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Research Article

Evidence in Kenya of Reassortment Between Seasonal Influenza A(H3N2) and Influenza A(H1N1)pdm09 to yield A(H3N2) Variants With the Matrix Gene Segment of A(H1N1)pdm09

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Background: Influenza viruses evolve rapidly and undergo frequent reassortment of different gene segments leading to emergence of novel strains with new traits possessing pandemic potential.

Objectives: To determine evidence of reassortment amongst A(H1N1)pdm09 and H3N2 co-circulating influenza virus subtypes and relate these to adamantine antiviral resistance.

Methodology: Nasopharyngeal swabs in virus transport medium were collected from patients with influenza-like illness. The presence of influenza was determined using real-time PCR followed by culture in MDCK cells. Haemagglutination inhibition was carried out to confirm the identity of the virus. Complete haemagglutinin (HA), matrix (M) and neuraminidase (NA) genes were sequenced and analyzed using a suite of bioinformatics tools.

Results: Influenza A(H3N2) was detected in 32 out of 708 samples collected between October and December 2010. Analysis of the HA gene confirmed it to be of the H3 subtype. However, analysis of the matrix gene showed that 28 of the isolates had the M gene of influenza A(H3N2) viruses while 4 had the M gene of the A(H1N1)pdm09 viruses.

Discussion: Our results show that four of the 32 influenza A(H3N2) viruses isolated had acquired the M gene segment of the A(H1N1)pdm09 virus by reassortment. This has implications in their transmissibility as the M gene is implicated in the increased transmissibility of the A(H1N1)pdm09 viruses.

Key words: Kenya; Influenza A(H3N2); reassortment; Matrix gene

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1. Introduction

Influenza vaccines and antiviral drugs are the mainstay for preventing influenza and reducing the impact of influenza epidemics. Currently, there are two classes of antiviral drugs available for preventing and treating influenza illness: M2 (matrix 2) ion channel blockers, (adamantanes: amantadine and rimantadine), and neuraminidase inhibitors (oseltamivir, zanamivir and peramivir) (Hill et al, 2009). Adamantanes inhibit viral replication by blocking the acid-activated ion channel formed by the virion-associated M2 protein encoded by

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the M gene. The neuraminidase inhibitors (NAI's) interrupt the replication cycle by preventing virus release and allowing progeny virus to clump (Monto et al, 2002). The rapid emergence of adamantine drug resistant influenza A virus strains has limited these drugs' clinical effectiveness.

The origin and evolution of antiviral drug resistance amongst influenza viruses can occur through different molecular mechanisms that also drive the evolution of the virus. The most crucial of these mechanisms result from the segmented nature of its genome. This permits the formation of new progeny viruses with novel combinations of segments through reassortment when two or more different virus subtypes infect a single cell, a phenomenon referred to as antigenic shift. This process is capable of introducing new genes in circulating viral populations that can drastically change the biological properties of the virus. Studies have shown that reassortment led to an increase in the frequency of amantadine-resistant seasonal influenza A(H1N1) viruses since the 2005-2006 season (Yang et al, 2011). This underscores the necessity to monitor genome dynamics in circulating influenza viruses because it is through such molecular surveillance that we are able to understand the evolution and mechanisms of the emergence and spread of antiviral resistance among influenza A viruses (Boni et al, 2010).

Thus, we set out to qualitatively analyze human influenza A(H3N2) viruses that circulated in Kenya in 2010, the period when influenza A(H3N2) and A(H1N1)pdm09 [previously referred to as swine flu] (WHO, 2011a) begun to co-circulate in the human population in the country, determine evidence of reassortment amongst the co-circulating subtypes and relate any such events to influenza antiviral resistance in the country. We applied the current laboratory testing protocol which involves routinely sequencing the HA, M and NA gene segments of the influenza viruses. These three gene segments were selected because they are the main antigens (NA & HA) and drug targets (M & NA) of the influenza A virus. Herein we provide evidence that indeed there was reassortment involving at least the M gene segment amongst co-circulating influenza A viruses in Kenya during this period.

2. Methods

2.1 Setting

Surveillance for patients with influenza-like illness (ILI) (defined as fever of ≥38 °C and either cough or sore throat) was conducted at one provincial hospital in Kisumu city in western Kenya, as well as in seven district hospitals in the cities of Nairobi & Mombasa and the towns of Malindi, Isiolo, Kericho, Kisii and Alupe.

2.2 Study Population

ILI study patients included anyone over two months of age presenting to a surveillance hospital outpatient clinic. We excluded presentations of ILI in patients with exudative pharyngitis or tonsillitis or symptom onset >72 hours prior to presentation. Surveillance officers enrolled a maximum of five ILI case-patients presenting to the each surveillance hospital daily.

2.3 Specimen collection

Surveillance officers collected duplicate nasopharyngeal (NP) samples from each ILI case-patient using Dacron flocked swabs. Swabs were placed in a 1 ml cryovial containing virus transport medium, kept at 4 °C, and stored in a liquid nitrogen dry shipper within eight hours of collection. All samples were transported from the surveillance sites to the National Influenza Center (NIC) laboratory within one week. The cold chain was maintained throughout.

2.4 Laboratory testing

Virus Isolation:

Influenza virus isolations were performed at KEMRI Influenza Laboratory in Nairobi, (designated Kenyan NIC) in Madin-Darby canine kidney (MDCK) cells followed by haemagglutination inhibition assay (HAI) using guinea pig red blood cells and reference antiserum in accordance with Centers for Disease Control and Prevention (CDC) protocols (WHO, 2011b). Inoculated cells were incubated at 37 °C with 5% CO_2 and observed daily for 10 days for visual cytopathic effect (CPE) using an inverted microscope. When CPE was observed, the supernatant fluid was collected and the haemagglutination titer measured. HAI testing was carried out on high titer samples. Isolates with low haemagglutination titer were re-passaged once.

RNA extraction

RNA extraction from clinical samples and isolates was performed using the viral RNA mini kit (Qiagen, Germany) according to the manufacturer's instructions.

Real-time RT-PCR

One step real-time reverse trancriptase PCR (RT-PCR) was performed on an ABI 7500 Fast platform using the CDC reagents (primers & probes) and protocol for the identification of influenza A viruses (CDC, 2009). The total reaction volumes were 25 µl, containing 0.5 µl superscript III/Platinum enzyme mix (Invitrogen, UK), 5.5 µl H_2O, 12.5 µl 2X buffer, 0.5 µl forward primer (10 µM), 0.5 µl reverse primer (10 µM), 0.5 µl probe (5 µM) and 5 µl of RNA template. Each RNA sample was tested for four sets of genes namely: matrix protein gene segment for the identification of influenza A viruses, nucleoprotein gene segment for the identification of swine A influenza viruses, haemagglutinin gene segment for the subtyping of swine A(H1) & human A(H3) viruses and lastly human ribonuclease P (RNP) gene segment was amplified to test the RNA extraction procedure. Reverse transcription was achieved at 50 °C for 30 min and 95 °C. PCR was achieved after 45 cycles of 95 °C for 15 sec and 55 °C for 30 sec.

Conventional RT-PCR

To study the genetic diversity of the isolated strains, full-length amplification of the HA and M genes of the strains was performed by conventional RT-PCR. Briefly, RNA from positive samples was reverse transcribed and

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HA and M gene amplification achieved with a one step RT-PCR using the Thermoscript one step RT-PCR kit (Qiagen, Germany). The reaction mixture contained 3 µl RNA, 12.5 µl 2X reaction mix, 0.5 µl of each primer (20 µM) (available on request), 1 µl of Thermoscript/Taq mix and 7.5 µl of water for a total volume of a 25 µl reaction. Reverse transcription included 50 °C for 30 min and 94 °C for 2 min. PCR amplification included 35 cycles of 94 °C for 30 sec, 55 °C for 30 sec and 68 °C for 60 sec. Amplicons were viewed under UV light after gel electrophoresis in 2% agarose gel.

Table 1: Haemagglutination inhibition results of a proportion of the isolates from this study in comparison with the reference strains. The four viruses later shown in this study to be reassortants are shown in bold.

Reference antigens	**Reference antisera**			
	A/Perth/16/2009 (H3N2)	**A/California/7/09A (H1N1)pdm09**	**A/Brisbane/59/07 (H1N1)**	**B/Brisbane/60/ 2008**
A/Perth/16/2009 (H3N2)	**2560**	<20	<20	<20
A/California/7/09A(H1N1)pdm09	<20	**1280**	64	<20
A/Brisbane/59/07(H1N1)	<20	64	**1280**	<20
B/Brisbane/60/2008	<20	<20	<20	**640**
Samples				
A/Kenya/152/2010	1280	<20	<20	<20
A/Kenya/153/2010	640	<20	<20	<20
A/Kenya/201/2010	**2560**	**<20**	**<20**	**<20**
A/Kenya/206/2010	**1280**	**<20**	**<20**	**<20**
A/Kenya/209/2010	**1280**	**<20**	**<20**	**<20**
A/Kenya/157/2010	1280	<20	<20	<20
A/Kenya/158/2010	1280	<20	<20	<20
A/Kenya/214/2010	**640**	**<20**	**<20**	**<20**
A/Kenya/160/2010	2560	<20	<20	<20
A/Kenya/161/2010	1280	<20	<20	<20

Nucleotide sequencing and genetic analyses

Amplicons were treated with shrimp alkaline phosphatase–exonuclease I (ExoSapI) (U.S Biologicals, Swampscott, MA, USA) and sequenced directly using the 3500 XL Genetic Analyzer (Applied Biosystems). Nucleotide contigs were assembled using the DNA Baser program (SRL, 2011). Homology searches of the resultant sequences against GenBank database were conducted online using BLAST (Altschul et al, 1997) applying the default parameters. Multiple sequence alignments of the Kenyan isolates and reference strains were performed with MUSCLE version 3.6 (Edgar, 2004a; Edgar, 2004b) and visualized using GeneDoc Multiple Sequence Alignment Editor & Shading Utility version 2.6.002 (Nicholas and Nicholas, 1997) while phylogenetic analyses were performed with MrBayes version 3.1 (Ronquist and Huelsenbeck, 2003). HA nucleotide sequences of reference strains A/California/07/2009 (for A(H1N1)pdm09) and A/Perth/16/2009 (for H3N2) were included in the phylogenetic analysis. Amino acid sequences of the Kenyan isolates have been deposited in the Global Initiative on Sharing of Avian Influenza Data (GISAID) database under accession numbers EPI357522 – EPI357544, EPI356800 – EPI356891, EPI356900 – EPI356917, EPI356816 – EPI356897, EPI356907 – EPI356926 and EPI357524 – EPI357545.

2.5 Ethical considerations

The Kenya Medical Research Institute (KEMRI) and the Walter Reed Army Institute of Research (WRAIR) institutional review boards reviewed and approved the study protocol with approval numbers **SSC#981** and **WRAIR#1267** respectively. Informed consent was obtained from patients prior to study participation.

3. Results

Real-time RT-PCR showed that 32 out of a total number of 708 samples collected between October and December 2010 contained the H3 variant of the haemaglutinin gene segment. These produced virus-specific CPE and haemagglutination identified them as Influenza. Haemagglutination inhibition tests carried out on the haemagglutinating isolates using reference antisera were used to confirm the rRT-PCR results. The identity of isolates was confirmed as A/Perth/16/2007 (H3N2)-like viruses. A proportion of these HAI results are shown in **Table 1**.

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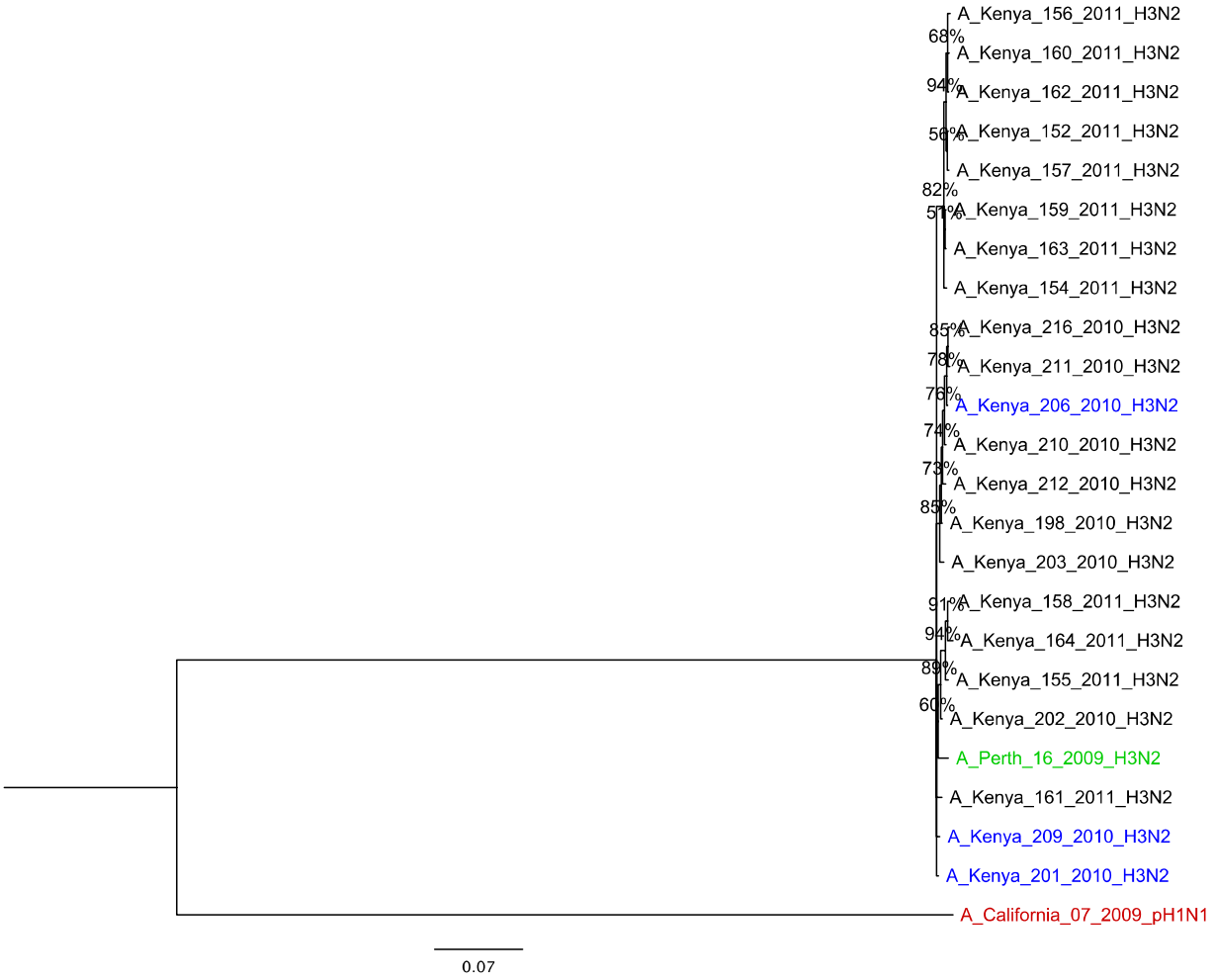

Figure 1: Phylogenetic relationship of the haemagglutinin genes of influenza viruses identified in this study compared two reference strains A/California/07/2009 (H1N1) and A/Perth/16/2009 (H3N2).

The tree was generated by Bayesian methods using MrBayes (Ronquist and Huelsenbeck, 2003).
The reference strains are A/California/07/2009 (for A(H1N1)pdm09) and A/Perth/16/2009 (for A(H3N2)); the reassortant A(H3N2) are A/Kenya/201/2010/H3N2 and A/Kenya/209/2010/H3N2.
The horizontal bar scale represents the number of nucleotide changes per 100 nucleotides. Bayesian posterior probabilities generated using MrBayes are indicated as percentages at the nodes.

Homology and phylogeny analysis of the HA nucleotide sequences of the 32 viruses showed that they all clustered together in a single clade together with the reference influenza A(H3N2) but separate from the influenza A(H1N1)pdm09 (**Figure 1**).

However, homology analysis of the M segment showed that 4 of the 32 isolates viz isolates A/Kenya/201/2010, A/Kenya/206/2010, A/Kenya/209/2010 and A/Kenya/214/2010 were homologous to the A(H1N1)pdm09 subtype. Phylogenetic analysis using the nucleotide sequence of the M gene segments from this study and A(H3N2) & A(H1N1)pdm09 reference strains showed that the four isolates clustered with the A(H1N1)pdm09 prototype strain [(A/California/07/2009(H1N1)] away from the A(H3N2) viruses (**Figure 2**).

In order to analyze amino acid changes associated with resistance to adamantanes, the nucleotide sequences were converted to protein code and a multiple sequence alignment of the matrix 2 proteins analyzed. This analysis included reference strains A/New York/32/2003 (H3N2) and /Virginia/04/2007 (H3N2) M2 proteins which are sensitive and resistant to adamantane drugs, respectively. The multiple protein sequence alignment showed that like A/Virginia/04/2007 (H3N2), all the isolates in this study had a mutation at position 31 where a serine amino acid present in A/New York/32/2003 (H3N2) was replaced by an asparagine. Thus because of the S31N amino acid change, all the Kenyan isolates were resistant to adamantanes (Grambas et al, 1992; Webster et al, 1992) (**Figure 3**).

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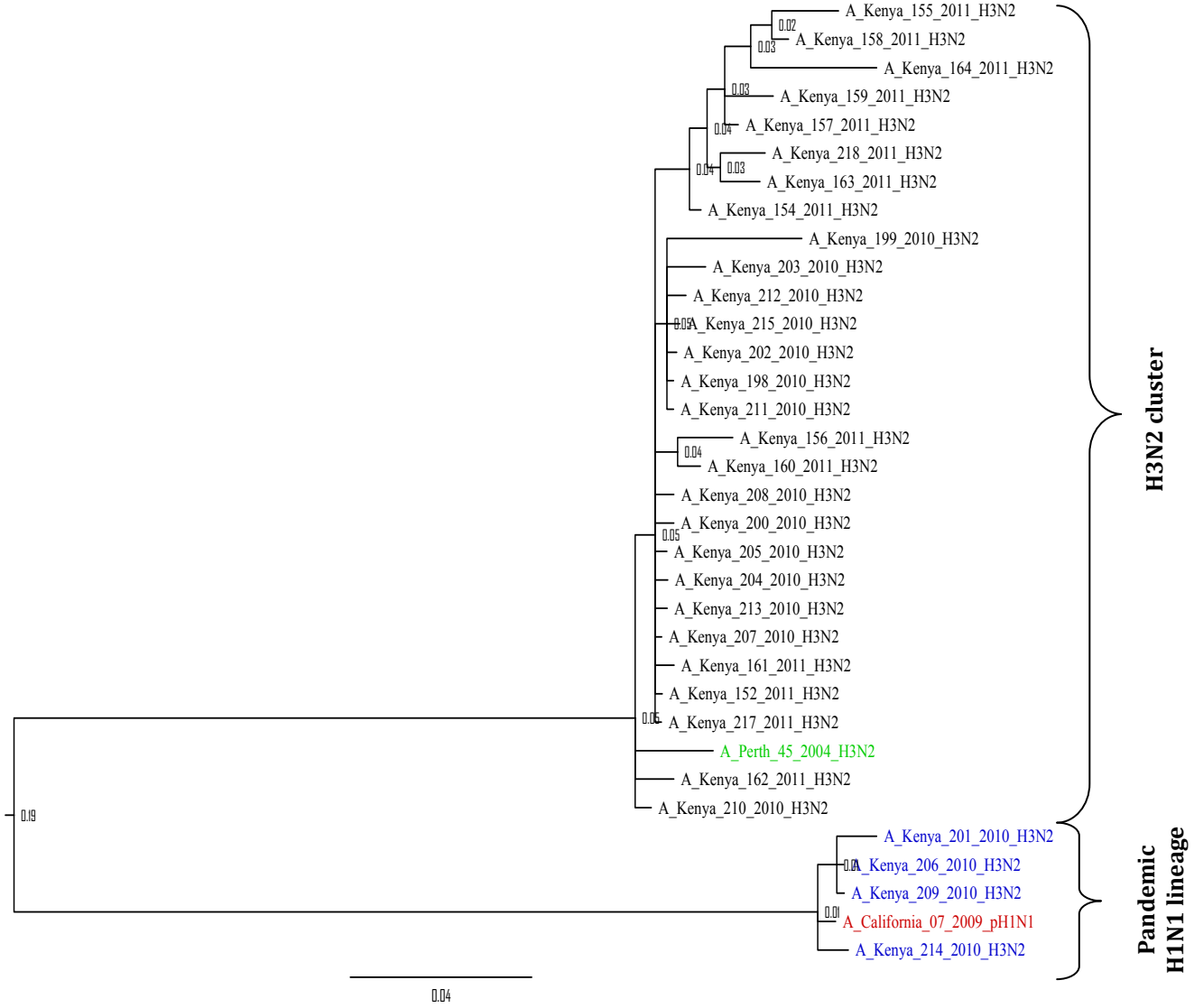

Figure 2: Phylogenetic relationship of the Matrix nucleotide-coding region of influenza viruses used in this study compared to two reference strains A/California/07/2009 (H1N1) and A/Perth/16/2009 (H3N2).

The tree was generated by Bayesian methods using MrBayes (Ronquist and Huelsenbeck, 2003).
The reference strains are A/California/07/2009 (for A(H1N1)pdm09) and A/Perth/45/2004 (for H3N2); the reassortant A(H3N2) are A/Kenya/201/2010/H3N2, A/Kenya/206/2010/H3N2, A/Kenya/209/2010/H3N2 and A/Kenya/214/2010/H3N2.
The horizontal bar scale represents the number of nucleotide changes per 100 nucleotides. Bayesian posterior probabilities generated using MrBayes are indicated as percentages at the nodes.

4. Discussion

This study isolated 32 influenza A viruses from a pool out of 708 patients presenting with influenza like illness in Kenya in 2010. These viruses were subtyped using real-time RT-PCR and shown to contain the H3 variant of the haemaglutinin gene segment. On further subtyping using the WHO reference ferret antisera, we discovered that all the viruses were of the A(H3N2) subtype. When the HA gene segment was sequenced and phylogenetically analyzed, all the 32 isolates clustered with other H3 genes including the A/Perth/16/2009 (H3N2), the 2010 vaccine reference strain. Thus, considered together, these results confirmed that the 32 isolates obtained were influenza A(H3N2) viruses. However, when we phylogenetically analyzed the matrix gene segments of the 32 isolates, twenty-eight were closely related to A/Perth/16/2009 (H3N2) vaccine reference strain. The other four isolates had matrix genes segments whose nucleotide sequences were very closely related to the matrix gene segment of the prototype A(H1N1)pdm09 virus, A/California/07/2009. Overall, these results show that the four influenza A(H3N2) viruses had acquired a M gene segment of the A(H1N1)pdm09 virus.

One important consequence of the segmented genome of influenza viruses is that the eight separate RNA

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segments can reassort when a single host cell is infected by two different viruses. Progeny viruses with novel antigenic and/or genetic features may be generated during the mixed infection. As described elsewhere, human A(H2N2) and A(H3N2) subtypes which emerged in the 1957 and 1968 pandemics respectively were generated by reassortment between human and avian influenza viruses(Xu et al, 2004). Thus our results show that four of the 32 (11%) patients who were ill with influenza had the etiology of a reassortant influenza A(H3N2) virus whose M gene segment was derived from influenza A(H1N1)pdm09 viruses.

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                                10        20        30        40        50        60        70        80        90
A/New_York/32/2003/H3N2 MSLLTEVETPIRNEWGCRCNDSSDPLVVAASIIGILHLILWILDRLFFKCVYRLFKHGLKRGPSTEGVPESMREEYRKEQQNAVDADDSHFVSIELE
A/Virginia/04/2007/H3N2 MSLLTEVETPIRNEWGCRCNDSSDPLVVAANIIGILHLILWILDRLFFKCVYRLFKHGLKRGPSTEGVPESMREEYRKEQQNAVDADDSHFVSIELE
A/Kenya/201/2010/H3N2   MSLVTEVEPPTRSEWECRCNDSSDPLVIAANIIGILHLILWITDRLFFKCIYRRFKYGLKRGPSTEGVPESMREEYQQEQQSAVDVDDGHFVNIELE
A/Kenya/206/2010/H3N2   MSLLTEVETPTRSEWECRCNDSSDPLVIAANIIGILHLILWITDRLFFKCIYRRFKYGLKRGPSTEGVPESMREEYQQEQQSAVDVDDGHFVNIELE
A/Kenya/209/2010/H3N2   MSLLTEVETPTRSEWECRCNDSSDPLVIAANIIGILHLILWITDRLFFKCIYRRFKYGLKRGPSTEGVPESMREEYQQEQQSAVDVDDGHFVNIELE
A/Kenya/214/2010/H3N2   MSLLTEVETPTRSEWECRCSDSSDPLVIAANIIGILHLILWITDRLFFKCIYRRFKYGLKRGPSTEGVPESMREEYQQEQQSAVDVDDGHFVNIELE
A/Kenya/199/2010/H3N2   MSLLTEVETPIRNEWGCRCNDSSDPLVVAANIIGILPLILWILDRLFFKCVYRLFKHGLKRGPSTEGVLFFLREEYRKEQQNAVDADDSHFVSIDFE
A/Kenya/203/2010/H3N2   MSLLTEVETPIRNEWGCRCNDSSEPLVVAANIIGILHLILWILDRLFFKCVYRLFKHGLKRGHSTEGVPESMREEYRKEQQNAVDADDSHFVSIELE
A/Kenya/200/2010/H3N2   MSLLTEVETPIRNEWGCRCNGSSDPLVVAANIIGILHLILWILDRLFFKCVYRLFKHGLKRGPSTEGVPESMREEYRKEQQNAVDADDSHFVSIELE
A/Kenya/198/2010/H3N2   MSLLTEVETPIRNEWGCRCNDSSDPLVVAANIIGILHLILWILDRLFFKCVYRLFKHGLKRGPSTEGVPESMREEYRKEQQNAVDADDSHFVSIELE
A/Kenya/202/2010/H3N2   MSLLTEVETPIRNEWGCRCNDSSDPLVVAANIIGILHLILWILDRLFFKCVYRLFKHGLKRGPSTEGVPESMREEYRKEQQNAVDADDSHFVSIELE
A/Kenya/204/2010/H3N2   MSLLTEVETPIRNEWGCRCNDSSDPLVVAANIIGILHLILWILDRLFFKCVYRLFKHGLKRGPSTEGVPESMREEYRKEQQNAVDADDSHFVSIELE
A/Kenya/205/2010/H3N2   MSLLTEVETPIRNEWGCRCNDSSDPLVVAANIIGILHLILWILDRLFFKCVYRLFKHGLKRGPSTEGVPESMREEYRKEQQNAVDADDSHFVSIELE
A/Kenya/207/2010/H3N2   MSLLTEVETPIRNEWGCRCNDSSDPLVVAANIIGILHLILWILDRLFFKCVYRLFKHGLKRGPSTEGVPESMREEYRKEQQNAVDADDSHFVSIELE
A/Kenya/208/2010/H3N2   MSLLTEVETPIRNEWGCRCNDSSDPLVVAANIIGILHLILWILDRLFFKCVYRLFKHGLKRGPSTEGVPESMREEYRKEQQNAVDADDSHFVSIELE
A/Kenya/210/2010/H3N2   MSLLTEVETPIRNEWGCRCNDSSDPLVVAANIIGILHLILWILDRLFFKCVYRLFKHGLKRGPSTEGVPESMREEYRKEQQNAVDADDSHFVSIELE
A/Kenya/211/2010/H3N2   MSLLTEVETPIRNEWGCRCNDSSDPLVVAANIIGILHLILWILDRLFFKCVYRLFKHGLKRGPSTEGVPESMREEYRKEQQNAVDADDSHFVSIELE
A/Kenya/212/2010/H3N2   MSLLTEVETPIRNEWGCRCNDSSDPLVVAANIIGILHLILWILDRLFFKCVYRLFKHGLKRGPSTEGVPESMREEYRKEQQNAVDADDSHFVSIELE
A/Kenya/213/2010/H3N2   MSLLTEVETPIRNEWGCRCNDSSDPLVVAANIIGILHLILWILDRLFFKCVYRLFKHGLKRGPSTEGVPESMREEYRKEQQNAVDADDSHFVSIELE
A/Kenya/215/2010/H3N2   MSLLTEVETPIRNEWGCRCNDSSDPLVVAANIIGILHLILWILDRLFFKCVYRLFKHGLKRGPSTEGVPESMREEYRKEQQNAVDADDSHFVSIELE
A/Kenya/216/2010/H3N2   MSLLTEVETPIRNEWGCRCNDSSDPLVVAANIIGILHLILWILDRLFFKCVYRLFKHGLKRGPSTEGVPESMREEYRKEQQNAVDADDSHFVSIELE
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Figure 3: Multiple Sequence alignment of the M2 proteins of the Kenyan isolates alongside two reference strains of known sensitivity to adamanatanes. A/NewYork/32/2003 is adamantane sensitive and A/Virginia/04/2007/H3N2 is adamantane resistant.

The alignment was performed using the default parameters of the Muscle version 3.8 (Edgar, 2004a; Edgar, 2004b) *software and visualized using GeneDoc* (Nicholas and Nicholas, 1997) *software.*

A hallmark of the A(H1N1)pdm09influenza virus is its efficient transmission in humans. A study to investigate the viral genetic determinants of the virus that confer its efficient transmission has implicated the M gene as the major determinant of transmissibility (Chou et al, 2011) and is thought to be involved in determining host tropism. As the 7th (second smallest) segment of the influenza virus, the M gene encodes 2 proteins. M1 is a matrix protein and M2 a membrane protein. The M2 protein, a product of a splice variation of the M gene, is largely responsible for uncoating the viral nucleoprotein during replication. Located in the viral envelope, M2 enables hydrogen ions to enter the viral particle (virion) from the endosome, thus lowering the pH inside of the virus, which causes dissociation of the viral matrix protein M1 from the ribonucleoprotein. This is a crucial step in uncoating of the virus and exposing its content to the cytoplasm of the host cell. Thus, M2 is an ideal target for anti-influenza drugs. Mutations in the M protein of influenza A viruses result in resistance to adamantane (amantidine and rimantidine) anti-influenza drugs (Webster et al, 1992).

While reassortment among influenza A viruses have been documented before, this is the first study that has documented the reassortment between A(H1N1)pdm09 and the seasonal human influenza A(H3N2) viruses. Some recent studies have reported the acquisition of the M segment of A(H1N1)pdm09 by swine influenza A(H3N2) viruses and the subsequent infection of humans by this reassortant (Nalluswami et al, 2011). We believe that the reassortant influenza A(H3N2) viruses reported in this study may be more efficiently transmitted in humans compared to the seasonal influenza A(H3N2) viruses due to the acquired M gene segment. However, these viruses may not have pandemic potential because they were spawned from influenza A(H3N2) and A(H1N1)pdm09, which are currently circulating in the human populations across the world and whose antigenic and virulence determinants are already familiar to the human immune system.

Analysis of M2 proteins of the reassortant viruses showed that similar to both parent influenza A viruses currently in circulation in Kenya [seasonal influenza A/H3N2 and A(H1N1)pdm09] the reassortants possess the S31N mutation that confers resistance to amantadine and rimantidine (Grambas et al, 1992; Webster et al, 1992). Furthermore, since the M segment acquired by these reassortants confers increased efficiency of transmission seen in A(H1N1)pdm09 viruses, we suspect that these reassortants should be better transmitted in the human population. Since these viruses have not been fully characterized, there is an urgent need to closely monitor their evolution and disease-potential dynamics. Owing to error-prone viral RNA polymerase activity, influenza virus genomes are subject to a very high rate of mutation (Webster et al, 1992). Emergence of mutations conferring virulence and drug resistance in a virus that is easily transmissible would be a serious global threat. There is therefore a need to closely monitor the genetic evolution of circulating influenza A viruses in order to monitor emerging variants with increased virulence and antiviral resistance patterns since these have the potential to become epidemic or pandemic strains.

This study has two major limitations. First, since influenza pathogenesis is polygenic and we were able to sequence only three of the eight genomic segments, the lack of genomic data of the other five gene segments of the reassortant viruses limits our understanding of the possibility of reassortment among these other gene segments. Secondly, we were not able to link the virus genetic data with the patients' clinical picture. This

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precludes any understanding of severity of the influenza disease due to these reassortant viruses. To mitigate the first shortcoming, our laboratory plans to carry out full nucleotide sequencing of the remaining gene segments to determine whether reassortments have occurred in them.

We conclude that influenza A(H3N2) have evolved in the country by reassorting with A(H1N1)pdm09 influenza viruses. Our findings thus reinforce the distinction of influenza viruses as both emerging and re-emerging pathogens that continue to pose a serious global health threat. We recommend that these reassortants be monitored closely in surveillance programs, particularly when changes in pathogenicity or transmission in humans become apparent.

Conflict of Interest declaration

The authors declare no conflict of interest

Disclaimer

The opinions stated in this paper are those of the authors and do not represent the official position of the U.S. Department of Defense.

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