the paramyoviridea family (White and Fenner, 1994).

The World Health Organization expert committee for the taxonomy and classification of Influenza viruses in its 1971 report established three genera of influenza as A, B and C genotypes (two other genera - thogoto and ISA (infectious salmon anemia) virus which are arthropod and fish borne were later added to the family) based on the antigenic relatedness of the nucleoprotein and matrix protein (WHO, 1971; OIE, 2009). Members within any of the three genera A, B and C of influenza viruses can undergo genetic reassortments, and thus readily exchange genetic information. However, reassortment between members of different genera has not been reported. The absence of genetic exchange between viruses of different genera is a manifestation of speciation as a result of evolutionary divergence.

Influenza A are further divided into subtypes based on the H and N surface proteins giving 18H and 9N subtypes in various combination i.e. H1N1, H1N2, H3N2, H5N1 etc (Webster et al., 1992). The 17^{th} and 18^{th} H were recently described in bats in Peru and Guatemala (Bao *et al*., 2008; Tamura *et al*., 2011; Promed, 2012). Different influenza virus strains are named according to their genus (type A, B or C), the species or host from which the virus was isolated (omitted if human and if the virus was isolated from inanimate object, the material is indicated), geographical location of isolate which could be country or state, the reference number of the isolate, the year of isolation, and, in the case of the influenza A viruses, the haemagglutinin (H) and neuraminidase (N) subtypes (White and Ferner, 1994). For example, the index case of Avian influenza isolate in Nigeria is designated: Influenza A/chicken/Nigeria/220/2006-H5N1 (Joannis *et al*. 2006).

This system of classification by WHO expert committee has not changed since 1981 (OIE, 2009).

Influenza B and C infects only humans and has not been described in animals, until Albert Osterhaus and fellow workers at Rotterdam University, detected influenza B in seals in the coast of Netherland (Osterhaus *et al.,* 2000). The genus thogotovirus contains two different species, Dhori virus and thogoto virus. Both viruses were isolated from ticks, and thus they are very different from influenza virus with respect to their host range. The genus Isavirus (Infectious salmon anemia virus of fish) is also very distinct from influenza viruses A, B, and C, although genetic, morphologic, and biochemical studies identify these isolates as belonging to the Orthomyxoviridae family (Fauquet, *et al*., 2004).

2.2.2 PHYSICO CHEMICAL PROPERTIES

The airborne particle size of influenza virus is ≤ 0.05 micron, which can be readily inhaled through the respiratory passages to initiate infection of host cell receptors (Brankston *et al*., 2007). Though climatic factors like ambient temperature and relative humidity affects its transmission and infectivity, variation in temperature, pH, water salinity, and UV irradiation etc affects environmental survival of influenza virus (Gurtler, 2006).

At 4^{o}C, the half-life of influenza virus infectivity is about 2-3weeks and the virus is readily destroyed in few seconds at temperatures above 70^{o}C and 30 minutes at > 60^{o}C, 56^{o}C for 3hours. The virus can survive for 4 days in water at 22^{o}C and for more than 30days at 0^{o}C and in the environment (at 28-37^{o}C) for 6 days. The virus remains viable at moderate temperature for long periods and can survive indefinitely in frozen material as was demonstrated by the survival of 1918 H1N1 virus in frozen human bodies at the arctic pole (Oxford, 2000). Virus buoyant density is about 1.19g/cm^{3} in aqueous sucrose and single virion molecular weight (Mr) is 250 x 104 (Cox *et al*, 2000).

Influenza virus is readily inactivated by acidic pH and dryness, organic solvents and detergents such as sodium desoxycholate and sodium dodecylsulfate, β-propiolactone, formalin and other disinfectants. This physico-chemical attributes affects the persistence of the virus in the environment as well as their integrity while being manipulated in the laboratory.

2.2.3 VIRUS STRUCTURE AND CHARACTERISTICS

Influenza A viruses like all viruses have a complex structure, appearing spherical, enlongated or pleomorphic in shape with an average diameter of 120nm (Figure 1). It consists of inner core of single stranded RNA enveloped by a lipid membrane derived from the host cell (White and Fenner, 1994). The influenza A and B virus genomes consist of 8 separate segments covered by the nucleocapsid protein. Together these form the ribonucleoprotein (RNP), and each segment codes for a functionally important protein(s): viz: 1. Polymerase B2 protein (PB2), 2. Polymerase B1 protein (PB1), 3. Polymerase A protein (PA), 4. Haemagglutinin (HA or H), 5. Nucleocapsid protein (NP), 6. Neuraminidase (NA or N), 7. Matrix protein (M), 8. Non-structural protein (NS) (Palese and Shaw, 2006).

Morphologically influenza A virus particles which are spherical or pleomorphic are characterized by distinct projecting surface spikes of glycoprotein that are readily seen in electron micrographs of negatively stained virus particles. These spike (HA and NA) are about 10 to 14 nm, in ratio of 4 HA to 1 NA (Compans *et al*., 1972). The spherical particles have a diameter of about 100 nm, but filamentous particles with elongated viral structures (more than 300 nm) have frequently been observed. However, virions freshly isolated from infected human or animals are often filamentous, with fairly uniform diameter (80) and greatly enlongated lengths (Jin *et al*., 1997).

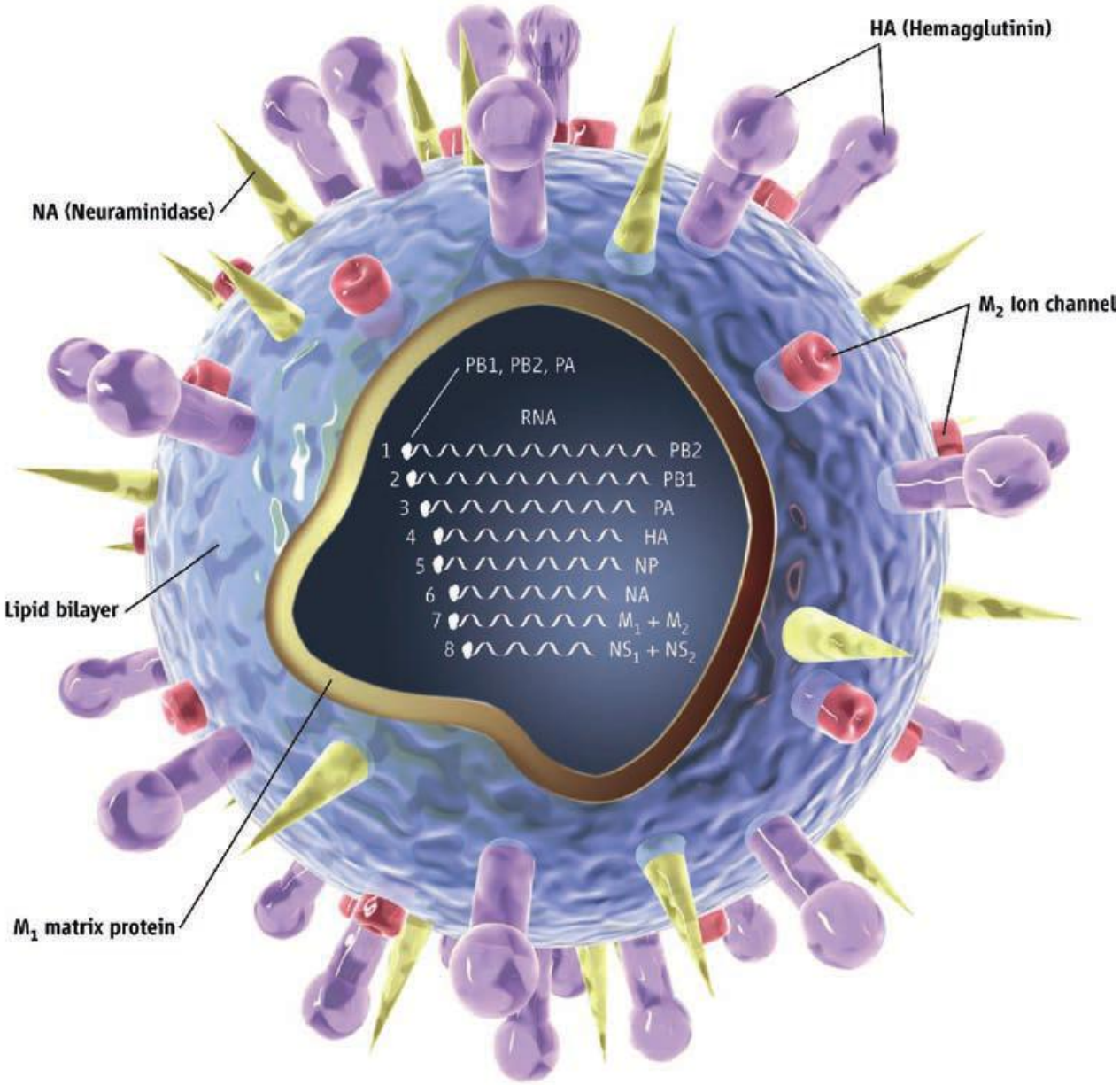

Figure 1: Structure of Influenza Virus showing the genome

Source: WHO Global Influenza Surveillance Network; Manual for the Laboratory Diagnosis and Virological Surveillance of Influenza (WHO, 2011)

2.2.4. INFLUENZA GENOME

Influenza A and B viruses possess eight segmented RNA genomes, while influenza C virus have seven segmented RNA genomes. Influenza A genome has a total length of approximately 13,600 nucleotides and encodes up to 13 proteins (McCauley et al. 2012**)**. Each gene codes for unique proteins that form the virus structure and proteins with each performing unique function in viral infection, replication and budding. Each viral segment contains noncoding regions at both 5' and 3' prime ends. The extreme ends are conserved among all segments, and this is followed by a segment-specific noncoding region. The influenza B virus genome is similar to that of influenza A virus with each of the eight RNA segments coding for one or more viral proteins viz:

1. Polymerase B2 protein (PB2)

The length of polymerase (basic) protein (PB2) nucleotides is 2341 and the polypeptide length amino acid is 759. It is a component of RNA transcriptase, mRNA synthesis and host cell capped RNA recognition and binding.

2. Polymerase B1 protein (PB1),

Polymerase (basic) protein (PB1) is also 2341 nucleotides long and 757 polypetide amino acid long. It is a component of RNA transcriptase, mRNA Synthesis, initiation of RNA transcription and possibly has an endonuclease activity. Recently, an 11th protein, the PB1-F2, has been found to be encoded by most influenza A viruses. In this case, an alternate open reading frame near the 5 prime end of the PB1 gene gives rise to the 87-amino acid long PB1-F2 polypeptide. It appears that this protein is an accessory protein because some human and animal influenza A virus strains lack this open reading frame. A cognate PB1-F2 protein in influenza B and C viruses has not yet been identified.

3. Polymerase A protein (PA)

Polymerase (Acidic) protein (PA) is 2233 nucleotide long and 716 polypeptide amino acids long . It is a component of RNA transcriptase, mRNA synthesis , initiation of RNA transcription and possibly has endonuclease activity.

4. Haemagglutinin (HA or H)

The Haemagglutinin (HA) is 1778 nucleotide long with 566 polypeptide amino acid. It is a surface glycoprotein, trimer in shape. It is cleaved into HA1 and HA2 just before virus entry following attachment to the host cell. It is the major antigenic determinant and performs multiple functions in virus adsorption and fusion (Figure 2 & 3).

5. Nucleoprotein (NP)

The nucleo-protein (NP) is 1565 nucleotide long and 498 polypeptide amino acids in length . It is associated with RNA segments to form ribonucleoprotein. It is a structural component of RNA transcriptase.

6. Neuraminidase (NA or N)

The neuraminidase (NA) is 1413 nucleotides and 240 polypetide amino acids in length. It appears as tetramer surface glycoprotein. It has neuraminidase activity and function in virus release from host cell.

7. Matrix protein (M)

The Matrix (M) is composed of M1, M2, and M3. It is 1027 nucleotides long and 252 polypetide amino acid. M1 is an important structural component of virus, underlining the lipid bilayer and may interact with HA and NA. M2 is spliced mRNA, it is non structural protein present on surface of infected host cells. M3 is spliced mRNA peptide predicted by nucleotides sequence only

8. Non-structural protein and nuclear export protein (NS1,NS2-NEP);

The Non-Structural protein has two component parts- NS1 and NS2. Made up of 890 nucleotides in length and 230 (NS1) and 121 (NS2) polypetide amino acid. Non structural protein may be involved in shut –off of the host cell protein synthesis and virus RNA synthesis. NS2 is spliced mRNA non structural protein and function is not clear. Graphical features of the HA protein an its antigenic binding domains is shown in Figure 2 and 3. On the bases of serologic properties and phylogenetic clusters 16 (18) HA and 9 NA subtypes were defined and can recombine in different HA and NA to give rise to 144 influenza A subtypes (Bao *et al.*, 2008; McCauley et al. 2012). Further subdivision of influenza virus subtypes into several distinct genetic lineages of each genome also contribute to viral delineation (Lu et al. 2007). These lineages evolved by accumulation of nucleotide substitutions (genetic drift) and virus circulation in restricted areas (geographic isolation).

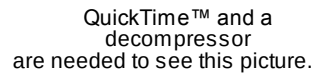

Figure 2: Structure of Haemagglutinin (HA) protein showing antigenic domains of the globular head and attachment to modeled human receptor.

Source: Imai and Kawaoka (2012)

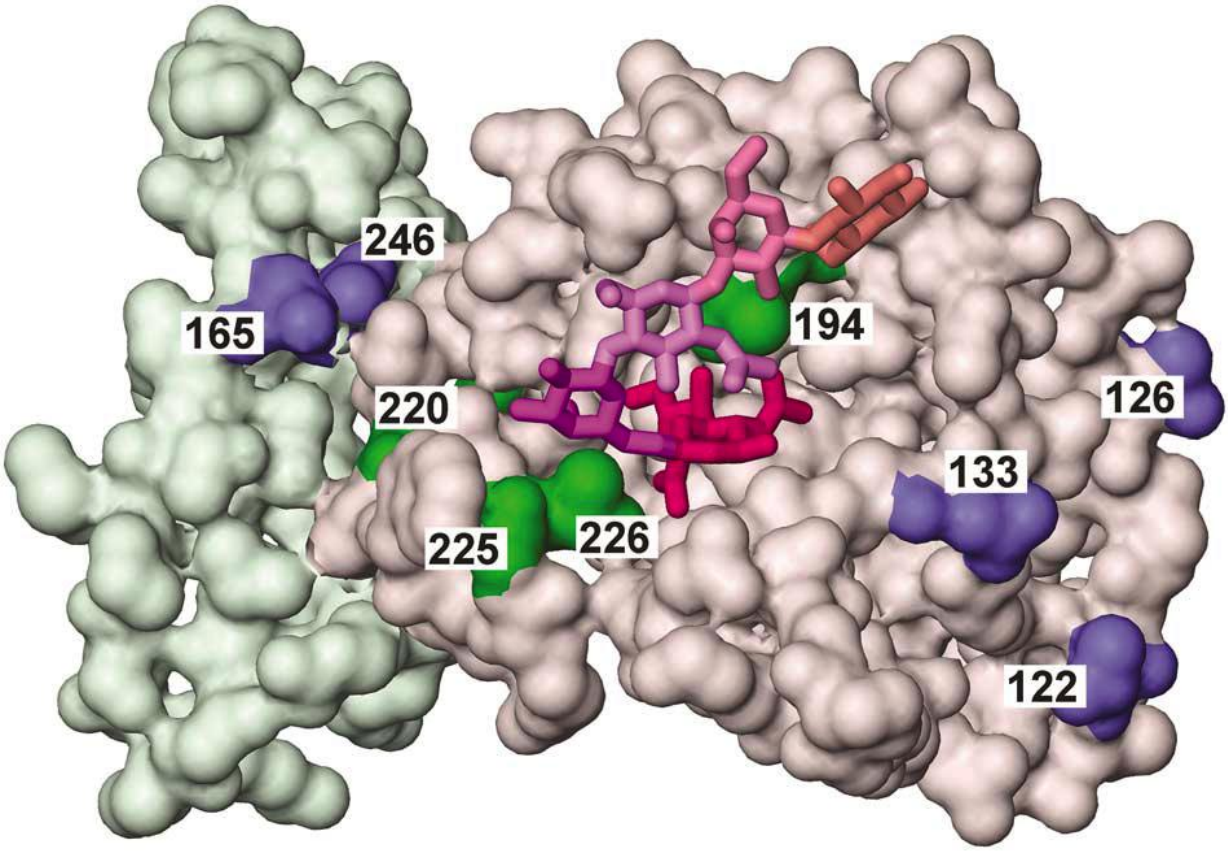

Figure 3: Three-dimensional model of the X-31 HA complexed with LSTc pentasaccharide (DS ViewerPro 5.0, Accelrys Inc. software).The glycosylated amino acids (purple) and amino acid substitutions appeared in HA of CE isolated human viruses (green) are shown on two neighboring HA1subunits (coloured by pale purple and mint green).

Source: Eisen *et al.*, 1997.

2.2.5. **VIRUS REPLICATION**

2.2.5.1 **Attachment**

Unlike most RNA viruses, the influenza virus RNPs are independently transcribed and replicated in the nucleus of the infected cells (Herz *et al*., 1981). Infection and replication is initiated when influenza viruses bind to N-acetylneuraminic acid (sialic acids) on the surface of host cells as illustrated in (Figure 4). Early reports indicated that viral RNA synthesis is associated to the nuclear cage and take place at fixed sites in the nucleus of infected cells (Jackson et al., 1982). The interaction of influenza viruses with ubiquitous molecule like sialic acid is constrained by the fact that the HAs of viruses that replicate in different species show specificity toward sialic acids (neuraminic acid) with different linkages. Human and swine viruses preferentially bind to sialic acid attached to the penultimate galactose sugar by an alpha 2,6 sialic acid linkage, whereas avian viruses mostly bind to 2,3 sialic acid linkage. In agreement with this finding is the fact that human and swine tracheal epithelial cells contain mostly alpha 2,6 Gal linked sialic acid, while the gut epithelium from ducks possesses mostly alpha 2,3 Gal sugar moieties linked sialic acid. However this viral specificity is not absolute as avian and human cells can contain both sialic acid linkages (2,3 and 2,6). Swine particularly are known to contain both types of sialic acid linkage in large proportion in their tracheal epithelium, hence are regarded as missing vessel because they can both be infected with avian or human type of the influenza virus. This demonstrates their importance in the epidemiology of influenza as mixing vessel (Newhouse *et al*., 2009; Viswanathan *et al*., 2010).

When viruses are passaged in a particular host they can adapt to that host by mutating the receptor-binding site in the viral HA. It was believed that the HA gene of the 1918 influenza virus has its origin in avian species and that a single amino acid change allowed the haemagglutinin to recognize the 2,6-linked sialic acids prevalent in human cells (Glaser *et al*., 2005; Stevens *et al*., 2006)

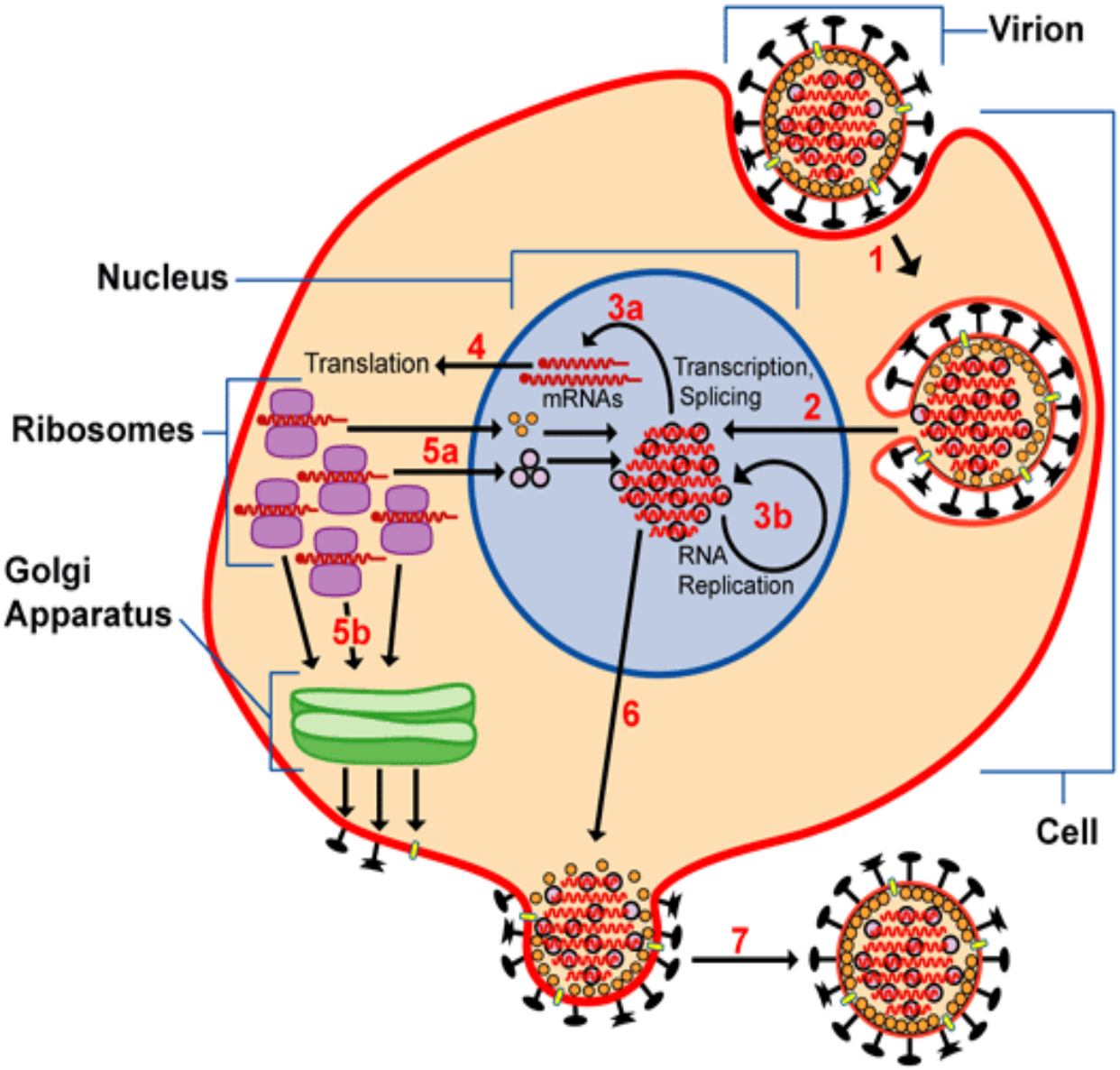

Figure 4: Influenza virus replication cycle

Source: National Council for Biotechnology Information (NCBI), Influenza resources. http://www.ncbi.nlm.gov/genomes/flu/html

2.2.5.2 FUSION AND UNCOATING

Although some viruses like the paramyxoviurses can enter cells directly through the plasma membrane by a pH-independent fusion process, influenza viruses and other envelope viruses like rabies require a low pH (acidic) to fuse with endosomal membranes and are therefore internalized by endocytosis. After binding to the target cell surface and endocytosis, the low pH of the endosome activates fusion of the viral membrane with that of the endosome. This fusion activity is induced by a structural change in the HA of influenza viruses, but in order for this to occur the HA0 precursor must first be cleaved into two subunits, HA1 and HA2. Once the virion is in the acidic environment of the endosome, the cleaved HA molecule undergoes a conformational change, and this exposes the fusion peptide at the N-terminus of the HA2 subunit, enabling it to interact with the membrane of the endosome. The transmembrane domain of the HA2 (inserted into the viral membrane) and the fusion peptide (inserted into the endosomal membrane) are in juxtaposition in the low pH-induced HA structure.

The concerted structural change of several hemagglutinin molecules then opens up a pore that releases the contents of the virion (i.e., viral RNPs) into the cytoplasm of the cell. The precise timing and the location of uncoating depends on the pH-mediated transition of the specific HA molecule involved (Wright and Webster, 2001).

2.2.5.3. TRANSCRIPTION

A minor virus envelope protein M2 acts as ion channel by making the inside of the virion more acidic. As a result, the major envelope protein M1 dissociates from the nucleocapsid and vRNPs are translocated into the nucleus via interaction between NP and cellular transport machinery. In the nucleus, the viral polymerase complexes transcribe and replicate the vRNAs.

2.2.5.4 TRANSLATION

Newly synthesized mRNAs migrate to cytoplasm where they are translated. Posttranslational processing of HA, NA, and M2 includes transportation via Golgi apparatus to the cell membrane.

2.2.5.5 ASSEMBLY, PROCESSING AND BUDDING

NP, M1, NS1 and NEP (nuclear export protein) move to the nucleus, where they bind freshly synthesized copies of vRNAs. The newly formed nucleocapsids migrate into the cytoplasm in a NEP-dependent process and eventually interact via M1 with a region of the cell membrane, where HA, NA and M2 have been inserted. Then the newly synthesized virions bud from infected cell. NA destroys the sialic acid moiety of cellular receptors and releases the progeny virions (Nicholson et al., 1998; Lamb and Krug 2001)

2.3.0 ECOLOGY AND EVOLUTION OF INFLUENZA

Human and classical swine viruses show common ancestry and American, Europe and Asian lineages have been described for swine influenza. Interaction between humans, pigs, birds and other mammalian species gave rise to a high potential for cross-species transmission of influenza viruses in nature such that species-specific lineages of viral genes have demonstrated that the prevalence of interspecies transmission depends on infected animal species (Webster *et al*., 1992). In avian viruses, two sublineages are American and Eurasia are defined for all eight segments suggesting an important role for host species in the evolution of influenza virus gene pools. Significant differences in evolutionary rates exist for influenza A, B, and C viruses. Type B viruses, but especially type C viruses, evolve more slowly than influenza A viruses. Type B and C viruses seem to be near or at an evolutionary equilibrium in human; genes of type A viruses were introduced into the human population less than 150 years ago, most likely from avian species but are yet to reach evolutionary equilibrium. Influenza A viruses in humans evolve along single-branch lineages, which suggests evolution by clonal selection and limited co-circulation of sublineages. Co-circulation of sub lineages has however been shown over limited periods. In contrast, the evolution of influenza B and C viruses is characterized by the co-circulation of antigenically and genetically distinct lineages for extended periods of time. For influenza B viruses, two lineages, the B/Victoria lineage (represented by B/Victoria/2/87) and the B/Yamagata lineage: (represented by B/Yamagata/16/88) have been co-circulating for about 25 years with changing patterns of prevalence and geographic distribution (Webster *et al*, 1992).

All mammalian influenza A viruses are derived from the avian influenza virus pool and are the natural reservoir of infection (Fouchier *et al*., 2005). At both the nucleotide and amino acid levels, the evolutionary rates are significantly lower for avian influenza viruses and in wild aquatic birds, influenza viruses seem to be in an evolutionary stasis, suggesting optimal adaptation of these viruses to their hosts. In this situation, amino acid substitutions may not provide selective advantages. Although mutations occur with similar frequencies, they do not result in amino acid changes. In contrast, all gene segments of mammalian (pigs, humans) and land-based poultry viruses continue to accumulate amino acid substitutions. Influenza viruses have a remarkable capacity to undergo variation in their surface glycoprotein (HA and NA) in two fundamental ways known as antigenic shift and antigenic drift (Katz *et al*., 2011).

2.3.1.0 INFLUENZA VIRUS GENETICS:

2.3.1.1 Reassortment

Reassortment is the rearrangement of viral gene segments in cells infected with two different influenza viruses (Zhou *et al*., 1999). Reassortment can theoretically be by various combination of H1 to H17 and N1 to N9 resulting in 256 different gene variations (i.e., the two parental genotypes and 254 new gene combinations). Reassortment occurs among influenza A, B, and C viruses, but has not been observed among or between the different types of influenza viruses. The importance of reassortment to the generation of new influenza virus strains is highlighted by the pandemics of 1957 and 1968, which were caused by reassortant viruses that contained HA, PB1, and NA or HA and PB1 segments of avian virus origin in a human genetic background. Moreover, reassortant H1N2 viruses emerged in humans in 2001 and have since become established in human populations. Further proof of the significance of reassortment comes from the emergence of a triple human/avian/swine reassortant that has spread throughout the North American pig population. The H5N1 viruses currently circulating in Asia, causing fatal infection in humans arose from multiple reassortment events among avian influenza viruses. More recently in the influenza A/H1N1 2009 pandemic was said to have originated from swine, combining avian, swine and human genetic materials (Garten *et al*., 2009)

2.3.1.2 Recombination:

Recombination occurs in influenza virus segments that contain genetic material from more than one origin. For negative-sense RNA viruses, homologous recombination is not a common event; however, recombination by template switching can take place and lead to increased biological fitness of the virus. For example, the insertion of 54 nucleotides of 28S ribosomal RNA into the A/turkey/Oregon/71 HA gene increased HA cleavability. Two avian influenza viruses of low pathogenicity have converted to high pathogenicity following the insertion of 21 nucleotides of the M segment or 30 nucleotides of the NP segment into the HA segment. Serial egg passages of an A/WSN/33 virus containing a 24-amino acid deletion in the NA stalk led to variants that replicated efficiently in eggs, the NA stalk of these variants contained sequences that originated from the PB1, PB2, or NP genes. The increased identification of recombinant viruses in recent years may indicate that recombination occurs more often than previously believed. In most cases, the event may be masked by the low biological fitness of the resulting viruses, whereas under selective pressure, recombination may give rise to a better fit virus (Suarez *et al*., 2004)

2.3.1.3 Antigenic Drift and Shift

(a) Antigenic drift

Antigenic drift results from a gradual accumulation of point mutations in the genes encoding the HA and NA. This can lead to emergence of variants that are no longer sufficiently inhibited by antibody to older strains, allowing the variants to predominate and become the next epidemic strain. In mammals, influenza A virus drift variants arise from the positive selection of spontaneous mutants by neutralizing antibodies. Antibodies to the parent strains can then no longer neutralize these variants. Antigenic drift has also been observed among influenza viruses in domestic poultry, although to a lesser extent than observed in humans. Mutations in the human virus HA or NA amino acid sequence occur at a frequency of less than 1% per year. Nevertheless, antigenic drift variants can cause epidemics and typically prevail for 2 to 5 years before being replaced by a different variant. This is the thrust of WHO continuous surveillance for human seasonal influenza so as to vary vaccine candidate virus to reflect circulating strains as illustrated in figure 5. (WHO, 2012).

(b) Antigenic Shift

Antigenic shift may occur either due to a reassortment event whereby human influenza viruses acquire a novel HA and one or more viral genes from an animal influenza virus, or by the introduction of a wholly animal virus into humans, followed by acquisition of point mutations to facilitate adaptation to human. If the new virus can have the capacity to spread efficiently and cause disease in humans, it results in a pandemic as was observed in H1N1 pandemic of 2009 (Garten *et al*., 2009). The HA protein is the major antigenic component of the virus, single point mutations in one HA antigenic site is sufficient to cause antigenic variation. Minor antigenic heterogeneity among the viruses is detectable at any time, while larger differences, detectable in hemagglutination-inhibition tests (WHO, 2012) usually require the accumulation of mutations over a 2- to 5-year period. Antigenic drift has also been reported for NA and correlated with amino acid differences in the molecule.

2.4.0. EPIDEMIOLOGY OF ANIMAL- HUMAN INFLUENZA

Prior to 1997, the transmission of avian influenza viruses to humans was not a serious public health threat. This assumption was based on findings that avian viruses do not replicate and transmit efficiently in humans and that no fatal cases of human infections had been reported during any outbreaks of highly pathogenic avian influenza (HPAI) (Peiris *et al.*, 2007) The differences in receptor-binding specificities between human and avian viruses were believed to provide a host range barrier that made the transmission of avian viruses to humans highly unlikely (Gambaryan *et al*., 2006). Until 1997, only three cases of direct avian-to-human transmission of influenza viruses are described: (a) an HPAI virus of the H7N5 subtype isolated from a patient with hepatitis in 1959, (b) an H7N7 HPAI virus isolated from a laboratory worker who developed conjunctivitis, and (c) an H7N7 virus of low pathogenicity isolated from a woman who developed conjunctivitis and was likely infected from ducks she kept. These cases, together with serologic studies demonstrated the potential for animal influenza viruses to be transmitted to humans; however, the threat was not fully realized until 1997, when avian H5N1 viruses were transmitted to humans in Hong Kong and killed 6 of 18 patients. The observed initial

mortality rate of 33% in 1997 has increased to 67% by 2012 and caused worldwide concern of a looming pandemic of avian origin (WHO, 2008; Keawcharoen *et al*., 2010).

The pandemic viruses emerging in human, called influenza A (H1N1) viruses contain genes from both Europe and North American lineage of swine influenza viruses (SIV). SIV is not the same in Europe, the United States and Asia in prevalence and genetic background. Not much data on African lineage is available in literature (Meseko *et al*., 2013). In China, mainly classical swine H1N1 and human-like H3N2 virus circulate in pigs. However, in recent years, SIV from Europe and North America have been introduced into Chinese pig herds, giving attention to the evolution of SIV in China. Worldwide, more than 50 cases of SIV infection in human have been documented since the 1970s, which indicate that SIV is also an important zoonosis, and the potential of SIV as human pandemic virus or genes donator in influenza ecology cannot be over emphasised (Qi *et al*., 2009; Kyriakis *et al*., 2009)

Influenza A viruses infect a wide variety of animals, including humans, birds, swine, horses, dogs, cats, whales, and seals. Viruses of all known HA and NA subtypes are maintained in aquatic birds and, therefore, they are considered the natural reservoir of influenza A viruses. In these hosts, influenza viruses are usually benign and in an evolutionary stasis (Alexander, 2000). Once introduced into land-based poultry or mammalian species, however, they evolve rapidly. Nonpathogenic viruses of the H5 and H7 subtypes can evolve into highly pathogenic avian strains that cause significant losses to the poultry industry. Viruses of the H9 and H6 subtypes have established or may be establishing lineages in chickens. Although human pandemics have been associated with viruses of the H1 to H3 subtypes, the ability of avian viruses of the H5, H7, and H9 subtypes to infect humans has flagged these subtypes as potential candidates of future influenza virus pandemics (Capua and Alexander, 2007). Influenza B viruses typically replicate in humans and rarely infect animals (Osterhaus *et al*., 2000).

The first report of interspecies transmission of influenza involving pigs dates back to 1934, when Shope presented serologic evidence for the transmission of a human virus to pigs (Shope, 1934). Further evidence for virus transmission between these two species came in 1976, when an H1N1 swine virus was isolated from a soldier who had died of

influenza at Fort Dix, New Jersey. This virus was subsequently isolated from five other soldiers, and serologic studies suggest that more than 500 personnel were infected. Numerous other reports have described the transmission of swine viruses to humans, some cases of which resulted in fatal outcomes and of the transmission of human viruses to pigs (Myer *et al*., 2007). An avian H9N2 virus was introduced into pigs in Southeast Asia, and H5N1 viruses were isolated from pigs in China and Vietnam. Recent reports of infection in pigs who had contact with human shedding influenza A/H1N1 2009 pandemic is a demonstration of the importance of studies in inter species transmission of influenza virus (Ma *et al*., 2008; Garten *et al*., 2009).

2.5.0 CLINICAL MANIFESTATION

2.5.1. CLINICAL MANIFESTATION IN PIGS

Influenza infection in pig is presented clinically in form of acute, severe respiratory illness often with high morbidity but less mortality rate. The sign of respiratory illness includes deep non-productive cough with abdominal effort and mild to moderate depression. The rate and quantity of feed consumption is reduced in mild illness, increases in percentage mortality may be observed in some pig production system from 0.43% to 0.87% and 0.19%to 2.04% (Howden *et al*., 2009). Infection can also be less severe and characterized by chronic respiratory disease syndromes affecting groups of pigs such as breeding pigs that may also suffer reproductive problems. Pigs may often be infected and not show signs of illness and recovery from non complicated co-infection can be quick (10-14 days. Infection is usually transmitted between pigs over short distances by aerosols generated by infected pigs coughing or sneezing, or by direct or indirect contact or movement of asymptomatic infected pigs. Presence of intercurrent infection can also make the disease appear more severe or seem to last longer.

Clinical signs of swine influenza in suckling pigs are unusual unless disease entered the herd for the first time. As maternally derived antibodies in sow colostrums may prevent infection during the sucking period. However where it occurs, high temperature, coughing, pneumonia are seen (Olsen *et al*., 2006).

When the virus first enters the herd two or three animals may be sick for the first two days, This is followed by rapid outbreak of inappetence and clinically ill pigs. The effects

on the reproductive system follow the sudden onset of a rapid spreading respiratory disease with coughing, pneumonia, fever and inappetence (Olsen *et al*., 2006).

Acute respiratory distress persists over a period of 7-10 days depending on the amount of contact between groups of sows. At a herd level the following may also be observed: sudden and rapid onset of acute illness in sows; coughing and pneumonia spreading rapidly, return to clinical normality over 7-10 days; delayed returns to heat after weaning; increased repeats at 21 days outside the normal cycle; increased numbers of sows coming through not in-pig; increased numbers of abortions, particularly late term; increased stillbirth rates, premature and slow farrowings; occasionally an increase in mummified fetuses which is a differential diagnosis of Porcine Respiratory and Reproductive Syndrome virus (PRRSV). Also during the phases of high temperatures other diseases present in the herd such as leptospiral infection associated with abortions may be triggered off (Lange, *et al*., 2009).

In weaners and growers, classically the pigs suddenly become prostrate, breathing heavily with severe coughing. Most pigs will look as if they are going to die but they mostly survive without treatment unless the herd already has a respiratory disease problem that could cause complications. Swine influenza causes severe pneumonia on its own but when it is combined with other infections like heamophilus suis a chronic respiratory disease syndrome can develop (Shope, 1931). In endemic situation, the virus remains in the herd, affecting small groups of pigs often weaners and growers that are responsible for continuing respiratory diseases with symptoms as in acute but less severe disease.

2.5.2. CLINICAL MANIFESTATION IN HUMANS.

WHO case definition of influenza or influenza like or severe acute respiratory disease (ART) include: sudden onset of fever usually greater than 38^0C, cough, and sore throat, shortness of breath or difficulty in breathing. In severe cases of pneumonia, vomiting, convulsion and lethargy may be accompanied by chest drawing or stridor in a calm child (Ortiz *et.al*, 2009). Following a brief incubation period of 1-3 days onset of the disease is usually abrupt with typical systemic symptoms of high fever and chills severe malaise, extreme fatigue and weakness headache or myalgia, as well as respiratory tract signs such as non-productive cough, sore throat, and rhinitis (CDC, 2005). Among children, otitis

media, nausea, and vomiting are also common (Peltola, 2003). In rare cases, the initial presentation may be a typical febrile seizures, and bacterial sepsis, (Dagan, 1984; Ryan-Power, 1995).

The severity of clinical presentation varies from a febrile respiratory symptom mimicking the common cold to severe prostration without major respiratory signs and symptoms, especially in the elderly. The severity of symptoms is related to the severity of the fever. Fever and systemic symptoms typically last 3 days, occasionally up to 4-8 days, and gradually diminishes, however, cough and malaise may persist for more than 2 weeks. Full recovery may take 1-2 weeks, or longer especially in the elderly.

Adults are infectious from as early as 24 hours before the onset of symptoms until about 7 days thereafter. Children are even more contagious: young children can shed virus for several days before the onset of illness and can be infectious for > 10 days (Frank, 1981). Several immunocompromised persons can shed influenza virus for weeks or months (Klimov, 1995; Boivin, 2002).

During non-epidemic periods, respiratory symptoms caused by influenza may be difficult to distinguish from symptoms caused by other respiratory pathogens. However, the sudden onset of the disease and rapid spread, fever, malaise, and fatigue are characteristically different from the common cold that can be cause by any or combination of over 200 upper respiratory tract viruses. Human influenza virus infections are frequently complicated by pneumonia, with secondary bacterial pneumonia being the most common form and primary influenza pneumonia the most severe. In addition, mixed viral and pneumonia frequently occurs during outbreaks and are the major cause of deaths. Influenza may exacerbate heart or lung diseases or other chronic conditions.

2.6.0 TRANSMISSION

Influenza can be introduced into animal herd by infected animals such as carrier pigs, poultry and feral birds and humans that are clinically sick particularly handlers and husbandry attendants. This scenario of multiplicity of infection is worse in a mix and intensive farm because swine influenza and influenza generally can be aerosolized, winds and other fomites can be carriers of the virus (Wen, *et al*., 2008). This is important where

neighboring farms are infected and where piggeries are located in urban or peri urban areas with high human population density. Though aquatic birds are reservoirs of infection and almost all H16 and N9 combinations of influenza are known to be naturally present in water fowls (Alexander 2000). Whereas pigs serve as mixing vessel for all subtypes, thus providing the condition for emergence by recombination, new subtypes including pandemic strains. Infected human can shed detectable amounts of influenzavirus the day before symptoms begin. Adults usually shed virus for 3–5 days (Murphy et al., 1973) whereas young children can shed virus for up to 3 weeks and immune compromised persons have been reported to shed influenza virus for even longer periods (Englund, *et al* 1998).

2.7.0 PATHOLOGY

2.7.1 PATHOLOGY OF INFLUENZA IN PIGS

Necropsy examinations on grower/finisher pigs grossly showed poorly collapsed lungs with a rubbery texture and mild interlobular edema in the dorso-caudal lobes. Multifocal disseminated, dark red-purple, shrunken and firm individual lobules, sharply demarcated from adjacent lobules and coalescing in cranio-ventral regions can be observed (Taubenberger and Morens 2008). A gross diagnosis of lobular to coalescing broncho-pneumonia are also seen in infected pigs with evidence of secondary bacterial infections and copious purulent exudate in airways. Also observe in the lung is severe consolidation with fibrin and abscessation in some pigs, though this is not a consistent feature (Landolt *et al*., 2003).

Histopathologic examination of the trachea and lungs shows mild, chronic, non-specific tracheitis moderate broncho-interstitial pneumonia with perivascular and peribronchiolarlymphoid hyperplasia, mild multifocal necrotizing and suppurative alveolitis, and subacute to chronic necrotizing to hyperplastic bronchiolitis. These lesions are compatible with mild to moderately severe infection with multiple respiratory pathogens including Porcine Reproductory and Respiratory Syndrome (PRRS) virus and secondary or opportunistic bacterial pathogens.

2.7. 2 PATHOLOGY OF INFLUENZA IN HUMAN

Influenza viruses infect the columnar epithelium lining the respiratory tract and can cause infection in both the upper and lower airways. Influenza A virus induces changes throughout the respiratory tract, but the most significant pathology is present in the lower respiratory tract. During bronchoscopy in uncomplicated influenza infections, acute diffuse inflammation of the larynx, trachea, and bronchi was observed with mucosal inflammation and edema (Taubenberger and Morens 2008). Light microscopic studies of infected cells show that columnar ciliated cells become vacuolated, edematous, and lose cilia before desquamating down to a one-cell-thick basal layer. Submucosal edema and hyperemia occur with an infiltration by neutrophils and mononuclear cells. In more severe primary viral pneumonia, there is an interstitial pneumonitis with marked hyperemia and broadening of the alveolar walls, with a predominantly mononuclear leukocyte infiltration, capillary dilation and thrombosis. Influenza virus-specific antigen is present in types 1 and 2 alveolar epithelial cells, as well as in intra-alveolar macrophages (Hamilton-Easton and Eichelberger, 1995). The pathological changes associated with the H5N1 virus include a hemophagocytic syndrome, renal tubular necrosis, lymphoid depletion, and diffuse alveolar damage with interstitial fibrosis. Necrotizing changes may occur with rupture of walls of alveoli and bronchioles (Chan *et al*., 2005).

At a cellular level, influenza virus shuts off cell protein synthesis and induces apoptosis as an additional mechanism of cell destruction (Hinshaw *et al*., 1994)

2.8.0 IMMUNOLOGY

The respiratory tract has multiple nonspecific protective mechanisms against influenza virus infection, including the mucin layer, ciliary action, and protease inhibitors, that may prevent effective cell entry and virus uncoating (Holmgren and Czerkinsky, 2005). The extremely short incubation period between infection and clinical illness implies that innate immunity or preformed cognate recognition components are very important in mounting defense against influenza invasion (Lessler *et al*., 2009). A role for neutrophils and alveolar macrophages in limiting virus replication has been suggested in murine infection

with a reconstructed 1918 virus. A transient increase in natural killer cell activity occurs after influenza virus infection.

2.8.1 INNATE IMMUNE RESPONSE

Once the epithelial cell is infected with influenza virus, a complicated series of intracellular events are triggered. At least two toll-like receptors, TLR 3 and 7, recognize influenza virus and trigger intracellular cascades leading to an innate resistance to infection. TLR3 is expressed on respiratory epithelial cells and recognizes dsRNA, presumably in the case of influenza virus in the form of a replicating intermediate. It acts through stimulation of the TRIF-IRF3 pathway to stimulate interferon (Iwasaki and Medzhitov, 2004). TLR 7 recognizes single-strand RNA, expressed on dendritic cells, and has the capacity to induce the interferon pathway and produce inflammatory cytokines on replication in these cells. Interferons may act in part through the induction of Mx protein, a large guanosine triphosphate related to dynamin (Staeheli and Haller, 1985).

2.8.2 HUMORAL AND CELLULAR IMMUNE RESPONSE

Antibodies are produced by plasma cells which are the final stage of B cell development, requiring that the B cells have recognized antigen and been stimulated by CD4 T cells and T cell-derived cytokines (Graham *et al*., 1993). Unlike T cells, B cells can recognize antigen in its naive form. The naive B cells then enter the circulation and travel via the blood stream and lymphatics through tissue and lymphoid organs. In the lymph nodes, naïve B cells recognize cognate antigen by their surface antibodies, become activated, switch from IgM to IgG production (class-switch), increase their immunoglobulin specificity and affinity, and differentiate into plasma cells or memory B cells as the cell continues to divide in the presence of cytokines (Graham *et al*., 1993). While IgA is transported across the mucosal epithelium of the upper airway, where it serves to neutralize and clear viral infection, IgG is primarily responsible for the protection of the lower respiratory tract (Palladino 1995, Renegar 2004). Antibodies act in immunity against influenza by neutralization of the virus or lysis of infected cells via complement or antibody dependent cellular toxicity. Hosts that survive an acute virus infection and clear the virus are in general immune to infections by the same virus (Welsh *et al*., 2003).

Nevertheless, acute infections caused by influenza virus occur repeatedly, despite active immune clearance. This is because influenza displays a structural plasticity as it can tolerate many amino acid substitutions in its structural proteins without losing its infectivity. As an example, the sialic acid receptor-binding molecule HA, responsible for entry of the virus into the target cell, is also a main target for neutralizing antibodies and cytotoxic T lymphocytes, which exhibit a continuous immunological pressure. This immune selection or diversity, which arises from copying errors, results in slight variations of HA over time that permit the virus to evade human immune responses (antigenic drift). These changes are the reason for the annual epidemic spread of influenza and require new vaccines to be formulated (Stöhr *et al*., 2012).

Dendritic cells that are sparsely distributed migratory group of bone marrow cells initiate T lymphocyte responses, derived leukocytes that are specialized for the uptake, transport, processing and presentation of antigens to T cells. The antigenic sample is processed and fixed on the dendritic cell surface as peptides which are presented by major histocompatibility complex (MHC) molecules (Silver *et al*., 1992). In the lymph nodes, the now mature dendritic cells efficiently trigger an immune response by any T cell with a receptor that is specific for the foreign-peptide-MHC complex on the dendritic cell surface (Shortman and Liu 2002). Endogenous antigens from the viral infection of dendritic cells are processed and presented to CD8 T cells on MHC I molecules. Exogenous antigens are presented via MHC II molecules to CD4 T lymphocytes.

The newly activated T cells acquire effector cell functions and migrate to the site of infection in the lung where they mediate their antiviral activities .Following recovery from an infection, a state of immunological memory ensues in which the individual is better able to control a subsequent infection with the same pathogen (Ahmed and Gray, 1996). During influenza infection, both CD4 and CD8 memory T cell subsets respond to, and mediate control of an influenza virus re-infection, which is in contrast to the primary infection where viral clearance depends on CD8 T lymphocytes (Woodland 2003).

2.9.0 DIAGNOSIS OF INFLUENZA

2.9.1 CLINICAL DIAGNOSIS

Clinical manifestation in either human and animals as enumerated earlier are diagnostic of influenza, but because over 200 other viruses can cause upper respiratory track infection, clinical signs and symptoms of influenza are normally combined with laboratory diagnosis for confirmation of influenza (Ong *et al*., 2009).

2.9.2 LABORATORY DIAGNOSIS

2.9.2 (a) SEROLOGY

Serology refers to the detection of influenza virus-specific antibodies in serum (or other body fluids). Serology can either detect total antibodies or be class-specific (IgG, IgA, or IgM). Different serological techniques are available for influenza diagnosis viz: haemagglutination inhibition (HI), complement fixation (CF), enzyme immunoassays (EIA) and indirect immunofluorescence. Serological diagnosis has little value in diagnosing acute influenza. In order to diagnose acute infection, at least four-fold rise in titre needs to be demonstrated (Gardner P.S. and McQuillin, 1968). This necessitates both an acute and a convalescent specimen. However it may have value in diagnosing recently infected patients. Serology is also used to determine the response to influenza vaccination (Prince 2003). Serology has greater clinical value in paediatric patients or young animals without previous exposure to influenza since previous exposure can lead to heterologous antibody responses also known as original antigenic sin (Steininger, 2002).

2.9.2 (a) i Haemagglutination inhibition (HI)

Haemaglutination Inhibition is the most widely used assay for detection of antibodies to influenza viruses. The test was developed by Hirst when he accidentally discovered the ability of influenza virus to agglutinate chicken red blood cells (Hirst, 1941). The demonstration that anitbodies in sera can inhibit agglutination of red blood cell by influenza virus led to the development of HI test with modifications by Salk (Salk, 1944). HI is labour intensive and time consuming assay that require several controls for standardisation. However the assay reagents are cheap and readily available. Various red blood cells such as guinea pig, chicken and human blood group O, erythrocytes are used

and 0.5 to 1% red blood cell dilution is generally used and serum is pre-treated to remove non-specific haemagglutinins and inhibitors (Balish *et al*., 2013). A viral haemagglutinin preparation that produces visible haemagglutination (usually 4/25ul or 8 /50ul haemagglutination units) is then pre-incubated with two-fold dilutions of the serum specimen. The lowest dilution of serum that inhibits haemagglutination is the HI titre. HI is more sensitive than complement fixation (Julkunen 1985, Prince 2003) and has the added advantage that it is more specific in differentiating between HA subtypes (Julkunen 1985).

2.9.2 (a) ii Complement fixation (CF)

Complement fixation test is an antibody mediated phenomenon based on the ability of antigen-antibody complexes to consume complement. This results in no complement being available to lyse sensitised sheep red blood cells (Friedewald, 1943). These assays are labour intensive and necessitate controls for each procedure but reagents are cheap and widely available. CF assays are less sensitive than HI both in the diagnosis of acute infection and the determination of immunity after vaccination (Prince 2003).

2.9.2 (a) iii Enzyme immuno assays (EIA)

The principle of EIAs is that virus or recombinant HA protein is absorbed to the wells of a microtitre plate followed by the sequential addition of serum sample, an enzyme-labelled antibody to detect immunoglobulin bound to the virus or HA, and substrate for colourimetric detection of binding. EIAs are more sensitive than HI or CF assays (Bishai 1978, Julkunen 1985). Various commercial EIAs are available. Assays that detect IgG and IgA are more sensitive than IgM assays (Julkunen 1985) but are not indicative of acute infection. ELISA measures antibodies that bind to influenza virions or to purified recombinant HA.. In ELISA antibody specific to influenza A or B can be discriminated but antibodies to influenza A subtpes cannot be distinguished. This lack of subtypes specificity is a shortcoming of traditional ELISA-based methods (Bishai *et al*., 1978; Hammond *et al*., 1980).

2.9.2 (b) Virus neutralization

Virus neutralization is highly sensitive and specific test for detecting strain specific antibodies that inhibit virus entry or block virus replication, including HA-mediated fusion of the viral envelop and the endosomal membrane (Zambon, 1998). The three steps involved in virus neutralization are 1. Virus-antibody reaction in which the virus is mixed with dilutions of the serum and allowed to react, 2. An inoculation step in which the virus-antibody mixture is inoculated into appropriate host system 3. A procedure to detect virus or antigen. The absence of infectivity by failure to detect virus, antigen or its pathological effect (CPE) means a positive neutralization test and indicate the presence of virus specific antibody in the serum sample (WHO, 2012b).

2.9.2 (c) Micronuetralization

The use of colorimetric detection of cell viability has recently been reported by Lehtoranta *et al*., (2009) Microneutralization combines the advantage of enzyme immuno assays with traditional virus neutralization test. More commonly ELISA has been used in Micronuetralization assays to detect viral antigen, typically the nucleoprotein (NP) expressed in infected cells following overnight culture. The presence of influenza virus is detected in fixed cells by a virus specific mouse monoclonal antibody followed by the addition of enzyme–labeled anti-mouse immunoglobulin antibody and a colorimetric substrate which can be either insoluble for detection of infected cells or soluble for spectrophotometric absorbance quantification of viral replication (WHO, 2010). Microneutralization assays using ELISA to detect viral-infected cells tend to be less variable than neutralization evaluated using cytopathic effect (CPE) (Stephenson *et al*., 2009). Studies have demonstrated the correlation between HI and VN or MN assays and had shown higher sensitivity of VN or MN over HI (Frank *et al*., 1980).

2.9.2 (d) SERO-ARCHAEOLOGY

Serology provides information about influenza virus outbreaks that preceded virologic techniques currently used to unequivocally identify infectious agents. Early studies suggested that the pandemic of 1889 to 1891 was caused by H2N2 subtype, whereas that of 1900 had been attributed to an H3N8 strain. More recent re-evaluation of data indicates that the 1889 to 1891 pandemic were caused by an H3-like virus, and there is no compelling evidence that links the H2 subtype to a pandemic other than that of 1957. The latter conclusion is substantiated by the lack of protection among those who were at least 80 years old during the 1957 H2 pandemic. Sero-archeology has also linked the 1918/1919 pandemic to an H1 virus, a finding that has been confirmed by molecular sequence determination of influenza virus RNA from the lung tissues of victims (Taubenberger, 1997). Studies using archived antibodies to the NA protein suggest that in the late 1800s, viruses of the N8 subtype were circulating and were later replaced by the N1 and N2 subtypes. Thus, during the 1900s, only a limited number of virus subtypes (H1N1, H2N2, H3N2, H3N8, and recently also reassortant H1N2 viruses) have become established.

2.9.2 (e) VIRUS ISOLATION

In 1930, Shope for the first time isolated the influenza virus, A/swine/Iowa/30, from pigs in a filteration experiments and by 1933 the first human virus was isolated by Wilson Smith, Sir Christopher Andrewes, and Sir Patrick Laidlaw of the National Institute for Medical Research in London, England (Smith *et al*., 1934). These investigators inoculated ferrets intranasally with human nasopharyngeal washes from an influenza patient. The animals exhibited an influenzalike disease, and the virus was transmitted to cage mates. One of their junior colleagues (late Sir Charles Stuart-Harris) became infected by these experimentally infected animals, and the virus was subsequently isolated from him. Because it was the first human influenza virus, it was termed influenza A virus. In 1940, an antigenically distinct virus was isolated and termed type B virus (B/Lee/40) and the first influenza C virus was isolated in 1947 (Buonagurio, 1986).

Influenza viruses were first propagated in embryonated hens eggs, which continue to be the system of choice for influenza research and growing large quantities of virus for

vaccine production. Avian, swine and equine strains of influenza A viruses can be isolated from the allantoic cavity of 10 to 12-day-old embryonated eggs after 2 to 3 days of incubation at 33°C to 37°C. Human influenza viruses have also been isolated from clinical samples on inoculation into the allantoic or amniotic cavity of eggs and incubation at 33°C to 37°C. Influenza virus growth in embryonated eggs leads to the selection of antigenic variants that are characterized by mutations in the HA protein (Bush *et al*., 1999). Influenza C viruses amplify in the amniotic, but not allantoic cavity of eggs and are usually grown for 5 days in 7 to 8-day-old embryonated eggs (WHO, 2011).

Influenza viruses can also be propagated in cell culture -Madin-Darby canine kidney (MDCK) cells which support the efficient replication of many (excluding avian) influenza A and B viruses and can be used to isolate viruses from humans. Although many influenza viruses can grow in African green monkey kidney (Vero) cells (Govorkova *et al*., 1996), they do so less efficiently than in MDCK cells. Cell culture systems based on MDCK and Vero cells are now being developed for influenza virus vaccine production. Influenza viruses also replicate in a number of primary cell cultures, including monkey, calf, hamster, and chicken kidney cells, as well as in chicken embryo fibroblasts and primary human epithelial cells. With the exception of primary kidney cells, most other cell culture systems require the addition of trypsin to cleave the HA protein of viruses (except highly pathogenic H5N1 viruses), which is a prerequisite for efficient replication. Replication of influenza viruses in eggs or cell culture is measured by testing the viruses ability to agglutinate erythrocytes of chicken, turkey, guinea pigs or human group O or by use of molecular biology techniques, such as reverse transcriptase (RT)-polymerase chain reaction (WHO, 2011).

2.9.2 (f) ELECTRON MICROSCOPY

Investigations of the structure of virions or of virus-infected cells can be carried out with the use of electron microscopy (Horne, 1960). Large magnifications are achievable with a transmission electron microscope but the specimen, whether it is a suspension of virions or an ultra thin section of a virus-infected cell, must be chemically treated so that details can be visualized. Negative staining techniques generate contrast by using heavy-metal-containing compounds, such as potassium phosphotungstate and ammonium molybdate. In

electron micrographs of virions the stains appear as dark areas around the virions, allowing the overall virion shape, size and structure to be determined. Further structural detail may be apparent if the stains penetrate any crevices on the virion surface or any hollows within the virion.

Negative staining techniques have generated many high quality electron micrographs, but the techniques have limitations, including structural distortions resulting from drying. Cryo-electron microscopy techniques are more recent. In these a wet specimen is rapidly cooled to a temperature below -160^0C, freezing the water as a glasslike material. The images are recorded while the specimen is frozen. They require computer processing in order to extract maximum detail, and data from multiple images are processed to reconstruct three dimensional images of virus particles (Goldsmith and Miller, 2009)

2.9.2 (g) POLYMERASE CHAIN REACTION

Polymerase Chain Reaction (PCR) is a biochemical technology whereby a single or few copies of DNA are replicated in several order of magnitude thereby generating millions of copies of a particular DNA sequence and was first developed in 1983 by Kary Mullis (Bartlett and Stirling, 2003). RT-PCR is a process whereby RNA is first converted to complementary DNA (cDNA) and a section of the genome is then amplified through the use of primers that bind specifically to this target area (WHO, 2011). This allows for exponential amplification of small amounts of nucleic acid, through the action of a thermo stable DNA polymerase enzyme, which enables highly sensitive detection of minute amounts of viral genome. Not only does RT-PCR have superior sensitivity (Steininger 2002) but it can also be used to differentiate between subtypes and conduct phylogenetic analysis (Allwinn, 2002). RNA degradation of archival samples can decrease the sensitivity of PCR (Frisbie 2004). Therefore specimens should be processed as fast as possible after collection.

The reverse-transcriptase-polymerase chain reaction (RT-PCR) can be used to determine minute amounts of mRNAs in tissue samples by co-amplification of a quantified amount of a competitive sequence, so called quantitative competitive (qc) RT-PCR. The first qRT-PCR was described by Wang *et al*. (1989). As an internal standard, an RNA template

with the same primer sites as the target mRNA was reverse-transcribed. The amplified PCR products differed in size and were separated by agarose gel electrophoresis. The presence of 32P-labeled 5' primer allowed quantification by scintillation counting. Determination of an unknown amount of any cDNA can also be performed by the use of an external standard, either based on a housekeeping gene or by serial dilutions of known amounts of an RNA standard quantified separately from the target RNA. Such approaches are subject to inter-sample variation in amplification efficiency.

To overcome these problems, a known amount of a standard is included in the sample, and competitive co-amplification of target RNA and standard is followed. The inclusion of an internal standard as DNA can control the PCR efficiency, but the inclusion of an internal standard as RNA can also control the reverse-transcription efficiency, and therefore provides more reliable quantitation. Construction of the competitor, the so-called internal standard, is the main part of the qRT-PCR technique. To act as competitor, it is important that the standard shows equivalence with the target mRNA with regard to primary and secondary structure, as well as primer affinity. This means that the length and sequence of the internal standard must be similar to the target, and contains only a slight modification to distinguish between the internal standard and target mRNA. To guarantee an optimal internal standard, it is advisable to produce a synthetic RNA (sRNA) standard by slight modification of the wild-type target cDNA sequence. Modification is achieved by either insertion or deletion of a few base pairs (bp), or manipulation of a restriction enzyme site.

The change of a restriction site makes post-PCR processing necessary, but a simple change of few bp (deletion or insertion) allows direct quantification of the PCR products. As an alternative to the modification procedure, some groups have used as an internal standard the homologous gene from another species based on interspecies sequence differences. Because of the high degree of homology between many interspecies sequences, it will be difficult to distinguish between the target mRNA and the internal standard after co-amplification. A simple detection based on fragment size differentiation is impossible, and therefore secondary structure differences are used to distinguish between the internal standard and the target mRNA sequence (Spackman *et al*., 2002).

2.9.2 (h) NUCLEOTIDE SEQUENCING

Dideoxy sequencing, also known as Sanger sequencing, proceeds by primer-initiated, polymerase-driven synthesis of DNA strands complementary to the template whose sequence is to be determined (Shendure *et al*., 2008). Numerous identical copies of the sequencing template undergo the primer extension reaction within a single microtitre scale volume. Generating sufficient quantities of template for a sequencing reaction is typically achieved by either:

(1) miniprep of a plasmid vector into which the fragment of interest has been cloned, or

(2) polymerase chain reaction (PCR) followed by a cleanup steps (Shendure *et al*., 2011).

In the sequencing reaction itself, both the natural deoxynucleotides(dNTPs) and the chain-terminating dideoxynucleotides (ddNTPs) are present at a specific ratio that determines their relative probability of incorporation during the primer extension. Incorporation of a ddNTP instead of a dNTP results in termination of a given strand. Therefore, for any given template molecule, strand elongation will begin at the 3' end of the primer and terminate upon incorporation of a ddNTP. In older protocols for dideoxy sequencing, four separate primer extension reactions are carried out, each containing only one of the four possible ddNTP species (ddATP, ddGTP, ddCTP, or ddTTP), along with template, polymerase, dNTPs, and a radioactively labeled primer. The result is a collection of many terminated strands of many different lengths within each reaction. As each reaction contains only one ddNTP species, fragments with only a subset of possible lengths will be generated, corresponding to the positions of that nucleotide in the template sequence (Shendure *et al*., 2011).

The four reactions are then electrophoresed in four lanes of a denaturing polyacrylamide gel to yield size separation with single-nucleotide resolution. The pattern of bands (with each band consisting of terminated fragments of a single length) across the four lanes allows one to directly interpret the primary sequence of the template under analysis.

Modern application of dideoxy sequencing differ in several key ways from earlier protocol. Only a single primer extension reaction is performed that includes all four ddNTPs. The four species of ddNTP are labeled with fluorescent dyes that have the same

excitation wavelength but different emission spectra, allowing for identification by fluorescent resonance energy transfer (FRET) (Winter *et al*., 1981; Didenko , 2007) .

To minimize the required amount of template DNA, a "cycle sequencing" reaction is performed, in which multiple cycles of denaturation, primer annealing, and primer extension are performed to linearly increase the number of terminated strands. This requires the use of engineered polymerases, such as ThermoSequenase, that are thermostable and that efficiently incorporate modified ddNTPs (Tabor and Richardson, 1995). The products of the cycle sequencing reaction are analyzed in an automated sequencing instrument via electrophoresis in a long capillary filled with a denaturing polymer that yields size separation with single-base-pair resolution. As fragments of each discrete length pass through a transparent component near the end of the capillary, a single wavelength of light excites the fluorophores linked to the ddNTPs. Labeled fragments fluoresce at one of four distinct wavelengths, revealing the identity of their terminal base via FRET. Simultaneous measurement of the emission spectra at these four wavelengths produces a four-color sequencing trace. Computer algorithms ("base callers") interpret the peak heights in these traces to produce a DNA sequence. Importantly, sophisticated algorithms exist that also define the accuracy with which individual base-calls are made. Although the per-base accuracy can vary substantially within a single sequencing read, the accuracy of the best base calls can be as high as 99.99%. (Ewing and Green, 1998; Ewing *et al*., 1998)

Nearly all dideoxy sequencing performed today makes use of automated capillary electrophoresis, which typically analyzes 96 to 384 sequencing reactions simultaneously via an array of capillaries. Major vendors include Applied Biosystems (e.g., the ABI 3730) and GE Healthcare (e.g., the MegaBACE instrument series). There is a trade off between long read lengths and the overall thoroughput of an instrument. Depending on which parameter is being optimized, conventional instruments are capable of reads just over 1000 base pairs in length, or production thoughputs of over 2.5 megabases per day (Shendure *et al*., 2011)

2.9.2 (i) PHYLOGENY, PHYLOGEOGRAPHY, AND BIO-INFORMATICS

Phylogenetic study of influenza virus is a useful tool in understanding the relationship between different isolates from across the world. With the aid of this molecular tool it has been possible to interpret that the novel 2009 influenza virus that emerge in Mexico shared genetic similarities with North America and European strains of classical swine influenza which informed initial nomenclature and the description that was used for the virus (Girard, 2009). Phylogenetic studies was also able to identify that the virus was a reassortant from avian, swine and human influenza viruses. (Garten, 2009)

The monitoring of influenza circulation from country to country and region to region across the globe defined clusters within and among countries hence the term phylogeography, by which different scientists are able to delineate the origin, spread and transmission of viruses from its initial source to other region. The collation of this genetic data synthesis and analysis has been made possible through the use of mathematical modeling in bio-informatics. This data are fed into computer programmes such as MEGA and Bayesian (Beast and beauti) that perform these analyses (Nelson *et al.*, 2011).

Bioinformatics tools involves the technology that uses computers for storage, retrieval, manipulation, and distribution of information related to biological macromolecules such as DNA, RNA, and proteins. The use of computers is absolutely indispensable in mining genomes for information gathering and knowledge building. Bioinformatics tool is extensively used in sequence, structural, and functional analysis of genes and genomes and their corresponding products. Analysing and comparing genetic material of different species is an important method for studying the functions of genes, the mechanisms of inherited diseases and species evolution. Bioinformatics tools can also be used to make comparisons between the numbers, locations and biochemical functions of genes in different organisms (Galperin and Koonin, 2010)

2.10.0 MANAGEMENT OF INFLUENZA INFECTIONS

2.10.1 BIOSECURITY

Recommendations for respiratory hygiene such as covering ones' mouth when coughing and avoid spitting, have been made more on the basis of plausible effectiveness than

controlled studies (CDC, 2003). Influenza virus can remain viable on environmental surfaces and is believed to be transmissible by hand or fomites (WHO, 2006). Most, but not all controlled studies show a protective effect of hand washing in reducing upper respiratory tract infections; most of the infections studied were likely viral, but only a small percentage were due to influenza (Fasley, 1999).

There is some evidence that containment and elimination of an emergent pandemic influenza strain at the point of origin is possible using a combination of antiviral prophylaxis and social distance measures. It should be noted that the idea of stopping a pandemic at its source or delaying its international spread, is an attractive, but as yet untested hypothesis. So far, no attempt has ever been made to alter the natural course of a pandemic once it has emerged in the human population (Ferguson, 2005).

Successful control of influenza in addition to chemotherapy, vaccination and hygiene also requires other interventions relevant for controlling emergent infectious diseases. These will involve community participation embracing public health workers, veterinary health workers, rural farmers, market women etc. Inter sectorial co-ordination involving other sectors especially agriculture, information, education, transportation, tourism and the military would need to be engaged in the planning and implementation of defenses against influenza (Jonas, 2013).

2.10.2 VACCINES AND VACCINATION

Vaccines are apathogenic entities that cause the immune system to respond in such a way, that when it encounters the specific pathogen represented by the vaccine, it is able to recognize it-and mount a protective immune response, even though the body may not have encountered that particular pathogen before (Stephen, 2006). The influenza vaccine is effective in preventing disease and death, especially in high-risk groups, and in the context of routine vaccination, the World Health Organization reports that the “influenza vaccine is the most effective preventive measure available” (WHO, 2005a; WHO, 2009). With regard to the present form of an imminent influenza pandemic, “Vaccination and the use

of antiviral drugs are two of the most important response measures for reducing morbidity and mortality rates during a pandemic" (WHO, 2005b).

In 1931, viral growth in embryonated hen's egg was discovered and in 1940s, the US military developed the first approved inactivated vaccines for influenza, which were used in the Second World War. (Hilleman, 2000; Baker, 2002). Greater advances were made in vaccinology and immunology, and vaccines became safer and mass-produced. Today, advances in molecular technology are on the verge of making influenza vaccines through the genetic manipulation of influenza genes (Couch, 1997; Hilleman, 2002).

The WHO global influenza surveillance network on influenza collates disease and epidemiological data from various eco-regions. Specimens are analyzed for virus isolation after which antigenic and genetic data form the basis for making recommendations on the influenza vaccine formulation for each season (Figure 5).

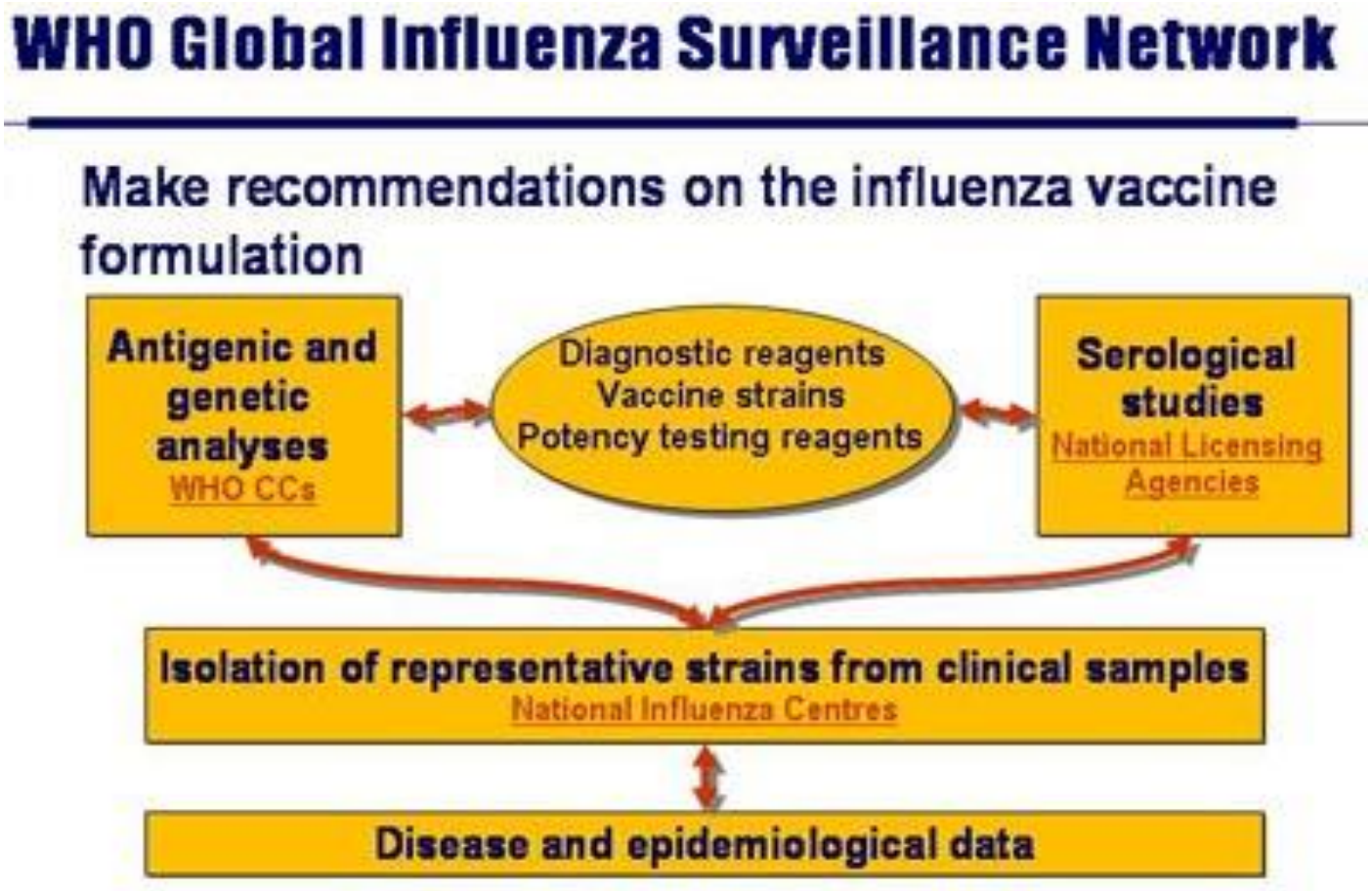

Figure 5: Flowchart of the WHO influenza surveillance (Source: © World Health Organization. www.who.int/csr/diseases/influential)

2.10.3 CHEMOTHERAPY

Influenza virus remains a constant health threat; major efforts have been directed at discovering effective antivirals over the past several decades. Fortunately, there are now four U.S. Food and Drug Administration (FDA)-approved drugs available for use in humans: amantadine, rimantadine, oseltamivir, and zanamivir (Long *et al*., 2000).

Although vaccination, in essence, targets the HA so that specific antibodies are generated that block attachment of the virus to the receptor, drugs that interfere with the HA-sialic acid interaction have not been successfully developed. Strategy, using sialic acid analogs (or polymers bearing sialic acids), has not yet advanced to human trials. Whether removal of receptor molecules from the respiratory tract by administration of exogenous sialidase/neuraminidase is a viable antiviral approach remains to be seen. It seems doubtful that receptors could be (safely) denuded for extended periods of time in order to prevent infection by influenza viruses (Skehel and Wiley, 2000).

Quinone derivatives that prevent the first stage of the conformational change of the HA and thus inhibit infection were discovered in 1993 (Bodian *et al.*, 1993) and other compounds with a similar mechanism showed inhibition for some strains but not for others. Some compounds have also been identified that appear to push the HA into an inactive state or that associate with the N-terminal heptad-repeat trimer, thus interfering with the trimer-of-hairpin (helix bundle) formation. This latter approach would be similar to that successfully applied for HIV using the fusion inhibitor T-20. So far, none of the influenza virus HA inhibitors has blocked all HA types or subtypes, and none has acceptable IC_{50} values in the submicromolar range (Cianci *et al*., 2004).

Amantadine, which has been known for many decades to inhibit most influenza A viruses, has been found to target the M2 ion channel. During uptake, virus enters endosomes where the acid pH activates the ion channel, resulting in the transport of protons into the viral interior. This process, which is required for the dissociation of the RNP complex from the M1 protein and subsequent release of the RNP into the cytoplasm, is blocked by amantadine and its derivatives (including rimantadine). In addition, amantadine can affect the pH regulation of vesicles involved in the transport of viral glycoproteins to the cell surface during assembly. Thus, there are two possible steps at which amantadine can exert

an antiviral effect: uncoating and HA stability (in some strains) during transport in vesicles. Unfortunately, resistance to amantadine and to its ten fold more active derivative, rimantadine, develops with increased use in humans and animals (Long *et al*., 2000. Hurt *et al*., 2006)

In a published report by Hastings *et al*., (1993), 2,4-dioxobutanoic acid and 2,6-diketopiperazine derivatives selectively inhibit the endonuclease activity (PB1) of the influenza virus polymerase. Ribavirin, an FDA-approved antiviral, and several other nucleotide analogs are also known to inhibit influenza in humans but its toxicity remains a problem. Also, capped and uncapped RNA fragments interfering with cap binding, capped-RNA primed transcription have been reported, but these compounds would have pharmacologic limitations because of difficulties in getting charged molecules into cells. Along with oseltamivir, zanamivir has become a highly important tool in efforts to curb the impact of epidemic and pandemic influenza and additional neuraminidase inhibitors are also in development (Moscana, 2005).

2.11.0 ONE HEALTH CONCEPT OF WHO/OIE/FAO

Zoonoses (diseases transmissible from animals to humans) account for approximately 60% of all infectious pathogens of human beings and 70% of all emerging infectious diseases (Woolhouse and Gowtage-Sequeria, 2005; Kahn *et al*., 2010). The origins of these diseases, and underlying factors (including man-made factors) in their emergence have been the subject of considerable interest (Kaplan *et al*., 2009). It is estimated that 56 different zoonotic diseases icluding influenza are responsible annually for 2.5 billion cases of human disease with 2.7 million deaths and substantial reductions in livestock production (Grace *et al*., 2013)

It is clear from epidemiological point of view, that as long as influenza virus circulates in animals, human contacts either directly in farms or indirectly in the environment are at a great risk of contracting the infection (Myers *et al*., 2007). Therefore, the principle of one health finds a lot of relevance in influenza epidemiology.

Primarily influenza virus is of avian origin, with adaptation to other hosts hence we have human, avian, swine, equine influenza based on a define lineages as a result of host receptor susceptibility (Alexander, 2000). But revelation with the HPAI H5N1 (1997-

2008) showed evidence of cross species transmission and susceptibility (van Reeth, 2007). Many species are now susceptible to avian influenza and were infected by H5N1 viz, human, dogs, cats, lions, tigers, leopard, seals, whales etc. (Cook, 2005). This may be as a result of antigenic changes in the HA glycoprotein of the virus and the sialic acid in the host receptor sites that promotes interspecies transmission (Nicholls, 2008). It has become clearer that genetic changes that eventually increase human susceptibility to new strain of influenza takes place many years before its clinical manifestation as was the case with 2009 pandemic (Aurora and Capua, 2011). A holistic approach to the surveillance and research of influenza across human and animal sectors is of utmost necessity as was suggested by Capua and Cattoli (2010). Analysis of the influenza gene pool as one entity irrespective of species and geography would improve understanding of the dynamics of the influenza virus in animals and humans and engender a greater understanding and information to support both public and animal health policy. The One Flu initiative could bring about international synergies and the bridging of gaps between medical and veterinary scientists, permanent monitoring of virus evolution and epidemiology, and investments in capacity building could also serve as models for other emerging diseases (Capua and Cattoli, 2010).

It is important that science and policy communities seek innovative ways to interdict zoonotic diseases by integrating human and veterinary medicine and vaccine development, and by creating new streams of funding aimed at the intersection of human and animal health. These aims are consistent with the Onehealth Initiative, which seeks to establish "collaborative efforts of multiple disciplines working locally, nationally and globally to attain optimal health for people, animals and the environment (Monath, 2013)

2.12. 0 INFLUENZA AND PIG HUSBANDRY IN AFRICA

Early domestication of pigs was introduced by the Portuguese to the West African coast (Blench, 2000). Early Africans often hunt wild pigs as bush meat. However at the turn of the 20th century, pig husbandry has become a major component of livestock farming in sub Sahara Africa raised under various husbandry practices including free range feeding, tethering,and confinement (Kimbi *et al*., 2001). Free roaming domestic pig abounds in

almost all the cities and villages including live pig market that serves as marketing outlet to homestead and free range pigs (Rekwot *et al*., 2003). Commercial pig husbandry is also popular with the introduction of exotic breeds like large white, landrace and duroc in institutional farms and commercial enterprise as investors in livestock farming found piggery very lucrative because of low over head cost and good turn over (Adesehinwa *et al.*, 2003; Faustin*et al.*, 2003). Intensive pig farm estates were established by Governments in Africa with Confinement Animal Feeding Operation (CAFO) (El–Hicheri, 1998, Saenz *et al*., 2006). CAFO operation increases because urbanization has increased the pressure on available space resulting in the intensification of agriculture in urban and peri urban areas (Saka *et al*., 2010). These urban and peri-urban livestock production is rapidly increasing in sub Saharan Africa not only because of the unavailability of land but also because of the need for livestock and economic activities in areas of high population density to supply much needed food (FAO, 2001; Dolberg, 2007). Expansive livestock farm operations intra city or in close proximity to urban areas with attendant poor biosecurity however, favours circulation of potentially dangerous viruses at the human and animal interface (Fasina *et al*., 2010).

Nigeria accounts for about 30% total pig production in Africa with over 10 million out of the 32 million quoted by FAO (FAOSTAT, 2009) and is clearly the most important country in Africa for swine influenza virus surveillance and research (Vincent *et al*., 2013; Figure 2.6). Domestic pigs are known to be endemic for different strains of swine influenza virus and clinical episodes of respiratory and reproductive losses are common (Smith *et al*., 2009). Pig farming, particularly in confinement is thus important in influenza epidemiology, not only to the animals but also for the health of the farmers and global epidemiology (Saenz, 2006).

Earlier surveillance and detection of influenza virus dates back to 1930, but in underdeveloped countries of Africa, surveillance activities was not noticeable in literature until the last half of the century.The burden of influenza in Africa was also thought to be negligible (Yazdanbakhsh and Krasner, 2009). However, sporadic reports from the Gambia (Mullholland, 1999), Senegal (Ndiaye *et al*., 2000), Congo and Madagascar (Nicholson *et al*., 2003), Kenya (Gachara *et al*, 2006), Ivory Coast (Akoua-Koffi *et al*,.

2006) and from Gabon (van Riet, *et al*, 2007), indicates influenza is circulating and may be causing regular epidemics, but are hardly reported and missing in the global data base because of the absence of sustainable surveillance system. The paucity of information on influenza is even worse with swine influenza compared to global spatial and temporal data on avian and human influenza (Finkelman *et al*, 2007; Russell, *et al*., 2008). Yet the potential exists for inter species circulation in some countries in the continent (Figure 6).

Globally, most surveillance systems are passive and are related only to specific disease of interest (Morse, 2007). A disease only gets significant mention and attention when its epidemic or pandemic level presents economic or public health risk as was the case with HPAI and SARS, which although may have existed earlier in animals but did not get mention until morbidity and mortality was observed in greater proportion (WHO, 2003). Diseases like polio, HIV/AIDS are accorded dedicated surveillance systems in Africa because of the prominence they assumed over the years and the problem associated with the continent being a reservoir of infection. Nevertheless, early detection, preparedness and control should be the guiding principle in swine influenza surveillance.

Different countries have their peculiar system of surveillance depending on public health and veterinary services priority (Yazdanbakhsh and Krasner, 2009). For instance, the outbreak of HPAI H5N1 in Nigeria with its attendant economic and public health risks brought much attention both locally and internationally to influenza surveillance in the region compelling a joint human and animal: (Avian Influenza Control and Pandemic Preparedness- AICP) surveillance (Tiongo, 2009). Generally, the structure of disease surveillance and control in Nigeria as with other countries in sub Saharan Africa operating a central or federal system of government is vested in the State or Local Governments, whose responsibility it is to ensure all reportable diseases as contained in the OIE list are passively or actively surveyed and documented with regular reports submitted to the Federal Department of Livestock. Attention of the Federal Government is brought in when a disease is of such magnitude beyond the capacity of the Local or State Councils to effectively control. Statutorily, all records of diagnosis at the private, local and State diagnostic facilities are expectedly referenced at the National Veterinary Research Institute or National level diagnostic facilities in other countries.

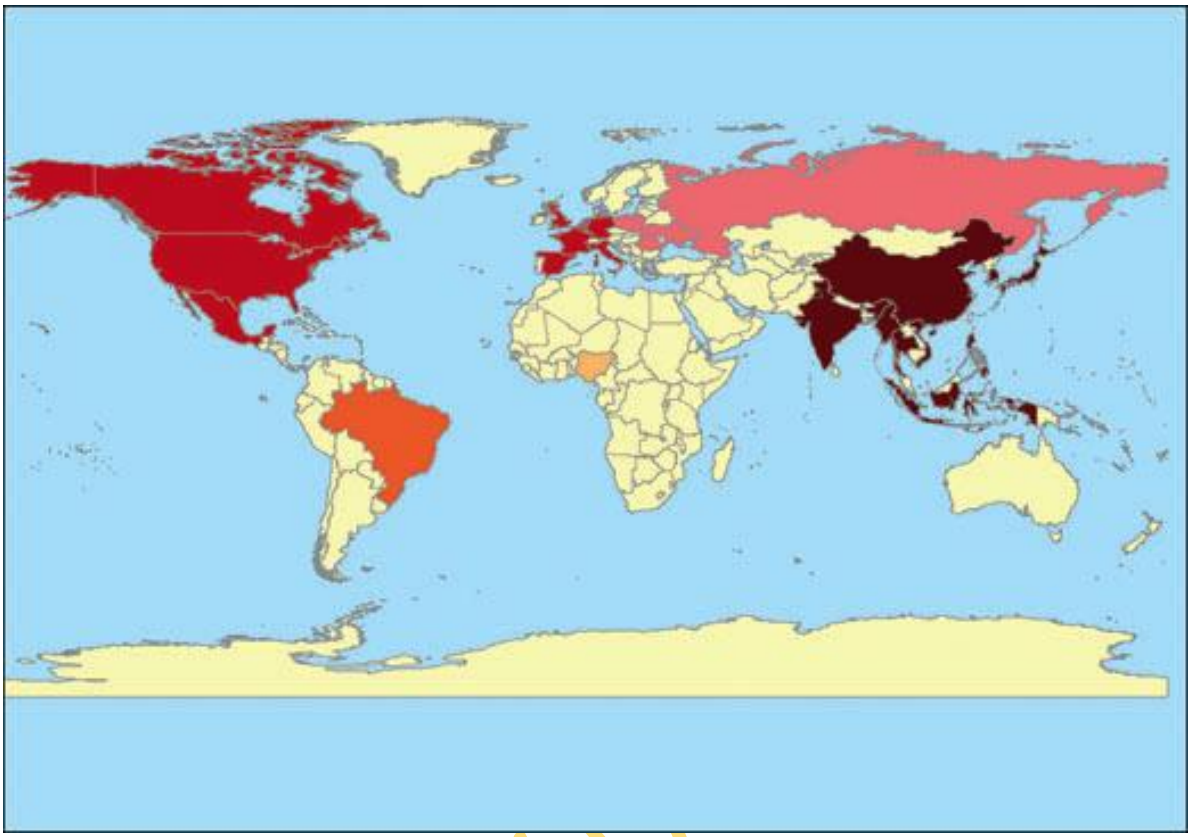

Figure 6: Countries with priority for targeted surveillance for influenza A virus (IAV) evolution or circulation in swine.

Colour Legend:

Brown- The highest priority for surveillance in swine is in countries of East and Southeast Asia due to higher rankings for spread and emergence of IAV in swine, with high population numbers of pig, poultry, and waterfowl.

Red- Western Europe and North America.

Pink- Countries of Eastern Europe.

Light orange- Nigeria ranked for potential reassortment between viruses circulating in pigs and birds.

Dark orange- Brazil ranked for outbreaks in pigs.

Source: Vincent *et al*., 2013

This tertiary institutes are saddled with the responsibility of disease investigation, diagnosis and research. The outcome of which is communicated to FLD who shares such information with OIE and FAO (Obi *et al*, 2008). This structure as plausible as it appears is however weak, which obviously is a reflection of the subsisting veterinary infrastructures and national development indices. Weakness of national surveillance is atimes attributable to political unrest, wars, terrorism (Al-Qeida, Boko haram), increasing global inequality, and cross-nation movement of people in regions of low income in Africa, Asia, and South America (Arita, *et al*., 2004).

Early description of influenza experiences in sub Saharan Africa was as far back as 1918-19 during the Spanish flu pandemic that diffused from the coastal cities of West Africa to the hinterland being brought by Ship from Europe and America (Patterson and Pyle, 1983). A sailing ship from Ghana reportedly took influenza virus to Lagos for the first time in 1918 (Patterson and Pyle, 1983). Following global remission of the pandemic, no other report was documented until the 1974 influenza A/H3 pandemic that was successfully isolated in chicken embryonated egg by Professor Tam David-West (1974) in the Virology Laboratory of the College of Medicine University of Ibadan. Probably this laboratory, famous for its discovery of many new viruses like Dugbe, Mokola, Sango, Shamonda, kotonkan etc in West Africa (Kemp *et al*., 1974) was more focused on arboviruses that had funding from the Rockefeller foundation not to have carried out extensive influenza surveillance prior to 1974. The work of David- West was prelude to serological investigations in human and animals that were to follow later in the 1989 and 1909. Olaleye *et al* 1989; 1990, 1994; Adeniji *et al.,* 1993; Adeyefa 1999).

Their works on serological investigation reported high degree of antibodies to influenza in camels, pigs, horses and human in Nigeria. The 1991 equine influenza A/H3N8, first in Africa caused high morbidity and mortality rates in the polo tournament in Ibadan (Adeyefa *et al*., 1996), the genetic information on the virus can be found in the GenBank (Bao *et al*., 2008).

Influenza surveillance and diagnosis came to a head in 2006, when Nigeria became the epicenter of avian influenza in West and Central Africa with the detection of HPAI H5N1 (Adene *et al.*,2006; Joannis *et al*., 2008 Fusaro *et al*, 2008). The virus was successfully isolated in chicken embryonated eggs and molecular characterization was carried out at the OIE/FAO Reference Laboratory for Avian influenza and Newcastle disease in Italy (De Benedictis *et al*., 2006; Monne *et al.*, 2007; Joannis *et al.*, 2008). Thereafter, in-depth research with the use of modern tools: viz, PCR, Bio-informatics provided more information about the influenza virus (Cattoli *et al*, 2010).

With enhance diagnostic tools available to the global scientific community, sub Saharan African countries are yet to take full advantage where other countries are regularly reporting situations with even seasonal influenza to the WHO flu network, sub Saharan Africa is still in lethargy and in the dark with respect to available global information. As at April 2012, there is only one swine influenza gene deposit in the GenBank in the whole of Africa and none from Nigeria (Bao *et al*., 2008). Effort by Adeola and co-workers (2008) to isolate swine influenza in embryonated chicken egg yielded 7 isolates that were identified by HI but the work is inconclusive as there are no record of the characterization and genetic evidence in global database (Bao *et al.*, 2008).

In a recent survey in free range pigs in Cameroun in 2011, pandemic swine influenza was detected by isolation and PCR with genetic evidence deposited in the GenBank (Njabo *et al.*, 2011) indicating the importance of sub Sahara African in the global burden of swine influenza. Further extensive and coordinated combination of sentinel and national surveillance is however required to appreciate the full extent of the ecology of classical and pandemic swine influenza in Africa.

CHAPTER THREE

MATERIALS AND METHODS

3.1 STUDY LOCATIONS AND POPULATION

Oke-Aro piggery estate in Lagos was established in 1984 and dedicated to pig husbandry by farmers in more than ten co-operative groups under the auspices of the Lagos State Ministry of Agriculture. The farm complex is located at Geographical Coordinate 6.413207 N and 3.193127 E at an attitude of 92 feet above sea level (Figure 7). It is situated at the interstate border between Lagos and Ogun and is approximately 50 kilometers distance to the international border with the Republic of Benin.

The aerial view (Figure 8) shows a dense linear farm plots that are divided by roads in rectangular fashion closely apposed along major farm road with major trunks designated A to H in location (I) and K to N in location (II). The entire area is adjacent a major water canal where liquid wastes from the pens are drained.

The farm estate is reputed to be the largest commercial pig concentration in West Africa. There are over 5000 registered farmers keeping about 800,000 pigs and more than 2000 farm hands and service providers within a land area of about 440,000 square meters. The pig population density of about 2 pigs per square meter creates an overpopulated condition that worsens sanitation and biosecurity.

Different breeds such as Duroc, Large White, Landrace and Hampshire are found in the farm and are inter crossed with occasional introduction of breeder boars from other farm locations in the country. Grower to finisher operation is the predominant practice with live pigs sold to other regions of Nigeria and neighboring West African countries. Fattened pigs are slaughtered daily in three slabs provided within the farm estate and fresh meat sold to dealers and middlemen who supplies open meat market and restaurant/hotels in Lagos metropolis and beyond.

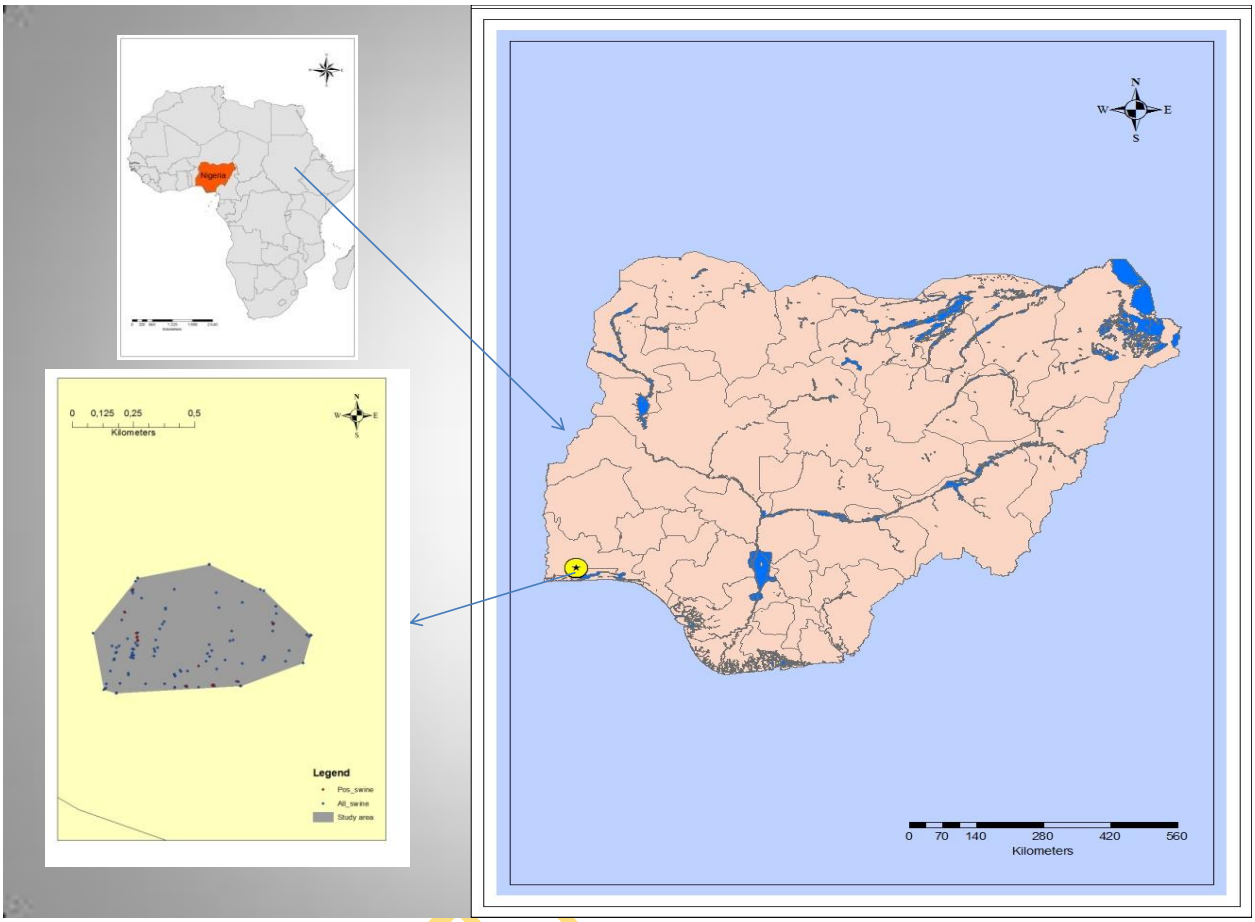

Figure 7: Oke-Aro study location: Geographical coordinate 6.413207 N and 3.193127 E at an attitude of 92 feet above sea level

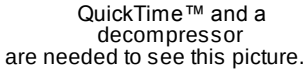

Figure 8: Aerial view of Oke-Aro sampling location showing the proximity between human and pig population.

Source: Google map 2010 digital globe. www.googlemaps.org

3.2 FIELD SAMPLING

Pig farms/husbandry units within the farm complex were visited to collect nasal/oropharyngeal swabs from pigs and human contacts showing respiratory signs. Any reported cases of influenza-like illness particularly cough within 10 days of visit was also sampled. Strict clinical cases of influenza-like illness based on case definition of fever, cough and respiratory distress in pigs and human handlers on a pen by pen basis were monitored for two years (July, 2010- June, 2012).

Nasal swab specimens were carefully collected into virus transport medium, kept on ice and transported in cold insulated box at about 4^0C to the laboratory where the samples were processed immediately or held at -80^0C until they were analysed. Farms were visited monthly for two years (24 months) during which period a total of 227 and 40 clinical cases in pigs and human were respectively identified and sampled.

3.2.1 SAMPLING MATERIALS

VIRUS TRANSPORT MEDIUM

Commercially prepared virus transport medium (VTM) produced by Copan Diagnostic CA USA described as universal transport medium (UTM) with insert polyester collection swab lot S8W599 (swab), 23Y383 (medium) were used. Other features of the VTM include:

- Room Temperature Stability, hence stable under controlled field conditions. UTM were conveniently stored at room temperature prior to use for collecting samples for virus detection and isolation.
- VTM system filled tubes in packs incorporating the combinations of swabs and media tubes as a unit.
- Safe, shatter proof polypropylene skirted tube with distinctive internal conical shape enabling centrifugation of samples and allowing tubes to stand upright on the laboratory bench
- Large 3ml medium fill volume enough for multiple tests and 3 aliquots of 1ml on the same specimen.

- Media formulation includes antibiotics to inhibit bacterial/fungal flora in samples.
- Three glass beads in each tube facilitates the release and dispersion of clinical sample/ materials and virus particles from the swab during vortexing
- All swabs featured unique molded breakpoint enabling safe, reliable break off and deposit of swab sticks into transport tubes

Cold boxes and ice

High quality cold boxes were procured for field work. Arrangement was made for sufficient ice gel, ice park and ice blocks to maintain all samples collected in cold chain during collection, after collection, in the course of transportation and before analysis or storage in the laboratory.

Personal Protective Equipment (PPE)

This included disposable laboratory coat, boots, hand gloves and nose masks and goggles in addition to freshly prepared 1% virkon as foot dip and general disinfectant for safety and biosecurity. Care was taken to disinfect before and after entry and handling of pigs in the pen. All specimen handling in the laboratory was carried out in biosafety cabinets for biosecurity containment (BSL 2) and the processing of animal and human samples were completely separated in accordance with WHO recommendation.

3.3. SAMPLE PROCESSING FOR RNA EXTRACTION

MATERIALS

- Qiagen RNA extraction kit
- 1.5 ml micro centrifuge tubes
- Sterile RNase- free pipette tips with aerosol barrier
- Voltex mixer and table top cold centrifuge
- Absolute alcohol (99-100% ethanol)
- Disposable gloves

- All samples and buffers were equilibrated to room temperature (25^0C)
- All wash buffers were prepared according to manufacturer's instruction as follows:

(1) Preparation of Carrier RNA:

Buffer AVL was checked for precipitate to be sure all precipitates were dissolved and 1 ml of buffer AVL was added to one tube of lyophilized carrier RNA. The content of carrier RNA tube was transferred to buffer AVL bottle and thoroughly mixed before each use.

(2) Preparation of Buffer AWI:

-150 ml of absolute ethanol was added to buffer AW1 as indicated on the bottle

(3) Preparation of Buffer AW2:

- 75 ml of absolute ethanol was added to AW2 as indicated on the bottle

3.3.1 NUCLEIC ACID EXTRACTION FROM SWAB SPECIMEN

Swab sample was centrifuged at 1500 rpm for 15 minutes to obtain clear supernatant. Subsequently, Qiagen RNAeasy commercial kit (Qiagen, Hilden Germany) was used for RNA extraction according to manufacturer's instruction (Templeton, 2004). Briefly, reagents and buffers were equilibrated to room temperature (25^0C) and wash buffers reconstituted accordingly. Thereafter, 140 ul of swab supernatant in VTM was added to buffer AVL –carrier RNA in micro centrifuge tubes. The tubes were mixed by pulse vortex for 15 seconds and incubated at room temperature for 10 minutes and briefly centrifuged to concentrate drops left inside the lid. Thereafter 560 ul of absolute ethanol was added to the samples and mixed by pulse vortexing for 15 seconds. Then 630 ul of the solution was added into the QIAamp minispin column in a 2ml collection tube and centrifuged at 8000rpm, for one minute. The QIAamp minispin column was transferred into 2ml collection tube for washing steps.

The first washing step involved dispensing 500ul of buffer AW1 into the minispin column and centrifuged at 8000rpm for one minute. Subsequently, 500ul of AW2 was also dispensed into the minispin column and centrifuged at 14000 rpm for 3 minutes for a second washing step. The minispin column was thereafter placed in a clean, sterile, RNAse free 1.5ul microcentrifuge tube and nucleic acid eluted by dispensing 60ul of RNA-free water and centrifuged at 8000rpm for one minute. Eluted (viral RNA) was either used immediately to run RT-PCR or stored at -20^0C until needed.

3.3.2 REAL TIME POLYMERASE CHAIN REACTION

Polymerase Chain Reaction (PCR) entails the amplification of a single or few copies of DNA or cDNA from few amount of nucleic acid in several order of magnitute thereby producing millions of copies of initial genetic material of the virus being investigated.

In this assay, one step RT-PCR for the detection of extracted RNA using real-time RT-PCR techniques was used (Gall *et al*., 2008). The system combines superscript III ReverseTranscriptase (RT) and platinum TaqDNA Polymerase in a single enzyme mix: both cDNA synthesis and PCR was performed in a single reaction. Gene specific primers and probes designed for generic matrix gene (Table 1.) was used for nucleotide amplification. Cocktail of master mix was prepared by dispensing 5.5ul nuclease free water, 0.5ul each of forward, reverse primers and probe, 0.5ul superscript Taq and 12.5ul 2x PCR master mix enzyme for a single reaction multiplied by the number of samples tested. Cycling condition was set at: reverse transcription 42°C for 30 minutes, RT inactivation/Taq activation 95°C 15 minutes, amplification (45 cycles), denaturation primer annealing/template extension at 95°C , 60°C at 15 seconds and 60 seconds respectively. Subtype identification with primers and probes designed for the Haemagglutination (HA) gene listed in Table 1 were used to identify swine influenza A and pandemic H1. Primers, probes were sourced from CDC, and using the WHO/CDC pandemic influenza A/H1N1 protocol. The real-time RT-PCR sample template on microplate is shown in Table 2.

Negative template controls (NTCs) was added first (to column 1) before any of the samples (S1–S9) was added to prevent any cross contamination of the master mix. Extract Control (EC) was added to column 11 after the samples have been added also minding cross contamination during sample preparation or addition. Positive test control (PTCs) was added last to column 12 after all the samples have been added and after all the wells containing samples and NTCs have been sealed.

The reaction was initiated after loading the test plate in the real time RT-PCR machine (Applied Biosystem 7500). Positive samples showed florenscence signal and standard curve at cycle threshold (CT) less than 30. Any CT more than 30 was considered nagative.

Table 1: Real time RT-PCR primers and probes sequences for matrix gene, flu A (pdm) and H1 (pdm)

RNA Detection		**Sequence bases***
	Matrix Gene	F: 5'-AGA TGA GTC TTC TAA CCG AGG TCG-3'
		R: 5'-TGC AAA GAC ACT TTC CAG TCT CTG-3'
		F: 5'- TCA GGC CCC CTC AAA GCC GA-3'
Subtyping	**Flu A pdm**	F: 5'- GCA CGG TCA GCA CTT ATY CTR AG-3'
		R: 5'- GTG RGC TGG GTT TTC ATT TGG TC -3'
		F: 5'- CYA CTG CAA GCC CAT ACA CAC AAG CAG GCA-3'
	H1pdm	F: 5'- GTG CTA TAA ACA CCA GCC TYC CA-3'
		R: 5'- CGG GAT ATT CCT TAA TCC TGT RGC-3'
		F: 5'- CA GAA TAT ACA "T"CC RGT CAC AAT TGG ARA A-3'

**Generic bases, Y represent C or T, R represent A or G in IUPAC nomenclature*

Table 2: Real time RRT-PCR sample set-up template, sample number in rows, negative and positive controls in the column

	Row										
Column											
1	2	3	4	5	6	7	8	9	10	11	12
A	NTC	S1	S2	S3	S4	S5	S6	S7	S8	S9	EC	PTC
B	NTC	S1	S2	S3	S4	S5	S6	S7	S8	S9	EC	PTC
C	NTC	S1	S2	S3	S4	S5	S6	S7	S8	S9	EC	PTC
D	NTC	S1	S2	S3	S4	S5	S6	S7	S8	S9	EC	PTC
E	NTC	S1	S2	S3	S4	S5	S6	S7	S8	S9	EC	PTC
F	NTC	S1	S2	S3	S4	S5	S6	S7	S8	S9	EC	PTC
G	NTC	S1	S2	S3	S4	S5	S6	S7	S8	S9	EC	PTC
H	NTC	S1	S2	S3	S4	S5	S6	S7	S8	S9	EC	PTC

3.4.0 VIRUS ISOLATION FROM SWAB SPECIMEN

All clinical swab specimens collected from pigs and human were processed for isolation in cell culture and embryonated chicken eggs.

3.4.1 VIRUS ISOLATION IN CELL CULTURE

Isolation of viruses in cell (or egg) culture is considered "gold standard" for virus diagnosis. The principle is that viable virus in specimens when inoculated in a live culture, acting as a host would permit the virus to replicate sufficiently to enable subsequent identification and other uses.

Madin-Darby canine kidney (MDCK) is typically the preferred cell line and was used in this study for influenza virus culture (Reina *et al*., 1997). Virus isolation procedures took minimum of 5 days or two weeks and minimum of two passages before a specimen was reported negative.

Materials

Equipment

-Incubator (37 °C)

- Centrifuge (low speed)

-Microscope (inverted)

-Biosafety cabinet

Consumables

-Tissue culture flasks (T-75 canted neck)

-Tissue culture flasks (T-25 canted neck)

-Filters (0.22 μm pore-size membrane) millipore

-Pipettes (1ml, 5ml, 10 ml) BD Biosciences (Falcon)

-Centrifuge tubes (conical, 15 ml)

- Cells, buffers, reagents and media

- MDCK cells

- Fetal bovine serum (FBS)

- DMEM high glucose (1x); liquid; with L-glutamine; without sodium pyruvate

- Trypsin- ethylenediamine tetra acetic acid (trypsin-EDTA) (0.05% trypsin; 0.53 mM EDTA · 4Na)

- Penicillin-streptomycin (stock solution contains 10,000 U/ml penicillin; and 10,000 µg/ml streptomycin sulfate)

-Trypsin L-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK-trypsin); treated (type XIII from bovine pancreas)

-HEPES (N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid)

- Buffer (1 M stock solution)

- Gentamicin reagent solution (50 mg/ml)

- Bovine albumin fraction V (7.5%)

Preparation of media and solutions

Complete D-MEM:

To obtain complete Dulbeco- mimimum essential medium (D-MEM), Penicillin-streptomycin 5.0 ml 100 U/ml penicillin;100 µg/ml streptomycin, Bovine albumin fraction V (7.5%) 12.5 ml 0.2%, HEPES buffer 12.5 ml 25 mM was added to 470 ml of D-MEM (10gm powder in 1000ml).

D-MEM medium for cell growth:

To 450 ml complete D-MEM (prepared as above), 50 ml of 10% Fetal bovine serum (FBS) added.

D-MEM medium for virus growth:

To 500 ml complete D-MEM, 0.5 ml TPCK-trypsin (2 mg/ml) was added to obtain a final concentration of 2 μg/ml

Preparation of TPCK-trypsin stock solution (2 mg/ml)

TPCK-trypsin (20 mg) was dissolved in 10 ml complete D-MEM and thereafter filtered through a 0.22 μm pore-size membrane and Stored in aliquots at -20 °C.

Preparation of MDCK cell suspension

MDCK cell suspension was prepared for use with confluent T-25 flasks with the volume adjusted for cell culture flasks.

In a complete procedure, one T-25 flask with a confluent monolayer of MDCK cells obtained contains approximately 10^7 cells.

- To detach confluent cells from the flask, 5 ml of trypsin-EDTA (pre-warmed to 37 °C) was added to each of the T-25 flasks containing the cell sheets and gently rocked for 1 minute for washing. Trypsin-EDTA was thereafter carefully removed with a pipette and the procedure was repeated. Finally, 1 ml trypsin-EDTA was added and distributed over entire cell sheet and the flask incubated at 37 °C until all cells detach from plastic surface at about 5 minutes. The flask was intermittently rocked to detach cells. Subsequently, 1 ml FBS was added to inactivate remaining trypsin and 8 ml complete D-MEM was then added.

To obtain a homogenous mixture, detached cells were gently pipette up and down to break up cell clumps after which 10 ml of the mixture was transferred to 90 ml D-MEM medium. The cell suspension obtained contains approximately 10^5 cells per ml.

In seeding each T-25 cell culture flask, approximately 6 ml (600 000 cells) cell suspension was added to an appropriate number of T-25 flasks. The remaining cell suspension was kept at +4 degree Celsius to be used to seed T-75 flasks for cell passage while seeded flasks were incubated at 37 °C.

Cell Quality control

Over a number of passages, MDCK cells can lose their susceptibility to respiratory viruses. Therefore, a stock of frozen cells at a low passage level was maintained. An aliquot of such low-passage cells was thawed each time and used during virus isolation process provided that the cells remain healthy and this did not exceed 25 consecutive passages or 3 months and adequate care was taken to avoid bacteria contamination especially mycoplasma.

Inoculation of cell cultures

The following steps were performed in a class-II biosafety cabinet to avoid the contamination of cells.

Cells were checked to ensure good health status and high degree of monolayer/ confluence using inverted microscope at 40X magnification. Thereafter, D-MEM medium for cell growth was discarded from the flasks and cells washed two times with 6 ml complete D-MEM or Phosphate Buffered Saline (PBS). Cell culture medium was carefully removed from the flask with a pipette after which 200 µl of each processed specimen were inoculated into a T-25 flask or tubes using pipettes. Subsequently, swab sample supernatant were allowed to adsorb for 30 minutes at room temperature (25 °C). Following which D-MEM medium (6 ml) containing 2 µg/ml of TPCKtrypsin was added to the flask. The inocculated flask(s) were thereafter incubated at 35°C and cells observed daily for evidence of cytopathic effect (CPE)

Harvesting virus

After sample inoculation, CPE was observed daily and at 3+ or 4+ (75–100%), virus harvest was carried out by the collection of the supernatant fluid after series of freezing and thawing. Thereafter 0.5% stabilizers such as glycerol, gelatin or bovine albumin fraction V (7.5%) were added. Virus harvests by day 6 or 7 were carried out even without CPE.

Virus Identification

Haemagglutination test was carried out on cell culture supernatant and samples that were positive for haemagglutinating agent in the absence of bacteria contamination was considered viral isolate and further tested by haemagglutination inhibition (HI).

In the absence of detectable haemagglutination, cell culture supernatants were passaged one or two more times before reporting an inability to recover virus from the specimen. Harvested viruses were centrifuged at 3000 rpm for 5 minutes to remove excess cells and then filtered with 0.45 micrometer Millipore filters before storage at -80°C within 24 hours of harvesting.

3.4.2 VIRUS ISOLATION IN EMBRYONATED CHICKEN EGGS

Only viruses grown in embryonated eggs can be used as seed viruses for the production of most influenza vaccines. For this reason, isolation of influenza viruses in eggs is usually encouraged to maintain this capacity. Secondly, egg culture compared to cell culture appeared to be more sensitive for the isolation of avian than other animal influenza. The procedure involved maintaining fertile eggs of the right age and condition, which though may be cumbersome. Egg culture, remained a good medium for virus isolation (Horimoto *et al.*, 2007). All the steps involved in embryonated egg inoculation were performed in a class-II Biosafety cabinet to avoid contamination and for personnel protection.

Materials required

Equipment:

-Egg candler

-Incubator (preferably humidified or provided with a bowl of water to create internal humidity of 60-80% in the incubator)

-Low speed refrigerated centrifuge

Consumables:

-Embryonated chicken eggs (9–12 day old)

- Non-toxic glue (cortex) or paraffin wax or paper seal

-70% ethanol as disinfectant

-Centrifuge tubes

-Needles; 22 gauge; 1½ inch (3.8 cm)

-Sharps safety container

-Pipettes (10 ml)

-Tuberculin syringe (1 ml)

-Forceps (sterile)

-Egg hole punch

-Stainless steel scissors

- Plastic egg trays

-Alcohol wipes and swabs

-Grease marker or pencils

Candling and inoculation of eggs

Eggs were examined with an egg Candler. Infertile, underdeveloped, cracked or porous shell eggs were discarded. Eggs were thereafter placed with the blunt end on the upper position and labeled with specific laboratory identification number, allocating 3-5 eggs for each specimen to be inoculated.

The blunt end of each egg was wiped or sprayed with 70% ethanol to decontaminate the surface before a hole was punched in the shell over the air sac. Thereafter, 0.2ml of nasal swab supernatant was aspirated into a tuberculin syringe with a 22 gauge, 1½ inch needle. Holding the egg up to the Candler, the embryo was located, inserting the needle into the

hole in the shell and, using a short stabbing motion, the amniotic membrane was pierced and 200 µl of the specimen was inoculated into the amniotic cavity.

Thereafter, the inoculating needle was slightly withdrawn by about ½ inch (1.25 cm) from the amniotic sac into the allantoic cavity and 200 µl of the specimen was inoculated into the allantoic cavity just below the air space before the needle was removed.

All other eggs allocated to the same specimen (5 eggs per sample) were inoculated in the same manner using the same needle and syringe. After which used needle and syringe were discarded safely in sharps container. Punched holes in the eggs were thereafter sealed with a drop of glue and incubated at 37^0C and eggs were observed daily for 5 days

Harvesting virus from inoculated chicken embryonated eggs

Dead eggs post incubation were chilled at 4 °C overnight before harvesting to freeze blood vessels and maintain low temperature for isolates. Alternatively eggs were rapidly chilled at -20 °C for 30 minutes. Centrifuge tubes (15 ml) were labeled with the specimen number for each sample for sets of egg.

The blunt end of each egg was cleaned with 70% ethanol thereafter, sterile forceps was used to break the shell over the air sac and cut to expose allantoic membrane

Using a 10 ml pipette, Pasteur pipette or 2ml syringe, allantoic fluid was collected and placed in the labeled centrifuge tubes. With a syringe and needle to pierce the amniotic sac, as much amniotic fluid as was possible to aspirate was collected and both amniotic as well as allantoic fluid were combined.

3.5 IDENTIFICATION OF INFLUENZA VIRUS

3.5.1 HAEMAGGLUTINATION TEST

Alpha Haemagglutination Inhibition (HI) test was carried out on isolates from egg culture based on the principle that red blood cells possess receptors that are recognized by the glycoprotein (HA) present on the surface of influenza virus. The interaction of the red blood cell receptor and HA glycoprotein results in the binding of the rbc in suspension.

Spot (qualitative) HA on white surface tiles was carried out by placing a drop of chicken red blood cells on the plate with a drop of harvested egg fluid dropped with a Pasteur pipette or wire loop and mixed thoroughly.

Quantitative HA was carried out on a V-bottom microtitre plate and haemagglutinating agents were tested by HI after which viral isolates were stored at 4°C (bacteria exclusion was carried out by culture in blood agar or broth).

In the absence of haemagglutination, the specimens were passaged twice before reporting an inability to recover virus from the specimen.

i) Chicken red blood cell used in this test was obtained by bleeding three different birds and pooling the blood. The rbc were washed by suspension in PBS and centrifuged at 1500rpm before various suspensions (10%, 1% and 0.5%) was prepared. Washed rbc was stored at 4°C for up to 1 week and discarded if haemolysis was observed.

ii) 25µl PBS was dispensed in a row of 8–12 wells on a 96-well V- bottom microtitre plate for each unknown virus except the first well that was left for undiluted virus. V-bottom plates are generally preferred over U-bottom plates for better agglutination. One additional row of wells was included for a positive control.

iii) 25µl of undiluted isolate was added to the first well of each corresponding row.

iv) 2-fold serial dilution of the isolate was performed with a micropipette set to deliver 25µl up to well 12. The last one or two row served as red blood cell (rbc) control and was not dispensed with virus

v) 25µl of 0.5% chicken erythrocyte suspension was added to each well and the plate rocked in microtitre plate shaker or manually tapped from side to side to mix the content thoroughly.

Note: RBC was always kept thoroughly suspended by agitation during the dispensing process to ensure homogenous cell suspensions.

vi) The microtitre plate (s) were covered with microtitre plate cover and incubated at room temperature until a distinct button was formed after 45 minutes in the red cell control wells.

vii) Wells with complete haemagglutination (positive HA, swine influenza virus present) have rbc spread throughout the well in a 'mat' type appearance or in suspension. Wells with a distinct button of erythrocytes at the bottom of the well are negative for haemagglutination activity (i.e negative for swine influenza virus). Incomplete HA activity was demonstrated by partial buttons characterized by fuzzy margins. When interpretation between negative and incomplete inhibition is doubtful, the microtitre plate was tilted to about 45^{o} angle for 20–30 seconds to observe lack of streaming or tear drop appearance and translucency around the cells in wells without haemagglutination. Wells with partial HA inhibition did not produce a tear drop.

3.5.2 HAEMAGGLUTINATION INHIBITION TEST

TYPING OF ISOLATES

Haemagglutination Inhibition test (alpha) was carried out based on the principle that glycoprotein (HA) on the surface of influenza virus binds to homologous antisera in the reaction. Subsequently rbc that was added later had no virus to bind and are described as inhibited.

The test was performed according to the following steps:

i) Reference HA antigens (H1 pandemic, H1 seasonal, H3 seasonal and H5 avian influenza etc.) were diluted to concentration of 4 HA units (HAU) per 25µl (4 HAU/25µl) in 0.01M PBS at pH 7.2.

ii) Standardize unknown influenza A viruses had 4 HAU of the virus in 25µl.

iii) Back titration (HA test) was carried out for all unknown isolates and the H subtype antigens to ensure that the correct HA units was present. The back titration was performed as previously described above in the HA procedures except that six well dilutions across roles were used instead of eleven.

iv) Each reference serum (specific for an individual HA subtype) was treated with RDE (receptor-destroying enzyme) by adding 50µl serum to 200µl RDE (1/10 dilution in calcium saline solution equaling 100 units per ml). This was incubated overnight in a 37°C water bath. 150µl 2.5% sodium citrate solution was added and heat inactivated at 56°C for 30 minutes. Treated sample (200µl) was combined with 25µl of PBS.

v) Removal of none specific agglutinins from the sera was achieved by treating diluted serum with 0.1 ml packed, washed erythrocytes per 1 ml diluted serum which was incubated for 30 minutes at room temperature with occasional mixing to keep the erythrocytes suspended. Treated serum was centrifuged at 1500rpm for 10 minutes and the serum supernatant retained.

vi) 25µl of standardized antigen (unknown isolate or positive control antigen) was dispensed into three wells of a 96-well V-bottom microtitre plate. PBS (25µl) was added to the wells to serve as erythrocyte cell control.

vii) 25µl of the appropriate standardized antiserum was added to the first well of the HA subtype being evaluated. Serial dilution of the antiserum in 25µl volumes in the antigen wells with a pipette set to deliver 25µl was performed. This procedure was repeated for each HA subtype being evaluated.

viii) plate (s) are covered and incubated at room temperature for 10–30 minutes.

ix) 25µl of 0.5% erythrocyte suspension was added to each well and plate rocked/agitated to thoroughly mix content.

x) plate(s) were covered and incubated at room temperature (25^0C) until a distinct button was formed in the positive control wells (usually 45 minutes). Plates were examined after 20 minutes of incubation for evidence of haemagglutination in order to observe isolates that may begin to elute (i.e detach from the rbc) before 45 minutes.

xi) test results were read as previously described for the HA test viz: a sample was considered positive for a specific HA subtype if haemagglutination is inhibited and the titre was measured in two folds according to the number of wells in which haemagglutination was observed. The test was also considered valid when the positive

reference antigen and its homologous antiserum demonstrated the expected HI titre and the back titration of each antigen (unknown and positive control) was 4 HA units.

Serological tests

The primary serological test for detection of SIV antibodies is the HI test and it is subtype specific, However Enzyme linked Immuno Sorbent Assay (ELISA) can also be used to initially screen for influenza A before subtype specific HI. Others are virus neutralisation, microneutralization, agar gel immuno-diffusion test and immunofluorescent antibody tests.

Haemagglutination inhibition test:

Influenza antibodies in serum can be detected using Haemagglutination Inhibition test based on the principle that homologous antiserum in the test sample could detect equivalent HA reference antigen. The binding of the test serum and reference antigen would thereafter inhibit rbc when it is added to the reaction.

The test procedure was carried out as follows:

i) Dilution of reference HA antigens (H1, H3 and H5) to a concentration of 4HAU/25µl in 0.01M PBS at pH 7.2 was carried out.

ii) ix) HA test was performed on reference antigens prior to and simultaneously to conducting the HI test to verify that antigen concentrations (4HA units) were appropriate.

iii) Treatment of test and reference (H1N1, H3N2 and H5) sera: sera were treated to remove non specific inhibitors and agglutinins by heat inactivation (30 minutes at 56°C) and addition of RDE. This was later diluted 1:10 in PBS. Thereafter, washed and packed rbc (0.2ml) was added to 1 ml of the serum and mixed. This was incubated at room temperature for 30 minutes with periodic shaking at every 10–15 minutes. Thereafter the mixture was centrifuged at 1500rpm for 10 minutes at 4°C to obtain clear supernatant.

iv) 25µl of PBS was dispensed in all wells except the first column

v) 25µl of treated sera was dispensed into two wells of the first and second column

vi) Positive, negative and serum control was dispensed into appropriate wells

vii) Two folds serial dilution of dispensed sera was carried out and then 25µl of appropriate antigen was added to all test wells except serum control and cell control wells.

viii) Microtitre plates were covered and incubated at room temperature for 45 minutes.

ix) Thereafter 25µl of 0.5% erythrocyte suspension was added to all wells, rocked then incubated at room temperature for 45 minutes until a distinct button was formed at the bottom of the cell control wells.

x) For a valid test, there was no haemagglutination in the serum control well, no inhibition of haemagglutination in the negative serum, the positive serum should had anticipated HI titre and the HA back titration indicated 4 HA units per 25µl.

3.6 NEGATIVE STAINING ELECTRON MISCROSCOPY

The principle of Negative staining electron microscopy (EM) for virus diagnosis is that a negative contrast stain is seen when electron beams are generated and passed through heavy metal compounds such as 2% potassium phosphotungstate or ammonium molybdate (negative contrast medium). The negative contrast medium around the viral particles does not allow penetration by electron beam but the beam passes through the viral particles and appears transparent while areas around the viral particles appear dark. This therefore allows the shape, size and structure of the virus to be determined. Further structural detail may be apparent if the stain penetrates any crevices or hollows on or within the virus.

EM does not require organism-specific reagents for recognizing the pathogenic agent and enable open view of whatever is present in the specimen. Negative contrast EM being a rapid procedure was used to view viral particles in fluids or suspensions of both swab specimen from the field and amplified egg culture.

The following procedure was carried out in the negative contrast staining of influenza virus isolates and nasal swab samples

- 2mls of nasal swab specimens and egg allantoic fluid positive for influenza by RT-PCR was prepared
- A grid of formav/carbon film was placed in the suspension
- This was ultra centrifuged at 90,000 rpm using Becham Airfuge for 5 minutes set at 20psi pressure
- A drop of Negative stain (phosphotunstinic acid) was placed on a slide
- The grid was removed after centrifugation and placed on the negative stain for 1 minutes contact time
- Grid was removed and placed in petri dish after one minute
- Grid was transfer into Electron microscope 'insert' and placed on EM machine
- Electron beams were accelerated through the grid for contrast imaging
- EM machine was operated by focusing, magnification, diffraction, and location adjustment of particles in the computer monitor
- Viral particles were observed for shape, size and structure to identify influenza virus and pictures taken and recorded.

3.7: VIRUS PURIFICATION AND ANTIGEN PREPARATION:

In order to produce large quantity of the isolate (A/swine/Nigeria), Serial passages and replication of virus in egg culture was carried out with HA test performed to determine the titre after each successive passage. Following series of passages in eggs, the allantoic fluid harvest was pelletized using high speed ultracentrifugation at 90,000 rpm to further concentrate the virus particles into pellets from allantoic supernatant fluid. The resultant suspension was inactivated with beta propiolactone and freeze dried.

3.8. NUCLEOTIDE SEQUENCING

Dideoxy sequencing:

The genome of all the eight gene segments of three representative isolates from samples collected in May 2011 was sequenced by Sanger's method using Big Dye Terminator v3.1 (Applied Biosystems, Foster City, CA). Partial sequencing of the HA gene of twelve of the positive swab samples was also carried out following detection by RT-PCR.

Sanger's sequencing is based on the principle that nucleotides start at a fixed point, random in a particular base and at the end. Attached to each base are fluorescent markers which are recorded, resulting in sequence of A, T, C, G presented as visible DNA base pattern which represent the constitution of the genome.

The complete process of DNA sequencing of influenza virus begin with the isolation of RNA as previously described but using Macherel-Nagel extraction kit according to the manufactural's protocol. This was followed by the RT-PCR desribed below with little modification. Reagents volume and reaction conditions are as shown in Tables 3 & 4.

Procedure

Master mix for the synthesis of cDNA was prepared. The master mix (13.5ul) obtained was incubated for 5 minutes at 65 degree centigrade using thermocycler and then cooled on ice for 5 minutes. Second mix containing first mix was incubated in the thermocycler set at 25 degree centrigate for 5 minutes and 50 degee centrigate for 60 minutes.

This was followed by the preparation of master mix for "full length PCR" (Table 5) in series of short fragments. Details of the forward and reverse primers used for PCR for full genome sequecing of HA gene is shown in Table 6. The rest of the swine influenza genome primers is provided in appendix vi to xii.

Cycling conditions:

The cycling condition for each step of the reaction was; 95^0C for 2 minutes, 95^0C for 45 seconds, 46^0C for 1 minutes, 72^0C for 4 minutes, 72^0C for 10 minutes and 4^0C (hold; infinity) for a total of 40 cycles was adjusted. When the full length PCR protocol was completed, the products were visualized and identified using agarose gel electrophoresis.

Table 3: Master mix (i) for the synthesis of cDNA:

Mix 1	**ul x 1 reaction**
RF 671 2UL	1
dNTPs mix 10ul	1
rRNAsin 40ul	0.5
Total volume	2.5ul
RNA (extract)	11.0ul
Final Volume of mix 1	**13.5ul**

Table 4: Mix (ii) containining cDNA

Mix 2	ul x 1
RT buffer 5x (FS,5X)	1
Ditetrazinetetroxide (DTTO)	1
rRNAsin	0.5
Superscript III RT	1
Total volume	6.5ul
Volume of mix 1 + RNA (carry over)	13.5ul
Final Volume of mix 2	**20.0ul**

Table 5: Master mix for full length sequencing reaction

REAGENTS	ul X 1 Reaction
Deionized water	15.875
PFU buffer 10x	2.5
DNTPs	0.625
primer forward	2.5
primer reverse	2.5
Turbo PFU (enzyme)	0.5
cDNA synthesised from previous prep	0.5
Final volume	**25.5 ul**

Table 6: Primers for sequencing reaction for swine influenza genome (HA) segment of influenza A/H1N1

Influenza A/H1N1 gene segment (HA)		**Primers sequence bases***
Fragment 1	1- 461-	F: 5'- tgt aaa acg atg gcc agt ata cga cta gta aaa gca ggg g - '3 R: 5'- cag gaa aca gct atg acc tca tga ttg ggt cay ga- '3
Fragment 2	351- 943-	F: 5'- tgt aaa acg acg gtt agt acr tgt tac ccw ggr gat ttc a -'3 F: 5'- cag gaa aca gct atg acc gaa akg gga grc tgg tgt tta -'3
Fragment 3	379- 1204-	F: 5'- tgt aaa acg acg gcc agt atg acg acc tra gag agc a -'3 F: 5'- cag gaa aca gct atg acc caa tgg crt tyt gtg tgc tc - '3
Fragment 4	736- 1340-	F: 5'- tgt aaa acg acg gcc agt agt atg rac tat tac tgg ac -'3 F: 5 - cag gaa aca gct atg acc ttc tkc att rta wgt cca aa -'3
Fragment 5	1124- 1541-	F: 5'- tgt aaa acg acg gtt agt tgg atg gta ygg tta yca yca g- '3 F: 5'- cag gaa aca gct atg acc tca taa gty cca ttt ytg a - '3
Fragment 6	1204- 1778-	F: 5'- tgt aaa acg acg gcc agt aag atg aay acr car ttc aca g- '3 F: 5'-cag gaa aca gct atg act gtg tca gta gaa aca agg gtg ttt -'3

**Generic bases- y=c or t, r=a or g, w=a or t in the IUPAC nomenclature*

Preparation of Agar gel:

Materials

1. Agarose powder
2. TBE buffer
3. Flask or bottle for gel mixing
4. Heat source (microwave oven)
5. Ethidium bromide (used with caution)
6. Reddish dye
7. DNA lambda
8. Gel tray and combs
9. Elecrophroresis buffer
10. Electrophoresis tank
11. Electric power voltage regulator
12. UV transilluminator
13. Gel Documentation set & Computer

Procedure

1. 1x running buffer was prepared from 5x stock
2. 0.6 gram of agar powder was mixed in 40-45 ml TBE to obtain 1.5% gel concentration
3. the slurry was microwave heated untill the powder was completely dissolved, usually about 5 minutes
4. Agarose solution was cooled to about 55 to 60^0C before adding 0.1 ul (4ul for 40ml) reddish dye (alternative to ethidium bromide) while in molten state
5. Agarose solution was poured into gel tray with appropriate comb size (3-5mm) in place
6. Gel was allowed to set for 30 minutes beferore careful removal of comb and the gel submerged in TBE electophoresis buffer
7. Loading of gel was achieved by initially mixing 1 ul loading buffer (bromophenol blue) with 5 ul of sample (from full length PCR protocol)

8. Loading with cappillary tips through the buffer and then discharged at the bottom of the wells
9. DNA Lambda, marker VI was loaded in the first or last well in the gel (100bp)
10. Tank was connected to power source and ran at 400 AM, 100 Volts for 30 minutes
11. Thereafter, the DNA bands are visualize under UV light and images were captured with camera or computer gel documentation system. Quality and estimate of DNA concentration was verified visually.

PURIFICATION OF AMPLICONS

ExoSAP-IT (for DNA purification) consists of two hydrolytic enzymes, Exonuclease I and Shrimb Alkaline Phosphatase (SAP), in a specially formulated buffer for the removal of unwanted primers and DNTPs from PCR product mixture without interference in downstream applications.

EXoSAP-IT was removed from freezer and kept on ice, it was directly added to the PCR product for purification after treatment (5ml of PCR products and 1-2 ml of ExoSAP given a total of 7ul per tube). Total quantity of EXOSAP used for both forward and reverse primers (double quantity) was estimated as 5ul of PCR products x 2 + 1 ul ExoSAP-IT x 2 = 12 ul for each sample in two tubes.

This mixture is incubated at room temperature for 5 minutes for the purification process. Thereafter ExoSAP was inactivated by heating in thermocycler at 80^0C for 15 minutes.

The treated PCR products was used immediately or stored at -20^0C for subsequent sequencing analysis.

SEQUENCING REACTION

Two tubes were prepared for each PCR product for forward and reverse sequences each containing reagent mix in Table 6. The tubes containing reagents and amplicons were briefly centrifuged to obtain homogenous solution and placed on thermocycler.

Amplification cycle condition for sequence reaction was set at:

96^0C for 10 seconds, 50^0C for 5 seconds and 60^0C for *2-4 minutes (25 cycles)

*Note: 2 minutes for small fragment and 4 minutes for long fragment timming was used.

Sequence clean- up

Colum Autoseq G-50 allowed rapid dye terminator removal from sequencing reactions by the process of gel filtration. Molecules larger than the largest pores in the sephadex are excluded from the gel and eluted first. Intermediate size molecules penetrate the matrix to varying extents, depending on their size and are retained.

The columns were designed for use in a microcentrifuge and for handling of small numbers of samples.

Procedure

- Resin column was resuspended by vortexing
- Column was placed in a collection tube (or 96 well plate)
- Column or tube was pre-spun for 1 minutes at 2000g (4800rpm)
- Sequenced sample was added (all 20 ul or 10 ul) from sequence reaction to the centre of the resin bed taking care not to touch the resin with tips)
- colunm was spun for 1 minutes at 2000g (4800rpm) / for 96 well plate 850g (1500rpm) for 5 minutes
- spun colunm was removed from the microcentrifuge tube and discarded.

Preparation of plate

- In a 96 well plate, 10 ul of sterile water was added to each well
- 10ul of purified/cleaned samples was added taking care to elimintae burbles and discharging in the correct order of sample arrangement
- Sequence analyzer with 16 cappillaries allows analyses of 16 samples in the same run therefore all the 16 wells contained samples or water to avoid drying up of the cappillary if empty.

Charging plate in genetic analyser

- On the computer, software (genetic analyser) GA instrument 3130 xl - plate manager- instrument protocol- analysis protocol- was selected and result group created and saved
- plate(s) were prepared and assembled and mounted on auto sampler
- plate was search and selected then linked to plate for recording

- then “okay” was clicked in dialogue box
- data was viewed during runs using the cappillary (cap/array)
- sequence products were saved in Abi files (fasta files)
- correction of sequences were performed in seqscape computer software for gene analysis
- alignment of sequences in MEGA version 5 was carried out
- phylogenetic trees were constructed using MEGA version 5 by the neigbour joining (NJ) and maximum likelihood (ML) methods

Gene sequences obtained were compared with corresponding genes of other influenza virus strains (swine and human) obtained from GenBank and Global Initiative on Sharing Avian Influenza Data (GISAID).

To infer the evolutionary relationship for each gene segment, maximum likelihood methods available through the PhyML program was empoyed, incorporating a GTR model of nucleotide substitution with gamma-distributed rate variation among sites and a heuristic SPR branch-swapping search. Parameter values for the GTR substitution matrix, base composition, gamma distribution of the rate variation among sites (with four rate categories) and proportion of invariant sites were estimated directly from the data using MEGA 5 computer software based.

A bootstrap resampling process (1,000 replications) using the neighbor-joining method and incorporating the ML substitution model defined above, was employed to assess the robustness of individual nodes of the phylogeny.

Table 7: Reaction mix for sequencing influenza A/H1N1

Reaction mix	ul x 1 reaction
Sterile water	3.0 (adjustable)
Sequencing buffer 5X	1.5
TRR mix u3.1	1.0
Primer (tag F, tag R)	0.5
DNA template	4.0 (adjustable)
Final Volume	**10**

INTERPRETATION OF SEQUENCING RESULT

BLAST program

A. BLAST site on the Internet was connected to access the home page of the National Center for Biotechnology Institute (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

B. Nucleotide blast or basic BLAST was entered.

C. Query sequence was entered.

D. Search set and program was selected.

Note: 'others (nr etc)' button and 'blastn' button were clicked

E. 'BLAST' button was clicked.

F. Analyze 'the blast result' was selected.

3.8. PHYLOGENETIC ANALYSIS

Three representative swine influenza A (H1N1) virus were phylogenetically analyzed for all 8 viral genes and compared with publicly available sequences. All sequences were assembled and edited with seqscape software.

Neighbor-joining tree analysis was conducted using ClustalW (www.clustal.org). Estimates of the phylogenies were calculated by performing 1000 Neighbor-Joining (NJ) bootstrap replicates. Subsequent sequence retrieval, editing, import and export of data to construct and modify trees were all carried out with combinations of MEGA software, and platform enabled functions on NCBI.

CHAPTER FOUR

RESULTS

4.1: SAMPLE COLLECTION

Clinical sample collection yielded 227 swabs in two years from July 2010 to June 2011, with the highest number of cases in January 2011. Monthly numbers of cases of cough observed and number positive by real time PCR are as shown in Figure 9.

Seasonal variation on observed cases of cough and virus recovery rate are shown in Figures 9 & 10. The highest number of 18 to 20 samples were collected during the dry cold harmattan months of November to January compared to lower number of cases (1 to 2) in February to September that is usually hot and humid. Conversely more samples were also collected between November and January. Out of the total number of 227 samples collected, 31 were positive by real time RT-PCR while 29 were positive by isolation in embryonated egg and serological tests.

4.2: MOLECULAR DETECTION OF PANDEMIC INFLUENZA A/H1N1

Thirty one (13.7%) samples were positive for influenza A virus by real time RT-PCR through tests to detect the matrix gene. Eighteen of the samples were subtype A/H1N1 pandemic influenza virus of swine origin (Figure 11). The 18 samples were positive for selectively designed swine A matrix and were also positive for pandemic AH1N1 as shown in both positive samples and control samples.

Seventy seven percent of positive specimens in this study were collected from weaners and growing pigs probably because of higher susceptibility in this category of naive population. Though pigs that were sampled and were positive for pandemic influenza exhibited severe clinical sign, only 2 (0.8%) deaths were recorded. This may be related to the observation that the 2009 influenza A/H1N1 virus was mild compared to 1918 pandemic (Spanish) influenza virus. Reported case-fatality rate in hospitalized infections

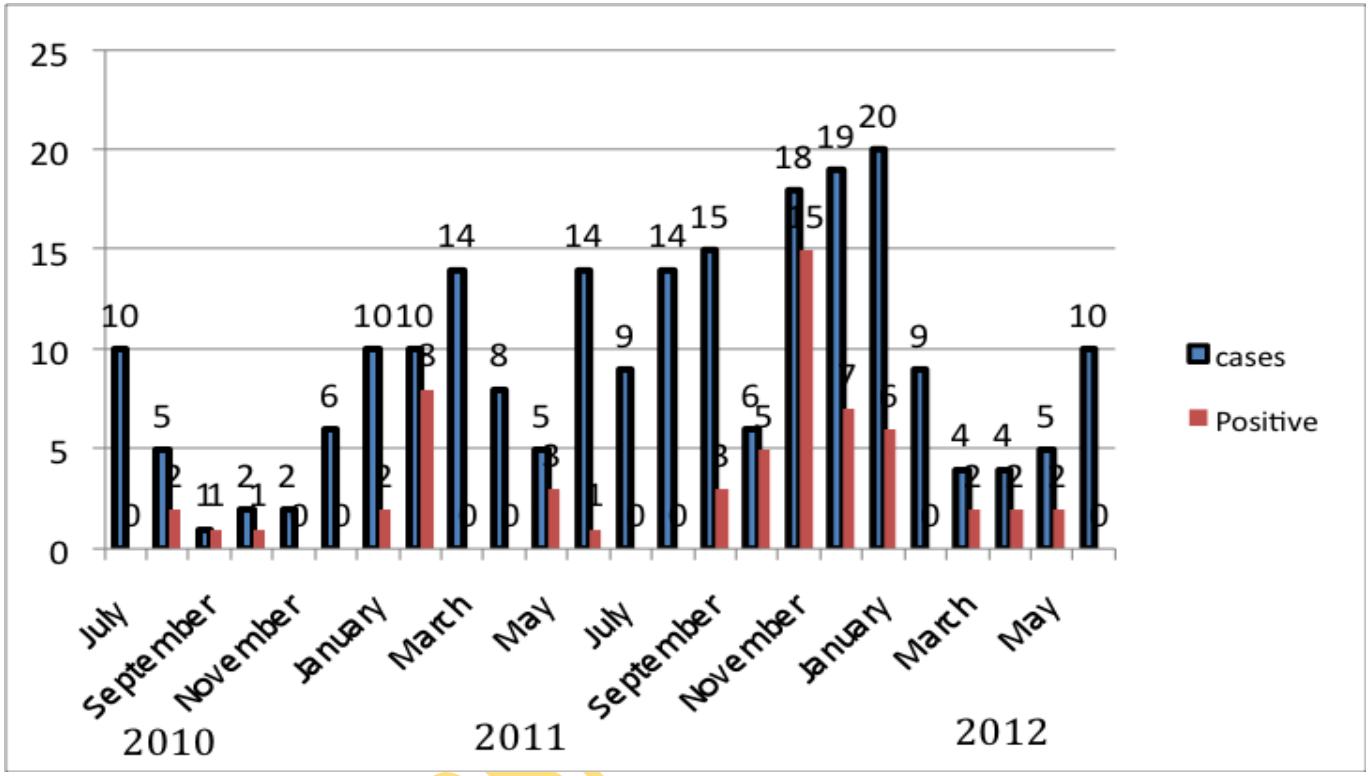

Figure 9: Monthly number of clinical cases of cough observed from 2010 to 2012 in the x-axis and number of positive and negative samples by real time RT-PCR and virus isolation in the y-axis

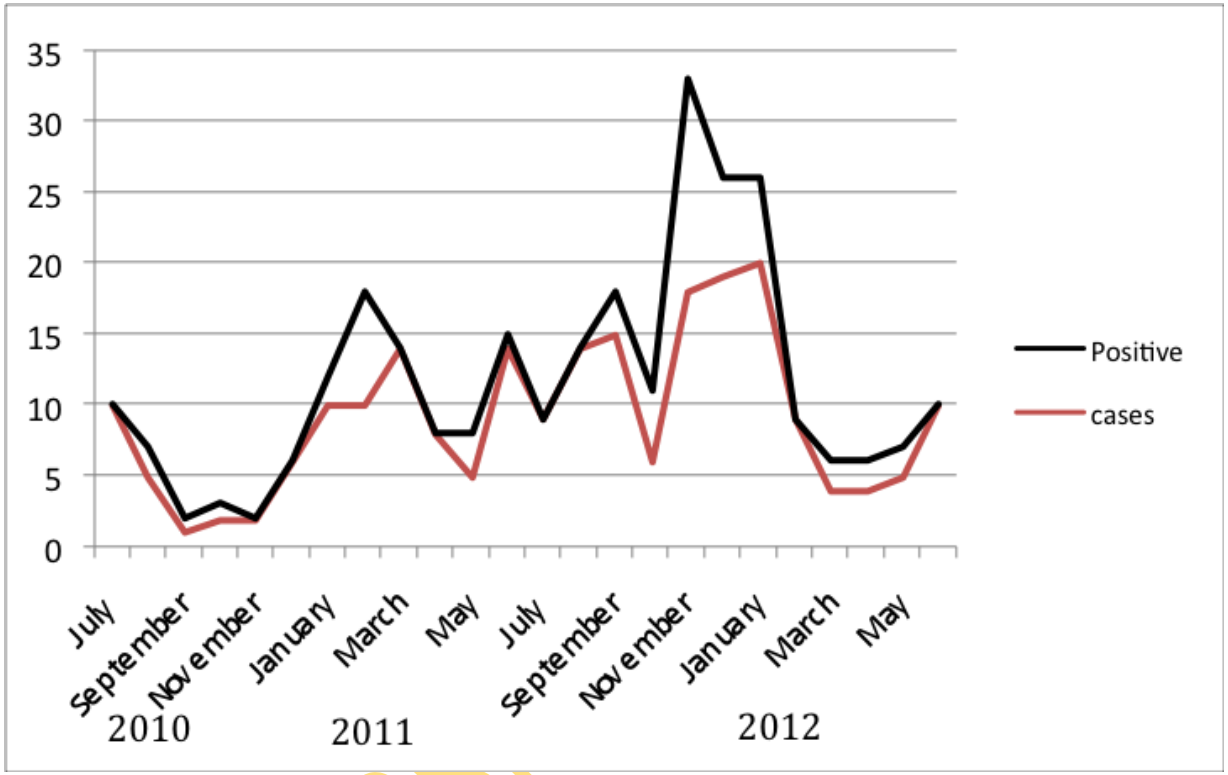

Figure 10: Seasonal variation on observed clinical cases of cough and virus recovery rate in pigs in the y-axis, while x-axis represent month of sample collection from 2010 to 2012.

of swine influenza in human was 5-20% compared with avian influenza H5N1 that was 65% (Padlan, 2010; Promed, 2013**).** Pigs that died during this study were disposed by burial before post mortem examination could be performed to determine actual cause/pattern of death. The necropsy picture and virus isolation in tissues was also not possible.

4.3: VIRUS ISOLATION AND IDENTIFICATION

Twenty nine out of the 227 clinical specimen were isolates from chicken embryonated eggs at first and second passages (Figure 15). These isolates were identified by heamaglutination (HA) and heamaglutination inhibition (HI) using homologous antisera to H1, pandemic H1N1, seasonal H3 and H5 avian influenza. The isolates were inhibited only by pandemic H1N1 with some degree of cross- reactivity to seasonal H1. The HA titre ranged from $\log2^4$ to $\log2^8$ (Figure 16 & 17). Attempt to isolate in MDCK cell culture was not successful.

4.4: ELECTRON MICROSCOPY AND ELECTRONMICROGRAPH

Virus inactivation and electron microscopy:

Viral isolates were concentrated and identified by negative staining (contrast medium) in an electron microscope. The physical structure of the virus as delineated by electron transparent structures and recorded in the computer imaging is shown in figure 18a and b. Characteristic spherical shape particles with projecting surface glycoprotein HA and NA on the envelope were observed. Nucleoprotein forms the core, which is delineated from the capsid layer. The shapes vary from spherical, elongated to pleomorphic. Viruses from swab specimen were scanty and more spherical (Figure 18a) whereas concentrated particles were observed for viruses grown and amplified in egg culture. In addition freshly isolated virus appeared elongated in shape (Figure 18b).

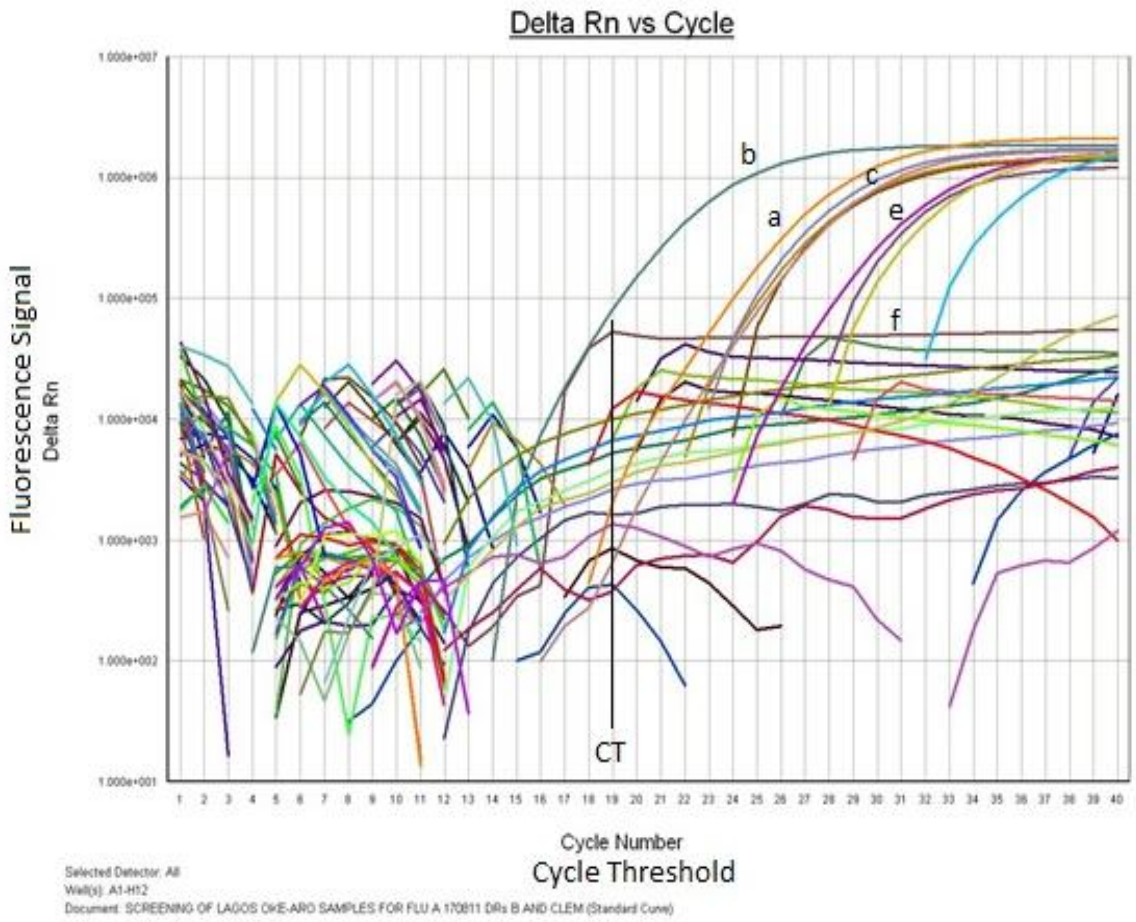

Figure 11: RT-PCR graphs showing some positive samples (b,c,d) including positive control (a), negative control (e) without exponential rise in signal, y-axis shows fluorescence signal strength. Cycle threshold (19) for positive sample (b) is shown on x-axis as seen on the real time RT-PCR GenAmp (Applied Biosystems 7500).

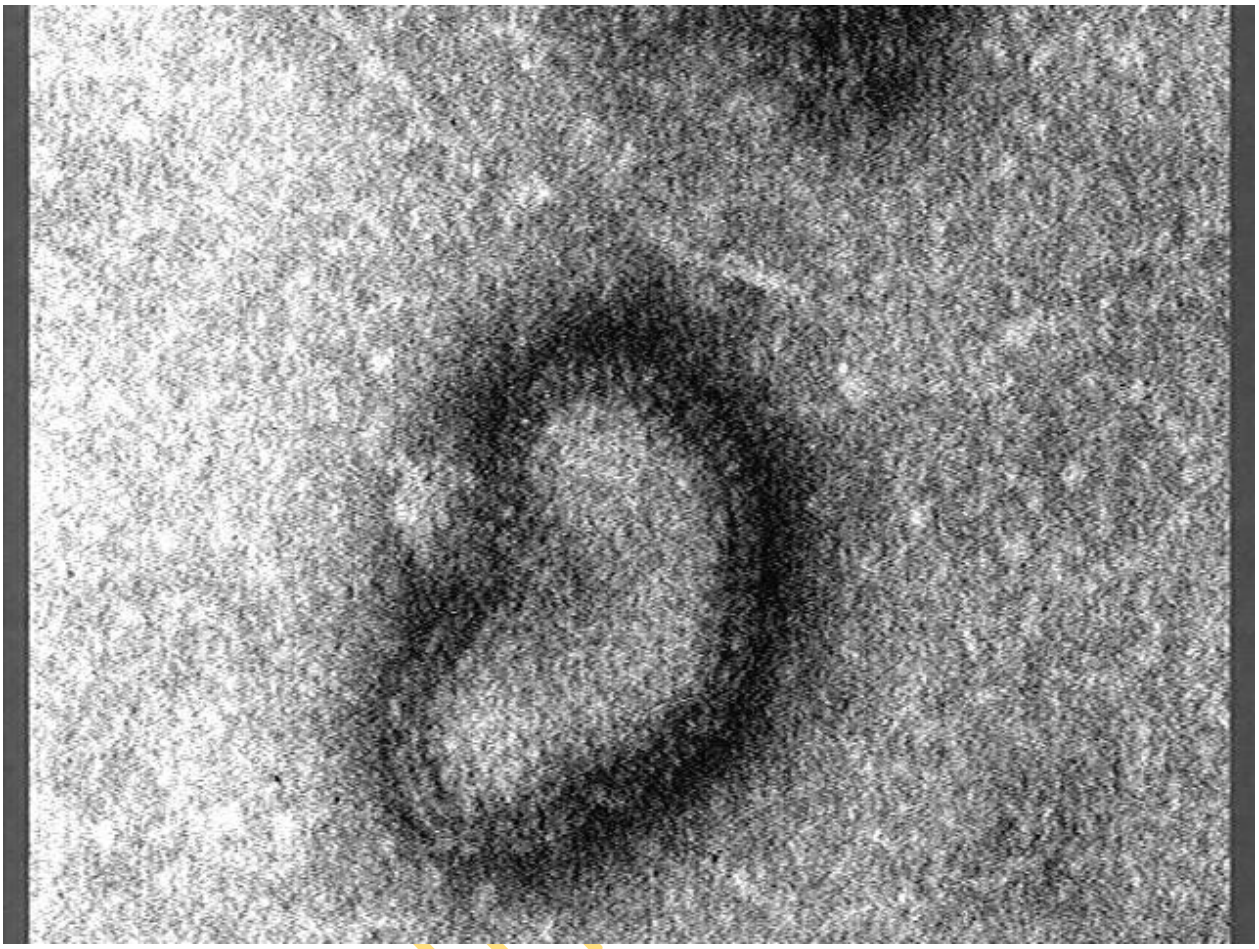

Figure 12a: Electron Micrograph of influenza A/H1N1pdm, A/swine/Nigeria x 55,000 (specimen from swab collected in pig)

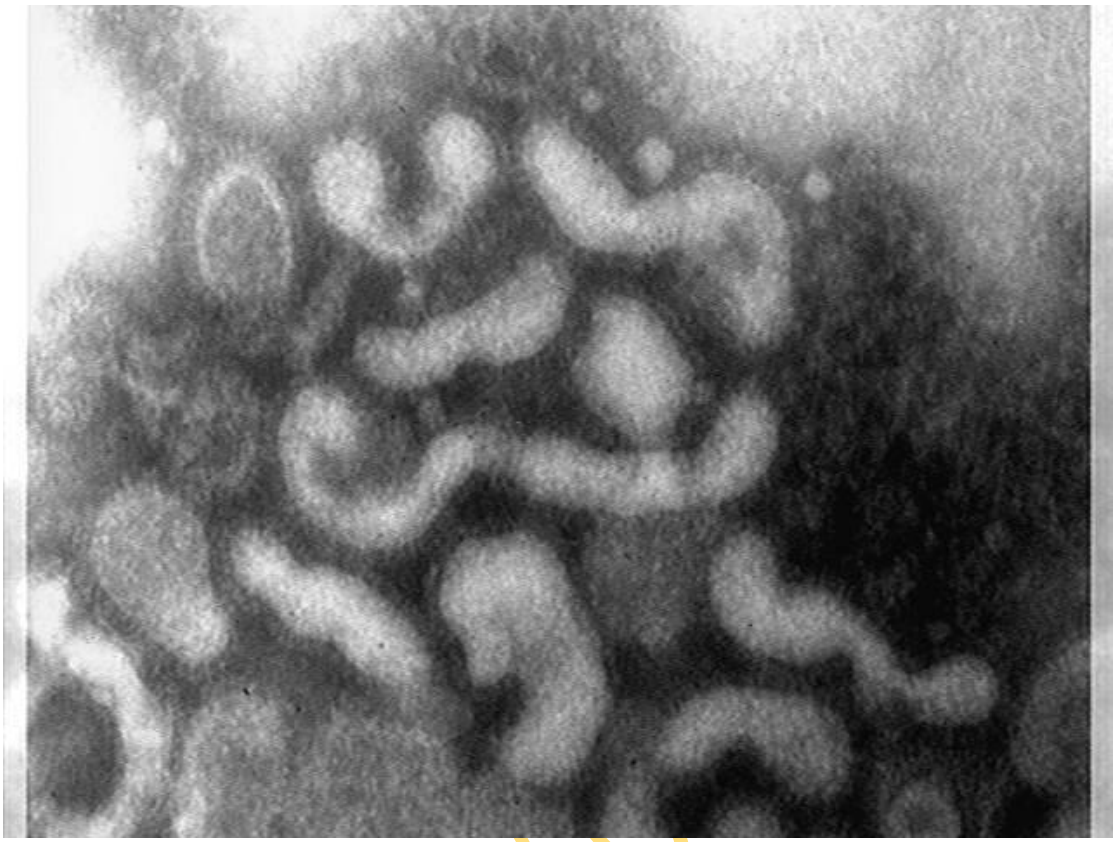

Figure 12b: Electron Micrograph of influenza A/H1N1pdm, A/swine/Nigeria x 28,000 (specimen from egg culture collected in pig)

4.5: VIRUS PURIFICATION AND ANTIGEN DEVELOPMENT:

Serial passages and replication of A/swine/Nigeria virus in egg culture yielded a HA titre of log 2^8. The pellets obtained and inactivated was freeze dried to be used as autologous diagnostic antigen (Appendix vii).

4.6: MOLECULAR CHARACTERISATION/ DNA SEQUENCING AND NUCLEOTIDE/ AMINO ACID ALIGNMENTS

Agar gel electrophoresis confirmed the presence of H1N1 DNA bands, with molecular band size at 350bp captured in Electrophoresis gel documentation system. This is within expected band size of influenza virus primers used (Figure 13). Full length sequences (in short fragments of the PB1) of virus isolates were electrophoresed in agar gel in short amplicon sizes in series (Figure 14). The short fragments were organized with seqscape software and the electropherograph of full length HA fragment is as shown in figure 15.

The full length nucleotide sequence of three selected HA gene of A/swine/Nigeria/2011 are as shown in Tables 7; 8 & 9.

MEGA 4 alignment of the nucleotides sequences with sequences in the GenBank is shown in Figure 25 & 26 and the amino acid alignment is as shown in Figure 27.

The full genome sequences of a representative isolate (A/swine/Nigeria/12VIR4047/2011), a total of 13600 nucleotides are deposited in the GenBank with accession number JX442481- HA, JX4442482- NA, JX482555-PB2, JX482556-PB1, JX482557-MA, JX482558-NP, JX482559-PA and JX48260-NS (Bao *et al*,.2009) access at www.ncbi.nlm.gov.genomes/FLU/FLU.html.

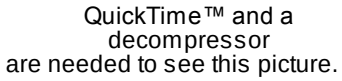

Figure 13: Gel electrophoresis of amplified product of swine influenza matrix gene with positive and negative controls, positive bands observed at 350 bp on the marker.

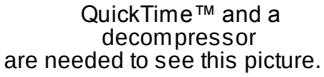

Figure 14: Gel electrophoresis of amplicons of fragments of the PB1 segment gene pre-sequencing

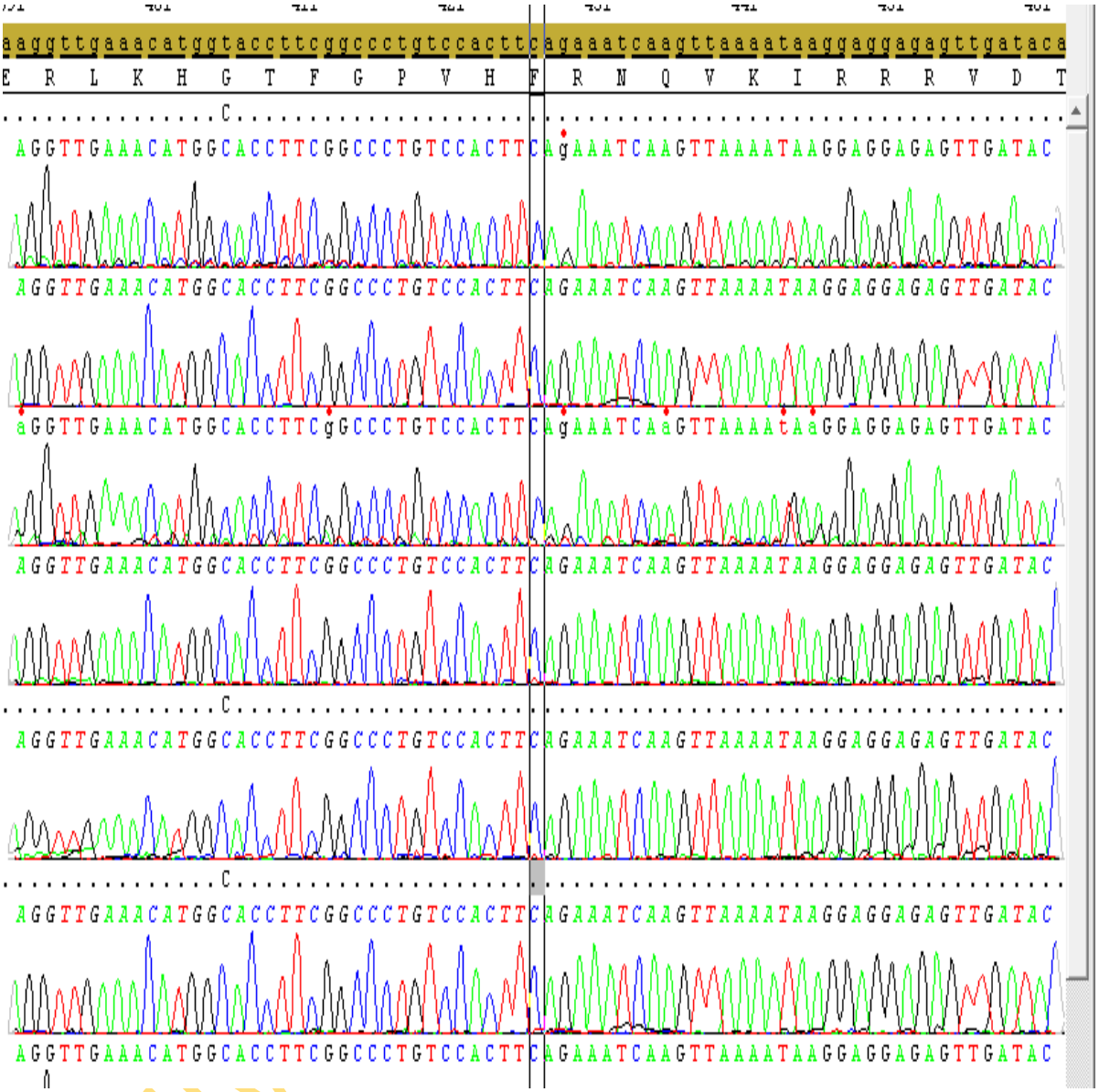

Figure 15: Electropherograph of swine influenza HA gene segment in Seqscape analyzer

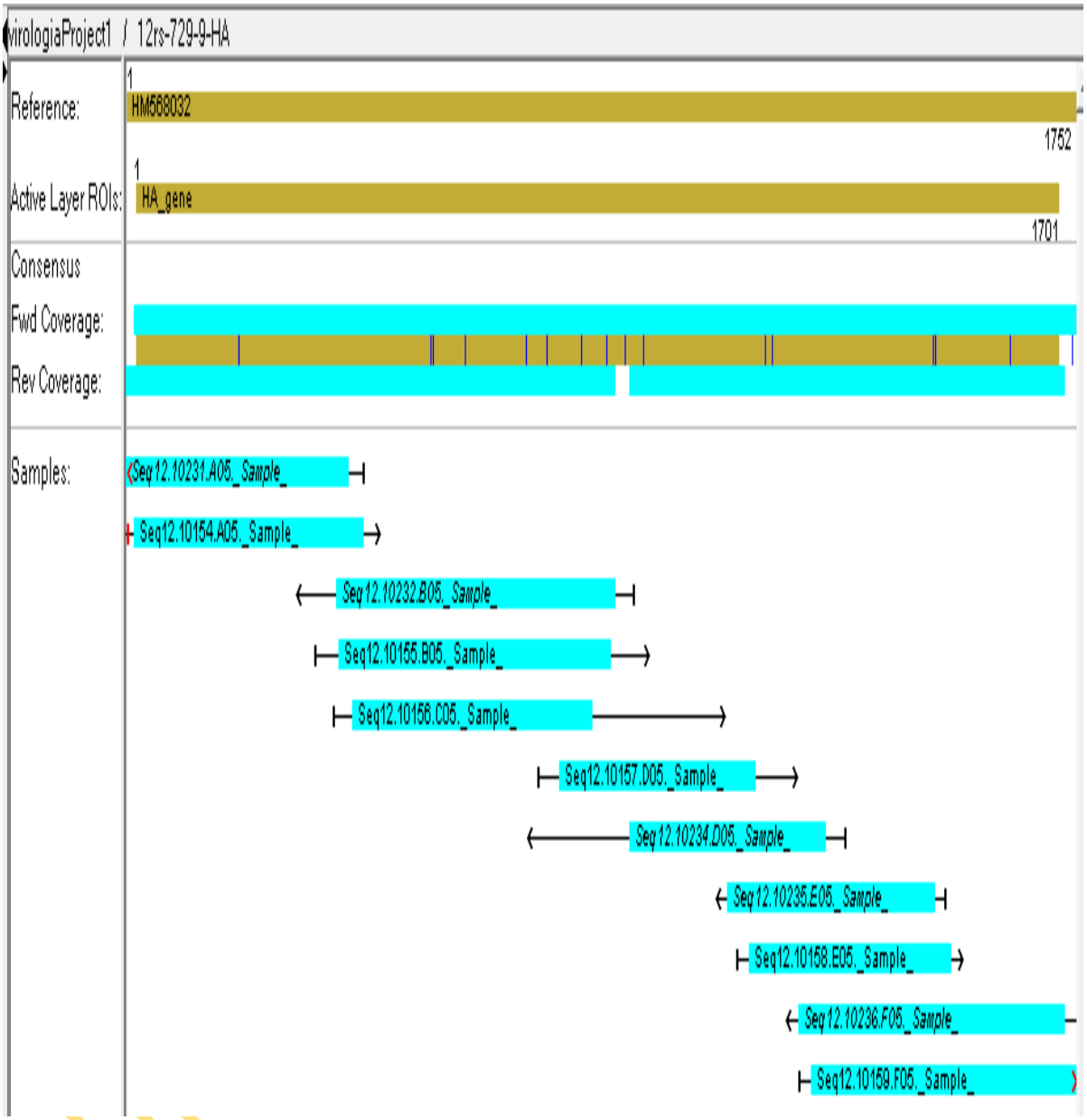

Figure 16: Seqscape computer software overlapping of the HA gene fragments for full consensus gene segment (1 to 1700 nucleotides)

Table 8: Gene sequence of Nigerian isolate of pandemic A/H1N1 influenza virus-07

>A/Swine/Nigeria/12VIR4047_07/2011 (H1N1) (HA)
QuickTime™ and a decompressor are needed to see this picture.

Table 9: Gene sequences of Nigerian isolate of pandemic A/H1N1 influenza virus-08

>A/Swine/Nigeria/12RS729_8_2011 (H1N1) (HA)
QuickTime™ and a decompressor are needed to see this picture.

Table 10: Gene sequences of Nigerian isolate of pandemic A/H1N1 influenza virus-09

>A/Swine/Nigeria/12VIR4047_09/2011 (H1N1) (HA)

```
          10        20        30        40        50        60        70        80
    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
1   ATGAAGGCAATACTAGTAGTTCTGCTATATACATTTGCAACCACAAATGCAGACACATTATGTATAGGTTATCATGCGAA

          90       100       110       120       130       140       150       160
    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
81  CAATTCAACAGACACTGTAGACACAGTACTAGAAAAGAATGTAACAGTAACACACTCTGTTAACCTTCTAGAAGACAAGC

         170       180       190       200       210       220       230       240
    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
161 ATAACGGGAAACTATGCAAACTAAGAGGAGTAGCCCCATTGCATTTGGGTAAATGTAACATTGCTGGCTGGATCCTGGGA

         250       260       270       280       290       300       310       320
    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
241 AATCCAGAGTGTGAATCACTCTCCACAGCAAGCTCATGGTCCTACATTGTGGAAACATCTAGTTCAGACAATGGAACGTG

         330       340       350       360       370       380       390       400
    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
321 TTACCCAGGAGATTTCATCGATTATGAGGAGCTAAGAGAGCAATTGAGCTCAGTGTCATCATTTGAAAGGTTTGAGATAT

         410       420       430       440       450       460       470       480
    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
401 TCCCCAAGACAAGTTCATGGCCCAATCATGACTCGAACAAAGGTGTAACGGCAGCATGTCCTCATGCTGGAGCAAAAAGC

         490       500       510       520       530       540       550       560
    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
481 TTCTACAAAAATTTAATATGGCTAGTTAAAAAAGGAAATTCATACCCAAAGCTCAGCAAATCTTATATTAATGATAAAGG

         570       580       590       600       610       620       630       640
    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
561 GAAAGAAGTCCTCGTGCTATGGGGCATTCACCATCCATCTACTAGTACTGACCAACAAAGTCTCTATCAGAATGCAGATG

         650       660       670       680       690       700       710       720
    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
641 CATATGTTTTTGTGGGGACATCAAGATACAGCAAGAAGTTCAAGCCGGAAATAGCAATAAGACCCAAAGTGAGGGATCGA

         730       740       750       760       770       780       790       800
    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
721 GAAGGGAGAATGAACTATTACTGGACACTAGTAGAACCGGGAGACAAAATAACATTCGAAGCAACTGGAAATCTAGTGGT

         810       820       830       840       850       860       870       880
    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
801 ACCGAGATATGCATTCGCGATGGAAAGAAATGCTGGATCTGGTATTATCATTTCAGATACACCAGCCCACGATTGCAATA

         890       900       910       920       930       940       950       960
    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
881 CAACTTGTCAGACACCCAAAGGTGCTATAAACACCAGCCTCCCATTTCAGAATGTACATCCGATCACAATTGGAAAATGT

         970       980       990      1000      1010      1020      1030      1040
    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
961 CCAAAATATGTAAAAAGCACAAAATTGAGACTGGCCACAGGATTGAGGAATGTCCCGTCTATTCAATCTAGAGGCCTATT
```

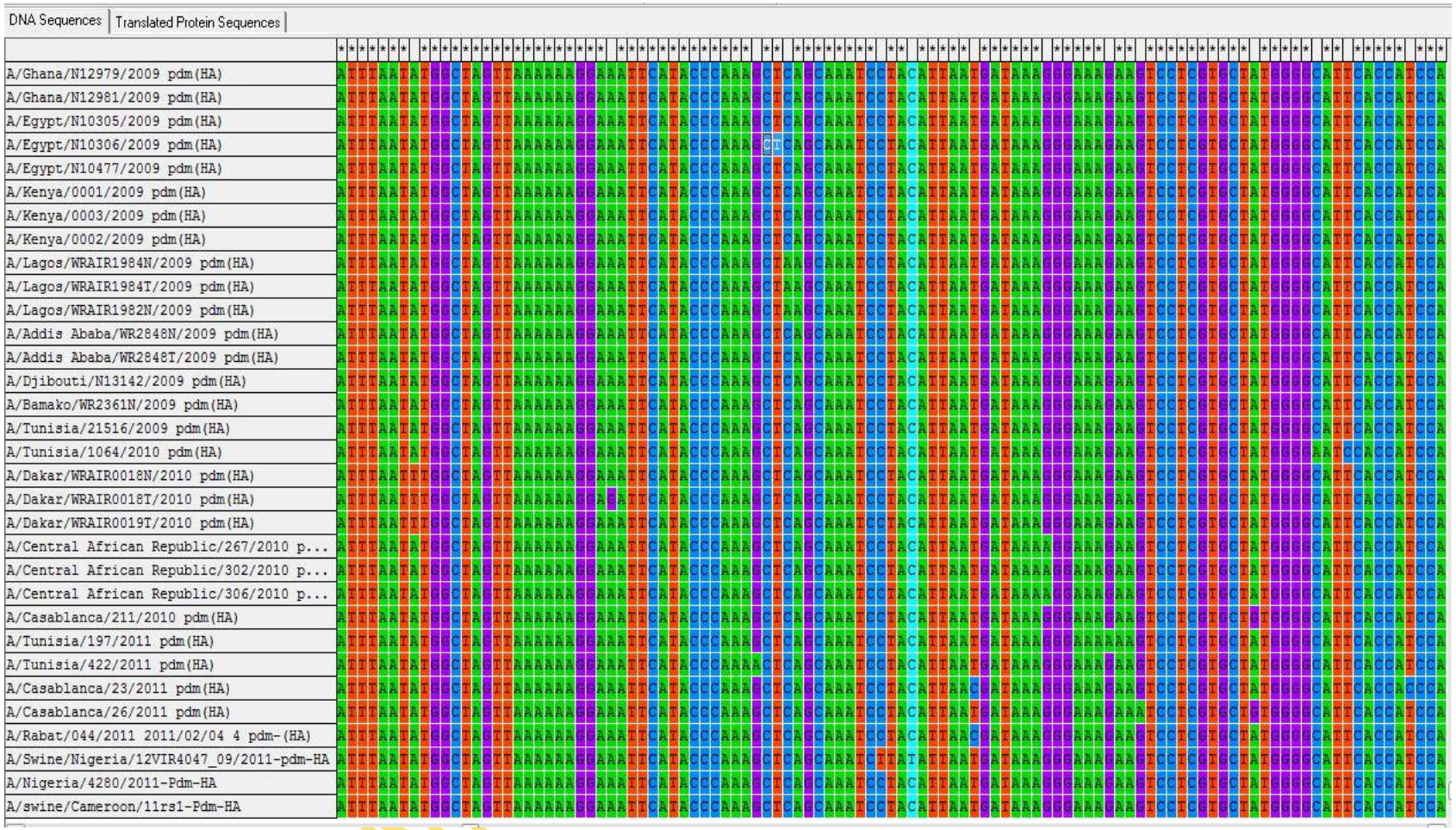

Figure 17: Nucleotide alignment of Nigerian isolates and selected sequences of pandemic A/H1N1 influenza virus using MEGA5

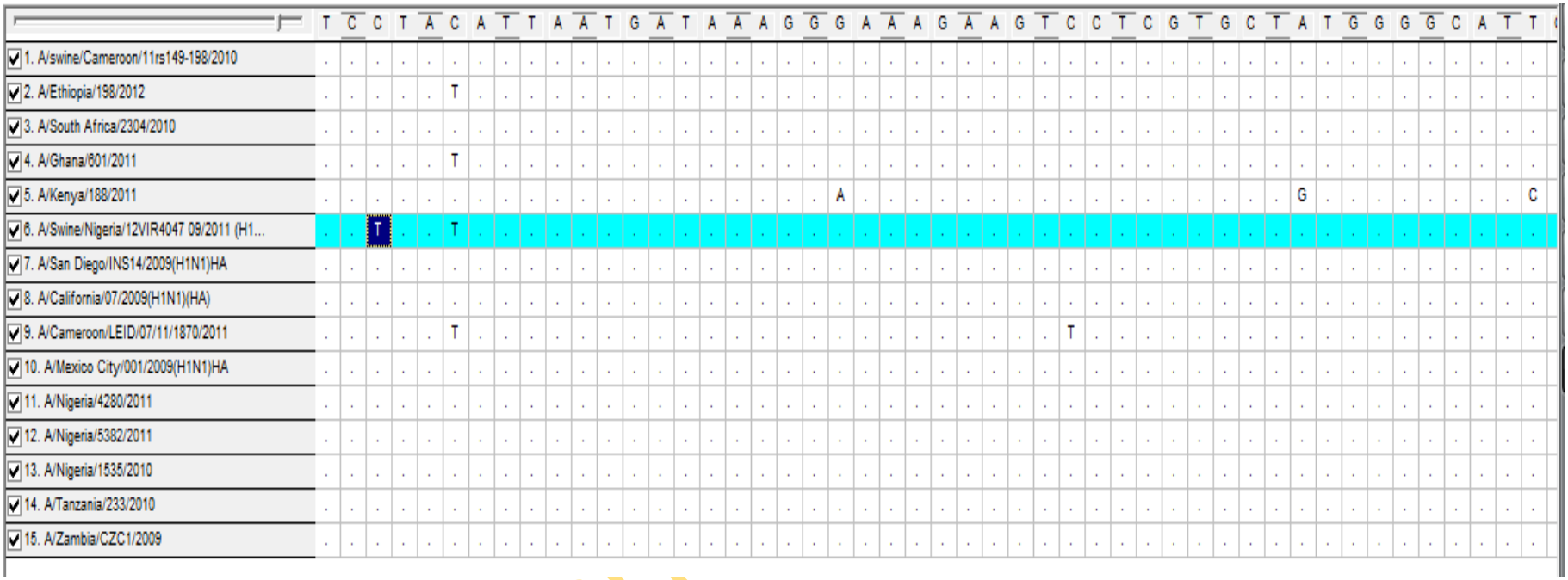

Figure 18: Nucleotide sequence alignment of Nigerian and selected sequences of pandemic A/H1N1 influenza virus

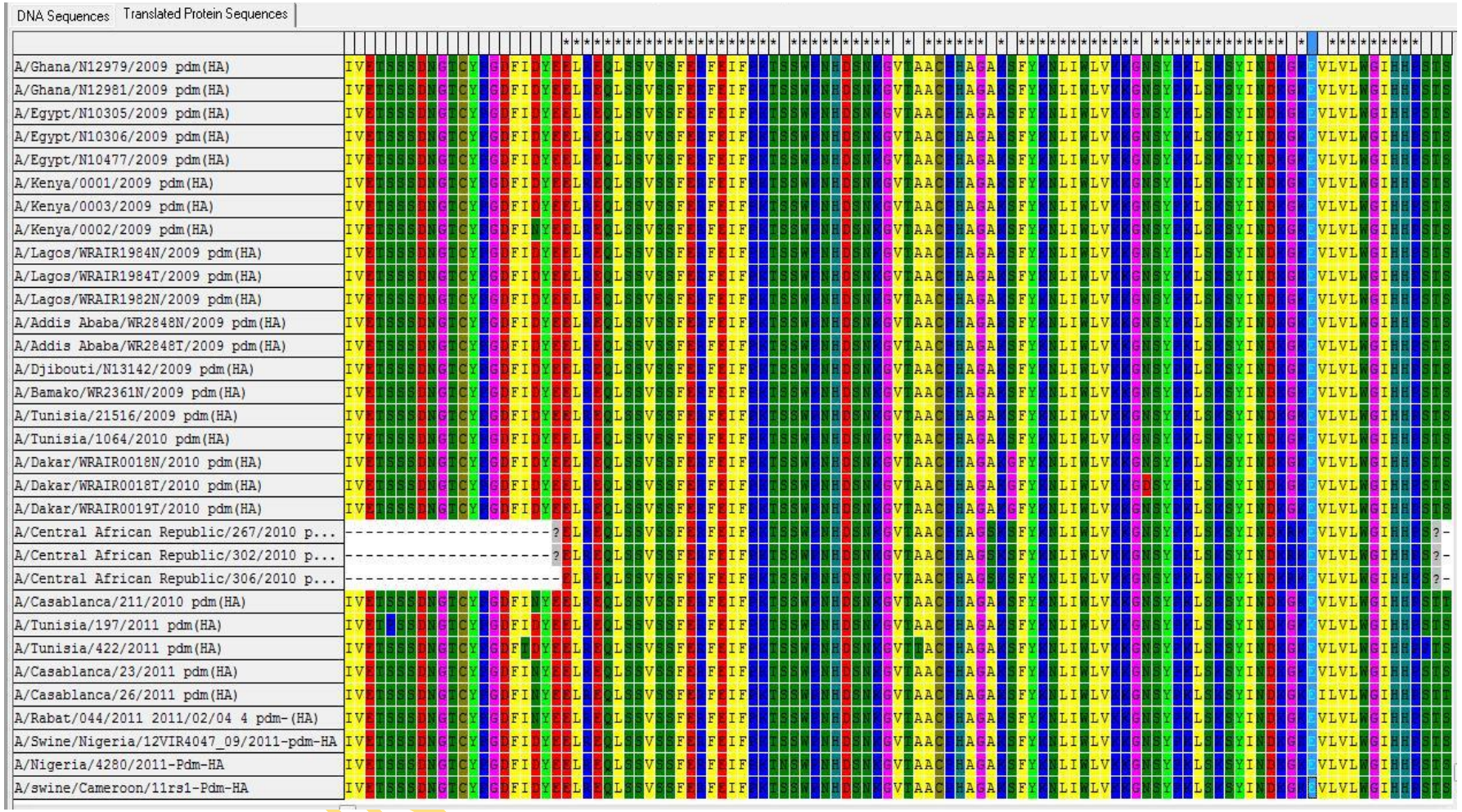

Figure 19: Amino acid alignment of A/swine/Nigeria HA segment and selected sequences from GenBank and GISAID

4.7: BASIC LOCAL ALIGNMENT SEARCH HOMOLOGY AND PHYLOGENY OF PANDEMIC A/H1N1 ISOLATES FROM NIGERIA

Basic Local Alignment Search Tool (BLAST) in the Genbank showed 99% congenetic with other pandemic influenza virus earlier detected in human. Initial Genebank BLAST of the HA gene showed that the closest virus related to Nigerian isolates was A/San Diego/2009 (Bao et al., 2009). However on further search in other genetic databank (GISAID), certain sequences, that are not available in Genbank from neighboring West African countries of Ghana, Cameroon and Nigeria were retrieved and added to the alignment and were found to be more related to A/Swine/Nigeria/VIR 4474-09/2011.

A/swine/Nigeria/2011 isolates obtained in this study are monophyletic with few amino acid substitutions between genes and are clustered with other 2009 A/H1N1 influenza pandemic (Figure 20). The results of Basic Local Alignment Search Tool (BLAST) homology for all the gene segments are presented in Tables 12; 13; 14; 15; 16; 17; 18; 19 while in Tables 20; 21; 22; 23; 24; 25 & 26 are shown amino acid substitution of surface and internal genes of A/Swine/Nigeria compared to closely related pandemic swine influenza virus in the GenBank.

A/Swine/Nigeria showed 13 nucleotide mutations and three amino acid substitutions in the HA gene. This was also translated to amino acid substitutions that also differed from the prototype A/California/07/2009 virus and West African human isolates at position 138, 240, 312 (Figure 21, Table 18).

The phylogenetic tree for pandemic A/swine/Nigeria isolated in this study was constructed for all influenza surface and internal genes segment by segment (HA; NA; MA; PB2; PB1; NP; PA and NS.) with related sequences retrieved from GenBank and GISAID and are presented in Figures 20; 21, 22; 23, 24; 25; 26; 27 and 28. The clustering on the phylogenetic tree shows that H1N1pdm isolates from pigs in Nigeria (cycle and red colour label) formed a sub cluster and had human isolates from Ghana and Cameroon close to it. The Nigeria human H1N1pdm virus (triangle and blue colour label) is further down the line on the tree and much farther is an isolate of H1N1pdm from pigs in Cameroon.

Table 11: BLAST homology results of HA sequences with Nigerian swine isolate (source NCBI, GenBank)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
JX442481.1	Influenza A virus (A/swine/Nigeria/12VIR4047-09/2011(H1N1)) segment 4 hemagglutinin (HA) gene, complete cds	3142	3142	100%	0.0	100%
CY083838.1	Influenza A virus (A/San Diego/INS14/2009(H1N1)) hemagglutinin (HA) gene, complete cds	3075	3075	100%	0.0	99%
CY055964.1	Influenza A virus (A/San Diego/INS03/2009(H1N1)) segment 4, complete sequence	3075	3075	100%	0.0	99%
CY066543.1	Influenza A virus (A/San Diego/INS197/2009(H1N1)) segment 4, complete sequence	3075	3075	100%	0.0	99%
JX309409.1	Influenza A virus (A/Singapore/SS13/2010(H1N1)) segment 4 hemagglutinin (HA) gene, complete cds	3070	3070	100%	0.0	99%

Table 12: BLAST homology results of NA sequences with Nigerian swine isolate (source NCBI, GenBank)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
JX442482.1	Influenza A virus (A/swine/Nigeria/12VIR4047-09/2011(H1N1)) segment 6 neuraminidase (NA) gene, complete cds	2604	2604	100%	0.0	100%
CY066281.1	Influenza A virus (A/California/VRDL113/2009(H1N1)) segment 6, complete sequence	2566	2566	100%	0.0	99%
CY063253.1	Influenza A virus (A/Wisconsin/629-D00692/2009(H1N1)) segment 6, complete sequence	2566	2566	100%	0.0	99%
JN381360.1	Influenza A virus (A/Taiwan/97674/2009(H1N1)) segment 6 neuraminidase (NA) gene, complete cds	2560	2560	100%	0.0	99%
CY123939.1	Influenza A virus (A/Singapore/ON361/2009(H1N1)) neuraminidase (NA) gene, complete cds	2560	2560	100%	0.0	99%

Table 13: BLAST homology results of MA sequences with Nigerian swine isolate (source NCBI, GenBank)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
JX482559.1	Influenza A virus (A/swine/Nigeria/12VIR4047-09/2011(H1N1)) segment 7 matrix protein 2 (M2) gene, partial cds; and matrix protein 1 (M1) gene, complete cds	1766	1766	100%	0.0	100%
CY118256.1	Influenza A virus (A/Malaysia/2089302/2009(H1N1)) matrix protein 2 (M2) and matrix protein 1 (M1) genes, complete cds	1755	1755	100%	0.0	99%
CY118243.1	Influenza A virus (A/Malaysia/2156787/2009(mixed)) matrix protein 2 (M2) and matrix protein 1 (M1) genes, complete cds	1755	1755	100%	0.0	99%
CY083895.1	Influenza A virus (A/San Diego/INS73/2009(H1N1)) matrix protein 2 (M2) and matrix protein 1 (M1) genes, complete cds	1755	1755	100%	0.0	99%
CY083370.1	Influenza A virus (A/South Carolina/WRAIR1660P/2009(H1N1)) matrix protein 2 (M2) and matrix protein 1 (M1) genes, complete cds	1755	1755	100%	0.0	99%

Table 14: BLAST homology results of NP sequences with Nigerian swine isolate (source NCBI, GenBank)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
JX482558.1	Influenza A virus (A/swine/Nigeria/12VIR4047-09/2011(H1N1)) segment 5 nucleocapsid protein (NP) gene, complete cds	2765	2765	100%	0.0	100%
CY061254.1	Influenza A virus (A/San Diego/INS104/2009(H1N1)) segment 5, complete sequence	2726	2726	100%	0.0	99%
CY071523.1	Influenza A virus (A/California/WR1319P/2009(H1N1)) segment 5 sequence	2721	2721	100%	0.0	99%
CY071667.1	Influenza A virus (A/Mexico City/WR1668T/2009(H1N1)) segment 5 sequence	2721	2721	100%	0.0	99%
CY058025.1	Influenza A virus (A/Wisconsin/629-D00401/2009(H1N1)) segment 5, complete sequence	2721	2721	100%	0.0	99%

Table 15: BLAST homology results of NS sequences with Nigerian swine isolate (source NCBI, GenBank)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
JX482560.1	Influenza A virus (A/swine/Nigeria/12VIR4047-09/2011(H1N1)) segment 8 nuclear export protein (NEP) gene, partial cds; and nonstructural protein 1 (NS1) gene, complete cds	1502	1502	98%	0.0	100%
CY120072.1	Influenza A virus (A/Hong Kong/H090-693-V20/2009(H1N1)) nuclear export protein (NEP) and nonstructural protein 1 (NS1) genes, complete cds	1467	1467	100%	0.0	99%
CY083850.1	Influenza A virus (A/District of Columbia/INS23/2009(H1N1)) nuclear export protein (NEP) and nonstructural protein 1 (NS1) genes, complete cds	1467	1467	100%	0.0	99%
CY123721.1	Influenza A virus (A/Singapore/ON202/2009(H1N1)) nuclear export protein (NEP) and nonstructural protein 1 (NS1) genes, complete cds	1461	1461	100%	0.0	99%
CY123528.1	Influenza A virus (A/Singapore/ON1802/2009(H1N1)) nuclear export protein (NEP) and nonstructural protein 1 (NS1) genes, complete cds	1461	1461	100%	0.0	99%

Table 16: BLAST homology results of PA sequences with Nigerian swine isolate (source NCBI, GenBank)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
JX482557.1	Influenza A virus (A/swine/Nigeria/12VIR4047-09/2011(H1N1)) segment 3 polymerase PA (PA) gene, complete cds	3973	3973	100%	0.0	100%
CY071521.1	Influenza A virus (A/California/WR1319P/2009(H1N1)) segment 3 sequence	3940	3940	100%	0.0	99%
CY083875.1	Influenza A virus (A/San Diego/INS49/2009(H1N1)) polymerase PA (PA) gene, complete cds	3934	3934	100%	0.0	99%
CY071601.1	Influenza A virus (A/Ft Carson/WR1446P/2009(H1N1)) segment 3 sequence	3934	3934	100%	0.0	99%
CY066444.1	Influenza A virus (A/California/VRDL133/2009(H1N1)) segment 3, complete sequence	3934	3934	100%	0.0	99%

Table 17: BLAST homology results of PB1 sequences with Nigerian swine isolate (source NCBI, GenBank)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
JX482556.1	Influenza A virus (A/swine/Nigeria/12VIR4047-09/2011(H1N1)) segment 2 polymerase PB1 (PB1) gene, complete cds; and nonfunctional PB1-F2 protein (PB1-F2) gene, complete sequence	4200	4200	100%	0.0	100%
CY056937.1	Influenza A virus (A/San Diego/INS65/2009(H1N1)) segment 2, complete sequence	4117	4117	100%	0.0	99%
CY083876.1	Influenza A virus (A/San Diego/INS49/2009(H1N1)) polymerase PB1 (PB1) gene, complete cds; and PB1-F2 gene, complete sequence	4111	4111	100%	0.0	99%
CY063305.1	Influenza A virus (A/Wisconsin/629-D00859/2009(H1N1)) segment 2, complete sequence	4111	4111	100%	0.0	99%
CY061177.1	Influenza A virus (A/Texas/JMS411/2010(H1N1)) polymerase PB1 (PB1) gene, complete cds; and PB1-F2 gene, complete sequence	4111	4111	100%	0.0	99%

Table 18: BLAST homology results of PB2 sequences with Nigerian swine isolate (source NCBI, GenBank)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
JX482555.1	Influenza A virus (A/swine/Nigeria/12VIR4047-09/2011(H1N1)) segment 1 polymerase PB2 (PB2) gene, complete cds	4211	4211	100%	0.0	100%
CY083877.1	Influenza A virus (A/San Diego/INS49/2009(H1N1)) polymerase PB2 (PB2) gene, complete cds	4150	4150	100%	0.0	99%
CY066590.1	Influenza A virus (A/San Diego/INS203/2009(H1N1)) segment 1, complete sequence	4150	4150	100%	0.0	99%
CY066582.1	Influenza A virus (A/San Diego/INS202/2009(H1N1)) segment 1, complete sequence	4150	4150	100%	0.0	99%
CY066286.1	Influenza A virus (A/California/VRDL113/2009(H1N1)) segment 1, complete sequence	4150	4150	100%	0.0	99%

Table 19: Amino acid sequence alignment of the HA protein of 2009 pandemic A/H1N1 compared

Virus strain	Amino acid position											
	100	138	203	219	220	222	**240**	251	279	289	312	338
A/California/07/2009	P	S	T	G	T	R	Q	V	G	A	I	I
A/Swine/Nigeria	**S**	**N**	**-**	**-**	**-**	**-**	**R**	**-**	**-**	**-**	**V**	**V**
A/Nigeria/4280/2011	S	-	-	-	-	-	-	-	-	-	-	V
A/Ghana/601/2011	S	-	-	-	-	-	-	-	-	-	-	V
A/Cameroon/07/1870/2011	S	-	-	E	T	-	-	-	-	V	-	V
A/San-Diego/INS 14/2009	S	-	-	-	-	-	-	-	-	-	-	V

A/Nigeria/5382/2011	S	-	A	-	-	-	-	-	-	-	-	V
A/Swine/Cameroon/2011	A	-	A	-	-	-	-	-	-	-	-	V

Table 20: Amino acid sequence alignment of the NA protein of 2009 pandemic A/H1N1 compared with prototype and selected strains showing amino acid substitutions

Virus strain	Amino acid position							
	84	106	248	340	351	389	439	442
A/California/07/2009	K	V	N	S	Y	I	S	S
A/Swine/Nigeria/2011	**-**	**I**	**D**	**-**	**F**	**-**	**G**	**-**
A/Nigeria/4280/2011	-	I	D	F	F	-	-	-
A/Nigeria/5382/2011	-	I	D	-	F	M	-	-
A/Ghana/763/2011	-	I	D	-	F	-	-	I
A/Cameroon/187	-	I	D	-	F	-	-	-
A/Mexico city/010/2009	T	I	D	-	F	-	-	-

Table 21: Amino acid sequence alignment of the PB2 protein of 2009 pandemic A/H1N1 compared with prototype and selected strains showing amino acid substitutions

Virus Strain (PB2)	Amino acid position				
	37	154	226	260	527
A/California/07/2009	G	L	S	I	D
A/Swine/Nigeria/2011	**-**	**-**	**-**	**-**	**G**
A/San-Diego/2009	-	-	-	-	-
A/Mexico city/2009	-	-	-	-	-

Table 22: Amino acid sequence alignment of the PB1 protein of 2009 pandemic A/H1N1 compared with prototype and selected strains showing amino acid substitutions

Virus Strain (PB1)	Amino acid position					
	111	126	636	643	736	738
A/California/07/2009	M	R	E	A	K	E
A/Swine/Nigeria/2011	**I**	**-**	**-**	**-**	**G**	**-**
A/San-Diego/NS849/2009	I	-	-	-	G	G
A/Mexico city/4/2009	-	H	G	Q	K	-

Table 23: Amino acid sequence alignment of the NS protein of 2009 pandemic A/H1N1 compared with prototype and selected strains showing amino acid substitutions

Virus Strain (NS)	Amino acid position								
	28	102	109	111	123	187	189	235	246
A/California/07/2009	G	W	Q	I	I	W	G	V	S
A/Swine/Nigeria/2011	**V**	**C**	**K**	**M**	**V**	**E**	**D**	**M**	**N**
A/Ivory coast/2009	-	-	-	-	-	-	-	-	-
A/Mexico city/4/2009	-	-	-	-	-	-	-	-	-

Table 24: Amino acid sequence alignment of the PA protein of 2009 pandemic A/H1N1 compared with prototype and selected strains showing amino acid substitutions

Virus Strain (PA)	Amino acid position						
	14	18	222	224	407	505	716
A/California/07/2009	V	E	N	P	V	I	K
A/Swine/Nigeria/2011	**I**	**-**	**-**	**S**	**-**	**V**	**Q**
A/San-Diego/NS84/2009	-	-	-	S	-	-	-
A/Mexico city/4/2009	-	-	K	S	I	-	-

Table 25: Amino acid sequence alignment of the NP protein of 2009 pandemic A/H1N1 compared with prototype and selected strains showing amino acid substitutions

Virus Strain (NP)	Amino acid position						
	23	100	114	122	373	375	396
A/California/07/2009	T	V	E	L	T	D	T
A/Swine/Nigeria/2011	**S**	**I**	**E**	**Q**	**-**	**N**	**A**
A/San-Diego/NS84/2009	-	I	E	Q	-	-	-
A/Mexico city/4/2009	-	-	E	Q	I	-	-

PHYLOGENETIC TREES

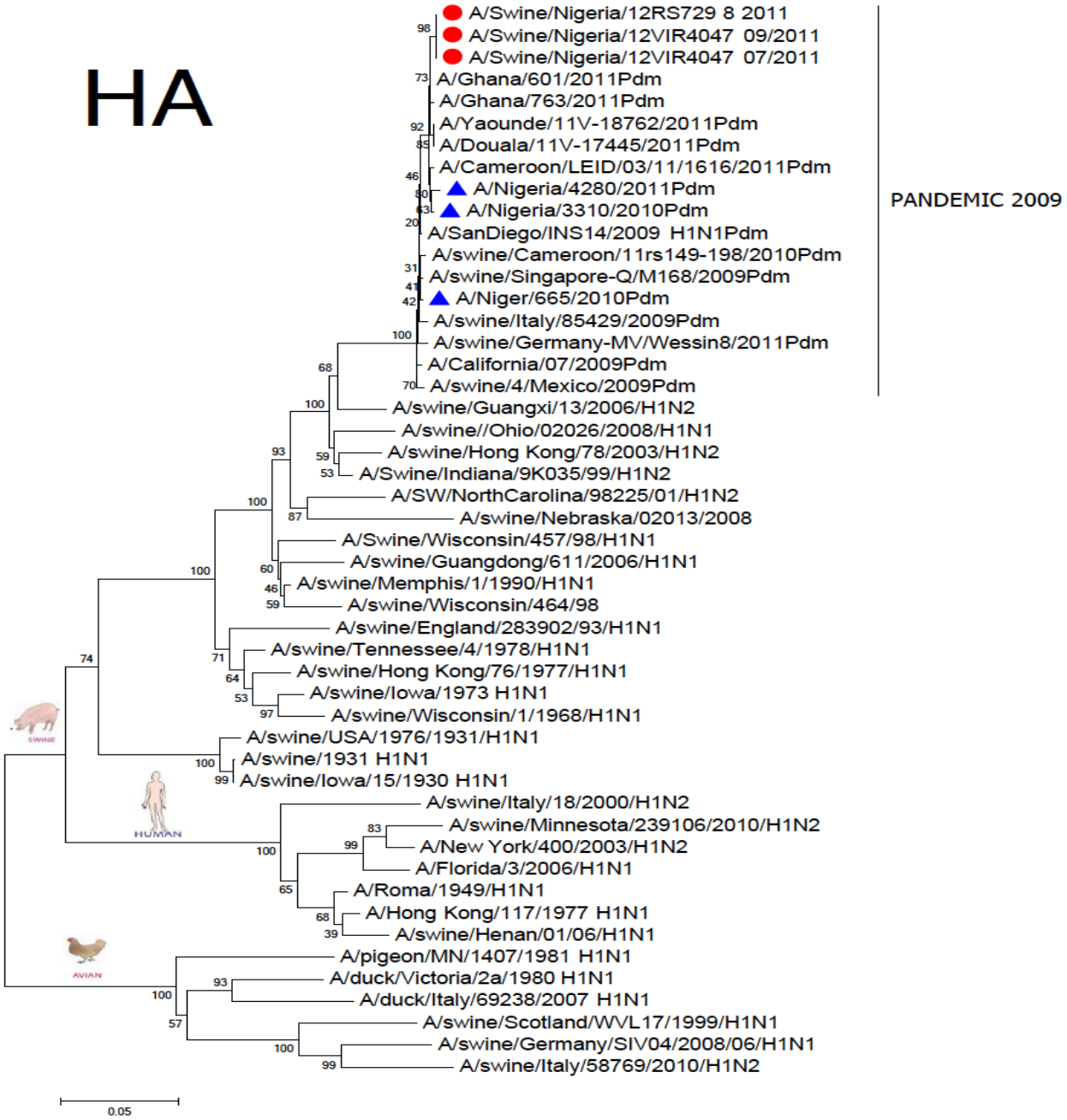

Figure 20: Phylogenetic tree of the HA gene of A/Swine/Nigeria/2011, 2009 pandemic A/H1N1 influenza virus

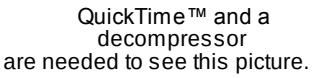

Figure 21: Phylogenetic tree of related and selected HA gene of 2009 pandemic A/H1N1 influenza virus with A/Swine/Nigeria/2011

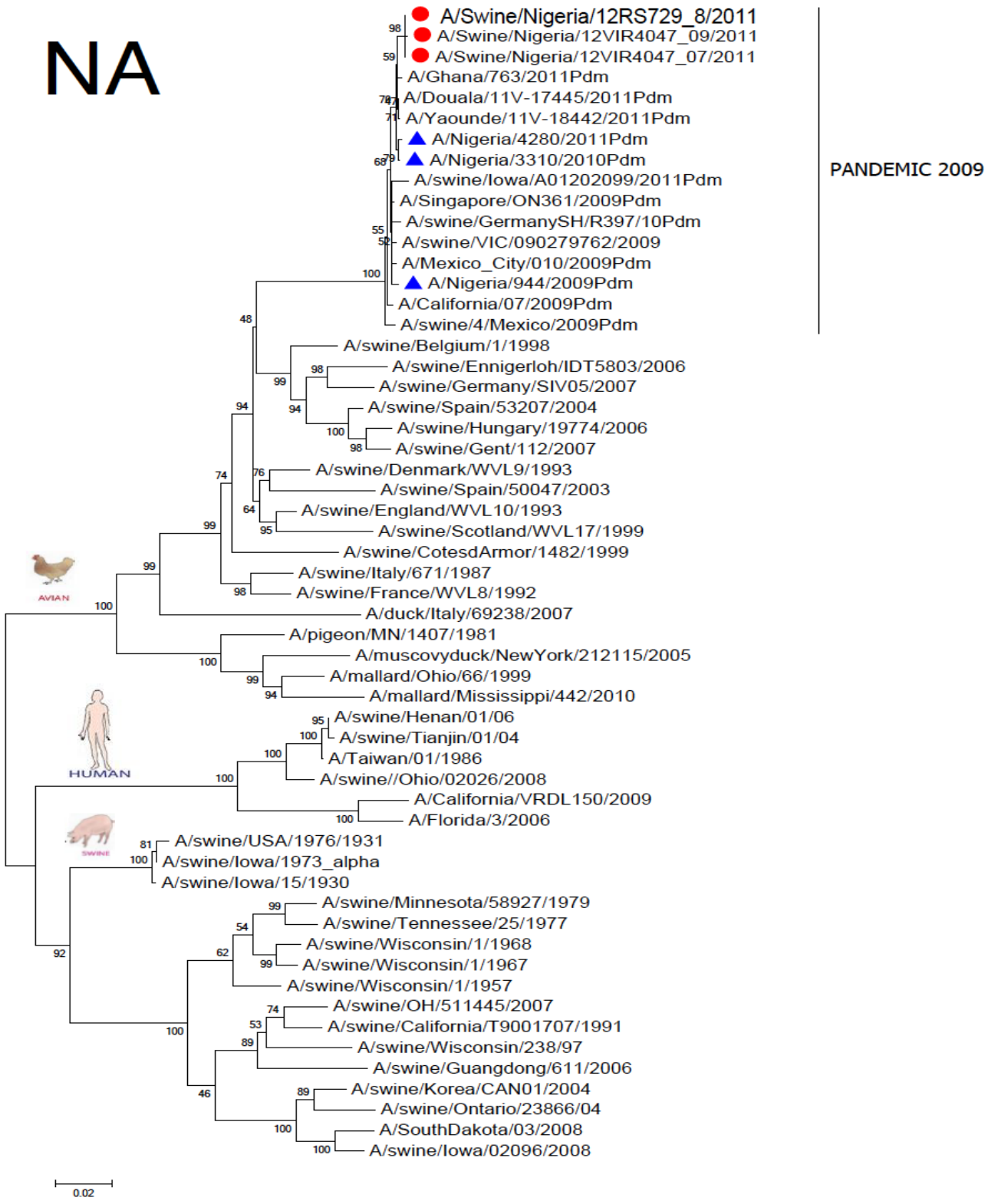

Figure 22: Phylogenetic tree of the NA gene of A/Swine/Nigeria/2011, 2009 pandemic A/H1N1 influenza virus

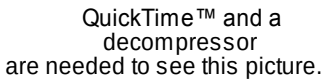

Figure 23: Phylogenetic tree of the MA gene of A/Swine/Nigeria/2011, 2009 pandemic A/H1N1 influenza virus

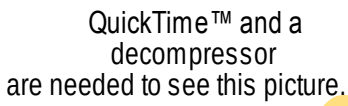

Figure 24: Phylogenetic tree of the PB1 gene of A/Swine/Nigeria/2011, 2009 pandemic A/H1N1 influenza virus

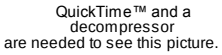

Figure 25: Phylogenetic tree of the PB2 gene of A/Swine /Nigeria/2011, 2009 pandemic A/H1N1 influenza virus

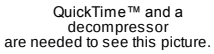

Figure 26: Phylogenetic tree of the NP gene of A/Swine /Nigeria/2011, 2009 pandemic A/H1N1 influenza virus

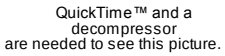

Figure 27: Phylogenetic tree of the PA gene of A/Swine /Nigeria/2011, 2009 pandemic A/H1N1 influenza virus

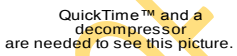

Figure 28: Phylogenetic tree of the NS gene of A/Swine /Nigeria/2011, 2009 pandemic A/H1N1 influenza virus

CHAPTER FIVE

DISCUSSION

This study established that pandemic influenza A/H1N1 virus circulates in pigs at an intensive piggery complex in Lagos, Nigeria. The influenza virus isolates from pigs in the study location showed amino acid substitutions, most importantly at the antigenic cleavage site of the haemagglutinin gene. Isolation, molecular subtyping and haemagglutination inhibition analysis of the virus confirmed circulation of 2009 pandemic influenza A/H1N1 in the study area. The virus was also identified by negative staining electron microscopy and inactivated for use as diagnostic antigen.

Isolation of pandemic 2009 influenza A/H1N1 from pigs represented the first time in Nigeria, and the second in Africa. Earlier deposit of pandemic A/H1N1 in the GenBank from Cameroon composed only partial sequences of the virus (Njabo *et al*., 2010). In this study, full genomic sequence data of pandemic H1N1 was determined and deposited in the GenBank (Bao *et al*., 2009). Phylogenetically, the virus and related viruses in the GenBank shares its HA gene with swine and human influenza. The NA gene is related to avian influenza virus while the internal genes were derived from swine and avian lineages of influenza A/H1N1 virus as was previously observed by Smith *et al*. (2009) and Garten *et al*. (2009).

There was no outbreak of swine influenza nor obvious influenza illness in the herd during the period of this study. It took careful observation to notice coughing pigs. Most of the specimens that were positive were collected from weaners and growing pigs, which suggest that younger pigs may be more susceptible to the virus. Adult pigs, like human hosts, might have developed cross-protective antibodies from previous exposure to strains of seasonal or classical influenza virus. Immune pressure has been suggested as an explanation for the reason why swine influenza virus lineage is more stable in pigs than in

humans (Yu *et al*., 2009). The implication is that variants and strains of influenza virus may emerge from pigs that could in turn become source of infection with the newly emergent strains. Interestingly, pig handlers in pens with positive swine samples in this study did not have any history or overt clinical signs of influenza. Samples collected from pig handlers based on subjective anamneses of minor febrile and respiratory illness were all negative. This may be due to certain yet to be determined host susceptibility factors as inability to detect influenza infection in human contacts was also prominently reported in Nigeria during avian influenza outbreaks in 2006-2008, where over 100 samples collected from poultry farmers all tested negative (Oritz *et al*., 2007). Earlier reports on pandemic A/H1N1 influenza virus showed that infected pigs present influenza-like signs, this was also evident in human cases that preceded swine infection during the 2009 pandemic (Girard *et al*., 2009). The virus spread across continents with efficient human-to-human transmission, prompted WHO to declare a phase 6 pandemic in June 2009 (WHO, 2012).

In this study, molecular detection to identify 2009 pandemic A/H1N1 influenza virus was achieved by real-time RT-PCR. The assay initially targeted the matrix gene that is common to all influenza A viruses. Thereafter, swine A matrix specifically designed for 2009 pandemic A/H1N1 influenza virus was used. This was because generic primers for matrix gene were not able to detect swine influenza A at the beginning of the pandemic, leading to false negative results (Garten *et al*., 2009). Subsequent subtyping also identified primer-specific 2009 pandemic influenza A/H1N1 virus. The viruses identified by RT-PCR were from samples collected from observed cases of influenza-like illness in pigs. This may likely be due to relative severity of H1N1 infection in the pig farm and viral load in the samples collected because classical influenza virus may circulate without overt clinical signs with less virus shedding and less sensitivity in RT-PCR assay (van Reeth, 2007).

Although the susceptibility of 2009 pandemic influenza virus and its ability to replicate in egg culture is not as efficient as was observed with highly pathogenic avian influenza (HPAI) H5N1, 29 (12.8%) viruses were successfully isolated in embryonated chicken eggs (Balish, 2013). Diagnostic HA titre was obtained compared with the use of MDCK

cells that proved to be less sensitive for field samples/specimen. Hence, embryonated eggs were the preferred method of isolation in replication-competent strains similar to observation by Chen *et al*. (2010). The host cell receptor and viral infection may not be unrelated to the adaptation of the HA genes over the years for better culture in avian tissue; although, certain strains of swine influenza may be lost after initial passage in eggs (Chen *et al*., 2010; Meseko, pers observation). The 2009 influenza pandemic has been described as the product of genetic combination of swine, avian and human influenza genes (Garten *et al*., 2009; Smith *et al*., 2009). The virus was also reported to be similar to the 1918 Spanish influenza virus, which was originally an avian influenza lineage. This may explain why embryonated egg was susceptible for virus isolation, and was efficient for the culture of the 2009 pandemic virus with 12.8% recovery rate observed in this study, similar to observations by Mochalova *et al*. (2003) and van Reeth (2007).

Though it has been previously observed that no pig farm is likely free from influenza virus either circulating clinically or sub-clinically (Kothalawala *et al*., 2011), the chances of virus isolation depend on whether animals in the herd are clinically shedding the virus or not, efficiency of sample collection and post-collection handling of samples that preserves its biological integrity. This is because enveloped and RNA virus degenerate more quickly by repeated cycles of freeze-thawing (Miller, *et al*., 1999). It is worthy of note that the viruses that were conclusively identified in this study were confirmed by both molecular test and virus isolation as 2009 pandemic influenza H1N1.

Phylogenetic analysis of the virus isolates showed that the HA gene of Nigerian swine influenza A/H1N1 are monophyletic and are clustered with global 2009 A/H1N1 influenza pandemic viruses. Initial GenBank BLAST of the HA gene showed the closest relation to Nigerian isolates was A/SanDiego/2009 (Bao *et al*., 2009). However, on further search in other genome databank (GISAID), certain sequences from Ghana, Cameroon and even Nigeria that were not available in GenBank but retrieved from GISAID were more related to A/Swine/Nigeria/VIR4474-09/2009. Importantly, all the strains also had their roots in A/SanDiego/2009. The HA gene of A/swine/Nigeria/12VIR4047/2011 is closely related to A/Nigeria/4280/2011, A/Ghana/601/2011 and A/Cameroon/EID/07/11/1870/2011 obtained from GISAID; all of which are 2009 human isolates clustering in the

phylogenetic tree with swine isolates from Nigeria. This reflects the transboundary nature of swine influenza virus as it is transmitted from different hosts. Interestingly, earlier detection of 2009 pandemic A/H1N1 influenza virus in pig was reported in Cameroon (Njabo *et al*., 2011), but the swine virus is less related to the isolates from pigs in Nigeria unlike the human isolates from Cameroon. That also is an indication of different introduction of the pandemic virus into the pig populations in the region (Cameroon and Nigeria).

The pandemic influenza virus (A/Nigeria/4280/2011(H1N1)), which also clustered with A/swine/Nigeria/12VIR4047/2011 on the phylogenetic tree, was detected in a six month old child that was traced to Kano in Northern Nigeria. Kano is a major commercial center in Nigeria with direct human and material traffic alongside Lagos in the southern part of the country where this study was carried out. This indicates a likelihood of virus introduction between the two cities. Similar human infection and detection of pandemic A/H1N1 were generally reported in Nigeria within the study period (2009 to 2011), suggesting prior circulation of the virus in humans before probable transmission of the virus to pigs in Nigeria (Dalhatsu *et al*., 2012; Meseko et al., 2014). This virus or its progenitors may have circulated either subclinically or mildly in human population to have enabled interspecies transmission to pigs. Further interspecies transmission and reassortment may result in the emergence of a new influenza strain with pandemic potential.

Similarly, phylogenetic analysis of the NA gene and all the other internal genes of analysed virus showed that it also clustered with 2009 pandemic H1N1 influenza virus. However, there were 13 nucleotide mutations and three amino acid substitutions in the HA gene in comparison with prototype A/California/07/2009. Significantly, aspartate (Q) to arginine (R) substitution at the antigenic/receptor binding domain of HA in position 240 (H1- numbering) 226 (H3 numbering) were observed. The importance of this mutation in virus transmission is that binding of influenza viruses to their target cells is usually mediated by HA glycoprotein, which recognises cell surface glycoconjugates containing terminal sialic acid residues (Zhang *et al*., 2012). This virus receptor binding specificity

plays significant role in its transmissibility, and was reported with 1918 Spanish influenza (Glaser *et al*., 2009). The 240 (H1) position of HA is usually occupied by either amino acid glutamine (Q) in 95 % of cases including H1, H3, H5, H7, H9 or arginine (R) in only about 5% of cases (Mastrosovich *et al*., 2000; Gambaryan *et al*., 2006). Importantly, four positions (204, 239, 240 and 242) have previously been found to be involved in the specific binding capacity of HA to the host cell receptor (Nobusama *et al*., 1991; Gamblin *et al*., 2004). Most 2009 pandemic A/H1N1 influenza virus circulating globally including those detected in Africa have Q240 in this position (Ding *et al*., 2010; Zhang *et al*., 2012).

The key amino acid substitution at position 240 found in this study has been shown to be important for host binding preference as was observed in A/Swine/Nigeria/12VIR4047/2011. Virus strain with Q240 has been reported to show preferential binding to alpha-2,6 receptors -human (Mastrosovich *et al*., 2000). Hence, glutamine to arginine substitution at the receptor binding site of HA may have been positively selected for increased transmissibility of the virus as it switched host from human (preference for alpha 2,6-linked sialic acid) to swine (possessing both alpha 2,3 and 2,6 sialic acid). Similar observation was also reported in a study by Xu *et al*. (2010), where a single amino acid substitution in similar domain Gln226Arg in the HA of a H1N1 virus strain resulted in the loss of binding to alpha 2,6 Gal sialic acid and replication competence.

This observation may also explain why there was scarcely any human infection or virus recovery from pig handlers in the farm. The mutated virus may be less adaptive to 2,6 sialic acid in humans, thus reducing the chances of human re-infection in the pig farm. However, if the mutated virus shows preference for alpha 2,3 receptors also found in pigs, given the presence and interaction of avian species (chicken, ducks, cattle egrets and bats) with pigs in the farms investigated, the risk of transmission of pandemic A/H1N1 to other suceptible hosts is high. There are evidences that a single amino acid substitution of Aspartate, with Glycine at residue 222 of the HA protein favours this type of adaptation where the virus switches host from mammalian to avian species (Xu *et al.*, 2010). In retrospect, the 1918 H1N1 pandemic switched from avian to human receptor specificity

through mutations at two positions (Glu187Asp and Glyc222Asp). This affects receptor binding and reduces alpha 2,6 preference and increase alpha 2,3 binding. This phenomenon was also noticed with A/New York/1/18 strain of the 1918 pandemic that had Glycine mutation at position 222, which markedly affected receptor binding, reducing alpha 2,6 preference and increasing alpha 2,3 avidity (Stevens *et al.*, 2006; Xu *et al.*, 2010).

In a similar study by Ding *et al.* (2010), point to point analysis of mutation in the HA protein was performed on isolates of 2009 pandemic A/H1N1. Among the mutations observed, T220 and E/G239 were positively selected over the course of global spread of the novel virus. Glu 239 had been previously found to be associated with the acquisition of SA α 2,6 Gal binding specificity (Matrosovich *et al*, 2000; Baigent *et al*., 2003). Both 239 and 240 amino acid positions observed in these studies are located in the highly variable epitope regions of HA1 at the antigenic site, where they play significant roles in the receptor–binding process and should be given due attention in the development of vaccines and therapeutics.

In another observation, the H7N9 avian influenza from China showed a Q226L mutation (same 240 position as with A/swine/Nigeria) in the HA receptor binding domain. The virus was described as low pathogenic avian influenza (LPAI) in view of the polybasic amino acid sequences at the cleavage site of the HA gene, which ordinarily makes it less symptomatic in poultry (Gao *et al*., 2013). However, it caused severe illness in humans, as the mutation in the amino acid at the antigenic site of HA confers a better adaptation for the replication of the virus in mammals over other LPAI viruses. This is because the mutation is associated with reduced binding of avian-like receptors bearing sialic acid linked to galactose by alpha 2,3 linkages, located in the human lower respiratory tract. On the other hand, it enhanced the ability of the virus to bind to mammalian-like receptors bearing sialic acids linked to galactose by alpha-2,6 linkages located in the human upper respiratory airway (Uyeki and Cox, 2013).

Mutations in position 240 of the HA gene was also predictive in the reverse genetic animal experiment by Kawaoka and co-workers with H5N1 in ferrets, where Q226L (H3 numbering) in the HA gene was found to be associated with increased transmission and pathogenicity in animal models (Imai *et al*., 2012). It is instructive that a Q240R antigenic mutation has been observed with swine influenza H1N1 in this study, either by natural selection and transmission or stimulated by culture in embryonated chicken egg. The implication is that mutations in the HA gene may lead to enhanced transmission and pathogenicity of swine influenza virus in different species of animals in the study area.

Furthermore, molecular analysis of the NA gene also revealed amino acid substitutions compared with prototype A/California/07/2009 at position 106 (V:I), 248 (N:D), 351 (Y:F), 439 (S:G). S439G substitution in the amino acid is also unique to A/Swine/Nigeria/VIR44-09/2009. All other reference viruses have S in this position. This may affect virus release by neuraminidase on host cell surface either positively or negatively. Antivirals that target neuraminidase such as aciclovir, amantadine and neuraminidase inhibitors could also be affected by this substitution and may need further study.

The matrix gene is highly conserved among influenza A viruses. In this study, no amino acid substitution was observed except in S31N that was reported for all 2009 pandemic influenza viruses (Garten *et al*., 2009). This mutation in the M2 protein, which confers resistance against the adamantanes, was also detected in most influenza H3N2 isolates in the United States (Bright *et al*., 2006). While related influenza strain had amino acid D in position 527, in the PB2 gene, A/Swine/Nigeria was substituted with G. In PB1, G replaced K in position 736 while in the NS gene there were several amino acid substitutions between A/Swine/Nigeria and prototype A/California viz: G28V, W103C, Q109K, I111M, I123V, W187E, G189D, V234M, S246N. The PA gene also had a few substitutions I; S; V; and Q in position 14; 224, 505 and 716 respectively. All these amino acid substitutions may have pathological and epidemiological implications that need to be further investigated in experimental studies.

In this study, interspecies transmission and subsequent mutations of swine influenza virus can be ascribed to the high concentration of intensively reared pigs under close confinement. This practice is a major risk factor for the emergence and circulation of swine influenza strains at the human-animal interface. This is corroborated by observations in Mexico during the 2009 pandemic (Saenz, 2006; Scoones, 2010). The pig density in Oke-Aro, and the observation that the distance between the farm area and human habitation is less than 10 meters in a city of over 15 million people (NPC, 2007; 2013) is a cause for concern. This is because the scenario presents a very complex epi-zone that portends high risk for continuous transmission of infectious pathogen as described by Scoones (2010).

The emergence of pandemic influenza virus and its transmission from humans to pigs and from pigs to humans has been attributed to unhindered circulation at the human-animal interface (Myer *et al.,* 2007). This is usually aggravated by the intermingling of humans and animals in a confined environment as was observed in this study and is presented in many commercial farming operations (Secka *et al*, 2010). This is even more relevant in view of the important role that pigs play as mixing vessels in influenza epidemiology (Ma *et al.,* 2008). In addition, poor attention to biosecurity and a lack of movement restriction may cause the swine influenza virus to circulate nationwide resulting in public health threat (Grace *et al*., 2012).

Earlier observation of human pandemic influenza virus being transmitted to pigs was reported in Canada (Pereda *et al*., 2010). Similar reverse zoonotic transmission episodes occurring where pigs are intensively raised has also been reported (Myer *et al*, 2007). In Cameroon, influenza H1N1 was detected in a free range pig but there was no evidence of further circulation or transmission (Njabo *et al.,* 2011). In this study, clinical signs related to swine influenza were observed in several cases within an epidemiological zone with widespread infection among farms.

This study also revealed the epidemiological intricacies in the circulation and transmission of influenza virus at the human-animal interface; whereas infectious pathogen like swine influenza can readily cross interspecies barrier and are either trasmitted from human to

animals (reverse zoonosis), with potentials for re-trasmission from animals to human (zoonosis). Uninhibited natural circulation of this virus in pigs may also lead to consequences where the pandemic A/H1N1 may reassort with seasonal and classical virus in humans and pigs or viruses from other species that are present in the farm environment including domestic chickens, ducks and wild birds which possess alpha 2, 3 receptors, resulting in the emergence of novel subtypes or strains.

The phenomenon of zoonotic reassortment of swine influenza virus was recently reported in the United States where variant influenza A/H1N1, H3N2 and H1N2 was detected in severe and fatal cases in persons who had close contacts with pigs and exhibited respiratory illness, tested positive for influenza A/H3N2 variant that had the M-gene of 2009 pandemic influenza A/H1N1 (Bowman *et al*, 2012; CDC, 2012). More studies for better understanding of swine influenza evolution and its biological or ecological impact on different hosts will require experimental infection of pigs or ferrets with A/swine/Nigeria H1N1 virus by repeated passages and reverse genetics akin to the work by Kawaoka and Ron Fouchier research groups (Herfst *et al*., 2012; Imai *et al*., 2013). In such experiment, it may be possible to predict mutations that may occur in nature and be better prepared to control yet to emerge viruses. This is also important because, historically, the classical swine influenza circulating as distinct genetic lineage in Asia, America, and Europe originates from reassortment with 1918 Spanish influenza. The current swine pandemic influenza virus that has adapted to pigs in Nigeria and may be other parts of Africa may become established and may be the origin of novel viruses of significance in future.

Primarily, all influenza A viruses are of avian origin with adaptation to other hosts based on defined lineages as a result of host receptor susceptibility (Alexander, 2000) and other factors, some of those known and others still to be elucidated. After HPAI H5N1 and H1N1pdm2009 showed evidence of cross species transmission and susceptibility, it has become clearer that genetic changes that eventually increase human susceptibility to new strain of influenza takes place many years before its manifestation (van Reeth, 2007; Fraser *et al*., 2009). A holistic approach to influenza surveillance and research across

various health and agricultural sectors is therefore a necessity as was suggested by Capua and Cattoli (2010). Unfortunately, influenza surveillance activities and research in sub-Saharan Africa is low compared with other infectious diseases (Meseko *et al*., 2014). The implication is that the infection may continue to circulate unreported and unchecked.

More implication of uninhibited circulation of influenza in pigs in Nigeria is that the pandemic A/H1N1 may reassort with seasonal and classical virus or viruses from other species like birds, resulting in the emergence of novel subtype or strain. Several human infections with swine-lineage AH3N2v viruses containing the 2009 influenza AH1N1 pandemic matrix gene were detected in 2011 in the United State (Kitikoon *et al*., 2012). Many commercial pig operations in Nigeria with attendant poor biosecurity measures are potential hotspots for the emergence of similar novel influenza virus or other infectious pathogens as observed in the study by ILRI (2012).

In this study, electron microscope (EM) was used for visualization and identification of the pandemic influenza virus from pigs. Importantly, this study corroborates observations that direct microscopic examination of swab suspension can also be used for influenza diagnosis with the added advantage of rapid execution (Goldsmith and Miller, 2009). The significance of that is the timely recognition of infectious agents that enables effective mobilization of control measures to curtail infections. In preparing diagnostic antigens, initial serial passages and replication of the virus in embryonated egg was necessary to obtain high virus titre. Subsequent inactivation and purification of the virus for the production of diagnostic antigen was achieved with the use of beta-propiolactone. Though formalin inactivation could be used, excessive tissue toxicity was considered, particularly when the antigen produced would be used to stimulate hyperimmune serum in live animals (King, 1991).

Translational research and product development, which in this case is the production of diagnostic antigen, could also form the basis for the development of vaccine and other biologicals in an expanded programme for the control of influenza in both veterinary and human health sectors. This is the first isolate that is fully characterized by molecular sequencing in Nigeria, with evidence of temporal circulation and high potential for spread

to other agro-ecological regions. Subsequent serological and virological investigation may rely on the diagnostic reagent here produced because autologous antigens have been described to be more sensitive in serological procedures (CDC, 2011). It is also anticipated that the antigen produced in this study will be used to raise hyperimmune serum in experimental animals like rabbits and goats. This is in order to produce complementary antigen/antiserum from original virus for diagnostic purposes. Therefore, one of the objectives of this study and the goal of WHO Global influenza surveillance has been achieved (WHO, 2009). Subsequently, the virus can be used also as vaccine seeds, which are ultimately important for influenza virus control and global pandemic preparedness.

In summary, this study shows for the first time, the circulation of pandemic influenza A/H1N1 virus in Nigeria and some molecular characteristics including amino acid substitution; most importantly at the antigenic cleavage site of the haemagglutinin gene which suggest human to swine host switch. Continuous circulation of this virus in intensive and widespread pig husbandry operations and at the human-animal interface in the country may result in increased mutations and substitution or re-assortment with classical or seasonal influenza virus, leading to the emergence of novel, zoonotic influenza viruses with pandemic potential. Epidemiological information, alongside the antigen and genetic data obtained in this study are useful for influenza virus diagnosis, control and pandemic preparedness in Nigeria and globally.

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

This study shows that virological surveillance at the human-animal interface is an important tool for the detection of circulating infectious viral diseases that may have economic and public health implications. Influenza virus surveillance is generally poorly organized in sub-Saharan Africa. This deficiency and paucity of data is even worse with animal influenza surveillance. Yet, the importance of zoonotic influenza surveillance particularly at the human-animal interface for early detection, early warning and pandemic influenza preparedness cannot be overemphasized. These weaknesses in regional or national surveillance programmes are largely due to poor infrastructural and capacity development in both human and veterinary services in most underdeveloped countries of Africa. To remedy this deficiency in the face of glaring emerging and re-emerging infectious diseases of economic and public health importance, targeted sentinel virological surveillance can be implemented with small focus groups or individual researchers, concentrating on important diseases or population foci. This would help to monitor disease situations more closely thereby generating epidemic and virological data for planning such interventions as biosecurity, vaccination and chemotherapy.

The effectiveness of sentinel surveillance has been shown in this study. Hitherto, few surveillance programmes for influenza in pigs in the country was carried out. One was organized by the Federal Livestock Department, which was a limited, nationwide survey for influenza in pig as part of the Avian Influenza Control and Pandemic Preparedness (AICP) of the Federal Government of Nigeria in 2008, and was partly sponsored by Support Programme for Integrated National Action Plans (SPINAP) under the auspices of AU-IBAR. The results were negative for swine influenza virus. Similarly, the Field Epidemiology and Laboratory Training Programme (FELTP), a CDC supported surveillance in the same geographical area as was monitored in this study did not show sufficient evidence of the circulation of swine influenza virus. The weaknesses in previous

surveillance programmes include: overtly expanded, cross-sectional surveillance in the country without focusing on the population groups at risk. Though the second attempt focused on a small population at risk, but surveillance activity was limited in time and space to enable any meaningful discovery. These weaknesses were corrected in this study whereas as focused sentinel surveillance and disease monitoring was consistent for 24 months. This, in effect, was able to identify a pandemic influenza virus of global economic and public health importance.

Detection, isolation, molecular characterization and phylogeny of swine influenza virus at the human-animal interface in this study are of international significance. The genomic study on the isolates revealed that some of the viruses are closely related to isolates in North America and neighbouring West African countries, and was probably introduced into Nigeria at the later phase of WHO declared pandemic of H1N1 in 2009. The virus was apparently transmitted to pigs in Lagos metropolis through the teeming urban population surrounding the pig farms.

The human-animal interface hypothesis of influenza circulation is corroborated by this study. Also, pre-existing immunity due to seasonal H1N1 circulation in humans may make symptoms of swine influenza virus less perceptible in human subjects, whereas young and growing pigs without pre-immunity continuously serve as naive hosts that are susceptible and can sustain influenza circulation in animals. When this biological phenomenon of zoonoses and reverse zoonoses is allowed to persist for a sufficiently long period, it may support virus re-assortment and mutation with other avian and human viruses that are present in the ecosystem. This could eventually result in the re-emergence of another subtype or strain of influenza virus that may be responsible for future pandemics. The effect on the society, economy, animal and public health could be devastating. To prevent, control or mitigate these consequences, it is thus recommended that we:

1. Re-organize surveillance efforts in Nigeria along the line of sentinel studies for closer monitoring of special populations of animals and humans i.e. intensive livestock farms, especially pig farms; and in pig farmers, foci of live animal marketing/fairs and among marketers, in animal butchering facilities and among butchers along the economic value chain.

2. Re-organize intensive pig production facilities to improve biosecurity and hygiene, for better economic prosperity, food sufficiency, biosafety and public health

3. Intensify sentinel surveillance in various epizones for the detection, not necessarily of influenza A/H1N1, but other subtypes and strains of influenza or other viruses that may be circulating in animal reservoir hosts and at the human-animal interface.

4. Develop and adopt locally produced, autologous and more sensitive diagnostic antigens, as in this study, based on influenza A/H1N1 virus isolates for surveillance of Influenza H1N1 across the country and in the sub-region.

5. Introduce occupational health protection programmes for pig farmers, veterinarians and farm workers by selective vaccination against swine influenza and other communicable diseases so as to break the infectious bridge or cycle between livestock keepers and the larger society.

6. Adopt preliminary research for a broad protective influenza vaccine with similar genetic features to be used in pigs and their human handlers.

7. Strengthen joint human–animal-ecosystem interface collaborative research and information sharing towards achieving the One Health initiative of the OIE/FAO/WHO.

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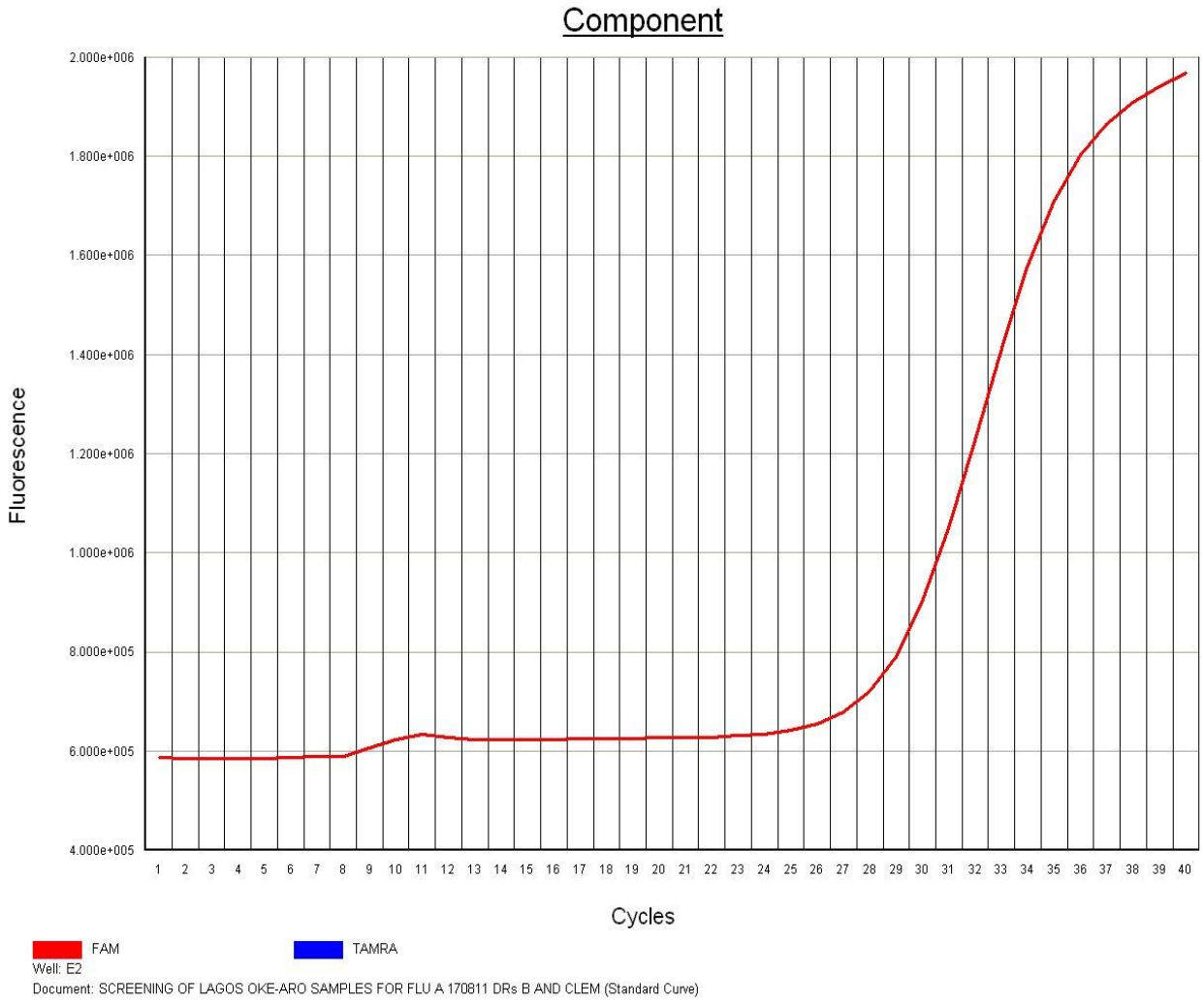
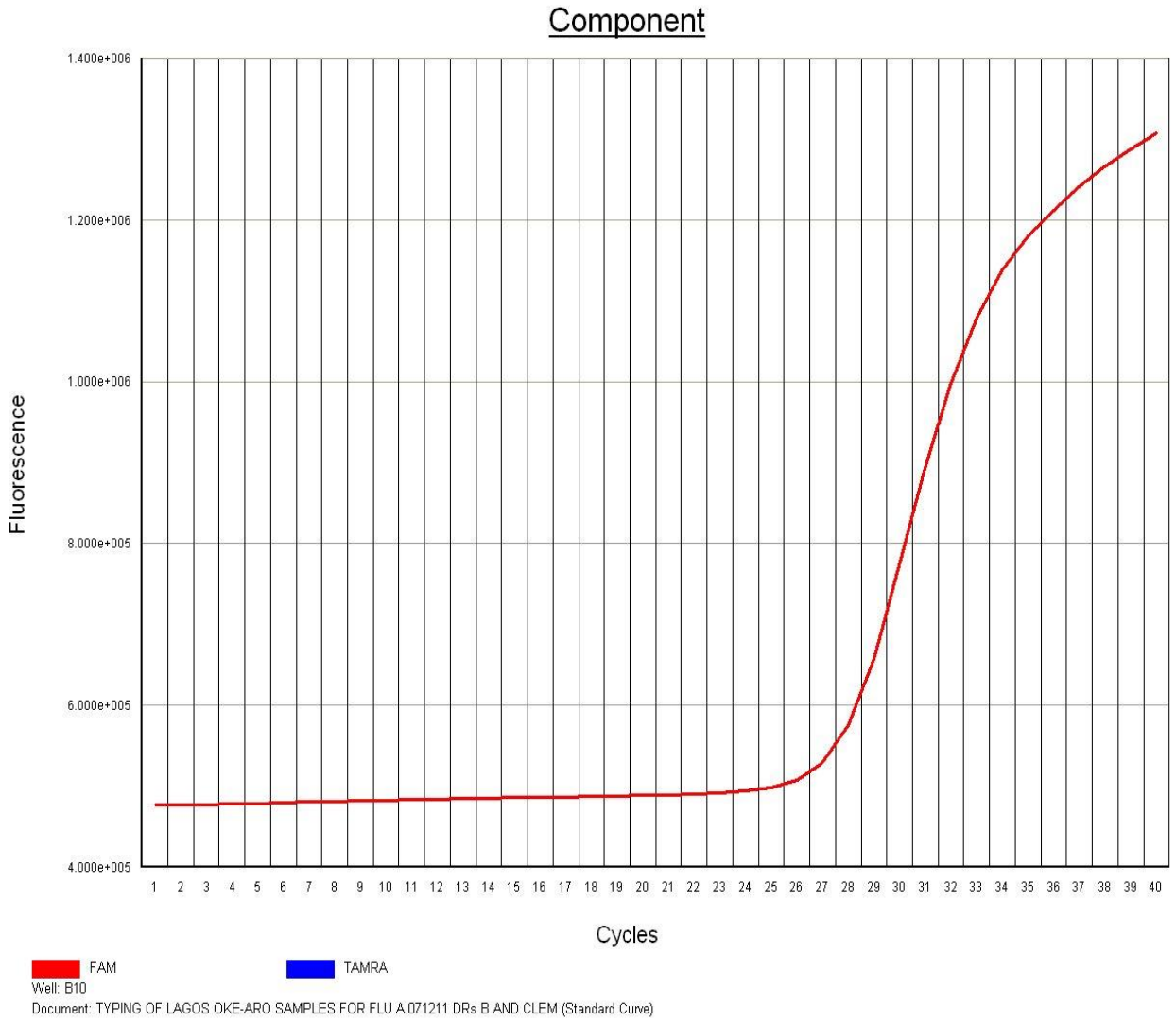
APPENDICES



Appendix I: Standard curve of sample screening for influenza A matrix gene by real time RT-PCR, fluorescence signal on the y-axis and cycles threshold on the x-axis.



Appendix II: Standard curve of sample subtyping for Influenza A subtype H1N1 by real time RT-PCR, fluorescence signal on the y-axis and cycles threshold on the x-axis

QuickTime™ and a
decompressor
are needed to see this picture.

Appendix III: Dead embryonated eggs with HA positive allantoic fluid of A/swine/Nigeria

(a) (b)

Appendix v: Rapid spot haemagglutination test for A/swine/Nigeria - Positive (a) and negative (b) plates

QuickTime™ and a
decompressor
are needed to see this picture.

Appendix v: Microtitre haemagglutination (HA) plate of swine influenza isolates as samples with positive, negative and RBC controls.

Appendix VI: Primers for sequencing reaction for swine influenza genome (PB2) segment of influenza A/H1N1

Influenza A/H1N1 gene Segment (PB2)		**Primers sequence bases***
Fragment 1	1- 538-	F: 5'- tgt aaa acg gcc agt agc aaa agc agg tca att- '3 R: 5'- cag gaa aca gct atg acc gct ttg rct aay atc rtc att- '3
Fragment 2	328- 816-	F: 5'- tgt aaa acg acg gcc agt gtr aca tgg tgg aay aga a-'3 F: 5'-cag gaa aca gct atg acc cca aar ctg aag gay gar ctg at -'3
Fragment 3	487- 1019	F: 5'- tgt aaa acg gcc agt cct ggt cay gca gat ctc ag-'3 F: 5'- cag gaa aca gct atg acc cct cta act gct ttt ayc cat - '3
Fragment 4	713- 1289	F: 5'- tgt aaa acg acg gcc agt ccr acw gaa gaa gct gt-'3 F: 5 – cag gaa aca gct atg acc cct cta act gct ttt ayc atg caa-'3
Fragment 5	946- 1509-	F: 5'- tgt aaa acg acg gtt agt ccr acw gaa gaa caa gct gt- '3 F: 5'- cag gaa aca gct atg acc gga gta ttc atc yat att cat- '3
Fragment 6	1169- 1740-	F: 5'- tgt aaa acg acg gtt agt aag caa cca gta gat tgt ttc a- '3 F: 5'- cag gaa aca gct atg acc ctg aga cca ytg aat ttt rat a-'3
Fragment 7	1447- 2186-	F:5'-tgt aaa acg acg gcc agt cca agy acc gag atg tca atg aga-'3 F:5'-cag gaa aca gct atg acc ttr ctc art tca ttg atg ct-'3
Fragment 8	1683- 2341-	F:5'-tgt aaa acg acg gcc agt caa tac cta yca rtg gat cat cag aa-'3 F:5'-cag gaa aca gct atg acc tag aaa caa ggt cgt t-'3

**Generic bases- y=c or t, r=a or g, w=a or t in the IUPAC nomenclature*

Appendix VII: Primers for sequencing reaction for swine influenza genome (PB1) segment of influenza A/H1N1

Influenza A/H1N1 gene segment (PB1)		**Primers sequence bases***
Fragment 1	22- 477-	F: 5'- tgt aaa acg acg gcc agt agt aaa agt agg tca att- '3 R: 5'- cag gaa aca gct atg acc ctr aaw act atr gtg tt- '3
Fragment 2	233- 843-	F: 5'- cag gaa aca gct atg acc gtt caa gct ttt crc awa tg-'3 F: 5'- cag gaa aca gct atg acc gaa akg gga grc tgg tgt tta -'3
Fragment 3	389- 1041-	F: 5'-tgt aaa acg acg gtt agt aca agr gtg gac aaa tra c -'3 F: 5'-cag gaa aca gct atg acc ctg aac cay tra ggy tga ttt - '3
Fragment 4	711- 1278-	F: 5'- tgt aaa acg gcc agt tga aca cca tga cca acg a-'3 F: 5 – cag gaa aca gct atg acc ttg aac atg ccc atc aty cca gg-'3
Fragment 5	974- 1566-	F: 5'-tgt aaa acy acg gcc agt aat caa aay cct mga atg tt - '3 F: 5'- cag gaa aca gct atg acc agc tcc atg ctr aaa ttr gc- '3
Fragment 6	1139- 1659-	F: 5'-tgt aaa acg acg gcc agt caa ata ccy gca gar atg ctagc - '3 F: 5'-cag gaa aca gct atg acc cca agr tca ctg ttt atc at –'3
Fragment 7	1489- 1954	F: 5'-tgt aaa acg acg gcc agt atg agy aaa aag aag tcy ta - '3 F: 5'- cag gaa aca gct atg acc tca aty tcy tta tgg gtg ac–'3
Fragment 8	1532 2321	F: 5'-tgt aaa acg acg gcc agt gcy aat tty agc atg gag ct - '3 F: 5'- cag gaa aca gct atg acc agt aga aac aag gca ttt–'3

**Generic bases- y=c or t, r=a or g, w=a or t in the IUPAC nomenclature*

Appendix VIII: Primers for sequencing reaction for swine influenza genome (PA) segment of influenza A/H1N1

Influenza A/H1N1 gene segment (PA)		**Primers sequence bases***
Fragment 1	0- 493-	F: 5'-tgt aaa acg acg gcc agt agc aaa agc agg tac tga t - '3 R: 5'-cag gaa aca gct atg acc tar tck gcc ttt gtg gcc att tt - '3
Fragment 2	235- 756-	F: 5'-tgt aaa acg acg gcc agt cca aat gca ctk tta aar cac aga tt R-'3 F: 5'-cag gaa aca gct atg acc tga gaa agc ctg ccc tca atg -'3
Fragment 3	361- 989-	F: 5'-tgt aaa acg acg gcc agt tat gay tac aar gag aa -'3 F: 5'- cag gaa aca gct atg acc ggt tct ttc cat cca aag aat gtt- '3
Fragment 4	702- 1292-	F: 5'- tgt aaa acg acg gcc agt tgc mtt gar aat ttt agr acc ta-'3 F: 5 –cag gaa aca gct atg acc tcr cak gcc ttg tgg aac tca tt -'3
Fragment 5	894- 1662-	F: 5'- tgt aaa acg acg gcc agt aaa ttr agc aat gar gay cca- '3 F: 5'-cag gaa ata gct atg acc tcm agt cty ggg tca gtg ag - '3
Fragment 6	1204- 2037-	F: 5'-tgt aaa acg acg gcc agt taa gcg att tra agt aat atg a - '3 F: 5'- cag gaa aca gct atg acc aay ccy tcy aat tgt ggm gat c–'3
Fragment 7	1444- 2057	F: 5'-tgt aaa acg acg gcc agt aat gca tcc tgt gca gca atg ga - '3 F: 5'-cag gaa aca gct atg acc ttg tcc cta aga gcc tga aca a–'3
Fragment 8	1787 2233	F: 5'- tgt aaa acg acg gcc agt atg aar tgg gga atg gag atg ag - '3 F: 5'-cag gaa aca gct atg acc agt aga aac aag gta cct ttt–'3

**Generic bases- y=c or t, r=a or g, w=a or t in the IUPAC nomenclature*

Appendix IX: Primers for sequencing reaction for swine influenza genome (NP) segment of influenza A/H1N1

Influenza A/H1N1 gene segment (NP)		**Primers sequence bases***
Fragment 1	1- 553-	F: 5'-tgt aaa acg acg gcc agt cag ggt aga taa tca ctc ac - '3 R: 5'-cag gaa aca gct atg acc aga gca cat yct ggg atc cat - '3
Fragment 2	296- 757-	F: 5'- tgt aaa acg acg gcc agt atg gtr ctc tct gct ttt gat ga -'3 F: 5'- cag gaa aca gct atg acc ttt gtc cag ctg ttt gaa att tyc ctt t-'3
Fragment 3	513- 1042	F: 5'-tgt aaa acg acg gcc agt tgg tat tch att ttr aat gat -'3 F: 5'-cag gaa aca gct atg acg ctg rct ctt gtg tgc dgg - '3
Fragment 4	619- 1177	F: 5'- tgt aaa acg acg gcc agt gct gca gtc aar gga rt-'3 F: 5 –cag gaa aca gct atg acc aag cra ttt gta cyc ctc tag t-'3
Fragment 5	925- 1565-	F: 5'- tgt aaa acg acg gcc agt cct gcy tgt gyg tam gga c- '3 F: 5'- cag gaa aca gct atg acc agg aga aac aag ggt att ttt c- '3

**Generic bases- y=c or t, r=a or g, w=a or t in the IUPAC nomenclature*

Appendix x: Primers for sequencing reaction for swine influenza genome (NA) segment of influenza A/H1N1

Influenza A/H1N1 gene segment (NA)		**Primers sequence bases***
Fragment 1	1- 600-	F: 5'-tgt aaa acg acg gcc agt agc aaa agc agg agt - '3 R: 5'- cag gaa aca gct atg acc ctg gat crg aaa ttc c- '3
Fragment 2	318- 740-	F: 5'- tgt aaa acg acg gcc agt tac aca aaa gac aay agc -'3 F: 5'- cag gaa aca gct atg aac ggr cca tcg gtc att atg -'3
Fragment 3	536- 1063-	F: 5'-tgt aaa acg acg gcc agt ggt cag caa gcg cat gyc atg a -'3 F: 5'-cag gaa aca gct atg acc cat aty tgt atg aaa acc - '3
Fragment 4	726- 1346-	F: 5'- tgt aaa acg acg gcc agt aat ggr car gcc tcr tac aa-'3 F: 5 –cag gaa aca gct atg acc gct gct ycc rct atg caa gat-'3
Fragment 5	941- 1452-	F: 5'- tgt aaa acg acg gcc agt tag gat aca tca gca gtg g- '3 F: 5'- cag gaa aca gct atg acc agt aga aac aag gag- '3

**Generic bases- y=c or t, r=a or g, w=a or t in the IUPAC nomenclature*

Appendix XI: Primers for sequencing reaction for swine influenza genome (M) segment of influenza A/H1N1

Influenza A/H1N1 gene segment (M)		**Primers sequence bases***
Fragment 1	0- 473-	F: 5'- tgt aaa acg acg gcc agt agc aaa agc agg tag - '3 R: 5'- cag gaa aca gct atg acc gca atc tgy tca cak gt- '3
Fragment 2	223- 750-	F: 5'- tgt aaa acg acg gcc agt cac cgt gcc cag tga gcg -'3 F: 5'- cag gaa aca gct atg acc tca ttt gaa ycg ctg cat-'3
Fragment 3	383- 1027-	F: 5'-tgt aaa acg acg gcc agt tcg gct ggm gca ctt gcc agt tg -'3 F: 5'-cag gaa aca gct atg acc agt agm aac aag gta gt - '3

**Generic bases- y=c or t, r=a or g, w=a or t in the IUPAC nomenclature*

Appendix XII: Primers for sequencing reaction for swine influenza genome (NS) segment of influenza A/H1N1

Influenza A/H1N1 gene segment (NS)		**Primers sequence bases***
Fragment 1	24- 482-	F: 5'-tgt aaa acg acg gcc agt agc aaa agc agg gtg aca aag aca - '3 R: 5'-cag gaa aca gct atg acc tcg gtg aaa gcc ctt a - '3
Fragment 2	250- 890-	F: 5'- tgt aaa acg acg gcc agt tga ggc ayt taa aat gat ca-'3 F: 5'- cag gaa aca gct atg acc agt aga aac aag ggt gtt ttt tat-'3
Fragment 3	418- 742-	F: 5'- tgt aaa acg acg gcc agt aaa gcc aay ttc agt tgg -'3 F: 5'- cag gaa aca gct atg acc ttc aat aag cca tct ta- '3

**Generic bases- y=c or t, r=a or g, w=a or t in the IUPAC nomenclature*

Appendix XIII: Full nucleotides sequences (1 to 1700) of HA segment of influenza A/H1N1 pdm, A/swine/Nigeria isolate

10 20 30 40 50 60 70 80
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
1
ATGAAGGCAATACTAGTAGTTCTGCTATATACATTTGCAACCACAAATGCAGACACATTATGTATAGG
TTATCATGCGAA
90 100 110 120 130 140 150 160
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
81
CAATTCAACAGACACTGTAGACACAGTACTAGAAAAGAATGTAACAGTAACACACTCTGTTAACCTTCT
AGAAGACAAGC
170 180 190 200 210 220 230 240
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
161
ATAACGGGAAACTATGCAAACTAAGAGGAGTAGCCCCATTGCATTTGGGTAAATGTAACATTGCTGGC
TGGATCCTGGGA
250 260 270 280 290 300 310 320
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
241
AATCCAGAGTGTGAATCACTCTCCACAGCAAGCTCATGGTCCTACATTGTGGAAACATCTAGTTCAGAC
AATGGAACGTG
330 340 350 360 370 380 390 400
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
321
TTACCCAGGAGATTTCATCGATTATGAGGAGCTAAGAGAGCAATTGAGCTCAGTGTCATCATTTGAAA
GGTTTGAGATAT
410 420 430 440 450 460 470 480
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
401
TCCCCAAGACAAGTTCATGGCCCAATCATGACTCGAACAAAGGTGTAACGGCAGCATGTCCTCATGCTG
GAGCAAAAAGC
490 500 510 520 530 540 550 560
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
481
TTCTACAAAAATTTAATATGGCTAGTTAAAAAAGGAAATTCATACCCAAAGCTCAGCAAATCTTATAT
TAATGATAAAGG
570 580 590 600 610 620 630 640
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
561
GAAAGAAGTCCTCGTGCTATGGGGCATTCACCATCCATCTACTAGTACTGACCAACAAAGTCTCTATCA
GAATGCAGATG
650 660 670 680 690 700 710 720
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
641

CATATGTTTTTGTGGGGACATCAAGATACAGCAAGAAGTTCAAGCCGGAAATAGCAATAAGACCCAAA
GTGAGGGATCGA
730 740 750 760 770 780 790 800
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
721
GAAGGGAGAATGAACTATTACTGGACACTAGTAGAACCGGGAGACAAAATAACATTCGAAGCAACTGG
AAATCTAGTGGT
810 820 830 840 850 860 870 880
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
801
ACCGAGATATGCATTCGCGATGGAAAGAAATGCTGGATCTGGTATTATCATTTCAGATACACCAGCCCA
CGATTGCAATA
890 900 910 920 930 940 950 960
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
881
CAACTTGTCAGACACCCAAAGGTGCTATAAACACCAGCCTCCCATTTCAGAATGTACATCCGATCACAA
TTGGAAAATGT
970 980 990 1000 1010 1020 1030 1040
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
961
CCAAAATATGTAAAAAGCACAAAATTGAGACTGGCCACAGGATTGAGGAATGTCCCGTCTATTCAATCT
AGAGGCCTATT
1050 1060 1070 1080 1090 1100 1110 1120
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
1041
TGGGGCCATTGCCGGTTTCATTGAAGGGGGGTGGACAGGGATGGTAGATGGATGGTACGGTTATCACC
ATCAAAATGAGC
1130 1140 1150 1160 1170 1180 1190 1200
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
1121
AGGGGTCAGGATATGCAGCCGACCTGAAGAGCACACAGAATGCCATTGACAAGATTACTAACAAAGTA
AATTCTGTTATT
1210 1220 1230 1240 1250 1260 1270 1280
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
1201
GAAAAGATGAATACACAGTTCACAGCAGTAGGTAAAGAGTTCAACCACCTGGAAAAAAGAATAGAGAA
TTTAAATAAAAA
1290 1300 1310 1320 1330 1340 1350 1360
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
1281
AGTTGATGATGGTTTCCTGGACATTTGGACTTACAATGCCGAACTGTTGGTTCTATTGGAAAATGAAA
GAACTTTGGACT
1370 1380 1390 1400 1410 1420 1430 1440
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
1361
ACCACGATTCAAATGTGAAGAACTTATATGAAAAGGTAAGAAGCCAGTTAAAAAACAATGCCAAGGAA
ATTGGAAACGGC
1450 1460 1470 1480 1490 1500 1510 1520

....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
1441
TGCTTTGAATTTTACCACAAATGCGATGACAAGTGCATGGAAAGTGTCAAAAATGGGACTTATGACTA
CCCAAAATACTC
1530 1540 1550 1560 1570 1580 1590 1600
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
1521
AGAGGAAGCAAAATTAAACAGAGAAGAAATAGATGGGGTAAAGCTGGAATCAACAAGGATTTACCAGA
TTTTGGCGATCT
1610 1620 1630 1640 1650 1660 1670 1680
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
1601
ATTCAACTGCCGCCAGTTCATTGGTACTGGTAGTCTCCCTGGGGGCAATCAGTTTCTGGATGTGCTCTA
ATGGGTCTCTA
1690 1700
....|....|....|....|.

Appendix XIV**:** Purified and freeze dried influenza A/H1N1pdm, A/swine/Nigeria isolate to be used as autologous diagnostic antigen