<u>Research Article</u>

Molecular Surveillance of Adamantane Resistance among Human Influenza A Viruses Isolated in Four Epidemic Seasons in Kenya

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Background: Adamantanes impede influenza A virus replication and are important in the treatment and prophylaxis of disease caused by these viruses. Genotypic characterization of influenza A viruses for mutations associated with resistance to adamantanes has not been fully investigated in Kenya.

Objective: To characterize susceptibility of influenza A virus subtypes that circulated in Kenya from 2008-2011 to adamantanes.

Methods: Archived influenza A virus strains obtained from 2008 to 2011 were propagated in MDCK cells prior to sequencing of the matrix and hemagglutinin gene segments, followed by bioinformatics analyses.

Results: Ninety two virus strains consisting of 21 A/H3N2, 18 A/H1N1 and 53 A/H1N1pdm09 were analyzed. All A/H3N2 and A/H1N1pdm09 viruses displayed resistance to adamantanes due to the S31N/S31D amino acid substitution. All A/H1N1pdm09 virus strains belonged to the N-lineage characterized by S203T amino acid substitution in the HA1. All A/H1N1 viruses were sensitive to adamantane and were characterized by K140E amino acid substitution in the HA1.

Conclusion: All Kenyan influenza A/H3N2 and A/H1N1pdm09 virus strains were resistant to adamantanes while seasonal A/H1N1 strains were sensitive to these drugs. During the study period, Amantadine and Rimantadine were inappropriate for prophylaxis and treatment of influenza disease caused by A/H3N2 and A/H1N1pdm09 virus subtypes in Kenya.

Key words: Kenya, influenza A/H3N2, A/H1N1pdm09, A/H1N1, adamantanes

1. Introduction

Influenza type A viruses cause serious respiratory disease. The illness can begin as sub-clinical infection and graduate to primary viral pneumonia. It can cause death in humans and this is determined by several factors including age of the patient, immune status of the patients, and virulence of the influenza A virus strain. The elderly aged > 65 years and children aged < 2 years are at a greater risk of developing complications from influenza(Rothberg et al, 2008). The disease manifests with severe outcomes in patients whose immune system is compromised as well as pregnant women. Certain strains with mutations in key virulence factors of the virus also lead to a severe outcome of diseases. Genes encoding the HA, NA, and PB1 proteins, significantly contribute to replication efficiency and virulence (Taubenberger et al, 2012). According to the CDC, the global annual morbidity and mortality rates due to influenza epidemics stand at 3.5 million cases of severe

illness and 300,000 to 500,000 deaths (CDC, 2015). In addition, influenza A viruses are responsible for infrequent but severe pandemics which can affect greater than 50% of the population in a single year (Palese & Lowen, 2007).

The use of antiviral drugs to treat illness and reduce transmission of the virus is essential in the early phase of a pandemic (Monto, 2003, Monto, 2006, Stohr, 2003) because it buys time prior to production and availability of a vaccine against the pandemic strain in sufficient quantities for mass vaccination. In fact, it has been shown through simulation of an influenza outbreak in Southeast Asia, that it may be possible to contain an emerging pandemic at the start if appropriate, targeted antiviral prophylaxis is applied (Ferguson et al, 2005, Longini et al, 2004). Thus the need for new and constant evaluation of efficacy of existing antiviral drugs through surveillance of resistance to such drugs cannot be overstated.

Characterization of human influenza A subtypes illustrated the dominance of two particular strains: A/H1N1 and A/H3N2 in seasonal epidemics and occasional pandemics within human populations prior to 2009. In 2009 a novel strain, influenza A/H1N1pdm09 emerged and this strain has continued to co-circulate and has completely replaced seasonal influenza A/H1N1(Broor et al, 2012). Therefore, the 2008-2011 time period provided a distinctive period when for the first time three human influenza A virus subtypes co-circulated. Antivirals are vital in the management of influenza infections because unlike vaccines, they have a direct effect on the viral agent making them important tools for mitigating influenza disease outcomes as well as prophylaxis (Moscona, 2008)**.** Two main classes of antivirals have been used in the control of influenza: Adamantane-derived M2 ion channel blockers (amantadine and rimantadine) which are only effective against influenza A viruses and neuraminidase inhibitors mainly oseltamivir and zanamivir (Okomo-Adhiambo et al, 2010) which are efficacious against both influenza A and B subtypes. M2 ion channel blockers inhibit the uncoating of the virus through acidification of the interior of the virion (Holsinger et al, 1994). Currently there is a major concern regarding increasing resistance to antivirals. Regular use of antivirals has been shown to promote the development of antiviral resistance in the population due to positive selection (Garcia & Aris-Brosou, 2013). Antiviral resistance can also be acquired via transmission in which resistant strains replace susceptible strains in the absence of drug pressure. This transmission has been observed in the global spread of adamantine resistant A/H3N2 viruses since 2003 (Hayden & de Jong, 2011). These premises provide the necessity for a better understanding and characterization of antiviral resistance to circulating influenza A viruses. The discovery of antiviral resistance to both classes of antiviral agents posed a serious threat to the management of influenza viruses not only because treatment was being rendered ineffective, but also because it was discovered that this resistance was being transmitted to other regions, even where occurrences of the virus had not been documented (Hayden & de Jong, 2011).

Currently, there exists no data on antiviral drug susceptibility patterns within the sub-Saharan Africa. Genotypic characterization of mutations causing antiviral resistance in these regions has not been investigated fully. To bridge this gap, we characterized antiviral susceptibility of influenza A viruses to M2 blockers using known molecular markers and report on susceptibility of the three major human influenza A subtypes to these drugs in Kenya between the years 2008 and 2011, a unique period when these three distinct human influenza A subtypes co-circulated in the country.

2. Materials and Methods

2.1 Specimen collection and transportation

The influenza A isolates were derived from samples obtained from outpatients in district hospitals constituting the sentinel sites of the USAMRU-K Influenza Surveillance Network in Kenya. Samples were obtained by trained clinicians after the necessary consenting process. The case definition consisted of being outpatients of age ≥ 2 months, having a fever of 38ºC and above, with a cough or sore throat and presenting within 72 hrs after the onset of symptoms. Duplicate nasopharyngeal swabs were collected and placed in the viral transport media and stored at 2 – 8ºC for not more than 8 hours. For midterm storage, samples were snap-frozen in liquid nitrogen and transported to the central National Influenza Center laboratory in Nairobi for analysis and storage in the ultra-low freezer (-65 to -85ºC).

2.2 Diagnostic test for influenza A subtypes by real-time reverse transcription polymerase chain reaction

Virus RNA was extracted from patient samples using QIAamp Viral RNA extraction kit (Qiagen, Germany) following the manufacturer's protocol. Briefly, 100μl of the NP sample was added to 500μl of lysis buffer per tube and allowed to incubate at room temperature for 10 minutes to allow for the lysis. 500μl of ethanol was then added and pulse vortexed for 15 seconds resulting in a homogeneous lysate. 630μl of the lysate was applied to the spin columns and centrifuged at 595(x-g) for 1 minute and the column placed in a clean collection tube. 500μl of Buffer AW1 (wash buffer 1) was added to the spin column and centrifuged at 595(x-g) for 1 minute and the column placed in a clean collection tube. The column was then washed with 500μl of Buffer AW2 (wash buffer 2) and centrifuged at 1620 (x-g) for 3 minutes. The spin column was then placed in a 1.5 ml micro centrifuge tube and 60 μl of Buffer AVE (elution buffer) added to the column and allowed to incubate for 1 minute. The column was then centrifuged at 595 (x-g) for 1 minute and the filtrate (RNA) stored at -20⁰C.

Presence of influenza A virus in patient samples was carried out by real-time reverse transcription polymerase chain reaction (RRT-PCR) assay, utilizing the Matrix gene-specific Uni12 primer set and probe (Hoffmann et al, 2001). Preliminary detection of seasonal influenza A/HI, seasonal influenza A/H3 and pandemic influenza A/H1 from viral laden nasopharyngeal swabs were determined by RRT-PCR

assay on an ABI Prism 9700 thermocycler (Applied Biosystems, Foster City, CA USA). This was performed using subtype-specific primer sets for hemagglutinin genes of influenza A viruses. All primer sets and probes were developed by the Centers for Disease Control and Prevention (CDC), Atlanta.

2.3 Virus isolation and serotyping

Viruses were isolated from NP samples that tested positive for influenza A viruses. This was performed in Madin-Darby canine kidney (MDCK) cells obtained from American Type Cell Culture (Manassas, VA. USA). The MDCK cells were cultured in Dulbecco's Modified Eagle's Medium (Sigma Aldrich, St. Louis, USA) supplemented with 4.5g/L glucose and 10% Fetal Bovine Serum (FBS), 1% sodium pyruvate, 1% penicillin-streptomycin solution and 1% glutamine (Sigma Aldrich, St. Louis, USA). The cells were grown as monolayers in flat sided tissue culture tubes. 100 µl of each sample were inoculated onto 70-90% confluent MDCK cells in flat sided tubes after pre-treatment with TPCK trypsin (2mg/ml) (Sigma, Missouri, USA) in order to facilitate virus entry into the cells. Tubes were incubated with the caps loose in a tissue culture incubator at 37°C under 5% CO_2. Tubes were observed daily for 10 days for visual cytopathic effect (CPE) by light microscopy using an inverted microscope (Olympus, Tokyo, Japan). When the CPE was clear, supernatant fluid was collected and tested for hemagglutination titer, and when sufficient, hemagglutination inhibition (HAI) testing was conducted in accordance with CDC protocols using guinea pig red blood cells and 2008 - 2011 reference reagents for influenza virus diagnosis from a WHO kit provided by the Center for Disease Control (CDC), Atlanta.

2.4 Sequencing of the M and HA gene segments of Influenza A viruses

To identify key mutations associated with adamantine resistance, we used genotypic methods. Preliminary genotyping involved targeted amplification of matrix (M) and HA genes by standard PCR method using RNA extracted from the human influenza A/H3N2, influenza A/H1N1 and influenza A/H1N1pdm09 isolates. Extraction of RNA from isolates was performed as outlined earlier using QIAamp Viral RNA extraction kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's protocol.

Reverse transcriptase PCR (RT-PCR) was performed using Superscript III One-Step RT-PCR system (Invitrogen, Carlsbad, USA)) with primers specific for M and HA genes of human A/H1N1pdm09, H3N2 and H1N1 subtypes. The primer sequences were provided by CDC Atlanta. A single reaction consisted of 12.5µl of 2x reaction mix; 0.5µl each of both sense and antisense primers reconstituted to 20µM concentration; 1.0µl of Superscript III RT/Platinum Taq Mix and 3µl of RNA template in a 25µl reaction mixture. All the RT PCR reagents were sourced from Invitrogen, Carlsbad, USA.

Thermocycling conditions on the 9700 Fast ABI thermocycler included 1 cycle of reverse transcription at 50°C for 30 minutes followed by denaturation at 94°C for 30 seconds, primer annealing at 55°C for 30 seconds and

strand synthesis at 68°C for 1 minute for 35 cycles. This was followed by a final strand extension at 68°C for 1min. Purification of sequencing amplicons was performed using ExoSAP-IT (USB Corporation, town city). Nucleotide sequences of the PCR amplicons were determined using the BigDye Terminator cycle sequencing Kit (version 3.1) (Applied Biosystems, Foster City, USA). Purification of labeled amplicons was performed using Sephadex gel (Sigma Aldrich, St. Louis, USA) filtration prior to loading and running on the 3500xL Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Sequence assembly was carried out using DNA Baser tool (DNA Baser Sequence Assembler v4). Raw sequence curation and preliminary alignment of M and HA gene sequences was carried out using the BioEdit suite run under default parameters. All the sequences reported here have been deposited in the GenBank and GISAID databases under accession numbers:

GenBank database

KX431150 - KX431168; HQ214406; HQ214407; HQ214418; HQ214422; HQ214423; HQ214429; HQ214432; HQ214437; HQ214441; HQ214444; HQ214449; HQ214453 for the M genes

KX431169 - KX431203; HM347404-HM347412; HM347414; HM347417-HM347422; HM347424; HM347361-HM347380; HM347382-HM347392 for the HA genes.

GISAID database

EPI400061; EPI356798; EPI356813; EPI356816; EPI356870; EPI356873; EPI356876; EPI356889 EPI356897; EPI356907; EPI356915; EPI356926; EPI357513 for the HA genes

EPI400063; EPI356800; EPI356815; EPI356818; EPI356872; EPI356875; EPI356878; EPI356891; EPI356900;EPI356909; EPI356917; EPI356929; EPI357518 for the M genes.

2.5 Genetic characterization and phylogenetic analysis

The nucleotide sequences of the M genes were spliced to give the M2 gene segments. Selection of global M2 and HA sequences from Gene bank for inclusion in the alignments was accomplished using the basic local alignment search tool (BLAST) with the default parameters of the program (Altschul et al, 1997). Nucleotide sequences of the gene segments and selected sequences were translated into protein code using discovery studio gene (DS Gene) V1.5 (Accelrys Inc., Oxford, UK) applying the universal genetic code. To determine presence or absence of known molecular markers of antiviral susceptibility, protein multiple sequence alignments of M2 proteins were performed using the Multiple Sequence Comparison by Log-Expectation (MUSCLE) V3.8 (Edgar, 2004a, Edgar, 2004c). Aligned sequences were visualized using GeneDoc (Nicholas & Nicholas).

The phylogenetic reconstruction was carried out using the Bayesian method of tree inference which makes use of the MrBayes program (Ronquist & Huelsenbeck, 2003). Aligned protein data in the fasta format was converted into the Nexus format using the Concatenator

program (Pina-Martins & Paulo, 2008). The nexus file incorporating the GTR model code was executed in MrBayes by running 1000000 Monte Carlo Markov Chains (MCMC) with a sampling frequency of 1000 trees. Consensus trees were generated and visualized using FigTree V1.3.1 (Rambaut, 2009)

2.6 Ethical considerations

All ethical requirements were adhered to during specimen collection as outlined in the parent protocols for Walter Reed Army Institute of Research (**WRAIR protocol # 1267**) and Kenya Medical Research Institute (**KEMRI SSC #981**).

Specimen processing and analysis was carried out using the guidelines from WRAIR protocol # 1990 (sub-project 4) and KEMRI SSC # 2243.

3. Results

Susceptibility of influenza A viruses circulating in Kenya 2008-2011 to M2-inhibitors

From the peculiar study period of 2008-2011 when the three human influenza subtypes co-circulated in Kenya, we analyzed 92 M2 protein sequences of influenza A viruses consisting of 21 fragments for influenza A/H3N2, 18 for seasonal influenza A/H1N1 and 53 for influenza A/H1N1pdm09. All the M2 proteins of influenza A/H3N2 and influenza A/H1N1pdm09 virus subtypes analyzed possessed an S31N substitution (Fig. 1). One virus isolate of influenza A/H3N2 subtype designated KEN/164/2011 possessed 4 mutations indicated as L26I, V27S, A30C and S31D substitution. Furthermore a single influenza Mutation analyses for M2 protein sequences amongst the 18 seasonal influenza A/H1N1 viruses showed absence of all drug-specific substitutions (**Figure 1**).

Isolate strains	4	9	11	13	14	16	20	21	23	24	25	26	27	28	29	30	31	36	43	48	49	50	51	52	54	57	58	59	61	63	77	78	82	86	89	93	95
KEN 2012010H3	V	P	T	S	E	E	N	D	S	D	P	L	V	I	A	A	N	L	T	F	K	C	I	Y	R	Y	G	L	R	P	Q	Q	S	V	G	N	E
KEN 29 2011P	L	T	.	.	.	.	S	.	.	N	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	.	.
KEN 10 2011P	L	T	.	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	.	.
KEN 62 2009P	L	T	.	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	.	.
KEN 32 2011P	L	T	.	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	.	.
KEN 105 2009P	L	T	.	.	.	.	S	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
KEN 206 2010H3	L	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
KEN 209 2010H3	L	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Kenya 196 2011P	L	T	.	.	.	.	S	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
KEN 125 2009	L	T	.	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	H	.	.	.	.	.	.	.	.	.	.	.	.	.
KEN 102 2009P	L	T	.	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	H	.	.	.	.	.	.	.	.	.	.	.	.	.
A California 07 2009P	L	T	.	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
KEN 61 2009P	L	T	.	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
KEN 63 2010P	L	T	.	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
KEN 96 2009P	L	T	.	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
KEN 87 2009P	L	T	.	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
KEN 121 2009P	L	T	.	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
KEN 108 2009P	L	T	.	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
KEN 100 2009P	L	T	.	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
KEN 97 2009P	L	T	.	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
KEN 126 2009P	L	T	.	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
KEN 71 2009P	L	T	.	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
KEN 118 2009P	L	T	.	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A Solomon Islands 3 2006H1	L	T	I	N	.	G	.	.	.	.	.	.	.	V	.	.	S	V	I	S	.	S	.	.	I	H	.	.	G	.	R	E	N	A	.	S	.
KEN 22 2008H1	L	T	I	N	.	G	.	.	.	.	.	.	.	V	.	.	S	V	I	S	Q	S	.	.	I	H	.	.	.	.	R	E	N	A	D	S	.
KEN 21 2008H1	L	T	I	N	.	G	.	.	.	.	.	.	.	V	.	.	S	V	I	S	Q	S	.	.	I	H	.	.	.	.	R	E	N	A	D	S	.
KEN 23 2008H1	L	T	I	N	.	G	.	.	.	.	.	.	.	V	.	.	S	V	I	S	Q	S	.	.	I	H	.	.	.	.	R	E	N	A	D	S	.
KEN 08 2009H1	L	T	I	N	G	G	.	.	.	.	.	.	.	V	.	.	S	V	I	S	.	S	.	.	I	H	.	.	.	.	R	E	N	A	D	S	.
A Brisbane 59 2007H1	L	T	I	N	.	G	.	.	.	.	.	.	.	V	.	.	S	V	I	S	.	S	.	.	I	H	.	.	.	.	R	E	N	A	D	S	.
KEN 18 2009H1	L	T	I	N	.	G	.	.	.	.	.	.	.	V	.	.	S	V	I	S	.	S	.	.	I	H	.	.	.	.	R	E	N	A	D	S	.
KEN 35 2009H1	L	T	I	N	.	G	.	.	.	.	.	.	.	V	.	.	S	V	I	S	.	S	.	.	I	H	.	.	.	.	R	E	N	A	D	S	.
KEN 06 2009H1	L	T	I	N	.	G	.	.	.	.	.	.	.	V	.	.	S	V	I	S	.	S	.	.	I	H	.	.	.	.	R	E	N	A	D	S	.
KEN 15 2009H1	L	T	I	N	.	G	.	.	.	.	.	.	.	V	.	.	S	V	I	S	.	S	.	.	I	H	.	.	.	.	R	E	N	A	D	S	.
KEN 25 2009H1	L	T	I	N	.	G	.	.	.	.	.	.	.	V	.	.	S	V	I	S	.	S	.	.	I	H	.	.	.	.	R	E	N	A	D	S	.
KEN 30 2009H1	L	T	I	N	.	G	.	.	.	.	.	.	.	V	.	.	S	V	I	S	.	S	.	.	I	H	.	.	.	.	R	E	N	A	D	S	.
KEN 39 2009H1	L	T	I	N	.	G	.	.	.	.	.	.	.	V	.	.	S	V	I	S	.	S	.	.	I	H	.	.	.	.	R	E	N	A	D	S	.
KEN 164 2011H3	L	T	I	N	.	G	.	.	N	E	V	I	S	V	S	C	D	.	L	.	.	.	.	.	V	.	L	H	.	.	R	K	N	A	S	S	.
KEN 203 2010H3	L	T	I	N	.	G	.	.	.	E	.	.	.	V	.	.	.	.	L	.	.	.	.	.	V	.	L	H	.	H	R	K	N	A	S	S	.
KEN 012008H3	L	T	I	N	.	G	.	.	.	.	.	.	.	V	.	.	.	.	L	.	.	.	.	.	V	.	L	H	.	.	R	K	N	A	S	S	K
A Perth 16 2009H3	L	T	I	N	.	G	.	.	.	.	.	.	.	V	.	.	.	.	L	.	.	.	.	.	V	.	L	H	.	.	R	K	N	A	S	S	.
A Brisbane 10 2007H3	L	T	I	N	.	G	.	.	.	.	.	.	.	V	.	.	.	.	L	.	.	.	.	.	V	.	L	H	.	.	R	K	N	A	S	S	.
KEN 154 2011H3	L	T	I	N	.	G	.	.	.	.	.	.	.	V	.	.	.	.	L	.	.	.	.	.	V	.	L	H	.	F	R	K	N	A	S	S	.
KEN 160 2011H3	L	T	I	N	.	G	.	.	.	.	.	.	.	V	.	.	.	.	L	.	.	.	.	.	V	.	L	H	.	F	R	K	N	A	S	S	.
KEN 1612011H3	L	T	I	N	.	G	.	.	.	.	.	.	.	V	.	.	.	.	L	.	.	.	.	.	V	.	L	H	.	F	R	K	N	A	S	S	.
KEN 152 2011H3	L	T	I	N	.	G	.	.	.	.	.	.	.	V	.	.	.	.	L	.	.	.	.	.	V	.	L	H	.	F	R	K	N	A	S	S	.
KEN 156 2011H3	L	T	I	N	.	G	.	.	N	.	.	.	.	V	.	.	.	.	L	.	.	.	.	.	V	.	L	H	.	F	R	K	N	A	S	S	.
KEN 159 2011H3	L	T	I	N	.	G	.	.	N	.	.	.	.	V	.	.	.	.	L	.	.	.	.	.	V	.	L	H	.	.	R	K	N	A	S	S	.
KEN 158 2011H3	L	T	I	N	.	G	.	.	N	.	.	.	.	V	.	.	.	.	L	.	.	.	.	.	V	.	L	H	.	.	R	K	N	A	S	S	.
KEN 157 2011H3	L	T	I	N	.	G	.	.	N	.	.	.	.	V	.	.	.	.	L	.	.	.	.	.	V	.	L	H	.	.	R	K	N	A	S	S	.

Figure 1: An alignment showing mutations in selected positions of M2 sequences of influenza A/H3N2, A/H1N1 and A/H1N1pdm09.

The S31N marker of drug resistance was dominant in the influenza A/H3N2 and A/H1N1pdm09 virus subtypes. Vaccine strains-A/California/07/2009, A/Solomon Island/3/2006, A/Brisbane/59/2007, A/Brisbane/10/2007 and A/Perth/16/2009- are included in the alignment.
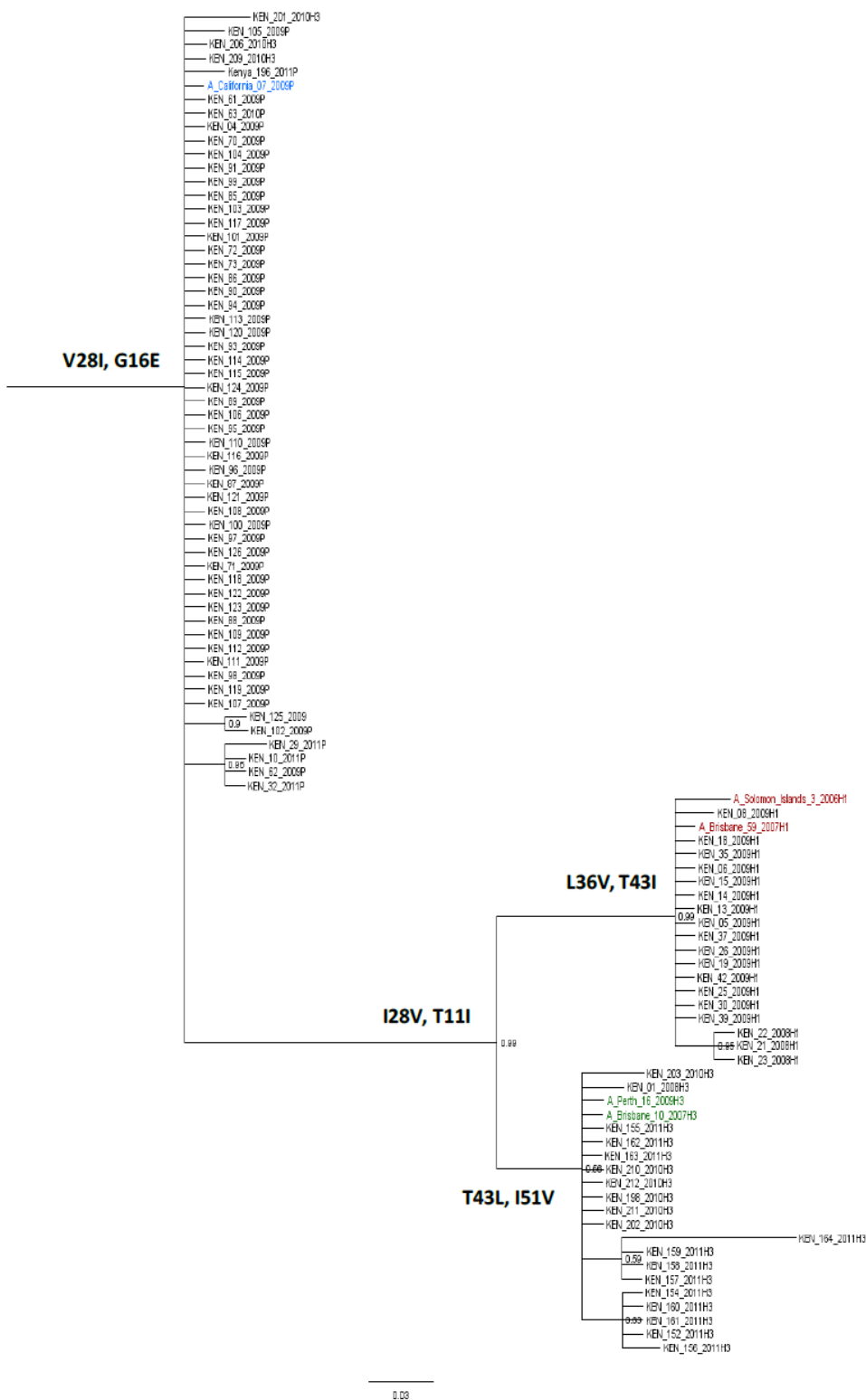
Figure 2: Phylogenetic tree analysis of M2 amino sequences of influenza A/H3N2, A/H1N1 and A/H1N1pdm09 virus isolates from Kenya.

The virus strains were obtained between 2009-2011. Amino acid substitutions determined are indicated at the node. Pyhlogenetic tree was inferred by running one million Monte Carlo Markov Chains (MCMC) with a sampling frequency of 1000 trees. A consensus tree for M2 sequences was generated and visualized using FigTree version 1.3.1. Reference vaccine strains for influenza A/H3N2, A/H1N1 and A/H1N1pdm09 virus subtypes are highlighted in green, brown and blue colours respectively. Three dominant clusters are evident: One consisting of influenza A/H1N1pdm09 and two influenza A/H3N2 virus strains characterized by V28I and G16E substitutions; second cluster formed by influenza A/H1N1 strains and possessing L36V and T43I amino acid changes; third cluster of influenza A/H3N2 virus strains distinguished by T43L and I51V substitutions.
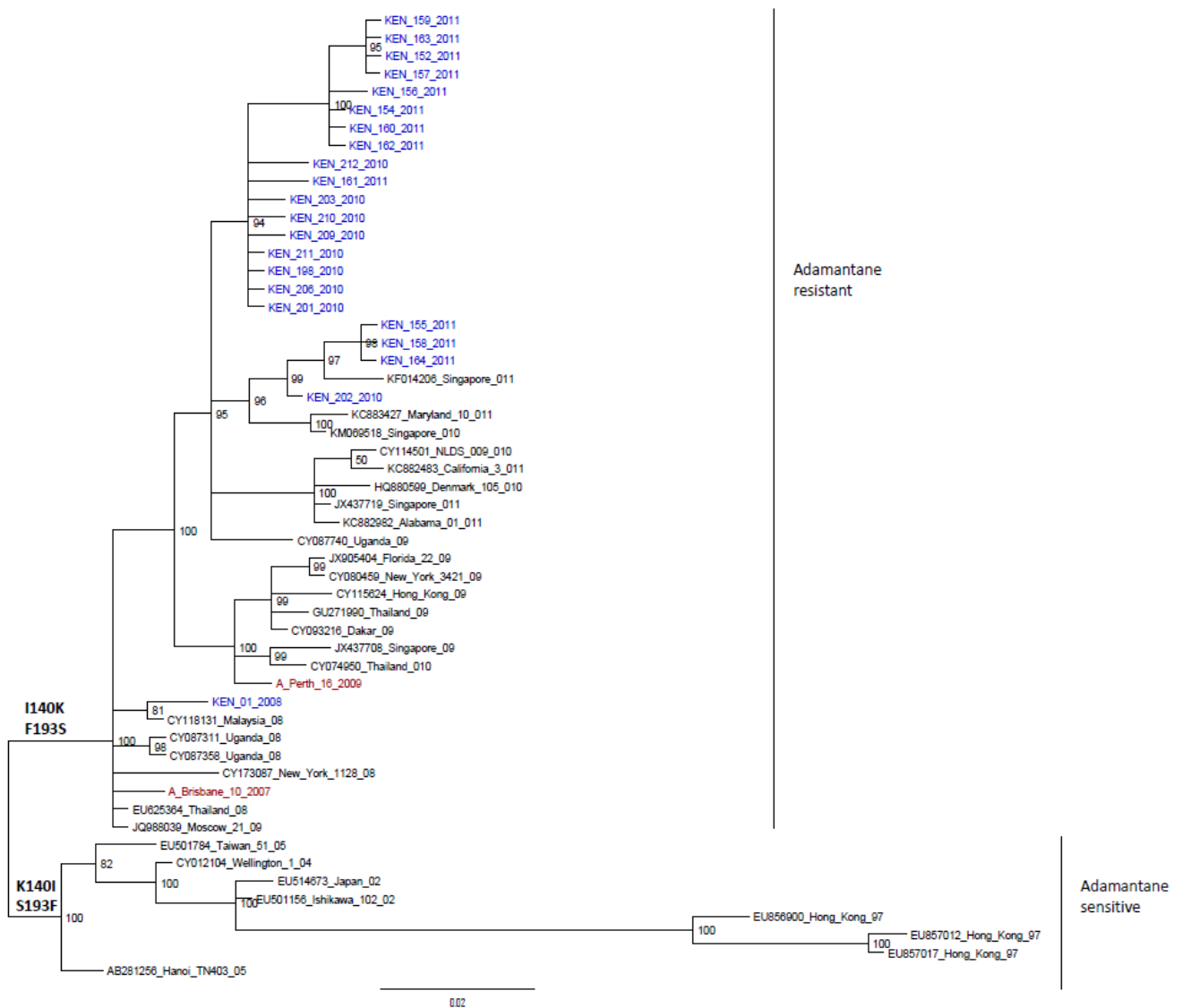
Figure 3: Phylogenetic tree of HA1 amino sequences of influenza A/H3N2 virus strains obtained between 2009-2011.

The vaccine strains A/Perth/16/2009 and A/Brisbane/10/2007 are highlighted in red. The strains from Kenya are highlighted in blue while global strains have been highlighted in black. All Kenyan viruses were defined by K140T and S193F amino acid substitutions in their HA1 gene.

The tree topology showing the genetic clustering of the M2 sequences (**Figure 2**) revealed three clusters with each virus subtype forming a distinct cluster. However, two virus strains of influenza A/H3N2 clustered with the A/H1N1pdm09 virus strains. Virus strains of influenza A/H3N2 and influenza A/H1N1 clustered on the same main branch as a result of sharing T11I and I28V substitutions. However, the two subtypes diverged from each other due to the presence of I15V, T43L & R54L amongst the A/H3N2 viruses and L36V & T43I substitutions amongst the A/H1N1 viruses. All the

Kenyan influenza A/H1N1pdm09 viruses, together with two influenza A/H3N2 virus strains: KEN_201_2010, KEN_206_2010 and KEN_209_2010 formed a separate cluster characterized by the amino acid substitutions I11T, N13S, G16E, V28I and I54R (**Figure 2**).

Phylogenetic analysis of HA1 protein sequences in relation to adamantane drug resistance.

To identify the origin and possible reassortment of adamantane resistance amongst the Kenyan viruses, we

performed phylogenetic analyses of the viruses using the HA1 gene segment of the viruses. HA1 is used to delineate the subtype assignment amongst influenza A viruses. Phylogenetic analysis of the HA1 protein of influenza A/H3N2 (Fig.3), revealed 2 distinct clades each consisting of one contemporaneous reference vaccine strain. Whereas all the Kenyan influenza A/H3N2 viruses spanning the four year period of 2008-2011 clustered with the WHO recommended vaccine strain A/Brisbane/10/2007, none of these Kenyan influenza A/H3N2 HA1 sequences clustered with the A/Perth/16/2009 vaccine strain. Furthermore, an interesting clustering pattern was noticed in the phylogenetic tree whereby adamantane-sensitive/resistant strains –previously defined by the N31S/S31N amino acid polymorphism in the M2 protein– formed distinct groupings characterized by the following parallel mutations in the HA protein: F193S & T140K for the sensitive strains and S193F & K140T for the resistant ones. In addition all the A/H3N2 virus strains from Kenya possessed D225N substitution (**Figure 3**).

Phylogenetic analysis of the of the 52 A/H1N1pdm09 Kenyan strains based on HA1 sequences in relation to global and reference viruses, (**Figure 4**) showed that all the Kenyan viruses, together with majority of the global strains, had S203T amino acid substitution which is a parallel mutation in the HA protein previously associated with adamantine resistance in this influenza subtype (Garten et al, 2009).

The tree topology for HA1 protein sequences of the Kenyan influenza A/H1N1 viruses (**Figure 5**) revealed 2 main clusters, one containing glutamic acid at position 140 (E140) of the HA1 protein and the other with a lysine substitution at this locus (E140K). All the influenza A/H1N1 virus strains obtained from Kenya between 2008 and 2009 clustered together with global viruses that circulated after 2007 and lacked this amino acid substitution. The vaccine strains, A/Solomon Island/3/2006 and A/Brisbane/59/2007, clustered together with the strains from Kenya characterized by the E140K change amongst other global strains. In this study, we found that the E140K in the HA is a parallel mutation with S31N in M2 of influenza A/H1N1 viruses characteristic of resistance to adamantane. The amino acid substitution R192H was common among all the influenza A/H1N1 virus strains obtained from Kenya.

4. Discussion

In Kenya, resistance profiles to adamantane drugs amongst human influenza A viruses have remained undocumented, prompting us to investigate these patterns. Whereas phenotypic and genotypic assays may be used for assessing resistance to adamantanes, the phenotypic assays are cumbersome because they first require viral propagation followed by determination of the drug 50% inhibitory concentration (IC50) values through plaque reduction assay (Zebedee & Lamb, 1988). However, genotypic analysis of resistance to adamantane is simple because it focuses on only a few substitutions occurring at five codons within the M2 gene (codons 26, 27, 30, 31 and 34) which are confirmed

amantadine/rimantadine resistance markers (Abed et al, 2005). While adamantane drug resistance is known to be conferred by a single mutation in one of these five positions of the M2 protein of influenza A viruses (Abed et al, 2005), a preponderance of resistant strains are due to possession of the S31N substitution (Saito et al, 2006b). In this study, we confirmed that where resistance occurred, the S31N / S31D mutation was the single most predominant marker of resistance found among influenza A virus strains that circulated in Kenya in the unique four year period between 2008-2011, when the three human influenza A subtypes co-circulated in the country. We found that following the global trend of decreasing resistance to adamantane, all Kenyan seasonal A/H1N1 viruses were sensitive to this class of drugs due to lack of all the molecular markers of drug resistance. In contrast we found that all influenza A/H1N1pdm09 and A/H3N2 viruses were resistant to adamantanes due to the presence of S31N/S31D mutation in the M2 protein. One unique A/H3N2 isolate had 80% substitutions in the region associated with adamantane resistance and is expected to have been highly resistant to adamantanes.

The incidence of adamantane resistance among influenza A/H3N2 and seasonal influenza A/H1N1 viruses has been on a rising trend (Hayden & de Jong, 2011) (Deyde et al, 2007) (Saito et al, 2008), while the influenza A/H1N1pdm09 subtype was resistant to this class of drugs from the onset (Hayden & de Jong, 2011). Amongst the two subtypes, the progression in resistance to adamantanes has remained significantly high reaching 90% among A/H3N2 obtained in Asia between 2005 and 2006 season and remaining at 100% for A/H1N1pdm09 since 2009 (Hayden & de Jong, 2011). Seasonal influenza A/H1N1 viruses showed a significant rise of up to 64% in terms of resistance in Asia to adamantanes during the same period. Beyond 2006, an inverse pattern has been seen worldwide with a decrease in the resistance pattern among seasonal influenza A/H1N1 viruses depicting a 15% in the antiviral resistance (Deyde et al, 2007) (Saito et al, 2008). This pattern of resistance for seasonal A/H1N1 showed a complete reversal to resistance where we have shown that by 2008, all seasonal A/H1N1 strains circulating in Kenya regained 100% sensitivity to adamantanes. Since the year 2009 resistance to adamantanes among influenza A/H1N1pdm09 has remained notably high (Hayden & de Jong, 2011). These trends were confirmed for the Kenyan A/H1N1pdm09 viruses where all the viruses tested had the S31N marker and were therefore resistant to adamantane.

The findings of the present study indicate a continuing trend of resistance to adamantane among A/H1N1pdm09 and A/H3N2 influenza virus strains globally. In fact, resistance to adamantane in Kenya peaked at 100% for both influenza A/H3N2 and influenza A/H1N1pdm09 from 2008-2011. In a previous Kenyan study, we reported that the seasonal influenza A/H1N1 was completely replaced by the A/H1N1pdm09 strain once the pandemic strain arrived in the country in mid 2008 (Majanja et al, 2013). By the time this subtype displacement occurred in Kenya, all the AH3N2 had become resistant to adamantane.
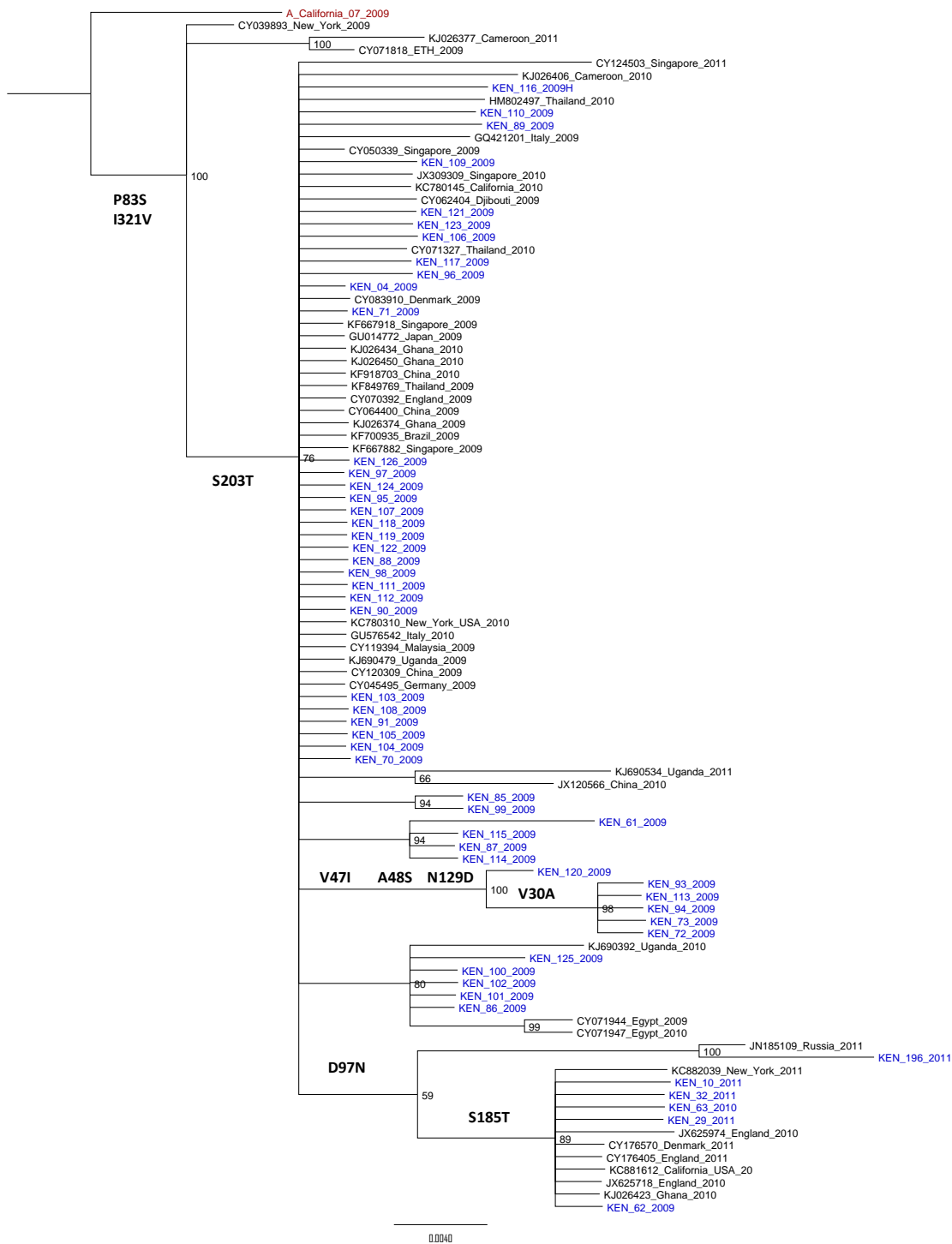
Figure 4: Phylogenetic tree analysis of HA1 amino acid sequences of influenza A/H1N1pdm09 strains obtained between 2009 and 2011.

The vaccine strain A/California/07/2009, global strains and local strains are indicated in red, black and blue colours respectively. Majority of the influenza A/H1N1pdm09 strains including those from Kenya possessed the S203T substitution common in all N-lineage influenza A viruses. Additional mutations V47I, A48S N129D, V30A, and S185T were noted among Kenyan strains and a few of the global strains.
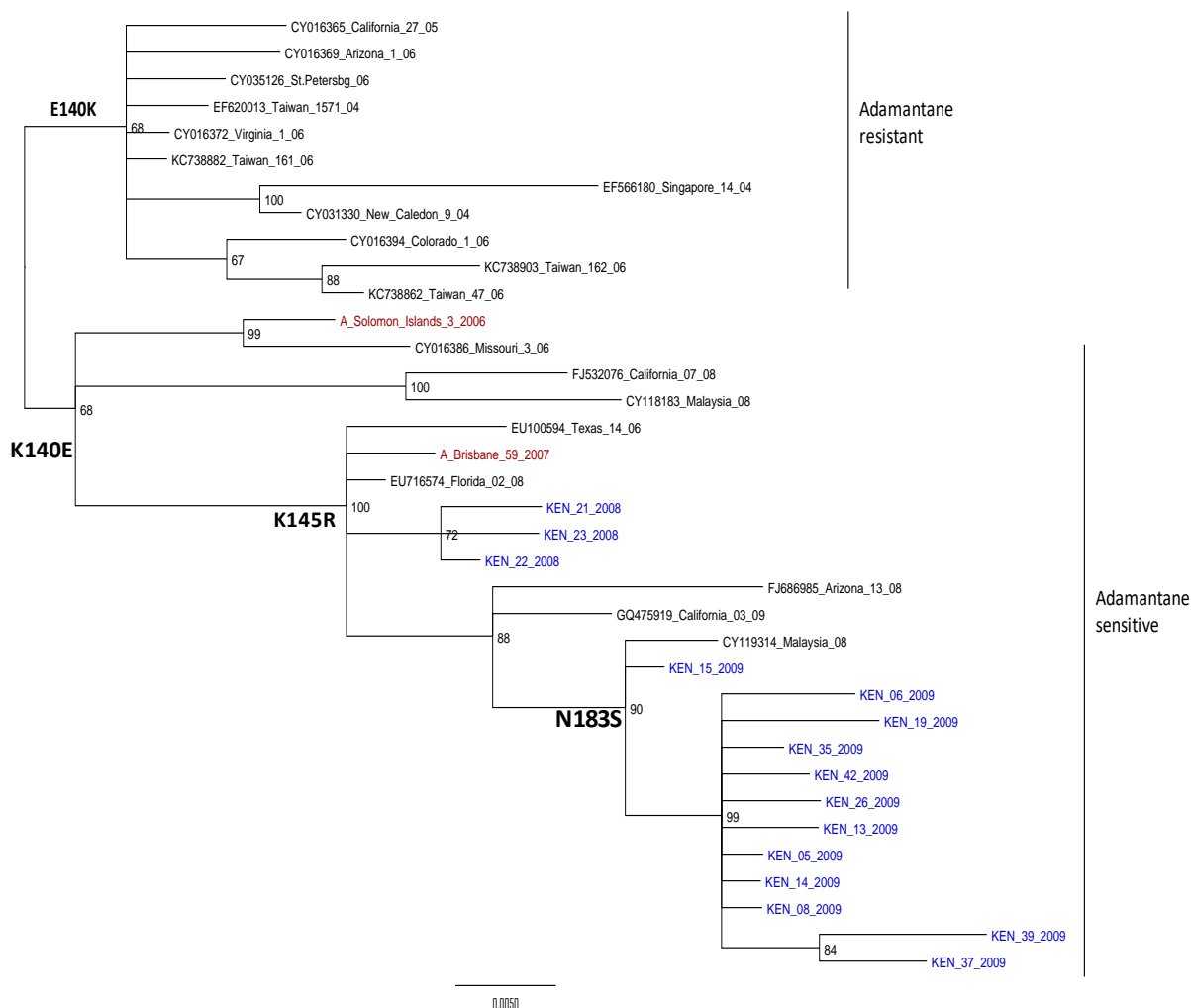
Figure 5: Phylogenetic tree of HA1 amino acid sequences of influenza A/H1N1.

The vaccine strains A/Solomon Island/3/2006 and A/Brisbane/59/2007 are highlighted in red. Global and local strains are shown in black and blue colours respectively. The tree topology for HA1 protein sequences of influenza A/H1N1 revealed 2 main branches: One branch with glutamic acid residue at position140 (E140) of the protein sequence and another with Lysine amino acid (E140K) substitution at the same locus. Influenza A/H1N1 virus strains from Kenya lacked the E140K amino acid substitution. In addition they did not possess S31N marker of adamantane drug resistance in the M2 protein sequence.

The antiviral resistance among seasonal influenza viruses has traditionally been attributed to the extensive use of antivirals in Asia and specifically in Japan due to easy access unlike in the United States where a physician's prescription is a requirement (Saito et al, 2006a). This, however, is not the case in Kenya and sub Saharan Africa where there is very limited use of antivirals to treat influenza due to the high cost of these drugs. In fact, in the sample collection questionnaire, when the antiviral drug usage question was posed to patients from whom the samples originated, all patients reported that they had no prior use of antivirals against influenza disease. Since the presence of resistance marker S31N in the Kenyan influenza A virus strains cannot be linked to unregulated antiviral use, we infer that the resistance traits are due to importations of strains carrying the resistance genotypes from

elsewhere into Kenya which then replace the sensitive strains due to their increased fitness. In fact, it has been documented that such drug resistance can emerge even in the absence of drug pressure due to transmission of resistant phenotype to naïve strains through reassortment (Hayden & de Jong, 2011).

Phylogenetic analyses of the influenza A viruses using the HA1 gene segment to identify the strain of origin and possible reassortment of adamantane resistance marker amongst the Kenyan viruses revealed that three of the influenza AH3N2 viruses clustered with and were virtually identical to the A/H1N1pdm09. Whereas this represents a reassortment of AH1N1pdm09 with seasonal A/H3N2, this was not unexpected because the M segment of the A/H1N1pdm09 strain was derived from the triple reassortant derived from a Eurasian

lineage of swine A/H3N2 appearing in a child in Hong Kong in 1999 (Gregory et al, 2001). Exchange of the M segment between the A/H1N1pdm09 is common and has previously been documented in Kenya (Bulimo et al, 2012). Analysis of HA1 protein segment of the Kenyan and global Influenza A/H3N2 viruses revealed distinct parallel S193F & K140T mutations for all virus strains (also referred to elsewhere (Saito et al, 2006a) as Clade N viruses) that also possessed the marker of drug resistance S31N in the M2 transmembrane protein sequence. These S193F and K140T mutations in HA1 protein of A/H3N2 viruses have previously been associated with adamantane-resistance (Saito et al, 2006a). Further phylogenetic analysis of the HA1 genes indicated presence of D225N substitution in all influenza A/H3N2 drug resistant virus strains from Kenya and other regions of the world. Strains that were depicted as sensitive in relation to adamantane possessed either the N225D or N225G substitution in the HA1 protein sequence. The findings from this study indicate a slight departure from those reported by Deyde (Deyde et al, 2007) in which both resistant and sensitive strains possessed N225 and G225 mutations. However, the findings in this study are in harmony with those of Saito et al, (Saito et al, 2006a) which indicated possession of S193F and D225N substitution in the HA1 protein segment of amantadine resistant viruses.

Analysis of the HA1 protein segment of all A/H1N1pdm09 virus isolates from Kenya from 2009 to 2011 showed that they belonged to Clade 7 characterized by signature S203T substitution (Garten et al, 2009). All the other regional and global virus sequences analyzed with the exception of only three strains: A/California/07/2009, KJ026377/Cameroon/2011, CY071818/ETH/2009 and CY039893/New York/2009 indicated presence of the same amino acid substitution. Significantly, the D222G and D239G substitutions in the HA1 protein associated with severe disease were not observed in any of the sequences analyzed (Kilander et al, 2010).

In a previous study, the S31N drug resistant genotype in the M2 gene was associated with R192M substitution in the HA1 gene of seasonal influenza A/H1N1 (Saito et al, 2008). Our findings regarding the Kenyan influenza A/H1N1virus strains in the study period indicated all the Kenyan isolates had the R192H amino acid change. This result negates the importance of a parallel mutation at position 192 in the HA1 as a contributor to resistance to adamantane in seasonal A/H1N1 strains. Overall, the study indicates that the codon 140 of HA1 gene in both seasonal A/H1N1 and A/H3N2 viruses is important in categorizing drug resistance to adamantane whereby E140K is associated with resistance and K140E is associated with sensitivity to adamantanes.

In assessing resistance to anti-influenza drugs, phenotypic assays are the gold standard. Such assays require viral culture prior to determination of the drug 50% inhibitory concentration (IC50) value. For the adamantane, IC50 values are assessed by the conventional plaque reduction assay. A major weakness of the present study was the lack of phenotypic data to correlate the drug sensitivities with the genotypic analyses of the M2 gene. Nonetheless, this is an important study because it for the first time, analyzed resistance to adamantane by the three human influenza A subtypes that circulated in the human population in Kenya in a time period of four years when for once, the three subtypes co-circulated.

5. Conclusion

All the seasonal influenza A/H1N1 isolates obtained from Kenya during the study period were sensitive to the adamantane class of drugs due to possession of serine amino acid residues at position 31 of the M2 proteins. In contrast both influenza A/H3N2 and A/H1N1pdm09 viruses were resistant to adamantane due to presence of asparagines at this locus. Thus, during the study period, Amantadine and Rimantadine were inappropriate for prophylaxis and treatment of influenza disease caused by A/H3N2 and A/H1N1pdm09 virus subtypes in Kenya. The findings of this study highlight the importance of continued surveillance of drug resistant genotypes amongst influenza A viruses. Continued surveillance of drug resistance is recommended in order to monitor emergence of dangerous resistant strains and inform epidemic/pandemic preparedness and drug use policy.

Conflict of Interest Declaration

The authors declare no conflict of interest.

Disclaimer

The views expressed in this work belong to the authors and should not be construed to represent the US Department of Defense, the Department of the Army, or the US Army Medical Department. The authors declare no conflict of interest in this work.

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