DETECTION AND CHARACTERIZATION OF HUMAN INFLUENZA A VIRUS ISOLATES FROM PATIENTS ATTENDING KAYUNGA AND MULAGO HOSPITALS IN UGANDA

BY

BLANCHE BYARUGABA KAIRA
(BSc. MAK)

A DISSERTATION SUBMITTED TO THE GRADUATE SCHOOL IN PARTIAL FULFILLMENT FOR THE AWARD OF A MASTER OF SCIENCE DEGREE IN MOLECULAR BIOLOGY OF MAKERERE UNIVERSITY

SEPTEMBER 2011
Declaration

I, **Blanche Byarugaba Kaira**, Registration number 2006/HD17/6735U and student number 206017717, do hereby declare that this is my own original work and has never been submitted to any other University/tertiary institution for the award of any academic qualification.

Signed…………………………………………Date………/……/………

This report has been prepared with the supervision of;

Dr. Denis K. Byarugaba (PhD),
Department of Microbiology and Parasitology,
School of Veterinary Medicine,
Makerere University,
P.O. Box 7062, Kampala, Uganda.

Signed………………………………….Date……../……/…………

Dr. Edison Arwanire Mworozzi,
Senior Consultant Paeditrician/ Honorary Lecturer,
Diplomate on Children’s Environmental Health,
Department of Paediatrics and Child Health,
Mulago Hospital/ Makerere University Medical School,
P.O. Box 7072, Kampala, Uganda.

Signed…………………………………………Date……../……/…………
Dedication

I dedicate this work to my family, especially my husband Robert Kaira as well as to my late parents, Paul and Constance Byarugaba. I also dedicate it to scientists, all over the world, devoted to laboratory research towards the design of efficacious vaccines against influenza viruses.
Acknowledgements

My sincere appreciation goes out to my study supervisors for the precious time spent giving me invaluable advice, knowledge, helpful scrutiny and corrections that have made this report contain what it has now. My gratitude also goes out to MUWRP-IRL for permitting me to use their space, materials and facilities during my research assistantship for my MSc project. The sequencing was done at St. Jude Children’s Research Hospital, in the USA, and they are greatly appreciated. My gratitude is extended to the MUWRP as a whole for the moral, psychological, material and financial support extended to me towards this achievement. This study was funded by the Global Emerging Infections Surveillance program of the US Army, through Makerere University Walter Reed Project, to which I extend my sincere gratitude. My gratitude further goes out to the patients who provided the samples. I thank my dear family for the patience you have exhibited during the course; your consistent prayers and constant encouragement have seen me through. Lastly, I acknowledge the hand of the Almighty God that has made possible the beginning and the end of this project against all odds.
# TABLE OF CONTENTS

**DECLARATION** .................................................................................................................. II

**DEDICATION** ...................................................................................................................... III

**ACKNOWLEDGEMENTS** ...................................................................................................... IV

**LIST OF ABBREVIATIONS AND SYMBOLS** ....................................................................... XII

**LIST OF AMINO ACID SYMBOLS AND CODONS** ............................................................... XIII

**ABSTRACT** .......................................................................................................................... XIV

**CHAPTER ONE** .................................................................................................................... 1

1.0 **INTRODUCTION** ........................................................................................................... 1

1.1 **Statement of the problem** ............................................................................................. 2

1.2 **Research questions** ....................................................................................................... 3

1.3 **Objectives of the study** ................................................................................................ 4

1.3.1 **General objective** ................................................................................................... 4

1.3.2 **Specific objectives** .................................................................................................. 4

1.4 **Justification of the study** .............................................................................................. 4

**CHAPTER TWO** ................................................................................................................... 5

2.0 **LITERATURE REVIEW** .................................................................................................. 5

2.1 **The influenza viral particle and genome** ....................................................................... 5

2.2 **Influenza diversity, epidemiology and prevalence** ....................................................... 6

2.2.1 **Diversity** ............................................................................................................... 6

2.2.2 **Epidemiology** ......................................................................................................... 7

2.2.2.1 **Predisposing factors** .......................................................................................... 7
2.6 Influenza vaccine research and development ................................................................. 28
2.6.1 Influenza vaccine strain selection ............................................................................. 28
2.6.2 Influenza vaccine design ......................................................................................... 28
2.6.3 Influenza vaccine types, administration and efficacy ............................................. 29
2.7 Human influenza A detection and characterization studies in Uganda ...................... 30

CHAPTER THREE .................................................................................................................. 31

3.0 MATERIALS AND METHODS .................................................................................... 31
3.1 Study design .................................................................................................................. 31
3.1.1 Study area ................................................................................................................. 31
3.1.2 Study population ...................................................................................................... 31
3.1.2.1 Target population ............................................................................................... 32
3.1.2.2 Accessible population ...................................................................................... 32
3.1.2.3 Study unit .......................................................................................................... 32
3.3 Sample size ................................................................................................................... 33
3.4 Sampling criteria .......................................................................................................... 33
3.4.1 Inclusion criteria ..................................................................................................... 33
3.4.2 Exclusion criteria .................................................................................................... 33
3.5 Sampling procedure ..................................................................................................... 34
3.5.1 Patient enrolment .................................................................................................... 34
3.5.2 Sample collection and transportation ..................................................................... 34
3.5.3 Sample handling ..................................................................................................... 35
3.6 Detection of influenza A viruses ................................................................................... 36
3.6.1 Screening of influenza A viruses by one-Step RT-PCR .......................................... 36
3.6.1.1 Amplification of influenza A matrix genes ......................................................... 36
3.6.1.2 Detection of PCR products on agarose gel ....................................................... 37
3.6.2 Virus isolation ........................................................................................................ 37
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.6.2.1 Observation of MDCK cells for CPE</td>
<td>38</td>
</tr>
<tr>
<td>3.6.2.2 Virus harvesting</td>
<td>39</td>
</tr>
<tr>
<td>3.6.3 Direct IFA</td>
<td>39</td>
</tr>
<tr>
<td>3.6.3.1 Preparation and staining of cells</td>
<td>39</td>
</tr>
<tr>
<td>3.7 Characterization of influenza A viruses</td>
<td>40</td>
</tr>
<tr>
<td>3.7.1 Sub-typing of influenza A HA and NA genes</td>
<td>40</td>
</tr>
<tr>
<td>3.7.1.1 Detection of PCR products on agarose gel</td>
<td>42</td>
</tr>
<tr>
<td>3.7.2 DNA sequencing</td>
<td>42</td>
</tr>
<tr>
<td>3.8 Data handling and analysis</td>
<td>43</td>
</tr>
<tr>
<td>3.8.1 Bioinformatic analyses</td>
<td>43</td>
</tr>
<tr>
<td>3.8.2 Statistical analyses</td>
<td>43</td>
</tr>
<tr>
<td>3.8.3 Homology analyses</td>
<td>43</td>
</tr>
<tr>
<td>3.8.4 Phylogenetic analyses</td>
<td>43</td>
</tr>
<tr>
<td>CHAPTER FOUR</td>
<td>45</td>
</tr>
<tr>
<td>4.0 RESULTS</td>
<td>45</td>
</tr>
<tr>
<td>4.1 The demographic characteristics of the study</td>
<td>47</td>
</tr>
<tr>
<td>4.1.1 Participant enrolment by month, site and gender</td>
<td>47</td>
</tr>
<tr>
<td>4.2 Influenza A virus detection results</td>
<td>48</td>
</tr>
<tr>
<td>4.2.1 One-step RT-PCR</td>
<td>48</td>
</tr>
<tr>
<td>4.2.2 Virus isolation</td>
<td>49</td>
</tr>
<tr>
<td>4.2.3 Direct IFA</td>
<td>49</td>
</tr>
<tr>
<td>4.3 Prevalence of influenza A viruses by month, site, gender and age-group</td>
<td>50</td>
</tr>
<tr>
<td>4.4 Influenza A virus characterization results</td>
<td>53</td>
</tr>
<tr>
<td>4.4.1 Influenza A HA / NA sub-typing by one step RT-PCR</td>
<td>53</td>
</tr>
<tr>
<td>4.4.1.1 Influenza A HA sub-typing</td>
<td>53</td>
</tr>
<tr>
<td>4.4.1.2 Influenza A NA sub-typing</td>
<td>54</td>
</tr>
</tbody>
</table>
LIST OF TABLES

Table 1: The role of influenza A virus genes in pathogenicity .................................................. 11
Table 2: Effects of amino acid changes within protein sequences of influenza A virus .......... 13
Table 3: History of Influenza A Virus Pandemics ...................................................................... 18
Table 4. Influenza A virus sequence characteristics and diversity indices ................................. 55
Table 5: Details for unique influenza A virus strain A/UgaKay/027/08(H3N2) ......................... 61
Table 6: The 31 influenza A virus PB2 gene strains detected in the study ............................... 65
Table 7: The 37 influenza A virus PB1 gene strains detected in the study ............................... 68
Table 8: The 31 influenza A virus PA gene strains detected in the study ................................. 71
Table 9: The 22 influenza A virus HA gene strains detected in the study ............................... 74
Table 10: The 4 influenza A virus NP gene strains detected in the study ............................... 77
Table 11: The 26 influenza A virus NA gene strains detected in the study ............................... 80
Table 12: The 21 influenza A virus M gene strains detected in the study ............................... 83
Table 13: The 23 influenza A virus NS gene strains detected in the study ............................... 86
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Schematic diagram of the influenza virion</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>Changes in human influenza A subtypes</td>
<td>19</td>
</tr>
<tr>
<td>3</td>
<td>Influenza A virus subtypes in the human population</td>
<td>20</td>
</tr>
<tr>
<td>4</td>
<td>Map of Uganda showing study participants’ districts of residence</td>
<td>46</td>
</tr>
<tr>
<td>5</td>
<td>Participant enrolment by month, site and gender</td>
<td>47</td>
</tr>
<tr>
<td>6</td>
<td>Gel image showing influenza A virus detection by RT-PCR</td>
<td>48</td>
</tr>
<tr>
<td>7</td>
<td>IFA slides showing influenza A virus detection in MDCK cells</td>
<td>49</td>
</tr>
<tr>
<td>8</td>
<td>Prevalence of influenza A virus by month and site</td>
<td>50</td>
</tr>
<tr>
<td>9</td>
<td>Prevalence of influenza A virus by month and gender</td>
<td>51</td>
</tr>
<tr>
<td>10</td>
<td>Prevalence of influenza A viruses by site and age-group</td>
<td>52</td>
</tr>
<tr>
<td>11</td>
<td>Gel images showing influenza A virus H1 and H3 sub-typing results</td>
<td>53</td>
</tr>
<tr>
<td>12</td>
<td>Influenza A virus strain distribution by month</td>
<td>57</td>
</tr>
<tr>
<td>13</td>
<td>Influenza A virus strain distribution per site by gene</td>
<td>57</td>
</tr>
<tr>
<td>14</td>
<td>The 28 unique sites in the 22 influenza A virus HA gene strains</td>
<td>58</td>
</tr>
<tr>
<td>15</td>
<td>The 44 unique sites in the 26 influenza A virus NA gene strains</td>
<td>59</td>
</tr>
<tr>
<td>16</td>
<td>The 24 unique sites in the 21 influenza A virus M gene strains</td>
<td>60</td>
</tr>
<tr>
<td>17</td>
<td>Un-rooted phylogenetic tree showing relationship of PB2 nucleotide sequences of the Ugandan isolates with those of the WHO reference and genbank strains for the year 2008</td>
<td>64</td>
</tr>
<tr>
<td>18</td>
<td>Un-rooted phylogenetic tree showing relationship of PB1 nucleotide sequences of the Ugandan isolates with those of the WHO reference and genbank strains for the year 2008</td>
<td>66</td>
</tr>
<tr>
<td>19</td>
<td>Un-rooted phylogenetic tree showing relationship of PA nucleotide sequences of the Ugandan isolates with those of the WHO reference and genbank strains for the year 2008</td>
<td>69</td>
</tr>
<tr>
<td>20</td>
<td>Un-rooted phylogenetic tree showing relationship of HA nucleotide sequences of the Ugandan isolates with those of the Kenyan, reference and genbank strains for the year 2008</td>
<td>72</td>
</tr>
<tr>
<td>21</td>
<td>Un-rooted phylogenetic tree showing relationship of NP nucleotide sequences of the Ugandan isolates with those of the WHO reference and genbank strains for the year 2008</td>
<td>75</td>
</tr>
<tr>
<td>22</td>
<td>Un-rooted phylogenetic tree showing relationship of NA nucleotide sequences of the Ugandan isolates with those of the Kenyan, reference and genbank strains for the year 2008</td>
<td>78</td>
</tr>
<tr>
<td>23</td>
<td>Un-rooted phylogenetic tree showing relationship of M nucleotide sequences of the Ugandan isolates with those of the Kenyan, reference and genbank strains for the year 2008</td>
<td>81</td>
</tr>
<tr>
<td>24</td>
<td>Un-rooted phylogenetic tree showing relationship of NS nucleotide sequences of the Ugandan isolates with those of the reference and genbank strains for the year 2008</td>
<td>84</td>
</tr>
<tr>
<td>25</td>
<td>Number of mutations per gene-site</td>
<td>87</td>
</tr>
</tbody>
</table>
# LIST OF ABBREVIATIONS AND SYMBOLS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMSF</td>
<td>Biological material shipping form</td>
</tr>
<tr>
<td>BSL-2</td>
<td>Bio-safety level 2</td>
</tr>
<tr>
<td>cDNA</td>
<td>Copy Deoxyribonucleic acid</td>
</tr>
<tr>
<td>CPE</td>
<td>Cytopathic effect</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
</tr>
<tr>
<td>dNTPs</td>
<td>Deoxyribonucleotide triphosphates</td>
</tr>
<tr>
<td>HA</td>
<td>Hemagglutinin</td>
</tr>
<tr>
<td>IFA</td>
<td>Immuno-fluorescence assay</td>
</tr>
<tr>
<td>IgA</td>
<td>Immunoglobulin A</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>M</td>
<td>Matrix gene</td>
</tr>
<tr>
<td>MDCK</td>
<td>Madin Darby Canine Kidney</td>
</tr>
<tr>
<td>mM</td>
<td>Milli molar</td>
</tr>
<tr>
<td>MP</td>
<td>Matrix Protein</td>
</tr>
<tr>
<td>MSc</td>
<td>Master of Science</td>
</tr>
<tr>
<td>MUWRP-IRL-1</td>
<td>Makerere University Walter Reed Project-Influenza Research Laboratory-1</td>
</tr>
<tr>
<td>NA</td>
<td>Neuraminidase</td>
</tr>
<tr>
<td>NP</td>
<td>Nucleoprotein</td>
</tr>
<tr>
<td>NS</td>
<td>Non-structural protein</td>
</tr>
<tr>
<td>P2</td>
<td>Passage 2</td>
</tr>
<tr>
<td>PA</td>
<td>Polymerase gene segment 3</td>
</tr>
<tr>
<td>PB2</td>
<td>Polymerase gene segment 1</td>
</tr>
<tr>
<td>PB1</td>
<td>Polymerase gene segment 2</td>
</tr>
<tr>
<td>RPM</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>TPCK</td>
<td>Tosyl phenylalanyl chloromethyl ketone</td>
</tr>
<tr>
<td>USA</td>
<td>United States of America</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>VTM</td>
<td>Virus Transport Media</td>
</tr>
<tr>
<td>μl</td>
<td>Micro liter</td>
</tr>
<tr>
<td>μM</td>
<td>Micro molar</td>
</tr>
</tbody>
</table>
# LIST OF AMINO ACID SYMBOLS AND CODONS

<table>
<thead>
<tr>
<th>Amino acids</th>
<th>Symbols</th>
<th>Codons</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>Ala</td>
<td>A</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GCA, GCC, GCG, GCT</td>
</tr>
<tr>
<td>Cysteine</td>
<td>Cys</td>
<td>C</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TGC, TGT</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>Asp</td>
<td>D</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GAC, GAT</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>Glu</td>
<td>E</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GAA, GAG</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>Phe</td>
<td>F</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TTC, TTT</td>
</tr>
<tr>
<td>Glycine</td>
<td>Gly</td>
<td>G</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GGA, GGC, GGG, GGT</td>
</tr>
<tr>
<td>Lysine</td>
<td>Lys</td>
<td>K</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AAA, AAG</td>
</tr>
<tr>
<td>Leucine</td>
<td>Leu</td>
<td>L</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TTA, TTG, CTA, CTC, CTG, CTT</td>
</tr>
<tr>
<td>Methionine</td>
<td>Met</td>
<td>M</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ATG</td>
</tr>
<tr>
<td>Asparagine</td>
<td>Asn</td>
<td>N</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AAC, AAT</td>
</tr>
<tr>
<td>Glutamine</td>
<td>Gln</td>
<td>Q</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CAA, CAG</td>
</tr>
<tr>
<td>Arginine</td>
<td>Arg</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AGA, AGG, CGA, CGC, CGG, CGT</td>
</tr>
<tr>
<td>Serine</td>
<td>Ser</td>
<td>S</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AGC, AGT, TCA, TCC, TCG, TCT</td>
</tr>
<tr>
<td>Threonine</td>
<td>Thr</td>
<td>T</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ACA, ACC, ACG, ACT</td>
</tr>
<tr>
<td>Valine</td>
<td>Val</td>
<td>V</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GTA, GTC, GTG, GTT</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>Tyr</td>
<td>Y</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TAC TAT</td>
</tr>
</tbody>
</table>
ABSTRACT

Human influenza A viruses are known to cause severe illnesses and fatalities. Therefore, their continuous surveillance is crucial for the early detection and appropriate interventions through treatment therapies and vaccination. The aim of this study was to detect and characterize human influenza A virus isolates from Kayunga and Mulago hospitals in Uganda. A total of 450 human respiratory samples were collected. All samples were screened for influenza A virus using a One Step RT-PCR Qiagen kit and M gene specific primers from Applied Biosystems, UK. A volume of 100 µl from each of the samples that tested positive for Influenza A viruses were cultured on MDCK (Madin Darby Canine Kidney) cell line passage 2 in T25 culturing flasks. The isolates that showed cytopathic effect (CPE) were subjected to immune-fluorescence assay (IFA) using a Light Diagnostics ™ Influenza A and B DFA kit. The samples confirmed positive by IFA were then sub-typed by One Step RT-PCR Qiagen kit using H1N1 and H3N2 specific primers from Applied Biosystems. This was followed by whole genome DNA sequencing of PCR products using the Illumina Genome Analyser IIe. There were 51 positive samples for influenza A viruses detected by One Step RT-PCR. The viruses from the samples all showed CPE when cultured on MDCK cell line and were further confirmed positive for influenza A viruses by IFA. The prevalence of the influenza A viruses was 11.3%. The participants who were of the 6 months to 5 years age-group had the highest infection rate, 80%. The infection rates were highest during November 2008 and the Mulago site registered more positive cases. All viruses detected were of the H3N2 subtype. The influenza A viral genes, PB2, PA, and NP, had significant amino acid changes that have been reported to be associated with host transmission and low virulence. All study isolates were of different strains that had nucleotide diversities by each of the eight genes. The human influenza A H3N2 viruses detected were already existent worldwide. Their gene sequences were 99% similar to those of other strain sequences of viruses detected in 2008 and submitted in the genbank database. The study findings indicated the presence of influenza A H3N2 viruses with a prevalence rate of 11.3% in the Ugandan human population. All the study strains were already existent worldwide.
CHAPTER ONE

1.0 INTRODUCTION

Influenza, commonly known as flu, is an infectious disease of birds and mammals caused by RNA viruses possessing negative single stranded genomes. It comprises of 5 genera namely influenza A, influenza B, influenza C, isavirus and thogotovirus. Of the 5, the influenza A, B and C virus types have been known to cause disease in humans. Influenza A virus can be subdivided into different serotypes based on the antibody responses to their HA and NA proteins (Webster et al., 1992). Although influenza A is the most common cause of human influenza illnesses, unknown proportions of cases are attributed to other types; B and C (Rendell, 2006). Primarily, influenza affects people in both developing and industrialized countries and is capable of killing healthy persons of all ages, mainly the patients aged 5 years and below, and those aged 65 Years and above (Zambon, 1999; Hilleman, 2002; Suarez et al., 2003; Nobusawa & Sato, 2006). Influenza A has a broad host range as it can cause disease in poultry, birds, pigs and humans, although its main reservoir is in wild birds (Simonsen, 1999). As such, Influenza A remains an important disease in many countries of the world.

In developed countries, influenza A infections have often been reported to cause significant illnesses (Simonsen, 1999). There is very little information available about influenza A infections in developing countries. However, the risk factors in both animals and humans are known to be close associations between healthy humans with infected humans and/or potentially infected animals such as pigs and poultry (Munier et al., 2010). Although influenza A viruses are endemic in wild birds, domestic reservoirs constitute important sources of infection that undercut control measures (Griot & Hoop, 2007). For example, in many African countries domestic
animals, such as poultry and pigs, are an integral part of human social life. As a result, humans are prone to influenza A virus infection through interspecies transmission (Sorell et al., 2007; Fasina et al., 2010). In addition, human to human transmission and the emergence of highly virulent influenza A viral strains has also been documented, raising infection control concerns in health settings. Other risk factors include food hygiene practices like consumption of infected raw poultry meat and pork, travelling with animals in public vehicles and immigration of infected people to areas of healthy populations. However, influenza A viruses are mostly transmitted by the aerosol route. As such, influenza A circulates within various human populations globally causing epidemics and pandemics (Tellier, 2006; Hall, 2007; Weber & Stilianakis, 2008).

Flu-like infections have been endemic in Uganda for no less than four decades (Montefiore et al., 1970). However, the country’s economic constraints have previously precluded the implementation of surveillance programs to determine which infections are due to influenza A viruses, let alone detect which particular strains are in circulation and/ or prevalent. This information would have contributed towards intervention efforts aimed at designing efficacious vaccines and anti-virals (Stohr, 2003). By October 2008, Uganda had not yet started any influenza A surveillance studies for the determination of circulating influenza A virus strains within the human population.

1.1 Statement of the problem

Flu-like infections have been endemic in Uganda for no less than four decades. As a result, many human patients in Uganda present with flu-like symptoms to health facilities. However, at the time of this study, it was not known which of these illnesses were due to influenza A viral
infections. Therefore, there has always been a risk of the frequent occurrences of seasonal influenza A and B viral epidemics which have posed as a health threat particularly in medically high-risk groups. Also, previous studies have indicated that influenza A viruses have a wide host range (Hayden & Palese, 1997) and that close association of humans with poultry and swine, have led to the risk of exposure to influenza A viruses and possibility of interspecies transmission (Griot & Hoop, 2007, Sorell et al., 2007). This may pose a possibility of emergence of new strains, with enhanced infectious ability and increased replication that can cause as high as 100% mortality of the infected populations (Drake, 1993; Lamb & Krugg, 1996; Zambon, 2001; Hilleman, 2002; Thompson et al., 2003; Peiris et al., 2004; WHO, 2005; Kash et al., 2006). If present within the human population in Uganda, the influenza A viruses pose as a serious health threat particularly in medically high-risk groups; such as the immunocompromised (Hilleman, 2002). Human influenza A viral disease is, thus, a public health problem of concern and constant challenge to both the medical and veterinary professions, hence, the need to maintain regular surveillance.

1.2 Research questions

1. Are there influenza A viruses circulating among human patients attending Mulago and Kayunga hospitals in Uganda?

2. How are influenza A viruses in Uganda related to other worldwide strains detected in 2008?
1.3 Objectives of the study

1.3.1 General objective

To detect the presence of human influenza A viruses in patients attending Mulago and Kayunga hospitals in Uganda.

1.3.2 Specific objectives

1. To establish the occurrence of human influenza A virus isolates from patients attending Kayunga and Mulago hospitals in Uganda.

2. To characterize the influenza A virus isolates genotypically by one-step conventional RT-PCR and DNA sequencing and establish the circulating serotypes

1.4 Justification of the study

Flu-like illnesses are common in Uganda with many patients presenting to health facilities with presumptive symptoms. Until this study, it was not known which influenza viruses were circulating in the Ugandan human population and responsible for the patients’ illnesses. This study was carried out to detect and characterize human influenza A viruses from patients attending Kayunga and Mulago hospitals in Uganda. The information obtained from the study will lead to better understanding of the contribution of influenza A viruses to flu-like illnesses in Uganda. This will also contribute to the existing scientific pool of knowledge of the circulating human influenza A viruses in Sub-Saharan Africa and globally. The information obtained could also be used to guide policy in the development of appropriate treatment for infected persons and vaccines for subsequent flu seasons.
CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 The influenza viral particle and genome

The Influenza virus particle is highly pleiomorphic, 80-120nm and has a total genome size of 12-15kb. It consists of 8 ss- negative sense RNA segments numbered 1 to 8 in decreasing order of electrophoretic mobility and are known to range from 2341 to 890 nucleotides.

<table>
<thead>
<tr>
<th>Genome segment</th>
<th>Poly-peptide(s)</th>
<th>Viral Proteins</th>
<th>Size (nt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>PB2</td>
<td>Transcriptase associate (polymerase) proteins</td>
<td>2341</td>
</tr>
<tr>
<td>2</td>
<td>PB1</td>
<td></td>
<td>2341</td>
</tr>
<tr>
<td>3</td>
<td>PA</td>
<td></td>
<td>2233</td>
</tr>
<tr>
<td>4</td>
<td>HA</td>
<td>Hemagglutinin</td>
<td>1778</td>
</tr>
<tr>
<td>5</td>
<td>NP</td>
<td>Nucleocapsid protein</td>
<td>1565</td>
</tr>
<tr>
<td>6</td>
<td>NA</td>
<td>Neuraminidase</td>
<td>1413</td>
</tr>
<tr>
<td>7</td>
<td>M1</td>
<td>Matrix protein</td>
<td>1027</td>
</tr>
<tr>
<td></td>
<td>M2</td>
<td>Matrix protein</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>NS1</td>
<td>Non-structural proteins 1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NS2</td>
<td>Non-structural proteins 2</td>
<td>890</td>
</tr>
</tbody>
</table>

Figure 1: Schematic diagram of the influenza virion.

Figure 1 shows the eight segments of single stranded genome RNA in the form of helical ribonucleoproteins within the envelope. The eight genome segments code for 10 proteins (helical in configuration and 9nm in diameter). Five of the genes encode structural proteins while three encode polymerase gene as shown in figure 1. The coding assignments of each of the eight segments are indicated on the left (Lamb & Choppin, 1983; Clancy, 2008). The proteins encoded by the genes are polymerase proteins (PB2, PB1 and PA genes); hemagglutinin proteins (HA gene), nucleo protein (NP gene), neuraminidase proteins (NA gene), matrix proteins (M
gene) and the non structural protein (NS gene). On the surface of the virion are the two surface glycoproteins, HA and NA. They are inserted through the lipid bilayer of the viral membrane. On the inner surface of the lipid bilayer is the membrane protein. The RNA in nucleocapsid that is RNase sensitive and undergoes viral transcription within the host cell nucleus (Smith & Helenius, 2004).

2.2 Influenza diversity, epidemiology and prevalence

2.2.1 Diversity

Influenza viruses belong to the family Orthomyxoviridae consisting of five genera; Influenza A viruses, Influenza B viruses, Influenza C viruses, Isaviruses and Thogotoviruses (Kawaoka, 2006). There are three main types of influenza viruses; A, B and C. However, the influenza viruses of public health concern are types A and B (Wright et al., 1995). Influenza type A viruses are divided into subtypes based on two proteins, HA and NA on the surface of the virus, and their responses to antibodies (Webster et al., 1992). These serotypes have been ordered by the number of known human pandemic deaths (Hilleman, 2002). Currently, there are sixteen HA (H1-H16) and nine NA (N1-N9) subtypes of influenza A viruses diverging by as much as 50% in their overall amino acid composition (WHO, 2002; Nicholson et al., 2003). Within each subtype, smaller amino acid substitutions (drifts) that enable influenza A viruses to evade preexisting immunity exist (Nicholson et al, 2003). Influenza A evolves 2-3 times faster than type B which evolves faster than type C, in humans (Yamashita et al., 1988; Fouchier et al., 2004). Influenza B is less genetically diverse, with only one serotype, which is not categorized by HA and NA subtypes and as a result a degree of immunity to influenza B is usually acquired at an early age but does not last due to frequent mutations (Kawaoka, 2006).
Influenza B almost only infects humans (Hay et al., 2001; Kawaoka, 2006). Its reduced rate of antigenic change, combined with its limited host range is the reason that pandemics of influenza B do not occur (Nobusawa & Sato, 2006). Both Influenza A and C infect multiple species. Type C is rarer than the other types and causes mild disease in children (Webster et al, 1992; Webster, 1997; Meltzer et al., 1999; Zambon, 1999).

2.2.2 Epidemiology

The critically important influenza virus types are A and B (Wright et al., 1995). On average, a sixth of samples collected from persons with influenza like illness (ILI) are positive for type A and/ or B viruses. These two types are responsible for the seasonal human influenza epidemics. However, of the three types, influenza A causes the most severe disease (CDC, 2004a).

2.2.2.1 Predisposing factors

The predisposing factors include: host factors such as age (patients aged 5 years and below and those aged 65 years and above) and immune status (individuals with immunosuppression), agent factors such as high genetic plasticity of the influenza A viruses and environmental factors such as increased humidity, decreased vitamin D levels from the sun; cold weather and crowding of persons especially during cold weather (Hilleman, 2002; Shek & Lee, 2003).

2.2.2.2 Transmission

Influenza virus is partially host restricted. However transmission may be interspecies or intra-species depending on which host cell receptors are present for viral attachment (Connor et al., 1994). Analyses of the genetic basis for virulence by using re-assortants that possess mixtures of genes from virulent and avirulent strains have identified various groupings of genes, which in aggregate implicate all eight genome segments (Brown et al., 2001, Tumpey et al., 2007).
2.2.2.2.1 Species and barriers

The transmission, of influenza A viruses from one host to another depends on the barriers of the latter that restrict viral transmission between some species. Birds possess α2,3 sialic acid receptors for viral attachment whereas humans possess α2,6 sialic acid receptors. The amino acids that make up the receptor-binding site are highly conserved, even among the HAs of different subtypes of avian influenza virus; however, those of human viruses display distinct variability. In particular, the residues at positions 138, 190, 194, 225, 226, and 228 are highly conserved in the avian receptor binding sites, whereas the HA ligands specific to human receptors harbor substitutions at these positions. In H2 and H3 influenza virus strains, residues at positions 226 and 228 in the HA correlate with the preferential recognition of the sialic acid-galactosidase (SA-Gal) linkage by HA and the host species from which the virus was isolated. HAs with Leu at position 226 (Leu-226) and Ser-228 (human viruses) preferentially recognize SAα2,6 Gal, whereas those with Gln-226 and Gly-228 (avian and equine viruses) recognize SAα2,3 Gal (Connor et al., 1994). There is, therefore, no direct means of transmission between humans and birds due to the barrier limitations. However, pigs possess both α2,3 and α2,6 sialic acid receptors in their respiratory tracts and, therefore, can be infected with either avian or human viruses (Neumann & Kawaoka, 2006). Within the pigs, genetic reassortments between segments of subtypes from the two hosts leading to the formation of new subtypes and, in some cases, new strains within the latter. These newly formed subtypes may then be transmitted to other hosts bearing α2,3 and/or α2,6 sialic acid receptors. Thus, pigs make the transmission of the virus be relatively easy (Yassine et al., 2007). Studies have indicated that changes at residues 226 and 228 in the HA of avian viruses that have transmitted to mammals such as pigs (Scholtissek et al., 1983; Guan et al., 1996) and horses (Webster & Guo 1991), show that the
viruses may have acquired the ability to recognize receptors in the human trachea (Vines et al., 1998).

Therefore, during the surveillance of viruses in nonhuman animals, it is particularly important to pay attention to the amino acid residues at these positions. Genes other than HA are also known to contribute to the host range restriction of influenza A viruses. Such genes include PB2 (Subbarao et al., 1993; Almond, 1977), NA (Hinshaw et al. 1983), and NP (Scholtissek et al., 1985). The influenza A viral amino acids that infer human specificity by gene are; PB2 (S199, T661, R702), PA (N409), NP (M136), (Zhou et al., 1999). However, the molecular mechanisms by which these other genes affect the host range of influenza viruses are not yet known.

2.2.2.2 Geographic differences

Phylogenetic maps indicate that human influenza A viruses are minimally impacted by geographic differences unlike swine and avian viruses that have appeared to be geographically dependent (Liu et al., 2009). Some lineages and sub-lineages of the virus emerge and may be more prevalent in certain locations. For instance, most human influenza outbreaks begin in Southeast Asia (Anon, 2004; Park & Glass 2007). This is because of crowded conditions in southeast and east Asia, where humans, pigs and poultry live in close quarters. These conditions enable the influenza virus to mutate into forms that infect humans more easily (Ferguson et al., 2005)

2.2.2.3 Pathogenicity factors

Molecular Epidemiology of influenza viruses reveals that the HA and NA genes are largely involved in pathogenicity (Shawn et al., 2009). The HA gene encodes the respective surface glycoprotein antigens. It is a lectin synthesized as a precursor protein, HA0, and is the critical
determinant of pathogenicity and restriction of interspecies viral transmission. The HA0 allows the recognition of target vertebrate cells and has to undergo proteolytic cleavage by a host cell protease into the disulfide linked subunits HA1 and HA2 that results into the conformational change in the HA molecule. This is a prerequisite for fusion which is accomplished through the binding of these cells' sialic acid-containing receptors. The infectivity of the virus follows, depending on the viral strain, host cell type and growth conditions which lead to the systemic spread of virus (Banks & Plowright, 2002; Palese, 2004). The presence and number of basic amino acid (R/K) sequences on the cleavage site region starting from HA1 (amino acid position 320) and ending HA2 (amino acid position 329) on the globular portion of the HA gene segment, are responsible for the degree of pathogenicity of the virus. A polybasic (4 or more basic amino acids) endoproteolytic cleavage site within the HA precursor protein is regarded as a reliable genetic marker for high virulence of a given influenza virus whereas the mono-/di-basic (1 to 2 basic amino acids) cleavage site is an indicator for its low virulence (Banks & Plowright, 2002; Shawn et al., 2009). The NA gene encodes an enzyme that digests sialic acid on most influenza A cellular receptors, assists in the mobility of virus particles through the respiratory tract mucus, enables the release of mature virions from an infected host cell which, and in turn, triggers the humoral immune response. It may also contribute to initial viral entry. However, the NA serves the opposite function to that of HA, and due to their opposite functions, mutations in HA and NA can suppress each other (Matrosovich et al., 2004). Besides the HA gene other influenza A viral genes have been found to play a role in pathogenicity (Banks & Plowright, 2002) as shown in table 1.
Table 1: The role of influenza A virus genes in pathogenicity

<table>
<thead>
<tr>
<th>Segment</th>
<th>Poly-peptide(s)</th>
<th>Mr Size (nt)</th>
<th>Amino acids</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>PB2</td>
<td>2341</td>
<td>780</td>
<td>enhances replication and viral spread -RNA Polymerase/Transcriptase: cap binding</td>
</tr>
<tr>
<td>2</td>
<td>PB1</td>
<td>2341</td>
<td>780</td>
<td>RNA Polymerase/Transcriptase: elongation of mRNA chain</td>
</tr>
<tr>
<td>3</td>
<td>PA</td>
<td>2233</td>
<td>744</td>
<td>RNA Polymerase/Transcriptase: protease activity</td>
</tr>
<tr>
<td>4</td>
<td>HA</td>
<td>77000</td>
<td>1778</td>
<td>592</td>
</tr>
<tr>
<td>5</td>
<td>NP</td>
<td>1565</td>
<td>521</td>
<td>Nucleoprotein: RNA binding; part of transcriptase complex; nuclear/cytoplasmic transport of vRNA protein</td>
</tr>
<tr>
<td>6</td>
<td>NA</td>
<td>56000</td>
<td>1413</td>
<td>471</td>
</tr>
<tr>
<td>7</td>
<td>M1</td>
<td>27861</td>
<td>1027</td>
<td>252</td>
</tr>
<tr>
<td></td>
<td>M2</td>
<td>15000</td>
<td>26000</td>
<td>230-237</td>
</tr>
<tr>
<td>8</td>
<td>NS1</td>
<td>890</td>
<td></td>
<td>Non-structural: nucleus; effects on cellular RNA transport, splicing, translation</td>
</tr>
<tr>
<td></td>
<td>NS2</td>
<td>26000</td>
<td>59-66</td>
<td>Non-structural: nucleus+cytoplasm, function unknown</td>
</tr>
</tbody>
</table>

Mr: Molecular Weight
nt - Nucleotide

(Source: Lamb & Choppin, 1983)

2.2.2.3.1 Infection, replication and spread

Influenza viruses infect both ciliated and non-ciliated epithelial cells of the trachea. Influenza viral replication peaks at approximately 48 hours after inoculation and declines slowly after 18 to 72 hours of incubation, with little virus shedding after 6 days (Petri et al., 2006). Viral entry is followed by spread within the epithelial cells by cell-to-cell contact, bypassing receptors and, eventually, leads to the shutdown of protein synthesis and the induction of apoptosis. The cells die, in part due to the direct effects of the virus on the cell and also, possibly, due to the effects of interferon as well as the actions of cytotoxic T-cells. As a result, the efficiency of ciliary clearance is reduced, leading to impaired function of the mucus elevator in the respiratory tract of the infected host. The removal of ciliated mucus producing epithelial cells impairs the body’s ability to clear the influenza virus (Gu et al., 2007). People who contract influenza are most infective between 48 to 72 hours post inoculation, when viral shedding is the highest, and
infectivity lasts for around 10 days (Petri et al., 2006). Children are notably more infectious than adults, and shed virus from just before they develop symptoms until 2 weeks after infection (Holmes et al., 2005).

2.2.2.3.2 Recovery and protection

The cell-mediated immune response is important in viral clearance whereas the humoral immune response, the main source of protection against re-infection, is usually not significant until after the virus has been cleared. The latter comprises of IgG which persists longer than the IgA immunoglobulin and, therefore, plays a more important role in long term immunity (Takahashi, 2007). Since the HA and NA proteins are antigenic, they are targeted during influenza A vaccine design (Hilleman, 2002; Ghedin et al., 2005). The antibody to the HA protein is of greatest important since it can neutralize the virus and prevent the virus from initiating an infection. The NA specific antibody slows viral spread (CDC, 1999). Virulence is the measure of the ability of a pathogen to damage its host. Human influenza A virus infection typically causes tracheobronchitis with a low incidence of fatal pneumonia. There is, thus, a need to understand the genetic basis for virulence in influenza virus variants, with the hope that specific mutations will be indicators and, thus, predictors of virulence (Kobasa et al., 2004). Virulence may be expressed by viral increased antigenicity, pathogenicity, replicative fitness, ease of transmission to different hosts, increased drug resistance, hosts (human passage) and change in host specificity as shown in table 2.
Table 2: Effects of amino acid changes within protein sequences of influenza A virus

<table>
<thead>
<tr>
<th>Gene segment</th>
<th>Amino acid change</th>
<th>Functional Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>PB2</td>
<td>E627K</td>
<td>Enhances HA cleavage</td>
</tr>
<tr>
<td>PB2</td>
<td>D701N</td>
<td>Enhances HA cleavage</td>
</tr>
<tr>
<td>PB2</td>
<td>A199S</td>
<td>Human Transmission</td>
</tr>
<tr>
<td>PB2</td>
<td>A661T</td>
<td>Human Transmission</td>
</tr>
<tr>
<td>PB2</td>
<td>V667I</td>
<td>Human Transmission</td>
</tr>
<tr>
<td>PB2</td>
<td>K702R</td>
<td>Human Transmission</td>
</tr>
<tr>
<td>PB2</td>
<td>S409N</td>
<td>Human Transmission</td>
</tr>
<tr>
<td>PB2</td>
<td>L136M</td>
<td>Human Transmission</td>
</tr>
<tr>
<td>PB1</td>
<td>N66S</td>
<td>Altered function-protein absent</td>
</tr>
<tr>
<td>PA</td>
<td>D at 556</td>
<td>Host specificity</td>
</tr>
<tr>
<td>PA</td>
<td>E133G</td>
<td>Replicative advantage</td>
</tr>
<tr>
<td>PA</td>
<td>N at 409</td>
<td>Host specificity</td>
</tr>
<tr>
<td>HA1</td>
<td>L at 226</td>
<td>Host specificity in humans</td>
</tr>
<tr>
<td>HA1</td>
<td>S at 228</td>
<td>Host specificity in humans</td>
</tr>
<tr>
<td>HA1</td>
<td>G218Y</td>
<td>Antibody escape</td>
</tr>
<tr>
<td>HA1</td>
<td>Polybasic cleavage site</td>
<td>High pathogenicity</td>
</tr>
<tr>
<td>HA1</td>
<td>Monobasic cleavage site</td>
<td>High pathogenicity</td>
</tr>
<tr>
<td>HA2</td>
<td>T156N</td>
<td>Loss of glycosylation site</td>
</tr>
<tr>
<td>NP</td>
<td>D at 34</td>
<td>Host specificity</td>
</tr>
<tr>
<td>NP</td>
<td>M at 136</td>
<td>Host specificity</td>
</tr>
<tr>
<td>NA</td>
<td>E119V</td>
<td>Resistance to Oseltamivir</td>
</tr>
<tr>
<td>NA</td>
<td>R292K</td>
<td>Resistance to Oseltamivir</td>
</tr>
<tr>
<td>M</td>
<td>C55F</td>
<td>Human Transmission</td>
</tr>
<tr>
<td>M</td>
<td>V28V</td>
<td>Human Transmission</td>
</tr>
<tr>
<td>M</td>
<td>A16G</td>
<td>Human Transmission</td>
</tr>
<tr>
<td>NS2</td>
<td>K88R</td>
<td>Modulating virulence</td>
</tr>
<tr>
<td>NS1</td>
<td>T92E</td>
<td>Increased disease severity</td>
</tr>
</tbody>
</table>

(Source: Connor et al., 1994; Barnett et al., 1999; Zhou et al., 1999; Brown et al., 2001; Carr et al., 2002; Jackson et al., 2005; Yen et al., 2005; Abed et al., 2006; Baz et al., 2006; Yen et al., 2006; Tumpey et al., 2007; Yen et al., 2007)

2.2.2.3.3 Influenza A virus disease diagnosis

Influenza like illness (ILI) is a description of flu-like symptoms such as; fever, body aches, fatigue, chills, sore throat, headache, coughing, weakness and general discomfort (Martin & Martin-Granel, 2006). However, the application of molecular tools during laboratory testing of samples from patients with ILI has been used to confirm the presence or absence of influenza A viruses (Taubenberger & Layne, 2001, Barry, 2010).
2.2.2.3.4 Challenges of influenza infections

Influenza viral infections are extremely difficult to resolve for three main reasons. First is the continued emergence of new strains due to the rapid and unpredictable mutations that lead to some form of antigenic drift over time resulting in the dominance of new strains with altered epitopes. Secondly, although the current antivirals such as the NA inhibitors are often effective against both influenza A and B, they do not eliminate viral loading but only halt the spread of the virus in the body, thereby, reducing the symptoms and complications (Glasgow and Middleton, 2001; Bootsma and Ferguson, 2007). Thirdly, due to the constant evolution of influenza viruses, differing degrees of resistance against these antivirals and vaccine mismatches have become a problem; whereby one of the strains selected for the vaccine doesn't optimally match and protect against the circulating influenza strains in vaccinated populations (Moscona, 2005; Recombinomics Commentary, 2008). Therefore, human populations, particularly the medically vulnerable groups, are at a high risk of infection by the constantly evolving viral strains in circulation that often cause annual seasonal epidemics (Ompad et al., 2006).

2.2.2.3.5 Management of influenza A infections

2.2.2.3.5.1 Treatment

Flu effects can be minimized using; antivirals such as NA inhibitors (Kiso et al., 2004). The NA inhibitors are useful for combating influenza infection, for example: zanamivir, administered by inhalation; oseltamivir, administered orally; and under research is peramivir administered parenterally, by intravenous or intramuscular injection. However, due to the constant evolution of influenza viruses over time, there have been reports of influenza A viral resistance to antivirals such as oseltamivir, with H1N1 showing resistance of up to 98% (Moscona, 2005; Webster & Govorkova, 2006; Racaniello, 2008). Recent studies have also shown that there has
been a huge increase in the amount of resistance to antiviral compounds such as, adamantanes. The incidence of adamantane resistance among the influenza A/H3N2 viruses has increased from 0.8% in the early 1990s to approximately 12.3% in 2004, reaching as high as 96% in certain regions of China (Bright et al., 2006; Deyde et al., 2007; Simonsen et al., 2007). However, recent studies have indicated that the proportion of adamantane's resistance among influenza A/H1N1 viruses was found to be at a global average of only 5.8% (Deyde et al., 2007). These records of built up resistance infer that drugs, such as adamantane, will not be useful against the influenza A H3N2 viruses in the future. Antibiotics are administered to prevent secondary infections due to bacteria (Shun-shin et al., 2009).

2.2.2.3.5.2 Vaccination

For large populations, the only effective preventive measure against influenza is by selective vaccination which is strongly recommended for high-risk groups in industrialized and developing countries (Villegas, 1998; Couch, 2000). Selective vaccination against influenza has proven to be the best option against influenza A viral infections. Influenza A evolution indicates that the HA gene undergoes minor and occasional major changes while NA undergoes some variation, hence, the continual need for updated influenza vaccines (Webster et al., 2006).

2.2.2.3.6 Adaptive mutations

Adaptation is an evolutionary process during which a population becomes better suited to its habitat. Antigenic mutants can evolve quickly due to the pressure of host immune system responses. These mutants have been found to undergo convergent and parallel forms of evolutions (Bull et al., 1997, Brown, 2000). The replacement of the parental influenza virus population by the adapted variants is due to their increased replication rate. The primary feature
of the dominant variant viruses that out-compete the less fit parental viruses is that their prevalence, within the host population, is increased because of improved replicative fitness. Other adaptive changes may lead to increased transmissibility and host specificity (Shaw et al., 2002, Miotto et al., 2010). The location of these mutations identifies novel modulators of virulence. The surface glycoprotein HA is under selective pressure to undergo mutation in order to evade the host's immune system (Scholtissek, 1995). Antibodies against the HA protein inhibit receptor binding and are very effective at preventing re-infection with the same strain. However, HA can change to evade previously acquired immunity either by antigenic drift, whereby mutations of the currently circulating HA gene disrupt antibody binding, or by antigenic shift, with the virus acquiring an HA of a new subtype by re-assortment of one or more gene segments (Scholtissek, 1995).

2.2.2.3.6.1 Antigenic variation

This is the process by which a given virus strain alters its surface glycoproteins in order to evade a given host’s immune response. The continuous evolution of influenza A viruses has led to a continuing battle for survival between host and parasite in which the host population updates the specificity of its pool of humoral immunity by contact with and response to infection with the most recent viruses which possess altered antigenic specificity in their HA ligand. The altered viral antigens then bind to the HA ligand on the cell to bring about infection (Scholtissek, 1995; Pfeiffer & Kirkegaard, 2006).
2.2.2.3.6.1.1 Genetic drifts

Antigenic drifts are the major source of genetic variation. These are caused by point substitutions within a given influenza virus gradually leading to the slight alteration of its antigenic epitopes. The viral RNA bears nucleotide insertions errors; one roughly every ten thousand nucleotides, and has slight alterations on their epitopes that have developed over time because their RNA is relatively error prone (Drake, 1993). The epidemic strains usually have HAs with monobasic cleavage sites associated with the development of mild disease within a given human population. When established subtypes exhibit antigenic drifts that cause influenza infections in a given population, frequent seasonal epidemics as well as large increases in deaths, in some inter-pandemic seasons occur (Simonsen et al., 1998). After recovering from one antigenic variant of the virus, a person is usually at least partially susceptible to new variants within a few years, due to gradual evolution of the virus (Cox & Bender, 1995).

2.2.2.3.6.1.2 Genetic shifts

These, on the other hand, are caused by genetic reassortments between two or more influenza viruses infecting the same host cell. The reassortment between these viruses has been enabled by the segmented nature of their genomes and also depends on how well the two viruses replicate within the host they have co-infected (Murphy & Webster, 1990). These reassortments have led to the emergence of novel influenza A viruses having new epitopes which have quickly spread through given immunely naïve human populations. Previous pandemics were caused by influenza A viruses formed after reassortments between human and avian sources (Gething et al., 1980; Fang et al., 1981). These in some cases, led to mortality of the infected persons with rates reaching as high as 100% (Drake, 1993; Lamb & Krugg, 1996; Zambon, 2001; Hilleman, 2002; Thompson et al., 2003; Kash et al., 2006).
Human influenza A pandemics were historically caused by subtypes of HA (H1, H2 and H3) and NA (N1 and N2) and were characteristically highly virulent because of the presence of the polybasic cleavage site on each of their HA genes (Qi et al., 2009, Taubenberger & Kash, 2010). Serotypes that have been confirmed in humans ordered by the number of known pandemic deaths are shown in table 3.

Table 3: History of Influenza A Virus Pandemics

<table>
<thead>
<tr>
<th>Year</th>
<th>Pandemic</th>
<th>Influenza A Subtype</th>
<th>Deaths</th>
</tr>
</thead>
<tbody>
<tr>
<td>1889-90</td>
<td>Asiatic (Russian) Flu</td>
<td>Possibly AH3N8</td>
<td>0.75–1 death per 1000</td>
</tr>
<tr>
<td>1918-20</td>
<td>Spanish flu</td>
<td>AH1N1</td>
<td>20-40 million</td>
</tr>
<tr>
<td>1957-8</td>
<td>Asian flu</td>
<td>AH2N2</td>
<td>1.0-1.5 million</td>
</tr>
<tr>
<td>1968-9</td>
<td>Hong Kong flu</td>
<td>AH3N2</td>
<td>0.75-1.0 million</td>
</tr>
<tr>
<td>2009</td>
<td>Swine flu</td>
<td>AH1N1</td>
<td>Over 18,209</td>
</tr>
</tbody>
</table>

(Source: Potter, 2001; Hilleman, 2002; Mils et al., 2004; WHO, 2009)

Table 3 shows that during the period 1889-90, there was a pandemic possibly caused by the influenza A virus H3N8 subtype. The next pandemic in 1918 was caused by the influenza A virus of the H1N1 subtype of avian origin. The virus must have directly infected and adapted to humans to cause human-to-human transmission. In 1957, when the H2N2 strain emerged as a re-assortant from a human and an avian strain, it replaced the previously circulating H1N1 strain (Hilleman, 2002). For the H2N2 subtypes; HA, NA and PB1 genes were from the avian H2N2 virus and the other five genes from the human H1N1 virus (Smith et al., 2009). Then in 1968, the H2N2 strain was replaced by the H3N2 strain, another re-assortant from a human and avian strain (Kawaoka et al., 1989). The H3N2 subtype received seven genes from the H2N2 circulating virus subtype through re-assortment events with an H3 containing virus, during or shortly before the 1967–1968 period. The HA and PB1 genes were from the avian virus and other six genes from the human H2N2 virus (Kilbourne, 2006). The reasons for the replacement of influenza virus strains during these two episodes of antigenic shift remain obscure (Smith et
However, it is thought that human vaccinations against the H2N2 subtype after the 1957 pandemic may have drastically led to its reduced prevalence in the human population and eventually killed it off as shown in the figure.

Figure 2: Changes in human influenza A subtypes

Figure 2 shows the successive recycling of only three of the 16 possible avian viral HAs; H1, H2 and H3, during past pandemics (Hilleman, 2002). After 2001, the H1 and H3 subtypes continued to circulate widely within the human population. The H1N2 subtype appeared to have emerged following the reassortment of genes of the H3N2 and H1N1 subtypes circulating at the time (Brown et al., 1998). However, because the influenza A vaccines contain strains with both H1 and N2 proteins similar to those in the new H1N2 strains, no unusual levels of disease have been associated with this subtype although its occurrence is still being tracked by WHO and US surveillance (Palese, 2004).
Figure 3: Influenza A virus subtypes in the human population.

In figure 3 above, the solid squares show the appearance of a new strain, causing recurring influenza pandemics. Broken lines indicate uncertain strain identifications (Source: Palese, 2004). Figure 3 shows that only the H3N2 and H1N1 subtypes rather than H2N2 influenza A subtype are currently responsible for the seasonal human influenza A epidemics since 1980 (Palese, 2004; Lewis, 2006; Mahsa et al., 2006, Manicassamy et al., 2010; Suwannakarn et al., 2010). These subtypes are associated with varying severity of influenza A epidemics (Simonsen et al., 1997). However, H3N2, a previously pandemic subtype (Hong Kong flu) but now, an epidemic strain is more prevalent than H1N1. This is because the H3N2 subtype undergoes faster evolution than the H1N1 subtype (Nelson et al., 2008). Some of the outcomes following the evolution of the H3N2 subtype are its acquisition of beneficial mutations that enable its relatively higher replicative rate and fitness than the H1N1. Also, H3N2 underwent mutations that enabled it to acquire higher drug resistance against certain standard antivirals compared to H1N1(Bright et al., 2006; Deyde et al., 2007; Simonsen et al., 2007). These mutations led to the establishment and higher prevalence of human influenza A H3N2 subtype than the H1N1. The H3N2 was associated with multiple reassortment events that contributed to the subtypes’ genetic
diversity. The subtype H3N2 has constituted the predominant influenza A strain during the last 20 years, with the exception of the 1988–1989 and 2000–2001 seasons when H1N1 infections were more prevalent (Lin et al., 2004).

Although influenza A is endemic in avian populations, more recently, human disease has been found to be caused by the additional H5, H7, H9, N3 and N7 (Palese, 2004; Allen et al., 2009). Such cases have been associated with exposure to infected birds and pigs (Scholtissek et al., 1985).

2.2.3 Prevalence

Human influenza A viral disease occurrence and statistics indicate that influenza A is responsible for frequent seasonal epidemics and large increases in deaths in some inter-pandemic seasons, when established subtypes exhibit antigenic drift (Simonsen et al., 1998). In a year's normal two flu seasons there is an average of approximately one billion cases of flu, around 3–5 million cases of severe illness and 300,000–500,000 annual influenza epidemic deaths worldwide which by some definitions is a yearly influenza epidemic (WHO Fact sheet, 2003; Cannel et al., 2006). An estimated 25–50 million cases of the flu is reported annually with about 200,000 people hospitalized and about 36,000 human deaths in America (Thompson et al., 2003; WHO Fact sheet, 2003; Linday et al., 2004). According to the WHO, 5-15% of a given population may be vulnerable to upper respiratory tract infections caused by influenza A viruses during annual epidemics (Hay et al., 2001; Kawaoka, 2006).

The disease is usually most severe in medically high risk groups such as children under five years (lacking prior exposure), the elderly aged above 50 years (with immunosenescence) and the immuno-compromised (Hilleman, 2002). Among the hardest hit are infants less than one year
of age and adults above 65 years of age (Zambon, 1999; Suarez et al., 2003; Nobusawa & Sato, 2006). Most flu fatalities, due to the seasonal epidemics, are in elderly patients that are 65 years and older. Their deaths account for more than 90% of fatalities attributed to pneumonia and influenza illnesses (Nicholson, 2003; Kiso et al., 2004; Moscona, 2005). Prevalence of the influenza A virus has been due to the ability of the virus to adapt to host environment usually by developing resistance against host immunity and anti-virals, due to genetic drifts but mostly due to genetic shifts (Horimoto & Kawaoka, 2005).

2.3 Surveillance of human influenza A viruses

Virological surveillance is the detection of recurrent and/or emerging new viral strains. It is also used to refer to the detection of characteristics associated with viral virulence such as; increased pathogenicity, human transmission, drug resistance and increased antigenicity. As such, surveillance studies are used to guide in the selection of vaccine candidate strains, expected to circulate in the subsequent flu seasons, and the timely design of vaccines (Sampath et al., 2007). Given that the genetic make-up of influenza A viruses changes every year, the continuous antigen and genome sequence surveillance of influenza A viruses is still a requirement. These surveillance studies also guide critical decision making during the update of the vaccines or during their use in combination with limited supplies of antiviral drugs (Cox, 2001; Suwannakarn et al., 2010).

2.3.1 Global surveillance of human influenza A viruses

Laboratory networks have led to generation of epidemiologic, phenotypic and genotypic data from surveillance of influenza A viruses (NRC, 2002). Epidemiologic data would relate to dates, locations, hosts, outcomes, histories, and exposures. Due to the fact that the occurrence of
influenza A virus human pandemics have mainly resulted from interspecies transmission, an understanding of the origin and evolution of the viral genome is of particular importance (Gorman et al., 1990; Webster et al., 1992; Neumann & Kawaoka, 2006; Yassine et al., 2007). Phenotypic data would pertain to viral antigenicity (HI titers). Genotypic data provides information about the exact sequence of nucleotides in all eight viral RNA segments and reveal significant genetic determinants encoding pathogenicity characteristics of the given influenza A strains in circulation (Sampath et al., 2007).

2.3.2 Surveillance of human influenza A viruses in Africa

By October 2008, limited studies had been carried out on human influenza studies in a few African countries such as; Cameroon (Njouom et al., 2010), Egypt (Schroedl, 2010), South Africa (Niman, 2007, Besselaar et al., 2008), Kenya (Bulimo et al., 2008), Tanzania (Montefiore et al., 1970) and Uganda (Montefiore et al., 1970). The study in Cameroon confirmed the presence of influenza A subtypes H1N1 and H3N2 and type B viruses in the human population in Central Africa. It also described the emergence of oseltamivir-resistant A H1N1 viruses in Central Africa (Njouom et al., 2010). The Egyptian studies revealed interspecies transmission of influenza A H5N1 viruses from birds to human and also described the increased case-fatality ratios from 2006 to 2008. The findings further indicated the decreasing virulence of H5N1 viruses as they circulated amongst children (Schroedl, 2010). The studies in South Africa reported the presence of oseltamivir-resistant H1N1 subtype viruses in Africa and the Southern hemisphere. The reports further indicated that resistant viruses had spread from the Northern hemisphere and had undergone widespread transmission within the human population. It also indicated the possibility of viral spread viruses to other countries within the Southern hemisphere (Besselaar et al., 2008). The Kenyan studies revealed genetic variations within the HA gene of
the influenza A virus isolates (Bulimo et al., 2008). The studies in Uganda reported the presence of influenza A and B viruses and indicated the possibility of trans-border events, such as travel, being one of the means of human-human viral transmission (Montefiore et al., 1970). The above studies in Africa highlighted the importance of maintaining careful and continuous surveillance of human influenza A viruses. By the time of this study, no complete genome characterization of human influenza A viruses had been carried out, neither were there any on-going surveillance studies to indicate the trends in the prevalence rate of human influenza A viruses in Uganda, over the years.

2.4 Detection and characterization of influenza A viruses

Human influenza A virus detection and characterization is crucial to determine the presence and characteristics of influenza viruses circulating within a given population relating to virulence, host specificity, pathogenicity and drug resistance, as well as to obtain baseline information required for the timely design of effective vaccines using rapid and inexpensive genomic analysis (Layne, 2006). The various steps performed would include; selection of optimal PCR primers, reverse transcription of influenza A viral RNA into cDNA, amplification of DNA by PCR, and analysis by DNA sequencers (Barr et al., 2003).

2.4.1 Phenotypic methods

Phenotypic characterization refers to the process of determining the observable physical or biochemical characteristics of an organism, as determined by both genetic makeup and environmental influences, of an individual with a biological assay.
2.4.1.1 Immunoassays

The immunoassays mainly refer to the HA and NA Inhibition Assays, IFA, ELISAs and other serological assays. Unfortunately, they are labor intensive, time consuming and expensive. Also, they require high technical competence and interpretive skills, relatively large quantities of virus and typing sera (Kendal et al., 1982). Some like IFA have low and variable sensitivity and specificity and thus may miss divergent strains (Ziegler et al., 1995).

2.4.1.2 Virus isolation

This is the process of growing viruses in a specific cell culture for purposes of identifying those missed by other tests as well as other respiratory viruses. Virus isolation is used to verify that influenza A and B antigens are present in samples after which the negative ones are set aside. Virus isolation has been used to multiply viruses for subsequent tests and to monitor new influenza strains and for vaccine development (Gaush et al., 1966). It is a broad range replicating system that recovers novel or highly divergent strains missed by other tests. Viruses from positive samples would be appropriately propagated on MDCK cell lines which are very sensitive to influenza viruses and ensure that the strains do not undergo any mutations within the HA gene during viral isolation. Also, the cell lines ensure a better yield of viral isolates and enable the faster and safer production of large quantities of vaccines (Gaush et al., 1966).

2.4.2 Genotyping methods

This refers to the process of determining the genotype of an individual with a biological assay, such as; PCR, DNA microarrays and DNA sequencing. Molecular genetic analysis provides researchers with the ability to relate genotypic information to host range or other clinical factors of influenza virulence (Subbarao et al., 1993; Horimoto & Kawaoka, 1995; Katz et al., 2000).
By the time of this study, there was no evidence of the application of any of the above methods in the detection of influenza A viruses in samples from human patients in Uganda.

2.4.2.1 One-step RT-PCR

One-step RT-PCR is a process in which single-stranded RNA is reverse transcribed into complementary DNA (cDNA), a reverse transcriptase enzyme, a primer, dNTPs and an RNase inhibitor. The reverse transcription reaction is also called first strand cDNA synthesis. The resulting cDNA can be used in a PCR reaction. PCR is the process of amplifying a specific gene segment of a DNA strand, *in-vitro*, by using primers that target a specific DNA sequence of interest. Both processes are carried out in a single step within a single tube (Bao et al, 2009)

2.4.2.2 DNA sequencing

Sequencing is the process of determining the nucleotide order of a given DNA fragment. Nucleic acid sequencing of amplified HA and NA genes is an important component of influenza surveillance because it allows rapid identification of novel or highly divergent strains, analysis of their variation, determination of their lineage and origin of outbreak as well as establishment of their pathogenicities and drug resistance (Ellis et al., 1997; Herrmann et al., 2001, Van et al, 2001). The various steps performed would include; selection of optimal PCR primers, transcription of viral RNA into cDNA, amplification of DNA by PCR, and analysis by DNA sequencers (Barr et al., 2005). Such efforts could help in the identification of vaccine candidates for the development of influenza A vaccines that protect against a wider range of variants and establish a more fundamental molecular basis for influenza surveillance (Ghendon, 1992; Cox, 2001; Nicholson et al., 2003; Barr et al., 2005).
2.5 Phylogenetic and homology studies

Phylogenetics is the study of evolutionary relatedness among various groups of organisms (for example, species or populations), which is discovered through molecular sequencing data and morphological data matrices. A clade is a group of organisms, within species, whose members share homologous features derived from a common ancestor. A strain is an isolate or group of isolates exhibiting specific phenotypic and/or genotypic traits which are distinctive from those of other isolates of the same species. A subclade is a subgroup of members with characteristics different from other members within a clade. A transversion refers to the substitution of a purine for a pyrimidine or vice versa. A transition is a point mutation that changes a purine nucleotide to another purine (A ↔ G) or a pyrimidine nucleotide to another pyrimidine (C ↔ T).

Phylogenetics can help determine past viruses, their patterns as well as a common ancestor of the virus (Gorman et al., 1990; Scholtissek, 1995). Past phylogenetic relationships of the influenza virus can provide information regarding treatment resistance, selecting vaccine strains, and geographical spread of influenza strains. Phylogenetic trees can help determine what codons in the HA gene of the influenza A virus have changed in past outbreaks (Fitch et al., 2000). Various lineages may continue their presence and reassort indicating the importance of a complete-genome approach to determine new influenza strains and future epidemics. Genetic factors identified may also help in identifying which viruses are of "human flu" and "avian flu" (Vana and Westover, 2008). The more mutations there are in a virus strain, the more likely that strain is to be a generator of a new lineage in future influenza seasons (Fitch et al., 2000). By the time of the study, there was no evidence indicating the use of PCR technology, virus isolation, nucleotide sequencing and phylogenetic tools in the analysis of human influenza A viruses in Uganda.
2.6 Influenza vaccine research and development

Partial control of influenza is through selective vaccination which relies on worldwide surveillance to provide early prediction of the altered immunologic specificity of the next virus to come.

2.6.1 Influenza vaccine strain selection

Annually, the WHO recommends the most suitable composition of influenza vaccine strains for both hemispheres, based on phylogenetic analyses of HA and antigenic characteristics of circulating viruses (Suwannakarn, et al., 2010). The critical influenza types of great public health concern are type A and B (Wright et al., 1995). The selection of vaccine strains favors human influenza A strains with antigenic drifts and shifts involving the HA and NA genes. This is because the surface proteins they encode are able to evade neutralizing antibody from prior infection or vaccination (CDC, 2004b). Hence, the primary focus on changes in HA and NA genes is critical because immunity against the encoded surface proteins is protective (Nicholson et al., 2003). Influenza strains selected for as vaccine candidates are those circulating human influenza A viruses with the highest prevalence and may be new or re-emergent strains. In terms of vaccine strain selection, antigenic clades evolve by reassortment, not by substitutions (Holmes et al., 2005).

2.6.2 Influenza vaccine design

Vaccines are designed in such a way that the selected strains bearing the detected divergent HA cleavage site sequences are inactivated and accurately matched with circulating strains within the population to be vaccinated. In Europe and Africa, inactivated vaccines are the ones licensed for use as recommended by WHO. They are preferred over the live attenuated vaccines because of
the risk of the possible reversion of the vaccine strain to its virulent form. The inactivated forms enable the elicitation of an optimal humoral immune response against the circulating strains, prior to a given influenza season. Influenza vaccines may be killed whole, subunit, or possible live virus vaccines. The sub-unit vaccines are usually used following extraction from recombinant viruses, have HA and/ or NA surface glycoprotein components, are in trivalent form, and consist of materials from the two selected influenza A H1N1 and H3N2 strains as well as one B strain in circulation (Horwood & Macfarlane, 2002; Palese & García-Sastre, 2002; Merck Manual-home edition for patients and caregivers, 2008).

Usually, two different vaccine formulations are made annually, one for each hemisphere. Vaccines for the northern hemisphere are usually from the period starting September of a given year to March of the following year whereas the southern hemisphere vaccines are designed for the period starting April of a given year to August of the same year (WHO, 2006). In the tropics, northern and southern hemisphere vaccines may be used in a given season.

### 2.6.3 Influenza vaccine types, administration and efficacy

The inactivated flu vaccine is administered as a single dose intramuscularly. The effectiveness of the vaccines that are currently available for influenza depends primarily on the antigenic “match” of the circulating strains used for vaccination, as well as on a subject’s age and immune status (Lewis, 2006). Influenza vaccines are 70- 90% effective when there is a good match between the vaccine and circulating influenza strains. These can elicit a long term protective humoral immune response that also prevents re-infection by previously infecting strains. The elicited immunity can last throughout the influenza season and re-immunization within 12 months is not usually necessary (Villegas, 1998; Couch, 2000).
Vaccine mismatch due to the continuous viral evolution usually occurs, whereby, the administered vaccine elicits antibodies that are not specific to the circulating strains, thereby, limiting the efficacy of the vaccines within a given vaccinated human population. It is, therefore, required that, regardless of whether it has been vaccinated before, a given population gets vaccinated each year with vaccine strains that closely match those circulating within the population to minimize vaccine mismatches and increase efficiency (Hilleman, 2002; Palese & García-Sastre, 2002; Jefferson, 2006). Vaccination against influenza is strongly recommended for high-risk groups in both industrialized and developing countries (WHO, 2005).

2.7 Human influenza A detection and characterization studies in Uganda

By the time of the study, a few efforts had been carried out in the detection of influenza A viruses (Montefiore et al., 1970). However, there were no published reports from on-going surveillance studies to determine the genetic characteristics and the trend in the prevalence rates of influenza A viruses circulating within the human population in Uganda. This knowledge is critical in order to determine the appropriate intervention strategies through design of treatment and vaccines for a given population.
CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study design

This was a cross-sectional study design that was carried out, for a period of 3 months from October to December 2008, among human patients attending Mulago and Kayunga hospitals in Uganda. It was part of a larger study titled “Surveillance of Human Influenza Viruses in Uganda” being conducted by MUWRP in collaboration with Makerere University, School of Veterinary Medicine and the Makerere University School of Public Health.

3.1.1 Study area

The study was carried out at Kayunga district- and the assessment centre of Mulago national referral hospitals located in Kayunga and Kampala districts in Uganda, respectively. Both hospitals provide preventive and curative services to both in- and out-patients. However, Kayunga hospital is a district referral hospital that is rurally based whereas Mulago is a national referral hospital in Kampala, an urban setting. Kayunga hospital is located 76 km north east of Kampala and receives patients from: Kayunga, Nakasongola, Kamuli, Kaliro, Pallisa, Namutumba, Iganga, Mukono and Luwero. Mulago hospital receives patients from all over Uganda. Both hospitals are located in the central region of Uganda.

3.1.2 Study population

The study population consisted of male and female human out-patients aged 6 months and above, who had influenza like illness (ILI). ILI was characterized by symptoms of fever equal to or above 38°C, cough and/ or sore throat with stuffy/ runny nose
3.1.2.1 **Target population**

All persons in Uganda, aged 6 months and above.

3.1.2.2 **Accessible population**

Patients aged 6 months and older with ILI, who had attended Kayunga and Mulago hospitals from October to December 2008.

3.1.2.3 **Study unit**

Patients aged 6 months and above, with ILI who attended Kayunga and Mulago hospitals from October to December 2008 and met the inclusion criteria.

3.2 **Ethical considerations**

This study was part of a bigger study that had been reviewed and approved by the Research and Ethics Committees of the School of Veterinary Medicine, Makerere University, Makerere University School of Public Health and approved by Uganda National Council for Science and Technology (Reference HS 377). This study also received approval from the School of Veterinary Medicine Higher Degrees and Research Committee before commencement. All volunteers were taken through a consent process. Parents/ care-takers of children aged between 6 months to 7 years signed a consent form on behalf of the eligible children. For children aged 8 to 17 years, their parents/guardians signed a consent form for their participation in the study but the children also signed an Assent form before enrolling and providing nasal and/or oral-pharyngeal swabs for the study. Consenting adults signed an Informed Consent form.
3.3 Sample size

The Kish Leslie formula for cross-sectional study was used to calculate the required sample size as indicated below:

\[ n = \left( \frac{Z_{\alpha/2}^2 PQ}{\delta^2} \right) \]

\( Z_{\alpha/2} \) = 1.96 (critical value of the normal distribution corresponding to error rate of \( \alpha/2 \))

P = Estimated prevalence of the problem under study

Q = 100% - P

\( \delta \) = Maximum error the investigator is willing to allow between the estimated prevalence and the true prevalence of the problem

(Kish, 1965)

Previous studies done in neighboring Kenya indicated the presence of human influenza A viruses, but they did not indicate their prevalence rate (Bulimo et al., 2008). Therefore, a prevalence rate of 50% was used to calculate the sample size of 384 for this study.

3.4 Sampling criteria

3.4.1 Inclusion criteria

Any individual who had ILI and met the following:

- Aged 6 months or older
- Attended an out-patient clinic at Kayunga or Mulago hospital
- Had had ILI i.e. fever equal to or above 38°C (axillary) plus, cough and/or sore throat with stuffy/runny nose within the previous 72 hours from the time of onset of symptoms.
- Had consented to participate in the study

3.4.2 Exclusion criteria

Any individual that met the above case definition but;

- Was not willing to provide a study sample
- Was too sick to respond to the questionnaire questions
Was unwilling or not able to provide consent (adults) or assent (children greater than 8 years of age) to participate in the surveillance study

3.5 Sampling procedure

3.5.1 Patient enrolment

Patients who were seated at the out-patients reception area were sensitized about the study and 10 volunteers who had ILI symptoms were identified and invited to the study specific area. At the study specific area, the patients were requested to sit and the study further explained to them. Those who were willing were taken individually to a separate room allocated for the study and taken through the consent process. The first five consenting volunteers who met the inclusion criteria were enrolled in the study after taking their medical and social histories, physical examination and providing a nasal and/or pharyngeal swab. The information obtained from history examination was recorded on the study questionnaire. From a given patient, one to two samples were taken; only one nasal swab was taken from the nostril and/or one pharyngeal from the pharynx per patient. A total of up to 10 respiratory samples were collected daily for five days in a week over three months from volunteering participants attending the hospital study sites.

3.5.2 Sample collection and transportation

Respiratory sample collection was done using the MUWRP SOPs in place. Briefly, for the nasal sample, a flexible, fine-shafted polyester swab was inserted into the nostril and back to the pharynx and left in place for 2 sec, and slowly withdrawn with a rotating motion. A pharyngeal swab was used for the second sample. For the pharyngeal swab, a similar polyester swab was inserted through the mouth to the back until it gently touched the pharyngeal lining and left in place for a few seconds. It was slowly withdrawn with a rotating motion. After the swabs were taken, they were each put into a 2ml cryovial containing 1.8 ml standard viral transport media.
(VTM) containing antibiotics (penicillin, streptomycin, gentamycin) and anti-fungals (amphotericin B) to avoid any degradation due to contaminating bacteria and fungi. The shafts were then cut off with a pair of scissors and cryovials immediately sealed. A field number was uniquely allocated to each of the cryovials. Cryovials with samples from the Mulago site were sealed in zip-lock bags and placed in cool boxes containing frozen ice-packs, whereas those with samples collected from the Kayunga site were placed onto cryocanes and stored in dry-shippers containing liquid nitrogen at -96°C. Upon completion of the sample collecting procedure, the details of eligible enrollees, such as age and sex, time of sample collection and name of the sample collecting field staff were recorded on the biological material shipment (BMSF) forms. The samples from the Mulago site were transported within 4 hours from the time of collection whereas those from the Kayunga site were transported within 4 days from the time of collection to the MUWRP –IRL-1 laboratory situated at the School of Veterinary Medicine at Makerere University, Kampala. It was ensured that all specimens arrived at the lab in time to avoid RNA degradation.

3.5.3 Sample handling

Upon arrival at the lab, the samples previously coded with field identification numbers were registered and baseline characteristics (age, sex, region) entered into a computerized laboratory information management system (LIMS) database. They were later allocated laboratory numbers before they were stored at -80°C before they were processed and analyzed.
3.6 Detection of influenza A viruses

3.6.1 Screening of influenza A viruses by one-Step RT-PCR

This was done to determine the presence of influenza A viruses in human respiratory samples (Bao et al, 2009). First, thawing of respiratory samples was done on ice inside a BSL-2 containment hood. This was followed by extraction of viral RNA from the nasal and oral pharyngeal swabs using QIAamp Viral RNA mini kit and in accordance with manufacturer’s instructions. Briefly, a 140μl sample volume was added to 560 μl buffer AVL-carrier RNA in a 1.5ml micro-centrifuge tube, mixed by pulse vortexing for 15 sec, incubated at RT for 10 min and the tubes briefly centrifuged at 1000 revolutions per minute (RPM) at room temperature for 15 seconds in a Bio-rad Model 16K Micro centrifuge to remove drops from the inside of the lid. A 560μl volume of ethanol (96%) was added to the sample, mixed by pulse vortexing for 15 secs and the tube briefly centrifuged at 1000 RPM at room temperature for 15 seconds to remove drops from inside the lid. Carefully, 630μl of the previously prepared sample was added to the QIAamp Min spin column, the cap closed and centrifugation done at 8000 RPM at room temperature for 1 min. The QIAamp spin column was placed into a clean 2ml collection tube and the tube with filtrate discarded. The column was washed twice with AW1 and AW2 buffers respectively, and RNA eluted using 60μl of AVE buffer. Known positive controls (A/Taiwan/1/86 (H1N1) and A/ShangDong/9/93 (H3N2) and negative controls (RNAse free water and PBS) were used during RNA extraction (Anon, 2006).

3.6.1.1 Amplification of influenza A matrix genes

This was done using a One-Step RT-PCR Qiagen kit to detect the presence of influenza A viruses using primers specific for the influenza A matrix gene. The primer sequences used were influenza M52C forward primer: 5’CTT CTA ACC GAG GTC GAA ACG-3’ and M52R
reverse primer: 5’-AGG GCA TTT TGG ACA AAK CGT CTA-3’. They were sourced from Applied Biosystems UK. Briefly, the reaction was constituted in a volume of 25µl containing 5µl of 5× PCR buffer, 13µl of RNAse free H₂O, 1µl of dNTPs, 1.5 µl of reverse primer, 1.5µl of forward primer, 1µl of enzyme mix (Taq DNA polymerase and reverse transcriptase) and 2µl of viral RNA extract.

The amplification was carried out in an Applied Biosystems Veriti 96 well Thermal Cycler and had a one-step reverse transcription step of 50ºC for 30 minutes followed by ‘‘hot start PCR’’ (95ºC) for 15 sec. The reverse transcription process was followed by one-step RT-PCR. The cycling conditions were 40 cycles for 30 seconds of denaturation at 95ºC, 30 sec of primer annealing at 55ºC and 1 min of extension at 72ºC. There was further extension for 10 mins at 72ºC. All samples were run simultaneously with sufficient positive and negative controls. Finally, PCR products were held until required at 4ºC.

3.6.1.2 Detection of PCR products on agarose gel

The PCR products yielded from the detection of the influenza A matrix gene, and a 50bp molecular marker were loaded into the wells of 1% agarose gel in a Biorad SUBCELL ® GT. They were later run at 100V for 2hrs 30mins, after which it was viewed using a Biorad Gel Doc XR imager. The gel bands that formed after the amplification of the targeted gene fragments were size analyzed for the presence of specific type PCR dsDNA product.

3.6.2 Virus isolation

Samples that were positive by one step RT-PCR were propagated on MDCK cells. This was done to confirm the presence of influenza viruses as well as propagate large quantities of viral RNA for subsequent characterization tests; influenza A H1,H3, N1 and N2 sub-typing by one
step RT-PCR as well as DNA sequencing. All culture tubes, each containing a clean sheet of MDCK cell monolayer of passage 2, had their growth media decanted off and were washed using 1 ml of washing media (sterile PBS containing trypsin, and is free of Ca<sup>2+</sup> and Mg<sup>2+</sup>). This excluded one culture tube in which the growth media was retained. The trypsin digestion enabled the digestion of surface proteins that the cells used to adhere to each other, enabling the exposure of MDCK cell receptors. The tubes were then incubated at 37°C in a 5% CO<sub>2</sub> incubator for 10 minutes after which the media was poured off. The procedure was repeated twice. Immediately afterwards, 100 µl of each sample, that had previously tested positive for influenza A virus was inoculated onto the 80% confluent MDCK cell sheet of each tube excluding one, the negative control, into which 100 µl of maintenance media was added instead. The same volume of known positives was inoculated alongside the samples into the respective culture tubes containing 80% confluent MDCK cell sheets. The culture tubes were then incubated at 37°C in a 5% CO<sub>2</sub> incubator for 1 hour in order to let the virus adsorb to the MDCK cell receptors. Rocking of each culture tube was done at 30 min intervals. At the end of the incubation time, 1 ml of the maintenance media was added to each culture tube which was then incubated at 37°C in a 5% CO<sub>2</sub> incubator.

### 3.6.2.1 Observation of MDCK cells for CPE

On day one, all culture tubes were observed macroscopically for contamination. The media from culture tubes showing contamination were each filter sterilized separately using micro filters with pore sizes of 0.2µm. The filtrate containing influenza A virus was passaged onto another 80% confluent MDCK monolayer of cells then cultured using freshly prepared growth media. However, cell culture tubes with samples that repeatedly (over two times) showed contamination were autoclaved and discarded. The inoculated tubes and controls were observed daily for a
CPE. The grade of CPE, varying from 25% to 100%, was recorded daily for all the cultures. When a CPE of 0-1 (0 to 25%) was observed, the cultures were left to continue until a CPE of 2-3 (50%–75%) occurred or until CPE stopped progressing. The CPE presumptive of influenza viruses is a one that appears within 1 to 3 days and will lead to the detachment of the monolayer from culture flasks. The culture tubes showing CPE of 3-4 (75% -100%) were frozen down in a -80°C freezer to avoid further depletion of the MDCK monolayer.

3.6.2.2 Virus harvesting

Following the freezing of isolates at -80°C, they were quick thawed in a 37°C CO₂ incubator for 5 mins. This was done to enhance the detachment of the MDCK cells from the culture tubes, and enable release of the virus. They were then centrifuged at 2000 RPM for 10 mins in a refrigerated Eppendorf centrifuge 5810R. The supernatant containing the virus was aspirated, transferred into a sterile labeled cryovial and stored in a -80°C freezer. The pellet was discarded.

3.6.3 Direct IFA

3.6.3.1 Preparation and staining of cells

The pellets were washed with 1ml of PBS and centrifuged at 2000 RPM for 10 min. After the wash, the PBS was slowly aspirated using a 1 ml serological pipette and discarded. The process of washing the pellet was repeated twice. Following the wash, the pellets were re-suspended in 1 ml of PBS. However, the volumes varied depending on the size of the pellets. Very small pellets were suspended in 0.2ml of PBS whereas moderately sized pellets were suspended in 0.5 ml of PBS. The resuspended cells were vortexed in a Denley Vibromix Vortexer to break any clumps formed by the cells. The IFA slides were labeled with the sample IDs. In the BSL-2, 25μl of the cell suspension was loaded onto each well on the labeled slide and left to dry overnight. They
were placed in acetone, that had been pre-chilled at 4°C for 15mins. The cells were then stained using a Light Diagnostics™ Influenza A and B DFA kit manufactured by Chemicon International, in accordance with manufacturer’s instructions. They were then examined under an IFA Olympus BX51 fluorescence microscope to observe for any fluorescence by cells specifically bound by fluorochrome conjugated antibodies (Kinney et al, 2005).

3.7 Characterization of influenza A viruses

The influenza A viruses detected were characterized to determine their subtypes. This was followed by whole genome sequencing

3.7.1 Sub-typing of influenza A HA and NA genes

Isolates positive for influenza A viruses by IFA were subjected to HA and NA subtyping using one step RT-PCR assay. This was done using a One Step RT-PCR Qiagen kit with H1 gene specific primers sequences; AH1 Fwd: 5’- AAC TAC TAC TGG ACT CTR CTK GAA-3’ and AH1 Rev:5’-CCA TTG GTG CAT TTG AGK TGA TG-3’. Briefly, the reaction was constituted in a volume of 25µl containing; 5µl of 5× PCR buffer, 13µl of RNAse free H2O, 1µl of dNTPs, 1.5 µl of reverse primer, 1.5µl of forward primer, 1µl of enzyme mix (Taq DNA polymerase and reverse transcriptase) and 2µl of viral RNA extract. The amplification was carried out in an Applied Biosystems Veriti 96 well Thermal Cycler and had a one-step reverse transcription step of 50°C for 30 minutes followed by ‘‘hot start PCR’’ (95°C) for 15 min. The cycling conditions were 40 cycles for 30 seconds of denaturation at 95°C, 1 min of primer annealing at 57°C and 2 min of extension at 72°C. Further extension was done at 72C for 7 mins. All samples were run simultaneously with sufficient positive and negative controls. Finally, PCR products were held until required at 4°C. The assay was repeated using H3 specific primer sets. The H3 subtype
specific primer sets used were: AH3F: 5’ AAG CAT TCC YAA TGA CAA ACC 3’ and AH3R: 5’ ATT GCR CCR AAT ATG CCT CTA GT 3’. The H1 and H3 primer sets used were sourced from Applied Biosystems UK. The same extracts of the known positive controls (A/Taiwan/1/86 (H1N1) and A/ShangDong/9/93 (H3N2) and negative controls (RNAse free water and PBS) and known were used (Anon, 2006).

The Influenza A NA Sub-typing was done using a One Step RT-PCR Qiagen kit. The N1 specific primers sequences used were; N1-F:AGR3CCTTGYTTCTGGGTTGA and N1-R:ACCGTCTGGCCAAGACCA. Briefly, the reaction was constituted in a volume of 25µl containing; 5µl of 5×PCR buffer, 13µl of RNAse free H2O, 1µl of dNTPs, 1.5 µl of reverse primer, 1.5µl of forward primer, 1µl of enzyme mix (Taq DNA polymerase and reverse transcriptase) and 2µl of viral RNA extract. The amplification was carried out in an Applied Biosystems Veriti 96 well Thermal Cycler and had a one-step reverse transcription step of 50ºC for 30 minutes followed by ‘‘hot start PCR’’ (95ºC) for 15 min. The cycling conditions were 40 cycles for 30 seconds of denaturation at 95ºC, 1 min of primer annealing at 57ºC and 2 min of extension at 72ºC. Further extension was done at 72C for 7 mins. All samples were run simultaneously with sufficient positive and negative controls. Finally, PCR products were held until required at 4ºC. The assay was repeated using N2 subtype specific primer sets. The N2 subtype specific primer sets used were: N2-F:5’-CATGGTCCAGYTCAAGYTG-3’ and N2-R:5’- CCYTTCCAGTTGTCTCTGCA-3’. The N1 and N2 primer sets used were sourced from Applied Biosystems UK. The same extracts of the known positive controls (A/Taiwan/1/86 (H1N1) and A/ShangDong/9/93 (H3N2) and negative controls (RNAse free water and PBS) and known were used (Anon, 2006).
3.7.1.1 Detection of PCR products on agarose gel

The PCR products yielded from the HA and NA sub-typing, and a 50bp molecular marker were loaded into wells of 1% agarose gel in a Bio-rad Power-Pac HC Firmware version 1.07 gel tank, which was run at 100V, for 2hrs 30mins, after which it was viewed using a Bio-rad Gel Doc XR imager. The gel bands that formed after the amplification of the targeted gene fragments were size analyzed for the presence of specific HA and NA subtype cDNA products. The influenza A virus testing and sub-typing results were entered into a computerized LIMS.

3.7.2 DNA sequencing

In this study, whole Genome Sequencing of all 51 influenza A virus isolates was done to: confirm PCR results, detect the presence and number of new and/ or re-emerging strains, determine any unique strain(s) and any significant amino acid changes that inferred specific characteristics such as host specificity of the study viral isolates. Sequencing was also done to determine the nucleotide diversity of the viral sequences by gene. The purified RT-PCR derived dsDNA products were used as the target for direct nucleotide whole genome sequencing using the Uni12 primer set and an illumina sequencing kit. DNA sequencing was done using the Illumina Genome Analyser IIe. The sequencing was carried out at the Hartwell Center in collaboration with the St. Jude Children’s hospital, Department of Infectious Diseases in Memphis, Tennesse. The complete genome nucleotide sequences of influenza A viruses detected in this study were subjected to bioinformatic, statistical, homology and phylogenetic analyses as indicated below:
3.8 Data handling and analysis

3.8.1 Bioinformatic analyses

Alignment of sequences was done using Clustal X version 2.0., (Larkin et al., 2007).

Editing of sequences was done using Bioedit sequence alignment editor 7.0.9.1 (Hall, 1999). The software helped in determining the open reading frame, sequence lengths, start and stop codons.

Translation of DNA sequences to amino acids was done using Mega version 4.0 (Tamura et al., 2007). The software enabled the identification of amino acid changes and the underlying nucleotide mutations.

3.8.2 Statistical analyses

Strain and nucleotide diversity was determined using Arlequin version 3.1.1 (Excoffier et al., 2005).

3.8.3 Homology analyses

This was done to determine how similar/different the nucleotide sequences of the study strains were. It was done using Arlequin 3.1.1 (Excoffier et al., 2005).

3.8.4 Phylogenetic analyses

After comparisons amongst the study isolates were done by month, site, gender and age-group, phylogenetic analyses were done to compare and determine how related the study strains were amongst themselves and other strains detected worldwide during 2008. The phylogenetic trees were built using Mega 4.0 (Tamura et al., 2007). A bootstrap test for phylogeny using Neighbor joining was done at 1000 replica. The trees helped in determining recent and past separations of the viral strain from their common ancestors. The actual number of mutations per site for each gene was determined. Sequences of study isolates were compared with the viral sequences
detected in the Kenyan human population in order to determine how related they were to viruses detected from neighboring Kenya in 2008. Further comparison of the sequences of the study isolates was done against genbank sequences in order to determine whether the former were new or already existent worldwide. Viral sequences from the study isolates were compared with those of the WHO 2008 reference strains to determine whether the study isolates had any significant sequence attributes similar to those of the latter.

In this study, Kenyan strain sequences refer to those gene sequences of the human influenza A viruses that were detected in different parts of Kenya during 2008 and which were submitted to the influenza A virus gene database. Genbank strain sequences refer to those gene sequences of the human influenza A viruses that were detected in different parts of the world during the year 2008 and which were submitted to the influenza A virus gene database. The WHO 2008 reference strain sequences refer to the sequences of those human influenza A viruses that were selected and used during vaccine design for the 2007-2008 and the 2008 flu seasons (northern and southern hemispheres respectively) and which were submitted to the influenza A virus gene database.
CHAPTER FOUR

4.0 RESULTS

A total of 450 participants were enrolled from both Kayunga district- and Mulago National Referral hospitals. Of the 450 participants, 274 (61%) were from Mulago hospital whereas 176 (39%) participants were from Kayunga hospital. Out of the 450 enrollees, 51 were positive for influenza A viruses.

Figure 4 shows that some of the study enrollees who attended Mulago and Kayunga hospitals, hailed from other neighboring districts. This observation was based on the fact that the two hospital sites, Mulago and Kayunga, were national referral and district referral hospitals respectively.
Map of Uganda showing distribution of study participants’ residences by district

Figure 4: Map of Uganda showing study participants’ districts of residence.
4.1 The demographic characteristics of the study

Of the 450 participants, 226 (52%) were female and 224 (48%) were male. The youngest of the participant was 6-months old and the oldest was 65 years old.

4.1.1 Participant enrolment by month, site and gender

Figure 5: Participant enrolment by month, site and gender

The figure 5 shows that during October, 49% participants were enrolled, followed by 30% in November and 21% in December 2008. Participant enrollment at the Kayunga site was 6% higher during October while there were 151% and 188% more enrollees at the Mulago site during November and December 2008 respectively. Mulago site had the higher number of enrollees constituting 61%. During November 2008, there were 28% more females enrollees while, in October and December 2008, there were 10% and 11% more male than female enrollees, respectively. At the Mulago site, the males enrolled constituted 58% while at the
Kayunga site, the males constituted 38% of enrolled participants. Overall, the females had a 0.4% higher enrollment rate.

4.2 Influenza A virus detection results

4.2.1 One-step RT-PCR

Of the 450 samples that were tested, 51 were positive for influenza A viruses (see Appendix II).

Figure 6: Gel image showing influenza A virus detection by RT-PCR.

Figure 6 shows that a 1kb molecular ladder (ML) was used in the determination of band sizes. Water was used as a negative control (NC) whereas A/ShangDong/9/93 (H3N2) was used as the positive control (PC). Besides the PC, the other band sizes indicate the presence of influenza A viruses. The lanes 13, 14 and 16 represent samples; A/ UgaKay /029/08(H3N2), A/ UgaMul
/030/08(H3N2) and A/ UgaMul /031/08(H3N2), that were found to be positive for influenza A virus.

4.2.2 Virus isolation

All 51 influenza A positive samples also showed CPE (pictures not shown) of varying grades ranging from than 25% to 100%, following viral infection of MDCK cell-line. The CPE was presumptive of influenza viruses because it appeared within 1 to 3 days and led to the detachment of the monolayer from culture flasks.

4.2.3 Direct IFA

All 51 cultures that showed CPE and which were subjected to IFA for further confirmation of the presence of influenza A viruses. They were observed under an IFA Olympus BX51 fluorescence microscope.

Figure 7: IFA slides showing influenza A virus detection in MDCK cells

Figure 7 shows IFA results. Whereas fig7(a) was a positive control, fig7(c) was one of the results that showed an apple green fluorescence, indicating that the respective study sample was positive for influenza A virus. This observation was an indication that the MDCK cells had been infected.
by the influenza A viruses, thereby, confirming the presence of influenza A viruses in the respective 51 samples (see Appendix II).

4.3 Prevalence of influenza A viruses by month, site, gender and age-group

Figure 8 shows that, during November, the highest number of the 51 influenza A virus positive cases, 25 (49%) and (5) 9.8%, were realized at Mulago and Kayunga sites, respectively. During October, the Mulago and Kayunga sites registered 13 (25.5%) and 1 (2%) positive cases respectively. December had the lowest prevalence of influenza A positive cases, 7 (13.7%), registered only at Mulago. On the whole, the Mulago site registered 45 (88%) while the Kayunga site had 6 (12%) out of the 51 cases positive for influenza A viruses.

Figure 8: Prevalence of influenza A virus by month and site
Figure 9 indicates that during October and December 2008, the prevalence of influenza A viral infection were higher in males. However, in November 2008, prevalence of influenza A viral infection was higher in females. On the whole, there were $3(6\%)$ more females that tested positive for the influenza A virus.

![Figure 9: Prevalence of influenza A virus by month and gender.](image)
Figure 10 shows patients of the 6-months-to-5-years age group had the highest prevalence, 41 (80%), of influenza A virus infection whereas those of the 31-35 years age group had none. The other age groups; 6yrs to 10yrs, 11yrs to 15yrs, 16 yrs to 20yrs, 21yrs to 25 yrs, 26yrs to 30yrs and 36yrs to 40yrs had influenza A viral prevalence rates of; 2 (4%), 1(2%), 2(4%), 3(6%), 1(2%) and 1(2%), respectively. The Mulago site had a higher influenza A virus prevalence rate compared to Kayunga site.

Figure 10: Prevalence of influenza A viruses by site and age-group
4.4 Influenza A virus characterization results

4.4.1 Influenza A HA / NA sub-typing by one step RT-PCR

The influenza A viruses were sub-typed using H3 and N2 specific primers

4.4.1.1 Influenza A HA sub-typing

The figures 11(a) and 11(b) show influenza A H1 and H3 sub-typing using 50kb molecular ladder respectively. In figure 11(a), the absence of 135 bp amplicons, in lanes other than the positive control, was an indicator for the absence of influenza A H1 subtypes. In figure 11(b), the presence of 146 bp amplicons were a positive indicator for the presence of influenza A H3 virus subtypes with respect to the specific HA primer sets used. Thus, all influenza A viruses detected in this study were of the H3 subtype (see AppendixII).

Figure 11: Gel images showing influenza A virus H1 and H3 sub-typing results.

The lanes numbered 3, 4, 5, 6 and 7 represent the samples; A/ UgaMul /036/08(H3N2), A/ UgaKay /037/08(H3N2), A/ UgaMul /038/08(H3N2), A/ UgaMul /039/08(H3N2) and A/ UgaKay
/040/08(H3N2), from Mulago and Kayunga hospital sites that were of the influenza A virus H3 subtype.

4.4.1.2 Influenza A NA sub-typing

Gel size analysis was done using a 50kb molecular ladder (gels not shown). The absence of 126 bp amplicons was an indicator of the absence of influenza A N1 subtypes. The presence of 362 bp amplicons was a positive indicator for the presence of influenza A N2 virus subtypes with respect to the specific NA primer sets used. All influenza A viruses detected were of the N2 subtype. Gel images for the N1 and N2 subtyping results are not shown. On the whole, all 51 samples were found to be of the influenza A H3N2 subtype (see Appendix II).

4.4.2 Whole genome sequencing

Of the 51 isolates, only 50 were subjected to whole genome sequencing. However, after sequencing was done, a total of 4, 1, 1 and 2 short sequences for the PB2, PB1, PA and NS gene segments of specific isolate, were eliminated, respectively. For the rest of the 50 isolates, all 8 genes were successfully sequenced. Table 4 shows the Influenza A virus sequence characteristics and diversity Indices;
Table 4. Influenza A virus sequence characteristics and diversity indices

<table>
<thead>
<tr>
<th>Segment</th>
<th>Gene</th>
<th>No. of gene sequences analysed</th>
<th>Sequence length</th>
<th>Start codon</th>
<th>Stop codon</th>
<th>Significant amino acid changes</th>
<th>Inferred function</th>
<th>No. of identified strain sequences by gene</th>
<th>% of strains by gene</th>
<th>Largest group of strain sequences by gene</th>
<th>No. of unique sites</th>
<th>N, % Approx. (10.5)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PB2</td>
<td>46</td>
<td>2280</td>
<td>ATG</td>
<td>TAA</td>
<td>All gene sequences had R at position 702</td>
<td>Both amino acids inferred human specificity</td>
<td>31</td>
<td>85%</td>
<td>67</td>
<td>5</td>
<td>980</td>
</tr>
<tr>
<td></td>
<td>PB1</td>
<td>49</td>
<td>2274</td>
<td>ATG</td>
<td>TAA</td>
<td>None</td>
<td>Low virulence</td>
<td>37</td>
<td>92%</td>
<td>76</td>
<td>5</td>
<td>1161</td>
</tr>
<tr>
<td></td>
<td>PA</td>
<td>49</td>
<td>2146</td>
<td>ATG</td>
<td>TAA</td>
<td>All except 2 had E at 133</td>
<td>Increased host specificity</td>
<td>31</td>
<td>80%</td>
<td>63</td>
<td>14</td>
<td>212</td>
</tr>
<tr>
<td></td>
<td>HA</td>
<td>50</td>
<td>966</td>
<td>ATG</td>
<td>TAG</td>
<td>None</td>
<td>None</td>
<td>22</td>
<td>44%</td>
<td>44</td>
<td>10</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td>NP</td>
<td>50</td>
<td>1497</td>
<td>ATG</td>
<td>TAA</td>
<td>None</td>
<td>None</td>
<td>4</td>
<td>52%</td>
<td>0.08</td>
<td>7</td>
<td>879</td>
</tr>
<tr>
<td></td>
<td>NA</td>
<td>50</td>
<td>1410</td>
<td>ATG</td>
<td>TGA</td>
<td>None</td>
<td>None</td>
<td>26</td>
<td>60%</td>
<td>52</td>
<td>46</td>
<td>44</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>50</td>
<td>948</td>
<td>ATG</td>
<td>TAA</td>
<td>None</td>
<td>None</td>
<td>21</td>
<td>42%</td>
<td>0.08</td>
<td>25</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>NS</td>
<td>48</td>
<td>843</td>
<td>ATG</td>
<td>TAG</td>
<td>None</td>
<td>None</td>
<td>23</td>
<td>63%</td>
<td>0.08</td>
<td>9</td>
<td>26</td>
</tr>
</tbody>
</table>

Ky: Kayunga
Ml: Mulago
N: Population size
Π: Π (Nucleotide diversity)
Σ: Σ (Sum)
Table 4 shows that all gene sequences had a starting codon of ATG and a stop codon of TAA except for NA and NS that had TGA. There was nothing significant about these variations. The PB2, PA and NP genes had significant amino acid changes.

PB2 genes for 46 isolates had arginine (R) at position 702 which have been reported to be associated with increased host specificity (Subbarao et al., 1993; Almond, 1977; Zhou et al., 1999). There were 47 influenza A viruses with PA gene sequences that had glutamic acid (E) at position 133, while the remaining two had glutamine (Q). Whereas the E amino acid was encoded by GAA, the Q was encoded by CAA, at positions 397, 398 and 399 respectively. All 50 isolates had the NP gene sequenced, and only one had aspartic acid (D) at position 34. The presence of this amino acid was encoded by the nucleotides GAT in positions 100, 101 and 102 respectively. The remaining NP, of the strain A/UgaMul/023/08(H3N2), instead had a T encoded by ACT nucleotides in the respective positions. For each of the gene segments, there was variation observed among the strains. Table 4 shows that PB1 gene had the highest number of strains (92%) followed by the PB2 (25%) and PA (80%) genes, the NA (60%) and NS (63%) genes, the NP(52%) and HA (44%) genes and lastly the M gene (42%), that had the lowest. The highest strain diversity was among the PB1 genes whereas the least diversity was within the M genes. However, although the PB1 gene sequences were the most diverse, they formed the smallest (n=4) group of strains. On the other hand, although the M genes sequences showed the least diversity, they had the largest (n=26) group of strains. The above two observations were with respect to the number of strains detected by gene.
4.4.2.1 Influenza A virus strain distribution by month and site

Figure 12 shows that the highest influenza A viral strains were detected in samples collected during November followed by October and the least, during December 2008. Figure 12 also indicates that some of the gene strains were detected in more than one patient over the three month period.

**Figure 12: Influenza A virus strain distribution by month**

Figure 13 shows that the highest number of influenza A viral strains were detected in samples collected from the Mulago site, for all genes.

**Figure 13: Influenza A virus strain distribution per site by gene**
Figures 14 shows unique sites within the HA gene of the influenza A virus isolates

<table>
<thead>
<tr>
<th>No.</th>
<th>Strain</th>
<th>Sequence</th>
<th>Site(s)</th>
<th>MULAGO</th>
<th>KAYUNGA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>UK2</td>
<td>AGATGGTCG GATGCACTGA CATAATAG</td>
<td>3</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>UK27</td>
<td>T...A ...T.............</td>
<td>-</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>UK37</td>
<td>T...A .G.............</td>
<td>6</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>UK40</td>
<td>T...A .G......C....</td>
<td>-</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>UM1</td>
<td>T...A ................</td>
<td>11</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>UM7</td>
<td>T...A .G............ T....G.</td>
<td>1</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>UM11</td>
<td>.A...TT..A .G.........</td>
<td>1</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>UM12</td>
<td>T...A .G..T........</td>
<td>1</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>UM15</td>
<td>.G..T.... .C.......G</td>
<td>3</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>UM16</td>
<td>T....... ............</td>
<td>2</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>UM18</td>
<td>T...A ..............A.</td>
<td>1</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>UM20</td>
<td>TT.AA .G.............</td>
<td>1</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>UM21</td>
<td>T..A .............T..</td>
<td>2</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>UM24</td>
<td>T....... ............</td>
<td>3</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>UM25</td>
<td>T...A .............C...</td>
<td>1</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>UM26</td>
<td>T...A .......G.......</td>
<td>1</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>UM39</td>
<td>CT...A .G.............</td>
<td>1</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>UM41</td>
<td>G...TT..A .G........</td>
<td>1</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>UM46</td>
<td>C.T...A ............GGC..</td>
<td>2</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>UM48</td>
<td>T.C.. .............C....</td>
<td>1</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>UM51</td>
<td>T...A .............G...</td>
<td>1</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>UM52</td>
<td>T....... .............C..T ..C...T</td>
<td>1</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

Number of bases 2222222222 2222222222 22222222 50 INFLUENZA A VIRUS ISOLATES

UM – A sample from Uganda, Mulago site
UK – A sample from Uganda, Kayunga site

**Figure 14: The 28 unique sites in the 22 influenza A virus HA gene strains**
Figure 15 shows unique sites within the NA gene of the Influenza A virus isolates.

<table>
<thead>
<tr>
<th>Number of bases</th>
<th>STRAINS</th>
</tr>
</thead>
<tbody>
<tr>
<td>11111111</td>
<td>MULAGO KAYUNGA</td>
</tr>
<tr>
<td>2447781</td>
<td>1237787859</td>
</tr>
<tr>
<td>6804891245</td>
<td>5326367331</td>
</tr>
<tr>
<td>5711528137</td>
<td>2128837919</td>
</tr>
<tr>
<td>5835799554</td>
<td>4659725688</td>
</tr>
<tr>
<td>9034</td>
<td>0507</td>
</tr>
</tbody>
</table>

UM – A sample from Uganda, Mulago site
UK – A sample from Uganda, Kayunga site

Figure 15: The 44 unique sites in the 26 influenza A virus NA gene strains
Figure 16 shows unique sites within the M gene of the influenza A virus isolates

UM – A sample from Uganda, Mulago site
UK – A sample from Uganda, Kayunga site

Figure 16: The 24 unique sites in the 21 influenza A virus M gene strains

The figures 14, 15, and 16 show the unique sites observed in the HA, NA and M genes for all the 50 study isolates respectively. The M gene had the least number of unique sites (21) compared to all the influenza A viral genes

There was a unique strain, A/UgaMul/027 /08(H3N2), that had an amino acid, T, at position 661 along its PB2 gene sequence. This amino acid was encoded by ACT nucleotides in the 1981, 1982 and 1983 nucleotide positions respectively. However, 44 of the other PB2 genes each had a K at position 661 while another strain had E at the same position. They were encoded by AAG and GAG nucleotide sequences in positions 1981, 1982 and 1983, respectively. The K and E amino acid differences were
due to a transition of A to G or vice versa in nucleotide position 1981. These two amino acids at the specific locations did not infer any significant viral functions. The unique strain had a different amino acid which was due to the presence of CT nucleotides in positions 1982 and 1983 different from the AG nucleotides in the respective positions within the PB2 sequences of the rest of the 45 viral gene strains. Hence, the uniqueness of A/UgaMul/027/08(H3N2) is because of the presence of T at position 661 of the PB2 gene which has been reported to infer with human specificity of the influenza A virus. Table 5 indicates that the strain was identified in a sample collected from a 20 year-old female patient, who had attended the Kayunga hospital site during November 2008.

Table 5: Details for unique influenza A virus strain A/UgaKay/027/08(H3N2)

<table>
<thead>
<tr>
<th>Genome size</th>
<th>12385bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subtype</td>
<td>H3N2</td>
</tr>
<tr>
<td>Year</td>
<td>2008</td>
</tr>
<tr>
<td>Host species</td>
<td>Human</td>
</tr>
<tr>
<td>Sex</td>
<td>Female</td>
</tr>
<tr>
<td>Age</td>
<td>20 years</td>
</tr>
<tr>
<td>Strain name</td>
<td>A/UgaKay/027/08(H3N2)</td>
</tr>
<tr>
<td>Date of collection</td>
<td>19/November/2008</td>
</tr>
<tr>
<td>Location of site of collection</td>
<td>Kayunga hospital</td>
</tr>
<tr>
<td>Country</td>
<td>Uganda</td>
</tr>
<tr>
<td>Flu season</td>
<td>Second</td>
</tr>
<tr>
<td>Geographical grouping</td>
<td>Northern hemisphere</td>
</tr>
</tbody>
</table>

4.5 Molecular Phylogeny of the influenza A virus isolates

Maximum likelihood phylogenetic trees for nucleotide sequences of the eight gene sequences for each of the human influenza A H3N2 virus isolates from the study were constructed. The robustness of individual nodes of the trees was assessed using a bootstrap test for phylogeny analysis (1000 replicates, with topologies inferred using the neighbor-joining method under the substitution model). Substitutions included transitions and transversions. These trees were used to compare the various nucleotide
sequences, by gene, to sequences of Kenyan strains, the WHO reference strains and highly similar (99%) genbank strains for influenza A viruses detected in 2008. For the various trees constructed, the clades indicated that, although they were closely related, there were differences in those relations. The tree scales represent time; with the different line-length representing a different time-length. The tree scale represents number of mutations per site. Below are the phylogenetic trees for all eight influenza A gene segments.
Nucleotide sequences of PB2 genes and their relationship with strains from elsewhere

Sub-clade 1
- A/UgaMul/024/08 H3N2
- A/UgaMul/048/08 H3N2
- A/UgaMul/025/08 H3N2
- A/UgaMul/042/08 H3N2
- A/UgaMul/043/08 H3N2
- A/UgaMul/016/08 H3N2
- A/UgaMul/017/08 H3N2
- A/UgaMul/020/08 H3N2
- A/UgaMul/032/08 H3N2
- A/UgaMul/013/08 H3N2
- A/UgaMul/003/08 H3N2
- A/UgaMul/021/08 H3N2
- A/UgaMul/045/08 H3N2
- A/UgaMul/046/08 H3N2
- A/UgaMul/018/08 H3N2
- A/UgaKay/037/08 H3N2

Sub-clade 2
- A/UgaMul/036/08 H3N2
- A/UgaMul/034/08 H3N2
- A/UgaMul/035/08 H3N2
- A/UgaMul/008/08 H3N2
- A/Arizona/WRAIR1142P/2009 H3N2
- A/UgaMul/033/08 H3N2
- A/UgaMul/039/08 H3N2
- A/UgaKay/040/08 H3N2
- A/UgaMul/038/08 H3N2
- A/UgaMul/041/06 H3N2
- A/Managua/4348.01/2007 H3N2
- A/Vermont/UR06-0471/2007 H3N2
- A/Brisbane/10/2007

Sub-clade 3
- A/UgaMul/011/08 H3N2
- A/UgaMul/015/08 H3N2
- A/UgaMul/050/08 H3N2
- A/Japan/WRAIR1059P/2009 H3N2
- A/Mississippi/UR07-6004/2008 H
- A/Florida/UR07-0102/2008 H3N2
- A/Pennsylvania/PIT43/2008 H3N2
- A/Ohio/UR07-0126/2008 H3N2
- A/California/VRDL.250/2009 H3N2
- A/New York/UR07-0160/2008(H3N2)
- A/Kansas/UR07-0135/2008 H3N2
- A/Kentucky/UR07-0068/2008 H3N2
- A/Boston/66/2008 H3N2
- A/Kyrgyzstan/WRAIR1121P/2008 H
- A/UgaMul/052/08 H3N2
- A/UgaKay/027/08 H3N2
- A/UgaMul/044/08 H3N2

Uganda strains
Genbank strains
2008 WHO reference strains
Figure 17: Un-rooted phylogenetic tree showing relationship of PB2 nucleotide sequences of the Ugandan isolates with those of the WHO reference and genbank strains for the year 2008.

In figure 17, the tree showed one clade, with five sub-clades, and one out-group with three Ugandan strains. However, some genbank, Ugandan and reference strains clustered separately from most of the other to form sub-clade 5. Four of the sub-clades were dominated by Ugandan strains while one was dominated by genbank strains. The Ugandan strains showed closer associations amongst themselves than to genbank strains and so they were grouped separately. However, the Ugandan strains showed more mutation events and variations amongst themselves and clustered nearer to the clade ancestor. Compared to the Ugandan strains, the genbank strains displayed no unique histories but formed a big cluster in sub-clade 5, that was further away from the common ancestor. There were 31 PB2 gene strains that were detected and presented in the phylogenetic tree in figure 17. The strains in figure 17 are shown in table 6.
Table 6: The 31 influenza A virus PB2 gene strains detected in the study

<table>
<thead>
<tr>
<th>31 influenza A virus PB2 gene Strains appearing in the clade</th>
<th>Strain(s) identical to clade strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/UgaMul /021/08 (H3N2)</td>
<td></td>
</tr>
<tr>
<td>A/UgaMul /017/08 (H3N2)</td>
<td></td>
</tr>
<tr>
<td>A/UgaMul /008/08 (H3N2)</td>
<td></td>
</tr>
<tr>
<td>A/UgaMul /011/08 (H3N2)</td>
<td></td>
</tr>
<tr>
<td>A/UgaKay /040/08 (H3N2)</td>
<td></td>
</tr>
<tr>
<td>A/UgaMul /041/08 (H3N2)</td>
<td></td>
</tr>
<tr>
<td>A/UgaMul /038/08 (H3N2)</td>
<td></td>
</tr>
<tr>
<td>A/UgaMul /035/08 (H3N2)</td>
<td></td>
</tr>
<tr>
<td>A/UgaMul /034/08 (H3N2)</td>
<td></td>
</tr>
<tr>
<td>A/UgaMul /036/08 (H3N2)</td>
<td></td>
</tr>
<tr>
<td>A/UgaMul /039/08 (H3N2)</td>
<td></td>
</tr>
<tr>
<td>A/UgaMul /018/08 (H3N2)</td>
<td></td>
</tr>
<tr>
<td>A/UgaMul /045/08 (H3N2)</td>
<td></td>
</tr>
<tr>
<td>A/UgaMul /013/08 (H3N2)</td>
<td></td>
</tr>
<tr>
<td>A/UgaMul /042/08 (H3N2)</td>
<td></td>
</tr>
<tr>
<td>A/UgaMul /043/08 (H3N2)</td>
<td></td>
</tr>
<tr>
<td>A/UgaMul /048/08 (H3N2)</td>
<td></td>
</tr>
<tr>
<td>A/UgaMul /024/08 (H3N2)</td>
<td></td>
</tr>
<tr>
<td>A/UgaMul /016/08 (H3N2)</td>
<td></td>
</tr>
<tr>
<td>A/UgaMul /050/08 (H3N2)</td>
<td></td>
</tr>
<tr>
<td>A/UgaKay /027/08 (H3N2)</td>
<td></td>
</tr>
<tr>
<td>A/UgaMul /052/08 (H3N2)</td>
<td></td>
</tr>
<tr>
<td>A/UgaMul /044/08 (H3N2)</td>
<td></td>
</tr>
<tr>
<td>A/UgaMul /032/08 (H3N2)</td>
<td></td>
</tr>
<tr>
<td>A/UgaMul /020/08 (H3N2)</td>
<td></td>
</tr>
<tr>
<td>A/UgaMul /046/08 (H3N2)</td>
<td>A/UgaMul /047/08 (H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /025/08 (H3N2)</td>
<td>A/UgaMul /014/08 (H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /033/08 (H3N2)</td>
<td>A/UgaMul /031/08 (H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /015/08 (H3N2)</td>
<td>A/UgaMul /012/08 (H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /014/08 (H3N2)</td>
<td>A/UgaMul /006/08 (H3N2)</td>
</tr>
<tr>
<td>A/UgaKay /028/08 (H3N2)</td>
<td>A/UgaMul /005/08 (H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /005/08 (H3N2)</td>
<td>A/UgaMul /026/08 (H3N2)</td>
</tr>
<tr>
<td>A/UgaKay /029/08 (H3N2)</td>
<td>A/UgaMul /049/08 (H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /018/08 (H3N2)</td>
<td>A/UgaMul /051/08 (H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /003/08 (H3N2)</td>
<td>A/UgaMul /001/08 (H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /007/08 (H3N2)</td>
<td>A/UgaMul /010/08 (H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /010/08 (H3N2)</td>
<td></td>
</tr>
</tbody>
</table>
Nucleotide sequences of PB1 genes and their relationship with strains from elsewhere

PB1

Figure 18: Un-rooted phylogenetic tree showing relationship of PB1 nucleotide sequences of the Ugandan isolates with those of the WHO reference and genbank strains for the year 2008.
In figure 18, the tree had one clade with five sub-clades and an out-group of three Ugandan strains. Sub-clade 1 showed dominance by reference strains. Sub-clade 2, 3, 4 and 5 consisted of Ugandan strain variants only. The genbank and Ugandan strains clustered separately. In the clade, Ugandan strains displayed unique histories amongst themselves while the genbank strains did not. Although most of the Ugandan strains showed unique histories, the genbank strains had none, clustered together and were nearer to the clade ancestor than the Ugandan strains. The genbank strains showed lower mutation rates and fewer variations amongst themselves compared to the Ugandan strains. There were 37 PB1 gene strains that were detected Ugandan isolates and presented in the phylogenetic tree in figure 18. The strains in figure 18 are shown in the table 7.
<table>
<thead>
<tr>
<th>37 influenza A virus PB1 gene strains appearing in the clade</th>
<th>Strain(s) identical to clade strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/UgaMul /034/08 (H3N2)</td>
<td></td>
</tr>
<tr>
<td>A/UgaKay /037/08 (H3N2)</td>
<td></td>
</tr>
<tr>
<td>A/UgaMul /036/08 (H3N2)</td>
<td></td>
</tr>
<tr>
<td>A/UgaMul /041/08 (H3N2)</td>
<td></td>
</tr>
<tr>
<td>A/UgaMul /008/08 (H3N2)</td>
<td></td>
</tr>
<tr>
<td>A/UgaMul /038/08 (H3N2)</td>
<td></td>
</tr>
<tr>
<td>A/UgaMul /050/08 (H3N2)</td>
<td></td>
</tr>
<tr>
<td>A/UgaMul /052/08 (H3N2)</td>
<td></td>
</tr>
<tr>
<td>A/UgaMul /047/08 (H3N2)</td>
<td></td>
</tr>
<tr>
<td>A/UgaMul /011/08 (H3N2)</td>
<td></td>
</tr>
<tr>
<td>A/UgaKay /040/08 (H3N2)</td>
<td></td>
</tr>
<tr>
<td>A/UgaMul /012/08 (H3N2)</td>
<td></td>
</tr>
<tr>
<td>A/UgaMul /026/08 (H3N2)</td>
<td></td>
</tr>
<tr>
<td>A/UgaMul /035/08 (H3N2)</td>
<td></td>
</tr>
<tr>
<td>A/UgaMul /019/08 (H3N2)</td>
<td></td>
</tr>
<tr>
<td>A/UgaMul /046/08 (H3N2)</td>
<td></td>
</tr>
<tr>
<td>A/UgaMul /051/08 (H3N2)</td>
<td></td>
</tr>
<tr>
<td>A/UgaMul /013/08 (H3N2)</td>
<td></td>
</tr>
<tr>
<td>A/UgaMul /001/08 (H3N2)</td>
<td></td>
</tr>
<tr>
<td>A/UgaMul /017/08 (H3N2)</td>
<td></td>
</tr>
<tr>
<td>A/UgaMul /049/08 (H3N2)</td>
<td></td>
</tr>
<tr>
<td>A/UgaMul /021/08 (H3N2)</td>
<td></td>
</tr>
<tr>
<td>A/UgaMul /015/08 (H3N2)</td>
<td></td>
</tr>
<tr>
<td>A/UgaMul /043/08 (H3N2)</td>
<td></td>
</tr>
<tr>
<td>A/UgaMul /039/08 (H3N2)</td>
<td></td>
</tr>
<tr>
<td>A/UgaMul /045/08 (H3N2)</td>
<td></td>
</tr>
<tr>
<td>A/UgaMul /042/08 (H3N2)</td>
<td></td>
</tr>
<tr>
<td>A/UgaKay /027/08 (H3N2)</td>
<td></td>
</tr>
<tr>
<td>A/UgaMul /044/08 (H3N2)</td>
<td></td>
</tr>
<tr>
<td>A/UgaMul /048/08 (H3N2)</td>
<td></td>
</tr>
<tr>
<td>A/UgaMul /020/08 (H3N2)</td>
<td></td>
</tr>
<tr>
<td>A/UgaMul /005/08 (H3N2)</td>
<td></td>
</tr>
<tr>
<td>A/UgaMul /032/08 (H3N2)</td>
<td></td>
</tr>
<tr>
<td>A/UgaMul /007/08 (H3N2)</td>
<td></td>
</tr>
<tr>
<td>A/UgaKay /029/08 (H3N2)</td>
<td></td>
</tr>
<tr>
<td>A/UgaMul /016/08 (H3N2)</td>
<td></td>
</tr>
<tr>
<td>A/UgaMul /006/08 (H3N2)</td>
<td></td>
</tr>
<tr>
<td>A/UgaMul /033/08 (H3N2)</td>
<td></td>
</tr>
<tr>
<td>A/UgaMul /024/08 (H3N2)</td>
<td></td>
</tr>
<tr>
<td>A/UgaMul /025/08 (H3N2)</td>
<td></td>
</tr>
<tr>
<td>A/UgaMul /031/08 (H3N2)</td>
<td></td>
</tr>
<tr>
<td>A/UgaKay /002/08 (H3N2)</td>
<td></td>
</tr>
<tr>
<td>A/UgaMul /003/08 (H3N2)</td>
<td></td>
</tr>
<tr>
<td>A/UgaMul /009/08 (H3N2)</td>
<td></td>
</tr>
<tr>
<td>A/UgaMul /014/08 (H3N2)</td>
<td></td>
</tr>
<tr>
<td>A/UgaMul /023/08 (H3N2)</td>
<td></td>
</tr>
<tr>
<td>A/UgaKay /028/08 (H3N2)</td>
<td></td>
</tr>
</tbody>
</table>
Nucleotide sequences of PA genes and their relationship with strains from elsewhere

Figure 19: Un-rooted phylogenetic tree showing relationship of PA nucleotide sequences of the Ugandan isolates with those of the WHO reference and genbank strains for the year 2008.
In figure 19, the tree had one clade with six sub-clades and an out-group with 5 Ugandan strains. Sub-clade 1 mainly comprised of genbank strains while sub-clade 2, 3, 4 and 5 comprised of Ugandan strains. Sub-clade 6 had genbank and Ugandan strains but was dominated by the former. The 2008 reference strains and a south East Asian strain were in the same sub-clade 1 as one Ugandan strain A/UgaMul/050/08 H3N2 and the genbank strains. The genbank and Ugandan strains were grouped separately, with most of the Ugandan strains displaying unique histories. The Ugandan strains were grouped nearer to the clade ancestor than the genbank strains. Also, the Ugandan strains showed more variations and higher mutation rates than the genbank strains. The Ugandan strains formed distinct clusters, in a few locations within the clade. There were 31 PA gene strains that were detected and presented in the phylogenetic tree in figure 19. The strains in figure 19 are shown in table 8.
Table 8: The 31 influenza A virus PA gene strains detected in the study

<table>
<thead>
<tr>
<th>31 influenza A virus PA gene Strains appearing in the clade</th>
<th>Strain(s) identical to clade strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/UgaKay /027/08 (H3N2)</td>
<td></td>
</tr>
<tr>
<td>A/UgaMul /009/08 (H3N2)</td>
<td></td>
</tr>
<tr>
<td>A/UgaMul /026/08 (H3N2)</td>
<td></td>
</tr>
<tr>
<td>A/UgaMul /034/08 (H3N2)</td>
<td></td>
</tr>
<tr>
<td>A/UgaMul /008/08 (H3N2)</td>
<td></td>
</tr>
<tr>
<td>A/UgaMul /015/08 (H3N2)</td>
<td></td>
</tr>
<tr>
<td>A/UgaMul /007/08 (H3N2)</td>
<td></td>
</tr>
<tr>
<td>A/UgaMul /048/08 (H3N2)</td>
<td></td>
</tr>
<tr>
<td>A/UgaMul /011/08 (H3N2)</td>
<td></td>
</tr>
<tr>
<td>A/UgaMul /012/08 (H3N2)</td>
<td></td>
</tr>
<tr>
<td>A/UgaMul /042/08 (H3N2)</td>
<td></td>
</tr>
<tr>
<td>A/UgaKay /028/08 (H3N2)</td>
<td></td>
</tr>
<tr>
<td>A/UgaMul /043/08 (H3N2)</td>
<td></td>
</tr>
<tr>
<td>A/UgaMul /044/08 (H3N2)</td>
<td>A/UgaKay /002/08 (H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /014/08 (H3N2)</td>
<td>A/UgaMul /025/08 (H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /001/08 (H3N2)</td>
<td></td>
</tr>
<tr>
<td>A/UgaMul /005/08 (H3N2)</td>
<td></td>
</tr>
<tr>
<td>A/UgaMul /006/08 (H3N2)</td>
<td></td>
</tr>
<tr>
<td>A/UgaMul /024/08 (H3N2)</td>
<td></td>
</tr>
<tr>
<td>A/UgaMul /035/08 (H3N2)</td>
<td></td>
</tr>
<tr>
<td>A/UgaMul /051/08 (H3N2)</td>
<td></td>
</tr>
<tr>
<td>A/UgaKay /037/08 (H3N2)</td>
<td></td>
</tr>
<tr>
<td>A/UgaMul /045/08 (H3N2)</td>
<td></td>
</tr>
<tr>
<td>A/UgaMul /049/08 (H3N2)</td>
<td></td>
</tr>
<tr>
<td>A/UgaMul /046/08 (H3N2)</td>
<td></td>
</tr>
<tr>
<td>A/UgaMul /039/08 (H3N2)</td>
<td></td>
</tr>
<tr>
<td>A/UgaMul /032/08 (H3N2)</td>
<td></td>
</tr>
<tr>
<td>A/UgaMul /019/08 (H3N2)</td>
<td></td>
</tr>
<tr>
<td>A/UgaMul /020/08 (H3N2)</td>
<td>A/UgaMul /052/08 (H3N2)</td>
</tr>
<tr>
<td>A/UgaKay /029/08 (H3N2)</td>
<td>A/UgaMul /016/08 (H3N2)</td>
</tr>
<tr>
<td>A/UgaKay /040/08 (H3N2)</td>
<td>A/UgaMul /031/08 (H3N2)</td>
</tr>
<tr>
<td></td>
<td>A/UgaMul /033/08 (H3N2)</td>
</tr>
<tr>
<td></td>
<td>A/UgaMul /050/08 (H3N2)</td>
</tr>
<tr>
<td></td>
<td>A/UgaMul /003/08 (H3N2)</td>
</tr>
<tr>
<td></td>
<td>A/UgaMul /010/08 (H3N2)</td>
</tr>
<tr>
<td></td>
<td>A/UgaMul /017/08 (H3N2)</td>
</tr>
<tr>
<td></td>
<td>A/UgaMul /023/08 (H3N2)</td>
</tr>
<tr>
<td></td>
<td>A/UgaMul /013/08 (H3N2)</td>
</tr>
<tr>
<td></td>
<td>A/UgaMul /018/08 (H3N2)</td>
</tr>
<tr>
<td></td>
<td>A/UgaMul /021/08 (H3N2)</td>
</tr>
<tr>
<td></td>
<td>A/UgaMul /036/08 (H3N2)</td>
</tr>
<tr>
<td></td>
<td>A/UgaMul /038/08 (H3N2)</td>
</tr>
<tr>
<td></td>
<td>A/UgaMul /041/08 (H3N2)</td>
</tr>
<tr>
<td></td>
<td>A/UgaMul /047/08 (H3N2)</td>
</tr>
</tbody>
</table>
Nucleotide sequences of HA genes and their relationship with strains from elsewhere

**Figure 20:** Un-rooted phylogenetic tree showing relationship of HA nucleotide sequences of the Ugandan isolates with those of the Kenyan, reference and genbank strains for the year 2008.
In figure 20, the tree comprised of one clade, four sub clades and an outgroup with five strains; three Kenyan and two 2008 reference strains. Most genbank and Ugandan strains were grouped separately. Sub-clade 1 mostly comprised of Ugandan strains. All strains were newly formed strains with very few unique histories. Sub-clade 2 had one Ugandan strain and one Kenyan strain. Sub-clade 3 had only Ugandan strains while sub-clade 4 comprised Kenyan and Ugandan strains. Ugandan strains were further away from the clade ancestor and displayed many unique histories and variation amongst themselves compared to the genbank strains. Some Kenyan strains such as the A/Kericho/6528/2008 H3N2 strain showed completely no relationship with the Ugandan strains although they were in the same clade. Others such as the A/Malindi/7579/2008 H3N2 shared a sub-clade with a Ugandan strain although the latter showed that it had separated from their common ancestor longer than the Kenyan strain. Other Kenyan strains such as the A/Malindi/7590/2008 H3N2 and A/Kisumu/7602/2008 H3N2 shared a common subclade with some Ugandan strains. There were 23 HA gene strains that were detected and presented in the phylogenetic tree in figure 20. The strains in figure 20 are shown in the table 9:
<table>
<thead>
<tr>
<th>22 influenza A virus HA gene Strains appearing in the clade</th>
<th>Strain(s) identical to clade strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/UgaMul /011/08(H3N2)</td>
<td>A/UgaMul /016/08(H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /020/08(H3N2)</td>
<td>A/UgaMul /049/08(H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /041/08(H3N2)</td>
<td>A/UgaMul /001/08(H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /007/08(H3N2)</td>
<td>A/UgaMul /003/08(H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /012/08(H3N2)</td>
<td>A/UgaMul /008/08(H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /039/08(H3N2)</td>
<td>A/UgaMul /009/08(H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /018/08(H3N2)</td>
<td>A/UgaMul /010/08(H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /026/08(H3N2)</td>
<td>A/UgaMul /014/08(H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /048/08(H3N2)</td>
<td>A/UgaMul /013/08(H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /052/08(H3N2)</td>
<td>A/UgaMul /019/08(H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /051/08(H3N2)</td>
<td>A/UgaMul /023/08(H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /019/08(H3N2)</td>
<td>A/UgaMul /025/08(H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /050/08(H3N2)</td>
<td>A/UgaMul /021/08(H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /046/08(H3N2)</td>
<td>A/UgaMul /047/08(H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /024/08(H3N2)</td>
<td>A/UgaMul /042/08(H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /015/08(H3N2)</td>
<td>A/UgaMul /043/08(H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /031/08(H3N2)</td>
<td>A/UgaMul /031/08(H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /033/08(H3N2)</td>
<td>A/UgaMul /028/08(H3N2)</td>
</tr>
<tr>
<td>A/UgaKay /027/08(H3N2)</td>
<td>A/UgaMul /006/08(H3N2)</td>
</tr>
<tr>
<td>A/UgaKay /002/08(H3N2)</td>
<td>A/UgaMul /022/08(H3N2)</td>
</tr>
<tr>
<td>A/UgaKay /044/08(H3N2)</td>
<td>A/UgaKay /029/08(H3N2)</td>
</tr>
<tr>
<td>A/UgaKay /037/08(H3N2)</td>
<td>A/UgaMul /005/08(H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /032/08(H3N2)</td>
<td>A/UgaMul /032/08(H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /034/08(H3N2)</td>
<td>A/UgaMul /034/08(H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /035/08(H3N2)</td>
<td>A/UgaMul /035/08(H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /036/08(H3N2)</td>
<td>A/UgaMul /036/08(H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /038/08(H3N2)</td>
<td>A/UgaMul /038/08(H3N2)</td>
</tr>
</tbody>
</table>
Nucleotide sequences of NP genes and their relationship with strains from elsewhere

**NP**

![Un-rooted phylogenetic tree showing relationship of NP nucleotide sequences of the Ugandan isolates with those of the WHO reference and genbank strains for the year 2008](image)

- **Uganda strains**
- **Genbank strains**
- **2008 WHO Reference strains**

**Figure 21:** Un-rooted phylogenetic tree showing relationship of NP nucleotide sequences of the Ugandan isolates with those of the WHO reference and genbank strains for the year 2008
In figure 21, the phylogenetic tree showed only one clade, without any sub-clades, and an out-group of 1 Ugandan and 1 2008 reference strain. The genbank strains comprised of US and south East Asian strains. Although clustered separately within the clade, the genbank and two Ugandan strains that had unique histories were newly formed strains and had minimal variation. Whereas the genbank strains neither displayed any unique histories nor variation, some Ugandan strains displayed unique histories that led to variation of the respective strains from the rest. There were 4 NP gene strains that were detected and presented in the phylogenetic tree in figure 21. The strains in figure 21 are shown in the table 10:
Table 10: The 4 influenza A virus NP gene strains detected in the study

<table>
<thead>
<tr>
<th>Strains appearing in the clade</th>
<th>Strain(s) identical to clade strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/UgaMul/023/08 (H3N2)</td>
<td>A/UgaMul/017/08 (H3N2)</td>
</tr>
<tr>
<td>A/UgaMul/012/08 (H3N2)</td>
<td>A/UgaMul/003/08 (H3N2)</td>
</tr>
<tr>
<td>A/UgaMul/008/08 (H3N2)</td>
<td>A/UgaMul/015/08 (H3N2)</td>
</tr>
<tr>
<td>A/UgaMul/002/08 (H3N2)</td>
<td>A/UgaMul/016/08 (H3N2)</td>
</tr>
<tr>
<td></td>
<td>A/UgaMul/025/08 (H3N2)</td>
</tr>
<tr>
<td></td>
<td>A/UgaMul/031/08 (H3N2)</td>
</tr>
<tr>
<td></td>
<td>A/UgaMul/033/08 (H3N2)</td>
</tr>
<tr>
<td></td>
<td>A/UgaMul/052/08 (H3N2)</td>
</tr>
<tr>
<td></td>
<td>A/UgaMul/045/08 (H3N2)</td>
</tr>
<tr>
<td></td>
<td>A/UgaMul/010/08 (H3N2)</td>
</tr>
<tr>
<td></td>
<td>A/UgaMul/011/08 (H3N2)</td>
</tr>
<tr>
<td></td>
<td>A/UgaMul/007/08 (H3N2)</td>
</tr>
<tr>
<td></td>
<td>A/UgaMul/026/08 (H3N2)</td>
</tr>
<tr>
<td></td>
<td>A/UgaMul/039/08 (H3N2)</td>
</tr>
<tr>
<td></td>
<td>A/UgaMul/021/08 (H3N2)</td>
</tr>
<tr>
<td></td>
<td>A/UgaMul/049/08 (H3N2)</td>
</tr>
<tr>
<td></td>
<td>A/UgaKay/029/08 (H3N2)</td>
</tr>
<tr>
<td></td>
<td>A/UgaMul/006/08 (H3N2)</td>
</tr>
<tr>
<td></td>
<td>A/UgaMul/044/08 (H3N2)</td>
</tr>
<tr>
<td></td>
<td>A/UgaMul/022/08 (H3N2)</td>
</tr>
<tr>
<td></td>
<td>A/UgaKay/037/08 (H3N2)</td>
</tr>
<tr>
<td></td>
<td>A/UgaMul/013/08 (H3N2)</td>
</tr>
<tr>
<td></td>
<td>A/UgaMul/019/08 (H3N2)</td>
</tr>
<tr>
<td></td>
<td>A/UgaMul/051/08 (H3N2)</td>
</tr>
<tr>
<td></td>
<td>A/UgaMul/046/08 (H3N2)</td>
</tr>
<tr>
<td></td>
<td>A/UgaMul/047/08 (H3N2)</td>
</tr>
<tr>
<td></td>
<td>A/UgaMul/014/08 (H3N2)</td>
</tr>
<tr>
<td></td>
<td>A/UgaMul/009/08 (H3N2)</td>
</tr>
<tr>
<td></td>
<td>A/UgaKay/028/08 (H3N2)</td>
</tr>
<tr>
<td></td>
<td>A/UgaKay/027/08 (H3N2)</td>
</tr>
<tr>
<td></td>
<td>A/UgaMul/018/08 (H3N2)</td>
</tr>
<tr>
<td></td>
<td>A/UgaMul/001/08 (H3N2)</td>
</tr>
<tr>
<td></td>
<td>A/UgaMul/005/08 (H3N2)</td>
</tr>
<tr>
<td></td>
<td>A/UgaMul/020/08 (H3N2)</td>
</tr>
<tr>
<td></td>
<td>A/UgaMul/024/08 (H3N2)</td>
</tr>
<tr>
<td></td>
<td>A/UgaMul/032/08 (H3N2)</td>
</tr>
<tr>
<td></td>
<td>A/UgaMul/034/08 (H3N2)</td>
</tr>
<tr>
<td></td>
<td>A/UgaMul/035/08(H3N2)</td>
</tr>
<tr>
<td></td>
<td>A/UgaMul/038/08 (H3N2)</td>
</tr>
<tr>
<td></td>
<td>A/UgaMul/040/08 (H3N2)</td>
</tr>
<tr>
<td></td>
<td>A/UgaMul/041/08 (H3N2)</td>
</tr>
<tr>
<td></td>
<td>A/UgaMul/043/08 (H3N2)</td>
</tr>
<tr>
<td></td>
<td>A/UgaMul/048/08 (H3N2)</td>
</tr>
<tr>
<td></td>
<td>A/UgaMul/050/08 (H3N2)</td>
</tr>
<tr>
<td></td>
<td>A/UgaMul/036/08 (H3N2)</td>
</tr>
<tr>
<td></td>
<td>A/UgaMul/042/08 (H3N2)</td>
</tr>
</tbody>
</table>
Nucleotide sequences of NA genes and their relationship with strains from elsewhere

Figure 22: Un-rooted phylogenetic tree showing relationship of NA nucleotide sequences of the Ugandan isolates with those of the Kenyan, reference and genbank strains for the year 2008.
In figure 2, the tree showed one large clade, with six sub-clades and an out-group of nine strains that included; the 2008 reference strains, three Ugandan, two Kenyan and three US strains. The Ugandan and genbank strains were distributed within the clade. Sub-clade 1 comprised of Kenyan strains. Sub-clades 2, 3, 4 and 5 were Ugandan strains while sub-clade 6 had two Kenyan and one Ugandan strain, and sub-clade 5 had both Kenyan and Ugandan strains. There was an European strain within the clade. Most of the Ugandan strains displayed unique histories; and did not cluster with the rest of the strains. They had more variations and mutation rates amongst themselves. The genbank strains showed very low variations amongst themselves and were more closely related to the clade ancestor than the rest of the strains. There were 26 NA gene strains that were detected and presented in the phylogenetic tree in figure 2. The strains in figure 2 are shown in the table 11.
Table 11: The 26 influenza A virus NA gene strains detected in the study

<table>
<thead>
<tr>
<th>Strains appearing in the clade</th>
<th>Strain(s) identical to clade strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/UgaMul /049/08(H3N2)</td>
<td>A/UgaMul /006/08(H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /046/08(H3N2)</td>
<td>A/UgaMul /044/08(H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /047/08(H3N2)</td>
<td>A/UgaMul /024/08(H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /026/08(H3N2)</td>
<td>A/UgaMul /010/08(H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /003/08(H3N2)</td>
<td>A/UgaMul /018/08(H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /023/08(H3N2)</td>
<td>A/UgaMul /017/08(H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /021/08(H3N2)</td>
<td>A/UgaMul /041/08(H3N2)</td>
</tr>
<tr>
<td>A/UgaKay /040/08(H3N2)</td>
<td>A/UgaMul /009/08(H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /052/08(H3N2)</td>
<td>A/UgaMul /011/08(H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /039/08(H3N2)</td>
<td>A/UgaMul /031/08(H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /020/08(H3N2)</td>
<td>A/UgaMul /033/08(H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /050/08(H3N2)</td>
<td>A/UgaMul /001/08(H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /025/08(H3N2)</td>
<td>A/UgaMul /013/08(H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /042/08(H3N2)</td>
<td>A/UgaMul /014/08(H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /012/08(H3N2)</td>
<td>A/UgaMul /045/08(H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /022/08(H3N2)</td>
<td>A/UgaMul /051/08(H3N2)</td>
</tr>
<tr>
<td>A/UgaKay /029/08(H3N2)</td>
<td>A/UgaKay /005/08(H3N2)</td>
</tr>
<tr>
<td>A/UgaKay /048/08(H3N2)</td>
<td>A/UgaMul /032/08(H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /043/08(H3N2)</td>
<td>A/UgaMul /034/08(H3N2)</td>
</tr>
<tr>
<td>A/UgaKay/027/08(H3N2)</td>
<td>A/UgaMul /035/08(H3N2)</td>
</tr>
<tr>
<td></td>
<td>A/UgaMul /036/08(H3N2)</td>
</tr>
<tr>
<td></td>
<td>A/UgaMul /007/08(H3N2)</td>
</tr>
<tr>
<td></td>
<td>A/UgaMul /016/08(H3N2)</td>
</tr>
</tbody>
</table>
Nucleotide sequences of M genes and their relationship with strains from elsewhere

Figure 23: Un-rooted phylogenetic tree showing relationship of M nucleotide sequences of the Ugandan isolates with those of the Kenyan, reference and genbank strains for the year 2008.
In figure 23, the tree had one clade, with two sub-clades with evenly distributed Ugandan and genbank strains. There was an out-group of only WHO 2008 reference strains. Each of the Ugandan strains, formed unique histories that were sufficiently different from the rest of the genbank strains. The Ugandan strains also showed more unique histories compared to the genbank strains. The genbank strains were all more related to the clade ancestor and clustered together nearer to it compared to the rest of the clade strains. There were 21 M gene strains that were detected and presented in the phylogenetic tree in figure 23. The strains in figure 23 are shown in the table 12.
Table 12: The 21 influenza A virus M gene strains detected in the study

<table>
<thead>
<tr>
<th>Strains appearing in the clade</th>
<th>Strain(s) identical to clade strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/UgaMul /016/08(H3N2)</td>
<td>A/UgaMul /021/08(H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /050/08(H3N2)</td>
<td>A/UgaMul /022/08(H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /048/08(H3N2)</td>
<td>A/UgaMul /044/08(H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /018/08(H3N2)</td>
<td>A/UgaMul /001/08(H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /036/08(H3N2)</td>
<td>A/UgaMul /007/08(H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /003/08(H3N2)</td>
<td>A/UgaMul /013/08(H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /005/08(H3N2)</td>
<td>A/UgaMul /014/08(H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /020/08(H3N2)</td>
<td>A/UgaMul /009/08(H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /012/08(H3N2)</td>
<td>A/UgaMul /010/08(H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /047/08(H3N2)</td>
<td>A/UgaMul /015/08(H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /011/08(H3N2)</td>
<td>A/UgaMul /019/08(H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /032/08(H3N2)</td>
<td>A/UgaMul /021/08(H3N2)</td>
</tr>
<tr>
<td>A/UgaKay /040/08(H3N2)</td>
<td>A/UgaMul /023/08(H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /043/08(H3N2)</td>
<td>A/UgaMul /024/08(H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /026/08(H3N2)</td>
<td>A/UgaMul /025/08(H3N2)</td>
</tr>
<tr>
<td>A/UgaKay /002/08(H3N2)</td>
<td>A/UgaMul /031/08(H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /006/08(H3N2)</td>
<td>A/UgaMul /033/08(H3N2)</td>
</tr>
<tr>
<td>A/UgaKay /027/08(H3N2)</td>
<td>A/UgaMul /034/08(H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /008/08(H3N2)</td>
<td>A/UgaMul /035/08(H3N2)</td>
</tr>
<tr>
<td>A/UgaKay /029 /08 (H3N2)</td>
<td>A/UgaMul /041/08(H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /037/08(H3N2)</td>
<td>A/UgaMul /017/08(H3N2)</td>
</tr>
<tr>
<td>A/UgaKay /028/08(H3N2)</td>
<td>A/UgaMul /022/08(H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /017/08(H3N2)</td>
<td>A/UgaMul /044/08(H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /016/08(H3N2)</td>
<td>A/UgaMul /001/08(H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /007/08(H3N2)</td>
<td>A/UgaMul /007/08(H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /013/08(H3N2)</td>
<td>A/UgaMul /014/08(H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /014/08(H3N2)</td>
<td>A/UgaMul /009/08(H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /009/08(H3N2)</td>
<td>A/UgaMul /010/08(H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /010/08(H3N2)</td>
<td>A/UgaMul /015/08(H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /015/08(H3N2)</td>
<td>A/UgaMul /019/08(H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /019/08(H3N2)</td>
<td>A/UgaMul /021/08(H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /021/08(H3N2)</td>
<td>A/UgaMul /023/08(H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /023/08(H3N2)</td>
<td>A/UgaMul /024/08(H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /024/08(H3N2)</td>
<td>A/UgaMul /025/08(H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /025/08(H3N2)</td>
<td>A/UgaMul /031/08(H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /031/08(H3N2)</td>
<td>A/UgaMul /033/08(H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /033/08(H3N2)</td>
<td>A/UgaMul /034/08(H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /034/08(H3N2)</td>
<td>A/UgaMul /035/08(H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /035/08(H3N2)</td>
<td>A/UgaMul /041/08(H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /041/08(H3N2)</td>
<td>A/UgaMul /017/08(H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /042/08(H3N2)</td>
<td>A/UgaMul /001/08(H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /038/08(H3N2)</td>
<td>A/UgaMul /007/08(H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /039/08(H3N2)</td>
<td>A/UgaMul /013/08(H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /046/08(H3N2)</td>
<td>A/UgaMul /014/08(H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /049/08(H3N2)</td>
<td>A/UgaMul /009/08(H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /045/08(H3N2)</td>
<td>A/UgaMul /010/08(H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /051/08(H3N2)</td>
<td>A/UgaMul /015/08(H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /052/08(H3N2)</td>
<td>A/UgaMul /019/08(H3N2)</td>
</tr>
</tbody>
</table>
Nucleotide sequences of NS genes and their relationship with strains from elsewhere

Figure 24: Un-rooted phylogenetic tree showing relationship of NS nucleotide sequences of the Ugandan isolates with those of the reference and genbank strains for the year 2008.
In figure 24, the phylogenetic tree consisted of one large clade with three sub clades; Sub-clades 1 comprised of genbank, a south east Asian strain and WHO reference strains for 2008. Sub-clades 2 and 3 were dominated by Ugandan strains most of which had unique histories. All sub-clade 2 strains did not display any unique histories and clustered together. Although the Ugandan strains displayed variation, they were grouped closer to the clade ancestor compared to the genbank strains. The genbank strains were more related to each other than to the Ugandan strains but were grouped further away from the clade ancestor compared to the Ugandan strain. There were 23 NS gene strains that were detected and presented in the phylogenetic tree in figure 24. The strains in figure 24 are shown in table 13.
<table>
<thead>
<tr>
<th>Strain(s) identical to clade strain</th>
<th>23 influenza A virus NS gene Strains appearing in the clade</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/UgaKay /028/08 (H3N2)</td>
<td>A/UgaMul /046/08 (H3N2)</td>
</tr>
<tr>
<td>A/UgaKay /040/08 (H3N2)</td>
<td>A/UgaKay /040/08 (H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /021/08 (H3N2)</td>
<td>A/UgaMul /021/08 (H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /049/08 (H3N2)</td>
<td>A/UgaMul /049/08 (H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /026/08 (H3N2)</td>
<td>A/UgaMul /026/08 (H3N2)</td>
</tr>
<tr>
<td>A/UgaKay /002/08 (H3N2)</td>
<td>A/UgaKay /002/08 (H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /043/08 (H3N2)</td>
<td>A/UgaMul /043/08 (H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /048/08 (H3N2)</td>
<td>A/UgaMul /048/08 (H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /050/08 (H3N2)</td>
<td>A/UgaMul /050/08 (H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /016/08 (H3N2)</td>
<td>A/UgaMul /016/08 (H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /032/08 (H3N2)</td>
<td>A/UgaMul /032/08 (H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /005/08 (H3N2)</td>
<td>A/UgaMul /005/08 (H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /007/08 (H3N2)</td>
<td>A/UgaMul /007/08 (H3N2)</td>
</tr>
<tr>
<td>A/UgaKay /037/08 (H3N2)</td>
<td>A/UgaKay /037/08 (H3N2)</td>
</tr>
<tr>
<td>A/UgaKay /027/08 (H3N2)</td>
<td>A/UgaKay /027/08 (H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /023/08 (H3N2)</td>
<td>A/UgaMul /023/08 (H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /020/08 (H3N2)</td>
<td>A/UgaMul /020/08 (H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /038/08 (H3N2)</td>
<td>A/UgaMul /038/08 (H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /047/08 (H3N2)</td>
<td>A/UgaMul /047/08 (H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /015/08 (H3N2)</td>
<td>A/UgaMul /015/08 (H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /034/08 (H3N2)</td>
<td>A/UgaMul /034/08 (H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /001/08 (H3N2)</td>
<td>A/UgaMul /001/08 (H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /025/08 (H3N2)</td>
<td>A/UgaMul /025/08 (H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /036/08 (H3N2)</td>
<td>A/UgaMul /036/08 (H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /052/08 (H3N2)</td>
<td>A/UgaMul /052/08 (H3N2)</td>
</tr>
<tr>
<td>A/UgaKay /029/08 (H3N2)</td>
<td>A/UgaKay /029/08 (H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /006/08 (H3N2)</td>
<td>A/UgaMul /006/08 (H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /022/08 (H3N2)</td>
<td>A/UgaMul /022/08 (H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /042/08 (H3N2)</td>
<td>A/UgaMul /042/08 (H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /044/08 (H3N2)</td>
<td>A/UgaMul /044/08 (H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /014/08 (H3N2)</td>
<td>A/UgaMul /014/08 (H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /003/08 (H3N2)</td>
<td>A/UgaMul /003/08 (H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /008/08 (H3N2)</td>
<td>A/UgaMul /008/08 (H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /009/08 (H3N2)</td>
<td>A/UgaMul /009/08 (H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /010/08 (H3N2)</td>
<td>A/UgaMul /010/08 (H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /013/08 (H3N2)</td>
<td>A/UgaMul /013/08 (H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /017/08 (H3N2)</td>
<td>A/UgaMul /017/08 (H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /018/08 (H3N2)</td>
<td>A/UgaMul /018/08 (H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /019/08 (H3N2)</td>
<td>A/UgaMul /019/08 (H3N2)</td>
</tr>
<tr>
<td>A/UgaMul /051/08 (H3N2)</td>
<td>A/UgaMul /051/08 (H3N2)</td>
</tr>
</tbody>
</table>
4.5.1 Number of mutations per gene-site as represented by the phylogenetic tree scales

Figure 25 shows the number of mutations per gene-site (0.0027, 0.16, 0.0016, 7.14, 0.043, 7.4, 2.105 and 4.76) by gene for the PB2, PB1, PA, HA, NP, NA, M and NS genes respectively. The HA and NA genes displayed the highest mutations per site while the polymerase and NP genes had the lowest.

Figure 25: Number of mutations per gene-site
CHAPTER FIVE

5.0 DISCUSSION

The detection of human influenza A viruses in 51 out of the 450 samples showed that the Ugandan population is exposed and susceptible to influenza A viruses. The influenza A viral infection of these persons was due to various factors discussed below. The patients who had ILI but were negative for influenza A viruses, were probably infected with influenza B viruses or other respiratory pathogens which were not tested for since they were not part of the study.

In this study, all the viruses detected were of the influenza A H3N2 subtype. These findings concurred with previous studies that reported the higher prevalence of H3N2 subtype over the H1N1 subtype after 2001 (Brown et al., 1998; Lin et al., 2004; CDC, 2007). Usually, the H3N2 and H1N1 human influenza A viral subtypes co-circulate in a given population leading to epidemic outbreaks (Lewis, 2006, Mahsa et al., 2006, Manicassamy et al., 2010; Suwannakarn et al., 2010). This may have been due to the possibility that most of the time the H1N1 strains formed after undergoing genetic reassortments, were inviable and were often killed off by the host’s immune response, while the H3N2 subtype viruses underwent faster evolution that enabled them to evade their hosts’ immune responses (Ferguson et al., 2003; Nelson et al., 2008). Thus, with higher replicative fitness, the H3N2 viruses established themselves over the H1N1 subtype viruses. Also, influenza A H1N1 and H3N2 viral subtypes have showed antiviral resistance of up to 98% and 96% to oseltamivir and adamantane, respectively (Deyde et al., 2007; Nelson et al., 2008). However, the H1N1 subtypes have an adamantane resistance of only 5.8% (Bright et al., 2006; Deyde et al., 2007; Simonsen et al., 2007; Racaniello, 2008). This suggests that any H1N1 viruses, present within a human populations treated with adamantane, may have been killed off before they
were spread by human to human transmission during transborder events, leaving the more resistant H3N2 strains. It is, therefore, probable that the H3N2 viruses detected in this study may have been strains resistant to adamantane that had been introduced into the Ugandan human population from other populations treated with adamantane. Thus the findings of this study concur with previous findings that reported higher association of influenza A virus H3N2 subtype with flu illnesses compared to other subtypes (Simonsen et al., 2005).

The differences in the significant amino acid changes along the PA gene were a result of a transversion at the first nucleotide position of the amino acid code leading to a change from G to C or vice versa. The presence of E in position 133 has been reported to be associated with low virulence of the influenza A viruses (Zhou et al., 1999). Hence, it is probable that these amino acid changes previously reported to be associated with low virulence of the viruses were the reason why there was no serious disease reported among any of the study participants that were positive for influenza A viruses. The presence of D at position 34 along the NP gene was due to a transition of A to G nucleotides and a transversion of A to C, or vice versa at nucleotide positions 100 and 101 respectively and has been reported to be associated with increased host specificity of the 49 influenza A viruses (Scholtissek et al., 1985; Zhou et al., 1999). The PB2 and NP proteins had significant amino acid changes that have been reported to infer host specificity (Hinshaw et al., 1983; Scholtissek et al., 1985; Subbarao et al., 1993; Almond, 1977; Zhou et al., 1999). These findings concur with reports of previous studies that the presence of viral ligands, on the influenza HA gene, that are specific to the α2-6 sialic acid receptors on the epithelial cells of the human hosts’ respiratory tract, is not an exclusive determinant of human transmission (Hinshaw et al., 1983; Scholtissek et al., 1985; Subbarao et al., 1993; Almond, 1977; Zhou et al., 1999). Although the PA, NP and PB2 influenza A virus genes had significant amino acids which have been reported to be associated with human transmission and low virulence (Hinshaw et al., 1983;
Scholtissek et al., 1985; Subbarao et al., 1993; Almond, 1977; Zhou et al., 1999), there was no severe disease reported among the patients. The PB1, NA, HA, M and NS gene segments did not have any significant amino acid changes.

The PB2, PB1 and PA genes had the highest strain diversities that resulted from accumulation of substitutions and deletions within the gene sequences. The highest and lowest strain diversities were observed in the polymerase and matrix genes respectively. The mutations in the polymerase genes may have been due to the high mutation rates due to along various sites as a result of substitutions and deletions. The low diversity observed among the matrix gene strains concurred with previous findings that it was highly conserved (Lamb & Choppin, 1983). The other genes displayed fewer strains. Although the HA and NA genes had the highest mutation rates, their diversities by gene were lower compared to the polymerase genes. This is because the HA and NA genes, unlike other genes, had higher mutations per site. There were 39 more influenza A virus strains detected at the Mulago site maybe because the number of positive cases reported had a P-value of 11.3% (n=450) and were significantly higher than those detected in Kayunga. This contributed to the increased probability of strain diversity by gene, among the viruses detected in patients attending Mulago hospital. November 2008 had the highest strain diversities for all the genes compared to the rest of the study period which may have been due to the highest number of positive cases that also provided for increased diversity during the month. The month, of November was a rainy season which was cold and highly humid. This enabled the efficient human to human transmission of the virus of the three months, November 2008 was the coldest month.

The polymerase and NP genes had displayed numerous unique sites because of very many mutations along their sequences. Although the HA and NA genes, have been previously reported to undergo constant evolution and are the major determinants of viral pathogenicity (Hilleman, 2002; Ghedin et al., 2005), they had few unique sites along their sequences. This
was probably due to higher mutations per site by gene (HA, NA) resulting from similar simultaneous substitution events which occurred at fewer specific locations of the various nucleotide sequences, respectively. Although there HA and NA genes had the highest mutation rates per site, they had significantly fewer unique sites observed than the rest of the genes. These mutations were vital to survival of the viruses and subsequently led to the enhanced immune evasion ability of the virus within the host. The relatively conserved NS gene was probably due to parallel evolution that resulted in relatively similar sites along most of the gene sequence (Lamb & Choppin, 1983). Also, in the area of overlap between the NS 1 and NS 2, the amino acid sequence of NS 2 is conserved at the expense of NS 1. The above factors may have significantly influenced the occurrence of few unique sites. The least number of unique sites shown by the matrix genes was consistent with the gene’s highly conserved nature, compared to the rest of the genes (Lamb & Choppin, 1983).

The influenza A virus gene sequences showed nucleotide diversity, $\pi$, as a result of substitutions and deletions at various sequence sites. The PB1 and M genes showed the highest and lowest $\pi$ respectively. This was consistent with their respective strain diversities as discussed above. The $\pi$ determined for all the strains from both study sites by gene was found to be relatively lower than when the individual site $\pi$ values were summed together, by gene. This was because the strains detected during the study appeared at both sites, hence, lower $\pi$ values observed when site values were combined, by gene.

A unique strain, A/UgaKay/027/08(H3N2) was detected during November 2008, when the highest number of influenza A virus positive cases were detected, its presence in a sample from the Kayunga site suggests that it had recently been introduced into the human population. Although unique to the study strains, it was highly similar (99%) to other strains
in the genbank, suggesting that it was a recurrent strain that may have been introduced into the Ugandan population through trans-border events.

Molecular phylogeny of the influenza A viruses: For all the genes, the presence of the Ugandan strains in the same clade as genbank strains indicated that the strains detected were already existent worldwide. Although the study isolates showed up to 99% similarity with genbank strains, the phylogenetic trees showed the nature of relationships amongst the study strains and with other strains worldwide. The higher number of mutations per site led to the development and progression of the various unique histories, with some strains showing recent separation from their clade ancestor, while others separated long ago. This was observed for the NA, HA, NS and M genes respectively. These strains with unique histories were sufficiently different from the rest of the strains that clustered by themselves because they were newly formed strains.

For the HA gene, the Kenyan HA strains clustered on their own because they were sufficiently different from the Ugandan strain, which showed unique histories. Two other Kenyan strains, A/Mbagathi/7593/2008 H3N2 and A/Kisumu/7605/2008 H3N2, also showed unique histories like the Ugandan strains. The strains that showed unique histories indicate that at one point, they shared a common ancestor with other strains within the clade. However, they underwent mutations that led to their diversifications genetically and eventual separations, forming unique histories. The strains that clustered together, were almost identical to the common ancestor they shared because they were sufficiently similar. For the NA genes, although all Kenyan strains showed unique histories, none shared a common ancestry with the Ugandan strains at sub-clade level except one, A/Malindi/7579/2008 H3N2. This shows that the other Kenyan strains apart from A/Malindi/7579/2008 H3N2 were separated from the common ancestor with the Ugandan strains whereas the
A/Malindi/7579/2008 H3N2 had undergone a recent separation from a common ancestor shared with the Ugandan strains, suggesting that it was more related to the Ugandan strains than the rest of the Kenyan strains. It’s probable that this strain, A/Malindi/7579/2008 H3N2, could have been introduced from Kenya to Uganda or the Ugandan strains with which it shared a common ancestry could have been introduced to Kenya through trans-border events.

Despite the 99% similarity between the Ugandan and genbank strains, the NA, HA, NS and M genes dominated the display of variations and unique histories for the Ugandan strains. For the HA and NA genes, this was probably due to the fact that the Ugandan human population has never been subjected to any form of flu vaccine, which usually target the HA and NA gene. Therefore, the rate of mutation of the viruses for these genes was much higher than those of the genbank strains that mainly clustered together. Besides the usual mutation of these error-prone RNA viruses, the results from this study suggest that after introduction into the Ugandan population, each time the viruses replicated, they underwent faster unhindered mutation compared to the viruses elsewhere in the world represented by the genbank sequences. It is probable that the genbank sequences showed less mutation and variation than the Ugandan strains because of previous exposure to the former to flu vaccines and antivirals that may have limited the mutation rates. On the contrary, the Ugandan HA and NA genes may have mutated faster alongside the NS and M genes because the human population in Uganda has not been exposed to anti-virals and vaccines in the past.

The high similarity of influenza A virus sequences to the genbank sequences suggests that the influenza A viruses detected in the study were introduced into the human population in Uganda through trans-border events. Although the Ugandan strains showed high similarity to Kenyan and genbank strains, most of the strains showed unique histories, some showing previous separations and others having separated from their ancestor long ago. This suggests
that the strains were from different hosts from human populations of different locations, hence, the variation in their mutation patterns. Some Kenyan strains, however, clustered with the genbank strains suggesting that they were almost identical to the genbank strains. As expected, the HA and NA genes, of the WHO reference strains, were not grouped with the rest of the clade strains because these genes were from WHO 2008 reference strains that were used in the vaccine design for the 2008 flu seasons and, therefore, did not appear in the clade because the vaccinated human populations represented by the genbank strains (from human populations in different parts of the world) had protection against them. The minimal mutation rate and variation as well as the absence of unique histories observed amongst the NP gene strains was probably because they underwent lower rates of divergent evolution. The strains of these genes were newly formed strains, some appeared un-noded and clustered together within the clade because they were almost identical and very closely related to their common clade ancestor. The genbank sequences of the influenza A strains show that the human populations, from which the respective samples had been collected, lacked protection against the influenza A viruses because none of them had been specifically targeted by vaccines of the 2008 flu season. Their relationship with the study strains within the clade suggests that there was human to human transmission during trans-border events that led to the introduction of strains from different parts of the world, into the Ugandan human population. The PB1 and NA genes had some strains from Australia and Europe respectively, indicating the presence of strains in those continents that were highly similar to the study strains by gene. All trees, apart from trees of the PB1, NA and M genes, displayed strains from south East Asia. Although dominated by US strains, the presence of genbank strains from south East Asia was very significant considering that many outbreaks have originated from there in the past (Anon, 2004; Fergusson et al., 2005; Viboud et al., 2006; Park & Glass 2007). The
appearance of strains from south East Asia, notably Japan and Taiwan, in the same clade as
the US strains suggests that the close vicinity of the former to the latter, may have led to the
human to human transmission of the influenza A viruses between the two population groups
through trans-border events. This was followed by the introduction and spread of the viruses
within the populations of the US and, later, different parts of the world such as Uganda,
mainly during subsequent trans-border events. For all the above genes, the out-groups formed
indicated that the strains within the same out-group had descended from another ancestor
different from the clade ancestor.

The study findings indicated the notably very high number of mutations per site displayed
by the HA and NA genes of the genbank and Uganda strains, which concurred with previous
findings that these genes were undergoing higher evolution rates per site (Hilleman, 2002;
Ghedin et al., 2005). However, the amino acid changes observed in this study were neither
significant enough nor did the influenza A viral strains have any attributes for selection as
vaccine candidates compared to the WHO 2008 reference strains.

Geographical epidemiology of the Influenza A viruses: In Uganda, the high human
population densities and the absence of significant topological barriers may have enabled the
easy human to human transmission and spread of influenza A viruses. Notably, the study
findings that the Ugandan and genbank strains from the US clustered together, suggests that
the strains detected during the study were introduced through trans-border events. Their
presence in Uganda and prevalence worldwide, notably the US, shows that human influenza
A viruses are geographically independent, unlike swine and avian flu viruses (Fergusson et
al., 2005; Park & Glass 2007). Globally, the presence of highly similar influenza A viruses
worldwide shows that the Ugandan strains were already existent worldwide and suggests that
human to human transmission of the viruses into and within the population was mainly
through trans-border events such as travel (Montefiore et al., 1970).
Disease clustering of the human influenza A virus: The higher disease clustering within and around Kampala district was due to the very high population density resulting from rampant rural-urban migration and subsequent inter-mingling of various influenza A virus infected persons from different destinations. With reference to previous studies, the findings of this study suggest that human to human transmission of various influenza A virus strains from infected to healthy persons may have resulted from inhalation of aerosols infected with influenza A viruses that had probably been exhaled by flu patients (Tellier, 2006; Hall, 2007; Weber & Stilianakis, 2008). Also, the influenza viruses may have also been spread through person to person contact or through the air by coughs or sneezes, creating particle aerosols containing the virus that can get into respiratory tract of a recipient healthy individual (Jiang et al., 2007). The higher rate of influenza human to human transmission observed in the urban based Mulago hospital may have been associated with the relatively higher occurrences of mingling of persons from different locations within Kampala district. Furthermore, Mulago, a well equipped national referral hospital run by professionals and a major communication centre was, understandably, preferred over other medical institutions by a majority of patients. This probably led to the higher influenza A viral prevalence registered at the Mulago site. The geographical epidemiology is further discussed in the ecology of the influenza A viruses and spatial analysis below.

Ecology of the Influenza A viruses: The rainy season within the southern region provided optimally inducive humid conditions for the survival, human to human transmission and spread of the influenza A viruses within the human population. Also, with cold weather, the cilia in the hosts’ upper respiratory tracts became static and resulted into the reduced efficiency of ciliary clearance and impaired function of the mucus elevator in the respiratory tract of the infected hosts (Jiang et al., 2007). Although reduced sun shine during the rainy season suggests that lower vitamin D levels impaired the immunity of influenza A virus
infected persons and led to disease development, there were viruses also detected in samples collected during the dry season prior to the onset of the rainy season. The latter concurs with previous findings that influenza A viruses can be spread during regularly warm and sunny weather (Montefiore et al., 1970). Thus, the prevalence of influenza A virus infection was highest in November 2008 when the rainy season was at its peak.

Spatial Analysis of the Influenza A viruses: Although 47% of the influenza A virus positive patients had recent histories of close associations with possible sources of exposure such as pigs and/or domestic poultry, interspecies transmission of influenza A viruses was ruled out. This was based on the findings that the study strains showed up to 99% similarity with genbank strains of human origin suggesting that the strains had been spread by human to human transmission. Although viral strains lacked aminoacids that encoded ligands that specifically bind to sialic acid α 2,6 human host receptors, they all had aminoacids in the PB2 and NP genes that inferred human transmission (Scholtissek et al., 1985; Subbarao et al., 1993; Almond, 1977; Zhou et al., 1999).

The higher number of child patients compared to adults was consistent with previous findings that influenza illnesses are more common in children aged 5 years and below as well as in the elderly aged 65 years and above. This is because the children lack prior exposure to influenza A virus infections and, hence, have naïve immunity to the viruses (Zambon, 1999; Hilleman, 2002; Suarez et al., 2003; Nobusawa & Sato, 2006). Another possible reason was the tendency for children to be hospitalized more than adults (Fasina et al., 2010). This tendency has been associated with a higher level of immunity in adults against the viruses due to previous exposure (Hilleman, 2002; Shek & Lee, 2003). Also in Uganda, most adults self-medicate especially using anti-pyretics, before visiting health facilities, which would reduce their temperature hence making them not be eligible for the study. Additionally, in
many parts of Africa, including Uganda, people have been known to visit the hospital less frequently as they advance in age, and supposedly non life-threatening conditions like seasonal influenza are often treated at home, leading to under-reporting of cases (Fasina et al., 2009). It is not clear why there were more females positive for influenza A virus.
CHAPTER SIX

6.0 CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

1. Influenza A viruses were detected and isolated from 11.3% (n=450) of samples collected from human patients at Kayunga and Mulago hospitals.

2. Influenza A viruses detected were found to be of the H3N2 subtype using RT-PCR. Further genetic characterization using DNA sequencing revealed wide nucleotide diversity among the human influenza A viruses isolated.

3. All of the influenza A viruses isolated were highly similar (99%) to other strains already detected worldwide in 2008.

4. A unique strain, A/UgaKay/027/H3N2, that had a significant amino acid change, T661 and which has been previously reported to be associated with increased human transmission, was identified.

6.2 Recommendations

1. It is recommended that HA immune responses are investigated for the strains detected in Uganda as well as those detected earlier from the northern hemisphere, in order to determine whether there is cross protection of the northern hemisphere vaccines for the Ugandan strains detected in the study. The relevance of this study is based on the finding that the strains in the northern hemisphere were found to be highly similar to those circulating in the southern hemisphere at the same time.

2. The samples that were negative for influenza A viruses should be screened for influenza B virus and other respiratory infections.

3. Further studies on the unique strain, A/UgaKay/027/H3N2, should be carried out.
REFERENCES


CDC. (1999) Morbidity and Mortality Weekly Report. Recommendations and Reports. 48(RR14);1-9 Division of Viral and Rickettsial Diseases, National Center for Infectious Disease, Neuraminidase Inhibitors for Treatment of Influenza A and B Infections


CDC. (2007) CDC Recommends against the Use of Amantadine and Rimantadine for the Treatment or Prophylaxis of Influenza in the United States during the 2005-06 Influenza


Hall, C.B. (2007) "The spread of influenza and other respiratory viruses: complexities and conjectures" Clinical Infectious Diseases;45 (3)353-9


105


Lewis, B. D. (2006) Avian Flu To Human Influenza Annual Review of Medicine, (57) 139–54


Change in the Hemagglutinin of the 1918 Influenza Virus Abolishes Transmission Science, 315(5812) 655-9.


Zambon, M.C. (1999) "Epidemiology and Pathogenesis Of Influenza". Journal of Antimicrobial Chemotherapy (44) supplementary 2, 3–9


APPENDICES

APPENDIX I  GLOSSARY OF TERMS

Adaptation is an evolutionary process during which a population becomes better suited to its habitat may refer to a feature important for an organism’s survival.

Antigenic variation is the process by which a given organism alters its surface glycoproteins in order to evade a given host’s immune response

Clade is a group of organisms within a species, whose members share homologous features derived from a common ancestor

Convergent evolution is the acquisition of the same biological trait in unrelated lineages

Cross-sectional study is epidemiological observational and analytical study of individual groups differing on the basis of specified criteria (for example, age) at the same point in time. It measures the prevalence of health outcomes or determinants of health or both in a point in time or over a short time

Cytopathic effect refers to degenerative changes in cells (especially in tissue culture) associated with the multiplication of certain viruses

Divergent evolution is the accumulation of differences between groups which can lead to the formation of new species, usually a result of diffusion of the same species to different and isolated environments which blocks the gene flow among the distinct populations allowing differentiated fixation of characteristics through genetic drift and natural selection

DNA sequencing is the process of determining the nucleotide order of a given DNA fragment

Endemic is the constant presence of a disease or infectious agent within a given population group or geographical area without the need for external inputs.

Epidemic occurs when new cases of a certain disease, in a given human population, and during a given period, substantially exceed what is expected based on recent experience

Human influenza surveillance. This is the continuous monitoring of influenza viruses within a given host population for the fast detection of their presence, typing of the viral strains and keeping a close watch on their evolution for the identification of antigenic changes in the circulating strains of

Interspecies transmission. Transmission of disease infectious agents from one host organism to another across a species barrier

Intraspecies transmission. Transmission of disease infectious agents from one host organism to another of the same species

Virus isolate is a population of viruses in pure culture derived from a colony of viruses and/or mixture of other organisms in a culture media, in which the original source of isolate was inoculated, and characterized by identification to the species level.
**Molecular epidemiology** is a branch of medical science that focuses on the contribution of potential genetic and environmental risk factors, identified at the molecular level, to the etiology, distribution and prevention of disease within families and across populations.

**Nucleotide diversity** is a concept in molecular genetics which is used to measure the degree of polymorphism within a population. It is a measure of genetic variation.

**Pandemic** is an epidemic of infectious disease that is spreading through human populations across a large region; for instance a continent, or even worldwide.

**Parallel evolution** is the development of a similar trait in related, but distinct, species descending from the same ancestor, but from different clades.

**Pathogenicity** is the ability of a pathogen to produce an infectious disease in an organism.

**Phylogenetics** is the study of evolutionary relatedness among various groups of organisms.

**Phylogeny** is the study of evolutionary relatedness among various groups of organisms (for example, species or populations), which is discovered through molecular sequencing data and morphological data matrices.

**Prevalence** is the total number of cases of a disease in a given population at a specific time.

**Reference strain** is a well characterized strain preserved for, and included in further studies for comparison purposes.

**Serotype** refers to a group of organisms, microorganisms, or cells distinguished by their shared specific antigens as determined by serologic testing.

**Strain** is an isolate or group of isolates exhibiting specific phenotypic and/or genotypic traits which are distinctive from those of other isolates of the same species.

**Subclade** is a subgroup of members with characteristics different from other members within a clade.

**Transversion** refers to the substitution of a purine for a pyrimidine or vice versa.

**Transition** is a point mutation that changes a purine nucleotide to another purine ($A \leftrightarrow G$) or a pyrimidine nucleotide to another pyrimidine ($C \leftrightarrow T$).

**Virulence** is the measure of the ability of a pathogen to damage its host.

**Virus isolation** is the process of growing viruses in a specific cell culture for purposes of identifying viruses missed by other tests as well as other respiratory viruses, and for the multiplication of viruses for further subsequent tests.
## APPENDIX II  TABLE SHOWING INFLUENZA A VIRUS SCREENING AND CHARACTERIZATION RESULTS BY DATE, SITE, GENDER AND AGE

<table>
<thead>
<tr>
<th>No.</th>
<th>Lab No.</th>
<th>Collection date</th>
<th>Site</th>
<th>Field Number</th>
<th>Patient Information</th>
<th>Influenza A Screening Results</th>
<th>Virus Subtyping by RT-PCR</th>
<th>Strain ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2008100020</td>
<td>02-Oct-08</td>
<td>Mulago Hospital</td>
<td>155265-0001</td>
<td>M 8Mths</td>
<td>+ + +</td>
<td>H3N2</td>
<td>A/UgaMul/001/08(H3N2)</td>
</tr>
<tr>
<td>2</td>
<td>2009100000</td>
<td>13-Oct-08</td>
<td>Kayunga Hospital</td>
<td>141993-001T</td>
<td>M 2Yrs</td>
<td>+ + +</td>
<td>H3N2</td>
<td>A/ UgaKay/002/08(H3N2)</td>
</tr>
<tr>
<td>3</td>
<td>2009100004</td>
<td>13-Oct-08</td>
<td>Mulago Hospital</td>
<td>142236-001T</td>
<td>F 4Yrs</td>
<td>+ + +</td>
<td>H3N2</td>
<td>A/ UgaMul/003/08(H3N2)</td>
</tr>
<tr>
<td>4</td>
<td>2009100033</td>
<td>16-Oct-08</td>
<td>Mulago Hospital</td>
<td>155146-0001</td>
<td>F 18Mths</td>
<td>+ + +</td>
<td>H3N2</td>
<td>A/ UgaMul/005/08(H3N2)</td>
</tr>
<tr>
<td>5</td>
<td>2009100042</td>
<td>17-Oct-08</td>
<td>Mulago Hospital</td>
<td>143209-0001</td>
<td>F 21Yrs</td>
<td>+ + +</td>
<td>H3N2</td>
<td>A/ UgaMul/006/08(H3N2)</td>
</tr>
<tr>
<td>6</td>
<td>2009100049</td>
<td>20-Oct-08</td>
<td>Mulago Hospital</td>
<td>142982-0001</td>
<td>F 1Yr</td>
<td>+ + +</td>
<td>H3N2</td>
<td>A/ UgaMul/007/08(H3N2)</td>
</tr>
<tr>
<td>7</td>
<td>2009100051</td>
<td>20-Oct-08</td>
<td>Mulago Hospital</td>
<td>143193-0001</td>
<td>F 21Yrs</td>
<td>+ + +</td>
<td>H3N2</td>
<td>A/ UgaMul/008/08(H3N2)</td>
</tr>
<tr>
<td>8</td>
<td>2009100054</td>
<td>21-Oct-08</td>
<td>Mulago Hospital</td>
<td>143122-0001</td>
<td>M 1Yr</td>
<td>+ + +</td>
<td>H3N2</td>
<td>A/ UgaMul/009/08(H3N2)</td>
</tr>
<tr>
<td>9</td>
<td>2009100094</td>
<td>27-Oct-08</td>
<td>Mulago Hospital</td>
<td>177582-0001</td>
<td>M 2Yrs</td>
<td>+ + +</td>
<td>H3N2</td>
<td>A/ UgaMul/010/08(H3N2)</td>
</tr>
<tr>
<td>10</td>
<td>2009100096</td>
<td>28-Oct-08</td>
<td>Mulago Hospital</td>
<td>141485-0001</td>
<td>M 2Yrs</td>
<td>+ + +</td>
<td>H3N2</td>
<td>A/ UgaMul/011/08(H3N2)</td>
</tr>
<tr>
<td>11</td>
<td>2009100102</td>
<td>28-Oct-08</td>
<td>Mulago Hospital</td>
<td>143900-0001</td>
<td>F 3Yrs</td>
<td>+ + +</td>
<td>H3N2</td>
<td>A/ UgaMul/012/08(H3N2)</td>
</tr>
<tr>
<td>12</td>
<td>2009100131</td>
<td>31-Oct-08</td>
<td>Mulago Hospital</td>
<td>177357-0001</td>
<td>M 3Yrs</td>
<td>+ + +</td>
<td>H3N2</td>
<td>A/ UgaMul/013/08(H3N2)</td>
</tr>
<tr>
<td>13</td>
<td>2009100132</td>
<td>31-Oct-08</td>
<td>Mulago Hospital</td>
<td>177399-0001</td>
<td>M 2Yrs</td>
<td>+ + +</td>
<td>H3N2</td>
<td>A/ UgaMul/014/08(H3N2)</td>
</tr>
<tr>
<td>14</td>
<td>2009100133</td>
<td>31-Oct-08</td>
<td>Mulago Hospital</td>
<td>177405-0001</td>
<td>M 12Yrs</td>
<td>+ + +</td>
<td>H3N2</td>
<td>A/ UgaMul/015/08(H3N2)</td>
</tr>
<tr>
<td>15</td>
<td>2009100158</td>
<td>06-Nov-08</td>
<td>Mulago Hospital</td>
<td>144333-0001</td>
<td>M 7Mths</td>
<td>+ + +</td>
<td>H3N2</td>
<td>A/ UgaMul/016/08(H3N2)</td>
</tr>
<tr>
<td>16</td>
<td>2009100161</td>
<td>06-Nov-08</td>
<td>Mulago Hospital</td>
<td>144481-0001</td>
<td>F 2Yrs</td>
<td>+ + +</td>
<td>H3N2</td>
<td>A/ UgaMul/017/08(H3N2)</td>
</tr>
<tr>
<td>17</td>
<td>2009100166</td>
<td>07-Nov-08</td>
<td>Mulago Hospital</td>
<td>144423-0001</td>
<td>M 2Yrs</td>
<td>+ + +</td>
<td>H3N2</td>
<td>A/ UgaMul/018/08(H3N2)</td>
</tr>
<tr>
<td>18</td>
<td>2009100167</td>
<td>07-Nov-08</td>
<td>Mulago Hospital</td>
<td>144436-0001</td>
<td>F 1Yr</td>
<td>+ + +</td>
<td>H3N2</td>
<td>A/ UgaMul/019/08(H3N2)</td>
</tr>
<tr>
<td>19</td>
<td>2009100170</td>
<td>10-Nov-08</td>
<td>Mulago Hospital</td>
<td>144375-0001</td>
<td>M 2Yrs</td>
<td>+ + +</td>
<td>H3N2</td>
<td>A/ UgaMul/020/08(H3N2)</td>
</tr>
<tr>
<td>20</td>
<td>2009100171</td>
<td>10-Nov-08</td>
<td>Mulago Hospital</td>
<td>144452-0001</td>
<td>F 1Yr</td>
<td>+ + +</td>
<td>H3N2</td>
<td>A/ UgaMul/021/08(H3N2)</td>
</tr>
<tr>
<td>21</td>
<td>2009100173</td>
<td>10-Nov-08</td>
<td>Mulago Hospital</td>
<td>144494-0001</td>
<td>M 2Yrs</td>
<td>+ + +</td>
<td>H3N2</td>
<td>A/ UgaMul/022/08(H3N2)</td>
</tr>
<tr>
<td>22</td>
<td>2009100207</td>
<td>17-Nov-08</td>
<td>Mulago Hospital</td>
<td>139445-0001</td>
<td>M 5Yrs</td>
<td>+ + +</td>
<td>H3N2</td>
<td>A/ UgaMul/023/08(H3N2)</td>
</tr>
<tr>
<td>23</td>
<td>2009100209</td>
<td>17-Nov-08</td>
<td>Mulago Hospital</td>
<td>144285-0001</td>
<td>M 2Yrs</td>
<td>+ + +</td>
<td>H3N2</td>
<td>A/ UgaMul/024/08(H3N2)</td>
</tr>
<tr>
<td>24</td>
<td>2009100216</td>
<td>18-Nov-08</td>
<td>Mulago Hospital</td>
<td>145502-0001</td>
<td>M 3Yrs</td>
<td>+ + +</td>
<td>H3N2</td>
<td>A/ UgaMul/025/08(H3N2)</td>
</tr>
<tr>
<td>25</td>
<td>2009100217</td>
<td>18-Nov-08</td>
<td>Mulago Hospital</td>
<td>145531-0001</td>
<td>F 25Mths</td>
<td>+ + +</td>
<td>H3N2</td>
<td>A/ UgaMul/026/08(H3N2)</td>
</tr>
<tr>
<td>26</td>
<td>2009100224</td>
<td>19-Nov-08</td>
<td>Kayunga Hospital</td>
<td>166708-0001</td>
<td>F 20Yrs</td>
<td>+ + +</td>
<td>H3N2</td>
<td>A/ UgaKay/027/08(H3N2)</td>
</tr>
<tr>
<td>27</td>
<td>2009100225</td>
<td>19-Nov-08</td>
<td>Kayunga Hospital</td>
<td>166711-0001</td>
<td>F 2Yrs</td>
<td>+ + +</td>
<td>H3N2</td>
<td>A/ UgaKay/028/08(H3N2)</td>
</tr>
<tr>
<td>28</td>
<td>2009100227</td>
<td>20-Nov-08</td>
<td>Kayunga Hospital</td>
<td>144915-0001</td>
<td>F 38Yrs</td>
<td>+ + +</td>
<td>H3N2</td>
<td>A/ UgaKay/029/08(H3N2)</td>
</tr>
<tr>
<td>29</td>
<td>2009100228</td>
<td>20-Nov-08</td>
<td>Mulago Hospital</td>
<td>145351-0001</td>
<td>M 2Yrs</td>
<td>+ + +</td>
<td>H3N2</td>
<td>A/ UgaMul/030/08(H3N2)</td>
</tr>
<tr>
<td>30</td>
<td>2009100231</td>
<td>20-Nov-08</td>
<td>Mulago Hospital</td>
<td>145573-0001</td>
<td>M 4Yrs</td>
<td>+ + +</td>
<td>H3N2</td>
<td>A/ UgaMul/031/08(H3N2)</td>
</tr>
<tr>
<td></td>
<td>Date</td>
<td>Age</td>
<td>Hospital</td>
<td>Gender</td>
<td>Clinic Code</td>
<td>Disease</td>
<td></td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>------------</td>
<td>------</td>
<td>--------------</td>
<td>--------</td>
<td>-------------</td>
<td>----------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>31</td>
<td>2009100232</td>
<td>20-Nov -08</td>
<td>Mulago Hospital</td>
<td>F</td>
<td>145634-0001</td>
<td>16Mths + + + H3N2 A/ UgaMul/032/08(H3N2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>32</td>
<td>2009100233</td>
<td>21-Nov -08</td>
<td>Mulago Hospital</td>
<td>F</td>
<td>139522-0001</td>
<td>3Yrs + + + H3N2 A/ UgaMul/033/08(H3N2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>33</td>
<td>2009100235</td>
<td>21-Nov -08</td>
<td>Mulago Hospital</td>
<td>F</td>
<td>145393-0001</td>
<td>3Yrs + + + H3N2 A/ UgaMul/034/08(H3N2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>34</td>
<td>2009100237</td>
<td>24-Nov -08</td>
<td>Mulago Hospital</td>
<td>F</td>
<td>139461-0001</td>
<td>3Yrs + + + H3N2 A/ UgaMul/035/08(H3N2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>35</td>
<td>2009100238</td>
<td>24-Nov -08</td>
<td>Mulago Hospital</td>
<td>F</td>
<td>139487-0001</td>
<td>26Mths + + + H3N2 A/ UgaMul/036/08(H3N2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>36</td>
<td>2009100243</td>
<td>24-Nov -08</td>
<td>Kayunga Hospital</td>
<td>F</td>
<td>146160-0001</td>
<td>19Yrs + + + H3N2 A/ UgaKay/037/08(H3N2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>37</td>
<td>2009100246</td>
<td>25-Nov -08</td>
<td>Mulago Hospital</td>
<td>F</td>
<td>144269-0001</td>
<td>3Yrs + + + H3N2 A/ UgaMul/038/08(H3N2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>38</td>
<td>2009100247</td>
<td>25-Nov -08</td>
<td>Mulago Hospital</td>
<td>F</td>
<td>144854-0001</td>
<td>2Yrs + + + H3N2 A/ UgaMul/039/08(H3N2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>39</td>
<td>2009100248</td>
<td>25-Nov -08</td>
<td>Kayunga Hospital</td>
<td>F</td>
<td>144902-0001</td>
<td>19Yrs + + + H3N2 A/ UgaKay/040/08(H3N2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>2009100250</td>
<td>25-Nov -08</td>
<td>Mulago Hospital</td>
<td>F</td>
<td>145599-0001</td>
<td>10Mths + + + H3N2 A/ UgaMul/041/08(H3N2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>41</td>
<td>2009100260</td>
<td>26-Nov -08</td>
<td>Mulago Hospital</td>
<td>M</td>
<td>146247-0001</td>
<td>3Yrs + + + H3N2 A/ UgaMul/042/08(H3N2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>42</td>
<td>2009100265</td>
<td>27-Nov -08</td>
<td>Mulago Hospital</td>
<td>M</td>
<td>172357-0001</td>
<td>7Yrs + + + H3N2 A/ UgaMul/043/08(H3N2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>43</td>
<td>2009100268</td>
<td>28-Nov -08</td>
<td>Mulago Hospital</td>
<td>M</td>
<td>146218-0001</td>
<td>1Yr + + + H3N2 A/ UgaMul/044/08(H3N2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>44</td>
<td>2009100269</td>
<td>28-Nov -08</td>
<td>Mulago Hospital</td>
<td>M</td>
<td>172344-0001</td>
<td>6Yrs + + + H3N2 A/ UgaMul/045/08(H3N2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>45</td>
<td>2009100299</td>
<td>03-Dec -08</td>
<td>Mulago Hospital</td>
<td>M</td>
<td>146829-0001</td>
<td>30Mths + + + H3N2 A/ UgaMul/046/08(H3N2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>46</td>
<td>2009100300</td>
<td>03-Dec -08</td>
<td>Mulago Hospital</td>
<td>M</td>
<td>146832-0001</td>
<td>4Yrs + + + H3N2 A/ UgaMul/047/08(H3N2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>47</td>
<td>2009100309</td>
<td>04-Dec -08</td>
<td>Mulago Hospital</td>
<td>M</td>
<td>146948-0001</td>
<td>17Mths + + + H3N2 A/ UgaMul/048/08(H3N2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>48</td>
<td>2009100318</td>
<td>09-Dec -08</td>
<td>Mulago Hospital</td>
<td>M</td>
<td>147027-0001</td>
<td>5Yrs + + + H3N2 A/ UgaMul/049/08(H3N2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>49</td>
<td>2009100330</td>
<td>11-Dec -08</td>
<td>Mulago Hospital</td>
<td>M</td>
<td>146591-0001</td>
<td>3Yrs + + + H3N2 A/ UgaMul/050/08(H3N2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>2009100332</td>
<td>11-Dec -08</td>
<td>Mulago Hospital</td>
<td>M</td>
<td>147416-0001</td>
<td>3Yrs + + + H3N2 A/ UgaMul/051/08(H3N2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>51</td>
<td>2009100335</td>
<td>12-Dec -08</td>
<td>Mulago Hospital</td>
<td>M</td>
<td>147429-0001</td>
<td>26Mths + + + H3N2 A/ UgaMul/052/08(H3N2)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>