THE PREVALENCE AND IDENTIFICATION OF INFLUENZA VIRUS SUBTYPES IN OLIVE BABOONS FROM SELECTED AREAS IN KENYA

Emmanuel Kulwa Bunuma

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for the award of the degree of Master of Science in Molecular Biology and Biotechnology of the Pan African University.

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other University.

Signature…………… Date……………….

Emmanuel Kulwa Bunuma.

MB 300-0005/16

This thesis has been submitted for examination with our approval as University Supervisors:

Dr. Andrew Nyerere

Jomo Kenyatta University of Agriculture and Technology (JKUAT), Kenya.

Signature:…………………………… Date:………………………..

Dr. Lucy Ochola

Institute of Primate Research (IPR), Nairobi Kenya.

Signature:…………………………… Date:………………………..

DEDICATION

To God the giver of all insight, strength and from whom all blessings flow, and to my family the close and dearest.

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TABLE OF CONTENTS

DECLARATION ii

DEDICATION iii

ACKNOWLEDGEMENTS iv

LIST OF TABLES x

LIST OF FIGURES xi

LIST OF ABBREVIATIONS AND A CRONYMS xii

ABSTRACT xiii

CHAPTER ONE 1

INTRODUCTION 1

1.1Background 1

1.2 Statement of the Problem 6

1.3 Justification 6

1.4 The overall objective 8

1.4.1 The specific objectives 8

1.5 Null hypothesis (Ho) 8

1.6 Significance of the study to the public health 8

CHAPTER TWO 9

2.0 LITERATURE REVIEW 9

2.1. Classification of Influenza Virus 9

2.2.Subtyping of influenza A virus 9

2.2.1. Haemagglutination inhibition (HAI) test 9

2.2.2.Real time reverse transcriptase -PCR...10

2.3 Structure of the Influenza Virus. 10

2.4 Organization of the segmented genome of Influenza 11

2.5 Haemagglutinin 12

2.6 The Matrix-2 13

2.7 Nucleoprotein 14

2.8. Viral polymérase 15

2.9 Non structural protein (NS 1A)..15

2.10 Neuraminidase-segment 6...16

2.11 M1 ma t rix g en e 17

2.12 V irus replication...17

2.12.1Receptor binding cell entry and budding. 17

2.13 .Influenza in Non-human primates...20

2. 14 Epidemiology of Influenza virus 21

2.14.1 Globally...21

2.14.2.In Africa 22

2.14.3 In Kenya...23

2. 15 Laboratory diagnosis of Influenza 23

2. 15.1 Haemagglutination inhibition (HAI) test 24

2. 15.2 Fluorescent - antibody staining 24

2. 15.3 Immunoassays 25

2. 15.4 Molecular methods 25

2.16.Treatment of Influenza virus 26

CHAPTER THREE 27

3.0 METHOD OLO GY 27

3.1 Study site 27

3.2 Study design 28

3.3 Ethics statement. 28

3.4 Clinical assessment of animals 28

3.5 Laboratory methods 29

3.5.1 RNA extraction from the viral nasal swabs samples 29

3.5.2 Real time RT-PCR 29

3.5.3 Conventional RT-PCR for genomic amplification 30

3.5.4 Visualization of the amplicons by Agarose Gel electrophoresis 31

3.5.5 Clean-up of PCR products using Exosap-IT 31

3.5.6 Cycle sequencing of the purified PCR products 32

3.5.7 Purification of the cycle sequencing products using sephadex spin columns.........32

3.5.8 Genetic analyzer procedure 33

3.5.9 Contiguous assembly 33

3.5.10.Similarity searches 34

3.6 Biosafety measures taken 34

3.7 Limitations of the study 34

CHAPTER FOUR...**35**

4:0 RESULTS...**35**

4.1 Real time Reverse Transcriptase PCR 35

4.2 Amplification of the positive samples from real time rt PCR..................................36

4.3 Sequences similarity searching in the gene bank 38

4.4 Clinical assessment of influenza positive P. anubis. 38

CHAPTER FIVE...**40**

5.0 Discussion 40

5.1 Conclusion 42

5.2 Recommendations 42

5.3 Acknowledgements 42

REFERENCES..**43**

APPENDICES **58**

Appendix 1: Ethical approval for the study. 58

Appendix 2: Primers used in this study 60

Appendix 3: Preparation of reagents 61

1% Agarose 61

Appendix 4:Physical examination forms 62

Appendix 5: Parasitology information form 63

Appendix 6 :Acquisition of the animal forms for vital events......................................64

Appendices 7:The real time RT-PCR screening results for M gene in P. anubis nasal samples. 65

Appendices 8 : The Origin of different segments of Kenyan baboon influenza isolates. 68

Appendices 9 :Kenyan baboon isolates sequences 69

LIST OF TABLES

Table.2.1: RNA segments and the corresponding gene products of influenza A virus ..12

Table 2.2: Natural hosts of Influenza A virus. 21

Table 4.1: The original location of P. anubis and percentage of influenza positive 36

Table 4.2: Clinical symptoms of baboons found to be positive with influenza A but yet they didn’t present the clinical influenza fever symptoms 39

LIST OF FIGURES

Figure 1.1: Host range and transmission pathways of Influenza Aviruses ……..………5

Figure 2.1: Influenza virion structure..11

Figure 2.2: IAV reprelication cycle 20

Figure 3.1: Map of Kenya showing the sites where the animals originated from. 27

Figure4.1: Gel photo showing the PCR amplification of the three influenza gene (HA, NA, genes)...37

Figure4. 2: Gel photo showing the PCR amplification of the three influenza gene (NA, and M genes)...37

LIST OF ABBREVIATIONS AND A CRONYMS

AI	Avian influenza
CDC	Center for Disease Control
ELISA	Enzyme linked immune-absorbent Assay
FAO	Food and Agriculture Organization
FNF	Fibronectin Fragments
HA	Hemagglutinin
HAI	Haemaglutination Inhibition
HPAI	Highly pathogenic Avian Influenza
IAV	Influenza A vireuses
IL-1	Interleukin 1
NHP	Non-Human Primates
RdRP	RNA dependent RNA-polymerase (RdRP)
RNA-	Ribonucleic Acid
RT PCR	Reverse transcription Polymerase Chain Reaction
Trp	Tryptophan
VNP	Viral Nucleus Proteins

ABSTRACT

Worldwide, infections with influenza A viruses are associated with substantial illness and death among mammals and birds ,in humans it accounts for 250,000 -500,000 deaths per year its continuous mutation in different hosts poses a threat that can result in the emergence of a novel virus with an ability to cause a widespread pandemic. Surveillance of Influenza A viral genome from diverse hosts and subtyping is critical in understanding of the antigenic shift and drift of the influenza virus especially in hosts that are closely related to human beings like the Non-Human Primates (NHPs),pigs and birds. This study therefore identified the influenza subtypes circulating in *Papio anubis* (Olive baboons) at the interface of human and NHPs in Kenya. Fifty nasal swabs samples were collected from baboons from the colony at the Institute of Primate Research (IPR), these animals were originally collected from Olorbototo, Yatta, Aberdares, Movoloni and Laikipia. The nasal swabs were collected in viral transport media using sterile dacron swabs and stored at −80°C. In this study, samples were screened initially using real time RT-PCR- CDC protocol for influenza A virus detection that targets the matrix gene and twenty five were found to be positive. The proportion positive were as follows , Olorbototo (75%), Ngurumani (44%) Aberdares (43%), Mavoloni (37.5%), Yatta (14%), and Laikipia (9%) . These samples were taken through conventional PCR to amplify the haemagglutinin, neuraminidase and the matrix genes and eight samples were successfully amplified and later sequenced through 24-capillaries ABI 3500 XL Genetic Analyzer. Upon BLAST of these sequences, influenza subtypes H1N1 and H3N2 were detected. It was observed that the subtypes in baboons were as follows Olorbototo H1N1,Yatta H3N2, Aberdares H3N2, Mavoloni H1N1, Ngurumani H1N1 and Laikipia H1N1. Upon further analysis, the influenza positive Olive baboons were found to have been reared in the colony at at IPR colony for between 1-2 years and were in close contact with personnel. Given the presence of H1N1 and H3N2 subtypes in baboons suggests that baboons can be naturally infected with seasonal endemic human influenza viruses , avian emerging pandemic or pandemic swine flu origin .

CHAPTER ONE

INTRODUCTION

1.1Background

Globally, influenza A virus infection is accompanied with significant sickness and death amongst mammals and birds (Karlson *et al.,* 2012)where as in human it is responsible for up to 5 million severe cases and between 250 000 to 500 000 deaths worldwide(WHO, 2009).The health of humans and animals is largely interlocked with 6 out of 10 emerging diseases originating from animals.(Leslie *et al.,* 2016).Many dynamics in relation to animals, environments and humans lead to the emergence of zoonotic diseases. The environments linked with pathogens and their reservoir hosts are continuously mutable and the rate of change is growing. The drivers of change include the transformation of farming practices, predominantly in the unindustrialized world, habitat obliteration; human invasion and climate change (David *et al*., 2013).

In sub-Saharan Africa there is paucity of data available on influenza due to inadequate surveillance systems (Matheka *et al*., 2013). However, Kenya is one of the few developing countries with an elaborate epidemiological influenza surveillance network and despite this; influenza is still the major cause of hospitalizations and deaths every year (Matheka et *al.,* 2013). In some other parts of Africa like Madagascar, influenza has led to a case-fatality rate of 3% which is 0.1% below other influenza epidemics,(WHO.,2010). In the Democratic Republic of Congo in 2002, influenza out break led to a case fatality rate of 3.5% among children below 5 years of age (WHO,2002).

Influenza viruses belong to the family of *Orthomyxoviridae* and are classified into three types, A, B and C they cause respiratory diseases in humans, avian and animals throughout the world, (Margine *et al.*, 2013). Influenza B and C viruses are normally found in humans but Influenza C has also been isolated from pigs (Osterhaus *et al.*, 2000). Animals like swine can transmit influenza to humans, but human to human transmission can occurs due to lack of protective acquired immunity against the specific pandemic strains.The pathogenesis starts by the viral HA binding to sialic acid receptors on the target cell surface and followed by the fusion of the viral envelope with the host cell membrane of the respiratory tract (Griffin et al., 2001).

It is by fusion process that the viral RNA gains access to the host cell cytosol. The RNA then enters the nucleus of the cell, where it replication occurs. Viral proteins are synthesized in the cytosol on ribosomes of the host cell, ultimately resulting in production of many new virus particles. During the production of progeny virus, the host cells own protein synthesis is effectively shut down. Once new viral genomes and proteins have been synthesized, they are assembled into new virions, this then "buds" through the membrane and are released into circulation. (Chieng *et al.,* 2004).

These cellular "factories" for virus production ultimately lead to of the epithelial cell and desquamation of the respiratory lining, which subsequently leads to secondary infections and pneumonia. At the same time, sensors of viral replication stimulate the production of pro inflammatory cytokines, including IL-1, IL-6 and TNF-α, which circulate in the body and which are responsible for the systemic symptoms associated with influenza illness, such as fever, muscle aches and malaise (Chien *et al.,* 2004).

There is always a public health concern of a novel influenza virus infection ,of animal origin (WHO, 2015). Given all the major pandemics that have occurred in the last 100 years which are 1918- (H1N1), 1957–1968 (H2N2), 1968- (H3N2) and 2009-(H1N1), were found to be involving influenza virus containing avian or porcine HA or neuraminidase genes (Taubenberger *et al*., 2010).

Circulating influenza virus pose a threat to human health because humans are susceptible to these viruses, such as avian influenza virus subtypes H5N1 and H9N2 and swine influenza virus subtypes H1N1 and H3N2 when in contact with these animals (WHO, 2010). All human infections with animal influenza viruses are of concern, not only because of the cases of disease and deaths in individual, but also because if these viruses become adaptive and able to spread from human and they could spark a pandemic. All of the past four pandemic influenza viruses have contained gene components originating from animals (WHO, 2010).

Circulating human influenza virus undergoes rapid mutation because RNA dependent RNA polymerase has a low fidelity and lacks3′-5′-exonuclease activity (Zhu *et al*.,2015). Antigenic drift occurs when the genes encoding the HA and or NA glycoproteins undergo stepwise mutations, resulting in variant viruses with amino acid changes at one or more antibody-binding sites of HA and/or NA (Lindstrom *et al.,* 2004 ; Holmes *et al.,* 2005 ; Schweiger *et al*., 2006)).. The changing antigenic composition of influenza viruses is one of their most important characteristics and plays a big role in determining their pathogenic potential and epidemiological behavior and prediction of future epidemics. (Wolfe *et al*..2007*).* The influenza virus A has its reservoir among

migratory wild waterfowl as low pathogenic strains but they have been isolated in other avian species like chickens while in mammalian species; there is a degree of host specificity to the virus subtypes that occur in natural infection. there is no doubt that there will be other pandemics in the future due to different infected hosts interactions and pathogen mutation and adaptability figure 1.1 (Webster *et al.*, 2004). The prevalence of the pathogen in various reservoirs can determine the dynamism of the epidemics at the animal-human interface which can cause spillover of infection in susceptible species that interact with the reservoir (Lloyd-Smith *et al.*, 2009), recently(Karlsson ,2012) observed that non- Human Primates (NHPs) that are in contact with humans can suffer from influenza virus by natural transmission. There is a close relationship between humans and NHPs in different parts of the world where avian and human influenza viruses co-circulate, further surveillance of these populations is warranted (Karlsson *et al.,* 2012). It is critical to assess and understand the influences of these changes on the interfaces between Influenza type A and their hosts and amongst the host and other species, plus other wildlife, livestock and humans.(Bryony *et al* .,2013). Nonhuman primates, the closest living relatives of humans, are susceptible to other respiratory virus like *paramyxoviruses* that cause respiratory disease in humans (Fuller *et al.,* 2013), these considerations prompted additional searches for species harboring novel influenza in Baboons. Mammals like Baboons are likely conduits for cross-species transmission of respiratory pathogens like influenza viruses because of their close and long term contact with their owners, scientists, audiences, domestic animals, wild animals, and birds.

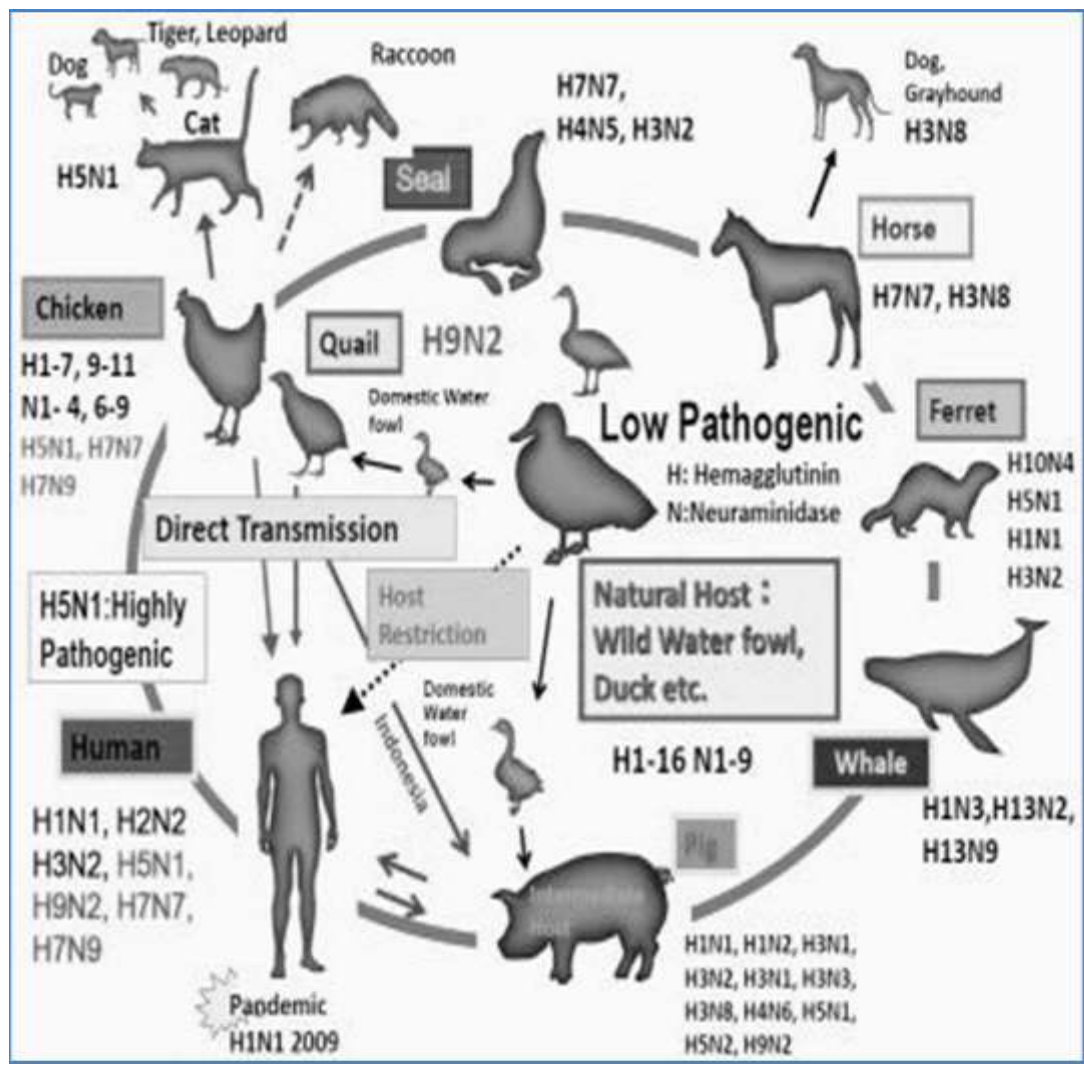

Illustration adapted from, (Suzuki, 2013)

Figure 1.1:Host range and transmission pathways of Influenza A viruses in different infected hosts, interactions that leads to pathogen adaptability and adaptability.

1.2 Statement of the Problem

Globally, infections with influenza A viruses are associated with substantial illness and death among mammals and birds(Karlson *et al.,*2012) in human it accounts for 500,000 deaths annually (Taubenberger *et al*.,2009).The presence of Influenza A Virus in diverse mammalian host species, including humans, domestic pigs, horses, dogs, non-human primates(NPHs) seals, cetaceans (toothed whales), mink and anteaters, bats among others , determine the dynamism of influenza epidemics of animal origin which can cause spillover of infection in susceptible species that interact with the reservoir ,the classical example being the outbreak of pig adapted H1N1pdm09 infecting human (Lloyd-Smith *et al*.,2009:Barrel et al., 2010). Hence newly emerging or `re-emerging' influenza virus from animal reservoirs such as avian, bats and NHPs continue to pose a significant global public health threat.

Nonhuman primates are widely distributed in Africa(Zinner *et al.,*2009),since they are susceptible to influenza virus infections(Karlson *et al.,*2012),it's important to have the subtypes identified in Kenyan baboons ,hence the aim of this study.

1.3 Justification

Influenza A viruses are zoonotic pathogens that continuously circulate and mutate in several animal hosts, including birds, pigs, horses and humans. The emergence of novel virus strains that are capable of causing human epidemics or pandemics is a possibility as observed in the swine flu pandemic of 2009.This was caused by flu virus that emerged from pigs likewise the Non Human Primates are susceptible

and therefore can naturally be infected with human and avian influenza viruses other respiratory viruses like human metapneumo virus that cause respiratory disease in human have aslo been found in Non human primates (Fuller *et al.,* 2013) these considerations prompted additional searches for other respiratory viruses in non human primates .

Information relating to Non Human Primates circulating influenza strains is unavailable in Africa. The evolution of the influenza virus is driven by adaptation mechanisms to its host immnunological pressure which can lead to antigenic shift and drift of the virus which can result to a novel influenza strain which the human race has no immunity over it .

Olive baboons are found across almost every habitat in Africa, they cohabitate with other mammals and avian species and they can naturally be infected with IAV (Karlson*.,* 2012, Sasaki *.,* 2013).

This work l therefore seeks to study and identify influenza viruses circulating in Olive baboons with the aim of providing answers to the risk of emergence of new strains that have the potential to cause a wide spread epidemic and which the humans race has no immunity over it. Its critical to understand the prevalence and the genomic subtypes of this virus in diverse hosts which is very important in drug and new generation vaccine development (ElHefnawi *et al.,* 2011), this would contribute to reducing the economy burden of the disease in terms of sicknesses and death.

1.4 The overall objective

To establish the presence and identify influenza virus subtypes in Olive baboons in selected areas in Kenya.

1.4.1 The specific objectives

1. To determine the presence of influenza A virus in nasal swab samples from baboons obtained from selected areas in Kenya.
2. To determine the influenza virus subtypes in positive nasal swabs from baboons obtained from selected areas in Kenya

1.5 Null hypothesis (Ho)

There is no Influenza A virus in Olive baboons in Kenya.

1.6 Significance of the study to the public health

This study will identify the IAV strains currently circulating in baboons in selected places in Kenya. Identifying circulating influenza virus strains in baboons will help determine new introductions of virus or new re-assortments occurring in animal hosts which could be of public health concern.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1. Classification of Influenza Virus .

Influenza viruses belongs to the family *Orthomyxoviridae*, they are enveloped and contain negative sense RNA 7-8 segments the five genera in the family include Influenza virus A, Influenza virus B, Influenza virus C, *Thogotovirus* and I*savirus* (Infectious Salmon Anemia virus) (Wright *et al.,* 2007). Influenza A viruses consists of 8 gene segments that encode 10 polypeptides, segment 4 encodes HA that has 16 known subtypes, segment 5 the nucleoprotein (NP), segment 6 encodes neuraminidase (NA) that has 9 known subtypes, segment 7 the M1, M2 matrix proteins (Griffin *et al.,*2001). The subtypes can be differentiated by serology and , there is no cross reactivity.

2.2.Subtyping of influenza A virus

2.2.1. Haemagglutination inhibition (HAI) test

When red blood cells are mixed with isolated influenza virus in the appropriate ratio, the virus bridges the red blood cells type O human blood,guinea pig or chicken and turkey blood resulting in agglutination of the cells (haemagglutination) and a change in their settling behaviour.Antibodies specific to the viral haemagglutinin interfere with the haemagglutinating activity and this is the basis of the HAI test which allows the identification by subtypes and the differentiation of the variant strains that frequently appear (Young, 2009). Some other rapid ELISA-based ‘flu/no flu’ assays have been developed and are commercially available. although fast and easily used, these assays

often exhibit specificity and sensitivity shortcomings and do not subtype influenza virus type A, forexample H1, H3, and H5. (Woolcock *et al.,*2005).

2.2.2.Real time reverse transcriptase -PCR

Traditional RT-PCR has become an effective methodology for viral detection, typing, and subtyping (Cattoli *et al.,*2004).Although RT-PCR exhibits high sensitivity and considerably shorter detection time than conventional virological methods, drawbacks, such as extraction complexity, post-PCR analysis, and increased potential for cross-contamination have been noted.This process requires the specicific primers for H1-H16 where by the real time amplification of the targeted HA genes will be noted and hence subtyping.(Daum *et al.,*2002: Poddar *et al.,*2002)

2.3 Structure of the Influenza Virus.

Influenza A virus is highly pleomorphic virus with particles exhibiting either spherical or filamentous morphology and their size ranges from 80 to 120 nm in diameter (Griffin *et al*.,2001). A characteristic feature of influenza virus particles is their external layer which is a lipid envelope with approximately 500 spike-like projections. These spikes represent the envelope glycoproteins HA (which are rod-like in shape) and NA (which are mushroom-shaped) The HA spike is a trimer consisting of three individual HA monomers, while the NA spike is a tetramer. (Suares ,2008) HA is about four times more abundant than NA. The total size of influenza genome is 13.5kbp.The typical structure is shown down on figure 2.1.

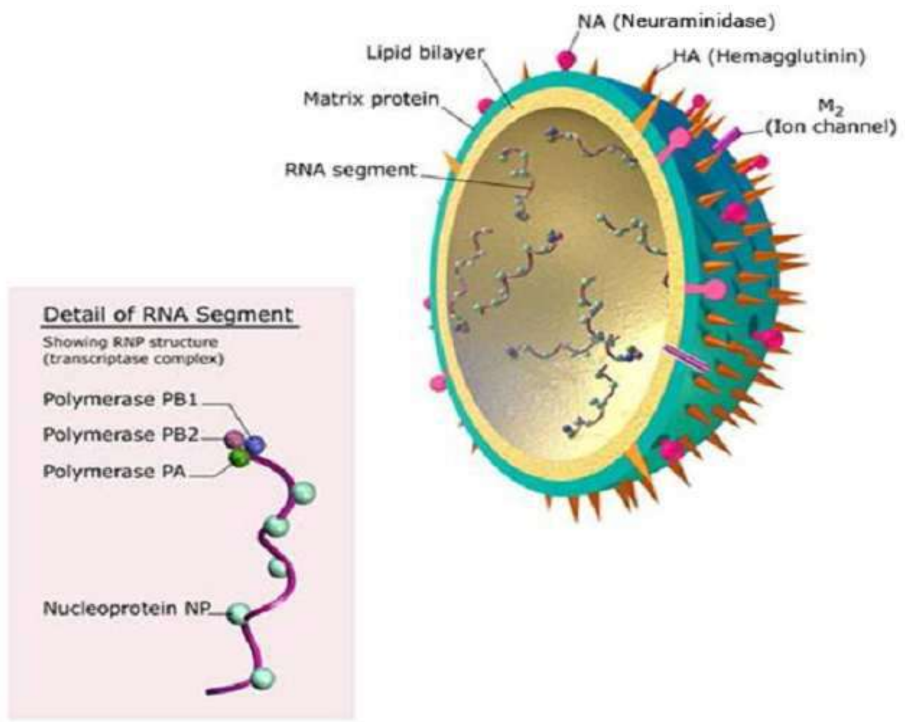

Figure 2.1:Influenza virion structure

Source: http://agrolink.moa.my/jph/epv/kertaskerja01.html

2.4 Organization of the segmented genome of Influenza

The influenza A virus genome encompasses eight negative sense RNA segments (Tong *et al.,* 2012) which are presented in table 1. Segments 1 to 6 are in monocistronic arrangement whereby segment 7 and 8 are intertwined to form mRNAs coding for two proteins (Lamb and Krug, 2001). The sizes of the viral RNA segments and the proteins encoded are summarized in Table 2.1, whereby of these NS1 protein from segment 8 (NS segment) is a non-structural protein.

Table.2.1: RNA segments and the corresponding gene products of influenza A virus

Influenza A virus RNA segments and the proteins they encode		
RNA segment (no. of nucleotides)	**Gene product(no. of amino acids)**	**Molecules per virion**
1 (2341)	Polymerase PB2 (759)	30–60
2 (2341)	Polymerase PB1 (757)	30–60
3 (2233)	Polymerase PA (716)	30–60
4 (1778)	Haemagglutinin (566)	500
5 (1565)	Nucleoprotein (498)	1000
6 (1413)	Neuraminidase (454)	100
7 (1027)	Matrix protein M1 (252)	3000
	Matrix protein M2 (97)	20–60
8 (890)	Non-structural proteins	-
	NS1 (230)	-
	NS2 (121)	130–200

Adapted from(Lamb RA and Krug RM,2001).

2.5 Haemagglutinin

Haemagglutinin (HA) is a trans-membrane glycoprotein (size 13.5nm, molecular weight 76kDA) and it attaches the virus to sialic acid receptors on the cell membrane. Once the virus is attached, it can easily release viral ribonucleoprotein particles (vRNPs) into the cytoplasm (Kalyad Das *et al.,* 2010).Two HA molecules

exist as a trimer the head of the HA1 chain, binds sialic acid receptors, and it has the important antigenic targets against which human antibodies are directed (Kalyad Das *et al.,*2010). The HA1 head region is closely associated with the central coiled-coil trimeric α-helices. During fusion the central coiled-coil is at a low pH and all three HA1 heads swing away from HA2 (Stevens *et al .,*2006).The refolding of the loops connecting the coiled-coil stem to the fusion peptides form the helical structures that extend and expose the fusion peptides at the N-terminal end of HA2 (NHA2), which then finally contacts the endosome membrane to infuse into the cytosol (Skehel *et al,* 2000).

The head of HA undergoes frequent antigenic changes and despite the vast majority of human antibodies directed against several regions of the head of the HA1 only two human monoclonal antibodies have been shown to neutralize diverse influenza A subtypes (Sui, *et al*, 2009).The HA has a conserved epitope near the base to which the antibody binds as indicated on (Figure2b). The low-pH conformational change of HA can be inhibited by binding to this potential target (TBHQ binding pocket). (Kalyan Das *et al.*2010).

2.6 The Matrix-2

This is the target for drugs like amantadine and remantadine. The M2 which consists of 97 amino acids residue has a trans membrane domain and a C-terminal cytoplasmic amphiphilic helix. The proton channel has a tetrameric structure of TM helices (Stouffer, 2008, Zaitseva *et al.,*2002). The M1 matrix attaches the vRNPs to the lipid bilayer membrane and the M2 releases the vRNPs by influx of protons from

endosome to the virus. On the M2 there are highly conserved residues His37 and Trp41 which are located in the proton channel and are very important in the proton transport phenomenon as shown in the part c of the fig 3 where by His at position 37 is protonated with hydrogen atoms and therefore makes the pH low this phenomenon enhances the proton flow and so the release of the vRNPs(Lamb *et al.,*2001).

2.7 Nucleoprotein

This is the viral single-stranded RNAs enclosed by Nucleoprotein molecules where by multiple copies of nucleoprotein molecules , ssRNA genome segment and a polymerase complex form a (P complex) and they are all packaged into each viral nucleoprotein complexes(vRNP) which is incorporated into the virus the nucleoprotein molecules assist in the nuclear import and export of vRNPs and viral replication, and they also interact with host proteins.(Kyalan Das *et al*,. 2010).A nucleoprotein molecule folds into a curved shape with a head and a body domain and so the ssRNA forms a binding groove that is located at the exterior surface between the two domains of the nucleoprotein, and has a tail loop that is inserted into the adjacent nucleoprotein molecule, this makes the oligomerization of the nucleoprotein phenomenon required for vRNP formation (Kyalan *et al*.,2010). So the Nucleoprotein is required for viral RNA replication and recent studies have found that the interaction of nucleoprotein with the viral polymerase initiates the switch from capped–primed viral mRNA synthesis to unprimed viral RNA replication(Ng *et al*.,2008).

2.8. Viral polymérase

Viral polymerase has a mass of ~250 kDa which forms a heterotrimer subunits namely P complex (PA, PB1 and PB2). The PA subunit performs the endonuclease and protease activities and it is also involved in viral RNA to complementary RNA (cRNA) promoter binding region and it interacts with the PB1 subunit. (Maier *et al.,*2008).

The PB1 subunit has the RNA dependent RNA-polymerase (RdRP) active receptor which interacts with both PA and PB2 at distinct position .Studies have indicated that mutations at this interface inhibit RNA synthesis, which proves that that if we have compounds that can dissociate the PB1–PB2 complex would be potential influenza A inhibitors and hence a drug target .(Kyalan Das *et al*.,2010).

2.9 Non structural protein (NS 1A)

The multifunctional NS1A protein has two proteins domains namely NS1AN and NS1AC. NS1AN has (residues 1–70) amino acids and it exists in a homodimeric state that binds double-stranded RNA (dsRNA) in a non–sequence specific manner while the NS1AC has (residues 86–230/237) amino acids ,also known as the effector domain, binds to numbers of cellular proteins like protein kinase R (PKR) and therefore helps inhibiting a PKR activation and also it binds the p85β subunit which can inhibit phosphatidylinositol-3 kinase (Min *et al*.,2007). The polyadenylation specificity factor (CPSF30) can bind the NSIA to inhibit 3′-end processing of cellular during the pre-mRNAs event , including IFN-β pre-mRNA. Therefore, based on this, influenza A

virus replication can be prevented by blocking the NS1AC-CPSF30 binding (Kyalan Das et al.,2010).

The basic function of the NS1AN proteins domain is to block the interferon (IFN)-α/β–induced 2′-5′-oligo A synthetase / RNase L pathway interaction by sequestering dsRNA away from 2′-5′- oligo synthetase enzymes (Min, *et a.l*, 2006). This indicate that drugs that interfere with NS1AN-RNA binding also can inhibit the virus replication.

The NS1AN dimer has a small cavity at the center of its two-fold axis, and the cavity opens toward the RNA-binding surface. Design of compound structure that can bind the cavity and interfere with the NS1AN-dsRNA binding may generate effective inhibitors of influenza A and it can be potential drug target for IAV.(Kyalan Das *et al.*, 2010).

2.10 Neuraminidase-segment 6

The neuraminidase (NA) is the glycoprotein on the surface of influenza viruses. It is the second most abundant glycoprotein on the surface of the virus , there are 9 different antigenic subtypes of influenza due to antigenic drift. Immunity due to NA protects against influenza virus infection because anti-NA antibodies works by preventing virus release from infected cells. (WHO.,2002). NA is a target of different drugs like oseltamivir and zanamivir. (Wang. *et al*. 2009The sialidase activity of NA is required to prevent the newly produced virion from attachment to the producer cell via HA interacting with surface sialic acid residues (Lamb R.A. & Krug, R.M. 2001).

2.11 M1 matrix gene

The membrane contains the viral ion channel M2 (Lamb *et al*., 1985) and is coated with a helical array of M1 proteins on its internal surface the M segment encodes for M1 and M2 ion channel.

,proteins, appears to contain genetic determinants of filamentous morphology (Calder *et al*..2010: Rossman *et al*.,2010). The M1 proteins works at the low pH in the endosomes, this triggers the flow of protons into the virus via the M2 ion channel, therefore disintegrates the vRNPs from M1 matrix proteins and so the release of the viral contents to the host cell (Kyalan *et al.,*2010).

2.12 V irus replication

2.12.1Receptor binding cell entry and budding.

The epithelial cells in the upper and lower respiratory tract are the primary targets for influenza virus. Humans and Non-human primates have same kind of sialic receptors which are α2,6 glycosidic linkages (Margine *et al*.,2014).The viral HA binds to sialic acid residues on glycoproteins or glycolipids on the cell surface (Blackwell Science, 2001). The fine specificity of HA's receptor binding depends on the nature of the glycosidic linkage between the terminal sialic acid and the penultimate galactose residue on the receptor sites (Watanabe , *et al*.,2010). In mammals,, influenza viruses preferentially bind to sialic acids attached to galactose in an α2,6 configuration, and in avian viruses have a preference for sialic acids attached to galactose in an α2,3 linkage (Margine *et al*.,2014). Pigs, on the other hand, have receptors with either type of linkage between sialic acid and galactose, and thus are readily susceptible to infection

with both human and avian viruses (Margine et al,.2014). When the glycoprotein HA molecules binds to the host cell sialic acid receptors molecules , and the virus crosses the membrane by endocytic vesicle. Then the endosomal low pH triggers a change in the form of the HA protein molecule which leads to fusion of the viral and endosomal membranes. The low pH also triggers the flow of protons flow into the virus through the M2 ion channel, therefore disintegrating the vRNPs from M1 matrix proteins attachment. (Kalyan et al,2010).The vRNPs that are released into the cytoplasm are taken into the nucleus by targeting of the nuclear localization sequences (NLSs) on nucleoproteins (Wu& Pante 2009) only when the M1 molecules are dissociated.In the nucleus, the viral polymerase starts the viral mRNA synthesis with 5′-capped RNA fragments cleaved from host pre-mRNAs. Then the PB2 subunit binds the 5′ cap of host pre-mRNAs(Schrenzel,*et al.,*2011) and the endonuclease domain in PA subunit cleaves the pre-mRNA 10–13 nucleotides downstream from the cap Viral mRNA transcription is then initiated from the cleaved 3′ end of the capped RNA segment Schweiger (*et al*.,2006).

This 'cap snatching' occurs on nascent pre-mRNAs. Viral mRNAs are taken to the cytosol of the host cell for translation into viral proteins. The surface proteins HA, M2 and NA are post- translational processed in the endoplasmic reticulum (ER), glycosylated in the Golgi apparatus of the host cell and then transported to the cell membrane. The NS1 protein of influenza A virus serves.

a critical role in lowering the production of host mRNAs segments by preventing the 3′-end processing of host pre-mRNAs(Venter et al.,2012)).The viral RNA polymerase

is responsible for the capped RNA-primed and unprimed replication of vRNAs (Kalyan *et al*.,2010). The nucleoprotein molecules are required for two steps of replication and are deposited on the cRNA and vRNA during RNA synthesis process (Newcomb *et al.,*2009).

The resulting vRNPs are then taken to the cytoplasm, a role mediated by a M1–NS2 complex that is bound to the vRNPs; NS2 interacts with human CRM1 protein that exports the vRNPs from the nucleus(Neumann *et al*.,2000) The vRNPs reach the cell membrane to be incorporated into new viruses (Nayak *et al*., 2004) that are budded out. The HA and NA proteins molecules in new viruses contain terminal sialic acids that causes the viruses to bind together and adsorb to the cell surface(Kalyan Das *et al*.,2010). The NA of newly formed viruses cleaves these sialic acid residues, thereby budding off and attach to new cells.

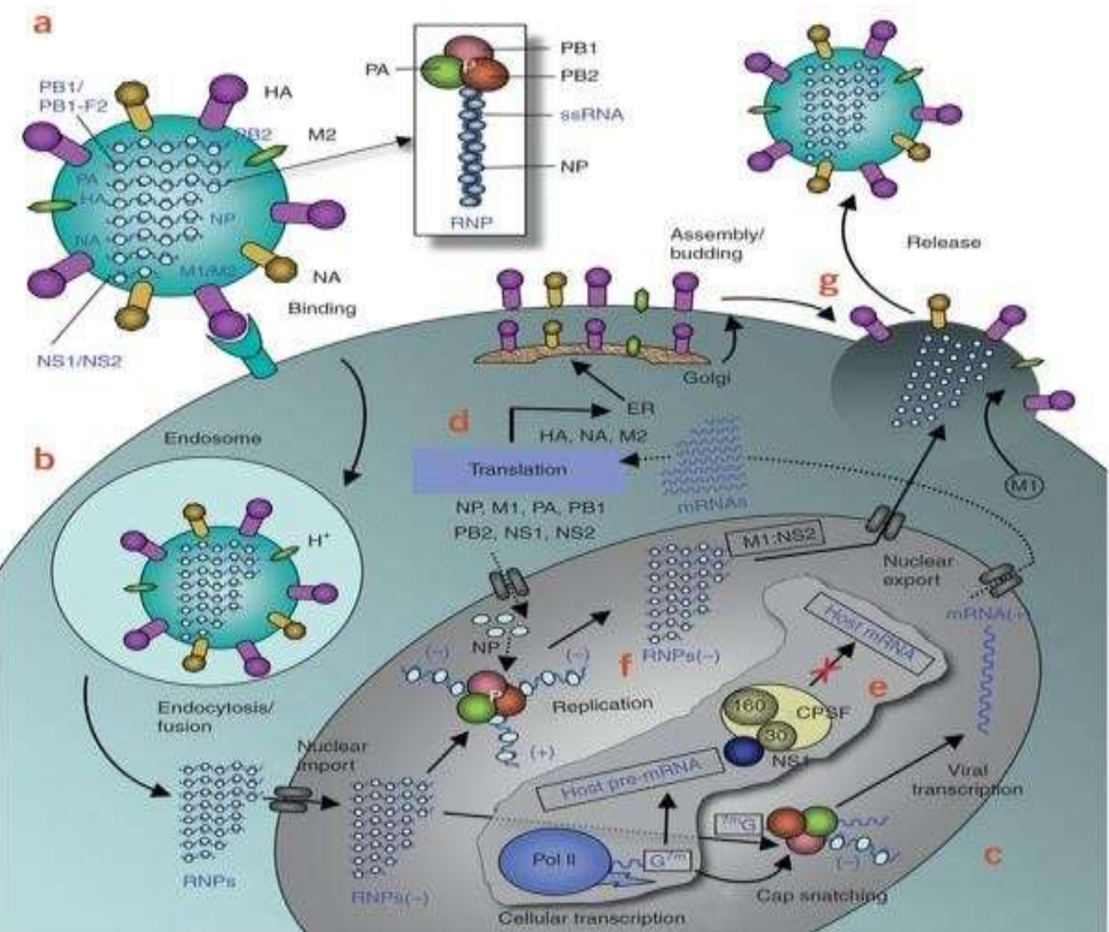

Figure 2.2. IAV reprelication cycle . (Kalyan Das *et al.,* 2010)

2.13 .Influenza in Non-human primates

A study done in East Asian showed that NHPs can be natural infected by humans that are under frequent contacts (Karlson *et al.,* 2012). Baboons and vervet monkeys live side by side with humans in game management areas in Zambia, and this situation often leads to high levels of human–baboon/monkey conflicts and there has been diseases like Human Parainfluenza 3 in baboons at the interface of humans and baboons .Monkeys trapped from the wild are used as pets or caged animals or sold in markets which can lead to outbreak of zoonotic diseases (Webster RG *et al.,* 2004).

Table 1.2 Natural hosts of Influenza A virus.

Natural hosts of Influenza A virus			
Haemagglutinin (H)		**Neuraminidase (N)**	
Subtype	**Predominant Host**	**Subtype**	**Predominant Host**
H1	Human, pig, birds	N1	Human, pig, birds
H2	Human, pig, birds	N2	Human, pig, birds
H3	Birds, human, pig,	N3	Birds
H4	Birds	N4	Birds
H5	Birds, (human)	N5	Birds
H6	Birds	N6	Birds
H7	Birds, horse,	N7	Horse, birds
H8	Birds	N8	Horse, birds
H9	Birds, (human)	N9	Birds
H10-H16	Birds		

Table adapted from (Lamb and Krug, 2001)

2. 14 Epidemiology of Influenza virus

2.14.1 Globally

Influenza occurs throughout the world ,surveillance indicates that in temperate regions, influenza activity peaks during winter months, in the northern hemisphere between November to March and in Southern hemisphere it occurs throughout the year ..Influenza remains an important emerging and reemerging infectious disease that causes high morbidity and mortality in communities and worldwide.

The influenza A viruses are at evolutionary equilibrium in their natural reservoir, which includes several wild avian species, and from this reservoir, viruses or viral genes are

occasionally transferred to new influenza virus strains and result in the infection of mammals, including humans.(Harimoto *et al*.,2005).These viruses undergo substantial and constant re-assortment or genetic shift in wild hosts, leading to the permanent possibility of the emergence of novel strains with epidemic or pandemic potential in humans.(García-Sastre ., 2011).

Influenza A virus has a unique characteristic that is not seen in other types of influenza virus which is the re-assortment and interspecies transmission which always results into a new strain of influenza virus with new HA, for example the swine origin influenza virus H1N1 pandemic of 2009 (; Neumann*, et al.,* 2009) .

2.14.2.In Africa

Data developing from Africa on influenza virus infection is revealing and often describes specific avian influenza virus outbreaks following considerable disease events. A study in Uganda between 2009 and 2011 reported an influenza A prevalence of 1% in poultry (chicken, ducks, geese and turkeys) and 1.4% in swine (Kirunda et al., 2014). In Nigeria, avian influenza H5N1 outbreaks were reported in poultry between 2006 and 2008 (WHO, 2006). In Egypt, HPAI H5N1 outbreaks have continued to occur in poultry and humans since the first report in 2006 and the virus has been declared endemic in poultry populations (Kandeel et al., 2010). In South Africa, infections with H5N2, H6N8 and H9N2 have occurred in Ostriches; H1N8 and H4N2 in Egyptian geese and H10N7 in ducks between 2004-2009 (Abolnik et al., 2010). In Mali, circulation of avian influenza viruses in domestic poultry has been documented (Molia et al., 2010). A study

done in Tunisia found up to 28% influenza A sero-prevalence in chicken and turkey flocks and H9N2 virus subtype isolated (Tombari et al., 2013).

There is limited data on influenza types circulating in pigs in Africa. Studies in Nigeria reported influenza A prevalence of 26.7% among pigs in 2010 and 14% virus isolation rate among pigs in 2008 (Oluwagbenga, 2009). The study in Nigeria reported co-circulation of two subtypes of influenza A, H1 and H3 in pigs (Oluwagbenga, 2009). A review of published data on Non human primates influenza in Africa is limited despite of increasing of anthropoject activities where by human beings are increasing growing towards the animals habitats and hence close interaction with the wild animals.

2.14.3 In Kenya

Influenza occurs in distinct outbreaks of varying extents every year in Kenya. The epidemiologic pattern reflects the changing nature of the antigenic properties of influenza viruses, and their subsequent spread depends upon multiple factors including transmissibility of the virus and susceptibility of the population. Influenza viruses circulate in humans throughout the year with increased activity in the colder months of the year (May-September) with slight variations through the years (Katz et al., 2012a) . Influenza in non-human mammalian hosts has not been described in Kenya previously.

2. 15 Laboratory diagnosis of Influenza

Diagnosis of influenza viruses can be done using samples from Nasopharyngeal or throat swabs combined or alone, nasopharyngeal aspirates for virus culture. For direct detection of viral antigens or nucleic acids after collection, these specimens should be transported to the laboratory without delay and preferably refrigerated at -70°C at all

times until processing for virus isolation or nucleic acid detection (Cox and Ziegler, 2003). Multiple freeze-thawing of samples should be avoided since they greatly reduce the virus yield. The following are the most used serological and molecular diagnostic tools for influenza viruses.

2. 15.1 Haemagglutination inhibition (HAI) test

Typing, subtyping, and further antigenic characterization of isolated influenza viruses generally is done by the HAI test.Influenza viruses agglutinate human type O, guinea pig, or chicken and turkey erythrocytes by binding on the sialic acid receptors on these cells The red blood cells are usually mixed with influenza virus in the applicable ratio, the virus bonds the red blood cells consequential in agglutination of the cells (haemagglutination) and a change in their settling behavior. Antibodies specific to the viral haemagglutinin interfere with the haemagglutinating activity and this is the basis of the HAI test which consents the identification and the disparity of the variant strains from H1-H16 (Young, 2009).

2. 15.2 Fluorescent - antibody staining

This method is deployed on screening exfoliated epithelial cells in respiratory specimens from clinically suspected individuals, it is a rapid and sensitive method for laboratory diagnosis of other respiratory virus as well like human metapneumo viruses infections. Where by the epithelial cells are washed free of mucus and fixed to microscope slides, and stained with exact, well-characterized monoclonal antibodies. In this method the specimens should be well collected suitably and frozen at all times, epithelial cells should be are handled carefully as they can be infective. The results are observed on a

fluorescent microscope, the sensitivity of this technique is up to 90% to that of standard virus isolation (Cox *et al .,* 2008).

2. 15.3 Immunoassays

There are several and specific radioimmunoassay's, enzyme immunoassays (EIAs) and fluoroimmunoassays for the detection of respiratory virus antigens in clinical specimens. These assays can produce a result within a few hours, but these tests have limited sensitivity and specificity compared with standard virus isolation (Cox *et al*.,2008). One cannot distinguish between seasonal influenza A virus and novel influenza A viruses using these methods. Due these factors, CDC does not recommend this technique for diagnostic purposes (Kim & Poudel, 2013).

The Enzyme-Linked Immunosorbent Assay (ELISA) can be used to screen all the sera for the presence of anti-influenza A nucleoprotein antibodies within 15 minutes, these assays are very fast but with false positive results sometimes and so they are not reliable (Robinson *et al.*, 2014).

2. 15.4 Molecular methods

Molecular methods are now widely used in the diagnosis of influenza virus isolates. The molecular methods are very sensitive and fast compared to old method of virus culture as the virus detection method. These molecular diagnosis are used to provide information's to the vaccine updated yearly and they can as well be employed on drug designing by using the bioinformatics tools .

In reverse transcriptase polymerase chain reaction (RT-PCR), viral nucleic acids are extracted from clinical samples, then a reverse transcription of viral RNA is done by

Transcriptase targeting the gene of choice using specific primers followed by amplification of the specific gene. The product can be detected by gel electrophoresis or fluorescence dye. The matrix gene, is conserved across all the subtypes and is widely used in the identification of influenza by real time PCR (WHO, 2002). The subsequent method which follows on that is the sequencing procedures.

2.16.Treatment of Influenza virus

Studies of influenza A virus molecular structure have helped in fighting the drug resistance and design of new drugs, for example the M2 protein which is the proton channel to the virus specific to influenza A viruses, is the target for the Adamantane and rimantadine (Kalyan *et al*.,2010).The viral enzyme function that the drug designers have invested in is the neuraminidase which functions by digesting the neuraminic acid in respiratory mucus, Drugs that targets the neuraminidase enzyme are Zanamivir and oseltamivir, these drugs bind to the active site on the viral neuraminidase enzyme and therefore inhibiting its activity(Grant Stiver *et al.,*2003).

CHAPTER THREE

3.0 METHOD OLO GY

3.1 Study site

The study was conducted on purposively wildly caught baboons from Olorbototo(Ngurumani), Aberdares, Ngurumani, Movoloni (Oldonyo sabuki) ,these baboons had stayed for 1-2 years at the Instittute of Primate Reseach at the time of this study . Figure 3.1 shows the location of the sites where the animals were caught within Kenya.

Figure 3.1 Map of Kenya showing the sites where the animals originated from.

3.2 Study design

This was a cross-sectional study carried out on two 50 hundreds nasal swab samples collected purposively from wild caught Olive baboons (*Papio anubis*) and from animals kept in the colony at the Institute of Primate Research (1°20'47.95"S, 36°42'51.13"E , Nairobi Kenya),where the presence and subtypes of influenza virus circulating in baboons from selected sites in Kenya was determined.

3.3 Ethics statement.

All procedures reported herein were performed in accordance with institutionally approved animal care and use protocols with reference number IERC/08/16 approved by the Institutional Scientific and Ethics review Committee of the Institute of Primate Research Kenya. The committee is guided by the institutional guidelines as well as the international regulations including those of WHO and Helsinki conventional on the humane treatment of animals for scientific purposes and GLP.(Appendix 1)

3.4 Clinical assessment of animals

Animals within the colony at IPR undergo physical examination daily. Information relating to physical and bio data collected are indicated in Appendices 4,5 and 6. These clinical assessments were compared to the available classical flu fever signs in human for host response variability between non-human primates to the response seen in humans.

3.5 Laboratory methods

3.5.1 RNA extraction from the viral nasal swabs samples

The virus RNA was extracted from the clinical nasal swabs of Baboons using the QIAamp® Viral RNA extraction kit (Qiagen, Germany) following the manufacturer's protocol. Briefly, 150µl of the viral transport media was added to 500µl of lysis buffer per tube and allowed to incubate at room temperature for 10 min to allow for the lysis.

A 500µl aliquot of ethanol was added and pulse vortex performed for 15s to give a homogeneous solution. A 650µl volume of the lysed solution was put to the spin columns and centrifuged at 8000 G for 1 minute and column placed in a clean collection tube. Then 500µl of Buffer AW1 was added to the spin column and centrifuged at 12000G for 1min in a Eppendorf 5415R centrifuge (Eppendorf AG, Barkhausenweg, Hamburg, Germany) and the column placed in a clean collection tube. Then the column was washed with 500µl of Buffer AW2 and centrifuged at 13,000 G for 3 min in a centrifuge.

Then finally spin column was positioned in a 1.5 ml micro centrifuge tube and 60µl of Buffer AVE added to the column and allowed to incubate at room temperature for 1 min. The column was then centrifuged at 8,000 G in the Eppendorf centrifuge at room temperature for 1 minute to the filtrate (RNA) then storage was done at -80°C.

3.5.2 Real time RT-PCR

Real-time PCR amplification and screening was performed on an ABI 7500 (Applied Biosystems, CA, USA) using the AgPath-ID one-step RT-PCR kit (Applied Biosystems, Foster City, CA, USA). The total 25µl reaction volume for each sample enclosed 5 µl of

extracted RNA, 12.5 µl of AgPath Kit 2X buffer, 1 µl of AgPath 25X enzyme mix, 5 pmol of Taqman probe, 10 pmol of each of the forward and reverse primers, and 6 µl of RNase-free water. Each RNA sample was tested by sets of matrix gene (Conserved gene across the subtypes) primers. For sample screening reverse transcription was achieved at 50°C for 30 min and 95°C for 15 min. PCR was achieved after 45 cycles of denaturation at 94°C for 15s and annealing at 55°C for 30s and final extension at 68°C for 5minutes the cycle threshold ≤40 was interpreted as positive.

3.5.3 Conventional RT-PCR for genomic amplification

The RT-PCR was performed using Superscript II One-Step RT-PCR system Platinum ™ /Taq mix (Invitrogen Corporation, NY, USA). The reaction mix was organized by mixing 12.5µl of the 2x reaction mix, 0.5 µl of the forward primer (20µM), 0.5µl the reverse primer (20µM) primers details and source are shown on the appendix 2-, 1.0µl Superscript II RT/Platinum Taq mix and this mixture was then capped using 7.5µl of distilled water to make a total of 22µl. 7µl of the RNA template was then added making the individual reaction volume to 29µl. Thermocycling conditions ware, 1 cycle of reverse transcription at 50°C for 30 min followed by an initial denaturation of 94°C for 2min. This was followed by 35 cycles of; denaturation at 94°C for 30s, annealing at 55°C for 30s and strand extension at 68°C for 1 min. Finally the reaction mixture was incubated at 68C for 1min to allow for extension of recessed ends of the amplicons. A final pause was set at 70 °C.

3.5.4 Visualization of the amplicons by Agarose Gel electrophoresis

The agarose prepared was 1% and was prepared in 1x TBE buffer. The solution was mixed by spinning gently and then heating in a microwave until all the agarose melted. Cooling was done for a few min at room temperature and then the gel was added at the ratio of 400mls of gel to 5µl of Ethidium bromide. Then the gel liquid at 37°c was then poured into an electrophoretic tank with a comb and left to set and solidify for 40 minutes at room temperature. The combs were then carefully removed. 5µl of the PCR samples were mixed with the 3µl of the blue orange gel loading dye (Invitrogen, NY, USA) and then loaded onto the wells. A 1kb DNA ladder marker (Invitrogen, NY, USA) was loaded on the first lane of each of the wells. The tank was run at 60 volts for about 90 minutes. The gel was then visualized and observed on the Alpha Imager gel documentation system (Alpha Innotech, CA, USA).

3.5.5 Clean-up of PCR products using Exosap-IT

Removing the dNTPs and primers from PCR product was done. PCR tubes covering 10µl of the PCR products to be purified were shortly spun then 3 µl of the ExoSap-IT enzyme (U. S Biological, Swampscott, MA, USA) was added to each of the PCR tubes, then followed by a brief vortex for 10s. They were then spun for 30s. The PCR tubes were then placed into Thermal Cycler, and incubated for 30 min at 37°C. Then inactivation of the ExoSap-IT enzyme by incubation for fifteen minutes at 80 °C before storing the product at 4 °C.

3.5.6 Cycle sequencing of the purified PCR products

PCR amplicons including fluorescent-labeled dideoxy-chain terminators were created using an ABI BigDye Terminator version 3.1 cycle sequencing Kit (Applied Biosystems, Forster City, USA). The reaction mixture for both the forward and reverse reactions were organized by adding 2µl of BigDye to 2µl BigDye 5X buffer then finally by addition of 1µl (4µM) of the M13R/F primers and 3µl of distilled water to make a total volume of 8µl. This reaction mixture was then loaded into each annotated well on the 96-well plate followed by addition of 2µl of the purified PCR product. The plates were enclosed with a sealing mat and were vortexed briefly.

The PCR running conditions were 1 cycle of initial denaturation at 95 °C for 5 min, followed by 30 Cycles of denaturation at 95 °C for 15 s, annealing at 45 °C for 30 s and strand extension at 68 °C for 2 min and 30 s. This was followed by a final incubation at 68 °C for 3 min to allow for extension of settled ends of the amplicons before storing the product at 4 °C.

3.5.7 Purification of the cycle sequencing products using sephadex spin columns.

®Sigma Dry sephadex G-50 medium powder was loaded into unused clean wells of 96-well Column Loader (Millipore, MA, USA). The 96 well Multi-Screen®-HV Plate Millipore was then placed upside down on top of the column loader, and both the Multi-Screen Plate and the column loader were held together and upturned. The top of the Column loader was selected to release the resin. Then 300µl of Milli-Q water was then

added to each well encompassing sephadex to swell the resin and the setup allowed to incubate at room temperature for 3 hours .The Multiscreen HV plate was then placed on top of a standard 96-well micro plate and then was spun at 8000rpm for 5 min on an Eppendorf 5810R bench top centrifuge (Eppendorf, Hamburg, Germany) using a two-tray rotor to eliminate extra water from the columns.

Extra water was then castoff. The sequencing products were sensibly added to the centre of each Sephadex well. The 96-well plate with the excess water was replaced with a new 96-well microplate (USA Scientific, FL, USA), and centrifugation done at 910× g. for 5 min guaranteeing that approximately 10μl of product came through the column. Then, 10μl of Hi-Di™ Formamide (Forster City, CA, USA) was added to guarantee the sequencing fragments were upheld as single strands and to hydrate any dry (empty) wells to circumvent extinguishing the capillaries.

3.5.8 Genetic analyzer procedure

The filtered PCR products and the Hi-Di in the 96 well plate were placed into 24-capillaries ABI 3500 XL Genetic Analyzer (Applied Biosystems). These were left to run and nucleotide sequences were obtained using the sequence analysis software (Applied Biosystems).

3.5.9 Contiguous assembly

To make contiguous nucleotide sequences from the reverse and forward sequence runs for each amplified segment from the Genetic Analyzer, the sequences were put into the contig assembly program (CAP) of DNA Baser Sequence Assembler v3 (Heracle

BioSoft SRL Romania, http://www.DnaBaser) and the consensus sequences were generated for BALST at https://blast.ncbi.nlm.nih.gov.

3.5.10.Similarity searches

To determine whether the obtained nucleotide sequences were similar to influenza A sequences deposited in genomic databases Blastn was performed at https://blast.ncbi.nlm.nih.gov.

3.6 Biosafety measures taken

Biosafety Level (BSL) 3 cabinet was used with protective equipment's worn all the time when handling the samples which includes laboratory coat, gloves and face masks ,appropriate sample labelling ,washing hands before and after every laboratory work.

3.7 Limitations of the study

This study could not culture the unsubtyped baboons influenza virus in avoidance of the risks that might have been associate with it ,and hence this made very low nucleic acid materials available for different target gene amplifications this factor also limited this study from doing serological tests as preliminary test and hence opted the real time PCR as the screening method as recommended by (WHO ,2012),limited time allocated for the study could not allow sampling of other species and hence data for avians and humans for this study are missing .

CHAPTER FOUR

4:0 RESULTS

4.1 Real time Reverse Transcriptase PCR

Real time RT-PCR of the Matrix gene was used to screen fifty nasal swab samples collected from the colony. Of the fifty samples we found that 25 were positive by RT-PCR (on the appendix 7) it was reported that the clony baboons hadstayed for 1-2 years at IPR. Results further indicated that majority of the positive animals were from Olorbototo (75%), Ngurumani (44%), Aberdares (43%), Mavoloni (37%), Yatta (14%) and Laikipia 9% (Table 4.1). This test was done in animals that ranged from 1–10 years of age at the time of sampling. Juvenile male baboons 1-4 years normally reach 5-7kgs in weight, while sub adult male 7-10 years reach 14-15kgs, juvenile female 3-4kgs and adult females reaches up to 13kgs. The results on the real time RT- PCR shows that juveniles are mostly infected compared to adults and sub-adults and that the total number of males infected were 11 and females were 12.

Table 2.1The original location of P. anubis and percentage of influenza positive samples.

Location	No. of Baboons	Type(s)	No of positive (%)
Olorbototo	8	Colony	6(75)
Yatta	7	Colony	1(14)
Aberdares	7	Colony	3(43)
Mavoloni	8	Colony	3(37.5)
Ngurumani	9	Colony	4(44)
Laikipia	11	Colony	1(9)

4.2 Amplification of the positive samples from real time rt PCR

The RT-PCR one step for HA fragment amplification was done in PAN3837, PAN3342, PAN3350, PAN3315 nasal swabs samples whereas the NA fragment amplification was done in PAN 3340, PAN3338, PAN3640 and PAN3201, finally the NA fragment was successfully amplified in PAN3350 and PAN3315 and Matrix genes was successfully amplified in PAN3640 and PAN 3201. The reason for missing bands and lack of multiple amplification of samples were due to low volume of nucleic acid present and hence unable to culture unsubtyped novel influenza virus from the baboons, the bands obtained are displayed on gel photos (Figure 4.1 and 4.2).The image was observed on Alpha Imager gel visualization system

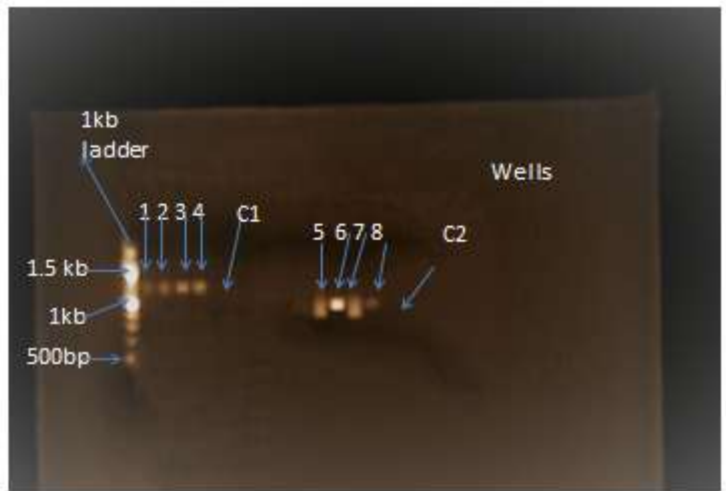

Figure4.1: Gel photo showing the PCR amplification of the three influenza gene

(HA, NA, genes)

Lane 1,2, 3, 4, is HA fragment for PAN3837, PAN3342, PAN3350, PAN3315 respectively , Lane C1 is negative control for HA ,Lane 5,6,7,8, is NA fragments for PAN 3340, PAN3338, PAN3640 and PAN3201respectively, Lane C2 is negative control for NA.

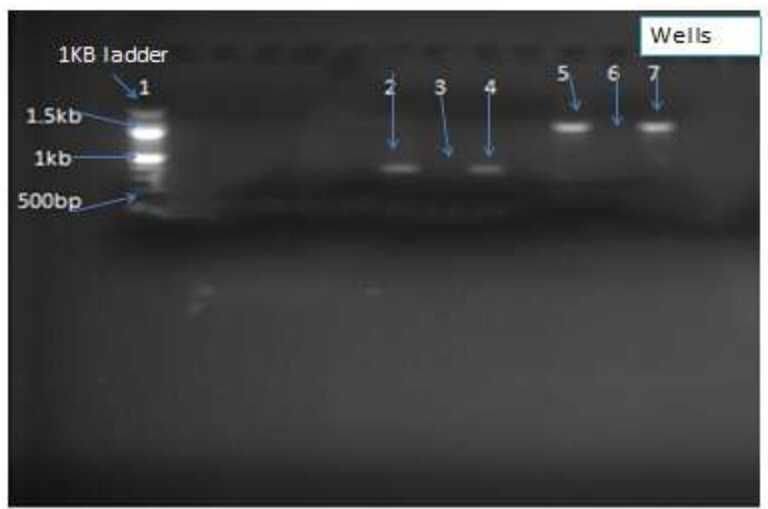

Figure4. 2 Gel photo showing the PCR amplification of the three influenza gene (NA, and M genes)

Lane 2 and 4 are NA fragments for , PAN3315 and PAN 3350 respectively and Lane 3 is the Negative control ,Lane 5 and 7 is MA fragment for PAN3640 and PAN 3201 respectively and lane 6 is the negative control.

4.3 Sequences similarity searching in the gene bank

To conclude whether the obtained nucleotide sequences from the Kenyan baboons were similar to other influenza sequences placed in genomic databases, a similarity search against sequences in the influenza virus resource was done using blastn (http://blast.ncbi.nlm.nih.gov/) (Appendix 8)which compares the query to the other sequences deposited in the Influenza genome database.

4.4 Clinical assessment of influenza positive *P. anubis*.

The age of the animals at the time of nasal swab collection was estimated to be between 1-10 years. Of these 80% of the animals were between 1-5 years of age and these were also positive for influenza infection Using amplification for the matrix gene by real time RT –PCR it was found that the positive samples were from Olorbototo (75%), Ngurumani (44%) Aberdares (43%), Mavoloni (37%), Yatta (14%), and Laikipia 9%.

These samples were taken through one step RT-PCR and only eight samples gave bands, which were then run through the 24-capillaries ABI 3500 XL Genetic Analyzer for sequencing and gave the following subtypes in baboons from Olorbototo H1N1,Yatta H3N2, Aberdares H3N2, Mavoloni H1N1, Ngurumani H1N1 and Laikipia H1N1.

The clinical signs of these subtyped baboon influenza isolates were documented as per the clinical information forms attached on appendices 5, 6 and 7, and presented on Table 4.2 below .

Table 4.2 Clinical symptoms of baboons found to be positive with influenza A but yet they didn't present the clinical influenza fever symptoms

Sample ID	Clinical information of the sampled Baboons
PAN3628	Thin, skin wounds on thighs and Respiratory system normal
PAN3624	Strongyloides species eggs, enlarged auxiliary inguinal nodes with respiratory system normal.
PAN3630	Cysts of Entamoeba were identified, alopecia on hind limbs with respiratory system normal.
PAN3350	Eggs of Trichuristrichura and Strongyloides, alopecic all over the body, with Respiratory system normal.
PAN3315	Was pregnant, cysts of Entamoeba and had a Normal respiratory system
PAN3340	Thin ,alopecic on thighs with normal respiratory system
	Eosinophilia, irregular Heart rates, with Inguinal lymph node swollen and Respiratory system normal
PAN3342	Had normal respiratory system, with swollen auxiliary lymph node

CHAPTER FIVE

5.0 Discussion

In Africa Non-Human Primates are widely distributed for example in Zambia Baboons and vervet monkeys live side by side with humans in game management areas and this situation often leads to high levels of human–baboon/monkey conflicts, in these settings, scientists have found Human Parainfluenza type 3 from Humans in baboons,(Sasaki et al. 2013) .Human metapneumo virus in wild great apes in Rwanda (Sasaki et al. 2013). In Kenya, studies have shown the presence of influenza in dogs ,but there is no data available on baboons yet there are found in large numbers and are widespread. Based on this , the baboons from a selected area in Kenya were screened to identify the influenza subtypes circulating in them.

Upon screening of fifty samples of the wildly caught colony kept baboons, 25 were found to be positive for the M-gene , of which 80% of the positive samples were from juvenile animals (1-5 years).These results are consisted with (Karlsson *et al.,*2012) who screened 48 non-human primates and found 14 positive animals , In 2005, Whittier *et al*. reported positive titers to influenza A and B viruses as part of a wider survey of seroprevalence of infectious agents in non-human primates .This shows that non-human primates are also infected by influenza virus.

Despite baboons being infected with influenza it was observed that they lack clinical signs, this was in agreement with earlier studies that observed the same that even though the viruses replicate well in the respiratory tract, animals do not generally develop any symptoms of disease (Margine *et al* .,2014) contrary of what is seen in humans,

normally accompanied with pneumonia acute respiratory failure and runny nose (Leslie et al., 2016) which is frequently complicated by bacterial co-infection This may suggest that baboons are either opportunistic hosts or infections with influenza viruses in baboons might exacerbate the development of other diseases such as active TB which they succumb to (Tarara *et al,* 1985). Viral infections induces the type I interferon's which inhibits the interferon-γ mediated immune responses which works to inhibit the development of active Mycobacterium tuberculosis (De Paus *et al,*.2013).

Two subtypes H1N1and H3N2 were identified in this study after sequencing and blastn .The H5N1 and other subtypes from avian species were not found which might indicate there were no avian species contact flu transmission . These observations further confirm results from an earlier study where seasonal subtype H1N1 and H3N2 influenza A strains were detected in performing macaques at frequencies of in Cambodia (29.2%), Singapore (16.7%), Sulawesi (16.1%), Bangladesh (13.3%), and Java (6.0%). (Karlson *et al.,* 2013). This study observed the following subtypes in baboons from Olorbototo H1N1,Yatta H3N2, Aberdares H3N2, Mavoloni H1N1, Ngurumani H1N1 and Laikipia H1N1.

The presence of influenza infections H1H1 and H3N2 subtypes in non-humans primates in Kenya confirms that baboons can also be infected with Influenza virus nevertheless with non-classical flu fever. Extra epidemiologic studies of humans and wild caught colony kept nonhuman primates are needed to determine whether Influenza virus is transmitted between humans and wild nonhuman primates or from other species like the porcine and avian.

5.1 Conclusion

Screening of nasal swabs by real-time reverse transcription PCR results indicated the presence of the conserved matrix gene which confirms the presence of influenza viruses in the baboons . Non-human primates can therefore naturally be infected with influenza viruses and the viruses replicate in the respiratory tract but without necessarily developing the clinical signs.This means that they can harbor viruses unnoticed if we only depend on clinical signs to diagnose The subtype H1N1 and H3N2 influenza A strains were identified circulating in non-human primates. These two subtypes have also been identified in human, so further epidemiologic studies can be done to search how baboons affect the transmission of these viruses .

5.2 Recommendations

There is a need for continuous surveillance and monitoring of genetic changes and virus evolution in wild and domesticated animal populations for example bats ,rodents ,camels .Continuous surveillance would also be critical in identifying other influenza viruses circulating in these animal populations in Kenya. Integrating this surveillance with on-going surveillance for influenza viruses in humans will provide useful information for public health action vaccine's, novel flu virus.

5.3 Acknowledgements

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APPENDICES

Appendix 1: Ethical approval for the study.

Institute of Primate Research

Address: P.O. Box 24481-00502 Karen Nairobi Kenya | Tel:+254 02 2606235 | Fax:+254 02 2606231
URL: www.primateresearch.org | Email: directoripr@primateresearch.org

P.O BOX 24481, KAREN, NAIROBI
TELEPHONE 254-20-882571/4
FAX: 254-20-882546
E-Mail: irc-secretary@primateresearch.org

INSTITUTIONAL SCIENTIFIC AND ETHICS REVIEW COMMITTEE
(ISERC)
FINAL PROPOSAL APPROVAL FORM

Our ref: **IERC/08/16**

Dear **Dr. Joseph Kamau,**
It is my pleasure to inform you that your proposal entitled **"PREDICT/KENYA-KENYA WILDLIFE AND DOMESTIC CAMEL SERVEILLANCE FOR EMERGING PATHOGENS"** in collaboration with **Dr. Suzan Murray, Dr. Kali Holder, Dr.Dawn Zimmerman and Dr. Devin Tunseth** of the Smithsonian Institution, United states of America; **Dr. Atunga Nyachieo and Dr. Daniel Chai** of IPR, Kenya has been reviewed by the Institutional Review Committee (IRC) at a meeting of 28th June 2016. The proposal was reviewed on the scientific merit and ethical considerations on the use of animals for research purposes. The committee is guided by the Institutional guidelines as well as International regulations, including those of WHO, NIH, PVEN and Helsinki Convention on the humane treatment of animals for scientific purposes and GLP

You are bound by the IPR Intellectual Property Policy.

Signed [signature] 20/7/2016. Chairman IRC: DR. I. O. FARAH

Signed [signature] Secretary IRC: DR. NGALLA JILLANI

INSTITUTE OF PRIMATE RESEARCH
INSTITUTIONAL REVIEW COMMITTEE
P. O. Box 24481-00502 KAREN
NAIROBI. 20/07/2016
APPROVED..........................

IPR is ISO 9001: 2008 Certified, a WHO Collaborating Centre, an ANDI African Centre of Excellence in Preclinical Research, an Associate Partner of the EUPRIM-Net and has Statutory Registration with the NIH-Office of Laboratory Animal Welfare.

Appendix 2: Primers used in this study

MATRIX GENE PRIMERS		
Gene fragment	Primer	Sequence
M	Fp	TATCCGTCAGGGAGCAAAAGCAGGTAGTC
	Rp	ATATCGTCTCGTATTAGTAGAAACAAGGTAGTTTTT

HA GENE PRIMERS		
Gene fragment	Primer	Sequence
HA	Fp	TATTCGTCTCAGGGAGCAAAAGCAGGGG
	Rp	ATATCGTCTCGTATTAGTAGAAACAAGGGTGTTTT

Gene fragment	Primer	Sequence
NA	Fp	TATTGGTCTCAGGGAGCAAAAGCAGGAGT
	Rp	ATATGGTCTCGTATTAGTAGAAACAAGGAGTTTTTT

Appendix 3: Preparation of reagents

1% Agarose

Weigh 2g of agarose and add it onto a 500ml flask then add 1x TBE slowly to the flask until the 200ml mark. Put to Microwave for 5 min making sure that the mixture does not boil over. Stop the microwave every 30s and spin the flask. Continue to heat until the agarose until its completely dissolved and colorless , then add 2.5 µl of ethidium bromide to the dissolved agarose and mix.

Preparation of 1% TBE

Add 900mls of distilled water , then add 100mls of concentrated TBE buffer to make 1% the finally shake well the mixture before using.

Appendix 4:Physical examination forms

INSTITUTE OF PRIMATE RESEARCH

PHYSICAL EXAM

Animal No__________ Sex_______ Age_______ Yr _____ Mo., ( ) Actual ( ) Estimated

Cage No__________ Generation (F0, F1, F2, etc) ________ Origin: ( ) IPR ( ) Trapped ( ) Purchased

Reason for Physical Exam: ( ) Routine health care ( ) Pre-shipment ( ) Experimental

( ) Quarantine screening ( ) Other________ Requested by:__________ Project: __________

General Condition: ( ) Excellent ( ) Good ( ) Fair ( ) Poor Current wt.__________ Kg

Check : N = Normal, A = Abnormal

Additional comments: Indicate number of system involved; include all systems checked abnormal

SYSTEMS

1. Digestive(upper)
   Teeth: ( ) N ( ) A
   Occlusion ( ) N ( ) A
   Gingiva: ( ) N ( ) A
   Calculi: ( ) Present ( ) Absent
   Missing teeth:__________
   Digestive (Lower): ( ) N ( ) A
2. Respiratory: ( ) N ( ) A
3. Circulatory: ( ) N ( ) A
4. Hemo/Lymph: ( ) N ( ) A
   Lymph nodes: ( ) N ( ) A
   Spleen: ( ) N ( ) A
5. Integument:
   Skin : ( ) N ( ) A
   Hair Coat : ( ) N ( ) A
6. Musc/Skel:
   ( ) Thin ( ) Obese
7. Nervous: ( ) N ( ) A ( ) Not examined
8. Special Senses : Eyes ( ) N ( ) A
   ( ) Opthalmoscopic exam performed
9. Urinary : ( ) N ( ) A
10. Reproductive - Male
    Testicles : ( ) N ( ) A
    Sperm : ( ) N ( ) A ( ) Not examined
11. Reproductive - Female
    A. Uterus : ( ) N ( ) A
    Pregnant : ( ) Yes ( ) No ( ) ?
    Shape : Size:_____ mm
    ( ) Pear
    ( ) Globoid
    ( ) Irregular
    Tone :
    ( ) Flaccid
    ( ) Soft
    ( ) Firm
    ( ) Hard
    ( ) Other
    ____ mm
    ____ mm
    Draw in any abnomalities
    B. Cervix : ( ) N ( ) A
    C. Vulva/Vagina:( ) N ( ) A ( ) Not examined
    D Ovaries : ( ) N ( ) A ( ) Not palped
    E. Nipples : ( ) N ( ) A

Laboratory samples (taken at exam) :
( ) CBC ( ) PCV + P.P. ( ) Clinical chemistry
( ) Serum ( ) Urinalysis ( ) Rental culture
( ) Stool sample ( ) Other __________

Examiner__________
Date __________

Appendix 5: Parasitology information form

INSTITUTE OF PRIMATE RESEARCH
NATIONAL MUSEUMS OF KENYA
PATHOLOGY AND DIAGNOSTICS, ANIMAL SCIENCES DEPARTMENT
P.O. Box 24481 - 00502, Karen, Nairobi. Tel: 020 - 2606235/6, Fax No. 020 - 2606231

PARASITOLOGY	FACILITY:	SEX:	ANIMAL IDENTITY		
	CAGE:	AGE:		SPECIES	NUMBER
INVESTIGATOR	PROJECT:	WEIGHT Kg	DATE	DAY	MONTH	YEAR

REQUESTED BY:	○ Routine preventive health
	○ Experimental protocol or health care due to experimental protocol. Charge to Grant:
CLINICAL HISTORY:	SPECIMEN:
Post R.: Yes ○ No ○	Drug/Dosage used:
Other:	

ANIMALS	Sex: ○ F ○ M	Generation: ○ F_0 ○ F_1 ○ F_2	Facility:

For laboratory use only

SPECIMEN DESCRIPTION			
DIRECT SALINE PREPARATION FINDINGS			
FORMOL-ETHER CONCENTRATION FINDINGS			
Reported by:		Date:	
DIAGNOSIS			
Diagnosis by:		Date:	

COPIES TO BE SENT

ANIMAL'S FILE   REQUESTOR   LABORATORY FILE

ISO 9001: 2008 Certified

Appendix 6 :Acquisition of the animal forms for vital events

INSTITUTE OF PRIMATE RESERCH

VITAL EVENTS - ACQUISITION

Acquisition Type:-

( ) PD: Purchased/Donated

( ) TD: Trapped by IPR

( ) CB: Colony Born

Delivery Type:

( ) V: Vaginal

( ) N: Nonvaginal

( ) A: Foetus at Necropsy

Animal No: ______ ______
Sp. No.

Sex: ______

Area/Cage: ______

Gen: ______

Age: ___/___/___
Yrs. Mos. A/E

Acquisition Date: ___/___/___
Day Mon Yr

Mother No: ______ ______
Sp. No.

Father No: ______ ______
Sp. No.

Geographic Origin: ____________________

Comments: ____________________

Appendices 7:The real time RT-PCR screening results for M gene in P. anubis nasal swabs samples.

Sample ID	CT Value	Area of collection	Sex	Weight	Age
PAN 3642	Undetect	Olorbototo	M	16	6
PAN3637	Undetect	Olorbototo	M	18	7
PAN 3837	38.1461	Yatta	F	12	5
PAN 3342	38.605	Olorbototo	F	9	4
PAN 3641	Undetect	Olorbototo	M	19	6
PAN 3625	Undetect	Olorbototo	F	14	5
PAN 3629	Undetect	Olorbototo	F	15	6
PAN3188	Undetect	Yatta	F	14	5
PAN 3340	38.099	Olorbototo	M	12	5
PAN3638	Undetect	Olorbototo	M	9	4
PAN 5536	36	Yatta	M	16	6
PAN3630	37.1206	Aberdares	F	15	5
PAN 3201	38.9381	Olorbototo	F	10	5
PAN3484	Undetect	Yatta	M	9	6
PAN 3585	Undetect	Olorbototo	M	13	5
PAN 3315	37.1621	Mavoloni	M	11	6
PAN 3197	Undetect	Olorbototo	F	10	4

PAN 3587	Undetect	Olorbototo	F	9	5
PAN 3335	Undetect	Olorbototo	M	19	6
PAN 3350	38.4184	Olorbototo	F	21	6
PAN 3632	Undetect	Olorbototo	F	17	5
PAN 3647	35.9276	Olorbototo	F	14	4
PAN 3623	Undetect	Olorbototo	F	11	6
PAN 3624	37.2694	Olorbototo	F	12	5
PAN 3464	Undetect	Olorbototo	F	24	7
PAN 3613	39.5136	Aberdares	M	18	6
PAN 3459	Undetect	Olorbototo	M	23	6
PAN 3181	38.8304	Aberdares	M	15	4
PAN 3540	37.157	Yatta	M	13	5
PAN 3560	Undetect	Orbototo	F	16	6
PAN 3646	39.3754	Orbototo	M	17	5
PAN 3479	Undetect	Olorbototo	F	10	4
PAN 3334	37.7283	Aberdares	F	11	5
PAN 3538	Undetect	Mavolani	F	9	4
PAN 3343	Undetect	Orbototo	F	10	4
PAN 3339	38.3343	Mavolani	F	11	5
PAN 3186	38.5032	Mavoloni	F	12	5
PAN 3631	Undetect	Mavoloni	M	13	4
PAN 3505	37.6242	Mavoloni	M	14	5

PAN 3621	Undetect	Yatta	M	15	4
PAN 3643	35.1316	Olorbototo	M	16	5
PAN 3837	37.4909	Laikipia	M	18	6
PAN3338	38.2143	Yatta	M	15	5
PAN 3640	38.5427	Ngurumani	F	13	4
PAN 3537	Undetect	Ngurumani	M	15	5
PAN 3634	Undetect	Olorbototo	M	16	6
PAN 3201,	38.5026	Ngurumani	F	18	7
VE +	28.17				
VE _	_VE				

Table 3The real time RT-PCR screening results for M gene in *P. anubis* nasal samples.

Cycle threshold value (CT Value) ≤40 was interpreted as positive in this study ,where by >40 was undetected (Undetected) and interpreted as negative result**.**

*Orbototo (within Ngurumani)

*Mavoloni (Oldonyo Sabuki)

Appendices 8 : The Origin of different segments of Kenyan baboon influenza isolates

		Origin of the segment	
Sample ID	**HA segment**	**NA Segment**	**M segment**
PAN3837	HA segment (H1N1) Swine origin 98%		
PAN3342			M1 segment(H3N2) human origin 98%
PAN3340		NA segment (H3N2) human origin 98%	
PAN3315	HA segment (H1N1) human origin98%		
PAN 3350		NA Segment(H1N1) human origin 98%	
PAN3338	HA Segment (H1N1) human origin98%		
PAN3640		NA segment(H3N2) human origin 98%	
PAN3201			M1 segment(H3N2) Human origin 98%

Appendices 9 :Kenyan baboon isolates sequences

>1 H1N1 2016 Isolates from Baboons

TTATTTTTTTTTTTGTCCTCAGGGAGCAAAAGCAGGGGTCAGGATATGCAGC

CGATCTGAAGAGCACACAAAATGCCATCGATAAGATTACTAACAAAGTAAA

TTCTGTTATTGAAAAGATGAATACACAGTTCACAGCAGTGGGTAAAGAGTTC

AACCACCTTGAAAAAAGAATAGAGAATCTAAATAAAAAAGTTGATGATGGT

TTCCTGGACATTTGGACTTACAATGCCGAACTGTTGGTTCTACTGGAAAATG

AAAGAAATTTGGACTATCACGATTCAAATGTGAAGAACTTGTATGAAAAAG

TAAGAAACCAGTTAAAAAACAATGCCAAGGAAATTGGAAACGGCTGCTTTG

AATTTTACCACAAATGCGATAACACATGCATGGAAAGTGTCAAGAATGGGA

CTTATGACTACCCAAAATACTCAGAGGAAGCAAAATTAAACAGAGAAAAAA

TAGATGGAGTAAAGCTGGAATCAACAAGGATCTACCATATTTTTCGTCTCAG

GGAGCAAAAGCAGGGGGACATAGTGTTGGTAATGAAACGAAAACGGGACT

CTAGCA

TACTTACTGACAGCCAGACAGCGACCAAAAGAATTTGGGATGGCCATCAAA

TAGTGTACGTTACTTTGTA

>2 H1N1 2016 Isolates from Baboons

ATAATAAAGTCTCTGTGTTGAGGAATGTGCATCCTCAACATCCTCGGGCT

CCTGCTTTTGCTCCCGGAGACGAATAAACCACAAAAGGATATGCTGCTCC

CGCTAGTCCAGATTGTGTTCTCTTCGGGTCGTCCTCTTATTAGTTCAACC

CAGAAGCAAGGTCTTATACAATCCAGCCCTGTTAGTTCTGGATGCTGAAC

AAAACTCCTGCTTTTGCTCCCTGAGACCAATAATACTACGATATCTTGCT

TTATTGAGAATTTATTGTCAGTCCCAGTCCATCCATTCGGATCCCAAATC

ATCTCAAAACCTTTTCTTGAACTAATGCTCTTAGTTCTCCCTATCCAAAC

ACCATTGCCGTATTTGAATGAAAATCCTTTTACTCCTGCTTTTGCTCCCT

GAAACCAATATAAA

>3 H3N2 2016 Isolates from Baboons

TTCCAAAAAGCGCAGGAGCATATATAAGCGTGACATTGGCGTCCCCCCCA

TCGGGCCGTGCCTCTGTTCCTTCTGTCCCTGAAGTGCCACAAACTCAAGA

TTTCTGTTTGAGGTCCACAAGTTTTCTTTTTCCTCTTTTCTTCCCCTAAT

CAACTCAACATAAAAGCACCGATTGATGCATTTTTTGCCTTCAACAGAAA

AAATACCAAAATACCCGGACCTATCAAGAGGGAAATTTGTGAGAAGAATC

ATATTGTGAAATTGGAGAGAGAAACAGACCCAATCGTTTTTATCTTTTGA

TTGGGATTCATCTTTACCCCTGTTTTTGCCCCCAGAGAAAAAAAAACACA

CGTCTTTTCCATCATCAAATGCCCACCCTTTCACTCCTTGAACCCCTTCT

TCATTGATAAAATAAAAACAATGACTACTGCTGGAGCTGTCTTTTTTTCG

GGGTGTGTCTCCAACAAGCCCTGAACACACATAACGGAAAACAATGCTAT

GACCCTTTATGTTTATATCTACTAGGGGCCGATTGGATCCTTTCCATTTG

TCTCTGCAAACACCTCTGACACCGGGATATCGGGGATAGAAAGAGCTCTC

TTCCACATGCTGAGCACTTCCTGACTATGTGCTAGTATAAAAAATTCTCC

CCTCCTCAGAGAATAATTTTATAGTATCAGCTTTTCCTGTGGCTTTTCTA

TCAGTCTTCTCTACTGTACAATTACTATTGATACAAACGCTTTCAGACTC

CTGGTTTCGTGATCATCTCTTGAAAACGATGAAAAAAACACTATCACAAA

CT

>3 H1N1 2016 Isolates from Baboons

AAAAGTTCGACACTAATTGATGGCCATCCGAATTCTTTTGGTCGCTGTCT

GGCTGTCAGTAAGTATGCTACAGTCCCGTTTTCGTTTCATTACCAACACT

ATGTCCCCCTGCTTTTGCTCCCTGAGACGAAAAATCTGGTAGATCCTTGT

TGATTCCAGCTTTACTCCATCTATTTTTTCTCTGTTTAATTTTGCTTCCT

CTGAGTATTTTGGGTAGTCATAAGTCCCATTCTTGACACTTTCCATGCAT

GTGTTATCGCATTTGTGGTAAAATTCAAAGCAGCCGTTTCCAATTTCCTT

GGCATTGTTTTTTAACTGGTTTCTTACTTTTTCATACAAGTTCTTCACAT

TTGAATCGTGATAGTCCAAATTTCTTTCATTTTCCAGTAGAACCAACAGT

TCGGCATTGTAAGTCCAAATGTCCAGGAAACCATCATCAACTTTTTTATT

TAGATTCTCTATTCTTTTTTCAAGGTGGTTGAACTCTTTACCCACTGCTG

TGAACTGTGTATTCATCTTTTCAATAACAGAATTTACTTTGTTAGTAATC

TTATCGATGGCATTTTGTGTGCTCTTCAGATCGGCTGCATATCCTGACCC

CTGCTTTTGCTCCCTGAGAACGAAAAAAAAAAAAA

>4 H3N2 2016 Isolates from Baboons

CTCAAGTTGCGAAGGCTTATATAAGCCTGACATTGACGTCCGCCCCATCA

GGCCATGACCCTGTTCCATCTGTACCTGAAGTGCCACGAACCACAAGATT

ACGGTTTGAGGTCCACAAGACTTCAGTTTCCTCTTTTCTTCCCCTAATCA

ACTCAACATAAAAGCACCGATTGATGCAGCTTTTGCCTTCAACAGAGAAA

ATACCAGAATAACCGGACCTATCAAGATGGCAATTTGCATGAAGAAGCAT

ATTGTGGAAATGGTGAGAGAAACAGAGCCAATCGTTATTATCTTTTGATT

TGGATTCATCTTTACTCCTGCTTTTGCTCCCTGAGACCAATAACCACACG

TCATTTCCATCATCAAATGCCCAGCCTTTCACTCCATGAACACCTTCTTC

ATTGTTAGAATTCAAACAATGACTACTGCTGGAGCTGTCGTTTTTTCTGG

GTGTGTCTCCAACAAGTCCTGAACACACATAACTGGAAACAATGCTATGA

TCCTTTATGTTTATATCTACTATGGGCCGATTGGATCCTTTCCAGTTGTC

TCTGCAGACACATCTGACACCAGGATATCGAGGATAGCAAGAGCACTCTT

CGACATGCTGAGCACTTCCTGACAATTTGCTAGTATGAACGATTTTCCCC

TCCTCAATGAATAGTATTTTAGTATCAGCTTTTCCTGTAGCATTTCCATC

AGTCATTACTACTGTACAAGTTCCATTGATACAAACGCATTCTGACTCCT

GGGTCCTGAGATATCTTTTGGAACATGAAAAACACTATCTACAAGCCTCC

CATTGTAAATGAAGCTAGCAGTTGCATTTTTATCATCCCCTGTTATACAA

ACATGCAGCCATGCTTTTCATCGTGACAACTTGAGCTAGGACCAAGGCTA

TTGCAACTGCCTGATCCAGAAATGGGAAGGAAACGCACCGAAGCACGACG

CTGCTGCTTGACGTA

>5 H3N2 2016 Isolates from Baboons

CTTCTCCTTACTGGCATCCTTCCACAGCCAGAAGATTCGAGCAGAGAACTTG

ATGAGTACTTGGCCTGGTATAACGACAGATCTTGAAGACTCTCGATGGAACT

GACATGCAAGACAAGATCAACTCTAGTCATCCTTCGACTTACGGGGATTTTA

TGGCCTGTTTTCACGGCTCACCGTGCCCAGTGAGCGAGGAGTGCAGCGTAGA

CTCCTTCGTCCAAAATGCCCTCAATGGGAATGGAGACCCAAATAACATGGAC

AAAGCAGTTCAAACTGTATAGGAAACTTAAGAGGGAGATAACGTTCCACGG

GGCCAAAGAAATAGCTCTTAGTTATTCTGCTGGTGCACTTGCCAGTTGCATG

GGCCTCATATACAATAGGATGGGAGCTGTAACCACTGAAGTGGCATTTGGCC

TGGTGTGTGCAACATGTGAGCAGATTGCTGATTCCCAGCACAGGTCTCATAG

GCAGATGGTGGCAACAACCAATCCATTAATAAAACATGAGAACAGAATGGT

CTTGGCCAGCACTACAGCTAAGGCTTTGGTTATCCGTGTCAGGGAGCAAAAG

CAGGTAGCGGAGGCCATGGAGATTGCTAGTCAGGCCAGGCAGATGGTGCAG

GCAATGAGAGCCATTGGGACTTATCCGAGTCAGGGAGCAAAAGCAAGAGAT

GATTTTAAAGAAAAGATGCAGTCCTATAAGAAATGAACGGGGGTGCAGTTG

CAACGATTCAAAAAGCCCGGATGTTGTTGCCGGGAAATTCAATGGGATCTTG

CACTCAGGCTTTTAGGGACAAACTCGAACCTTGTCCCATTGATCTATGGACT

CTTCAAACACGGCCGAGGAAGACGCCCTTAATGAGACGGGGGTCTTCTCAA

TGGGTGGAAGTTCAACTCATTCAACACCCAACATTTAAAATGTCAAGCACAA

AATCATGTAGCAGACGATCAGTTTCT

>6 H3N2 2016 Isolates from Baboons

TGTATACATGTTATCTTTCGCATCGTCATCAGACTCTCATGCCGAGATCGAGC

AGAGACTTGCAGATGTACTTTGCTTGGTATAACACAGATCCTGAGGCTCTCA

TGATGACTGAGACAAGACCAATCTGTCACCTTTGACTAAGGGATTTAGGGTT

TGTTTTCACGCTCACCGTGCCCAGTGAGCGAGGAGTGCAGCGTAGACGCTTT

GTCCAAAATGCCCTCAATGGGAATGGAGACCCAAATAACATGGACAAAGCA

GTTAAACTGTATAGGAAACTTAAGAGGGAGATAACGTTCCACGGGGCCAAA

GAAATAGCTCTTAGTTATTCTGCTGGTGCACTTGCCAGTTGCATGGGCCTCAT

ATACAATAGGATGGGAGCTGTAACCACTGAAGTGGCATTTGGCCTGGTGTGT

GCAACATGTGAGCAGATTGCTGATTCCCAGCACAGGTCTCATAGGCAGATG

GTGGCAACAACCAATCCATTAATAAAACATGAGAACAGAATGGTCTTGGCC

AGCACTACAGCTAAGGCTATGGTTCTAAGTGTCAGGGAACAAAAGCAGGTA

GCGGAGGCCATGGAGATTGCTAGTCAGGCCAGGCAGATGGTGCAGGCAATG

AGAGCCATTGGGACTTATCCGAGTTCCGGAGCAAAAGCAAGAGATGATTTT

AAAGAAAATATGCAGACCTATCAGAAATGAACGGGGGTGCAGATGCAACGA

TTCAAGTGACCCGGATGTTGTTGCCGGGAAAATCAATGGGATCTTGCACTCG

AGCTTGTGGATTCAAAATCGTCTCCTTGTCCAATGGATCTATGGACTCTTCAA

ACACGGCCGAGGAGACGCCCTTAATGGGCCGGAGGACCTCTCAAAGGAGGG

AAGAATAACTCAAGCAACACCCAACATTTAAAATGTCAAGCCCAAAATCAT

GTAGCAGACGTCCTGTACCT

>7 (H3N2) 2016 Isolates from Baboons

CTATAAAATCTTTATCGGAGATATAGCAATTGCCTCTGTTTCTCTCACCA

TTTCCACAATATGCTTCTTCATGCATATTGCCGTCTTGATAAACCCGGTG

TTTCGGGAATTTTATACGTTGAAGGCAAAAGCTGCATCAATCGATGCTTT

TATGTTGAGTTGATTAGGGGAAGAAAAGAGGAAACTGAAGTCTTGTGGAC

CTCAAACAGTATTGTTGTGTTTTGTGGCACTTCAGGTACATATGGAACAG

GCTCATGGGCTGATGGGGCGGACCTCAATCTCATGCTTATATAAGCTTTC

GCAATTTTAGAAAAAACTCCTTGTTTCTACTAATACAAGACCATATGACA

AGAGAACCTTATGTGTCATGCGATCCTGACAAGTGCTATCAATTTGCTCT

CGGACAGGGAACAACACTAAACAACGTGCATTCAAATAACACAGTACGTG

ATAGGACCCCTTATCGGACTCTATTGATGAGTGAGTTGGGTGTTCCTTTC

CATCTGGGGACCAAGCAAGTGTGCATAGCATGGTCCAGCTCAAGTTGTCA

CGATGGAAAAGCATGGCTGCATGTTTGTATAACAGGGGATGATAAAAATG

CAACTGCTAGCTTCATTTACAATGGGAGGCTTGTAGATAGTGTTGTTTCA

TGGTCCAAAGATATTCTCAGGACCCAGGAGTCAGAATGCGTTTGTATCAA

TGGAACTTGTACAGTAGTAATGACTGATGGAAATGCTACAGGAAAAAGCT

GATACTAAAATACTATTCATTTGAGGAGGGGGAAAAATCGTTACATACTA

GCAAATTGTCAGGAAGTGCTCAGCATTGTCGTAGAGTGCTCATGCTATTC

CTCGATATCCCTGGTGTCAAATGGGTTCTGCAGAGAGCAACTGGGAAAGG

ATCCCGATACGCGGC

>8 (H1N1) 2016 Isolates from Baboons

ATGCAAGGGTTGCGAAAACACACATGATCTCTTTGTTATACTAACACATT

ATAGACATAGTAAATTCTGTTATTGAAAAGATGAATACACAGTTCACAGC

AGTGGGTAAAGAGTTCAACCACCTTGAAAAAGAATAGAGAATCTAAATA

AAAAAGTTGATGATGGTTTCCTGGACATTTGGACTTACAATGCCGAACTG

TTGGTTCTACTGGAAAATGAAAGAACTTTGGACTATCACGATTCAAATGT

GAAGAACTTGTATGAAAAAGTAAGAAACCAGTTAAAAAACAATGCCAAGG

AAATTGGAAACGGCTGCTTTGAATTTTACCACAAATGCGATAACACATGC

ATGGAAAGTGTCAAGAATGGGACTTATGACTACCCAAAATACTCAGAGGA

AGCAAAATTAAACAGAGAAAAAATAGATGGAGTAAAGCTGGAATCAACAA

GGATCTACCAGATTTTGGCGATCTATTCAACTGTCGCCAGTTCATTGGTA

CTGGTAGTCTCCCTGGGGGCAATCAGCTTCTGGATGTGCTCTAATGGGTC

TCTACAGTGTAGAATATGTATTTAACATTAGGATTTCAGAATCATGAGAA

AAACACCCTTGTTTCTACTAATACGAGACAGATAATAGATAATAA