Research Article

Serotype Diversity of Respiratory Human Adenoviruses amongst Pediatric Patients from Western Kenya, 2010-2012

Fredrick M. Mbui [a], Rachel A. Achilla [b], Rodney L. Coldren [b], and Wallace D. Bulimo [a,b],*

[a] *Department of Biochemistry, School of Medicine, University of Nairobi, Nairobi, Kenya*
[b] *Department of Emerging Infectious Diseases, US Army Medical Research Unit – Kenya*
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* **Corresponding author:** Department of Biochemistry, School of Medicine, University of Nairobi, P.O. Box 30127-00100 GPO, Nairobi, Kenya. **Tel**: +254-73-3616602. **E-mail**: wallace.bulimo@usamru-k.org

Background: Respiratory illnesses are common among pediatric patients in Kenya, and many are attributed to viral causes. However, there is limited knowledge of the diversity of viral etiologies associated with these illnesses.

Objective: To characterize respiratory adenoviruses isolates using serological and molecular approaches.

Methods: A total of 1,879 samples were collected from symptomatic pediatric patients seeking medical care at New Nyanza Provincial General Hospital during the period of June 2010 to June 2012 and screened for adenoviruses as well as other respiratory viruses. Sixteen respiratory human adenoviruses (HAdVs) were isolated in Hep2 cell culture and characterized them using Immunofluorescence Assay, viral DNA amplification, sequencing and phylogenomics.

Results: Phylogenetic characterization of the HAdVs using the hyper variable region 7 of the hexon gene identified HAdV B and C as the major species associated with respiratory infections during the study period. Amongst these, a single B-type and four C-type serotypes were identified. The serotype distribution consisted of 31% HAdV B7, 25% HAdV C1, 25% HAdV C2, 6% HAdV C5, and 13% HAdV C6. Positive selection was observed in the nucleotide sequences from HAdV B7 and HAdV C5 signaling evolution of these two serotypes.

Conclusion: These finding may be useful to policy makers regarding appropriate strain selection for vaccination in Kenya.

Keywords: Respiratory Human adenovirus, Kenya, Pediatric, Serotype, Hexon, HVR-7

1. Introduction

Adenovirus is a double stranded DNA virus first isolated in 1953 from adenoid tissue–derived cell cultures (Enders et al, 1956). Human adenovirus (HAdV) belongs to the genus *Mastadenovirus* in the Adenoviridae family. It is a non-enveloped virus with an icosahedral structure and a genome size of between 26-45kb. Currently, there are 60 known serotypes of HAdVs which are grouped into seven species A-G (Robinson et al, 2013; Walsh et al, 2010). Species B, C and E are associated with approximately 10 % of pediatrics respiratory illnesses globally, whereas the other species are associated with gastrointestinal, genitourinary and ocular infections (Sharma et al, 2009; Tebruegge and Curtis, 2012).

Amongst adults, respiratory adenoviruses have been a serious concern in military training camps and are major cause of morbidity and have occasionally been associated with mortality (Potter et al, 2012).

Each species of adenovirus is associated with different spectra of diseases and severity of infection, therefore, rapid identification of a particular virulent serotype can help in prevention and disease management strategies. Initially, diagnosis and determination of adenovirus type involved virus isolation in cell culture followed by antibody/antigen detection by immunofluorescence or neutralization assays. However, molecular methods have replaced these serological approaches of detecting and serotyping adenoviruses (Madisch et al, 2005; Sarantis et

al, 2004). The molecular approaches are rapid and specific in detection and classification of adenoviruses (Madisch et al, 2005). These methods are based on the nucleotide sequencing of adenovirus genome followed by bioinformatics analysis of the sequences (Madisch et al, 2005).

Here, we used the adenovirus classification scheme reported by (Madisch et al, 2005) together with the method described by (Sarantis et al, 2004) to molecularly identify species and serotypes of respiratory human adenoviruses isolates from pediatric patients in western Kenya. We show the serotype diversity of these viruses in Kenya and that two species, B and C, represented by five serotypes predominated during the study period.

2. Materials and Methods

2.1 Study population and case definition

We used previously collected and archived nasopharyngeal specimens (Bulimo et al, 2008). The study population comprised persons from two months to 17 years presenting with influenza like illnesses (ILI) to the New Nyanza Provincial General Hospital. The ILI cases were identified according to the WHO manual for influenza surveillance (W. WHO, 2013). Briefly, the case definition included patients presenting with cough and fever of above 38^0C within 3 days prior to onset of illness.

2.2 Virus stocks, cell cultures and viral DNA extraction

We studied HAdV isolated from nasopharyngeal swab specimens from an ongoing influenza virus surveillance program at the United States Army Medical Research Unit – Kenya (Bulimo et al, 2008). The isolates were obtained from symptomatic pediatric patients seeking medical care at New Nyanza Provincial General Hospital located in the western part of Kenya during the period of June 2010 to June 2012. During this period, a total of 1,879 samples were collected and screened for adenoviruses as well as other respiratory viruses. The viruses were isolated and archived at -80ºC prior to serological and molecular characterization.

Serological characterization was carried out using a HAdV-specific fluorescent monoclonal antibody (DFA) kit (Millipore, Temecula, CA, 92590, USA) according to the manufacturer's instructions. Prior to molecular characterization, viral genomes were extracted from 200 μl of infected cell lysate using a QIAamp Viral DNA mini kit (Qiagen, Valencia CA, USA) according to the manufacturer's instructions. Extracted viral DNAs were re-suspended in 100 μl elution buffer and stored at -80ºC until use.

2.3 PCR and sequencing

We used a molecular typing scheme based on PCR amplification and sequencing of portions of hexon epsilon determinant loop-2 region (Madisch et al, 2005). The loop-2 fragment of hexon gene was amplified using primers pair described previously (Sarantis et al, 2004). The forward primer sequence was 5'-GCG TTG CGG TGG TGG TTA AAT GGG TTT ACG TTG TCC AT-3' while the reverse primer sequence was 5'-CTG ATG TAC TAC AAC

AGC ACT GGC AAC ATG GG-3'. These primers flank the hyper-variable region 7 (HVR-7) of loop-2 of the hexon gene and yield PCR products ranging in size from 510 to 530bp depending on the HAdV serotype and species.

PCR amplification was performed in a 50μl reaction volume containing 1X PCR Buffer, 3μM MgSO4, 200μM concentrations of each deoxynucleotide triphosphate, 0.5μM concentrations of each primer, 2.5U of high fidelity *Taq* DNA polymerase (all reagents from Invitrogen, Carlsbad CA, 92008 USA), and 5μl of DNA template. Gene Amp PCR Systems 9700 Thermo cycler (Life Technologies, Grand Island, NY, USA) was used with the following settings: an initial denaturation at 94°C for 2 minutes, followed by 30 cycles of denaturation at 94°C for 1 minute, annealing at 54°C for 1 minute, and elongation step at 72°C for 3 minutes, with a final extension at 72°C for 7 minutes. Virus amplicons were separated using 1% agarose gel electrophoresis and visualized under UV light by staining with ethidium bromide (0.5μg/ml).

Following amplification, PCR products were purified using the ExoSAP-IT method according to the manufacturer's instruction (Affymetrix, Inc. USA). Bidirectional sequencing was performed using the amplification primers and the Terminator Cycle Sequencing Ready Reaction Kit version 3.1 (Applied Biosystems, Carlsbad CA, USA), prior to running on the 3500xL Genetic analyzer (Applied Biosystems, Carlsbad CA, USA). Nucleotide sequence contigs were assembled and edited using DNA Baser Sequence Assembler version 10 (Heracle BioSoft, Pitesti, Romania) available at http://www.dnabaser.com.

2.4 Bioinformatics analyses

The assembled nucleotide sequences were used to query for similar sequences in the GenBank non-redundant nucleotide sequences database using BLAST (Altschul et al, 1997). The GenBank nucleotide sequences displaying the highest sequence identities to each of the sixteen HAdV sequences as well as reference hexon sequences representing each serotype of respiratory human adenovirus were retrieved from GenBank for multiple sequence alignment and inference of phylogeny. The sequences identified had accession numbers KC747631.1, AF515814.1, HQ535662.1, AF053085.1, AB436561.1, FJ943599.1, KC747641.1, JQ407712.1, AJ293903.1, AY008279.1, KC689915.1, KC551973.1, AB330115.1, AB330116.1, AB330131.1, AB330097.1, AB330095.1, AB330092.1, AB330088.1, AB330087.1, AB330086.1, AB330085.1, AB330084.1, AB330082.1.and K01264.1. K01264.1 is a bovine adenovirus 3 sequence and was used as the out group in the phylogenetic analyses.

Multiple sequence alignment was perfomed using Muscle version 3.8.3.1 (Edgar, 2004) and edited and visualised by GeneDoc version 2.6.002 (Nicholas and Nicholas, 1997). Phylogenetic analysis was perfomed using Bayesian inference of phylogeny executed in MrBayes version 3.2.2 (Huelsenbeck and Ronquist, 2001). The General Time reversible (GTR) model (Tavare, 1986) with gamma distributed rate variation across site and a proportion of invariable sites was used. For phylogenetic reconstruction, 1,000,000 Markov chain Monte Carclo (MCMC) generations were

performed with a sampling frequency after every 1000 trees. The results of phylogenetic analyses were visualized with Figtree version 1.4.0 (Rambaut, 2007).

Synonymous and nonsynonymous mutations were calculated using MEGA version 5.2 (Tamura et al, 2011). The analysis involved computation of the overall mean distance in a set of aligned sequences of the same serotype together with corresponding reference sequences. Two substitution models were used separately for comparison purpose; the Nei-Gojobori (Jukes-cantor) and the Kumar (Kimura-2-parameter) methods. Nonsynonymous-synonymous ratio (dN/dS) of <1 for sequences of the same serotype was used to imply a purifying selection of the sequences. Likewise dN/dS ratio of >1 implied that the sequences were under a positive selection pressure.

## 2.5 Ethical considerations

Informed consents were obtained from parents or guardians of children involved in the study. Adults >18 years consented for themselves. The subjects also consented that samples collected from them or their children could be stored and be used for other subsequent studies. Two ethical review boards, the Walter Reed Army Institute of Research (WRAIR) Institutional Review Board (IRB) and the Kenya Medical Research Institute (KEMRI) Ethics Review Committee (ERC) approved this study and consent procedure under protocol **WRAIR#2014** and **KEMRI SSC#2499** respectively. The informed consent process covered the archiving and use of samples for future molecular studies.

3. Results

Clinical picture of patients infected with HAdVs

All the patients infected with HAdV presented with fever and cough. Other symptoms associated with infection with HAdV included nasal stuffiness (63 %), runny nose (56 %), chills (25%) and vomiting (19 %). Malaise (13 %), sore throat (13 %) headache (6 %) and retro-orbital pain (6 %) were reported less frequently (**Figure 1**).
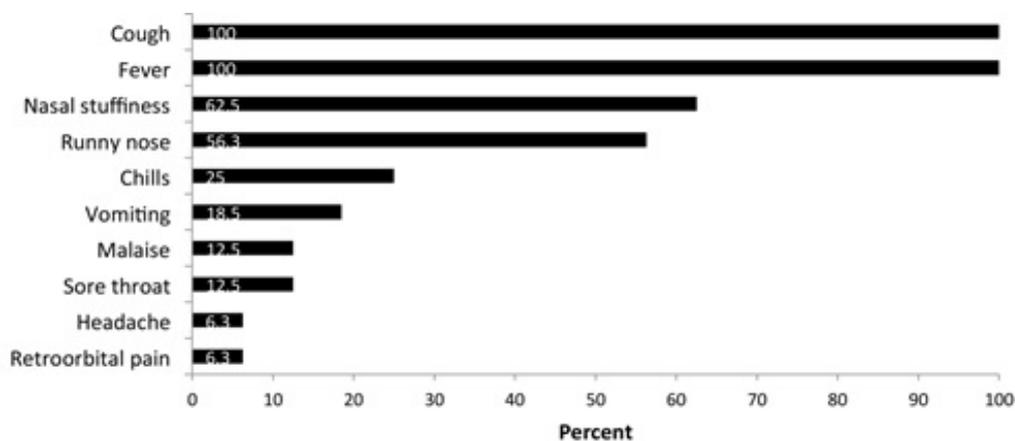
Figure1. Summary of % distribution of presenting symptoms of pediatric patients with human adenovirus infection during the period of June 2010- June 2012

Serological and nucleotide sequences analysis

In the period of June 2010 to June 2012, 1879 nasopharyngeal swab specimens from symptomatic patients were collected from New Nyanza Provincial General Hospital through the influenza surveillance program at USAMRU-K. Serological examination of patient's samples revealed 16 HAdV cases amongst these. BLAST analyses for the isolated nucleotide sequences revealed that the sixteen sequences had very high similarities to human adenovirus with expectation (E) values of zero and nucleotide identities of 98-99% compared to the next homologous reference sequence in the GenBank database.

Phylogenetic analyses

Phylogenetic reconstruction of the sixteen HAdV based on reference sequences from respiratory HAdV species B, C and E yielded the phylogentic tree shown in **Figure 2**. The sequences grouped into two main genetic clusters belonging to HAdV B and C species. Six sequences (isolate 02, isolate 05, isolate 06, isolate 11, isolate 13, and isolate 14,) clustered among the HAdV B7 reference serotypes while the remaining ten clustered with HAdV C reference serotypes. The grouping in the HAdV C genetic clusters further segregated into four distinct clades representing serotype 1 (isolate 01, isolate 08, isolate 09) serotype 2 (isolate 07, isolate 10, isolate 15, isolate 16) serotype 5 (isolate 12) and serotype 6 (isolate 03, isolate 04). Overall, the HAdV distribution was HAdV C serotype 1 at 25%, HAdV C serotype 2 at 25%, HAdV C serotype 5 at 6%, and HAdV C serotype 6 at 13%. HAdV B serotype 7 accounted for 31% of the isolates. The nucleotide sequences from this study were deposited in GenBank database under the accession numbers: KJ527469 to KJ527484.

### Natural selection within Hexon loop2

The synonymous (dS) and non-synonymous (dN) rates of nucleotide substitutions in the loop-2 sequences were used to evaluate natural selection amongst the viruses. **Table 1** shows a summary of the dN/dS ratio as calculated following two substitution models; Nei-Gojobori and Kumar substitution models (Kimura, 1980; Nei and Gojobori, 1986). Sequences in the HAdV C serotype 1 and serotype 6 gene clusters had dN/dS ratio of 1. The dN/dS nucleotide substitutions ratio of HAdV species C serotype-2 genetic clusters was less than 1 while in HAdV species C serotype 5 and HAdV species B serotype 7 was greater than 1.

4. Discussion

HAdVs cause morbidity and mortality, especially in pediatric & immune compromised patients and in the military recruits living in close quarters (Potter et al, 2012). Specific HAdV serotypes are associated with distinct clinical conditions (Sharma et al, 2009) and therefore an understanding of HAdV serotypes circulating in a population is crucial in planning and management of the disease caused by these viruses and ensuring timely intervention, policy decision making and public health preparedness.

Serological and molecular characterization of HAdV is important in understanding the epidemiology of the disease caused by this virus. Therefore, we sought to bridge the gap in the knowledge from a Kenyan context by systematically carrying out serological and molecular characterization of those 16 identified respiratory human adenoviruses. The 16 cases reported here show that the prevalence of respiratory HAdV at this hospital during the study period was approximately 1%. This prevalence is very low compared to prevalence of infection by influenza viruses at the same hospital during the same time, which was approximately 25% (Kenyan National Influenza Centre laboratory reports). Furthermore, the prevalence of respiratory HAdV elsewhere ranges from 5-10% (Yan, 2014). Thus, respiratory disease burden at this hospital due to respiratory HAdV was very low.
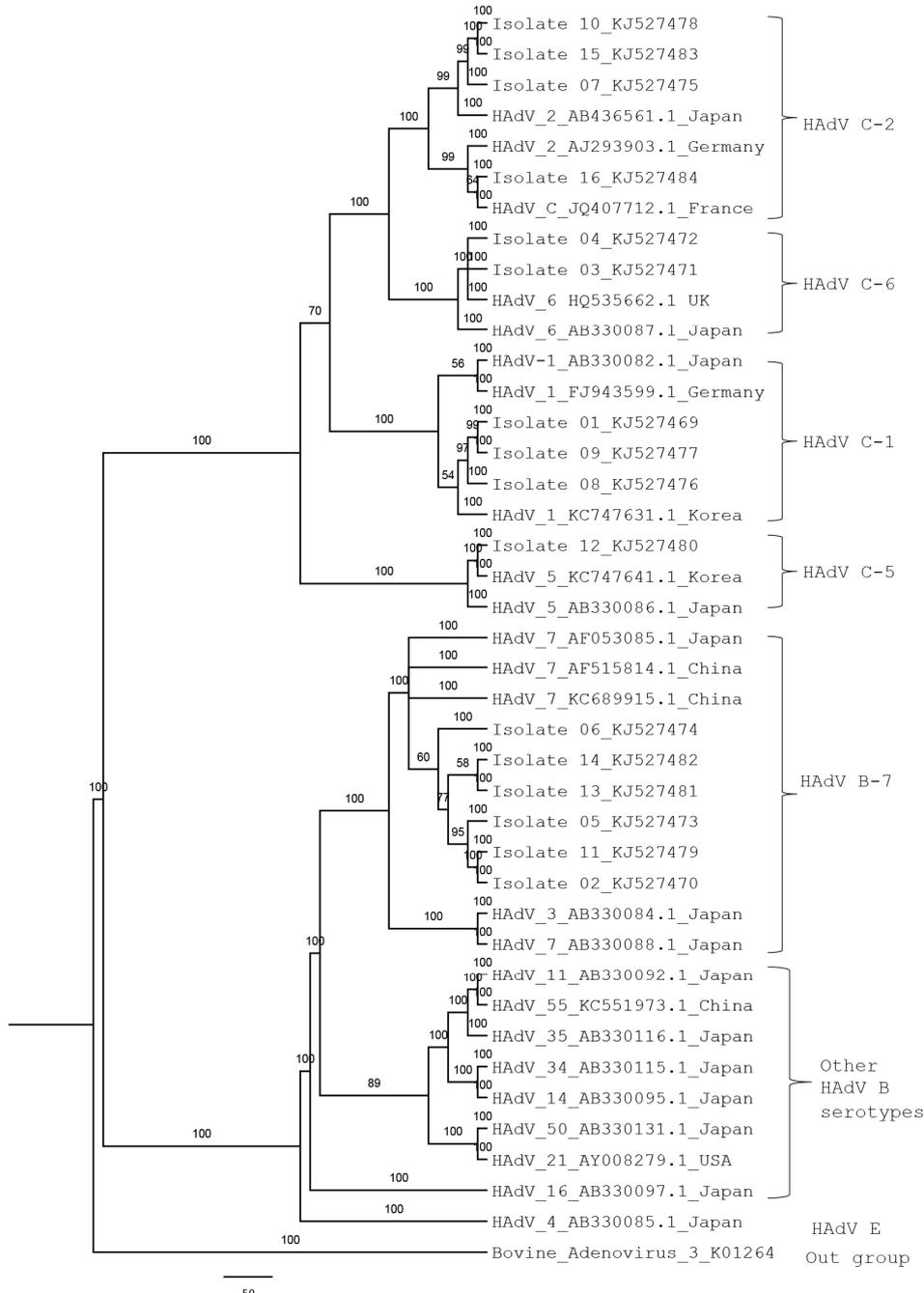
Figure 2. Phylogenetic analysis using the nucleotide sequences of the ε determinant loop2 from HAdVs. Phylogeny was inferred by a Bayesian method and visualized using FigTree v1.4.0. Reference HAdV species B, C and E are identified with their GenBank accession numbers. Bovine Adenovirus 3 was used as an out-group. Sequences belonging to the same species or serotype clusters are highlighted with brackets. The posterior probability values are expressed as percentages on each branch.

Table 1. Summary of nucleotide sequences distance estimation by synonymous and non-synonymous substitutions

	Nei-Gojobori substitution model			Kumar substitution models		
	dS	**dN**	**dN/dS**	**dS**	**dN**	**dN/dS**
HAdV C serotype 1 genetic cluster	0.2052	0.2746	1.0	0.2291	0.2523	1.0
HAdV C serotype 2 genetic cluster	0.0208	0.0133	0.64	0.0335	0.0136	0.4
HAdV C serotype 5 genetic cluster	0.0067	0.0417	6.2	0.0026	0.046	17.7
HAdV C serotype 6 genetic cluster	0.0109	0.0118	1.0	0.01	0.0109	1.0
HAdV B serotype 7 genetic cluster	0.013	0.0283	2.2	0.0185	0.0263	1.4

The high sequence homology of isolated nucleotides sequences confirmed the serological results that indeed the virus stocks belonged to adenovirus group of viruses. Indeed, past studies have demonstrated that nucleotide sequence homology of more than 97.5% is a valid criteria for determining the identity of adenovirus isolates (Madisch et al, 2005).

The Hexon epsilon fragment is one of the species and serotype-specific determinant regions in adenovirus genome. Other serotype-specific regions are found in the penton and fiber genes (Xu et al, 2000). Previous studies have demonstrated that analyzing either loop1 or loop2 regions of hexon epsilon determinant can classify adenoviruses serotypes (Madisch et al, 2005). The Hexon loop2 region used in this study has been described as the most simple, yet sufficient approach for molecular typing of human adenoviruses (Madisch et al, 2005). Thus using the nucleotide sequences of loop-2 hyper-variable region 7 (HVR 7) in a phylogenetic reconstruction analysis, the 16 virus isolates clustered into distinct clades with high (100%) overall posterior probability values. The general overall high posterior probabilities values indicated that the viruses were adequately identified as belonging to a particular species or serotype. The posterior probability values were, however, low (e.g. 54%) at nodes separating putative strains of the same serotype, indicating that strain separation may be more difficult to achieve using molecular phylogenetic analyses.

Phylogenetic analyses using the hexon loop-2 nucleotide sequences demonstrated that during the study period, HAdV C was the predominant species accounting for 69% of the reported cases. During the study period, four serotypes of species C (i.e. serotypes C1, C2, C5 and C6) were identified in circulation. However, for HAdV B, only a single serotype 7 was identified. Interestingly, despite being the only species B serotype in circulation during this period, HAdV 7 was the predominant serotype accounting for 31% of all isolates. The HAdV B serotype 7 has been associated with outbreaks and in certain cases fatalities and therefore prevalence of this serotype in the Kenyan population has public health significance (Kim et al, 2003; O'Flanagan et al, 2011). Vaccination

against HAdVs and in particular serotype 7 is therefore recommended.

HAdV species E was not observed among the isolates, suggesting that this species was not in circulation during the study period. Previous studies have suggested that HAdV E might have emerged through recombination involving strains of HAdV B serotypes14 and 16 (Shenk, 2001). Since only HAdV B7 serotype was observed amongst the Kenyan isolates during the study period, the absence of serotypes 14 and 16 may explain the absence of HAdV E among the Kenyan isolates.

The dN/dS ratio amongst sequences constituting HAdV C1 and HAdV C6 genetic clusters had a value = 1, showing that those viruses were not under any selection pressure. The HAdV C2 genetic cluster yielded dN/dS ratio of less than 1 indicating the sequences were under a purifying selection pressure. This means that from an evolutionary perspective HAdV C2 have interacted with the human host for long and are probably an old serotype. The dN/dS ratio of sequences constituting the HAdV C5 and HAdV B7 genetic clusters was greater than 1 implying that these sequences were under positive selection pressure and that these two are the most recently emerged serotypes and are expected to be more virulent.

Normally genes exhibiting positive selection pressure indicate that the organism is continually undergoing evolution in order to adapt to changes in the environment. Consequently, the Kenyan HAdV C5 and HAdV B7 were found to be undergoing an evolutionary process which signifies instability in their genomes. HAdV B7 was also the predominant serotype circulating during the study period. These serotypes are of great significance from a clinical perspective. Both HAdV B7 and HAdV C5 serotypes have been implicated in adenovirus outbreaks and in some cases fatalities (Kim et al, 2003; O'Flanagan et al, 2011; Seto et al, 2010) while HAdV C5 was a vaccine vector for Prime Boost HIV vaccine that was tested in clinical trials in Kenya (Kibuuka et al, 2010). Positive selection pressure occurring in Kenyan HAdV C5 leading to an activated immune response may explain the failure of the Prime

Boost HIV vaccine in Kenya. In future, HAdV C5 vectored vaccines may not be appropriate for Kenya.

There is need to continually carry out surveillance of these adenovirus serotypes in order to track their evolution. Drastic evolution of these serotypes could eventually result in emergence of more virulent adenoviruses which can be difficult to manage in case of severe outbreaks. Policy makers in the Kenyan health sector may do well to consider introducing the adenovirus vaccine currently used by the US military to the Kenyan population especially among the most vulnerable in the general population like respiratory disease pediatric patients in wards and military recruits living in close quarters. The adenovirus vaccine used by the US military is a divalent vaccine with components of B7 and B14 serotypes. A Kenyan specific vaccine should, perhaps, include the C5 component. Adenovirus vaccination programs have been successful in reducing the prevalence of adenovirus infections in other parts of the world (Potter et al, 2012) and therefore such efforts would be prudent in Kenya.

Whereas this work has contributed to closing the knowledge gap that existed regarding some molecular characteristics of respiratory human adenoviruses found in Kenya, like most studies, it has limitations. For example, only one of the three diagnostically useful genes in detection and genotyping of adenoviruses, the hexon gene, was used. Further studies utilizing nucleotide sequences of the penton and fiber genes, preferably full-length genes and even better full genome sequences of the adenoviruses will afford a more comprehensive picture of the genetic characteristics and serotype diversity of the Kenyan respiratory human adenoviruses. Additionally, since adenovirus was such an uncommon cause of ILI in the population under study, the number of specimens available for genetic analysis was low and may not represent a complete picture of diversity of these viruses in Kenya.

In conclusion, we have identified and classified respiratory human adenoviruses using the HVR 7 region of Hexon gene for the first time in Kenya.

Conflict of Interest Declaration

The authors declare no conflict of interest.

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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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