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Research paper

Whole genome analyses of DS-1-like Rotavirus A strains detected in children with acute diarrhoea in southern Mozambique suggest several reassortment events

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ABSTRACT

We report the first whole genome constellations of Mozambican rotavirus A strains detected between 2012 and 2013 in the Mavalane General Hospital in Maputo city and Manhiça District Hospital in the Manhiça district. Consensus sequences for ten DS-1-like strains (G2P[4] and G8P[4]) were identified with an Illumina Miseq platform using cDNA prepared from dsRNA extracted from stool samples, without genome amplification or prior adaptation to cell culture. Comparison of previously reported genotyping results and the consensus sequences described in this study, indicated that the genotype primers specific for G12 and P[4] might require revision. Phylogenetic analyses indicated diversity among the G2P[4] Mozambican strains and suggested reassortment between G2P[4] and G8P[4] Mozambican strains, as well as the intragenogroup reassortment of all the genome segments encoding VP1, 2, 3 and 6 for strain RVA/Human-wt/MOZ/0045/2012G8P[4]. These results highlight the necessity to determine whole genome constellations to confirm surveillance data in Africa and to monitor the growing diversity in DS-1-like strains.

1. Introduction

Rotavirus (RV) is an important enteric pathogen in the young of many mammalian and avian species. The virus belongs to the *Reoviridae* family and contains an 11-segmented double-stranded RNA (dsRNA) genome. The dsRNA segments encode six structural (VP1–4, 6 and 7) and six non-structural (NSP1–5/6) proteins. The viral particle consists of outer capsid (VP4 and VP7), inner capsid (VP6) and core (VP2 encasing VP1 and VP3) proteins which encloses the nucleic acid material (Estes and Greenberg, 2013). Based on genotyping targeting genome segment 6 which encodes VP6, RVs are classified into eight groups (RVA – RVH) with an additional two proposed groups, I and J (Bányai et al., 2017; Matthijnssens et al., 2012; Mihalov-Kovács et al., 2015).

Surveillance studies of the public health important RVA, usually make use of a dual typing system targeting partial genome segments encoding VP7 (G-type) and VP4 (P-type) encoding genome segments (Guerra et al., 2015; Hokororo et al., 2014; Seheri et al., 2014, 2017; Shoeib et al., 2015). However, whole genome characterisation is now widely used to describe and understand the origins of strains detected in humans and animals (Matthijnssens and Van Ranst, 2012; Nyaga et al., 2015; Phan et al., 2016; Steyer et al., 2013). Prevalent human RVA strains have G1, G2, G3, G4, G9 and G12 (VP7) genotypes in combination with P[4], P[6] and P[8] (VP4) genotypes (Heiman et al., 2008; Matthijnssens et al., 2010). The genotypes of the remaining nine genome segments (VP6-VP1-VP2-VP3-NSP1-NSP2-NSP3-NSP4-NSP5/6) for human RVAs often have a Wa-like (I1-R1-C1-M1-A1-N1-T1-E1-H1) or DS-1-like (I2-R2-C2-M2-A2-N2-T2-E2-H2) origin (Matthijnssens et al., 2008).

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The DS-1-like backbone was typically associated with G2P[4] combinations (Matthijnssens et al., 2008). However, in recent years, non G2P[4] DS-1-like strains have been reported in Africa such as G3P [6] (Bwogi et al., 2017; Heylen et al., 2013) and G8P[4] (Nyaga et al., 2014), Asia (G1P[4] and G12P[6]) (Agbemabiese et al., 2017; Saikruang et al., 2016) and Europe (G3P[8]) (Arana et al., 2016). Evolutionary studies of G2P[4] DS-1-like strains have also shown emergence of distinct strains after 2000 (Dennis et al., 2014; Giammanco et al., 2014). Furthermore, stepwise changes between lineages of all the genome segments, as well as intra-genotype re-assortment which resulted in emerging lineages in genome segments encoding VP7, VP3 and NSP4 have been identified (Doan et al., 2015). Specifically, the E2 genotype of NSP4 encoding genome segment has greater sequence diversity compared to the other RV genome segments (Nyaga et al., 2014) and lineage IX and X are evidence of continuous diversification of this genotype. A possible explanation of the multiple NSP4 lineages is intra-genotype reassortment with non G2P[4] DS-1-like strains such as G8P[4], G8P[6], G2P[6], G3P[6] and G6P[6] (Agbemabiese et al., 2016).

A surveillance study in southern Mozambique during 2012 and 2013 indicated a predominance of G2P[4] strains (42.3%), whereas G8P[4] strains were detected at a lower frequency (4.5%). Phylogenetic analysis of the VP8* encoding genome segments revealed possible re-assortment. In addition, various mixed infections and partially typed strains were identified in the surveillance study (João et al., 2018). In the current study, it was attempted to identify the unknown genotypes reported for the partially typed strains as well as characterise the genotypes in previously identified mixed infections (João et al., 2018) using whole genome analysis. Results revealed no mixed infections, albeit identified G8P[4] and G2P[4] strains on a DS-1-like backbone that suggested various reassortment events. This is the first report on Mozambican rotavirus strains that describe whole genome constellations.

2. Materials and methods

2.1. Rotavirus strains

The study was approved by the National Committee on Bioethics from Mozambique in 2010 (reference N°286/CNBS/10, IRB 00002657) and by the Ethical Committee of the University of Free State (ECUFS 201/2013).

A surveillance program during February 2012 to September 2013 at Mavalane General Hospital in Maputo city and Manhiça District Hospital in the Manhiça district identified a prevalence for rotavirus infections in under five year old children in southern Mozambique (de Deus et al., 2018). The genotypes of selected strains were confirmed with partial sequencing of the VP8* and VP7 encoding segments (João et al., 2018). In the present study, samples previously identified to contain mixed and untyped strains by João and colleagues, were selected for whole genome analyses. In total, six samples from Manhiça (0043, 0044, 0045, 0052, 0126 and 0144) and four samples from Mavalane (0257, 0308, 0314 and 0440) were selected (Table 1).

2.2. RNA extraction and cDNA synthesis

Total RNA was extracted from stool samples with TRI-reagent (Sigma) and single-stranded RNA was precipitated with lithium chloride as described before (Potgieter et al., 2009). In order to obtain full-length sequences, an anchor primer (PC3-T7loop; Integrated DNA Technologies) was ligated to the dsRNA of specific samples (0308 and 0440) as previously described (Potgieter et al., 2009). Complementary DNA (cDNA) was synthesised using the Maxima H Minus Double Stranded cDNA kit (ThermoFisher Scientific). Random hexamer primers in combination with the annealed self-priming anchor primer were employed for cDNA synthesis for samples 0308 and 0440, while only random hexamer primers were used for the remaining samples (0043, 0044, 0045, 0052, 0126, 0144, 0257, and 0314). The manufacturer's instructions were followed with the following minor modifications. Firstly, the dsRNA was first denatured at 95 °C for 5 min and secondly, the first strand synthesis was carried out for two hours at 50 °C.

Table 1
Comparison of PCR genotyping and on-line webtools using NGS-derived consensus sequences.

Sample	Date	Area	Genotyping PCR	NGS
0043	2012	Manhiça	G2G8G9P[X]	**G2P [4]**
0044	2012	Manhiça	G8G12P[X]	**G8P [4]**
0045	2012	Manhiça	G8G12P[X]	**G8P [4]**
0052	2012	Manhiça	G8G12P[X]	**G8P [4]**
0126	2013	Manhiça	GXP[4]	**G2**P[4]
0144	2013	Manhiça	GXP[X]	**G2P [4]**
0257	2012	Mavalane	G8G12P[6]	**G8P [4]**
0314	2012	Mavalane	G2G9P[8]	**G8P [4]**
0308	2012	Mavalane	G2P[4]	G2P[4]
0440	2013	Mavalane	G2P[4]	G2P[4]

X: Unknown.
Underlined: False genotyping result.
Bold: Not previously identified with genotyping PCR (João et al., 2018), but identified in current study using BLASTn and ViPR analyses of NGS-derived consensus sequences.

2.3. Next generation sequencing

MiSeq Illumina (Illumina, Inc.) sequencing was done at Inqaba Biotec, South Africa (0043, 0044, 0045, 0052, 0126, 0144, 0257, 0314) and at the Next Generation Sequencing Unit at the University of the Free State (NGS-UFS) (0308 and 0440). Sequencing was completed using the NextEra XT DNA library Preparation Kit (Illumina, Inc.). Briefly, cDNA was standardized to 0.2 ng/µl and tagmented at 55 °C for 5 min. Illumina indexes were then ligated by integrating the sheared DNA templates, Illumina sequencing adapters P5 and P7, NextEra PCR Master Mix, NextEra PCR primer cocktail and an indexing primer set (Integrated DNA Technologies) to a total volume of 50 µl per reaction. Libraries were purified and size selected with AMPure XP beads (Beckman Coulter, Inc.) and 100% ethanol. Validation of the libraries was done using a Bioanalyzer 2100 (Agilent Technologies) and normalized to a final concentration of 8 pM, prior to sequencing using the MiSeq Reagent Kit V3 (600 cycles), with 301 bp paired-end reads, or the MiSeq Reagent Nano Kit V2 (500 cycles) with 251 bp paired end reads.

2.4. Data analyses

The data was assembled in CLC Genomics Workbench (8.5.1; Qiagen). A *de novo* assembly was done for all samples and the resulting contigs were identified with Nucleotide Basic Local Alignment Search Tool (BLASTn). These results were used to choose reference strains for reference mapping of the data. The curated assembled sequences were used to derive consensus sequences for the respective genome segments. Specific genotypes were analysed using BLASTn and confirmed with the online database Virus Pathogen Database and Analysis Resource (ViPR) (Pickett et al., 2012). The sequences were submitted to GenBank and accession numbers MG891933-MG891965; MG891977-MG892020 and MG926720-MG926752 were assigned. Genotyping PCR results determined by João and colleagues were compared to the P-type and G-type identities obtained with the above-described analyses. Alignment of genotype specific primers to the Mozambican rotavirus sequences in Multiple Alignment using Fourier Fast Transform (MAFFT) were used to determine if primer binding played a role in unidentified or false positive genotyping PCR results.

Phylogenetic analysis was done in MEGA 7.0.143. DS-1-like strains with G2P[4], G2P[6], G8P[4], G8P[6] and G9P[4] combinations were

chosen for analysis following identification with BLASTn and ViPR analyses. Strains representing the different lineages of all the genome segments described in Doan et al. (2015) and Agbemabiese et al. (2016) were also included. Alignment of the reference strains, obtained from GenBank and the study strains, were done with a Multiple Sequence Comparison by Log Expectation (MUSCLE) alignment and the best model was determined for each segment. The Tamura 3-parameter (Tamura, 1992) model was used for segments 3 to 11, the Tamura-Nei (Tamura and Nei, 1993) was used for segment 2 and the General Time Reversible model (Kumar et al., 2016) was used for segment 1. Maximum Likelihood trees were inferred for each segment and the reliability of the branching order and partitioning were assessed by performing 1000 bootstrap replicates. Pairwise distance matrixes were obtained in MEGA 7.0.143, using the corresponding models for each segment as for the phylogenetic analysis. Visualisation of the aligned sequences were done with *mVISTA* and the LAGAN alignment tool (Brudno et al., 2003; Frazer et al., 2004).

3. Results

3.1. Genome assembly

Ten rotavirus genome constellations were assembled from the sequencing data, with average coverage ranging from 101 to 7962,7 per sequence (Suppl Table 1). Partial genome segments of eight Mozambican strains (RVA/Human-wt/MOZ/0043/2012/G2P[4] (0043), RVA/Human-wt/MOZ/0044/2012/G8P[4] (0044), RVA/Human-wt/MOZ/0045/2012G8P[4] (0045), RVA/Human-wt/MOZ/0052/2012/G8P[4] (0052), RVA/Human-wt/MOZ/0126/2013/G2[4] (0126), RVA/Human-wt/MOZ/0144/2013/G2P[4] (0144), RVA/Human-wt/MOZ/0257/2012/G8P[4] (0257) and RVA/Human-wt/MOZ/0314/2012/G8P[4] (0314) were assembled. These strains contained between 70 and 99.9% of the open reading frames (ORF) (Suppl Table 1). Ligation of the anchor primer (TC3-T7loop; Potgieter et al., 2009) to the dsRNA prior to cDNA synthesis resulted in full-length sequences for 21 of 22 genome segments for strains RVA/Human-wt/MOZ/0308/2012/G2P[4] (0308), RVA/Human-wt/MOZ/0440/2013/G2P[4] (0440).

3.2. Genome constellations

The genotypes of 102 genome segment sequences were identified using ViPR, whereas six segment 10 (NSP4) sequences (0043, 0044, 0126, 0144, 0314 and 0440) and two segment 11 (NSP5) sequences (0044 and 0045) were too short to be identified using this online genotyping tool (Suppl Table 1). However, it was possible to identify the closest strains, and possible genotypes (either E2 or H2) for these untyped genome segments using BLASTn analysis (Table 2). All ten Mozambican strains contained DS-1-like backbones with five G8P[4] combinations, (0044, 0045, 0052, 0257 and 0314) and five G2P[4] combinations (0043, 0126, 0144, 0308 and 0440). The G2 and G8 strains were detected in both Manhiça and Mavalane in 2012. Three G2P[4] strains were detected in 2013: two in Manhiça and one in Mavalane.

3.3. Analysis of primer binding sites

Using G- and P-genotyping based on PCR, six samples (0043, 0044, 0045, 0052, 0257 and 0314) were previously identified as mixed infections (João et al., 2018). During the current study, these six samples were identified with whole genome analysis using NGS on an Illumina MiSeq platform, as single-strain containing samples with DS-1-like backbones. Five of these strains had a G8P[4] combination and the remaining strain (0043) a G2P[4] combination (Table 2). In addition, the P-type could not be determined for five samples (0043, 0044, 0045, 0052, 0144), and the G-type for a further two samples (0126 and 0144) (Table 1). It was, however, possible to identity the genotypes for these samples using BLASTn and ViPR analyses of the NGS-generated consensus sequences as G2 and P[4].

During genotyping PCR analysis four of the samples containing G8P[4] strains (0044, 0045, 0052 and 0257) were shown to contain both G8 and G12 rotavirus strains (João et al., 2018). The G12 specific primer (G12b, 20 bp) (Aladin et al., 2010a) aligned to the nucleotide sequences of the Mozambican G8 strains at position 536–554, with only four mismatches (Fig. 1A). The remaining G8 strains identified in (João et al., 2018) (0258, 0297, 0310 and 0314) were not mistyped as mixed G12G8 strains (Fig. 1A). Amplification of the G8 sequences with the G12 primer is predicted to form a 527 bp amplicon, a difference of only 32 bp to the intended 559 bp amplicon with the G12-specific primer.

The genotyping PCR also failed to identify the P[4] genotype in six of the 10 Mozambican P[4] strains included in the study (0043, 0044, 0045, 0052, 0257 and 0314; João et al., 2018). Alignment of the P[4] primer (2 T-1, 18 bp) (Gentsch et al., 1992) to the P[4] sequences indicated that the primer aligned at the intended position (474–494). However, a base pair mismatch at the 5′ end (A474G) between the primer and the unidentified six P[4] Mozambican sequences was observed (Fig. 1B). Two strains, one (0314) identified using genotyping PCR as P[8] and the second (0257) as P[6], was revealed with sequencing as P[4] strains. Both these strains contained the A474G variation. No discrepancies were observed for the remaining genotypes that were incorrectly identified during the previous study and their corresponding primer binding sites (results not shown).

3.4. Phylogenetic analyses of DS-1-like Mozambican strains

Phylogenetic analysis of the Mozambican rotavirus strains revealed strain diversity and indicated several reassortment events (Table 2, Suppl Fig. 1). Visualisation of the alignment in *mVISTA* confirmed the diversity between the 2012 and 2013 G2P[4] Mozambican strains and furthermore supported reassortment suggested by the phylogenetic analysis (Fig. 2).

The three G2P[4] strains detected in 2013 (0126, 0144 and 0440) clustered with strains detected in the Philippines (RVA/Human-wt/PHI/TGO12-003/2012/G2P[4]) and Ghana (RVA/Human-wt/GHA/GHPML1989/2012/G2P[4]) for all the genome segments. However, these 2013 strains never grouped with the Mozambican G2P[4] strains detected in 2012 (0043 and 0308). Instead, the 2012 G2P[4] Mozambican strains grouped with two South African G2P[4] strains (RVA/Human-wt/ZAF/MRC-DPRU82/2012/G2P[4] and RVA/Pig-wt/ZAF/MRC-DPRU1533/2007/G2P[4]) and a Malawian strain (RVA/Human-wt/MWI/BID115/2012/G2P[4]) for all the genome segments (Table 2). Segment 9 of the 2013 G2 and 2012 G2 strains only share an average of 95.90% nucleotide identity (Suppl Table 2).

All the Mozambican G8P[4] strains grouped in the same lineages except for segment 3, where 0045 grouped with the 2013 G2P[4] strains in lineage VII and segment 10, where 0045 and 0052 did not group in lineage VII with the remaining G8P[4] strains. Strain 0045 only share 85.32% nucleotide identity with the other G8P[4] strains for segment 3 and both 0045 and 0052 share 83.30 and 83.54% with the remaining G8P[4] strains for segment 10 (Suppl Table 2). Interestingly, the G8P[4] strains (0044, 0257 and 0314), also clustered with the 2012 G2P[4] strains for segments 1, 3, 4, 5, 7, 10 and 11. Analysis based on segment 4 indicated that the 2012 G2P[4] and G8P[4] strains clustered in lineage II of P[4], whereas the 2013 Mozambican G2P[4] strains clustered in lineage III (Suppl Fig. 2). Here, the G8P[4] strains (0044, 0045, 0257 and 0314) and the 2012 G2P[4] (0043 and 308) had nucleotide similarities above 99.23% whereas, 0052 shared 98.34% with the other G8P[4] strains. The 2013 G2P[4] strains only shared 91.81% with the 2012 G2P[4] strains (Suppl Table 2).

Mozambican strain, 0045 (G8P[4]), grouped with the G8P[4] (0044, 0257 and 0314) strains and the 2012 G2P[4] (0043 and 0308) strains for segments 4, 5 and 7 (Fig. 2). The G8P[4] Mozambican strains 0044, 0257, 0314 and 0045 grouped for most of the segments with

Table 2
Colour representation of phylogenetic clustering between Mozambican study and reference strains. Identified lineages are indicated in brackets for each genotype.

Strain	VP7 (9)	VP4 (4)	VP6 (6)	VP1 (1)	VP2 (2)	VP3 (3)	NSP1 (5)	NSP2 (8)	NSP3 (7)	NSP4 (10)	NSP5/6 (11)
RVA/Pig-wt/ZAF/MRC-DPRU1533/2007/G2P[4] RVA/Human-wt/ZAF/MRC-DPRU82/2012/G2P[4] RVA/Human-wt/MWI/BID115/2012/G2P[4]	G2	P[4]	I2	R2	C2	M2	A2	N2	T2	E2	H2
RVA/Human-wt/ZMB/MRC-DPRU3463/2009/G8P[4] RVA/Human-wt/ZWE/MRC-DPRU3347/2010/G8P[4]	G8	P[4]	I2	R2	C2	M2	A2	N2	T2	E2	H2
RVA/Human-wt/UGA/MUL-13-427/2013/G8P[4] RVA/Human-wt/KEN/MRC-DPRU1606/2009/G8P[4] RVA/Human-wt/TZA/MRC-DPRU4576/2010/G8P[4]	G8	P[4]	I2	R2	C2	M2	A2	N2	T2	E2	H2
RVA/Human-wt/PHI/TGO12-003/2012/G2P[4] RVA/Human-wt/GHA/GHPML1989/2012/G2P[4]	G2	P[4]	I2	R2	C2	M2	A2	N2	T2	E2	H2
RVA/Human-wt/MWI/BID11S/2012/G2P[6] RVA/Human-wt/MWI/BID15I/2012/G2P[6]	G2	P[6]	I2	R2	C2	M2	A2	N2	T2	E2	H2
RVA/Human-wt/IND/Kol-065/2013/G9P[4] RVA/Human-wt/USA/LB1562/2010/G9P[4]	G9	P[4]	I2	R2	C2	M2	A2	N2	T2	E2	H2
MOZ/0043/2012/G2P[4]	G2 (IVa-1)	P[4] (II)	I2 (V)	R2 (V)	C2 (IV)	M2 (V)	A2 (IVa)	N2 (V)	T2 (V)	E2* (VII)	H2 (IVa)
MOZ/0308/2012/G2P[4]	G2 (IVa-1)	P[4] (II)	I2 (V)	R2 (V)	C2 (IV)	M2 (V)	A2 (IVa)	N2 (V)	T2 (V)	E2 (VII)	H2 (IVa)
MOZ/0044/2012/G8P[4]	G8 (II)	P[4] (II)	I2 (V)	R2 (V)	C2 (IV)	M2 (V)	A2 (IVa)	N2 (V)	T2 (V)	E2* (VII)	H2* (IVa)
MOZ/0257/2012/G8P[4]	G8 (II)	P[4] (II)	I2 (V)	R2 (V)	C2 (IV)	M2 (V)	A2 (IVa)	N2 (V)	T2 (V)	E2 (VII)	H2 (IVa)
MOZ/0314/2012/G8P[4]	G8 (II)	P[4] (II)	I2 (V)	R2 (V)	C2 (IV)	M2 (V)	A2 (IVa)	N2 (V)	T2 (V)	E2* (VII)	H2 (IVa)
MOZ/0045/2012/G8P[4]	G8 (II)	P[4] (II)	I2 (V)	R2 (V)	C2 (IV)	M2 (VII)	A2 (IVa)	N2 (V)	T2 (V)	E2	H2* (IVa)
MOZ/0052/2012/G8P[4]	G8 (II)	P[4] (II)	I2 (V)	R2 (V)	C2 (IV)	M2 (V)	A2 (IVa)	N2 (V)	T2 (V)	E2	H2 (IVa)
MOZ/0126/2013/G2P[4]	G2 (IVa-3)	P[4] (IVa)	I2 (V)	R2 (V)	C2 (IV)	M2 (VII)	A2 (IVa)	N2 (V)	T2 (V)	E2* (VI)	H2 (IVa)
MOZ/0144/2013/G2P[4]	G2 (IVa-3)	P[4] (IVa)	I2 (V)	R2 (V)	C2 (IV)	M2 (VII)	A2 (IVa)	N2 (V)	T2 (V)	E2* (VI)	H2 (IVa)
MOZ/0440/2013/G2P[4]	G2 (IVa-3)	P[4] (IVa)	I2 (V)	R2 (V)	C2 (IV)	M2 (VII)	A2 (IVa)	N2 (V)	T2 (V)	E2* (VI)	H2 (IVa)

*Genotype could not be identified with ViPR. The genotype identity of the closest relative in a BLASTn analyses was recorded. Phylogenetic clustering supported the BLASTn-determined genotype identities.

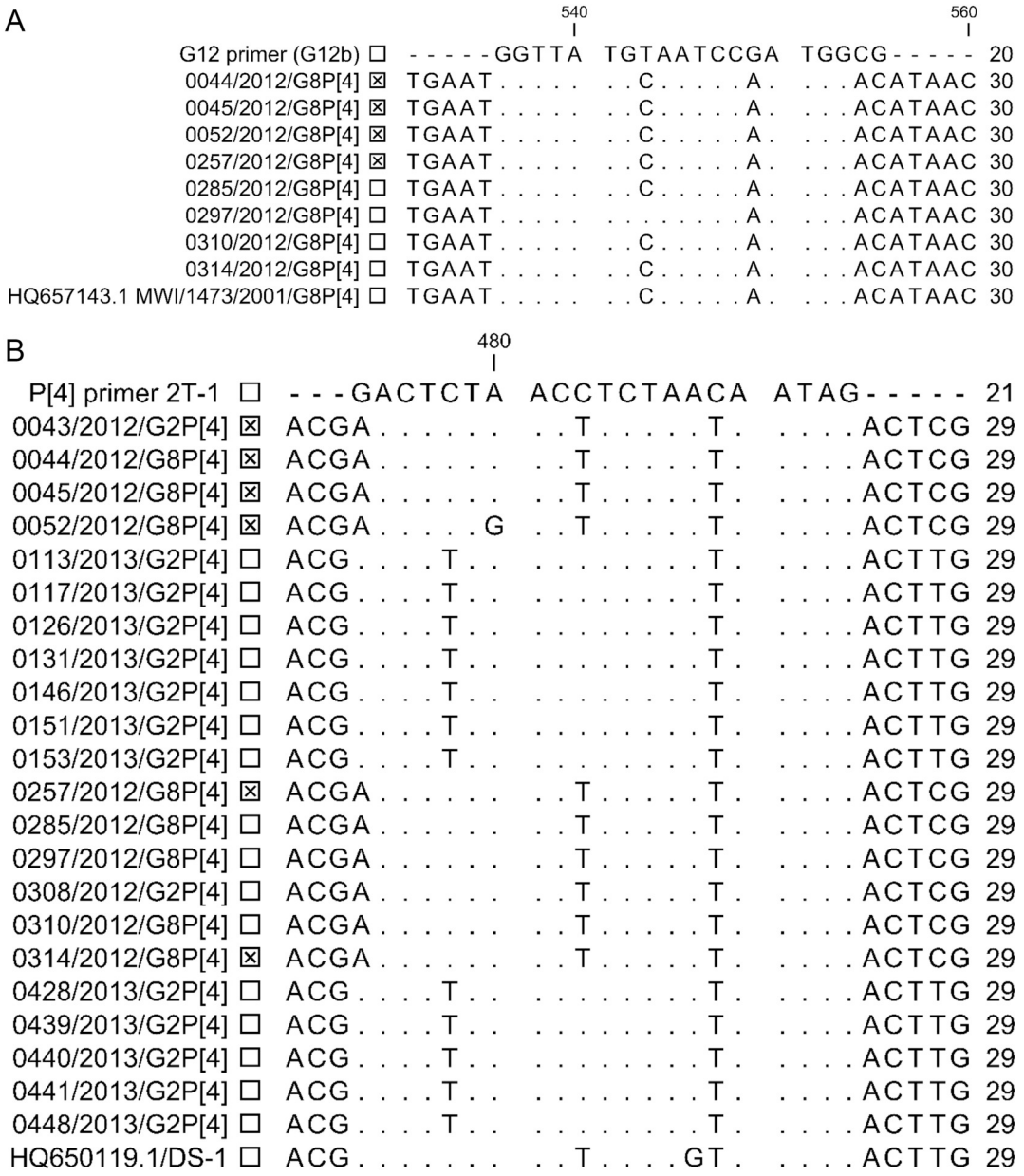

Fig. 1. A: Alignment of G12 specific genotyping primer (G12b) (Aladin et al., 2010a, 2010b) to Mozambican G8 sequences. Additional G8 strains previously identified with Sanger sequencing (João et al., 2018), were included in the analysis. Strains that were identified as mixed G12 and G8 samples are indicated with an X. Strain RVA/MWI/1473/2001/G8P[4] is used as a reference. Identical nucleotides are indicated with a dot. B: Alignment of P[4] specific genotyping primer (2T-1) (Gentsch et al., 1992) to Mozambican P[4] sequences. Strains that were not identified with genotyping PCR are indicated with an X. Strain RVA/USA/DS-1/1976/G2P[4] is used as a reference. Identical nucleotides are indicated with a dot. Additional P[4] strains previously identified with Sanger sequencing (João et al., 2018) were included in the analysis.

African G8P[4] and G2P[4] strains detected in South Africa and Zambia (RVA/Human-wt/ZMB/MRC-DPRU3463/2009/G8P[4], RVA/Human-wt/ZAF/MRC-DPRU2716/2008/G8P[4]P[8], RVA/Human-wt/ZAF/MRC-DPRU82/2012/G2P[4] and RVA/Pig-wt/ZAF/MRC-DPRU1533/2007/G2P[4]). An exception for strains 0044, 0257 and 0314 was that for segment 6 these strains grouped with a G2P[6] strain from Malawi (RVA/Human-wt/MWI/BID15I/2012/G2P[6]) (Table 2, Suppl Fig. 1). Strain 0045 had an average of 97.00% nucleotide similarity to the G8P[4] strains for segment 6, but a 98.44% with the 2013 G2P[4] strains, supporting the phylogenetic clustering. Additionally, 0044, 0257 and 0314 shared < 97.17% nucleotide similarity with all the G2P[4] strains for segment 6 (Suppl Table 2). Nucleotide analysis of the DS-1-like segment 6 sequences (phylogenetic analysis, *mVISTA* alignment, distance matrixes; Table 2, Fig. 2, Suppl Fig. 1 and Suppl Table 2), suggested difference between these segments and the question arises if reassortment from G2 (0043 and 0308) to G8 (0044, 0257 and 0314) required a different segment 6 (Table 2). However, aligning the amino acid sequences derived from the segment 6 sequences, indicated only two differences at positions 255 and 286 (Suppl Fig. 2). Additionally, these differences were not located at the interaction sites between VP7 and VP6 (data not shown).

Analyses based on nucleotide segments 1, 2, 3 and 6 indicated that strain 0045 clustered with two strains from India (RVA/Human-wt/IND/RV11/2009/G9P[4] and RVA/Human-wt/IND/Kol-065/2013/G9P[4]) and one strain from the United States (RVA/Human-wt/USA/LB1562/2010/G9P[4]) (Table 2, Suppl Fig. 1). This observation is also supported by the *mVISTA* alignment (Fig. 2) and in the distance matrixes where 0045 have a 94.21% similarity to the G8P[4] strains for

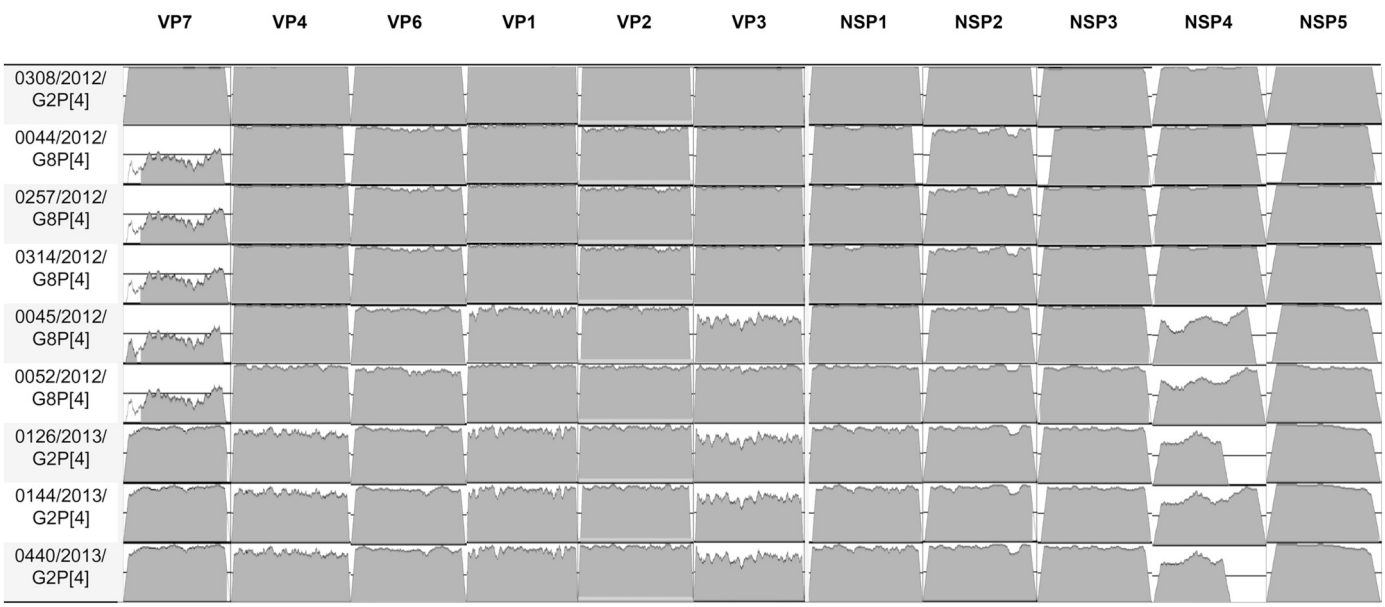

Fig. 2. *mVISTA* visualisation of an alignment of the Mozambican DS-1-like nucleotide sequences. The strains are compared to strain RVA/Human-wt/MOZ/0043/2012/G2P[4], which is not shown and the strains are listed in the same order as in Table 2.

segment 1 and 96.98% for segment 2 (Suppl Table 2). Strain 0052 (G8P[4]) clustered for all the genome segments with strains detected in Tanzania (RVA/Human-wt/TZA/MRC-DPRU4576/2010/G8P[4]), Uganda (RVA/Human-wt/UGA/MUL-13-427/2013/G8P[4]) and Kenya (RVA/Human-wt/KEN/MRC-DPRU1606/2009/G8P[4]).

4. Discussion

The present work characterizes the whole genome constellations of rotavirus strains detected in Mozambique. Ten rotavirus genome constellations were identified from Maputo city and Manhiça district, both located in the Maputo province in southern Mozambique between 2012 and 2013. Initial synthesis of cDNA was not sufficient to obtain full-length sequences of the genome segments, prompting the introduction of a self-priming anchor primer (Potgieter et al., 2009). Ligation of the PC3-T7loop primer resulted in full-length sequences for the majority of segments. Although eight genome segments were too short to be identified in ViPR, BLASTn results, and phylogenetic clustering indicated that these were six E2 and two H2 segments. Failure to obtain full-length sequences for these segments might be due to quality of the extracted RNA or viral load in the samples.

4.1. Failed genotyping PCR

Identification of rotavirus strains in surveillance studies are mainly done with genotyping PCR (Esona et al., 2010a, 2010b; Page et al., 2017; Seheri et al., 2017). Mistyping with genotyping methods has been reported before. These include failure to identify mixed infections (Jere et al., 2011; Nyaga et al., 2015), G8 strains identified as G12 strains (Aladin et al., 2010a), failure to identify P[8], P[6] and G1 strains (Esona et al., 2010a, 2010b; Iturriza-Gómara et al., 2000) and failure to detect G9, G10 and P[11] strains (Iturriza-Gómara et al., 2004).

The primer used to identify G12 genotypes (G12b) (Aladin et al., 2010b) is a cause for concern as this primer aligned to the G8 Mozambican strains close to the intended binding site for G12 sequences. With only four mismatching base pairs, this could be the reason for the four false positive results in the genotyping PCR. It is, however, not clear why only 50% of the sequences with the proposed G12 primer binding site was identified as G12 and others not (Fig. 1A). The original G12 specific genotyping primer (nt 548–567) was based on emerging G12 strains in southern India (Banerjee et al., 2007). The G12b primer (nt 504–524) was re-designed when G8 strains were mistyped as G12 strains during surveillance of the European Rotavirus Network (Aladin et al., 2010a). The results in this study suggest that the G12 specific genotyping primer should again be revised to prevent incorrect typing, specifically in southern Africa.

The inability of the genotyping PCR to identify seven of the Mozambican P[4] sequences may be due to a mismatch at the 5′ end. A similar problem was previously observed in identifying G8 strains with genotyping PCR (Aladin et al., 2010a). The mismatch might prevent proper annealing of the primer and subsequent amplification of the P[4] encoding sequences. Since the majority of the sequences with the A474G variation is found in Africa, revision of this 2T-1 primer (Gentsch et al., 1992) should be considered for rotavirus surveillance in Africa.

The sequencing data in this study disproved the mixed infections results of the genotyping PCR (João et al., 2018) in samples 0043, 0044, 0045, 0052, 0257 and 0314. It is therefore clear from the current study that the 11.7% rate of mixed infections reported by João and colleagues was over reported. The rates reported in the previous study correlated, however, with previously reported rates of mixed infections from the African continent (Mwenda et al., 2014; Seheri et al., 2014) raising the question whether mixed infections reported from other regions of the continent might also be over reported. It is therefore important to validate genotyping results with sequencing and to update the genotyping methods as required.

4.2. Reassortment in DS-1-like Mozambican strains

Phylogenetic analyses revealed diverse DS-1-like Mozambican rotavirus strains. No association was seen between rotavirus strains and the detection area, indicating that these strains were circulating in the whole region. The phylogeny clearly indicated that diverse G2P[4] strains circulated in 2012 (0043 and 0308) and 2013 (0126, 0144 and 0440). We previously reported a high detection rate for G2P[4] strains from these sites in 2013 (João et al., 2018). The three G2P[4] strains detected in 2013, clustered separately from the other Mozambican as well as most African strains with DS-1-like backbones. Their closest relatives were strains from the Philippines, Australia and Ghana, suggesting transmission between Africa and the East. The relationship with the Philippine strains was also previously observed for the 2013 detected G2P[4] strains during phylogenetic analysis of the VP8*

encoding gene (João et al., 2018).

The two Mozambican G2P[4] strains (0043 and 0308) detected in 2012, were closely related to a human and an animal strain detected in 2012 and 2007 (RVA/Human-wt/ZAF/MRC-DPRU82/2012/G2P[4] and RVA/Pig-wt/ZAF/MRC-DPRU1533/2007/G2P[4]), as well as a Malawian strain detected in 2012 (RVA/Human-wt/MWI/BID115/2012/G2P[4]). Strain MRC-DPRU82 was detected during a surveillance study in Eastern, Southern and Western Africa during 2007–2012 (Nyaga et al., 2014). In this study, the five G2P[4] strains (0043, 0308, BID115, MRC-DPRU1533 and MRC-DPRU82) also clustered with G8P[4] strains in lineage II of P[4] and not with the other G2P[4] strains in lineage IVa. These G8P[4] (0044, 0257 and 0314) and G2P[4] (0043 and 0308) strains also clustered together in the phylogenetic analyses based on segments 1, 3, 4, 5, 7, 10 and 11. Previously it was speculated that segment 4 of MRC-DPRU82 might be a reassortant (Nyaga et al., 2014) and similarly, the Mozambican strain 0308 (G2P[4]) was also thought to have the same reassortment (João et al., 2018). However, based on the phylogenetic relatedness of the genome segments, segment 4 of the 2012 G2P[4] strains is closer related to that of the G8P[4] strains than the 2013 G2P[4] strains. The closest relatives to these Mozambican strains (G8P[4] (0044, 0257 and 0314) and G2P[4] (0043 and 0308)) were detected in four countries that border Mozambique namely Malawi, Zambia, Zimbabwe and South Africa, as well as other regional countries: Uganda and Kenya in Eastern Africa. An interesting observation was that a change in G-type was accompanied with a change in segment 6. It is tempting to speculate that this was necessary for structural compatibility. The segment 6 nucleotide sequences of the Mozambican strains (G8P[4] (0044, 0257, 0314 and 0045) and G2P[4] (0043 and 0308)) are diverse as seen in the separate phylogenetic clustering and *mVISTA* alignment (Table 2, Fig. 2). However, the encoded VP6 proteins are highly conserved and would not be a structural requirement for the change in VP7 genotype.

Strain 0052 (G8P[4]) grouped in the same lineages as the other G8P[4] Mozambican strains (0044, 0257 and 0314), but formed smaller clusters with other cognate African strains from Tanzania, Kenya and Uganda. For segment 10, strains 0052 and 0045 clustered separate from lineage VII which contains the remaining G8P[4] strains (Doan et al., 2015). This group of African G8P[4] strains, including 0045 and 0052, as well as RVA/Human-tc/KEN/AK26/1982/G2P[4] have not yet been assigned a lineage. The DS-1-like segment 10 has been shown to have greater sequence diversity compared to the other RV genome segments (Nyaga et al., 2014) with evidence of continuous diversification of this genotype (Agbemabiese et al., 2016). The segment 10 sequences determined in this study support these previous observations as seen in the phylogenetic analysis, *mVISTA* alignment and distance matrixes.

Mozambican strain 0045 (G8P[4]) grouped with G9P[4] strains during analyses based on segments 1 (VP1), 2 (VP2), 3 (VP3) and 6 (VP6). Two of these G9P[4] strains were detected in India in 2011 and 2013 (Doan et al., 2017) and one was detected in the USA in 2010 (Lewis et al., 2014). This might indicate that the genome segments encoding the double-layered particle of strain 0045 (G8P[4]) reassorted with strains similar to G9P[4] strains. The Indian strains were described with complex genome constellations, likely carrying reassortment events of segments 5 (NSP1), 9 (VP7) and 10 (NSP4) and highlighted the spread of unusual rotavirus strains in Asia (Doan et al., 2017). Interestingly, these strains grouped in lineage VII of segment 3 with the 2012 G2P[4] strains. Based on the whole genome analysis, it is clear that the study strains contain closely related DS-1-like backbones probably differentiated by multiple reassortment events. These results are similar to a study of G8 strains, detected over a ten-year period in Malawi, which indicated reassortment between co-circulating human DS-1-like rotavirus strains (Nakagomi et al., 2013).

5. Conclusion

Whole genome characterisation through the Illumina MiSeq next generation sequencing platform was used to determine the whole genome constellations using cDNA prepared from dsRNA that was extracted from rotavirus-positive stool samples without prior genome amplification or adaptation to cell culture. Diverse DS-1-like strains were detected at two hospitals during 2012 and 2013 in the south of Mozambique. Two distinct G2P[4] strains were identified and possible reassortment events, specifically between 2012 G2P[4] and G8P[4] strains, were observed. Furthermore, segment 6 of strains 0044, 0257 and 0314 as well as segments 1, 2, 3, and 6 of strain 0045 are possible reassortants. The study also showed that the rate of mixed infections in the preceding study was over reported due to false PCR genotyping results. The genotype specific primers used in this study for G12 and P[4] detection, might need revision. These results highlight the necessity to determine whole genome constellations to confirm surveillance data in Africa and to keep track of the growing diversity in DS-1-like strains.

Ethical approval

Informed consent was obtained from the parents or guardians of the children and the ethical principles of Helsinki Declaration guided all procedures. The study was approved by the National Committee on Bioethics from Mozambique in 2010 (reference N°286/CNBS/10, IRB 00002657) and by the Ethical Committee of University of Free State (UFS) (201/2013).

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