Identification and Characterization of Influenza A Viruses among Domestic Animals in Selected Sites in Kenya

A thesis submitted in fulfillment of the requirements for Doctor of Philosophy degree of the University of Nairobi (Epidemiology)

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DECLARATION

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

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DEDICATION

To God the giver of all wisdom, strength and from whom all blessings flow, and to my family--

the nearest and dearest.

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LIST OF ABBREVIATIONS AND ACRONYMS

AIV — Avian Influenza viruses

AI — Avian influenza

BSL 3 — Biosafety Level 3

CDC — Centers for Disease Control and Prevention

EMPRES — Emergency Prevention System for Transboundary Animal and Plant Pests and Diseases

ELISA — Enzyme linked immune-absorbent Assay

FAO — Food and Agriculture Organization

GISN — Global Influenza Surveillance Network

HA — Hemagglutinin

HPAI — Highly pathogenic Avian Influenza

KEMRI — Kenya Medical Research Institute

LPAI — Low Pathologic Avian Influenza

MMWR — Morbidity Mortality Weekly Report

NA — Neuraminidase

OD — Optical Density

PDA — Personal Digital Assistant

RNA — Ribonucleic Acid

rtRT PCR — Real time Reverse transcription Polymerase Chain Reaction

SSC — Scientific Steering Committee

USA — United States of America

WHO — World Health Organization of the United Nations

ABSTRACT

Influenza A virus has been described in multiple mammalian including humans, and in most domestic and wild *avian* species. Complex interspecies transmission of influenza A virus subtypes have been reported between and within avian and mammalian hosts. While there is evidence of influenza A virus circulation in avian hosts in Kenya, influenza A subtypes circulating in non-human mammalian hosts has not been described.

The current study was carried out in Kibera in Nairobi County, Asembo in Siaya County and Ndumbu-ini slaughterhouse in Kiambu County between August 2011 and December 2012 through a series of cross-sectional studies based at the household and slaughterhouse level. Kibera and Asembo were selected because they provided an ideal ecosystem with human and animal interaction and livestock diversity. In addition the sites provided logistical advantage of an existing database of households that were available for random selection of households. Ndumbu-ini slaughterhouse was selected based on the catchment area for small-holder pig farms. The objectives of the study were to 1) determine and characterize influenza viruses circulating in pigs, dogs, cats, chicken, turkeys, ducks and geese in the study sites and, 2) characterize the genome of the influenza virus isolates obtained from the study sites. Nasal swabs were collected from pigs, dogs and cats, oropharyngeal swabs from chicken, ducks, geese and turkeys and blood samples from all the species. All specimens were tested for presence of virus and anti-influenza A antibodies and virus isolation and subtyping carried out on all positive specimens.

A total of 8246 specimens comprising of 5110 (62.0%) swabs and 3134(38.0%) sera were tested. Of these, 3837 (46.5%), 2702 (32.8%) and 1705 (20.7%) were from Asembo, Kibera and

Ndumbu-ini, respectively. A total of 143 sera (4.6%) were positive for anti-influenza A antibodies. Influenza A sero-prevalence was highest in pigs 17.1% (n=136) followed by cats 1.5% (n=1), dogs 0.8% (n=3), ducks 0.6% (n=1) and chicken at 0.1% (n=2) while all sera from geese and turkeys were negative. In addition, while 14/986 (1.4%) from Kibera were sero-positive none of the 1389 from Asembo were sero-positive. For the slaughterhouse specimens, influenza A sero-prevalence was significantly (p<0.05) higher for the sampling periods in 2011 compared to the sampling periods in 2012. On serology subtyping by hemagglutination inhibition, close to half (48.5%; n=67), of the influenza A sero-positive sera by ELISA were positive for the A/California/04/2009(H1N1) pdm09.

A total of 19 of 5110 (0.4%) specimens were positive for influenza A M-gene by reverse transcriptase real time polymerase chain reaction (rt RT-PCR). Influenza A virus prevalence was 0.8% in pigs and dogs, 0.3% in ducks and 0.2% in chicken while none of the specimens from cats, turkeys and ducks were positive. Eight virus isolates were obtained from swabs collected in pigs and subtyped as A(H1N1) pdm09. Full genome sequencing was conducted for four of the isolates and sequences deposited in Gene bank (Accession numbers KJ680515 to KJ680545). On phylogenetic analysis, the hemagglutinin segments of the swine isolates clustered together and closely to human influenza isolates that circulated contemporaneous in Kenya.

This is the first report of circulation of influenza virus strain A (H1N1) pdm09 in pigs in Kenya between 2011 and 2012. Molecular analysis of the swine influenza virus isolates suggested that the pandemic virus strain was introduced into the pig population from humans an observation that has been reported in multiple countries globally. Influenza A was also detected in respiratory

swabs in a limited number of dogs, chicken and one duck in Kibera and Asembo. The identity of other circulating influenza virus strains among pigs, dogs, chicken and ducks was not elucidated.

Continuous monitoring of influenza virus circulation in domestic animals and monitoring of emergence of new strains of human, swine, and avian influenza viruses is key in providing data to assist future emergence of novel virus strains with pandemic potential.

CHAPTER 1

GENERAL INTRODUCTION

1.1   Background

Influenza A virus has been described in multiple mammalian including humans, and in most domestic and wild avian species (Webster *et al.*, 1992 ; Brown, 2000a ; Cardona *et al.*, 2009). Influenza disease in mammalian species is primarily a mild respiratory disease. In avian species, two forms of the disease have been described; a highly pathogenic form that causes high mortalities in affected birds and a low pathogenic form that causes a mild disease with low mortality in affected birds. Interspecies transmission of influenza viruses including humans has been documented (Webster *et al.*, 1992).

The recent influenza pandemics and epizootics in various parts of the world have continued to highlight the public health importance of influenza viruses. The influenza virus subtypes of global concern include H5N1, H7N2, H9N2 in avian species that were first reported over 10 years in Asia (GISN, 2005 ; Sims *et al.*, 2005), the 2009 pandemic Influenza A H1N1 that was first reported in April of 2009 in the USA (MMWR, 2009b) and most recently the H7N9 infections in China in 2013 (Gao *et al.*, 2013 ; Liu *et al.*, 2013).

The four human influenza pandemics that occurred in the 20th Century were caused by viruses that contain genetic components from avian influenza viruses (AIVs) (Cox and Uyeki, 2008). In addition at the global level, in the last decade, Food and Agriculture Organization (FAO) reported an increase of the number of influenza A virus subtypes and genotypes circulating in farm animals (FAO, 2010). In sub-Saharan Africa, seven countries had cases of highly pathogenic avian influenza (HPAI) H5N1 in poultry and wild birds

(EMPRES, 2010). By July 2014, the HPAI H5N1 had resulted in the death or killing of over 200 million birds globally following the infection or for control efforts.

Monitoring of the involvement of animals in the emergence and global spread of pandemic influenza A H1N1pdm09 in the human population suggests that animals have not played any role in the global spread of the virus in the human population (EMPRES, 2009 ; WHO, 2009b) but virus infection has been reported in pigs, turkeys, ferrets, cats, dogs and cheetahs in a growing number of countries around the world (WHO, 2009b ; Sponseller *et al.*, 2010 ; Mathieu *et al.*, 2010 ; Schrenzel *et al.*, 2011).

As of February of 2014, Avian Influenza H5N1 virus had caused 650 laboratory confirmed human cases in 16 countries worldwide with resultant 386 mortalities representing a case fatality rate of 59.3% (WHO, 2014). Three African countries (Nigeria, Egypt and Sudan) reported cases of human infections to WHO (WHO, 2014). Human cases in the on-going outbreak of H7N9 in China have been associated with contact with poultry mainly at the live bird markets suggesting direct contact with infected poultry as the main source of infection (Chen *et al.*, 2013).

The global influenza pandemics have raised the public health importance of influenza virus circulation and cross-transmission between humans and animals. In particular, pigs play a critical role in the evolution of influenza viruses with potential to cause pandemics in humans due to the inherent ability to allow multiplication of swine, avian and human influenza viruses (Brown, 2000a ; Vijaykrishna *et al.*, 2010). Systematic surveillance of influenza viruses in domestic animals in Kenya, especially in places with close animal-human interactions has not been carried out previously.

This study was designed to identify and characterize influenza A virus subtypes circulating in domestic animals (chicken, ducks, geese, turkeys, pigs, dogs and cats). Identifying circulating influenza virus strains would help to determine new introductions of virus or new re-assortments that can occur in the animal hosts which could be of public health concern. In addition, data on prevalence could be used to generate quantitative transmission models for influenza in the country to further refine guidelines on influenza surveillance and control activities and provide factual evidence to base development of biosecurity guidelines and messages.

This study was carried out in two sites; a rural site in Siaya county and a high human population density slum area in Nairobi county where human population-based infectious disease surveillance has been on-going for over 5 years (Feikin *et al.*, 2011). The two sites however had few pig-owning household and hence due to the central role that pigs play in the evolution of animal influenza viruses, a pig slaughterhouse in the outskirts of Nairobi was identified as a suitable site to provide data for describing influenza infection in pigs in Kenya since the slaughterhouse is the second largest facility and receives pigs from a wide catchment area. Assessing the occurrence of the pandemic strain in the animals would give an indication whether influenza virus transmission was occurring from humans to animals and *vice versa*. The results of this study provide a basis for influenza surveillance and control measures in animals in Kenya.

1.2 Problem statement

All human influenza pandemics have been caused by viruses that contain viral genetic components from AI viruses (Cox and Uyeki, 2008). At the global level, FAO has reported an increase of the number of influenza A virus subtypes and also genotypes circulating in farm animals (FAO, 2010). Unfortunately, there is a paucity of data on the influenza viruses

circulating in domestic animals in Africa. A live bird market survey in Kenya carried out in 2011 identified influenza A virus in 0.8% of chicken, geese and turkey, (Munyua *et al.*, 2013) suggesting that influenza viruses do circulate in avian species. In addition, qualitative risk assessment carried out following the 2005 threat of introduction of HPAI in the country suggested a significant risk of introduction and transmission of avian influenza into Kenya's poultry population (Omiti and Okuthe, 2008). Presence of low pathogenic avian influenza (LPAI) viruses in susceptible species has been recognized as a risk factor to occurrence of HPAI outbreaks as a result of virus mutations (Kawaoka and Webster, 1985 ; Banks *et al.*, 2001). Thus, studies of influenza virus gene pools in farm animals and humans are critical to help better understand virus genetic exchanges and disease dynamics in animal and human host populations (FAO, 2010).

1.3   Study objectives

The overall objective of this study was to determine the occurrence of influenza viruses in selected domestic animals in different sites in Kenya.

The specific objectives of the study were:

1. To determine and characterize circulating influenza A subtypes in pigs, dogs, cats, chicken, turkeys, ducks and geese at household level in Kibera and Asembo

2. To characterize swine influenza viruses circulating in pigs presented for slaughter at Ndumbu-ini slaughter house

3. To compare isolated influenza viruses with other viruses in GenBank

1.4   Significance of the study

Influenza A viruses circulating in domestic animals in Kenya have not been characterized. The results of this study will provide a basis for surveillance and control measures of

influenza in animals in Kenya. Identifying circulating influenza virus strains will help determine new introductions of virus or new re-assortments occurring in animal hosts which could be of public health concern. In addition, data on influenza sero-prevalence could be used to generate quantitative transmission models for influenza in the country to further refine guidelines on influenza surveillance and control activities and provide factual evidence on which to base development of biosecurity guidelines and messages. Assessing the occurrence of the pandemic strain in the animals would provide an indication of whether influenza virus transmission occurs from humans to animals and vice-versa.

1.5 Thesis structure

Chapter 1 provides back ground information on influenza virus occurrence globally and locally and the study objectives. Chapter 2 discusses the literature relevant to this study. This study was implemented in two study designs described in Chapter 3 and 4 over 18 months period of sampling at three months interval. Chapter 3 describes the longitudinal household-based follow-up animals in Kibera and Asembo while chapter 4 describes cross-sectional surveys of pigs presented for slaughter in Ndumbu-ini slaughter house at different points in time. Chapter 5 describes the molecular characterization of the influenza virus isolates obtained in this study.

1.6 Study approval

The study protocol was reviewed and approved by the animal care and use committee and the ethical review board at Kenya Medical Research Institute (KEMRI SSC # 1191.

2. CHAPTER 2

GENERAL LITERATURE REVIEW

2.1 Spatial distribution of chicken and pigs in Kenya

Rearing domestic animals is practiced around the world for food security, economic and social reasons (LID, 1999 ; Randolph *et al.*, 2007 ; FAO, 2009). Food and Agriculture organization (FAO) estimates that livestock contributes food security to a Billion people globally (FAO, 2009, 2011). Livestock rearing contributes to the wellbeing of households in many ways: providing a source of protein, diversifying livelihoods and improving social status. These benefits are particularly important in poorer households throughout most African communities (Randolph *et al.*, 2007). While they play an important role, animals are also reservoirs of multiple pathogens including viruses, bacteria, and helminthes and protozoa species, some of which are zoonotic and can be transmitted to humans (Cleaveland *et al.*, 2001 ; LeJeune and Kersting, 2010).

FAO estimates global livestock numbers at 1.8 billion cattle and buffalo, 2.5 billion sheep and goats , 1.5 billion pigs and 30.6 billion poultry by 2012 (FAO, 2012a). There is uneven distribution of different domestic animal species where 57.3% of all pigs are found in Asia and 2.8% in Africa. For poultry, 69.5% and 2.9% of turkeys are found in the Americas and Africa respectively, while 88.6% and 1.9% of ducks and 52.5% and 8.2% of chickens are found in Asia and Africa, respectively http://faostat3.fao.org/browse/Q/QA/E (FAO, 2012a). These livestock population estimates are conservative and attempts to utilize spatial modelling techniques of livestock distributions have been suggested to provide accurate estimates and minimize missing data in certain regions particularly in Africa (Prosser *et al.*, 2011 ; Robinson *et al.*, 2014).

According to the Kenya 2009 livestock census, there were approximately 335, 000 pigs and close to 32 Million indigenous and commercial chicken (KNBS, 2011) Figure 2.1. There are no official estimates of total population of dogs and cats in Kenya. While majority of livestock (cattle, sheep, goats and poultry) are kept in rural areas, urban livestock keeping is a growing practice which is driven by the ready demand for livestock products in urban areas (Randolph *et al.*, 2007 ; Anonymous, 2010a) as well as the need to provide for protein in the households, provide security and supplement income among the urban poor living in informal settlements (Richards and Godfrey, 2003). A survey on urban agriculture conducted in six towns in Kenya –Isiolo, Kakamega, Kisumu, Kitui, Mombasa and Nairobi - found that 50% of urban households kept livestock in the urban areas, back in rural areas or both and overall 17% kept livestock in urban areas (Memon and Lee-Smith, 1993). Poultry were the most common species kept in all towns with other species kept being goats, cattle, sheep, pigs, donkeys and rabbits (Memon and Lee-Smith, 1993).
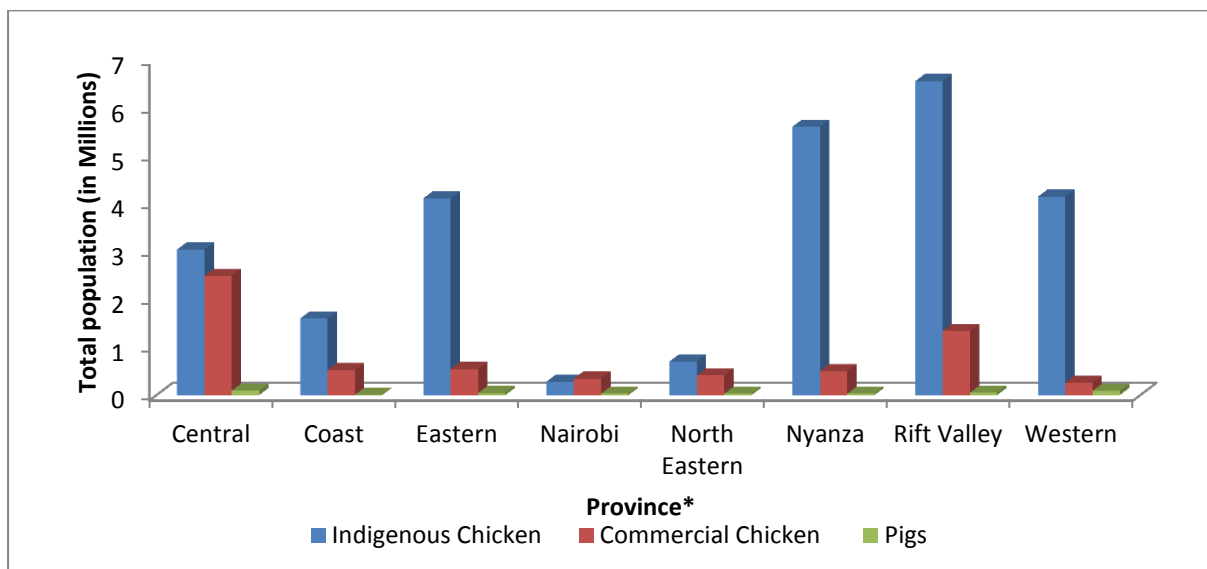
*Data provided by provinces; Study was carried out in Ndumbu-ini in Central Province, Kibera in Nairobi county and Asembo in Siaya county

† Central Province the location of the pig slaughterhouse holds 25% of the pig population while Nyanza and Nairobi Province each holds 8% total pig population.

Figure 2.1: Distribution of chicken and pigs† by provinces in Kenya, 2009 (Source: KNBS, 2009)

2.2 Importance of animals as reservoirs of zoonotic pathogens

Animals serve as important reservoir for pathogen amplification and dissemination to the environment, highlighting the need to determine the prevalence and range of pathogens that circulate in domestic animals especially zoonotic ones. Multiple host, pathogen and environment factors influence the probability of developing disease in a population and the magnitude of pathogen transmission in a specified population (Lloyd-Smith *et al.*, 2009 ; Engering *et al.*, 2013). Occurrence of zoonoses in the agricultural environment is influenced by the animal species raised, level of veterinary care, husbandry practices and farm location (LeJeune and Kersting, 2010 ; Engering *et al.*, 2013). The contribution of these factors, particularly limited veterinary care and poor husbandry practices that do not observe good biosecurity practices which is common in small holder farms in Kenya, could promote incidence of zoonoses. Lloyd-Smith *et al* (2009) suggests that for infection to occur among different species, factors including human susceptibility, risk behaviors, infections prevalence, transmission routes and pathogen specific biology influence the rate of transmission. Ultimately, presence of the reservoir and population of the susceptible hosts are key determinants of emergence and transmission of a zoonotic pathogen (Cleaveland *et al.*, 2001 ; Lloyd-Smith *et al.*, 2009 ; Engering *et al.*, 2013). Studies comparing disease prevalence in susceptible animal species in rural and urban settings provide important insights in understanding disease transmission dynamics.

2.3   Epidemiology of influenza infections in domestic animal species

2.3.1   Aetiology

Influenza A viruses are segmented, negative sense, single strand RNA viruses belonging to the *Orthomyxoviridae* family. The *Orthomyxoviridae* family consists of five genera including influenza A, B and C; Isavirus and Thogotovirus differentiated on the basis of the identity of the major internal protein antigens, the nucleoprotein and matrix proteins (Suarez, 2008). Only viruses of the Influenza A genus are known to infect birds whereas influenza type B and C are predominantly human pathogens that have also been isolated in seals and pigs respectively (Guo *et al.*, 1983 ; Osterhaus *et al.*, 2000). Influenza A viruses are further divided into subtypes on the basis of antigenic relationships in the surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA) (Figure 2.2) and are clustered into 16 HA subtypes (H1-H16) and nine NA (N1-N9). Recently, an influenza virus isolate from a bat in Guatemala was designated as H17 and N10 (Tong *et al.*, 2012).

PB1, PB2, PA
(RNA polymerase)

HA (hemagglutinin)

M2 (ion channel)

M1 (matrix protein)

NA (neuraminidase)

Lipid bilayer

NEP

NP (nucleocapsid protein)

Segmented (−) strand RNA gene
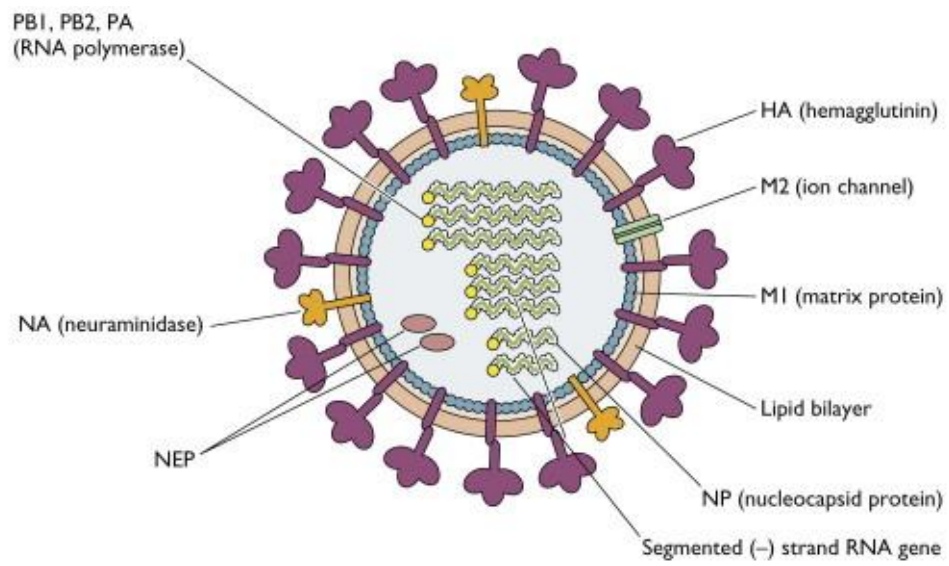
Figure 2.2 Influenza A virus structure.

(Adapted from google.com; 2014)

2.3.2 Host range

Influenza A viruses have a wide host range and have been isolated in a variety of animals including pigs, dogs, horses, birds, minks, seals and humans (Webster *et al.*, 1992). Wild birds predominantly the aquatic species form the reservoir of influenza A viruses in nature and the 16 hemagglutinin (H1-H16) and the 9 neuraminidase (N1-N9) subtypes have been isolated from these bird species (Webster *et al.*, 1992 ; Fouchier *et al.*, 2003) (Figure 2.3). It is expected that the recent H17N10 will also be isolated in aquatic birds. In mammals, there is a degree of host specificity to influenza viruses that occur in natural infections which in part is associated with the virus receptor binding sites on the host (Ito and Kawaoka, 2000).
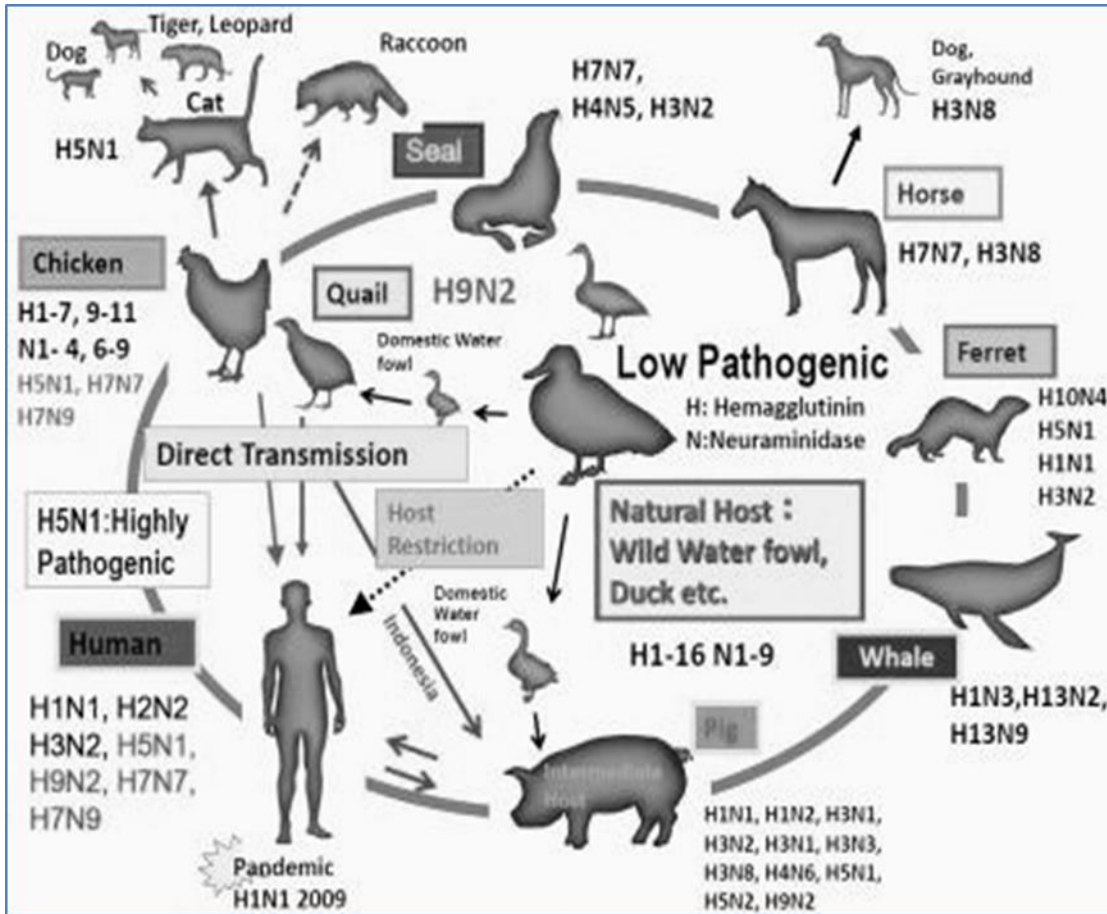
(Illustration adapted from, (Suzuki, 2013)

Figure 2.3: Host range and transmission pathways of Influenza A viruses

Note: For transmission pathways, bold lines represent pathways and direction of transmission while broken lines represent sporadic infections that have been reported The natural host of influenza A viruses (subtypes: H1-H16, N1-N9) is wild waterfowl. Multiple hemagglutinin and neuraminidase types have been isolated in avian species while in mammalian species; there is a degree of host specificity to the virus subtypes that occur in natural infection.

2.3.3   Influenza in domestic poultry

Multiple Influenza virus subtypes infect a variety of domestic poultry such as chicken, turkeys, ducks and geese (Capua and Alexander, 2008), (Figure 2.3). Two groups of influenza viruses have been isolated from domestic poultry; the highly pathogenic avian influenza (HPAI) viruses and low pathogenic avian influenza viruses (LPAI), a classification based on the pathogenicity of influenza virus subtype in poultry. The HPAI are mainly H5 and H7 subtypes that cause a severe systemic disease with high mortality in natural infections in chicken (Alexander, 2000a) and at least 75% mortality in experimentally infected 4-8 weeks old chickens. They have characteristic multiple basic amino acid at the cleavage site of the HA molecule while LPAI viruses are all the other avian influenza (AI) viruses of any subtype that are not of HPAI pathotype (Alexander, 2000a ; Suarez, 2008).   Natural infections with LPAI viruses are characterized by high morbidity of >50% but low mortality rates < 5% (Swayne and Pantin-Jackwood, 2008). Outbreaks of HPAI H5 and H7 subtypes have been reported in the US since 1924, Canada in 1966, Mexico since 1994 and Chile in 2002 (Swayne, 2008).

 The HPAI influenza A H5N1was isolated in a goose in Guangdong Province in China in 1996 (Xu *et al.*, 1999). Subsequently, between 2003 and February of 2014, the virus was detected in poultry in 52 countries in Asia, Africa and Europe (OIE, 2014) . However, in many of the countries where the virus was introduced, control efforts were instituted and outbreaks either prevented or significantly mitigated.   In Asia, multiple sub-lineages of the H5N1 virus have been described (Chen *et al.*, 2006 ; Smith *et al.*, 2006) and outbreaks continue to be reported in a number of countries including Vietnam, Bangladesh, Cambodia, China, India, Indonesia, Japan, South Korea, and Russia (OIE, 2014).   The virus became established in poultry and was declared endemic in Egypt and Indonesia, where it continues

to cause serious socio-economic losses to poultry producers and infections in humans (Smith *et al.*, 2006 ; Kayali *et al.*, 2011a ; OIE, 2014).

An outbreak of HPAI H7N3 virus that was previously LPAI occurred in Mexico in 2012 and resulted in loss of up to 4.9 million birds due to natural infections and concomitant depopulation efforts to control the outbreak (Wainwright, 2012). Genetic analysis of the HPAI H7N3 virus isolate from the Mexican outbreak suggested the insertion of part of chicken genome into the HA binding site of the H7 gene may have been responsible for increased infectivity and change from LPAI to HPAI (Wainwright, 2012).

Multiple LPAI virus subtypes occur widely in poultry populations globally with sporadic infections in humans in direct contact with poultry (Alexander, 2000a ; Brown, 2010) . The LPAI H7N9 subtype that was first detected in February of 2013 and has been causing human infections is asymptomatic in poultry (Wang *et al.*, 2014). The H7 virus subtypes have been known to evolve to highly pathogenic forms through some unknown process (Monne *et al.*, 2014) necessitating the need for controlling transmission in poultry. Since the first report of H7N9 in February of 2013 to date, the virus has been confined to the South East China region (Monne *et al.*, 2014).

2.3.4   Influenza in mammals

Multiple influenza virus subtypes have been isolated in a variety of mammalian species where they cause primarily a mild disease. Pigs play a key role in the evolution of influenza virus subtypes since they were first described in pigs in the US during the 1918 influenza pandemic (Webster *et al.*, 1992). Since 1918, multiple antigenically different Influenza A H1N1 and H3N2 subtypes have been isolated in pigs in the last century worldwide. These subtypes cause one of the most prevalent respiratory disease in pigs in the United States and

Europe (Webster *et al.*, 1992 ; Brown, 2000a ; Kuntz-Simon and Madec, 2009). Novel influenza subtypes that have previously caused outbreaks and pandemics in humans have quickly got established in pig population where they circulate with resultant genetic reassortment (Cox and Uyeki, 2008 ; Ducatez *et al.*, 2011 ; Liu *et al.*, 2012). This is due to the efficient transmission pathways of influenza viruses from humans to pigs coupled with the presence α2-3Gal-binding receptors that allow for transmission of influenza viruses of human-origin (Rogers and Paulson, 1983 ; Rogers and D'Souza, 1989).

In dogs, natural influenza A virus infections with H3N8 and H3N2 circulate efficiently causing severe and even fatal clinical disease in America, Europe and Asia (Harder and Vahlenkamp, 2010 ; Barrell *et al.*, 2010). In horses, influenza A virus subtype H3N8 and H7N7 have been endemic across the world where they often cause a mild disease (Webster *et al.*, 1992 ; Daly *et al.*, 1996 ; Swayne and Pantin-Jackwood, 2008).

2.3.5   Interspecies transmission of influenza viruses

The epidemic dynamics at the human-animal interface is determined by the prevalence of the pathogen in the reservoir, which can cause spillover infection in susceptible species that come in contact with the reservoir (Lloyd-Smith *et al.*, 2009). The ability of influenza virus to infect and persist in a different host particularly from birds to human is dependent on mutations at the virus receptor binding sites that confers the virus the ability to bind to the host receptor binding sites (Webster *et al.*, 1992). The HA protein of avian-origin influenza virus strains preferentially bind to oligosaccharides that terminate with sialic acid linked to galactose by α2-3-linkages (α2-3Gal; avian-type receptor), whereas the HA protein of human influenza virus strains prefer oligosaccharides that terminate with a sialic acid linked to galactose by α2-6-linkages (α2-6Gal; human-type receptor) (Rogers and Paulson, 1983 ; Rogers *et al.*, 1983 ; Rogers and D'Souza, 1989).

For avian origin influenza virus to be transmitted and cause infection in humans, a critical adaptation involving a shift from α2-3Gal binding to α2-6 Gal-binding specificity is critical. Human respiratory system possess the α2-6 Gal-binding receptors, avian species possess α2-3 Gal-binding receptors while pigs possess both the α2-3Gal and α2-6 Gal-binding receptors hence ability of transmission of influenza viruses of human-origin and avian-origin making the pigs a preferred host of virus re-assortment (Rogers and Paulson, 1983 ; Rogers *et al.*, 1983 ; Rogers and D'Souza, 1989).

Interspecies transmission of influenza viruses has been observed among many animal species involving different influenza A subtypes that often result in limited transmission in the new host (Webster *et al.*, 1992). Human influenza viruses containing avian influenza-like genes have been detected in influenza outbreaks in humans where direct contact with poultry is the likely source of infection. The AI subtypes implicated include the H7N7 (Banks *et al.*, 1998), H3N2 in the Netherlands in pig populations (Claas *et al.*, 1994) and H9N2 in Hong Kong in 1999 (Peiris *et al.*, 1999) and most recently H7N9 in China (Gao *et al.*, 2013 ; Chen *et al.*, 2013 ; Liu *et al.*, 2013).

Transmission of swine influenza viruses to humans occurs sporadically and is associated with occupational and environmental exposures but often resulting in spread to in-contact family members (Zimmer and Burke, 2009). A New Jersey outbreak of respiratory disease among soldiers with a novel swine influenza A H1N1 in 1976 resulted in localized transmission (Gaydos *et al.*, 1977 ; Gaydos *et al.*, 2006) . Sporadic cases of possible influenza A subtypes transmission from human to animal species has been reported such as H3N2 in cats (Paniker and Nair, 1970) where the virus resulted in a mild clinical illness. Since the emergence of the influenza A H1Npdm09 in humans in North America and its rapid spread across the world, the virus has been detected in animals including pigs, turkeys, ferrets, cats, dogs and cheetahs

(Mathieu *et al.*, 2010 ; Sponseller *et al.*, 2010 ; Schrenzel *et al.*, 2011) . In most of these cases, direct contact of the pigs with infected persons was documented as the most likely source of infection to the pigs (Hofshagen *et al.*, 2009 ; Deng *et al.*, 2012).

Pigs play a key role in evolution of influenza viruses hence transmission and maintenance of the human origin influenza virus strains in pig populations raises the possibility of genetic re-assortment with swine influenza virus (Ducatez *et al.*, 2011 ; Liu *et al.*, 2012) that could result in emergence of novel viruses of pandemic potential. Most recently in 2011-2012, variant strains of H3N2v and H1N2v that typically occur in pigs were detected in humans where they caused a mild illness with limited human to human transmission reported in the United States (MMWR, 2012).

2.3.6 Genetic evolution of Influenza A virus

Influenza A viruses evolve rapidly through various pathways that involve frequent genetic reassortments, antigenic drifts and natural selection in different hosts. First, genetic reassortment occurs when two or more different influenza A virus strains co-infect the same host cell. During the virus replication process, there is exchange of gene segment resulting in new virions with RNA from a combination of the parent strains (Lindstrom *et al.*, 2004 ; Holmes *et al.*, 2005 ; Schweiger *et al.*, 2006) . Secondly, Influenza A viruses are characterized by a very high mutation rate (close to one error per replication) resulting in evolution of the virus glycoproteins the neuraminidase and hemagglutinin in a process called antigenic drift (Drake, 1993 ; Drake and Holland, 1999 ; Nobusawa and Sato, 2006). Third, the ability of influenza viruses to be cross-infect different hosts could result in influenza viruses of avian or swine origin jump to humans resulting in reassortment with human viruses (Gorman *et al.*, 1990b ; Gorman *et al.*, 1990a ; Gammelin *et al.*, 1990 ; Vijaykrishna *et al.*,

2010) . This ability of influenza A to cross-infect different hosts with genetic reassortment resulted in the influenza pandemic in 1918, 1957 and 1968 (Cox and Uyeki, 2008).

2.3.7 Influenza in animals in Africa

Data emerging from Africa on influenza virus infection is scanty 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, 2006a ; Joannis *et al.*, 2008 ; Fusaro *et al.*, 2009). 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 ; Kayali *et al.*, 2011b). 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 ; Anjorin, 2012). The study in Nigeria reported co-circulation of two subtypes of influenza A, H1 and H3 in pigs (Oluwagbenga, 2009). A concurrent study in Nigeria among pig handlers reported 96.7% sero-prevalence of multiple influenza A subtypes (Adeola and Adeniji, 2010).   The 2009 pandemic virus was reported in pig populations in Cameroon in 2011 (Njabo *et al.*, 2012) and in Nigeria sentinel surveillance

over two years recorded a 13% influenza A prevalence as well as 18% AH1N1 pdm09 virus in pigs (Meseko *et al.*, 2014) .

A review of published data on swine influenza in Africa highlighted the limited surveillance and paucity of data from Africa despite the growing evidence of multiple cross-transmission of influenza viruses between humans and pigs globally, fairly large pig populations and low biosecurity practices in most small-scale holder farms (Capua and Munoz, 2013 ; Meseko *et al.*, 2013).

2.4   Influenza viruses in Kenya

Surveillance for influenza viruses in poultry presented for sale at five live bird markets in Kenya found an influenza A prevalence of 0.8% (95% CI 0.6-11) in chicken, geese and turkeys while all duck samples were negative (Munyua *et al.*, 2013). However, this study did not identify the subtypes or isolate the virus. Systematic surveillance for influenza viruses in humans has been carried out in Kenya in the last ten years and Influenza A H1N1, H3N2, A H1N1pdm09 and influenza B have been documented (Bulimo *et al.*, 2008 ; MMWR, 2009a ; Katz *et al.*, 2012b ; Katz *et al.*, 2012a). 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.

2.5   Risk factors of influenza infection in animals

A review of factors associated with outbreaks of HPAI H5N1 in poultry identified poultry and livestock factors (such as presence and density); anthropogenic factors (such as human population density, road networks); environmental factors (including husbandry practices and wild bird populations) and socio-economic factors (Kaoud, 2007 ; Fasina *et al.*, 2010b ; Gilbert and Pfeiffer, 2012).

However, factors associated with LPAI infection in poultry are similar to those of HPAI as highlighted in studies done in the US, Africa and Asia where outbreaks have occurred (McQuiston *et al.*, 2005 ; Woo and Park, 2008 ; Tombari *et al.*, 2013). Generally, improved biosecurity practices such as limiting access and number of visitors to the poultry houses, enclosed poultry houses were associated with low avian influenza virus (AIV) occurrence. Factors that were associated with high AIV occurrence included farms hiring one or more workers, older layer flocks, farms where farmers noted clinical signs of reduced egg production and low biosecurity measures (McQuiston *et al.*, 2005 ; Woo and Park, 2008 ; Tombari *et al.*, 2013). However, the measurement and description of factors associated with AIV infection in different studies are highly variable and tend to be specific to local conditions such as production systems and practices present (Gilbert and Pfeiffer, 2012). Older pigs, bigger pig herds, purchasing of pigs, low biosecurity practices and proximity to other pig farms are associated with increased risk of influenza A infection. In contrast, hiring of external workers was associated with decreased sero-positivity (Suriya *et al.*, 2008 ; Simon-Grife *et al.*, 2011 ; Trevennec *et al.*, 2012).

2.6   Public health Implications of influenza viruses

The twentieth century has seen four global human influenza pandemics in 1918, 1968-69, 1977 and 2009 arising from novel influenza viruses (Webster *et al.*, 1992 ; MMWR, 2009b). In each of these pandemics, the new pandemic virus subtype was derived from AI viruses either after re-assortment with a circulating strain or by direct transfer from a non-human animal species (Webster *et al.*, 1992 ; Cox and Uyeki, 2008) and resulted in high mortalities in 1918 (Zimmer and Burke, 2009) and high morbidity in 2009 (WHO, 2010). A small number of human infections with LPAI and HPAI viruses of different subtypes have been

involved in sporadic human illness often linked to direct contact with sick or healthy poultry (Cox and Uyeki, 2008 ; Brown, 2010 ; Wang *et al.*, 2014 ; Monne *et al.*, 2014 ; WHO, 2014). The infections exert pressure on public health systems where they occur, cause morbidities and mortalities in humans and have potential to cause socio-economic disruption. In addition, control efforts aimed at limiting the viruses at source have resulted in huge investments in influenza and pandemic preparedness planning. More importantly, evolution pathways for animal origin influenza viruses to adapt to new hosts including humans is not well understood and extensive research is being undertaken to clarify the role of animal influenza and interspecies transmission in emergence of influenza strains capable of sustained transmission in humans (Capua and Munoz, 2013).

2.7   Control of influenza in animals

Influenza is a highly contagious infection of the respiratory tract in animal host causing a mild to severe clinical disease. The severity of infection is influenced by multiple factors including age, viral strain, concurrent infections, and immune status of the susceptible host. In pigs in the US, control of influenza is often accomplished by the use of vaccines using both the inactivated licensed commercial vaccines and autogenous licensed inactivated vaccines (USDA, 2007 ; Romagosa *et al.*, 2011). The subtypes composition in the vaccine in each season is dependent on the circulating swine influenza strains hence the need for continuous surveillance to identify the candidate strains for optimum protection.

In dogs, an inactivated-Canine Influenza Virus H3N8 vaccine has been licensed for use in the US since 2010 (Larson *et al.*, 2011). In horses where equine influenza often leads to disruption of equestrian activities, rapid diagnosis, movement restrictions and vaccination are the key control measures (OIE, 2012). Inactivated equine influenza vaccines containing the

H3N8 strains are available and routinely used in Asia, Europe and the America's (Bryant *et al.*, 2009 ; Bryant *et al.*, 2010 ; OIE, 2012).

In general, the influenza vaccines in animals do not prevent occurrence of disease but they significantly reduce the severity of clinical disease and virus shedding hence reducing the morbidity and opportunity for interspecies transmission (Deshpande *et al.*, 2009b ; Deshpande *et al.*, 2009a ; Larson *et al.*, 2011 ; Romagosa *et al.*, 2011).

3. CHAPTER 3

PREVALENCE OF INFLUENZA VIRUSES IN DOMESTIC ANIMALS IN KIBERA
AND ASEMBO

3.1 Introduction

Influenza A viruses circulate widely in domestic animals including pigs, dogs, cats, poultry
(chicken, ducks, geese and turkeys) and man (Webster *et al.*, 1992 ; Tong *et al.*, 2013). The
wild water birds are the natural reservoirs of influenza viruses with all the 16 HA and 9NA
subtypes that infect all mammalian and avian species having been detected in the species
(Webster *et al.*, 1992). More recently, HA17 &18 and NA10 & NA11 have been identified in
bat samples (Tong *et al.*, 2013).

Interspecies transmission of influenza viruses occurs through direct or indirect transmission
pathways (Webster *et al.*, 1980 ; Webster *et al.*, 1992). In direct virus transmission, new virus
subtype not naturally occurring in the infected species is transmitted from one species to the
other with no antigenic differences of the virus in the resulting host (Webster *et al.*, 1992).
For example, influenza A H1N1pdm09 originally detected in humans has been detected in
dogs, cats, turkeys and pigs in various parts of the world and the virus in the animals has been
found to be antigenically similar to the A/California/04/2009(H1N1) pdm09 that was isolated
in humans (Hofshagen *et al.*, 2009 ; Sponseller *et al.*, 2010).

In most of these cases, direct contact of the animals with humans was the most likely source
of infection. In Addition, human infections with influenza A H5N1 and influenza A H7N9
after direct contact with sick poultry have been reported where the virus isolated in humans
was found to be antigenically similar to the virus circulating in infected birds in the same
environments (Fasina *et al.*, 2010 -a ; Chen *et al.*, 2013 ; Liu *et al.*, 2013 ; Wang *et al.*, 2014).

This study sought to determine the occurrence of influenza A viruses in pigs, poultry, dogs and cats at household level.

3.2 Objectives

The overall objective was to identify and characterize influenza viruses circulating in selected domestic animals in Kibera and Asembo.

The specific study objectives were:

1. To determine the subtype of circulating influenza A subtypes in pigs, dogs, cats, chicken, turkeys, ducks and geese in the study households.

2. To determine the sero- prevalence of influenza A viruses and subtypes circulating in pigs, dogs, cats, chicken, turkeys, ducks and geese in the study households.

Null Hypothesis (Ho)

There is no occurrence of influenza A in pigs, dogs, cats, chicken, turkeys, ducks and geese in Kibera and Asembo.

Alternate Hypothesis (Ha)

There is occurrence of influenza A in pigs, dogs, cats, chicken, turkeys, ducks and geese in Kibera and Asembo.

3.3 Materials and Methods

3.3.1 Study sites

The study was conducted in two research sites where the International Emerging Infections Program (IEIP) of the Kenya Medical Research Institute/Centers for Disease Control–Kenya (KEMRI/CDC) has conducted population-based infectious disease surveillance since 2007: Asembo, a rural location in western Kenya along Lake Victoria; and Kibera, an urban

informal settlement (slum) in Nairobi (Adazu *et al.*, 2005 ; Feikin *et al.*, 2010). Figure 3.1 shows the location of the two sites within Kenya. The two sites were selected because they provided an ideal ecosystem with human and animal interaction and livestock diversity. In addition the sites provided logistical advantage of an existing database of households that were available for random selection of households.
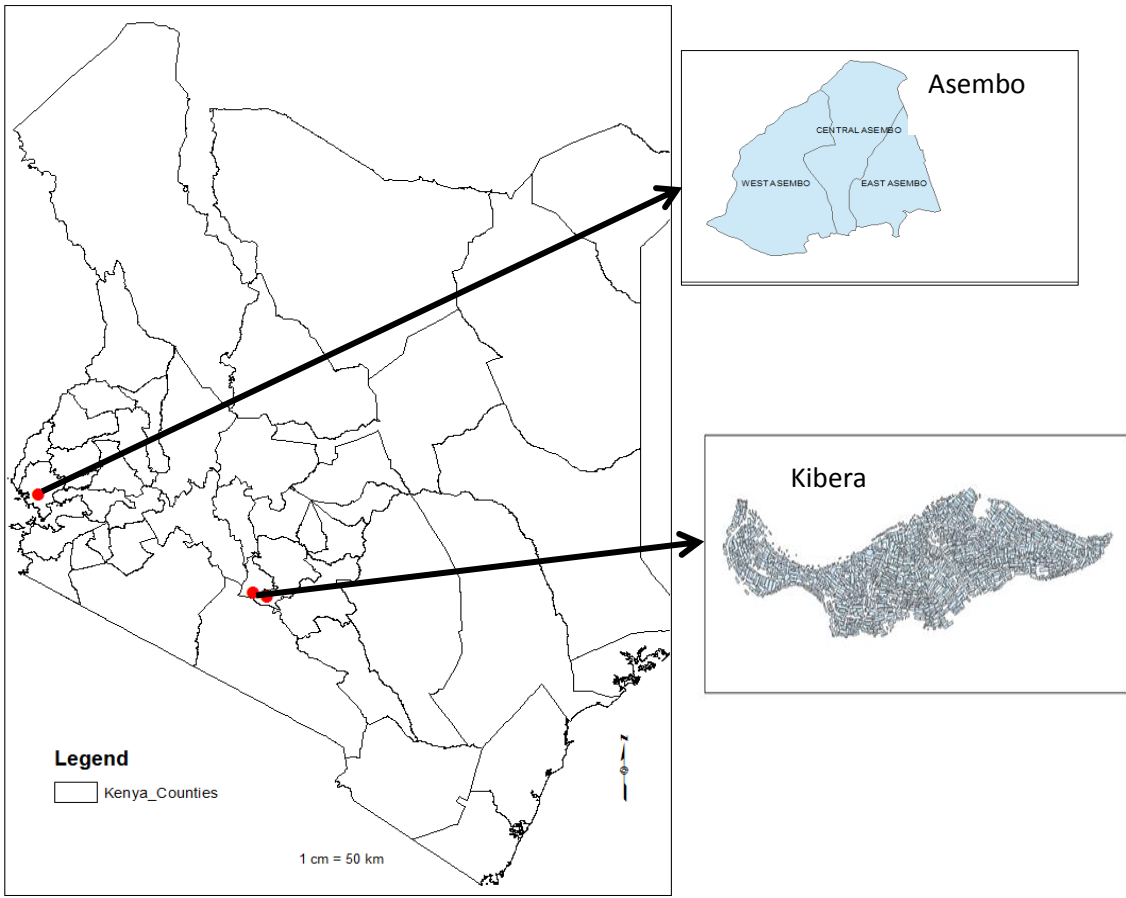
Figure 3.1: Map of Kenya counties showing inset map of the two KEMRI CDC population based study sites, Kibera in Nairobi County and Asembo in Siaya County.

Kibera – Urban site

Kibera is a large informal urban settlement located within Nairobi, with an estimated human population of 170,070 people (2009, Kenya population and housing data). There are 13 villages and like any other urban slum, Kibera is characterized by poor housing, poor sanitation and lack of access to adequate water. The CDC/KEMRI PBIDS has conducted population based studies on infectious diseases in two of these villages—Gatwikira and Soweto since 2006 (Feikin *et al.*, 2010). The two villages lie in the extreme south-western area of Kibera, with estimated human populations of 28,000 people in approximately 6,000 households (total area, 0.4 km^2; population density, 77, 000 persons/km$^{2)}$ (Feikin *et al.*, 2010) . All households have been mapped using a differential global positioning system and house doors marked with unique location codes based on cluster, structure and house number.

Asembo –Rural site

Asembo is a rural location in Rarieda sub-county of western Kenya along Lake Victoria. The population is predominantly subsistence farmers and fishermen. Houses are widely dispersed in a bushy landscape cultivated with small fields. The area occupies approximately ~100 km^2 with an overall human population density of about 325 persons/ km^2 (Feikin *et al.*, 2010). There are 6,000 homes enrolled in the human study spread over 33 villages. In the health and demographic surveillance system (HDSS) all compounds have been mapped using a differential global positioning system and house doors marked with unique location codes based on village, compound and house number.

3.3.2   Study design

To identify influenza subtypes circulating in domestic animals in the two sites, a longitudinal study design was adopted using single-stage cluster sampling. In this case, selected

households at the start of the study were considered as sampling units and were followed-up for a one-year period with repeated quarterly sampling for disease outcomes. On each visit, a maximum of three animals for each of the species of interest –chicken, ducks, turkeys, geese, cats, dogs and pigs present on the farm were selected and sampled. From this study, influenza viruses were identified and subtyped and prevalence of influenza viruses in different domestic animals in the two ecosystems determined.

3.3.3   Sample size determination

To calculate the number of households to include in the study site, it was assumed that 50% of the households would have an animal test positive for Influenza A at one point during the follow-up period and at 90% confidence level. A household was considered as a sampling unit and more than one animal (to a maximum of 3) of each species were sampled per household where available. Sampling more than one individual in a sampling unit leads to loss of effectiveness due to the relatedness of subjects in one cluster (clustering) as compared to simple random sampling of individual animals in the population (Katz and Zeger, 1994).

To account for the effect of clustering at household level that would be expected for an infection such as influenza that is spread through direct contact, a design effect which is the ratio of the actual variance, under the sampling method actually used, to the variance computed under the assumption of simple random sampling needs to be applied (Henry, 1990 ; Katz and Zeger, 1994 ; Lemeshow, 1999). Design effect is a factor of the cluster size (number of subjects sampled in each cluster) and the intraclass correlation coefficient (ICC) (Lemeshow, 1999). The ICC of influenza in animals was not available hence an assumed design effect of 1.5 was applied to calculate sample size according to the method of Schaeffer et al, (1990) .

n = [DEFF*Np(1-p)]/ [(d^2/Z$^2_{1-\alpha/2}$*(N-1)+p*(1-p)] ,

Where, n= Sample size required

Z = Value of Z which provides 95% confidence interval (1.96)

p = *A priori* estimate of the prevalence (50%),

d = Precision (allowable error) - 10%

 Deff= design effect (1.5)

The resulting sample size of 101 households was increased by 10% to adjust for households that were likely to be lost to follow-up due to lack of animals or other reasons. Using the sampling frame of all households owing animals in Kibera and Asembo identified by unique household identification, random sampling was used to select households to be sampled based on the number of households owning chicken, dogs, and ducks.   In total, 110 households each in Kibera and Asembo were enrolled for the one-year follow-up.

3.3.4   Identification of participating households

In Kibera, data from an animal census in all households enrolled in the Kibera study conducted in February, 2010 was used. The data set had type and number of animals owned by each household. The households were identified by a unique household identification number assigned to all the participating households.

In the Asembo, a data set with type and number of animals per households collected in December of 2010 was obtained. The households were identified by a unique household identification number assigned to all the participating households.

3.3.5   Selection of participating households, enrollment and follow up

Households were randomly selected from animal-owning households of those enrolled in the two study sites. From a list of all animal owning households, a command in stata was used to select 110 households from the list using the assigned unique household IDs. Households were enrolled into the study after consenting for administration of the questionnaire and collection of samples from their animals. The enrolled households were visited after every four months for one year between July 2011 and August 2012 and all the eligible animals sampled on each visit.

3.3.6   Sample collection, processing and storage

All animals were manually restrained. Blood samples were obtained from pigs and cats from the jugular vein, in poultry from the brachial vein and dogs from the cephalic vein. For adult pigs blood was collected into plain 10 ml BD Vacutainer® glass serum tube; for young pigs, dogs, cats and poultry, blood was collected into 5 ml BD Vacutainer ® glass serum tube without EDTA and transported on ice to the KEMRI/CDC Biosafety Level (BSL) 3 laboratory in Kisumu for samples from Asembo and Nairobi for samples from Kibera for sera harvesting on each day of collection.

Cloacal swabs were collected from poultry species by inserting plastic shafted polyester tipped swab into the cloacal cavity, twisting it once and withdrawing the swab. Oropharyngeal swabs were collected from the poultry species by swabbing the oropharyngeal area near the opening of the trachea using plastic shafted polyester tipped swab. Nasal swabs were collected from dogs and pigs by inserting plastic shafted polyester swabs and in cats using a nylon shafted polyester tipped swabs into the nasal canal. All swabs were then put in 2 ml cryo-vials (Greiner bio-one, Germany) containing 1ml of viral transport media with antibiotics and transported on ice to the laboratory on each day of collection. All samples

were labeled using bar code labels at the point of collection. Due to difficulties of capturing cats, only a small proportion of the cats in the households were sampled.

Blood was allowed to clot and serum separated. Serum aliquots were dispensed into 2 ml cryovials (Greiner bio-one, Germany) and stored at -20^0C prior to testing. Swabs were stored at -80^0C prior to testing.

3.3.7 Data collection and management

A standardized questionnaire was administered to the household head or an adult relative who was present at the household at the time of visit using a personal digital assistant (PDAs). Data on animal demographic (flock and individual level) in the enrolled households and husbandry practices included animal population dynamics (e.g. flock sizes, flock composition, age, and flock movement), animal species, and husbandry practices (Appendix 1).

3.3.8 Laboratory methods

All specimens were tested at the BSL 3 KEMRI/CDC laboratory in Kisumu. Laboratory testing included antigen detection test on the swab specimens and antibody detection tests on the sera specimens.

3.3.8.1 Detection of influenza A antibodies

The Enzyme-Linked Immunosorbent Assay (ELISA) was used to screen all the sera for the presence of anti-influenza A nucleoprotein antibodies (Robinson *et al.*, 2014) using the IDEXX FlockChek® Avian Influenza MultiS-Screen Antibody test kit (IDEXX laboratories, Westbrook, Maine, USA. www.IDEXX.com). The test detects antibodies against the nucleoprotein common to all influenza A viruses and is not subtype specific. The test was originally developed for detecting anti-influenza A nucleoprotein in sera from avian species

(chicken, turkey, duck, ostrich, and goose). (IDEXX, 2014). Samples were assayed in duplicate into 96-well plates according to the manufacturer's protocol for testing by using 15 μl of sera diluted 10-fold in diluent provided by the manufacturer. The diluted serum was dispensed onto the nucleoprotein-coated (NP) plate and allowed to bind to the antigen. An anti-NP antibody conjugate with horseradish peroxidase (HRP), followed by 3,39,5,59-tetramethyl benzidine (TMB) substrate, was then added as a color indicator. If present, antibody in the sample competitively bound to the epitope and blocked binding of the HRP conjugate. Color development, that is inversely proportional to the amount of anti-influenza virus antibodies in the test sample, was determined using an ELISA plate reader using a filter with a wavelength of 650 nm. Data readings and calculations were performed by using software provided by the manufacturer. Results were reported as the ratio of the sample optical density (OD) reading to the kit negative control OD reading (S/N). The manufacturer recommended cut-off of ≤ 0.5 for positive sera was applied for poultry, cats and dogs. The test has been validated for use with swine sera with an adjusted cut-off of S/N ratio ≤ 0.673 applied since this was determined to increases test sensitivity to 72% and specificity to 99% in pigs sera (Ciacci-Zanella *et al.*, 2010).

3.3.8.2 Detection of subtype specific antibodies

To detect antibodies to specific swine influenza subtypes, all ELISA-positive sera were tested by hemagglutination inhibition (HI) for antibodies to three influenza A virus strains: A/California/04/2009(H1N1) pdm09, A/Swine/Texas/4199-2/98 triple-reassortant H3N2, A/Swine/Iowa/15/30 H1N1 obtained from St. Jude Children's Research Hospital, TN, USA. The method described in the WHO manual on Influenza Diagnosis and Surveillance http://www.who.int/csr/resources/publications/influenza/en/whocdscsrncs20025rev.pdf was used. Briefly, sera were treated with receptor-destroying enzyme (Denka Seiken Co., Ltd.

Japan) and haemadsorbed on guinea pig red blood cells. Serial two-fold dilutions from 1/2 to 1/2048 of test sera were then mixed with 25µl of standardized viral antigen. Endpoints of serum dilutions that showed complete inhibition were determined and the HI titer was expressed as the reciprocal of the highest dilution of serum where hemagglutination was inhibited. Titers greater than or equal to 1:80 were considered positive. For sera with polytypic cross reactions, the antigen with a HI titer fourfold or higher was considered the positive antigen.

3.3.8.3 Detection of influenza A virus by real time reverse transcriptase polymerase chain reaction (rtRT-PCR)

Nasal and bronchiole swabs, were screened for influenza A virus by real time reverse transcription-polymerase chain reaction (rtRT-PCR) using the CDC protocol for influenza A virus detection that targets the matrix gene (Whiley *et al.*, 2009). The matrix gene is a highly conserved region that is present in all influenza A viruses. The total RNA from nasal/bronchiole swab were extracted using the QIAamp RNA extraction kit (Qiagen Inc.,Valencia, CA) following the manufacturer's instructions. The RNA carrier was added to the AVL kit-supplied buffer (Qiagen Inc., Valencia, CA) and 400 µL of this mixed with 100 µL of each sample and left to incubate for 10 minutes at room temperature. 400µL of absolute ethanol was added and this mixture applied to a QIAamp spin column. The column was washed with kit-supplied buffers and the RNA eluted in 60 µL of elution buffer. Thereafter, one step rtRT-PCR was carried out on an ABI 7500 Fast platform using the protocol for identification of influenza A viruses (Whiley *et al.*, 2009). The RT-PCR assays was performed using the AgPath-ID RT-PCR kit (Ambion, Austin, TX). The primers used for all animal specimens were 5' AGA TGA GTC TTC TAA CCG AGG TCG 3' as the forward, and 5' TGC AAA AAC ATC TTC AAG TCT CTG 3' as the reverse primer. The detection

probe was 5' TCA GGC CCC CTC AAA GCC GA 3'. Fluorescence was read at the annealing/extension step and results recorded as cycle threshold (C_T) values with values ≤39.9 recorded as positive. Appropriate negative and positive control specimens were run alongside each reaction.

3.3.8.4   Influenza virus isolation

Virus isolation was attempted on all influenza A positive swabs and a proportion of influenza A negative swabs as described in the WHO manual on Influenza Diagnosis and Surveillance http://www.who.int/csr/resources/publications/influenza/en/whocdscsrncs20025rev.pdf.

Briefly, 200 µL of nasal or bronchiole swab sample was placed on confluent Madin- Darby canine kidney (MDCK) cells in 24-well plates to incubate for 1 hour. After the incubation, the sample was removed and 400µL of MEM w/TPCK trypsin was added. The plate was checked at 24 and 48 h for cytopathic effects. After 48 h, 200 µ of cell culture supernatant from each well of the 24-well plate was subsequently passed onto a confluent 48-well plate. After 48 h, evidence of cytopathic effects was evaluated and presence of virus antigen confirmed by rapid hemagglutination (HA) assay. Post culture RT PCR was done on all HA positive isolates. Virus titers in virus isolates were determined on MDCK cells in 96-well plates.

3.3.8.5   Subtyping of influenza virus isolates

Identification of the influenza isolates was done by rt RT-PCR. Total RNA was extracted from the virus isolates as described above. Subsequently, the RNA was sub typed with CDC primers and probes (pandemic influenza A and pandemic H1) for detection of the influenza A matrix gene, the pandemic influenza A and pandemic influenza H1 and (Richt *et al.*, 2004) primers and probes for detection of Swine N1, H1,N2 and H3 were used to complete the

subtyping as per the protocol for the identification of influenza A viruses (WHO, 2009a). Gene sequences for the primers and probes are presented in Appendix 2.

The RT-PCR assays were performed using the AgPath-ID RT-PCR kit (Ambion, Austin, TX). Briefly, 25µl reaction mixtures were made containing: 12.5µL of 2x Taqman one-step RT-PCR master mix, 1µl 40x MultiScribe and RNase inhibitor mixture, 0.25 µl of 0.8µM forward primer, 0.8µM reverse primer and 0.2µM probe, 5.75 µl nuclease free water and 5µL of purified RNA. Amplification and detection were performed on a 7500 Fast Real-Time PCR platform (Applied Biosystems, Foster City, CA). The following thermal cycling conditions were used: 50°C for 30 min (reverse transcription), 95°C for 10 min (reverse transcriptase enzyme inactivation). PCR was achieved after 45 cycles of 95°C for 15 sec (denaturation) and 55°C for 60 sec (annealing and signal acquisition).

3.3.9   Data management and analysis

All data were collected using PDAs and downloaded and stored in an MS Access database. Each household had an assigned unique household identification (ID) number that was used to link all animals from a household as well as the results from the four sampling periods. On the other hand, each animal that was sampled was assigned unique ID to link the serology and virus detection results. In addition, all samples were assigned unique sample IDs using pre-printed bar code labels that were placed on the sample collected and on sample tracking sheet submitted to the laboratory. The unique id sample ID was used to link the epi data with the laboratory test results. Global positioning coordinates for each household were collected using Global positioning receiver. The latitudes and longitudes were downloaded and stored in MS excel sheet.

Descriptive statistics for demographic characteristics of the enrolled households and sampled animals were computed using STATA 13.1 statistical software (StataCorp, College Station, TX, USA). Comparisons were made to show differences in proportions of animals kept for each household between the two sites and the Z-score test statistic and p-value were reported in tables. Loss to follow up was calculated by households was calculated based on the number a household no samples were collected from animals for the four times each household was visited and compared for the two sites. The study outcome was detection rates of influenza A virus and serological evidence of infection with influenza A viruses by animal species for each of the sampling period. Influenza A prevalence and sero-prevalence by species, site and sampling month for each year was computed at 95% confidence intervals using the exact binomial method and presented in tables and figures. The spatial occurrence of influenza A by household and animal species was displayed by mapping the enrolled households by ArcGIS® 10.2 (Redlands, California, USA). An existing shapefile of the study site and the world street map from ArcGIS online database were used as the base layers and maps exported as tagged image file format for display.

Exact logistic regression in STATA ® was used to model binary outcome variables (influenza positivity by either influenza A RNA detection or sero-positivity) in which the log odds of the outcome was modeled as a linear combination of the predictor variables (animal age and sex of the animal) for each animal species. Exact logistic regression was used since the sample size was small (less than 30 observations for some species) for a regular logistic regression (which uses the standard maximum-likelihood-based estimator) and some of the cells formed by the outcome and categorical predictor variable had no observations. Odds ratios with 95% confidence intervals were reported and a P value <0.05 was considered significant.

3.4 Results

3.4.1 Animal ownership of households

A total of 221 households were enrolled in Kibera (n=110) and Asembo (n=111). The proportion of enrolled households owning different animal species and household mean ownership are shown in Table 3.1 . In Asembo, almost all (96.4%; 107/111) the enrolled households owned chicken while over half of the households owned cattle, goats and dogs, (Table 3.1). None of the households owned pigs in Asembo. In Kibera, 75% (83/110) of all enrolled households owned chicken. A significantly (p=0.05) higher proportion of enrolled households in Asembo owned cattle, goats, dogs, cats and chicken than Kibera, while a significantly (p=0.05) higher proportion of enrolled households in Kibera owned ducks. The proportion of enrolled households that owned turkeys was not significantly different. The mean ownership of chicken in Asembo (11.5) was almost double the mean number of chickens owned in Kibera (6.2).

Table 3.1: Comparison of proportion of households owning different animals in Asembo and Kibera, 2011

Animal type	Study site				Test of proportions (95% CI)	
	Asembo n=111		Kibera n=110			
	Total number owning n(%)	Mean owned(sd)	Total number owning n(%)	Mean owned(sd)	Z	p-value
Cattle	77(69.4)	7.6(10.0)	2(1.8)	4.0(1.4)	10.48	0.000
Goats	68(61.3)	7.3(12.3)	2(1.8)	4.5(2.1)	9.5	0.000
Dogs	64(57.7)	1.8(1.1)	27(24.5)	2.9(2.5)	5.0	0.000
Cats	54(48.6)	1.5(1.5)	32(29.1)	1.3(0.7)	2.98	0.003
Chickens	107(96.4)	11.5(11.3)	83(75.5)	6.2(7.3)	4.48	0.000
Ducks	9(8.1)	22.3(28.3)	22(20.0)	15.0(4.3)	-2.55	0.011
Turkeys	6(5.4)	24.8(28.4)	4(3.6)	17.2(28.5)	0.63	0.527
Pig	0(0)	-	4(3.6)	27.8(1.4)	-2.03	0.043

3.4.2 Farm/household husbandry practices

Most of the households (87%) kept chicken and ducks for food and for sale. There were differences in the rearing of poultry (chicken and ducks) whereby in Asembo almost all (> 99%) were kept under free-range while in Kibera, 51.2% and 86.4% of chickens and ducks respectively, were kept under free range. Among those who vaccinated their poultry, 45.4% (50/110) and 16.5% (vaccinated against Newcastle disease virus) in Asembo and Kibera, respectively. Among dog owners, 77.8% and 58.8% did not confine the dogs during the day and 82.1% and 92.3% of them did not confine their dogs during the night in Kibera and Asembo, respectively. Among cat owners, 93.7% and 100 % did not confine their cats during the day while 71.9% and 90.7% of households did not confine their cats at night in Kibera and Asembo, respectively. All the cats were kept by their owners to keep away rodents. Of the four households that owned pigs in Kibera, one had the pigs under zero-grazing while three reared the pigs under free ranging.

3.4.3 Losses to follow up

An enrolled household was considered lost to follow-up if no animal was sampled during a sampling period. The proportion of households lost to follow up was significantly (p=0.05) higher in Kibera (19.1%) than Asembo (6.5%). Lack of animals in subsequent visits after initial household enrolment was the most common reason of loss to follow up in Kibera while absence of the household head was the most common reason for non-sampling in Asembo. The percent loss to follow-up by sampling period and site is presented in Table 3.2.

Table 3.2: Proportion of households (HH) lost to follow-up in Asembo and Kibera in 2011-2012

Month, Year of sampling	Total HHs sampled by site		% HHs lost to follow-up	
	Asembo	Kibera	Asembo	Kibera
July, 2011	111	110	-	-
Dec, 2011	97	89	12.6	19.1
March, 2012	106	76	4.5	30.9
July, 2012	101	81	9.0	26.4
Total HH visits	**415**	**356**	**6.5**	**19.1**

Key: HH = Households

3.4.4   Samples collected

Between July 2011 and July 2012, a total of 6824 samples were collected of which 6539(95.8%) were tested. Table 3.3 shows the distribution of samples collected by species, and site. Over a half (58.6%) of the samples were collected in Asembo. The majority (76.3%) of the samples collected were from chicken.   For the animals sampled, there was no significant difference in the mean age in months of cats in Asembo (mean=24.9, SD=24.3) and Kibera (mean= 24.1, SD=20.1), t (41.9) = 0.17, p=0.864 and turkeys in Asembo (mean =38.5, SD= 33.7) and Kibera (mean=16, SD=6.6), t(11.9)=2.1 p=0.055   . The mean age in months for dogs was significantly higher (p=0.014) and significantly lower for chicken (p= 0.00) and ducks (p=0.014) in Kibera (Table 3.4).

Table 3.3: Distribution of samples collected by species in Asembo and Kibera in 2011-2012

Species	Study site		
	Asembo n (%)	Kibera n (%)	Total and proportion (%)
Dogs	538 (13.4)	276 (9.8)	814 (11.9)
Cats	31(0.8)	110 (3.9)	141(2.1)
Chicken	3306 (82.5)	1901 (67.4)	5207 (76.3)
Duck	97 (2.4)	427 (15.1)	524 (7.7)
Geese	0(0.0)	3 (0.1)	3(0.0)
Turkey	33 (0.8)	23 (0.8)	56(0.8)
Pig	0 (0.0)	79 (2.8)	79 (1.2)
Total	4005(58.7)	2819(41.3)	6824

Table 3.4: Comparison of mean age of animals sampled by site, Kenya 2011-2012

| | Study site | | | | Test statistic | |
Species	Count	Asembo Mean (SD)	Count	Kibera Mean (SD)	t (df)	p-value
Dogs	253	30.5(22.8)	128	37.6(27.6)	-2.5 (206)	0.014
Cats	27	26.0(25.4)	52	24.5(21.9)	0.26(47.8)	0.795
Chicken	1052	21.3(18.1)	587	15.5(13.3)	7.4(1521)	0.000
Duck	24	28.1(18.1)	130	17.8(16.2)	2.6(30.8)	0.014
Geese	0	-	1	9(-)	-	
Turkey	11	38.5(33.7)	5	16.0(6.6)	2.1(11.9)	0.055
Pig	0	-	35	17.8(14.0)	-	

3.4.5   Sero-prevalence of influenza A

3.4.5.1   Sero-prevalence of influenza A by species and study site

In total, 2375 sera comprising of 1389 (58.4%) from Asembo and 986 (41.5%) from Kibera were screened for anti-influenza A antibodies using ELISA. Overall, 14 (1.4%) sera all collected from Kibera tested positive while none of the 1389 sera collected from different animals in Asembo tested positive. The highest sero-prevalence was observed in pigs at 18.9% (7/37) followed by dogs (2.2%), cats (1.9%), ducks (0.7%) and chicken (0.3%) while none of the sera collected from geese and turkeys were positive (Table 3.5)

Table 3.5: Sero-prevalence of influenza A antibodies by species in Kibera, 2011-2012

Species	Study site		
		Kibera	
	Total samples tested	No. positive and proportion (%)	Binomial Exact 95% CI
Pig	37	7(18.9)	7.9, 35.1
Dog	135	3(2.2)	0.5, 6.3
Cat	52	1(1.9)	0.04, 10.2
Chicken	613	2(0.3)	0.04, 1.2
Duck	143	1(0.7)	0.02, 3.8
Turkey	5	0(0)	-
Geese	1	0(0)	-
Total	986	14(1.4)	0.8, 2.4

3.4.5.2   Temporal variation of influenza A sero-prevalence

In Kibera the overall influenza sero-prevalence in all species sampled observed over this period was 1.4 % but was variable for each sampling time with the highest prevalence observed in samples collected in August 2011 (2.4%) and lowest in samples collected in December 2011 (0.4%) (Figure 3.2). There was no significant difference in the observed sero-prevalence by the sampling periods. In dogs, influenza A antibodies were detected in three of 135 dogs sampled in December 2011 and April 2012 while in chickens, two of 613 chickens sampled in April and July 2012 were positive. In pigs, two thirds (25/37) of the samples collected over this period were collected in July 2011 and all the sero-positives were detected from these samples. The low number of pigs sampled in subsequent periods was due to low number of pigs in the enrolled households due to a reported outbreak of African Swine Fever that killed most of the pigs. Controlling for the number of pigs sampled, the influenza A sero-prevalence observed in July of 2011(28.0%) was significantly higher than for the other sampling periods.
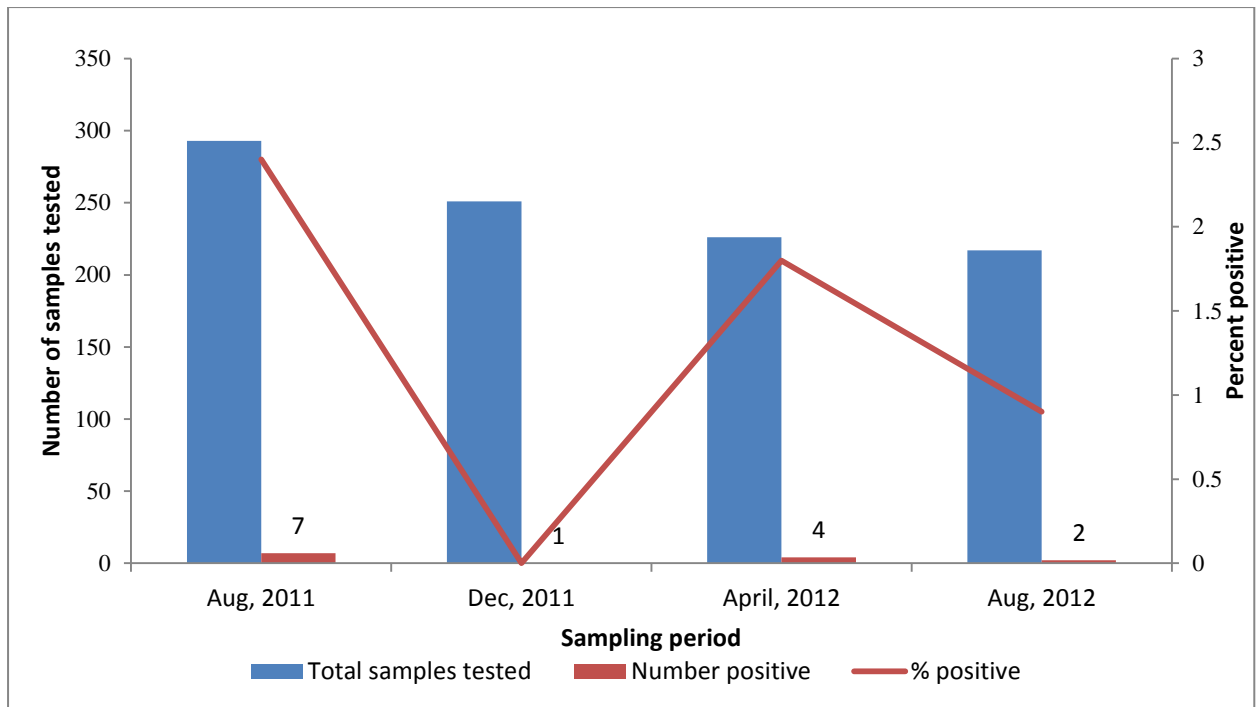
Figure 3.2: Temporal variation in influenza A seroprevalence in serum samples collected in animals in Kibera, Nairobi 2011-2012

3.4.6 Subtype specific anti-influenza antibodies

Eleven of the 14 (78.5%) influenza A seropositive sera were tested by hemagglutination inhibition (HI) assay. These sera (7 from pigs) were tested against a panel of swine influenza reference antigens A/California/04/2009(H1N1) pdm09, A/Swine/Texas/4199-2/98 triple-reassortant H3N2 and A/Swine/Iowa/15/30 H1N1. Suitable reference antigens could not be obtained for dog (n=2), chicken (n=1) and duck (n=1) sera hence the sera were only tested against the A/California/04/2009(H1N1) pdm09. There was an insufficient serum for the HI assay for one positive serum each from cat, chicken and dog.

Of the 7 pig sera that were positive, all (mean HI titer 274.3) were positive to the A/California/04/2009(H1N1) pdm09 antigen while one serum (14.3%) was also positive to A/Swine/Iowa/15/30 and H1N1 A/Swine/Texas/4199-2/98 triple-reassortant H3N2. When the criteria for determining the positive antigen in sera with polytypic cross-reactions was applied, the antigen with a HI titer fourfold or higher was considered the positive antigen, the sero-prevalence to A/California/04/2009(H1N1) pdm09 was 85.7% (n=6), while 14.3% (n=1) had polytypic reactions with all the three antigens hence the subtype could not be determined (Table 3.6).

For the two dog sera that were tested by HI against the A/California/04/2009(H1N1) pdm09 antigen, one was positive (HI titer 160) and one did not cross-react (HI titer 1:<10). The duck and chicken sera did not cross-react with A/California/04/2009(H1N1) pdm09 antigen, HI titers 1:10 and 1:40, respectively (Figure 3.3 and Table 3.6).
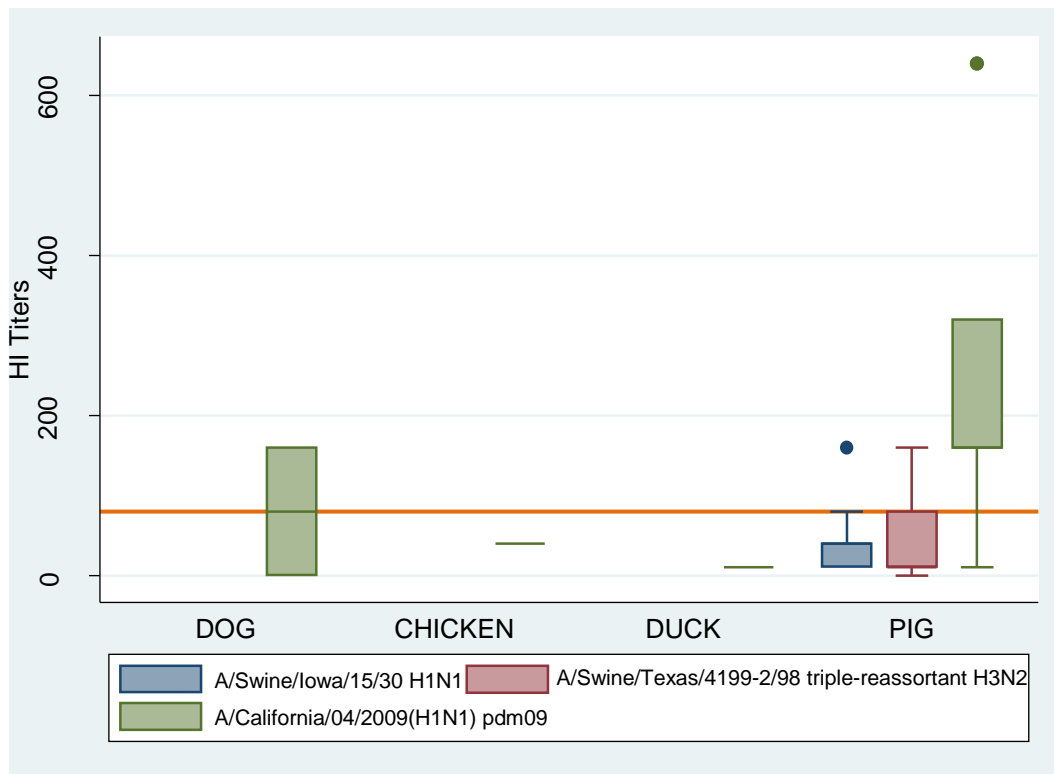
Figure 3.3: A box plot showing the distribution of HI titers for the sero-positive sera against three antigens for pig sera (n=7) and against the 2009H1N1pdm09 for chicken (n=1), duck (n=1) and dog (n=2). The orange line represents the HI cut off of 1:80.

Table 3.6 Hemagglutination Inhibition cross-reactivity proportions by animal species

Species	Number of sera tested	Hemagglutination Inhibition assay: Sera reactive‡ to test reference antigens†		
		A/California/04/2009(H1N1) pdm09	All†	Inconclusive*
		No. positive (%) [Binomial Exact 95% CI]	No. positive (%) [Binomial Exact 95% CI]	No. positive (%) [Binomial Exact 95% CI]
Pig	7	6(85.7) [42.1, 99.6]	1(14.3) [0.4, 57.9]	1(14.3) [0.4, 57.9]
Dog	2	1(50.0) [1.3, 98.7]	-	-
Chicken	1	0	-	1(100)
Duck	1	0	-	1(100)

‡ HI titers ≥ 1:80 were considered positive. For pig sera with polytypic reactions, the antigen with four fold or higher HI titer was considered the positive antigen
† Reference antigens used on the HI assay panel were: A/California/04/2009(H1N1) pdm09, A/Swine/Texas/4199-2/98 triple-reassortant H3N2 and A/Swine/Iowa/15/30 H1N1 for pigs sera while only A/California/04/2009(H1N1) pdm09 was used for dog, chicken and duck sera
*subtype could not be determined using the reference antigens in the HI panel

3.4.7 Prevalence of influenza A

A total of 4164 nasal and oropharyngeal swabs comprising of 2448 (58.8%) from Asembo and 1716 (41.2%) from Kibera were screened for influenza A nucleoprotein by rtRT PCR. Of these, 15/4164 (0.4%) were positive (Table 3.7). Overall 0.2% (4/2448) and 0.6% (11/1716) of swabs collected from animals in Asembo and Kibera respectively were positive for influenza A. Influenza A prevalence was highest at 10.0% (n=4, 95% CI 2.8%, 23.7%) in pigs followed by dogs at 0.8% (n=4, 95% CI 0.2%, 2.2%), ducks at 0.3% (n=1, 95% CI 0.01, 1.7) and chicken at 0.2% (n=7, 95% CI 0.1%, 0.4). None of the swabs collected from turkeys, cats and geese were positive (Table 3.7).

Of the influenza A positive swabs by rtRT PCR, 11/15 (73.3%) were from Kibera. Influenza A prevalence was highest in pigs 10% (95% CI, 2.8, 23.6), followed by chicken at 0.5% and ducks at 0.4%. Of the six positive swabs from chicken, 5 were oropharyngeal swabs and one was a cloacal swab; the proportion of the oropharyngeal swabs that were positive was statistically (p=0.03) higher than cloacal swabs that were positive at a level greater than 6.6%. In Asembo, influenza A was detected in dogs with a prevalence of 1.2% (95% CI 0.2, 3.4) and chicken with a prevalence of 0.05% (95% CI, 0.001, 0.3).

Table 3.7 Influenza A prevalence on rt RTPCR by study site and animal species, Kenya 2011-2012

	Kibera			Asembo		
	Number of samples tested	No. positive n(%)	Binomial Exact 95% CI	Number of samples tested	No. positive n(%)	Binomial Exact 95% CI*
Pig	40	4(10.0)	2.8, 23.6	-	-	-
Dog	141	0(0)	2.6*	256	3(1.2)	0.2, 3.4
Cat	52	0(0)	6.8*	15	0(0)	21.8*
Chicken	1191	6(0.5)	0.2, 1.1	2098	1(0.05)	0.001, 0.3
Duck	274	1(0.4)	0.01, 2.0	59	0(0)	6.1*
Turkey	16	0(0)	20.6*	20	0(0)	16.8*
Geese	2	0(0)	84.1*	-	-	-
Total	**1716**	**11(0.6)**	**0.3,1.1**	**2448**	**4(0.2)**	**0.04, 0.4**

*one-sided, 97.5% confidence interval reported

3.4.8   Temporal distribution of influenza A

In Kibera, Influenza A was detected in all the sampling periods and was highest in August of 2011 at a prevalence of 0.9% (95% CI, 0.3, 2.2) and lowest in August of 2012 at a prevalence of 0.3% (95% CI 0.001, 1.7) (Table 3.8). In pigs and ducks, influenza A was detected only once in August and December of 2011, respectively. There was no significant difference at 5% level in the prevalence of influenza A by sampling period in chicken. None of the swabs collected in dogs, cats, turkeys and geese in Kibera were positive for influenza A over this period.

In Asembo, influenza A was only detected in two of the sampling periods in 2012 (April and August) where 3 nasal swabs from dogs and one from chicken were positive, (Table 3.8). There was no significant difference at 5% level in the prevalence of influenza A in dogs between the two sampling periods. None of the swabs collected in August and December of 2011 were positive and none of the swabs collected from ducks, turkeys and cats were positive.

Table 3.8: Temporal distribution of Influenza A by study site and animal species, Kenya, 2011-2012

		Asembo			Kibera		
Sampling Period	Species	Total tested	Number positive n (%)	95% CI	Total tested	Number positive n (%)	95% CI
Aug, 2011	Chicken	545	0(0)	0.7	331	1(0.3)	0.01, 1.7
	Ducks	20	0(0)	16.8	92	0(0)	3.9
	Turkeys	10	0(0)	30.8	10	0(0)	30.8
	Dogs	78	0(0)	4.6	43	0(0)	8.2
	Cats	1	0(0)	97.5	18	0(0)	18.5
	Pigs	0	-	-	25	4(16.0)	4.5, 36.1
	Geese	0	-	-	2	0(0)	84.1
	Total	**654**	**0(0)**	**0.6**	**521**	**5(0.9)**	**0.3, 2.2**
Dec, 2011	Chicken	411	0(0)	0.9	321	2(0.6)	0.1, 2.2
	Ducks	15	0(0)	21.8	85	1(1.2)	0.01, 6.4
	Turkeys	0	-	-	6	0(0)	45.9
	Dogs	48	0(0)	7.4	39	0(0)	9.0
	Cats	0	-	-	9	0(0)	33.6
	Pigs	0	-	-	5	0(0)	52.2
	Total	**474**	**0(0)**	**0.8**	**465**	**3(0.6)**	**0.1, 1.9**
April, 2012	Chicken	608	1(0.2)	0.004, 0.9	297	2(0.7)	0.1, 2.4
	Ducks	12	0(0)	26.5	65	0(0)	5.5
	Turkeys	6	0(0)	45.9	0	-	-
	Dogs	67	1(1.5)	0.04, 8.0	27	0(0)	12.8
	Cats	1	0(0)	0	15	0(0)	21.8
	Pigs	0	-	-	8	0(0)	36.9
	Total	**692**	**2(0.3)**	**0.03, 1.0**	**412**	**2(0.5)**	**0.1, 1.7**
Aug, 2012	Chicken	534	0(0)	0.7	242	1(0.4)	0.01, 2.3
	Ducks	12	0(0)	26.5	32	0(0)	10.9
	Turkeys	4	0(0)	60.2	0	-	-
	Dogs	63	2(3.2)	0.4, 11.0	32	0(0)	10.9
	Cats	13	0(0)	24.7	10	0(0)	30.8
	Pigs	0	-	-	2	0(0)	84.2
	Total	**626**	**2(0.3)**	**0.04, 1.1**	**318**	**1(0.3)**	**0.00, 1.7**
Total		**2448**	**4(0.2)**	**0.04, 0.4**	**1716**	**11(0.6)**	**0.3, 1.1**

3.4.9 Spatial distribution households with influenza A positive animals

In Kibera, all the 23 animals that tested positive for Influenza A RNA or anti-influenza A antibodies were drawn from 13 households (Figure 3.4). Of these, ten households had one of dog, cat, chicken, duck or pig test positive; one household had two chicken testing positive, one household had seven pigs test positive and one household had three pigs and one dog testing positive for influenza A. In pigs there was clustering of influenza A occurrence at household level such that the four pigs that were positive for influenza A virus RNA were all from the same household. Clustering in influenza A virus occurrence was not evident for chicken, ducks and dogs that had more than one animal testing positive. In Asembo, four animals (three dogs and one chicken) tested positive for influenza A RNA from four households (Figure 3.5).
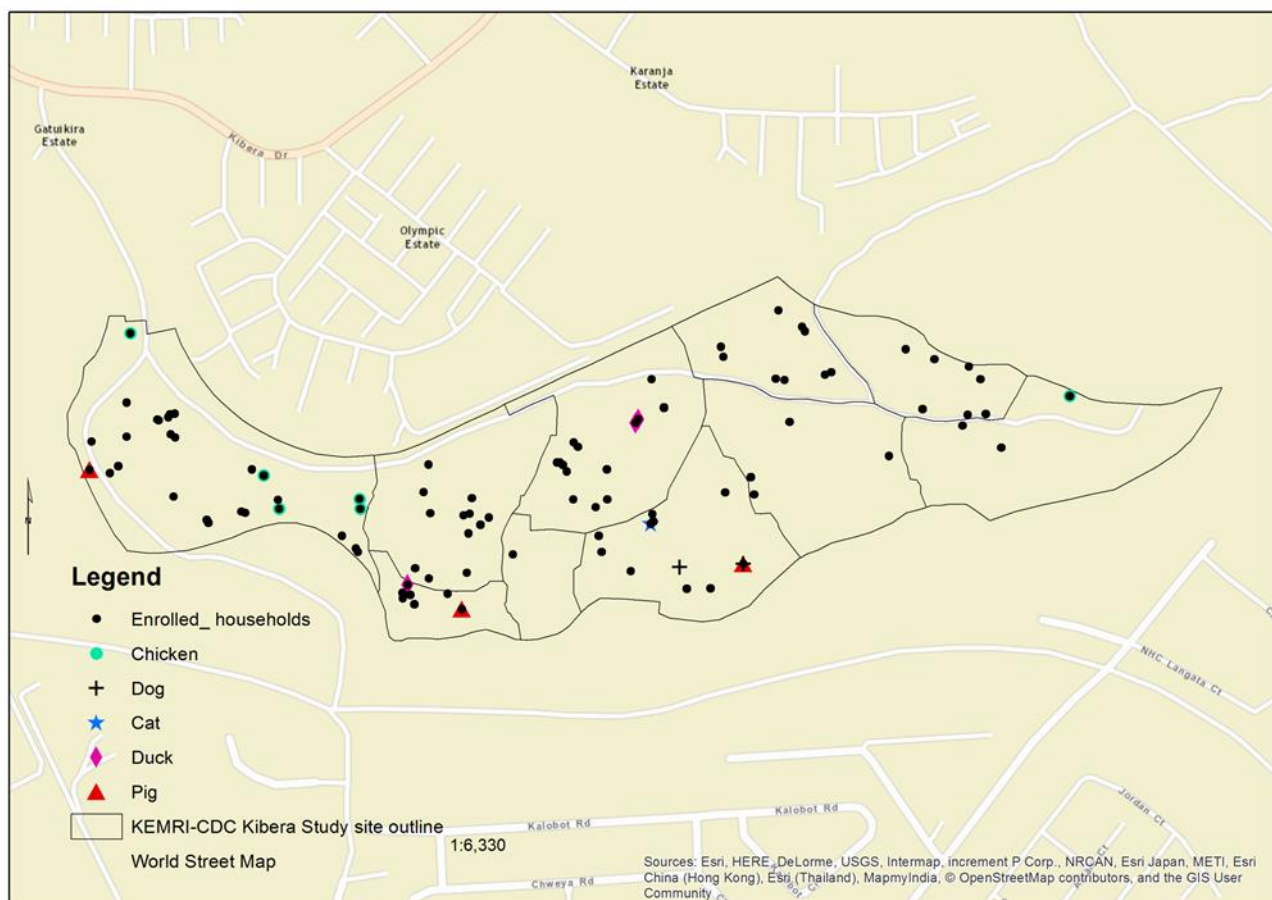
Figure 3.4: Spatial distribution of enrolled households and households with influenza A positive animals in Kibera, 2011-2012
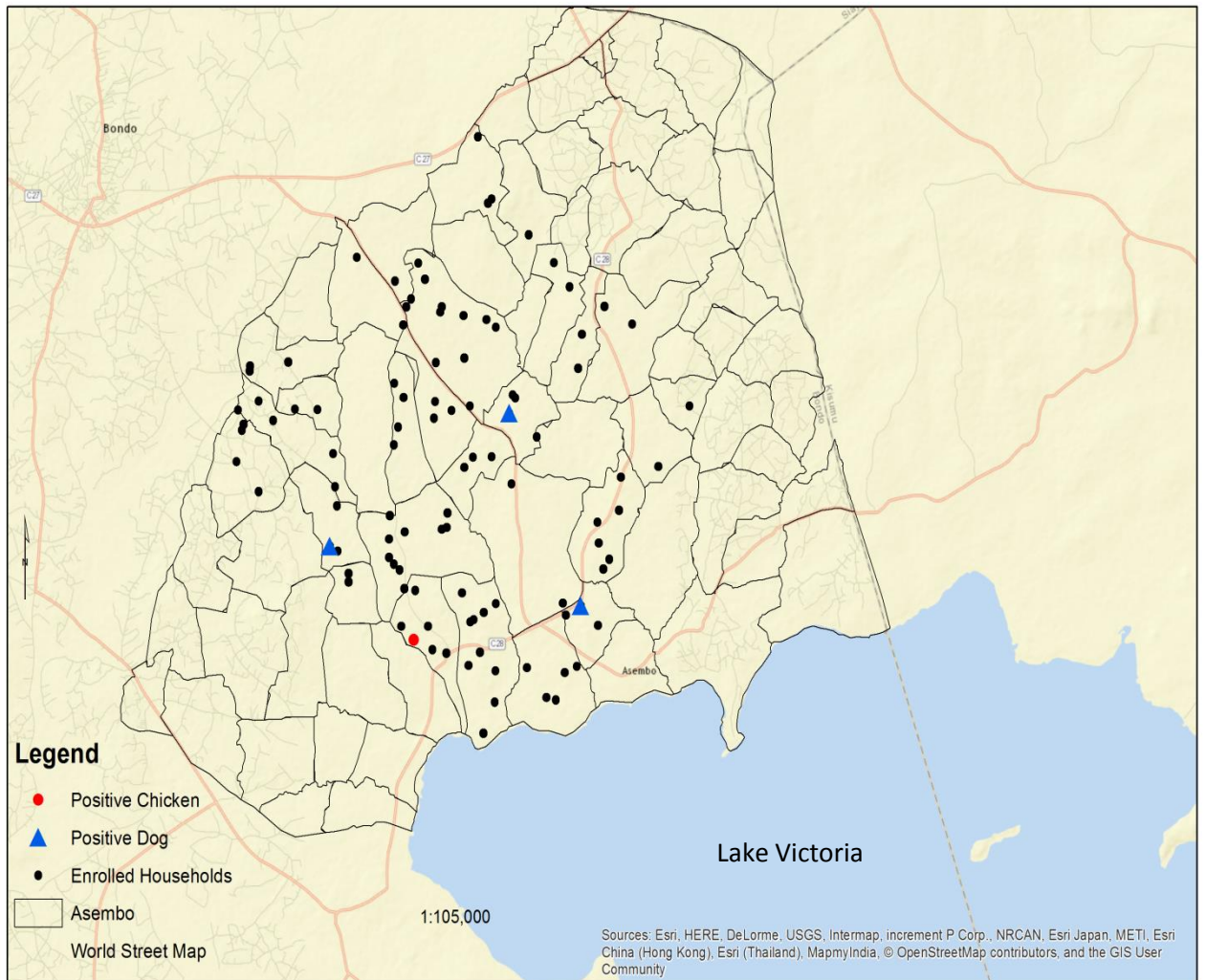
Figure 3.5 Spatial distribution of enrolled households and households with influenza A positive animals in Asembo, 2011-2012

3.4.10 Influenza virus isolates and subtypes

Virus isolation in MDCK cells was attempted for 6 of 7 [pig n=4; dog n=2] influenza A positive swabs and 12 influenza A negative nasal swabs from pigs. There was insufficient volume for cell culture for one dog nasal swab. Five virus isolates were obtained from pig nasal swabs collected on July 25th 2011 in Kibera. All four positive nasal swabs as well as one nasal swab that had tested negative for influenza A by rtPCR (CT values = 43.5) had virus isolated. All five isolates were identified as A (H1N1) pdm09 on subtyping using rtRT-PCR. No virus growth was observed from the two dog nasal swabs.

Virus isolation in embryonated chicken eggs was attempted in 7 of 8 Influenza A positive chicken and duck swabs [chicken n=6 and duck n=1]. There was no virus growth observed.

3.4.11 Animal level factors associated with influenza A positivity

On univariate analysis, neither age nor sex of the animal was found to be associated with influenza A positivity in all the species (Table 3.9).

Table 3.9 Association between Influenza A positivity and demographic characteristics by animal type and site, 2011-2012

Animal type	Combined		Asembo		Kibera	
	OR (95% CI)	p-value	OR (95% CI)	p-value	OR (95% CI)	p-value
Age in months						
Pig	1.0(0.9-1.1)	0.23	-	-	1.02(0.98-1.08)	0.23
Dog	0.9 (0.9-1.0)	0.97	0.9(0.9-1.0)	0.9	1.0(0.9-1.0)	0.89
Cat	1.0(0.9-1.1)	0.25	-	-	1.0(0.9-1.1)	0.2
Chicken	0.9(0.8-1.0)	0.1	0.8(0.4-1.0)	0.2	0.9(0.8-1.0)	0.5
Duck	0.9(0.6-1.1)	1.0	-	-	0.96(0.6-1.1)	1.0
Sex						
Pig	1.3 (0.1-78.6)	1.0	-	-	1.3(0.1-78.6)	1.0
Dog	0.9 (0.8-1.0)	0.9	0.9(0.9-1.0)	1.0	1.0(0.9-1.1)	0.9
Cat	0.9(0.9-1.1)	0.8	-	-	0.9(0.9-1.0)	0.9
Chicken	0.9(0.8-1.0)	0.5	0.8(0.4-1.0)	0.6	0.9(0.8-1.0)	0.8
Duck	0.9(0.8-1.0)	1.0	-	-	0.9(0.8-101)	1.0

OR- Odds ratio; CI – Confidence intervals

3.5   Discussion

This study reports, for the first time, isolation of influenza virus in pigs in Kenya. Five influenza viruses were isolated from nasal swabs collected from pigs from two households in Kibera in July 2011. The influenza viruses were identified as A/California/07/2009-like (H1N1) subtype on antigenic characterization, suggesting that the pandemic influenza virus that was first introduced in Kenya through humans in July 2009 (MMWR, 2009a) was circulating in pig populations. These results are consistent with the finding of the 2009 influenza pandemic strain in multiple countries in the US, Britain, Australia, Asia and in Africa (Hall, 1999 ; Anonymous, 2010b ; Ducatez *et al.*, 2011 ; Deng *et al.*, 2012 ; Njabo *et al.*, 2012). In Australia, influenza virus circulation in pigs was first reported with the introduction of pandemic virus into the pig population in 2010 (Deng *et al.*, 2012).

Overall, influenza A virus antibodies were detected in pigs, dogs, chicken and ducks but not in turkeys, geese and cats. Pigs had the highest prevalence of 18%, followed by dogs at 2.2%, ducks at 0.7% and chicken at 0.3%. The results in poultry are similar to those from Uganda where they reported influenza A prevalence of 0.4% in chickens and 2.7% in ducks (Kirunda *et al.*, 2014) who reported influenza A prevalence of 0.4% in chickens and 2.7% in ducks and in Mali where they reported prevalence of 1.2% in chicken but a higher prevalence of 5.2% in ducks were reported (Molia *et al.*, 2010). The study in Uganda reported influenza A prevalence of 2.6% in turkeys in contrast to this study where all the turkeys were negative. A previous study in Kenya among poultry at live bird markets reported slightly higher influenza A detection rates in chicken, geese and turkeys of between 1.6-2.4% while none of the ducks were positive (Munyua *et al.*, 2013). The higher prevalence reported in Kenya and Uganda in chicken, geese and turkeys sampled in live bird markets compared to the poultry sampled at

household level may have been due to increased potential for infection in poultry during transportation and housing in the market where birds from different farms are mixed and housed together (Webster, 2004). Studies in Taiwan, Vietnam and Hong Kong reported up to 30% influenza A prevalence in ducks in live poultry markets (Nguyen *et al.*, 2005).

The influenza A prevalence seroprevalence reported in dogs of 2.2% is comparable to that reported in dogs in China of 1.5% , (Sun *et al.*, 2014). Influenza A H3N8 and H3N2 and the 2009 pandemic virus strain have been reported in dog populations in Asia, Europe and the US (Harder and Vahlenkamp, 2010 ; Sun *et al.*, 2014). It's important to note that the sero-positive dog was sampled in the same household as the pigs that were positive for influenza A suggesting possible inter-species transmission. This study however, did not subtype the influenza virus circulating in dogs.

In pigs, influenza A prevalence observed in this study (10%) was higher compared to 1.4% reported in the Uganda study (Kirunda *et al.*, 2014). Studies from Nigeria reported prevalence of between 13.2% and 26.7% in pigs (Anjorin, 2012 ; Meseko *et al.*, 2014). The above two were field based surveillance studies. The higher prevalence observed in this study may have been due to the small sample size of the pigs sampled in this study and clustering of influenza infection in herds since all the pigs sampled were from two households.

The overall influenza A positivity by either test was significantly higher in Kibera (0.9%) than Asembo (0.1%). While mean age for dogs and chicken was significantly different between the two sites, age was not associated with influenza A positivity in either species. Considering influenza A positivity by either test in dogs, influenza A prevalence was 1.1% and 0.5% and 0.4% and 0.03% in chicken for Kibera and Asembo, respectively. It is likely that the animals

reared in Kibera had higher opportunity of virus transmission from humans given the high population density in this site. A study of incidence of influenza in humans in Kibera and Asembo found high rates of influenza in the two sites and slightly higher number of patients who presented with influenza like illness in Kibera tested positive for influenza A than Asembo (Katz *et al.*, 2012a) suggesting that perhaps there are factors that could be favoring influenza transmission in this site.

Among pigs, the observed sero-prevalence was higher than sero-prevalence reported in Uganda (4.6%) but lower than sero-prevalence reported in Nigeria (35%) (Kirunda *et al.*, 2014 ; Meseko *et al.*, 2014) . In many European countries where influenza is endemic in pig populations, >50% sero-prevalence was reported between 2002 and 2003 (Harrington, 2005). It is likely that influenza virus transmission from humans to pigs in Kibera contributed to the high sero-prevalence given that the influenza A (H1N1) pdm09 was most prevalent in these pig populations and in humans in Kenya during the same period (Katz *et al.*, 2012a ; Majanja *et al.*, 2013). The pandemic virus strain has been shown to be efficiently transmissible from humans to pigs through direct contact (Nelson *et al.*, 2012) and it is likely that for these pigs reared in Kibera that has a high human population density, there is opportunity for virus direct transmission from humans to pigs.

4. CHAPTER 4

PREVALENCE OF INFLUENZA VIRUSES AMONG PIGS PRESENTED FOR
SLAUGHTER, KIAMBU, KENYA

4.1   Introduction

Pig population in Kenya has been growing in the recent years. In 2009, pig population was
estimated at 364,645 (Kenya National Bureau of Statistics, 2009). About half of the total pig
population is found in Central (25.2%) and Western (24.1%) Provinces with Coast (1.4%) and
the North Eastern (7.6%) Province having the least number of pigs. There are two distinct
pig production systems in Kenya; intensive and semi-intensive that differ largely in pig
production intensity, accessibility to markets accessibility and availability of production
resources (Kagira *et al.*, 2010 ; Mutua *et al.*, 2011 ; Mbuthia *et al.*, 2015) .

Pigs reared under the intensive production system are confined maximizing on space with
more investment in commercial feed to attain market weight and increase the herd size
(Kagira *et al.*, 2010 ; Mutua *et al.*, 2011 ; Mbuthia *et al.*, 2015). Pigs reared in the free-range
system roam freely around the household and surrounding area, scavenging and feeding in the
street, from garbage dumps or from neighboring land or forests around villages (Kagira *et al.*,
2010 ; Mutua *et al.*, 2011 ; FAO, 2012b ; Thomas *et al.*, 2013 ; Mbuthia *et al.*, 2015).
Housing is dependent on prevailing weather conditions and mostly night shelter is provided to
protect them against theft and predators.

While both production systems are found in same geographical areas, the intensive production
system is more common in Central Kenya largely in response to market access and demand
for pork while the semi-intensive system is common in Western Kenya (Mutua *et al.*, 2011 ;

Mbuthia *et al.*, 2015). High cost of feeds and pig diseases in particular ecto-parasites (*Haematopinus suis* , *Sarcoptes scabiei* and ticks) , worms have been highlighted the greatest production challenge (Kagira *et al.*, 2010 ; Kagira *et al.*, 2013 ; Mbuthia *et al.*, 2015). Respiratory diseases affecting pigs in Kenya has not been studied.

The emergence of the 2009 influenza A H1N1 (pH1N1) in humans in 2009 in North America and its rapid spread across the world was accompanied by detection of the virus in animals including pigs, turkeys, ferrets, cats, dogs and cheetahs (Sponseller *et al.*, 2010 ; Mathieu *et al.*, 2010 ; Schrenzel *et al.*, 2011).   In most of these cases, direct contact of the pigs with infected persons was documented as the most likely source of infection to the pigs (Hofshagen *et al.*, 2009 ; Deng *et al.*, 2012).

Majority of cases where swine influenza viruses have been transmitted to humans has been associated with occupational and environmental exposures (Gaydos *et al.*, 2006 ; Peiris *et al.*, 2009 ; Terebuh *et al.*, 2010 ; Wong *et al.*, 2012), raising the need to identify and monitor circulating influenza strains in pig populations. Most recently between August 2011-2013 in the US, variant strains of H3N2v that typically occur in pigs were detected in humans where they caused a mild illness in 339 patients with limited human to human transmission reported (MMWR, 2012 ; CDC, 2013). The infections in humans were mainly associated with direct contact with pigs at state fairs (MMWR, 2012 ; Feng *et al.*, 2013).

Pigs play a key role in evolution of influenza viruses hence transmission and maintenance of the human origin influenza virus strains in pig populations raises the possibility of genetic reassortment with swine influenza virus that could result in emergence of novel viruses of pandemic potential (Brown, 2000a ; Vijaykrishna *et al.*, 2010 ; Ducatez *et al.*, 2011).

However, evolution pathways for influenza viruses to adapt to new hosts including humans is not well understood and extensive research is being undertaken to clarify the role of animal influenza and interspecies transmission in emergence of influenza strains capable of sustained transmission in humans (Capua and Munoz, 2013).

In Africa, there are limited data on influenza types circulating in pigs. Swine A (H1N1) influenza virus (SIV) subtypes have been reported historically in Africa (Brown, 2000b) although information regarding these earlier detections is very scanty. In Nigeria, high seroprevalence of 86% and 87% for H3N2 and H1N1 human influenza, respectively, in pigs was reported in 1990 (Olaleye *et al.*, 1990). More recently, limited 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 ; Anjorin, 2012). The study in Nigeria reported co-circulation of two subtypes of influenza A, H1 and H3 in pigs (Oluwagbenga, 2009). A concurrent study in Nigeria among pig handlers reported 96.7% (n=30) seroprevalence of multiple influenza A subtypes (Adeola and Adeniji, 2010) highlighting the possibility of transmission of swine viruses to humans.

Influenza A(H1N1)pdm09 virus was reported in limited pig populations in Cameroon in 2011 (Njabo *et al.*, 2012). A review of published data on swine influenza in Africa highlighted limited surveillance and paucity of data despite the growing evidence of multiple cross transmission of influenza viruses between humans and pigs globally, fairly large pig population and low biosecurity practices in most small scale holder farms that could promote influenza transmission between humans and pigs (Capua and Munoz, 2013 ; Meseko *et al.*, 2013).

Surveillance for influenza viruses can be carried out at farm or slaughterhouse level. While the farm level surveillance would provide detailed information on classes of pigs (piglets, growers, weaners and adults) that could be maintaining infection at farm level, surveillance at slaughterhouse level provides an opportunity to sample pigs from multiple farms across a wide geographic region, depending on the catchment area of the facility. Sampling a bigger population of pigs was also desirable to increase the opportunity of virus isolation, a major objective of this study.

The epidemiology of influenza viruses in pigs in Kenya is not known. In Kenya, pigs are not vaccinated against influenza viruses and the vaccine is not commercially available or licensed for use in the country. This study sought to identify influenza viruses circulating in pigs in Kenya where majority of pigs are raised in small holder farms that have close interaction with humans at farm level hence possible transmission pathways exist for influenza virus transmission from humans to animals.

4.2   Objectives

The main objective was to identify and characterize influenza viruses circulating in pigs presented for slaughter at the Ndumbu-ini slaughter house, in Kiambu County.

The specific objectives were to:

1. To determine the subtype influenza A viruses in pigs in Ndumbu-ini slaughterhouse
2. To estimate the sero- prevalence of influenza A viruses and subtypes circulating in pigs at Ndumbu-ini slaughterhouse

4.3   Materials and Methods

4.3.1   Description of Study Site

Ndumbu-ini slaughterhouse is the second largest pig slaughterhouse in Kenya after Farmers choice and is located 20 kilometers west of Nairobi in Kikuyu sub-county of Kiambu County (FAO, 2012b). It was established in 1972 and receives pigs mainly from small holder farms in Nairobi, Kiambu and other neighboring counties. It has an average kill of 50 pigs per day. This informed the decision to use the slaughterhouse as a site for this study. The slaughterhouse provided the opportunity to sample pigs over a period of time hence providing temporal and spatial distribution of influenza A virus infection in pigs. However, reliable data on the demographic characteristics of the animals at the farm of origin such as number of pigs by age-group could not be obtained from the traders. Data on individual demographics were not collected because the main objective was to determine the seroprevalence levels.

At the slaughterhouse, pigs are presented to the slaughterhouse in groups by the traders or individual farmers. A group of pigs may be coming from the same farm or from different farms where a trader buys pigs from different farms and pools them together as they are transported to the slaughterhouse. The pigs were delivered at different times and the slaughter process continues such that pigs were slaughtered as they were delivered.

4.3.2   Sample size determination

Influenza A sero-prevalence in pigs was estimated to be 10%, precision level of 5% and at 95% confidence level for sample size calculation. Using the formula described in section 3.3.3 and a design effect of 1.5 to account for clustering, the sample size for each sampling was determined to be 163 pigs.

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4.3.3 Animal sampling and selection of pigs

Sample collection was carried out in July and December 2011, and April, August and December 2012. Slaughter pigs were brought in groups from a common farm or pooled from several farms and brought by a trader. Pigs are slaughtered as they are delivered to the slaughterhouse sometimes without first being held at the holing pen prior to slaughter. Hence it was difficult to attain a random selection of the groups of animals to be sampled, all groups of pigs that were presented for slaughter were targeted for sampling and a convenient sample of four pigs from each group was selected for sampling. For groups where less than four pigs were delivered for slaughter, samples were collected from all pigs. To attain the required sample size for each sampling period, sampling was carried out on each consecutive day until the sample size was achieved.

4.3.4 Sample collection, processing and storage

Blood samples were collected immediately after stunning and sticking of the animals by collecting blood flowing from the jugular vein into plain 10 ml BD Vacutainer® glass tube and transported on ice to the KEMRI/CDC Biosafety Level (BSL) 3 laboratory in Nairobi for sera harvesting on each day of collection. Nasal swabs were collected by inserting a plastic shafted polyester swabs into the nasal canal of the pig. The swabs were then put into 2 ml cryo-vials (Greiner bio-one, Germany) containing 1ml of viral transport medium with antibiotics and transported on ice to the laboratory on each day of collection. Bronchiole swabs were collected from a proportion of pigs slaughtered by inserting a swab into the bronchiole of lungs of pigs after evisceration. All samples were clearly labeled using bar code labels at the point of collection.

Blood was allowed to clot and serum separated. Serum aliquots were dispensed into 2 ml cryo-vials (Greiner bio-one, Germany) and stored at -20^0C prior to testing while swabs were stored at -80^0C.

4.3.5 Data collection

A standardized questionnaire (Appendix 4) was administered to the person delivering the pigs to the slaughterhouse and information on size of the herd where the pigs came from, size of the group of pigs delivered and source of pigs was recorded. In some instances, this information was not available. Each animal that was sampled was assigned a unique identification number (ID) that was recorded on the questionnaire to identify animals sampled from the same group. In addition, all samples were assigned unique sample IDs using pre-printed bar code labels that were placed on the sample collected and the questionnaire. The unique sample ID was used to link the epi data (district of source of the pig, number delivered from the same farm and number at the source farm) with the laboratory test results. All data were collected using scannable teleforms. The teleforms were scanned and data stored in an MS Access database.

4.3.6 Influenza virus molecular and serological assessments

The procedures for virus identification, virus subtyping and serology described in section 3.3.8 were followed.

4.3.7 Data management and Analysis

Descriptive statistics were computed for number of pigs owned at the farm of origin and number of pigs sampled per group delivered using STATA 13.1 statistical software (StataCorp, College Station, TX). The study outcome was detection of influenza A virus and

serological evidence of infection with influenza A viruses in pigs for each of the sampling period. The overall sero prevalence and the sero prevalence by sampling period for both influenza A and by Influenza A subtypes were computed using the exact binomial method and reported with the associated confidence intervals in tables and figures. Mean titers for each of the reference antigens used in the HI assay were computed and reported in tables and figures.

To analyze for differences in the observed sero-prevalence by sampling period, individual seroprevalences were assessed for equality of variances across sampling periods using the Bartlett's test for equal variances. After running the test, the test statistic was larger than the critical value, hence the null hypotheses was rejected at the 0.05 significance level and concluded that at least one sampling period variance was different from the others. Subsequently, a non-parametric method, Kruskal-Wallis rank test was used to test whether the five independent groups had equal medians. The Kruskal-Wallis test compares the mean rank in each of the five groups against the overall mean rank, based on all combined values. If there is a significant difference among the five groups, the mean rank differs considerably from group to group.

Pos hoc analysis of pairwise comparisons was done to identify which sero-prevalence was significantly different using Bonferroni correction. Bonferroni correction was used to reduce the chances of obtaining false-positive results (type I errors) since 10 pair wise tests were performed on a single set of data. To perform a Bonferroni correction, the critical P value (α) was divided by 10 (the number of comparisons being made) and the new critical P value was 0.05/10. The statistical power of the study is then calculated based on this modified P value (0.005).

4.4   Results

4.4.1   Samples collected from pigs

Between August 2011 and December 2012, samples were collected from 778 pigs delivered to the slaughterhouse in 611 different groups as shown in Table 4.1. The average number of pigs sampled on each sampling period was 155 and each sampling period lasted on average 8 days.

Table 4.1: Summary of pigs sampled and other characteristics by sampling period at Ndumbu-ini slaughterhouse, 2011-2012

| Characteristics | | Sampling period Month, Year | | | | | |
		Aug, 2011	Dec, 2011	April, 2012	Aug, 2012	Dec, 2012	Total
Total no. of sampling days	n	8	6	8	9	8	39
Total no. of pigs sampled	n	162	151	155	160	150	778
Average no. of pigs sampled per day	n	20.2	25.1	19.3	17.7	18.7	19.9
Total number of groups sampled	n	133	126	122	100	130	611
No. of pigs at the farm of origin	Mean (n)*	18.2(4)	18.5(2)	78.5(2)	16(2)	95.6(6)	54.5(16)
	Median	19	18.5	78.5	16	19.5	17
	Min, Max	6,29	7,30	7, 150	8,24	2,500	2,500
No. of pigs per group delivered to the slaughterhouse	Mean (n)†	1.5(120)	1.8(106)	2.4(87)	1.6(97)	1.2(130)	1.4(540)
	Median	1	1	1	1	1	1
	Min, Max	1,10	1,11	1,13	1,6	1,7	1,13
No. of pigs sampled per group	Mean	1.3	1.2	1.3	1.6	2.3	1.5
	Median	1	1	1	1	2	1
	Min, Max	1,3	1,4	1,4	1,4	1,3	1,4

*n =mean number of pigs at the farm of origin for groups whose the data were available
†n = mean for pigs per group delivered to the slaughterhouse for groups whose data were available

Data regarding the number of pigs brought to the slaughterhouse was available for 540/611 (88.4%) of the groups. On average traders and farmers delivered pigs in groups of two and the mean number of pigs sampled per group was 1.5 (Table 4.1). Data regarding the number of pigs at homesteads were available for 16 of 611 (2.6%) groups: of these, the median number of pigs at homesteads was 17 (range = 2-500).

Of the 512 (65.8%) pigs sampled whose source district was known, 501 (97.8%) were from Kiambu County.

Figure 4.1: Map of Kenya showing the location of Ndumbu-ini slaughterhouse (red dot) and the source of pigs that were sampled

4.4.2 Seroprevalence of influenza A in pigs

A total of 759 of 778 pig sera collected were screened for anti-influenza A antibodies by ELISA. Overall, 129 of 759 pigs (16.9%) were positive for influenza A antibodies. The influenza A seropositive pigs were drawn from 114 of the 591 (19.3%) groups (Table 4.2). Majority of the groups (482/591; 81.5%) had only one pig sampled per group of which 17.8% were positive for influenza A antibodies (Table 4.2). Of the 109 groups of pigs delivered to the slaughterhouse that had two or more pigs tested, 8 (7.3%) had all the animals in the group seropositive. The intra-class correlation for positivity based on the total number of pigs tested for the 109 groups was low [ICC 0.06; se 0.11; 95% CI 0.0, 0.27].

Table 4.2: Frequency distribution of number of pigs sampled and percent testing positive per group in Ndumbu-ini slaughterhouse, 2011-2012.

Total number of pigs per group sampled	No. of groups (%)	Number of pigs per group testing positive n (%)			
		0	1	2	3
1	482 (81.5)	396 (82.2)	86 (17.8)	-	-
2	53 (8.9)	42 (79.2)	7 (13.2)	4 (7.5)	-
3	54(9.1)	39 (72.2)	9 (16.7)	2 (3.7)	4 (7.4)
4	2(3.7)	0 (0)	1 (50.0)	1(50.0)	0 (0)
Total	591	477 (80.7)	103 (17.4)	7 (1.2)	4 (0.7)

Only one pig was sampled in 82% of all pigs sampled and of these, 17.8% were positive.

4.4.2.1 Comparison of influenza A sero-prevalence by period of sampling

The highest influenza A seroprevalence (42.2%) was recorded during the July/August 2011 sampling period and the lowest (4.4%) recorded during the March/April 2012 sampling period. Generally, the influenza sero-prevalence seemed to decline from December 2011 and remained at low levels till December 2012 (Table 4.3).There was significant difference in the mean ranks of sero-prevalence recorded among the sampling periods (χ^2= 27.2, df =4, p=0.0001).

The sero-prevalence recorded by day of sampling varied greatly with the highest recorded in August 2011 where 19 of 20 samples (95%) collected were sero-positive. However, from the 39 days of sampling, none of the samples collected in 8 non-consecutive days (3 days in April 2012, 3 days in August 2012 and 2 days in December 2012) tested positive for influenza A (Figure 4.2).

Table 4.3: Influenza A seroprevalence by sampling period

| Sampling (Month, Year) | Influenza A ELISA | | | 95% confidence interval | |
	Total samples tested	No. Influenza A pos	Sero-prevalence (%)	Lower bound	Upper bound
Aug, 2011	161	68	42.2	34.5	49.9
Dec, 2011	150	35	23.3	16.4	30.1
April, 2012	150	7	4.7		8.1
Aug, 2012	157	8	5.1	1.6	8.5
Dec, 2012	141	11	7.8	3.3	12.2
Total	**759**	**129**	**16.9**	**14.3**	**19.6**
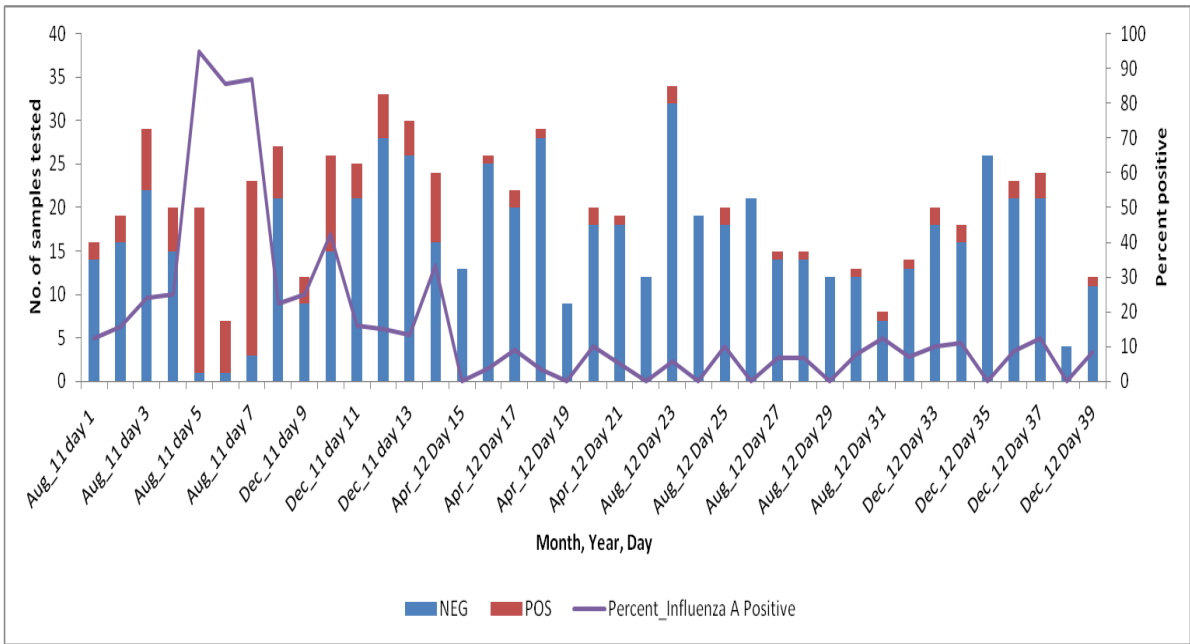
Figure 4.2: Number of samples tested and percent positive by day of sampling for a total of 39 non-contiguous sampling days.

4.4.3   Influenza A subtyping

In total, 126 of 129 (97.7%) influenza A seropositive sera by ELISA were tested by HI assay. Of those tested, 63.5% (80/126) were positive to the A/California/04/2009 (H1N1) pdm09 antigen compared to 25.4% (32/126) that were positive to the A/Swine/Texas/4199-2/98 triple-re-assortant H3N2 and 19.0% (24/126) that were positive to the A/Swine/Iowa/15/30 H1N1(Table 4.4). Generally, the mean HI titers for the pandemic virus strain were five times higher compared to the other two virus strains used in the HI panel which could reflect the antigenic similarity of the virus strain to the influenza virus strain circulating in pigs during this period (Table 4.4). The distribution of the HI titers for the three antigens by the sampling period is shown in Figure 4.3. None of the 8 sera collected in August 2012 cross-reacted with the three reference antigens used in the HI assay panel.

Generally, the median HI titers to the pH1N1 were higher in December of 2011 and December of 2012 compared to the other sampling periods, (Figure 4.3). The interquantile ranges for four sampling periods were reasonably similar, though overall, the range of the HI titers was greater for samples collected in August and December of 2011 and December 2012.

Table 4.4: Distribution of HI titers for the three reference antigens used in the HI panel

Reference antigen used in the HI panel	No. positive (%)* [Binomial Exact 95% CI	Mean HI titers (sd)	Min, Max
A/California/04/2009 (H1N1) pdm09	80 (63.5) [54.4, 71.9]	208.2 (285.1)	10,1280
A/Swine/Texas/4199-2/98 triple-re-assortant H3N2	32 (25.4) [18.1, 33.9]	39.0 (49.2)	10,320
A/Swine/Iowa/15/30 H1N1	24 (19.0) [12.6, 27.0]	39.7 (68.3)	10,640

*percentages do not add up 100% due to polytypic cross-reactions with two or all three antigens
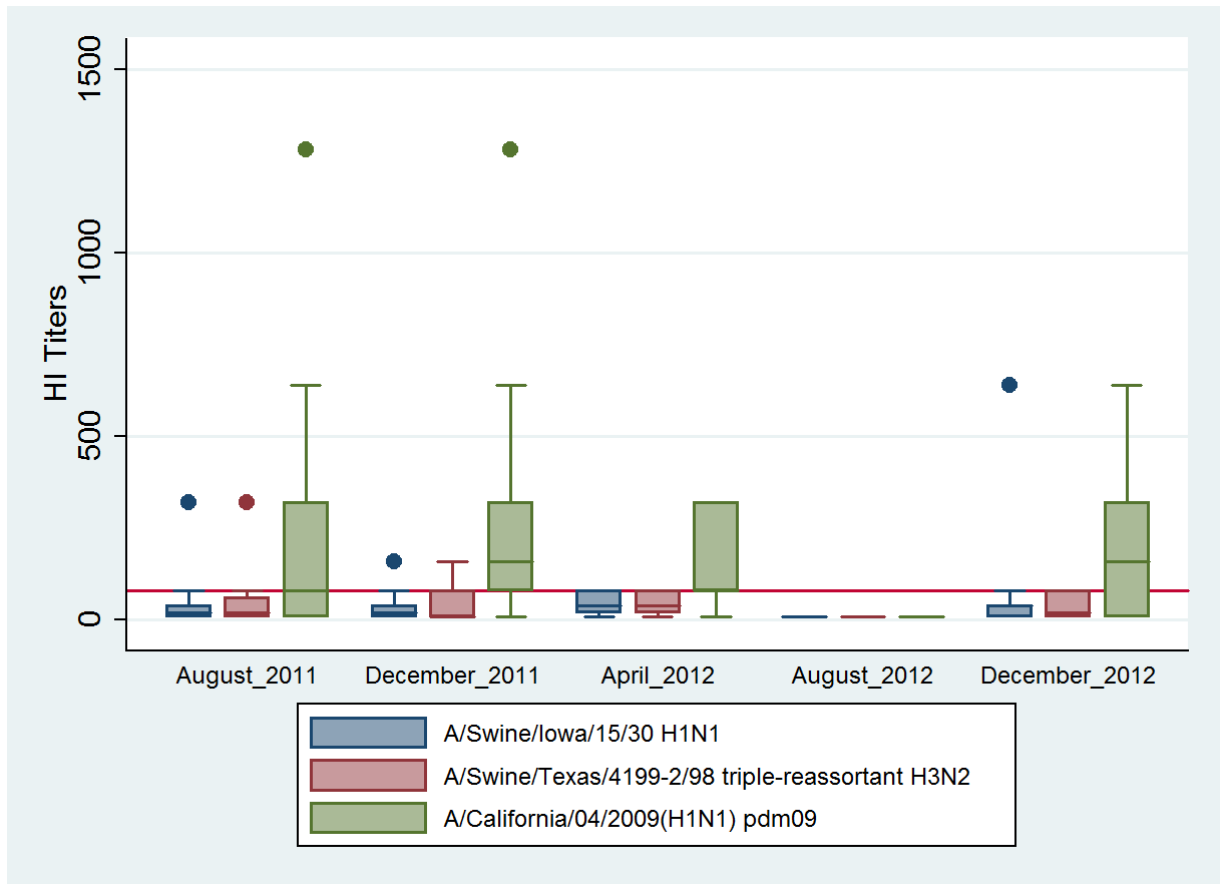
Figure 4.3: A box plot showing the distribution of HI titers for ELISA sero-positive sera against three influenza antigen by period of sampling.

The red line represents the HI cut-off of 1:80. Note only the one reference antigen (A/California/04/2009(H1N1) pdm09 had positive responses in four of the five sampling periods.

When the criteria for determining the positive antigen in sera with polytypic cross reactions was applied, the antigen with a corresponding fourfold or higher HI titer was considered the positive antigen, the sero-prevalence to A/California/04/2009(H1N1) pdm09 was 47.6% (n=60), 0.8% (n=1) to the A/Swine/Texas/4199-2/98 triple-reassortant H3N2 and none to the A/Swine/Iowa/15/30 H1N1. In total, 17.4% (n=22) had polytypic reactions with two or all the three antigens (Table 4.4). However, 34.1% (n=43) of the sera did not cross-react with any of the antigens used and hence the subtype could not be determined (Table 4.5).

Table 4.5: Prevalence of influenza A subtypes in pigs at different sampling times in Ndumbu-ini slaughterhouse Kiambu, Kenya

Sampling Period (Month, Year)	Number of samples tested by HA/HI	Hemagglutination Inhibition assay: Sera reactive‡ to test reference antigens† n (%)						
		A(H1N1)pdm09	A(H3N2)	A(H1N1)pdm09 & A(H1N1)	A(H1N1)pdm09 & A(H3N2)	A(H1N1) & A(H3N2)	A(H1N1)pdm09 & A(H3N2)& A(H1N1)	Inconclusive*
Aug, 2011	68/68	27(39.7)	1(1.5)	0(0.0)	4(5.9)	2(2.9)	7 (10.3)	27(39.7)
Dec, 2011	35/35	25(71.4)	0(0.0)	0(0.0)	2(5.7)	0(0.0)	2(5.7)	6(17.1)
April, 2012	6/7	3(50.0)	0(0.0)	1(16.7)	0(0.0)	0(0.0)	1(16.7)	1(16.7)
Aug, 2012	6/8	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	6(100.0)
Dec, 2012	11/11	5(45.5)	0(0.0)	2(0.0)	1(9.1)	0(0.0)	0(0.0)	3(27.3)
Total	**126/129**	**60(47.6)**	**1(0.8)**	**3(2.4)**	**7(5.5)**	**2(1.6)**	**10(7.9)**	**43(34.1)**

‡ HI titers ≥ 1:80 were considered positive. With polytypic reactions, the antigen with four fold or higher HI titer was considered the positive antigen
† Reference antigens used on the HI assay panel were: A/California/04/2009(H1N1) pdm09, A/Swine/Texas/4199-2/98 triple-re-assortant H3N2 and A/Swine/Iowa/15/30 H1N1
*Sera were not reactive to any of the three antigens used in this panel

4.4.4 Sero-prevalence of the influenza A(H1N1) pdm09 across sampling periods

For pigs that were influenza A positive, the influenza A/California/04/2009(H1N1) pdm09 sero-prevalence was 47.6% (n=60), (Table 4.6). Comparison of the observed sero-prevalence for each sampling period revealed that at least one of the sero-prevalence were significantly different (χ^2 with ties 27.21, df =4, p=0.0001). Results of post hoc analysis of the sero-prevalence between different sampling periods showed that there was no significant difference in the sero-prevalence by month of sampling for the sampling periods in 2012 (April, August and December) and for the sampling periods in 2011 (August and December). However, the sero-prevalence was significantly higher for the sampling periods in 2011 compared to the sampling periods in 2012 (p<0.005), (Table 4.6 and Table 4.7).

Table 4.6:   Seroprevalence of A/California/04/2009(H1N1) pdm09 in pigs by sampling periods

Sampling Period (Month, Year)	Total tested	Number positive n(%)	95% confidence Interval	
			Lower bound	Upper bound
Aug, 2011	68	27 (39.7)	28.0	52.3
Dec, 2011	35	25 (71.4)	53.6	85.3
April, 2012	6	3 (50.0)	11.8	88.1
Aug, 2012	6	0 (0.0)	-	-
Dec, 2012	11	5 (45.4)	16.7	76.6
Total	**126**	**60 (47.6)**	**38.6**	**56.7**

Table 4.7: Pairwise† comparisons of sero-prevalence by sampling period

Comparison sampling month and year	Comparison sero-prevalence	Chi-square with ties	p-value
Dec 2012 & Aug 2012	7.8; 5.1	1.15	0.283
Dec 2012 & April 2012	7.8; 4.7	1.78	0.182
Dec 2012 & Dec 2011	7.8; 23.3	9.62	0.001*
Dec 2012 & Aug 2011	7.8; 42.2	10.97	0.0009*
Aug 2012 & April 2012	5.5; 4.7	0.621	0.430
Aug 2012 & Dec 2011	5.5; 23.3	10.261	0.0014*
Aug 2012 & Aug 2011	5.5; 42.2	11.755	0.0006*
April 2012 & Dec 2011	4.7; 23.3	9.685	0.0019*
April 2012 & Aug 2011	4.7; 42.2	11.361	0.0008*
Dec 2011 & Aug 2011	23.3; 42.2	0.505	0.477

Key
† Using Kruskal Wallis equality-of-populations rank test and Bonferroni correction was used to determine the critical p-values at alpha = .05/10 = .005
*Statistically significant different sero-prevalence between the sampling periods

4.4.5 Influenza A virus RNA in animals

Influenza A was detected in 4/946 (0.4%) of nasal (n=2) and bronchiole (n=2) swabs (Table 4.8). The influenza A was detected in two sampling periods of August 2011 and April 2012. The four pigs that were positive for influenza came from different groups. Virus isolation was done in all four positive swabs. Three isolates were obtained from nasal swabs collected in August 2011. The isolates were identified as A (H1N1) pdm09 on subtyping using rtRT-PCR.

Table 4.8: Prevalence of influenza A by rtRT PCR in pigs by sampling periods in Ndumbu-ini slaughterhouse

Sampling Period (Month, Year)	rtRT PCR testing				Total bronchial swabs tested	No. positive n (%)	Virus isolation
	Number of nasal swabs tested	No. positive n (%)	Virus isolation	Influenza A subtype			
Aug, 2011	161	2(1.2)	Pos	A(H1N1) pdm09	-	-	-
Dec, 2011	150	0(0.0)	-	-	51	0(0.0)	-
April, 2012	154	0(0.0)	-	-	58	2(3.4)	Neg
Aug, 2012	157	0(0.0)	-	-	65	0(0.0)	-
Dec, 2012	149	0(0.0)	-	-	1	0(0.0)	-
Total	**771**	**2(0.26)**	**3***		**175**	**2(1.1)**	**0**

Key:

*one virus isolate was obtained from a nasal swab that had tested negative (CT value 43.0) for influenza A on rt RT-PCR.

4.5   Discussion

This study reports circulation of pandemic virus A (H1N1) pdm09 (pH1N1) among pigs presented for slaughter in Kiambu, Kenya. In this study, 16.9% of the pigs sampled over a fifteen month period had antibodies against influenza A. Over half (63.5%) of influenza A seropositive pigs had antibodies against pandemic H1N1 (2009) confirming the widespread introduction and spread of the virus after its introduction into the Country in July 2009 (MMWR, 2009a ; Majanja *et al.*, 2013). The high seroprevalence of the pandemic H1N1 (2009) virus in the pigs in Kenya in the period following introduction of the pandemic virus in the country is consistent with studies that have demonstrated high transmissibility of the virus in naïve pig populations as demonstrated in infection and transmission study with A California (H1N1) (Brookes *et al.*, 2010).

There were minimal reactivity with the swine A/Swine/Texas/4199-2/98 triple-reassortant H3N2 and A/Swine/Iowa/15/30 H1N1. This was not surprising given that these North American swine influenza strains have not been detected in Kenya or Africa in general.

Other unidentified swine influenza virus (SIV) strains could be circulating in pigs in Kenya given that 34.1% of the influenza A positive sera were non-reactive to pandemic H1N1 (2009), North America H3N2 and H1N1, and swine A/Swine/Texas/4199-2/98 triple reassortant. In addition, antigenic variants of the AH1N1pdm09 circulating in this population cannot be ruled out since HI assay were not carried using the local A (H1N1) pdm09 isolates obtained in the pig population over the sampling period. Further, sporadic infections in pigs with prevailing human influenza viruses that may not persist is known to occur (Brown, 2000a) hence it is probable that a proportion of the HI non-reactive ELISA-positive sera may have been from infections with human influenza A(H1N1) and A(H3N2) that was circulating

in the human population during this study period (Majanja *et al.*, 2013). Ultimately, the lack of data on circulating strains of influenza virus in pigs in Kenya and other African countries made it difficult to identify appropriate SIV antigens for complete serology (Meseko *et al.*, 2013 ; IRD, 2013).

Influenza virus RNA was detected in nasal swabs in only two of the five sampling periods and Influenza A (H1N1) pdm09 was isolated from nasal swabs of three pigs in 2011. Little is known about the influenza subtypes circulating in African pig populations (Meseko *et al.*, 2013). The findings of this study are comparable to results from Nigeria and Cameroon where exposure to pH1N1 was detected and virus isolated in pig populations (Oluwagbenga, 2009 ; Anjorin, 2012 ; Njabo *et al.*, 2012) and in multiple other countries globally (Ducatez *et al.*, 2011). However, our study demonstrated persistent circulation of virus over a two year period. Since the first detection of A (H1N1) pdm09 in pigs in Canada primarily associated with human to pig transmission (Howden *et al.*, 2009) and subsequently in multiple countries around the world, establishment and evolution of the pandemic virus in pig populations with or without significant antigenic changes observed over the years has been reported (Vijaykrishna *et al.*, 2010 ; Brookes *et al.*, 2010 ; Ducatez *et al.*, 2011).

 In this study, exposure to the pandemic virus strain was observed in five of the six sampling periods in over half of the sero-positive pigs suggesting establishment in this population. It is important to note that influenza vaccines are not used in pigs in Kenya hence all the sero-positives are believed to be due to natural virus infections.

In Kenya smallholder pig production system rear less than 10 pigs per farm that mature for slaughter in 8-12 months. The existing farm to slaughterhouse marketing networks provides

opportunity for transmission of viruses across farms due pooling of pigs from different farms and low biosecurity standards on the farms such that as traders move from farm to farm in search of pigs, there is potential for spread of the virus through indirect contact. Pigs that were sero-positive for A (H1N1) pdm09 were from 74 different groups sampled over the 15 months period. This finding suggests widespread spatial and temporal exposure of the local Kenyan pig population to the pandemic virus either through persistent circulation throughout the year within the species or through repeated introductions from the humans. Influenza viruses have been shown to circulate throughout the year in the human population in Kenya (Bulimo *et al.*, 2008 ; Majanja *et al.*, 2013) and results of this study suggest that SIVs could be circulating in the pig population throughout the year.

This study had some limitations. Clinical data from the animals in the study were not collected and cannot therefore examine the association of illness with influenza infection or previous exposure. In addition, only mature pigs presented for slaughter were slaughtered hence cannot determine variability in influenza sero-prevalence among different pig age-groups. Furthermore, as discussed above, complete serology was not possible due to lack of data on circulating SIVs in the region. Regardless of these limitations, these findings provide critical knowledge on the epidemiology of swine influenza viruses in a tropical ecology.

Pigs are known to play an important role in the epidemiology of influenza A viruses and the A(H1N1)pdm09 specifically (Brown, 2000b ; Smith *et al.*, 2009) and the findings of this study highlight the need for continued monitoring of influenza strains circulating in pigs as well as transmission of influenza strains between humans and pigs in this region. These findings contribute in part to the understanding of the epidemiology of influenza viruses in pigs in Africa, a region where despite the economic importance that pigs play in the

livelihoods of rural populations, limited or no surveillance is being carried out on this disease of great global public health importance. Such data are very useful in assessing the risk posed by pigs in regard to zoonotic influenza including pandemic strains in this region.

5. CHAPTER 5

MOLECULAR CHARACTERIZATION OF SWINE INFLUENZA VIRUSES FROM
KIBERA AND NDUMBUINI SLAUGHTERHOUSE, 2011

5.1   Introduction

Phylogenetic analysis of the pandemic virus strain A (H1N1)pdm09 found that the virus was
a swine–human–avian triple-reassortant virus with gene segments of the polymerase basic
protein 2 and polymerase acidic protein from avian, polymerase basic protein 1 from human
H3N2, hemagglutinin, nucleoprotein and nonstructural protein from the classical swine
lineage, and the neuraminidase and matrix protein from Eurasian avian-like swine H1N1
lineage (Garten *et al.*, 2009). Subsequent to introduction in humans in many countries, the
virus had been isolated in pigs with occasional mutations and reassortment with other
circulating subtypes in human and pig populations (Vijaykrishna *et al.*, 2010 ; Ducatez *et al.*,
2011 ; MMWR, 2012 ; CDC, 2013 ; Feng *et al.*, 2013).

The pandemic virus influenza A(H1N1)pdm09 was first detected in Kenya in humans in July
2009 (MMWR, 2009a). Subsequently, the virus strain has been shown to co-circulate in
humans together with influenza AH3N2 and influenza B (Majanja *et al.*, 2013 ; Rajao *et al.*,
2013). The isolation of the virus in pigs suggests that the virus was transmitted to and became
established in local pig population similar to observations in other countries. In addition,
subsequent cross transmission between humans and pigs could be occurring.

To determine genetic changes among the swine virus isolates, phylogenetic analysis of
influenza A(H1N1)pdm09 strains obtained from the swine were compared to virus isolates

that circulated in humans between July 2009 and December 2012 as well as select swine isolates from Africa, Europe and North America. The NA and HA segments were selected for this analysis since they are the main antigens of the influenza viruses.

5.2 Objectives

The objective was to compare isolated influenza viruses with other viruses from Kenya and globally in Gen Bank.

5.3 Materials and methods

5.3.1 Whole genome sequencing

Four swine influenza isolates obtained for this study underwent whole genome sequencing and analysis. Briefly, RNA was extracted using the Qiagen QIAamp RNA extraction kit (Qiagen Inc., Valencia, CA) following the instructions of the manufacturer and as described in section 3.4. Amplicons were obtained using primer Uni 12 (M) 5'-AGCRAAAGCAGG-3'which is complementary to the conserved 3' end of all influenza A virus RNA segments in 25 μL reactions using the One Step RT-PCR Kit (QIAGEN, Valencia, CA). Amplicons were treated with shrimp alkaline phosphatase–exonuclease I (ExoSapI) (U.S Biologicals, Swampscott, MA, USA) and sequences were obtained using an ABI 3130xl Genetic Analyzer (Life Technologies).

5.3.2 Nucleotide sequence accession numbers

Complete sequences of four of the eight viruses isolated from swine in this study were submitted in GenBank. Accession numbers assigned to the sequences determined in this study were KJ680515 to KJ680545 (Table 5.1). The sequences for the HA and NA segments are presented in Appendix 3.

Table 5.1: List of the Kenya swine isolates strain names and GenBank accession numbers

Date of collection	Site collection	Influenza subtype by antigenic characterization	Sequence data	Strain Name	GenBank accession number (Reference)
7/25/2011	Kibera	A/California/07/2009-like (H1N1)pdm09	Complete	A/swine/Kenya/9455/2011	KJ680515 for S1, KJ680519 for S2, KJ680523 for S3, KJ680527 for S4, KJ680531 for S5, KJ680535 for S6, KJ680539 for S7, KJ680543 for S8
7/25/2011	Kibera	A/California/07/2009-like (H1N1)pdm09	Complete	A/swine/Kenya/9469/2011	KJ680516 for S1, KJ680520 for S2, KJ680524 for S3, KJ680528 for S4, KJ680532 for S5, KJ680536 for S6, KJ680540 for S7, KJ680544 for S8
7/25/2011	Kibera	A/California/07/2009-like (H1N1)pdm09	Complete	A/swine/Kenya/9470/2011	KJ680517 for S1, KJ680521 for S2, KJ680525 for S3, KJ680529 for S4, KJ680533 for S5, KJ680537 for S6, KJ680541 for S7, KJ680545 for S8
8/5/2011	Ndumbu-ini Slaughterhouse	A/California/07/2009-like (H1N1)pdm09	Complete	A/swine/Kenya/1613/2011	KJ680514 for S1, KJ680518 for S2, KJ680522 for S3, KJ680526 for S4, KJ680530 for S5, KJ680534 for S6, KJ680538 for S7, KJ680542 for S8

5.3.3   Phylogenetic analysis of the HA genes

A Basic Local Alignment Search Tool (BLAST) search was performed using the HA nucleotide sequences of the four swine isolates from this study to identify sequences with high homology deposited in Influenza Research Database at http://www.fludb.org/brc/influenza_sequence_search_segment_display.spg?method=ShowCleanSearch&decorator=influenza. A total of 52 (23 from the North America, 15 from Europe, 12 from Africa and 2 from Asia) human H1N1 collected between 2009 and 2011 were identified. For detailed phylogenetic analysis, 22 influenza virus isolates were selected, including   12 isolates from Kenya, and 7 were from Europe and 3 from USA, (Appendix 6). In addition, the original pandemic influenza strain A/California/04/09 was included in the analysis.

Since no swine isolates were identified through BLAST, a sequence search in Influenza Research Database was conducted to identify HA segments of influenza A H1N1pdm09 swine influenza isolates collected between 2009 and 2012 from Africa, Europe and North America as the search criteria. In Europe, 49 isolates were found and five of these were randomly selected; from North America, a total of 197 HA isolates were found and 2 were randomly selected and the only two swine isolates from Africa were included, (Appendix 6).

A BLAST was performed using the NA nucleotide sequences of the four swine isolates and 100 isolates were identified comprising of 3 swine and 97 human isolated collected in North America (n=40), Europe (n=36), Russia (n=16), Asia (n=7) and Australia (n=1) between 2010 and 2011. From these, 22 isolates comprising of the 3 swine isolates from North America

(n=2) and Asia (n=1) and a random selection of 19 human isolates of which 10 were from

North America , seven from Europe and two from Asia. Since no Kenya NA isolates were

identified through BLAST, a sequence search in Influenza Research Database was conducted

to identify NA segments of influenza A H1N1pdm09 influenza isolates collected in humans in

Kenya between 2009 and 2012. Nine human isolates collected in 2009 were identified. In

addition, two isolates from Africa and the A\California\04\09 were included, (Appendix 6).

Using web browser to access NCBI BLAST at http://blast.ncbi.nlm.nih.gov/Blast.cgi ,

selected isolates were added using the Accession numbers to the Alignment Explorer window.

Multiple sequence alignments were performed in BioEdit (Hall, 1999). Phylogenetic analyses

were performed with MrBayes version 3.1 with a general time-reversible (GTR) substitution

model (Ronquist and Huelsenbeck, 2003). The Bayesian posterior probability is the

probability that the phylogenetic tree is correct, given the observed data (nucleotide

sequences) and existing knowledge (Huelsenbeck and Rannala, 2004). Posterior probabilities

were reported for each node of the tree branch. The phylogenetic tree was edited in Inkscape

ver 1.1(Harrington, 2005).

5.4   Results

The four hemagglutinin gene segments of the Kenya swine H1N1 influenza isolates had 1710 nucleotides each. On Multiple sequence alignments of the four HA genes, A/swine/Kenya/9469/2011, A/swine/Kenya/9455/2011 and A/swine/Kenya/9470/2011 isolated from pigs in Kibera had 100% nucleotide identity while A/Swine/Kenya/1613/2011 had three nucleotide substitutions; adenine to cytosine at position 1038, guanine to adenine at position 1265 and adenine to guanine at position 1412 of the HA gene segment.

On BLAST, 52 human pandemic H1N1 viruses collected between 2009 and 2011 had high nucleotide sequence identity (approximately 99%) with the Kenya swine isolates. On constructing the phylogenetic tree, the hemagglutinin genes (HA) of the four Kenya swine H1N1 isolates clustered together, branching out with the human pandemic H1N1 (2009) influenza viruses isolated in Kenya during the same period (Figure 5.1). The posterior probability of the branch for the Kenya 12 human and 4 swine influenza viruses was 100% with all the viruses having been collected in 2011. Within that branch, the four swine viruses and one human isolate (A/Kenya/134/2011) branched out with 100% posterior probability to form one cluster. Among the four swine viruses, the three isolates from pigs in Kibera branched separately with 94% posterior probability from A/Swine/Kenya/1613/2011 from the slaughterhouse pigs.

 The Kenya swine H1N1 viruses were more distant from the two other African H1N1 swine isolates from Nigeria. The Kenya swine viruses were also distant from   human and swine H1N1 viruses from Europe, North America and Asia. The tree was rooted with A/California/04/09 isolated from human at the start of the 2009 influenza pandemic.

The four Neuraminidase genes (NA) gene segments had 1410 nucleotides each. On Multiple sequence alignments of the four NA genes, overall there was 99% nucleotide sequence identity. A/swine/Kenya/9455/2011 and A/swine/Kenya/9470/2011 had 100% sequence identity. A/Swine/Kenya/1613/2011 had a thymine to cytosine substitution at position 563 while A/swine/Kenya/9469/2011 had adenine to purine substitution at position 991 of the NA gene.

On BLAST, 100 viruses comprising of 97 human and 3 swine pandemic H1N1 viruses collected between 2010 and 2011 had 99% nucleotide sequence identity with the Kenya swine isolates. The Kenya swine NA of the influenza virus isolated in pigs formed a cluster with 99% posterior probability for the branch. Within that branch, the A/Swine/Kenya/1613/2011 isolated from a pig in Ndumbu-ini slaughterhouse showed slightly longer genetic distance compared to the three swine isolates from Kibera pigs (Figure 5.2). The Kenya swine isolates clustered with other global swine and human NA sequences with 100% posterior probability but far from the Kenya human isolates from 2009 and the two swine isolates from Africa, A/Swine/Cameroon/11rs149-198/2010 and A/Swine/Nigeria/12VIR4047-09/2011. From the phylogenetic tree, the Kenya human isolates from 2009 showed shorter genetic distance from the parent pandemic strain A/California/04/2009 in comparison to global viruses isolated in 2010 and 2011.
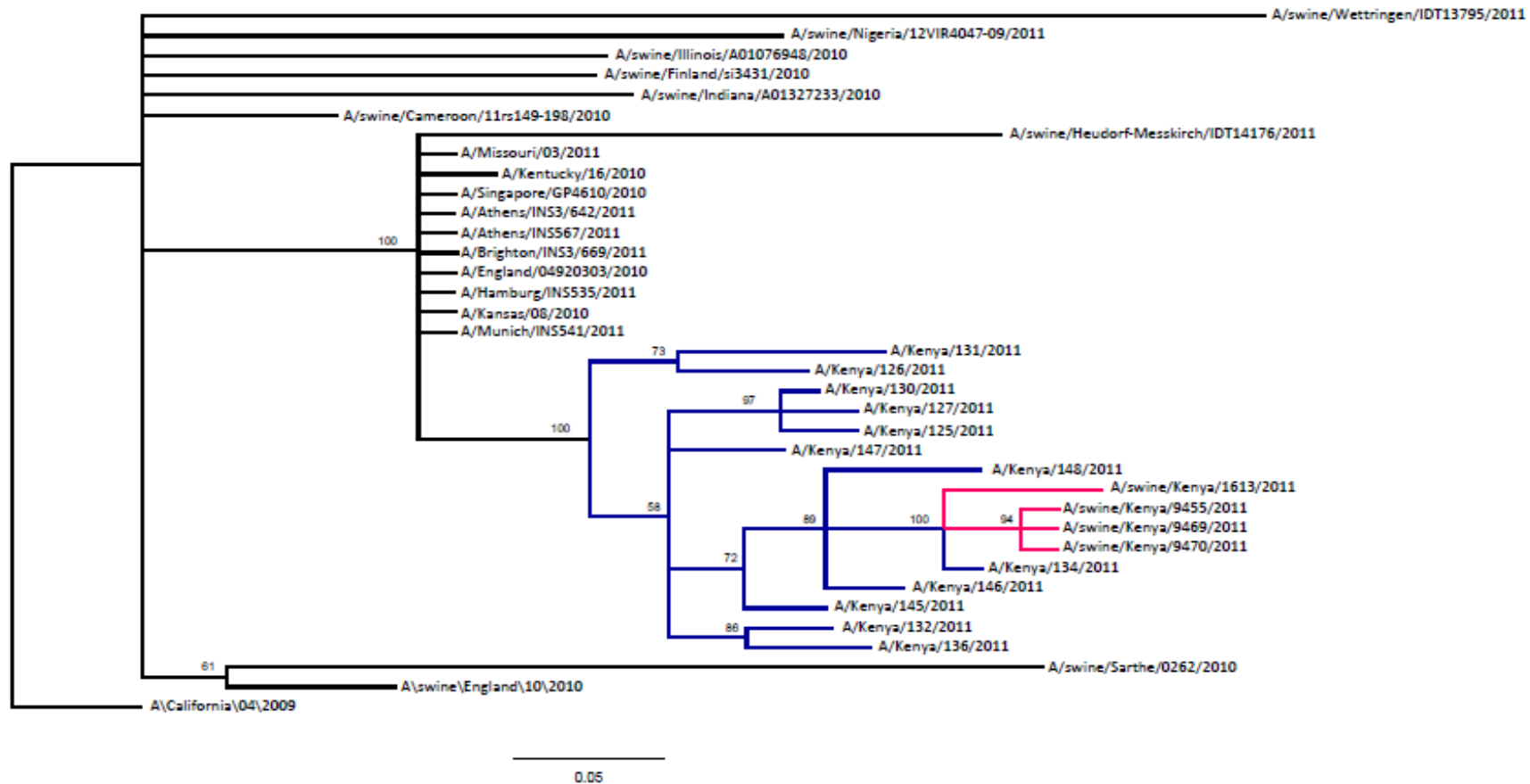
Figure 5.1: Phylogenetic comparison of haemagglutinin (HA) genes from the swine influenza H1N1 viruses isolated from Kenya with other human and swine H1N1 viruses isolated elsewhere.

Note: Posterior probabilities are shown as percentages on each node. Branches highlighted in red refer to Kenya swine isolates while those in blue are Kenya human isolates circulating in 2010-2011. Other human and swine isolates are in black branches. The tree is rooted on the A\California\04\2009. The horizontal scale bar represents the number of nucleotide substitutions per 100 nucleotides.

A\Kenya\0020\2009

96 ⎯ A\Kenya\0045\2009
A\Kenya\0007\2009

81
A\Kenya\0061\2009
A\Kenya\0071\2009

88
A\Kenya\0065\2009
A\Kenya\0015\2009

A\Kenya\0012\2009
A\Kenya\0011\2009
A\swine\Cameroon\11rs149-198\2010

A\swine\Nigeria\12VIR4047-09\2011

A\Ghom\167\2010
A\swine\Thailand\RY227\2010

A\India\NIV30784\2010
A\Boston\DOA44\2011
A\Athens\INS566\2011
A\Aarhus\INS610\2011
A\Nizhnii \Novgorod\CRIE-CEN\2011
A\Moscow\oblast\CRIE-131\2011
A\Moscow\oblast\CRIE-6\2011
A\England\05040482\2010
A\England\05160856\2010

100
A\Wisconsin\09\2010
A\District\Of\Columbia\01\2011
50
A\Virginia\02\2011
A\Rhode\Island\03\2010
A\Kentucky\18\2010
A\Alaska\02\2011

A\swine\Kenya\1613\2011
99
A\swine\Kenya\9455\2011
A\swine\Kenya\9469\2011
A\swine\Kenya\9470\2011

78
A\swine\Minnesota\A0113481
A\swine\Minnesota\A0113483
A\Texas\10\2011
76
A\Kentucky\10\2010
A\Arizona\15\2010

A\California\04\2009

0.08
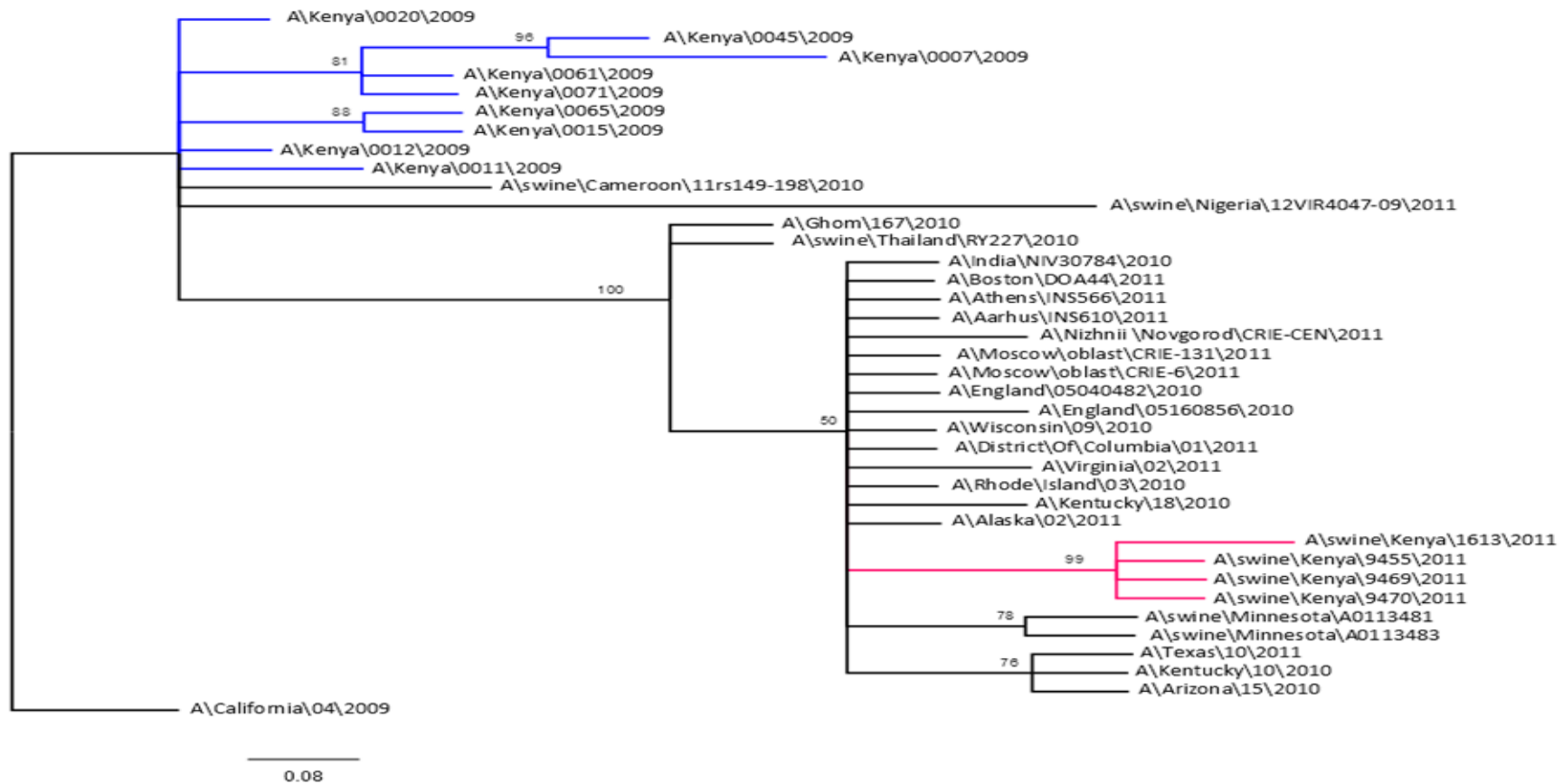
Figure 5.2: Phylogenetic comparison of neuraminidase (NA) genes from the Kenya swine H1N1 influenza viruses with other human and swine H1N1 viruses isolated elsewhere.

Note: Posterior probabilities are shown as percentages on each node. Branches highlighted in red refer to Kenya swine isolates while those in blue are Kenya human isolates circulating in 2009. Other global human and swine isolates are in black branches.

A\California\04\2009 is used to root the phylogenetic tree. The horizontal scale bar represents the number of nucleotide substitutions per 100 nucleotides

5.5   Discussion

The HA genes of the Kenya swine viruses clustered together and in close association with Kenya human isolates from the same year. These findings re-affirm the epidemiological data suggesting rapid local transmission of the viruses from human to pigs following its introduction in different countries (Nelson *et al.*, 2012 ; Trevennec *et al.*, 2012 ; Njabo *et al.*, 2012 ; Rith *et al.*, 2013 ; Rajao *et al.*, 2013 ; Nokireki *et al.*, 2013 ; Meseko *et al.*, 2014). The evolution pattern on the phylogenetic tree also suggests genetic reassortment with local swine influenza strains and/or antigenic drifts of the swine viruses away from the human isolates circulating in the same season (Drake, 1993 ; Drake and Holland, 1999 ; Rabadan and Robins, 2007 ; Vijaykrishna *et al.*, 2010). The Kenya swine H1N1 viruses were more distant from the two other African H1N1 swine isolates from Nigeria, suggesting that the introduction of this virus from North America to the African continent was through multiple routes and timelines, likely associated with human travelers as opposed to pig to pig transmission across countries through direct and indirect transmission.

 The phylogenetic analysis suggests that the Kenyan pigs most likely acquired the infections from the humans after the initial introduction in Kenya in July 2009 (MMWR, 2009a). This observation has been reported globally (Nelson *et al.*, 2012 ; Rajao *et al.*, 2013). Specifically, the evolution pattern of Kenya swine isolates HA segments as captured by this phylogenetic analysis, suggested that the swine virus isolates arose from at least a single but not more than two source introductions from humans. The Kenya swine viruses were also distant from human and swine H1N1 viruses from Europe, North America and Asia. The tree was rooted with   A/California/04/09 isolated from human at the start of the 2009 influenza pandemic.

The observed pattern of the swine isolates from different regions included in this analysis infer localized evolution patterns of the pandemic virus strain in different ecologies where after the initial introduction of virus from humans to pigs, sustained transmission was achieved with localized genetic reassortments with local swine strains or with influenza strains of human origin circulating in pigs (Nelson *et al.*, 2012). This observation has been reported in many countries around the world including in Brazil (Rajao *et al.*, 2013).

On the other hand, Kenya swine Neuraminidase genes (NA) of the influenza virus isolated in pigs formed a cluster with other global NA sequences suggesting global diversity of this segment away from the human NA sequences from Kenya. However, the Kenya human isolates included in this NA phylogenetic analysis were from 2009, soon after the initial introduction in human in Kenya since no NA segment were deposited in GeneBank between 2011-2012 with lower homology of nucleotide sequences to the Kenya swine isolates that circulated two years later. These Kenyan 2009 human sequences also clustered further away from the more recent human and swine sequences, illustrating genetic drifts that may have occurred over time in the NA segment of the more recent viruses. The genetic distance of the Kenya human viruses from 2009 was comparable to the parent A/California/04/2009 virus.

Overall, there was minimal antigenic drifts (point mutations) observed in the HA and NA genes of the swine influenza A isolates from this study. However the virus isolated from the slaughterhouse pig had more antigenic drift compared to the other three isolates from Kibera pig. Antigenic drift is a random process and one of the evolution pathways for influenza A viruses (Rabadan and Robins, 2007 ; Rabadan *et al.*, 2008). The low rate of antigenic drift was likely due to the fact that the viruses were related in temporal and spatial scale hence the low variability (Drake, 1993 ; Drake and Holland, 1999 ; Rabadan and Robins, 2007).

Monitoring of genetic evolution in influenza viruses circulating in different hosts is a key tool in studying transmission patterns and emergence of influenza virus strains that could have altered transmissibility or severity in human hosts (Brown, 2000a ; Brown *et al.*, 2008 ; Nelson *et al.*, 2012). Influenza viruses are continually evolving giving rise to new lineages of human, swine, and avian influenza viruses that have public health implications. In an ecosystem where there is close interaction between humans and pigs coupled with low biosecurity practices in most small holder farms and in slaughterhouses, there exists opportunity for influenza virus transmission from humans to pigs and from pigs to humans. These transmission pathways present opportunity for virus evolution and possible emergence of novel influenza virus strains with zoonotic potential.

6. CHAPTER 6

    CONCLUSIONS AND RECOMMENDATIONS

6.1   Conclusions

1. There was evidence of exposure to influenza viruses in a limited number of chicken, ducks, dogs and one cat in Kibera site while the rural site (Asembo) had no evidence of circulating influenza A antibodies in all the species sampled

2. One dog showed evidence of past infection with 2009 pandemic influenza virus strain and it is likely the dog could have been infected through contact with humans or with pigs in the same household.

3. In pigs sampled from the slaughterhouse, Influenza A sero-prevalence of 16.9% was observed. Over half (63.5%) of the influenza A positive pigs had previous exposure influenza A/California/04/2009(H1N1) pdm09 virus strain.

4. Eight swine influenza isolates were obtained from this study and were all identified as influenza A(H1N1)pdm09 the pandemic strain that was first detected in humans in Kenya in July 2009.

5. After the initial introduction, the virus may have become established and continued to circulate in the pig populations but there exists possibilities of repeated introductions as well as human to pig to human transmission pathways. Other swine influenza virus strains were circulating in pigs sampled in this study were not identified. The sero-prevalence data suggest that the virus continued to circulate in pig populations after initial introduction.

6. Genetic evolution pattern on molecular analysis of the hemagglutinin and Neuraminidase segments of the four Kenya swine isolates from this study, Kenya

human isolates in GenBank and other global human and swine viruses suggested that the pandemic influenza strain was likely introduced into the pig population in Kenya from humans. There was minimal antigenic variation among the influenza A isolates from swine for the HA and NA. However, the HA segment showed genetic variation from contemporary human influenza A isolates.

6.2   Recommendations

The following recommendations can be made from the findings of this study:

1. The subtypes of influenza A viruses circulating in chicken, ducks, cats and dogs were not clarified. The animal types showed evidence of previous infection with influenza A and influenza A RNA was detected in respiratory swabs. Additional surveillance is needed to identify the circulating influenza subtypes, reassortment of influenza subtypes and monitor introduction of new influenza subtypes.

2. Though the molecular analysis of influenza A (H1N1) pdm09 virus isolated from pigs in this study inferred a human to swine transmission, the exact transmission pathway either from direct contact with infected humans and the mechanism of spread after initial introduction in the pig populations was not investigated. Studies focusing on pigs and persons who work closely with pigs at household level or along the pig value chain would be useful in establishing transmission pathways as well as estimating the frequency of virus transmission events. This would be useful in designing interventions to reduce interspecies transmission of influenza virus between humans and pigs.

3. The identity of other swine influenza viruses circulating in pigs was not achieved. Due to the likelihood of genetic reassortment that could result when influenza viruses of human origin circulate in pig populations, there is a need for continuous surveillance and monitoring of genetic changes and virus evolution in local pig populations. Continuous surveillance would also be critical in identifying other swine influenza viruses circulating in pig populations in Kenya need to be investigated through systematic surveillance. Integrating this surveillance with on-going surveillance for influenza viruses in humans will provide useful information for public health action including recommendations in targeting these at risk populations for seasonal influenza vaccination.

4. Factors associated with the level of influenza infection in pig farms were not investigated in this study. There is therefore need to conduct systematic cross-sectional studies to identify factors associated with influenza infection in pigs under different production systems in Kenya.

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8. Appendices

Appendix 1: Data collection questionnaire administered to enrolled households in Kibera and

Asembo

Initial visit study questionnaire – Study site Kibera ☐ Asembo ☐
To be filled for each household visited for animal sampling
Purpose: Collect animal use and husbandry practices data
Household ID Code: ☐☐/☐☐☐/☐☐
household_id
Visit Date: ☐☐/☐☐/☐☐☐☐   3. Interviewer: ☐☐
visit_date   DD/MM/YYYY   interviewer

How many of the following animals are owned by households in this compound?
Adult = weaned (mammals); adult plumage (birds)

Species	Adult		Juvenile		Total	Kept at compound?
	Male	Female	Male	Female		
Cattle						
Goats						
Sheep						
Pigs						
Chickens						
Ducks						
Guinea Fowl						
Dogs						
Cats						

      Yes No
Do you keep cattle? If no, skip to question 5)   ☐   ☐
What is the primary reason for keeping cattle?
Source of cattle?
How are cattle managed during the day?
Free-ranging (no herdsman)   ☐
Herded   ☐
    If yes:
By whom?
        Paid herdsman   ☐
        Family member   ☐
           Male adult   ☐
           Female adult   ☐
           Male child   ☐
           Female child   ☐
Tethered grazing   ☐
    If yes:
    Where? _____
Zero-grazing   ☐
    If yes:
    Where? _____
How are cattle managed at night?
Not confined   ☐
Penned   ☐

Tethered ☐
In house ☐

Distance to human sleeping quarters:_____
Husbandry activities:

Activity	Frequency	Carried out by whom?			
		Male adults	Female adults	Male children	Female children
Herding					
Supplementary feeding					
Cleaning of overnight area					
Manure removal					
Milking					
Medication					
Veterinary intervention					
Manual tick removal					
Assistance with calving					
Slaughtering					
Butchering					
Skinning					

Veterinary care:
Ectoparasite control
      Frequency: _____
Dipping ☐ Product: _____
      Spraying ☐ Product: _____
      Manual removal ☐ Product: _____
Deworming
      Product: _____
Frequency _____
Source _____
      Administration_____
Vaccination
      Product: _____
Frequency _____
Source _____
      Administration_____
Distribution/disposal of animal products:
Live animals:
Milk _____
Manure _____
Foetus/stillborn _____
Afterbirth
Slaughtered animals:
Meat _____
Hide _____
Offal _____
Blood _____
Bones _____
      Dead animals:
Meat _____
Hide _____

Offal   _____

Blood   _____

Bones   _____

Do you keep goats? If no, skip to question 6)   ☐   ☐

What is the primary reason for keeping goats?

Source of goats?

How are goats managed during the day?

Free-ranging (no herdsman)   ☐

Herded   ☐

       If yes:

By whom?

                  Paid herdsman   ☐

                  Family member   ☐

                      Male adult   ☐

                      Female adult   ☐

                      Male child   ☐

                      Female child   ☐

Tethered grazing   ☐

       If yes:

       Where? _____

Zero-grazing   ☐

       If yes:

       Where? _____

How are goats managed at night?

Not confined   ☐

Penned   ☐

Tethered   ☐

In house   ☐

Proximity to human sleeping quarters:_____

Husbandry activities:

Activity	Frequency	Carried out by whom?			
		Male adults	Female adults	Male children	Female children
Herding					
Supplementary feeding					
Cleaning of overnight area					
Manure removal					
Milking					
Medication					
Veterinary intervention					
Manual tick removal					
Assistance with birth					
Slaughtering					
Butchering					
Skinning					

Veterinary care:

Ectoparasite control

       Frequency: _____

Dipping   ☐   Product: _____

       Spraying   ☐   Product: _____

       Manual removal   ☐   Product: _____

Deworming

Product: _____
Frequency _____
Source _____
Administration_____
Vaccination
Product: _____
Frequency _____
Source _____
Administration_____
Distribution/disposal of animal products:
Live animals:
Milk _____
Manure _____
Foetus/stillborn _____
Afterbirth
Slaughtered animals:
Meat _____
Hide _____
Offal _____
Blood _____
Bones _____
Dead animals:
Meat _____
Hide _____
Offal _____
Blood _____
Bones _____
Do you keep pigs   If no, skip to question 7)   ☐   ☐
What is the primary reason for keeping pigs?
Source of pigs?
How are pigs managed during the day?
Free-ranging (no herdsman)   ☐
Herded   ☐
        If yes:
By whom?
                Paid herdsman   ☐
                Family member   ☐
                        Male adult   ☐
                        Female adult   ☐
                        Male child   ☐
                        Female child   ☐
Tethered grazing   ☐
        If yes:
        Where? _____
Zero-grazing   ☐
        If yes:
        Where? _____
How are pigs managed at night?
Not confined   ☐
Penned   ☐
Tethered   ☐
In house   ☐

Proximity to human sleeping quarters:_____
Husbandry activities:

Activity	Frequency	Carried out by whom?			
		Male	Female	Male	Female

		adults	adults	children	children
Herding					
Supplementary feeding					
Cleaning of overnight area					
Manure removal					
Milking					
Shearing					
Medication					
Veterinary intervention					
Manual tick removal					
Assistance with lambing					
Slaughtering					
Butchering					
Skinning					

Veterinary care:
Ectoparasite control
      Frequency: _____
Dipping ☐ Product: _____
      Spraying ☐ Product: _____
      Manual removal ☐ Product: _____
Deworming
      Product: _____
Frequency _____
Source _____
      Administration_____
Vaccination
      Product: _____
Frequency _____
Source _____
      Administration_____
Distribution/disposal of animal products:
Live animals:
Milk _____
Manure _____
Foetus/stillborn _____
Afterbirth
Slaughtered animals:
Meat _____
Hide _____
Wool _____
Offal _____
Blood _____
Bones _____
      Dead animals:
Meat _____
Hide _____
Wool _____
Offal _____
Blood _____
Bones _____
Do you Keep Chickens If no, skip to question 8) ☐ ☐

What is the primary reason for keeping chickens?
Source of chickens?
How are chickens managed during the day?

Free-ranging ☐
In a coop/cage ☐
In the house ☐
How are chickens managed at night?
Free-ranging ☐
In a coop/cage ☐
In the house ☐

Proximity to human sleeping quarters:_____
Husbandry activities:

Activity	Frequency	Carried out by whom?			
		Male adults	Female adults	Male children	Female children
Supplementary feeding					
Cleaning of overnight area					
Manure removal					
Egg collection					
Medication					
Veterinary intervention					
Manual tick removal					
Slaughtering					
Plucking					
Butchering					

Veterinary care:
Ectoparasite control
        Frequency: _____
Dipping   ☐   Product: _____
        Spraying   ☐   Product: _____
        Manual removal   ☐   Product: _____
Deworming
        Product: _____
Frequency   _____
Source   _____
        Administration_____
Vaccination
        Product: _____
Frequency   _____
Source   _____
        Administration_____
Distribution/disposal of animal products:
Live animals:
Eggs   _____
Manure   _____
Slaughtered animals:
Meat   _____
Feathers   _____
Offal   _____
Blood   _____
Bones   _____
        Dead animals:
Meat   _____
Feathers   _____
Offal   _____
Blood   _____
Bones   _____

Do you Keep ducks If no, skip to question 9)   ☐   ☐

What is the primary reason for keeping ducks?
Source of ducks?
How are ducks managed during the day?
Free-ranging   ☐
In a coop/cage   ☐
In the house   ☐
How are ducks managed at night?
Free-ranging   ☐
In a coop/cage   ☐
In the house   ☐

Proximity to human sleeping quarters:_____
Husbandry activities:

Activity	Frequency	Carried out by whom?			
		Male adults	Female adults	Male children	Female children
Supplementary feeding					
Cleaning of overnight area					
Manure removal					
Egg collection					
Medication					
Veterinary intervention					
Manual tick removal					
Slaughtering					
Pluckng					
Butchering					

Veterinary care:
Ectoparasite control
        Frequency: _____
Dipping   ☐   Product: _____
        Spraying   ☐   Product: _____
        Manual removal   ☐   Product: _____
Deworming
        Product: _____
Frequency   _____
Source   _____
        Administration_____
Vaccination
        Product: _____
Frequency   _____
Source   _____
        Administration_____
Distribution/disposal of animal products:
Live animals:
Eggs   _____
Manure   _____
Slaughtered animals:
Meat   _____
Feathers   _____
Offal   _____
Blood   _____
Bones   _____
        Dead animals:

Meat   _____
Feathers   _____
Offal   _____
Blood   _____
Bones   _____
Do you keep dogs If no, skip to question 10)   ☐   ☐

What is the primary reason for keeping dogs?
Source of dogs?
Where are dogs kept during the day?
Not confined   ☐
Confined to compound   ☐
In the house   ☐
In a kennel/cage   ☐
Tied/chained   ☐
Where are dogs kept at night?
Not confined   ☐
Confined to compound   ☐
In the house   ☐
In a kennel/cage   ☐
Tied/chained   ☐
Proximity to human sleeping quarters:_____
Veterinary care:
Ectoparasite control
       Frequency: _____
Dipping   ☐   Product: _____
       Spraying   ☐   Product: _____
       Manual removal   ☐   Product: _____
Deworming
       Product: _____
Frequency   _____
Source   _____
       Administration_____
Vaccination
       Product: _____
Frequency   _____
Source   _____
       Administration_____
Do you keep cats If no, skip to question 11)   ☐   ☐

What is the primary reason for keeping cats?
Source of cats?
Where are cats kept during the day?
Not confined   ☐
Confined to compound   ☐
In the house   ☐
In a kennel/cage   ☐
Tied/chained   ☐

Where are cats kept at night?
Not confined   ☐
Confined to compound   ☐
In the house   ☐
In a kennel/cage   ☐
Tied/chained   ☐

Proximity to human sleeping quarters:_____

Veterinary care:
Ectoparasite control
     Frequency: _____
Dipping ☐ Product: _____
     Spraying ☐ Product: _____
     Manual removal ☐ Product: _____
Deworming
     Product: _____
Frequency _____
Source _____
     Administration_____
Vaccination
     Product: _____
Frequency _____
Source _____
     Administration_____

Do you keep other species of animals? Please specify types
Influenza study Initial visit household questionnaire
To be filled in all households visited for animal sampling
Household Level Data
Study site Kibera ☐ Asembo ☐
 Household ID Code: __ __ / __ __ __ / __ __
Interview Date: _____ 3: Interviewer: _____
Head of Household Name: _____
Respondent Name:
     _____
Please write "As above" if respondent is the head of household
How many of the following animals are present at this household today?
Please ask for each species in turn and record 0 if no animals of a given age and species are present.

	Pigs	Goats	Dogs	Cats	Ducks	Chickens	Turkeys	cattle
Adults (older than 3mo)								
Young (3mo and younger)								

	Others 1	Others 2	Others 3
Adults (older than 3mo)			
Young (3mo and younger)			

If other what species?
 Have any of the animals that belong to this household been sick in the past four months? This includes animals that were sick and then recovered as well as animals that were sick and died.
     No Other Animals ☐
Yes ☐ No ☐ Don't Know ☐

If Yes complete table below. If No Other Animals, No or DK go to question 7.

9A: Species	9B: Number Died	9C: Number Sick	9D: Clinical Signs/Details	9E: Sickness When?
1=Pigs 2=Goats 3=Dogs 4=Cats 5=Chickens 6=Ducks 7=Turkeys 8=Geese 9=Other (Specify)				Please record weeks or months since event

Have any new animals been born at the household or bought into the household in the past four months?

Yes ☐ No ☐ Don't Know ☐

If Yes complete table below. If No or DK go to question 8. Complete 1 row for each age (or purchase) group of each species.

10A: Species	10B: Number Born/ Brought In	10C: Source	10D: Born / Brought In When?
1=Pigs 2=Goats 3= Dogs 4=Cats 5=Chickens 6=Ducks 7=Turkeys 8=Geese 9=Other (Specify)		1=Bought inside Kibera 2=Bought outside Kibera 3=Gift inside Kibera 4=Gift outside Kibera 5=Adopted off the street. 6=Market (specify name) 7=Born at this Household 8=Other (specify)	Record number of weeks since born or acquired

What do you do with sick livestock?
Sell them ☐ ☐
Attempt to treat them
Slaughter and eat them ☐ ☐
Nothing ☐ ☐
Other (please specify) _____ ☐ ☐

If you have birds what do you do with sick birds? If no birds skip to question 11
Sell them ☐ ☐
Attempt to treat them ☐ ☐

Slaughter and eat them   ☐ ☐
Feed to the dogs   ☐ ☐
Other (please specify) _____   ☐ ☐
What do you do with dead animals/birds?
Eat them   ☐ ☐
Burn or bury them   ☐ ☐ ☐
Throw them in the nearest pond/lake/river/ bush/trash heap   ☐ ☐
Feed them to the dogs (uncooked)   ☐ ☐
Sell them   ☐ ☐
Other (please specify) _____   ☐ ☐

 How are your animals restrained?
Fill out for day and night

Species	During the day? (During daytime hours – daylight)	During the night? (During night time hours – darkness)
1=Pigs 2=Goats 3= Dogs 4=Cats 5=Chickens 6=Ducks 7=Turkeys 8=Geese 9=Other (Specify)	1=In a coup/Kennel/cage 2=Restricted to household/compound (within walls, secure fence) 3=Free, but stay at home/close 4=Free, roaming away from home 5=Free, unknown 6=In the family house	1=In a coup/Kennel/cage 2=Restricted to household/compound (within walls, secure fence) 3=Free, but stay at home/close 4=Free, roaming away from home 5=Free, unknown 6=In the family house

 Do you house your birds with other domestic animals? If no or DK, please go to Q #
Yes   ☐   No   ☐   Don't Know   ☐
If yes, please complete the table below. If no or DK, please go to Q #

	During the day? (During daytime hours – daylight)	During the night? (During night time hours – darkness)
With other bird types		
With cats		
With dogs		
With small ruminants		
With pigs		
Don't know		

If the household has no poultry, skip to question#
 Have your birds been vaccinated against any diseases?

Yes ☐　　　　　　No ☐　　　　　　Don't Know ☐

If Yes, what disease or diseases?

Which person/people in the household is/are responsible for looking after the birds (for example, feeding them)? Please record the their relationship to the head of household e.g. head of household, wife, son/daughter name, age and gender of the person or people. If no-one takes responsibility for the birds write 'no-one'.

15A: Relationship to head of household	15B: Age Class	15C: Sex
	1 = 0-5 yrs 2 = 6-19 yrs 3 = 20-49 yrs 4 = 50 yrs and older	M = Male F = Female= DK = Don't know

Are your birds slaughtered here at the house?

Yes ☐　　　　　　No ☐　　　　　　Don't Know ☐

If Yes, go to questions 17 & 18. If No, where are the birds slaughtered?

.Which person/people in the household slaughter your birds? Please record the their relationship to the head of household e.g. head of household, wife, son/daughter name, age and gender of the person or people.

17A: Relationship to head of household	17B: Age Class	17C: Sex
	1 = 0-5 yrs 2 = 6-19 yrs 3 = 20-49 yrs 4 = 50 yrs and older	M = Male F = Female= DK = Don't know

Where do you dispose of the bird carcass and entrails when you slaughter birds at the house?

_____
_____

pecies type and management

Complete for each of the households to capture all the species owned

	18 A Species	18B No. Of animals	18C: Sex	18D: Origin	18E: Duration in the	18F: Feed Type	18G: Feed Source	18H: Feed Other	18I: Feed Du	18J: Water sources
A										
B										
C										
D										
E										
F										
G										
H										
I										
J										
K										
L										
		Record the number of animals per species and sex where applicable	M= Male - F= Female - DK=Don't Know	1=Born in the household 2=Bought inside Kibera - 3=Bought outside Kibera - 4=Gift inside Kibera 5=Gift outside Kibera - 6=Adopted off the street - 7=Other (specify) 8=Unknown	Record in months the duration the household has had the species.	1=Commercial 2=Home-made 3=Leftovers 4=None 5=DK	1=House 2=Other Houses/Hotels 3=Market (specify name) 4=Butchery 5=Shops 6=Other (specify)	Y= Yes N= No DK= Don't Know	Y= Yes N= No DK = Don't Know	1=Well water/tap water 2=River/pond/lake water 3=Collected rain water 4=Scavenge 5=Other (please specify) ____

18A   Species   What is the species of the animal
18B   No. of animals   What is the number of each of the species owned by sex
18C   Sex: What is the sex of this animal? Fill out different rows for the same
18D   Origin: Where did this individual animal originally come from?
18E   Duration in household: Record in months the duration the household has kept that species
18F   Feed Type: What type of feed do you give do this animal?
18G   Feed Source: Where does the food that you give this anima come from? (record up to two options)
18H   Feed Other Houses: Does this animal ever feed at other houses?
18I   Feed Dump: Does this animal ever feed at rubbish dumps and in the streets?
18J   Water source   Where does this animal get its drinking water

Animal sampling data
Complete for each of the animals sampled in this household

	19A: Animal ID	19B: Species	19C: Age	19D: Sex	19E: Sickness Since Last Questionnaire Visit?	19F: Clinical Signs/Details	19G: Sickness When?
A							
B							
C							
D							
E							
F							
G							
H							
			To the nearest month if <12mo and nearest yr if older.	M= Male F= Female DK= Don't Know	Y= Yes N= No DK= Don't Know New = NA as New Dog		Record month and year or time since sickness

19A   Animal ID: Record the individual ID for each mammal owned by or present at the household
19B   Species   Record the species of the animal sampled
19C   Age: What is the age of this animal now?
19D   Sex: What is the sex of this animal?
19E   Sickness Since Last Questionnaire Visit:
Has this animal been sick at any time since since we last visited and carried out a questionnaire at the household?
19F   Clinical Signs/Details: If Yes to Q#; what were/are the signs of this sickness?
19G   Sickness When?:   If Yes to Q#; When was this animal sick?Thank you for your time and cooperation. Do you have any questions or comments?
Record any questions or additional information provided by the respondent.

Influenza study follow up visit household questionnaire
To be filled for all households visited in the follow-up phase-1 per household
Household Level Data
Study site Kibera ☐ Asembo☐
1. Household ID Code: __ __ / __ __ __ / __ __
2. Interview Date: _____
3. Interviewer code: _____
4. Head of Household Name:
   _____
5. Respondent Name:
   _____
Please write "As above" if respondent is the head of household
6. Have any new animals been born at the household or bought into the household since we last visited and carried out a questionnaire?
   Yes ☐ No ☐ Don't Know ☐
If Yes complete table below. If No or DK go to question #.

6A Species	6B Number Born/ Brought In	6C Source	6D Born/ Brought In When?
1=Sheep *2=Goats* *3=Chickens* *4=Ducks* *5=Cats* *6=Other* *(Specify)*		*1=Bought inside Kibera* *2=Bought outside Kibera* *3=Gift inside Kibera* *4=Gift outside Kibera* *5=Adopted off the street* *6=Market (specify name)* *7=Born at this Household* *8=Other (specify)*	*Record weeks since born or acquired*

7. Have any of the animals that belong to this household been sick since we last visited and carried out a questionnaire? This includes animals that were sick and then recovered as well as animals that were sick and died.
   Yes ☐ No ☐ Don't Know ☐
If Yes complete table below. If No Other Animals, No or DK go to question #.

8A Species	8B Number Died	8C Number Sick	8D Clinical Signs/Details	8F Sickness When?
1=Sheep *2=Goats* *3=Chickens* *4=Ducks* *5=Cats* *6=Other* *(Specify)*				*Record weeks since event*

Animal Data

8. Since we last visited and carried out a questionnaire, did any animals that the household owned die or leave the household?

Yes ☐   No ☐   Don't Know ☐

If Yes complete table below. If No or DK go to question 9.
Complete for each species

9A **Species**	9B Number	9C Sex	9D Fate		9E Time since last visit
		M= Male *F= Female* *DK= Don't Know*	*1= Sold* *2= Given Away* *3= Killed by owner* *4= Killed by authorities* *5= Killed by someone else* *6= Died in accident*	*7= Died of disease/parasite* *8= Died through starvation* *9= Died other* *10= Disappeared* *11= Abandoned* *12= Stolen* *13=Unknown*	*Duration in months the animal left the last visit*

9. What do you do with sick animals?
    a. Sell them   ☐ ☐
    b. Attempt to treat them   ☐ ☐
    c. Nothing   ☐ ☐
    d. Other (*please specify*) _____   ☐ ☐

10. If you have birds what do you do with sick birds?
    a. Sell them   ☐ ☐
    b. Attempt to treat them   ☐ ☐
    c. Slaughter and eat them   ☐ ☐
    d. Feed to the dogs   ☐ ☐
    e. Other (*please specify*) _____   ☐ ☐

11. What do you do with dead birds?
    f. Eat them   ☐ ☐
    g. Burn or bury them   ☐ ☐ ☐
    h. Throw them in the nearest pond/lake/river/ bush/trash heap ☐ ☐
    i. Feed them to the dogs (uncooked)   ☐ ☐
    j. Sell them   ☐ ☐
    k. Other (*please specify*)   _____   ☐ ☐

12. How are your animals restrained?
Fill out for day and night

Species	During the day? (During daytime hours – daylight)	During the night? (During night time hours – darkness)
1=Pigs 2=Goats 3= Dogs 4=Cats 5=Chickens 6=Ducks 7=Turkeys 8=Geese 9=Other (Specify)	1=In a coup/Kennel/cage 2=Restricted to household/compound (within walls, secure fence) 3=Free, but stay at home/close 4=Free, roaming away from home 5=Free, unknown 6=In the family house	1=In a coup/Kennel/cage 2=Restricted to household/compound (within walls, secure fence) 3=Free, but stay at home/close 4=Free, roaming away from home 5=Free, unknown 6=In the family house

13. Do you house your birds with other domestic animals? *If no or DK, please go to Q #*

Yes ☐　　　　　No ☐　　　　Don't Know ☐

If yes, please complete the table below. If no or DK, please go to Q #

	During the day? (During daytime hours – daylight)	During the night? (During night time hours – darkness)
With other bird types		
With cats		
With dogs		
With small ruminants		
With pigs		
Don't know		

14. Have your birds been vaccinated against any diseases?

　　　　Yes ☐　　　　No ☐　　　　Don't Know ☐

If Yes, what disease or diseases?

_____

15. Which person/people in the household is/are responsible for looking after the birds (for example, feeding them)?

Please record the their relationship to the head of household e.g. head of household, wife, son/daughter name, age and gender of the person or people. If no-one takes responsibility for the birds write 'no-one'.

15A: Relationship to head of household	15B: Age Class	15C: Sex
	1 = 0-5 yrs 2 = 6-19 yrs 3 = 20-49 yrs 4 = 50 yrs and older	M = Male F = Female= DK = Don't know

16. Are your birds slaughtered here at the house?

Yes ☐   No ☐   Don't Know ☐

If Yes, go to questions 17 & 18. If No, where are the birds slaughtered?

17. .Which person/people in the household slaughter your birds?

Please record the their relationship to the head of household e.g. head of household, wife, son/daughter name, age and gender of the person or people.

17A: Relationship to head of household	17B: Age Class	17C: Sex
	1 = 0-5 yrs 2 = 6-19 yrs 3 = 20-49 yrs 4 = 50 yrs and older	M = Male F = Female= DK = Don't know

18. Where do you dispose of the bird carcass and entrails when you slaughter birds at the house?

    a. _____
        _____

Animal Sampling Data

19.

Complete for all animals sampled during the visit

	#: Animal ID	**Species**	#: Age	#: Sex	#: Sickness Since Last Questionnaire Visit?	#: Clinical Signs/Details	#: Sickness When?
A							
B							
C							
D							
E							
F							
G							
H							
			To the nearest month if <12mo and nearest yr if older.	*M= Male F= Female DK= Don't Know*	*Y= Yes N= No DK= Don't Know New = NA as New Dog*		*Record month and year or time since sickness*

\#   Animal ID: Record the individual ID for each mammal owned by or present at the household

\#   Species   Record the species of the animal sampled

\#   Age: What is the age of this animal now?

\#   Sex: What is the sex of this animal?

\#   Sickness Since Last Questionnaire Visit:

           Has this animal been sick at any time since since we last visited and carried out a questionnaire at the household?

\#   Clinical Signs/Details: If Yes to Q\#; what were/are the signs of this sickness?
\#   Sickness When?:   If Yes to Q\#; When was this animal sick?

Thank you for your time and cooperation. Do you have any questions or comments?

Appendix 2: CDC PCR Primer and Hydrolysis Probe Sequences used for influenza A RT

PCR (Matrix gene) and influenza A H1N1pdm09

Matrix Gene
M-F 5" GAC CRA TCC TGT CAC CTC TGAC 3"
M-R 5" AGG GCA TTY TGG ACA AAK CGT CTA 3"
M-Probe 5" FAM-TGC AGT CCT CGC TCA CTG GGC ACG3"
Pandemic influenza H1
H1-F 5" GTG CTA TAA ACA CCA GCC TCC CATT 3"
H1-R 5" AGA CGG GAY ATT CCT CAA TCC TG 3"
H1-Probe5" FAM -ATA CAT CCG A"T"C ACM ATT GGA AAA TGT CC 3"

Appendix 3: Primer and Hydrolysis Probe Sequences used for RT PCR subtyping of

influenza A Swine N1, H1,N2 and H3(Richt *et al.*, 2004)

Specificity and size Primer/Probe Sequence*
Swine H1, 102 base pairs
H1-F 5"GTT TAC ATA GTT TYC CRT 3"
H1-R 5"AAT AAT TCA ACY GAC ACT G 3"
H1-Probe 5"TXRED-AAG AAT GTA ACM GTA ACA CAC TCT G-BHQ2- 3"
Swine H3, 244 base pairs
H3-F 5" AAA TTG AAG TGA CTA ATG CTA C 3"
H3-R 5" TGA GGC AAC TAG TGA CCT AAG 3"
H3-Probe 5"FAM-CAA CAG GTA GAA TAT GCG ACA GTC C-TAMRA-3"
Swine N1, 267 base pairs
N1-F 5" GTA ATG GTG TTT GGA TAG GAA G 3"
N1-R 5" ATG CTG CTC CCA CTA GTC CAG 3"
N1-Probe 5"FAM-TGA TTT GGG ATC CTA ATG GAT GGA CAG-TAMRA-3"
Swine N2, 233 base pairs
N2-F 5" TGG ACA GGG AAC AAC ACT AAA C 3"
N2-R 5" ACA AGC CTC CCA TCG TAA AT 3"
N2-Probe5" TXRED-CAA ATG AAA TGG AAC ACC CAA CTC AT-BHQ2-3"
* Y 5 C, T; M 5 A, C; R 5 A, G; FAM 5 6-carboxyfluorescein; TAMRA 5 6-carboxytetramethylrhodamine; TXRED 5 Texas Red;

Appendix 4: Data collection questionnaire administered to pig owners or traders at the

slaughter house

Uthiru Influenza Project
Animal sampling visits

Visit Date ☐☐ ☐☐ ☐☐☐☐   Interviewer code ☐☐
            dd   mm   yyyy

A: Individual Animal Record

   1. **Animal ID** ☐☐☐☐

   2. **Group ID** ☐☐☐☐

   3. **Source of pigs (District)** ☐☐☐☐☐☐☐☐☐

   4. **Number of pigs in the group brought to the slaughterhouse**
      Number☐☐ ☐☐   Don't know☐☐

   5. **Number of pigs in the homestead**
      Number☐☐ ☐☐   Don't know☐☐

B: **Sample record**

   6. **Blood   Yes**☐ No ☐   Sample Label ------------   Bar ccode
      ID☐☐☐☐☐☐☐☐

   7. **Bronchial swab Yes**☐ No ☐ Sample Label ------------   Bar ccode
      ID☐☐☐☐☐☐☐☐

   8. **Nasal Swab Yes**☐ No ☐   Sample Label ------------   Bar ccode
      ID☐☐☐☐☐☐☐☐

Appendix 5: Nucleotide sequences of the Haemagglutinin and Neuraminidase genes of four

swine influenza isolates

Hemagglutinin nucleotide sequences

>A/swine/Kenya/9455/2011_4
ATGAAGGCAATACTAGTAGTTCTGCTATATACATTTACAACCGCAAATGCAGACACATTGTGTATAGGTTATCA
TGCGAACAATTCAACAGACACTGTAGACACAGTACTAGAGAAGAATGTAACAGTAACACACTCTGTTAACCTT
CTAGAAGACAAGCATAACGGGAAACTATGCAAACTAAGAGGGGTAGCCCCATTGCATTTGGGTAAATGTAACA
TTGCTGGCTGGATCCTGGGAAATCCAGAGTGTGAATCACTCTCCACAGCAAGCTCATGGTCCTACATTGTGGAA
ACATCTAGTTCAGACAATGGAACGTGTTACCCAGGAGATTTCATCAATTATGAGGAGCTAAGAGAGCAATTGA
GCTCAGTGTCATCATTTGAAAGGTTTGAGATATTCCCCAAGACAAGTTCATGGCCCAATCATGACTCGAACAAA
GGTGTAACGGCAGCATGTCCTCATGCTGGAGCAAAAAGCTTCTACAAAAATTTAATATGGCTAGTTAAAAAAG
GAAATTCATACCCAAAGCTCAGCAAATCCTACATTAATGATAAAGGGAAAGAAGTCCTCGTGCTGTGGGGCAT
TCACCATCCATCTACTACTGCAGACCAACAAAGTCTCTATCAGAATGCAGATGCATATGTTTTTGTGGGGACAT
CAAGATACAGCAAGAAGTTCAAGCCGGAAATAGCAATAAGACCCAAAGTGAGGGATCAAGAAGGGAGAATG
AACTATTACTGGACACTAGTAGAGCCGGGAGACAAAATAACATTCGAAGCAACTGGAAATCTAGTGGTACCGA
GATATGCATTCGCAATGGAAAGAAATGCTGGATCTGGTATTATCATTTCAGATACACCAGTCCACGATTGCAAT
ACAACTTGTCAGACACCCAAGGGTGCTATAAACACCAGCCTCCCATTTCAGAATATACATCCGATCACAATTGG
AAAATGTCCAAAATATGTAAAAAGCACAAAATTGAGACTGGCCACGGGATTGAGGAATGTCCCGTCTATTCAA
TCTAGAGGCCTATTTGGGGCCATTGCCGGCTTCATTGAAGGGGGGTGGACAGGGATGGTAGATGGATGGTACG
GTTATCACCATCAAAATGAGCAGGGGTCAGGATATGCAGCCGACCTGAAGAGCACACAGAATGCCATTGACAA
GATTACTAACAAAGTAAATTCTGTTATTGAAAAGATGAATACACAGTTCACAGCAGTAGGTAAAGAGTTCAAC
CACCTGGAAAAAAGAATAGGGAATTTAAATAAAAAAGTTGATGATGGTTTCCTGGACATTTGGACTTACAATG
CCGAACTGTTGGTTCTATTGGAAAATGAAAGAACTTTGGACTACCACGATTCAAATGTGAAGAACTTGTATGA
AAAGGTAAGAAACCAGTTAAAAACCAATGCTAAGGAAATTGGAAACGGCTGCTTTGAATTCTACCACAAATGC
GATAACACGTGCATGGAAAGTGTCAAAAATGGGACTTATGACTACCCAAAATACTCAGAGGAAGCAAAATTA
AACAGAGAAGAAATAGATGGGGTAAAGCTGGAATCAACAAGGATTTACCAGATTTTGGCGATCTATTCAACTG
TCGCCAGTTCATTGGTACTGGTAGTCTCCCTGGGGGCAATCAGTTTCTGGATGTGCTCTAATGGGTCTCTACAGT
GTAGAATATGTATTTAA
>A/swine/Kenya/1613/2011_4
ATGAAGGCAATACTAGTAGTTCTGCTATATACATTTACAACCGCAAATGCAGACACATTGTGTATAGGTTATCA
TGCGAACAATTCAACAGACACTGTAGACACAGTACTAGAGAAGAATGTAACAGTAACACACTCTGTTAACCTT
CTAGAAGACAAGCATAACGGGAAACTATGCAAACTAAGAGGGGTAGCCCCATTGCATTTGGGTAAATGTAACA
TTGCTGGCTGGATCCTGGGAAATCCAGAGTGTGAATCACTCTCCACAGCAAGCTCATGGTCCTACATTGTGGAA
ACATCTAGTTCAGACAATGGAACGTGTTACCCAGGAGATTTCATCAATTATGAGGAGCTAAGAGAGCAATTGA
GCTCAGTGTCATCATTTGAAAGGTTTGAGATATTCCCCAAGACAAGTTCATGGCCCAATCATGACTCGAACAAA
GGTGTAACGGCAGCATGTCCTCATGCTGGAGCAAAAAGCTTCTACAAAAATTTAATATGGCTAGTTAAAAAAG
GAAATTCATACCCAAAGCTCAGCAAATCCTACATTAATGATAAAGGGAAAGAAGTCCTCGTGCTGTGGGGCAT
TCACCATCCATCTACTACTGCAGACCAACAAAGTCTCTATCAGAATGCAGATGCATATGTTTTTGTGGGGACAT
CAAGATACAGCAAGAAGTTCAAGCCGGAAATAGCAATAAGACCCAAAGTGAGGGATCAAGAAGGGAGAATG
AACTATTACTGGACACTAGTAGAGCCGGGAGACAAAATAACATTCGAAGCAACTGGAAATCTAGTGGTACCGA
GATATGCATTCGCAATGGAAAGAAATGCTGGATCTGGTATTATCATTTCAGATACACCAGTCCACGATTGCAAT
ACAACTTGTCAGACACCCAAGGGTGCTATAAACACCAGCCTCCCATTTCAGAATATACATCCGATCACAATTGG
AAAATGTCCAAAATATGTAAAAAGCACAAAATTGAGACTGGCCACGGGATTGAGGAATGTCCCGTCTATTCAA
TCTAGAGGCCTCTTTGGGGCCATTGCCGGCTTCATTGAAGGGGGGTGGACAGGGATGGTAGATGGATGGTACG
GTTATCACCATCAAAATGAGCAGGGGTCAGGATATGCAGCCGACCTGAAGAGCACACAGAATGCCATTGACAA
GATTACTAACAAAGTAAATTCTGTTATTGAAAAGATGAATACACAGTTCACAGCAGTAGGTAAAGAGTTCAAC
CACCTGGAAAAAAGAATAGAGAATTTAAATAAAAAAGTTGATGATGGTTTCCTGGACATTTGGACTTACAATG
CCGAACTGTTGGTTCTATTGGAAAATGAAAGAACTTTGGACTACCACGATTCAAATGTGAAGAACTTGTATGA
AAAGGTAAGAAACCAGTTAAGAACCAATGCTAAGGAAATTGGAAACGGCTGCTTTGAATTCTACCACAAATGC
GATAACACGTGTATGGAAAGTGTCAAAAATGGGACTTATGACTACCCAAAATACTCAGAGGAAGCAAAATTAA
ACAGAGAAGAAATAGATGGGGTAAAGCTGGAATCAACAAGGATTTACCAGATTTTGGCGATCTATTCAACTGT
CGCCAGTTCATTGGTACTGGTAGTCTCCCTGGGGGCAATCAGTTTCTGGATGTGCTCTAATGGGTCTCTACAGT
GTAGAATATGTATTTAA
>A/swine/Kenya/9469/2011_4
ATGAAGGCAATACTAGTAGTTCTGCTATATACATTTACAACCGCAAATGCAGACACATTGTGTATAGGTTATCA
TGCGAACAATTCAACAGACACTGTAGACACAGTACTAGAGAAGAATGTAACAGTAACACACTCTGTTAACCTT
CTAGAAGACAAGCATAACGGGAAACTATGCAAACTAAGAGGGGTAGCCCCATTGCATTTGGGTAAATGTAACA
TTGCTGGCTGGATCCTGGGAAATCCAGAGTGTGAATCACTCTCCACAGCAAGCTCATGGTCCTACATTGTGGAA

ACATCTAGTTCAGACAATGGAACGTGTTACCCAGGAGATTTCATCAATTATGAGGAGCTAAGAGAGCAATTGA
GCTCAGTGTCATCATTTGAAAGGTTTGAGATATTCCCCAAGACAAGTTCATGGCCCAATCATGACTCGAACAAA
GGTGTAACGGCAGCATGTCCTCATGCTGGAGCAAAAAGCTTCTACAAAAATTTAATATGGCTAGTTAAAAAAG
GAAATTCATACCCAAAGCTCAGCAAATCCTACATTAATGATAAAGGGAAAGAAGTCCTCGTGCTGTGGGGCAT
TCACCATCCATCTACTACTGCAGACCAACAAAGTCTCTATCAGAATGCAGATGCATATGTTTTTGTGGGGACAT
CAAGATACAGCAAGAAGTTCAAGCCGGAAATAGCAATAAGACCCAAAGTGAGGGATCAAGAAGGGAGAATG
AACTATTACTGGACACTAGTAGAGCCGGGAGACAAAATAACATTCGAAGCAACTGGAAATCTAGTGGTACCGA
GATATGCATTCGCAATGGAAAGAAATGCTGGATCTGGTATTATCATTTCAGATACACCAGTCCACGATTGCAAT
ACAACTTGTCAGACACCCAAGGGTGCTATAAACACCAGCCTCCCATTTCAGAATATACATCCGATCACAATTGG
AAAATGTCCAAAATATGTAAAAAGCACAAAATTGAGACTGGCCACGGGATTGAGGAATGTCCCGTCTATTCAA
TCTAGAGGCCTATTTGGGGCCATTGCCGGCTTCATTGAAGGGGGGGTGGACAGGGATGGTAGATGGATGGTACG
GTTATCACCATCAAAATGAGCAGGGGTCAGGATATGCAGCCGACCTGAAGAGCACACAGAATGCCATTGACAA
GATTACTAACAAAGTAAATTCTGTTATTGAAAAGATGAATACACAGTTCACAGCAGTAGGTAAAGAGTTCAAC
CACCTGGAAAAAAGAATAGGGAATTTAAATAAAAAAGTTGATGATGGTTTCCTGGACATTTGGACTTACAATG
CCGAACTGTTGGTTCTATTGGAAAATGAAAGAACTTTGGACTACCACGATTCAAATGTGAAGAACTTGTATGA
AAAGGTAAGAAACCAGTTAAAAACCAATGCTAAGGAAATTGGAAACGGCTGCTTTGAATTCTACCACAAATGC
GATAACACGTGCATGGAAAGTGTCAAAAATGGGACTTATGACTACCCAAAATACTCAGAGGAAGCAAAATTA
AACAGAGAAGAAATAGATGGGGTAAAGCTGGAATCAACAAGGATTTACCAGATTTTGGCGATCTATTCAACTG
TCGCCAGTTCATTGGTACTGGTAGTCTCCCTGGGGGCAATCAGTTTCTGGATGTGCTCTAATGGGTCTCTACAGT
GTAGAATATGTATTTAA
>A/swine/Kenya/9470/2011_4
ATGAAGGCAATACTAGTAGTTCTGCTATATACATTTACAACCGCAAATGCAGACACATTGTGTATAGGTTATCA
TGCGAACAATTCAACAGACACTGTAGACACAGTACTAGAGAAGAATGTAACAGTAACACACTCTGTTAACCTT
CTAGAAGACAAGCATAACGGGAAACTATGCAAACTAAGAGGGGTAGCCCCATTGCATTTGGGTAAATGTAACA
TTGCTGGCTGGATCCTGGGAAATCCAGAGTGTGAATCACTCTCCACAGCAAGCTCATGGTCCTACATTGTGGAA
ACATCTAGTTCAGACAATGGAACGTGTTACCCAGGAGATTTCATCAATTATGAGGAGCTAAGAGAGCAATTGA
GCTCAGTGTCATCATTTGAAAGGTTTGAGATATTCCCCAAGACAAGTTCATGGCCCAATCATGACTCGAACAAA
GGTGTAACGGCAGCATGTCCTCATGCTGGAGCAAAAAGCTTCTACAAAAATTTAATATGGCTAGTTAAAAAAG
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Neuraminidase gene sequences

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Appendix 6: List of influenza virus isolates included in the phylogenetic analysis of the HA segment

No	Strain Name	Subtype	Segment	Country	Host species	Year of Collection	GenBank accession number (Reference)
1	A/Kenya/125	H1N1	HA	Kenya	Human	2011	JQ396224
2	A/Kenya/126	H1N1	HA	Kenya	Human	2011	JQ396225
3	A/Kenya/127	H1N1	HA	Kenya	Human	2011	JQ396226
4	A/Kenya/130	H1N1	HA	Kenya	Human	2011	JQ396229
5	A/Kenya/131	H1N1	HA	Kenya	Human	2011	JQ396230
6	A/Kenya/132	H1N1	HA	Kenya	Human	2011	JQ396231
7	A/Kenya/134	H1N1	HA	Kenya	Human	2011	JQ396232
8	A/Kenya/136	H1N1	HA	Kenya	Human	2011	JQ396233
9	A/Kenya/145	H1N1	HA	Kenya	Human	2011	JQ396238
10	A/Kenya/146	H1N1	HA	Kenya	Human	2011	JQ396239
11	A/Kenya/147	H1N1	HA	Kenya	Human	2011	JQ396240
12	A/Kenya/148	H1N1	HA	Kenya	Human	2011	JQ396241
13	A/swine/Cameroon/11rs149-198	H1N1	HA	Cameroon	Swine	2010	JF707784
14	A/swine/England/10	H1N1	HA	United	Swine	2009	CY115943
15	A/swine/Finland/si3431	H1N1	HA	Finland	Swine	2009	KC336410
16	A/swine/Heudorf-	H1N1	HA	Germany	Swine	2010	KC631888
17	A/swine/Illinois/A01076948	H1N1	HA	USA	Swine	2010	CY114613
18	A/swine/Indiana/A01327233	H1N1	HA	USA	Swine	2010	JX463281
19	A/swine/Nigeria/12VIR4047-09	H1N1	HA	Nigeria	Swine	2011	JX442481
20	A/swine/Sarthe/0262	H1N1	HA	France	Swine	2011	FR871195
21	A/swine/Wettringen/IDT13795	H1N2	HA	Germany	Swine	2010	KC222548
22	A/Athens/INS3_642	H1N1	HA	Greece	Human	2011	CY176482
23	A/Athens/INS567	H1N1	HA	Greece	Human	2011	CY129467
24	A/Brighton/INS3_669	H1N1	HA	United	Human	2011	CY176405
25	A/California/04	H1N1	HA	USA	Human	2009	FJ966082
26	A/England/04920303	H1N1	HA	United	Human	2010	JX625758
27	A/Hamburg/INS535	H1N1	HA	Germany	Human	2011	CY129598
28	A/Kansas/08	H1N1	HA	USA	Human	2010	KC882013
29	A/Kentucky/16	H1N1	HA	USA	Human	2010	KC882263
30	A/Missouri/03	H1N1	HA	USA	Human	2011	KC882295
31	A/Munich/INS541	H1N1	HA	Germany	Human	2011	CY129638
32	A/Singapore/GP4610	H1N1	HA	Singapore	Human	2011	CY091674
33	A/India/NIV30784	H1N1	NA	India	Human	2010	CY084252
34	A/Boston/DOA44	H1N1	NA	USA	Human	2011	CY111296
35	A/Athens/INS566	H1N1	NA	Greece	Human	2011	CY129461
36	A/Aarhus/INS610	H1N1	NA	Denmark	Human	2011	CY129920

No	Strain Name	Subtype	Segment	Country	Host species	Year of Collection	GenBank accession number (Reference)
37	A/Kenya/0020	H1N1	NA	Kenya	Human	2009	HQ214271
38	A/Kenya/0045	H1N1	NA	Kenya	Human	2009	HQ214275
39	A/Kenya/0065	H1N1	NA	Kenya	Human	2009	HQ214278
40	A/Kenya/0061/	H1N1	NA	Kenya	Human	2009	HQ214289
41	A/Kenya/0012	H1N1	NA	Kenya	Human	2009	HQ214296
42	A/Kenya/0011	H1N1	NA	Kenya	Human	2009	HQ214302
43	A/Kenya/0007	H1N1	NA	Kenya	Human	2009	HQ214306
44	A/Kenya/0015	H1N1	NA	Kenya	Human	2009	HQ214317
45	A/Kenya/0071	H1N1	NA	Kenya	Human	2009	HQ214318
46	A/Ghom/167	H1N1	NA	Iran	Human	2010	JF500425
47	A/Nizhnii Novgorod/CRIE-	H1N1	NA	Russia	Human	2011	JN714540
48	A/Moscow oblast/CRIE-131	H1N1	NA	Russia	Human	2011	JN714545
49	A/Moscow oblast/CRIE-6	H1N1	NA	Russia	Human	2011	JN714549
50	A/swine/Minnesota/A01134815	H1N1	NA	USA	Swine	2011	JQ809782
51	A/swine/Minnesota/A01134830	H1N1	NA	USA	Swine	2011	JQ809783
52	A/England/05040482	H1N1	NA	United	Human	2010	JX625832
53	A/England/05160856	H1N1	NA	United	Human	2010	JX625944
54	A/Wisconsin/09	H1N1	NA	USA	Human	2010	KC781302
55	A/swine/Thailand/RY227	H1N1	NA	Thailand	Swine	2010	KC859104
56	A/Texas/10	H1N1	NA	USA	Human	2011	KC881590
57	A/District Of Columbia/01	H1N1	NA	USA	Human	2011	KC881763
58	A/Kentucky/10	H1N1	NA	USA	Human	2010	KC881825
59	A/Arizona/15	H1N1	NA	USA	Human	2010	KC881867
60	A/Virginia/02	H1N1	NA	USA	Human	2011	KC881869
61	A/Rhode Island/03	H1N1	NA	USA	Human	2010	KC882158
62	A/Kentucky/18	H1N1	NA	USA	Human	2010	KC882230
63	A/Alaska/02	H1N1	NA	USA	Human	2011	KC882282
64	A/swine/Cameroon/11rs149-198	H1N1	NA	Cameroon	Swine	2010	JF707786
65	A/swine/Nigeria/12VIR4047-09	H1N1	NA	Nigeria	Swine	2010	JX442482
66	A/California/04	H1N1	NA	USA	Human	2009	FJ969517