RESEARCH ARTICLE

Molecular characterization of rotavirus group A strains circulating prior to vaccine introduction in rural coastal Kenya, 2002-2013 [version 1; referees: awaiting peer review]

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Abstract

Background: Kenya introduced the monovalent Rotarix® rotavirus group A (RVA) vaccine nationally in mid-2014. Long-term surveillance data is important prior to wide-scale vaccine use to assess the impact on disease and to investigate the occurrence of heterotypic strains arising through immune selection. This report presents baseline data on RVA genotype circulation patterns and intra-genotype genetic diversity over a 7-year period in the pre-vaccine era in Kilifi, Kenya, from 2002 to 2004 and from 2010 to 2013.

Methods: A total of 745 RVA strains identified in children admitted with acute gastroenteritis to a referral hospital in Coastal Kenya, were sequenced using the di-deoxy sequencing method in the VP4 and VP7 genomic segments (encoding P and G proteins, respectively). Sequencing successfully generated 569 (76%) and 572 (77%) consensus sequences for the VP4 and VP7 genes respectively. G and P genotypes were determined by use of BLAST and the online RotaC v2 RVA classification tool.

Results: The most common GP combination was G1P[8] (51%), similar to the Rotarix® strain, followed by G9P[8] (15%), G8P[4] (14%) and G2P[4] (5%). Unusual GP combinations—G1P[4], G2P[8], G3P[4,6], G8P[8,14], and G12P[4,6,8]—were observed at frequencies of <5%. Phylogenetic analysis showed that the infections were caused by both locally persistent strains as evidenced by divergence of local strains occurring over multiple seasons from the global ones, and newly introduced strains, which were closely related to global strains. The circulating RVA diversity showed temporal fluctuations both season by season and over the longer-term. None of the unusual strains increased in frequency over the observation period.

Conclusions: The circulating RVA diversity showed temporal fluctuations with several unusual strains recorded, which rarely caused major outbreaks. These data will be useful in interpreting genotype patterns observed in the region during the vaccine era.
Keywords
Rotavirus, epidemiology, genotype, strains, diversity

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Introduction
Rotavirus group A (RVA) infection is a leading cause of childhood severe dehydrating acute diarrhoea, which can lead to death. The 2016 estimates show that, annually, RVA is responsible for 128,500 deaths globally, with the highest burden occurring in sub-Saharan Africa and South-East Asia countries. In 2009, the World Health Organization (WHO) recommended the inclusion of either of the two licensed RVA vaccines (Rotarix® and RotaTeq®) into national immunization programmes (NIPs) of all countries to curb RVA associated disease burden. Kenya introduced the monovalent Rotarix® vaccine (based on the G1P[8] strain) into its NIP in July 2014.

In Africa, the introduction of the Rotarix® vaccine into the NIPs of several countries has been associated with a marked reduction in hospitalization caused by RVA infection. For instance in Malawi, Burkina Faso and Tanzania, the vaccine effectiveness against hospitalization was estimated at 62%, 58% and 53%, respectively. However, this effectiveness is lower than that observed in developed countries; for example in Belgium, vaccine effectiveness of Rotarix vaccine was estimated at 90%. Furthermore, concerns remain that in time, given the high diversity of RVA strains, vaccine immunity escape variants could emerge which may undermine the gains from the vaccination programmes. Such a scenario was observed in Japan where, a G8P[8] RVA strain appeared to emerge and caused acute gastroenteritis disease in up to 66% (53/80) of children attending a pediatric clinic. Similarly, the predominance of non-vaccine type G2P[4] strains was observed in Rotarix® vaccinated populations of Belgium and Brazil, raising concerns of the effect of the vaccine on circulating non-vaccine strains.

The rotavirus genome is comprised of 11 segments of double-stranded RNA, which encode 12 proteins (VP1-4, VP6, VP7, NSP1-6). The VP7 and VP4 proteins independently elicit neutralizing antibodies and specify the G (glycoprotein) and P (protease-sensitive) genotypes, respectively. Molecular characterization of the VP7 and VP4 proteins encoding regions is commonly used to investigate local and global RVA molecular epidemiology and is the basis of the dual genotype classification of this virus. Up to 36 different G and 51 P RVA genotypes have been identified worldwide in animals and humans. Globally, G1P[8], G2P[4], G3P[8], G4P[8] and G9P[8] (in decreasing order) have been identified as the most common genotypes causing disease in children, although their distribution can vary considerably from region to region and from one season to the next. While these genotypes are similarly dominant in Africa, understanding of their local natural seasonal fluctuations and intra-genotype diversity in the pre-vaccine introduction era is incomplete despite importance to vaccine impact evaluation.

The current study presents molecular analysis of historical RVA strains from coastal Kenya detected between 2002–2004, reported in Nokes et al., which we refer to as phase I, together with more recent RVA strains detected between 2010–2013, referred to as phase II. We present findings from partial sequence analysis of these longitudinally collected RVA strains identified at the Kilifi County Hospital (KCH), Kilifi, Kenya, and phylogenetically compare these with those deposited in public databases across the globe. The typing of phase I strains was previously performed by nested multiplex PCR using genotype-specific VP7 and VP4 primers. We utilize these extensive sequence data to illuminate on local RVA genotype circulation characteristics and provide baseline information on natural patterns of RVA genotype diversity in coastal Kenya prior to vaccine introduction.

Methods

VRA surveillance in Kilifi County Hospital
VRA surveillance in KCH reported in this analysis was conducted from January 2002 to December 2004, and from January 2010 to December 2013. Study subject recruitment criteria and sample collection methods are as previously described. The study targeted children aged less than 13 years admitted with acute diarrhoea defined as three or more watery stools passed during a 24-hour period. The KEMRI Scientific and Ethics Review Unit (SERU) in Kenya approved the study protocol.

Detection of RVA
Stool samples were screened for RVA using an enzyme immunoassay (ELIA) kit, marketed under two different names in the two periods: IDEIA (DAKO Rotavirus IDEIA™, Oxoid, Ely, United Kingdom) in phase I and ProSpect™ (Oxoid, Basingstoke UK) in phase II, following the manufacturer’s instructions.

Partial sequencing of RVA positive samples in VP4 and VP7 segments
Sequencing was conducted on 272 (46%) of 558 positive samples detected in phase I, and all positive samples identified in phase II (n=473). The phase I samples were selected to represent common RVA genotypes (>70%) observed throughout the surveillance period from each year. Partial fragments of the VP4 and VP7 genes, were amplified in a one-step reverse transcriptase PCR reaction using the following primer pairs: VP4F 5’-TATGCCTCCAGTNAATTTGG-3’, VP4R 5’-ATTTGTAGTTGAATATACCCAC-3’, VP7F 5’-ATGTATGGTATTGAATATACCAC-3’, VP7R 5’-AAGTTGCCACACTTTCCTTG-3’, as previously described by Simmonds et al. and Gomara et al. To confirm successful amplification of the targeted genomic area the products were checked (VP7, 881 bp, VP4, 660 bp) by electrophoresis in a 2% agarose gel. Products of samples that showed presence of the expected band size on gels were purified using GFX DNA purification kit (GFX-Amersham, UK) following the manufacturer’s instructions. These were then sequenced using Big Dye Terminator 3.1 (Applied Biosystems, Foster City, California, USA) chemistry and the same primers as in PCR amplification on an ABI Prism 3100xl Genetic Analyser (Applied Biosystems, Foster City, California, USA).

RVA genotyping and sequence analysis
The sequence reads were assembled into contigs using Sequencher version 5.4.6 (Gene Codes Corp Inc., Ann Arbor, MI, USA). The nucleotide sequences were aligned using MAFFT version 7.222 and visualized in Aliview version 1.8 and further trimmed to remove sequence overhangs, resulting in
Table 1. A summary of diarrhea cases, the number of samples tested, the proportion of RVA cases observed in the entire surveillance period and the number of samples sequenced from each phase from childhood admissions to KCH, Kenya, between 2002–2004 and 2010–2013.

<table>
<thead>
<tr>
<th>Period</th>
<th>Admissions, n</th>
<th>Diarrhoea, n</th>
<th>Samples tested, n</th>
<th>RVA cases, n</th>
<th>Proportion, %</th>
<th>Sequenced, n (%)</th>
<th>Successfully assembled, n (%)</th>
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<td></td>
<td></td>
<td></td>
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<td>VP7</td>
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<td>3296</td>
<td>2039</td>
<td>558</td>
<td>27.2</td>
<td>272 (48)</td>
<td>192 (71) 218 (80)</td>
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<td>(Phase I)</td>
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<td></td>
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<td>VP4</td>
<td>VP7</td>
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<tr>
<td>2010–2013</td>
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<td>473 (100)</td>
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<td>(Phase II)</td>
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<td>VP4</td>
<td>VP7</td>
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<td>3779</td>
<td>1031</td>
<td>27.3</td>
<td>745 (72%)</td>
<td>569 (76) 572 (77)</td>
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Table 2. Frequency and proportions of RVA strains observed in Kilifi County Hospital between 2002–2004 and 2010–2013.

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<th>2003</th>
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<th>2010</th>
<th>2011</th>
<th>2012</th>
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<td>26 (100)</td>
<td>16 (100)</td>
<td>32 (100)</td>
<td>101 (100)</td>
</tr>
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NT, non-typable; Gx and Px, undetermined G and P genotypes, respectively.
Phylogenetically, the P[8] strains showed a close association among themselves with high sequence homology of >92% at nt level. Despite, the high homology, majority of the P[8] strains observed in phase I formed separate clusters from those observed in phase II. Unlike P[8] strains, P[4] strains occurred less frequently, with high prevalence observed in 2004 in phase I and 2010 and 2012 in phase II. These strains formed three clusters, with one cluster containing both phase I and II strains while the other two clusters containing only phase II strains. Despite the distinct clustering, P[4] strains showed a high sequence similarity of 95–100% at the nucleotide level.

**Phylogenetic placement of Kilifi strains in the global context**

The placement of Kilifi strains in the global context is shown in Figure 3 and Figure 4. Tree clusters leading to Kilifi strains are
Figure 2. Maximum likelihood phylogenetic trees of G1, G2, G8, G9, P[4] and P[8] genotypes inferred in MEGA v7, with taxa stratified in 3 groups, black for 2002–2004, green for 2010–2011 and red for 2012–2013, from viruses detected in childhood diarrhea admissions to KCH, Kenya. The bar graphs represent frequency of the same genotypes between 2002–2004 and 2010–2013. Only bootstrap values ≥70% are shown. This figure excludes the infrequent genotypes G3, G10, G12, G29 and P[6]. The scale bars indicate nucleotide substitutions per site.
Figure 3. Maximum likelihood tree for VP7 G1 genotype showing the relationship between G1 genotypes from viruses detected in childhood admissions to KCH, Kenya, and to other global G1 genotypes detected between 2002 and 2013. Tree clusters (branches) including Kilifi strains are shown in the expanded boxes. Taxa for Kilifi strains are stratified in three groups, black for 2002–2004, green for 2010–2011 and red for 2012–2013. Only bootstrap values ≥70% are shown. The scale bars indicate nucleotide substitution per site.
Figure 4. Maximum likelihood tree for VP4 P[8] genotype showing the relationship between P[8] genotypes detected in childhood admissions to KCH, Kenya, and to other global P[8] genotypes detected between 2002–2013. Tree clusters (branches) including Kilifi strains are shown in the expanded boxes. Taxa for Kilifi strains are stratified in three groups, black for 2002–2004, green for 2010–2011 and red for 2012–2013. Only bootstrap values ≥70% are shown. Scale bar represents nucleotide substitutions per site.
shown in the expanded boxes. A majority (85%) of the observed G1 strains, clustered away from the other global strains, clustering closely to strains detected in Africa, specifically in Kenya, South Africa and Togo. The second cluster comprised only strains from 2010–2012 which distinctively clustered with strains from Belgium and Ethiopia. The last clusters which had only single strains grouped together with strains from Japan and Pakistan. The Kilifi P[8], (Figure 5) strains were placed into four clusters, where the largest group comprised of Kilifi strains observed in both phase I and II, with external strains observed in Kenya, South Africa, Tanzania, Ireland and Russia. The second cluster included Kilifi strains from phase I and II with strains from Belgium, Brazil and Ethiopia. The last minor clusters, each made of a single virus, showed a close similarity to strains isolated in Pakistan, Denmark, Ecuador and Belgium.

Phylogenetic analysis of the rare G8P[14] strain

Whilst several rare GP combinations (not commonly detected) were observed during the study period, G8 associated with a P[14] genotype has overall been rarely detected in human population. Additionally, there has been an increasing number of human P[14] rotavirus strains globally, which are associated with rabbits, cattle, sheep and guanacos. We therefore sought to investigate the probable origin of the observed G8P[14] detected in a 14 months old infant in 2010. All cognate sequences for genotypes G8 (n=71) and P[14] (n=47) isolated by 2013 were retrieved from GenBank and phylogenetically compared to the observed genotypes. Duplicate sequences from strains isolated from the same country were removed. The G type in this samples (G8) clustered closely to other G8 strains isolated from humans with a nucleotide and amino acid (aa) identity of 95% and 99%, respectively, and G8 strains isolated from camel showing a nucleotide and aa identity of 94% and 98%, respectively (Figure 5A). The P[14] genotype showed a high sequence similarity to other P[14] strains isolated from humans and bovine with a nucleotide similarity of 96% and 93% respectively and aa identity of 98% (Figure 5B).

Discussion

The present study provides insight into the molecular epidemiology and phylogenetic relatedness of distant (7 years or more) and recent pre-vaccine introduction RVA strains detected in Kilifi, Coastal Kenya. This is the first detailed study on prevalence of RVA genotypes causing diarrhoea in children in rural coastal Kenya spanning over a decade before introduction of the nationwide routine RVA vaccination programme. The work builds on a previous study, which highlighted the importance of genotypes G1, G8 and G9 in sub-Saharan Africa during the pre-vaccine introduction period. During phase I surveillance period, genotype-specific primers were used to characterize the strains into different G and P genotypes. In the present analysis, a fraction of phase I (46%) and all phase II RVA samples were sequenced and GP annotations assigned as per the guidelines of the Rotavirus Classification Working Group.

In this analysis, strains G1P[8], G9P[8], G8P[4] and G2P[4] were the most common RVA strains, accounting for over 70% of the infections. These strains have also been observed in studies conducted elsewhere in Kenya and the world. Genotype G8P[4] was the third most important strain after G1P[8] and G9P[8] accounting for 15% of RVA infections. The G8 genotype is mostly found in combination with P[4], P[6] and P[8] VP4 specificities. In this study, the majority (83%) of the G8 strains combined with P[4] types, while only 16% combined with P[8] types. The increase in prevalence of this strain in phase II supports the notion of G8 strain regarded as an unusual and newly emerging strain in the world. Genotype G3P[8] is also among the common genotypes causing infections in children, and is the second most important strain in Africa and fifth most important globally. Here, G3P[8] was detected at a low frequency, accounting for only 1% of all the cases. Genotype G12 detection has increased in Africa and has also been observed in Kenya and for the first time in Kilifi (2010–2013).

The detection of atypical GP combinations; G1P[4, 6], G2P[6], G3P[4,6], G10P[8], and G8P[14], albeit at low levels, raises interest in their origins. Despite such atypical strains being less frequent, strains G3P[4] and G2P[6] were found to be the most important causes of diarrhoea in the late 1990s in Ghana. Genotype G10 has long been reported to infect calves, pigs or cattle but recently has sporadically been reported in humans in several studies. Similarly strain G8P[14] has recently been detected in humans and is thought to have originated from animals. In this study, the close association of the observed strain G8P[14] with strains from both humans and animal origins shows a possibility of zoonotic transmission. The increase in diversity of RVA in this setting could be attributed to the emergence of such unusual strains which might have arisen due to zoonotic transmission or re-assortment cases within and between RVA genotypes.

Post-vaccine surveillance studies have reported shifts in the prevalence of RVA genotypes. Recent post-vaccine introduction reports from Kenya have indicated an increase in prevalence of uncommon genotypes G3, G9 and G12. Similarly, data from the USA depicted an increase in prevalence of G3P[8] in post-vaccine era relative to G1P[8] in the pre-vaccine period. In contrast, surveillance studies in Australia and Belgium revealed the dominance of G2 strains in post-vaccine period, relative to G1P[8] in pre-vaccine period. Similar studies in Ghana reported an increase in prevalence of G12P[8] and G10P[6] in the post-vaccine era. This shift in distribution of genotypes post-vaccine introduction might be associated with either selective vaccine pressure or the natural fluctuations of RVAs, although these are not evidently supported. The emergence of uncommon genotypes and increased prevalence of non-vaccine strains warrants close monitoring to determine their circulation in the post-vaccine introduction period and their probable effect on performance of the vaccine.

Overall, the observed strains showed a high nucleotide sequence homology of up to 100%, as observed in the different genotypes. The close genetic relationship of strains observed in phase I and phase II suggest a persistence in circulation of these RVA strains to continuously cause the observed epidemics. In addition, the exclusive clustering of majority of Kilifi strains
Figure 5. Maximum likelihood phylogenetic tree showing the relationship of the rare G8P[14] strain detected in a child admitted to KCH, Kenya, and other similar strains detected in humans and animals retrieved from GenBank. (A) shows the phylogenetic relationship of the VP7 G8 genotype to other G8 genotypes. (B) Phylogenetic relationship of the VP4 P[14] genotype to other P[14] genotypes. Sequences for the strains identified in this study are marked by the black filled circle and the arrows. Only bootstrap values ≥70% are shown. Scale bar represents nucleotide substitution per site.
from the global strains shows that these strains might have been localized in Kilifi over a long period of time. However, few strains that formed three distinct clusters in both G1 and P[8] global trees, supports the notion of separate introductions and persistence of possibly foreign strains in this setting. Although cases of re-assortment and possible introductions is evident, partial data from only two genes is insufficient in providing a complete understanding of the genetic diversity of such common and not common genotypes. Full genome sequencing will thus illuminate on the complete genomic constellations of these strains and provide data on their evolutionary dynamics. The marked seasonal and longer-term changes in genotype distribution observed in this pre-vaccine surveillance should be considered when interpreting changes to genotype patterns that may follow the introduction of rotavirus vaccine in any setting.

This study had several limitations, e.g. firstly, by use of partial sequencing method, we were unable to identify mixed infections in phase II, which were previously identified in phase I using other primer-based methods. Partial sequencing only identifies the dominant genotype in mixed infections resulting to one genotype. The sequencing chromatograms of samples identified as mixed infections in phase I, appeared clean and mono-infected, with no background indicators of co-infections. Secondly, the classification of the strains into lineages and sub-lineages was limited due to the short consensus sequences, since only ~23% and ~67% of the VP4 and VP7 genes were sequenced, respectively. Thirdly, it was not possible to perform comparative analysis of the rare genotype G29 due to unavailability of cognate sequences in GenBank. Only a single reference sequence for genotype G29 had been deposited in GenBank by the time of this analysis.

In conclusion, this study shows that most of the pre-vaccine RVA infections and epidemics have been caused by a diverse range of RVA strains which fluctuated in prevalence from season to season, with some persistent in circulation for a long period. Additionally, new strains might have been introduced in this population and contributed significantly to the epidemics experienced in the pre-vaccine period. The recommendation by WHO for countries to vaccinate infants against rotavirus infection led to the inclusion of Rotarix™ vaccine in the childhood immunization programme in Kenya. In addition to reducing hospitalization caused by RVA diarrhoea, the vaccine has been reported to offer protection against both homotypic and heterotypic RVA strains. With the increase in diversity of circulating strains and emergence of rare strains in Kilifi, continuous monitoring will help evaluate the performance of this vaccine against the circulating strains.

Data availability
The replication data and analysis data for this manuscript are available from the Harvard Dataverse: https://doi.org/10.7910/DVN/LVGYYW.

Owing to data personal protection concerns, these data are restricted, but will be made available to researchers who meet the criteria for access to confidential data. Details of the criteria for sharing data and the conditions under which data are made available can be found in the KEMRI-Wellcome data sharing guidelines. Users who wish to use the data should send a request to the KEMRI Wellcome Trust Research Programme data governance committee, which can be contacted by emailing: dgc@kemri-wellcome.org.

Nucleotide sequence accession numbers
Partial sequences for the VP7 and VP4 genes reported in this work were deposited in the GenBank database under the sequential accession numbers MH402005-MH402781 and MH402782-MH403560 for the VP7 and VP4 genes, respectively.

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pie charts. The others category represents genotypes that circulated in low proportions. This included G3, 10, G12, G29 for panel a, P[6] and P[14] for panel b and G1P[4], G2P[8], G3P[4,6,8], G8P[6,8,14], G9P[4,6], G12P[4,6,8] for panel c.

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Supplementary Figure 2. Pairwise distances within the common genotypes indicating the level of identity within genotypes. The y axis indicates genetic distances determined in MEGA v7 by calculating the proportions of pairwise nucleotide differences between sequences.

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Supplementary Table 1. Evolutionary models used in construction of phylogenetic trees for Kilifi sequences and both Kilifi and Global sequences. The models were tested using the maximum likelihood method in MEGA v7.

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Supplementary File 1. Accession numbers of VP7 G1 global sequences (list 1) and VP4 P[8] global sequences (list 2) used in phylogenetic comparison with the local strains.

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References


