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Characteristics of a pandemic clone of O3 : K6 and O4 : K68 *Vibrio parahaemolyticus* isolated in Beira, Mozambique

M. Ansaruzzaman,[1] Ashrafuzzaman Chowdhury,[1] Nurul A. Bhuiyan,[1] Marzia Sultana,[1] Ashrafus Safa,[1] Marcelino Lucas,[2] Lorenz von Seidlein,[3] Avertino Barreto,[2] Claire-Lise Chaignat,[4] David A. Sack,[1]† John D. Clemens,[3] G. Balakrish Nair,[1]‡ Seon Young Choi,[3,5] Yoon Seong Jeon,[3,5,6] Je Hee Lee,[3,5] Hye Ri Lee,[3] Jongsik Chun[3,5,6] and Dong Wook Kim[3]

Correspondence
Dong Wook Kim
dwkim@ivi.int

[1]ICDDR,B (International Centre for Diarrhoeal Disease Research, Bangladesh), Mohakhali, Dhaka 1212, Bangladesh

[2]Ministry of Health, Maputo, Mozambique

[3]International Vaccine Institute, Seoul, Republic of Korea

[4]World Health Organization, Geneva, Switzerland

[5]School of Biological Sciences, Seoul National University, Seoul, Republic of Korea

[6]Interdisciplinary Program of Bioinformatics, Seoul National University, Seoul, Republic of Korea

The genetic characteristics of *Vibrio parahaemolyticus* strains isolated in 2004 and 2005 in Mozambique were assessed in this study to determine whether the pandemic clone of *V. parahaemolyticus* O3 : K6 and O4 : K68 serotypes has spread to Mozambique. Fifty-eight *V. parahaemolyticus* strains isolated from hospitalized diarrhoea patients in Beira, Mozambique, were serotyped for O : K antigens and genotyped for *toxR*, *tdh* and *trh* genes. A group-specific PCR, a PCR that detects the presence of ORF8 of the filamentous phage f237, arbitrarily primed PCR, PFGE and multilocus sequence typing were performed to determine the pandemic status of the strains and their ancestry. All strains of serovars O3 : K6 (n=38) and O4 : K68 (n=4) were identified as a pandemic clonal group by these analyses. These strains are closely related to the pandemic reference strains of O3 : K6 and O4 : K68, which emerged in Asia in 1996 and were later found globally. The pandemic serotypes O3 : K6 and O4 : K68 including reference strains grouped into a single cluster indicating emergence from a common ancestor. The O3 : K58 (n=8), O4 : K13 (n=6), O3 : KUT (n=1) and O8 : K41 (n=1) strains showed unique characteristics different from the pandemic clone.

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INTRODUCTION

Vibrio parahaemolyticus is a seafood-borne pathogen that can cause gastroenteritis in humans. It is a Gram-negative, halophilic bacterium inhabiting marine and estuarine environments. *V. parahaemolyticus* was first isolated in Japan in 1950 as a cause of food-borne illness. To date, 75 different combinations of the O and K serotypes of *V. parahaemolyticus* have been recognized (Nasu *et al.*, 2000). Beginning in February 1996, a newly recognized clone of *V. parahaemolyticus* of the O3 : K6 serovar was found to be responsible for a dramatic increase in the number of diarrhoeal cases in Calcutta, India (Okuda *et al.*, 1997). Analysis of these strains by molecular methods revealed that a unique clone with an identical genotype and arbitrary primed PCR (AP-PCR) profile, not previously isolated during the surveillance in Calcutta, accounted for 50–80 % of the infections. All of the post-1995 O3 : K6 strains were genetically indistinguishable and showed

†Present address: Johns Hopkins Bloomberg School of Public Health, Baltimore, MD, USA.

‡Present address: National Institute of Cholera and Enteric Diseases, Kolkata, India.

Abbreviations: AP-PCR, arbitrarily primed PCR; GS-PCR, group-specific PCR; MLST, multilocus sequence typing; ST, sequence type; UPGMA, unweighted-pair grouping with mathematical averaging.

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clonality by AP-PCR, ribotyping, PFGE and multilocus sequence typing (MLST), but they were significantly different from the genetically variable pre-1995 O3 : K6 strains (Chowdhury *et al.*, 2004; Matsumoto *et al.*, 2000; Okuda *et al.*, 1997). Since the finding of the clonal O3 : K6, serotypes O4 : K68, O1 : K25 and O1 : KUT have been recognized as having the same genetic characteristics as the pandemic O3 : K6 strains, and currently 21 serotypes are reported to belong to the same genetic lineage of pandemic O3 : K6 and are believed to have emerged from a single origin (Nair *et al.*, 2007).

No information on *V. parahaemolyticus* infections in Mozambique or sub-Saharan Africa was available until 2004. During surveillance for cholera in Beira, Mozambique, from December 2003 to January 2004, a large number of *V. parahaemolyticus* infections were reported (Ansaruzzaman *et al.*, 2005). In this report, 32 isolates of O3 : K6 and two isolates of O4 : K68 serotype were compared with six isolates of O3 : K58 and one isolate of O4 : K13 serotype of *V. parahaemolyticus*. Using group-specific (GS)-PCR and a PCR that detects the presence of ORF8 of the filamentous phage f237, the presence of the pandemic *V. parahaemolyticus* in sub-Saharan Africa was described (Ansaruzzaman *et al.*, 2005). In the present study, we investigated the pandemic specificity and molecular diversity (using AP-PCR, PFGE and MLST) of these Mozambican *V. parahaemolyticus* strains including these 2004 isolates and a further 16 isolates collected in 2005 (six isolates of O3 : K6, five isolates of O4 : K13, two isolates of O3 : K58, two isolates of O4 : K68 and one isolate of O8 : K41) to determine whether these strains shared a common lineage with the pandemic strains that emerged globally after the first reports in 1996 from India.

METHODS

Bacterial strains. A total of 58 suspected *V. parahaemolyticus* isolates (n=42 in 2004 and n=16 in 2005) were grown as pure cultures on taurocholate tellurite gelatin agar and thiosulphate citrate bile salt sucrose agar (Eiken) plates. Isolates with a characteristic colony morphology were transported to ICDDR,B: Centre for Health and Population Research, Dhaka, Bangladesh, and identified as *V. parahaemolyticus* by standard methods (Ansaruzzaman *et al.*, 2005). For comparison of clonal variation, reference strains of *V. parahaemolyticus* serotype O3 : K6 (ATCC BAA-239) and O4 : K68 (ATCC BAA-241) isolated in 1996 and 1998, respectively, in India were included (Matsumoto *et al.*, 2000). Pandemic strains of *V. parahaemolyticus* of serotype O3 : K6 (AN7410 isolated in 1998 from Bangladesh) and serotype O4 : K68 (AN11790 isolated in 1998 from Bangladesh) characterized previously were also included for comparison (Bhuiyan *et al.*, 2002). *Salmonella* Braenderup strain H9812 was used as a molecular size marker (Hunter *et al.*, 2005). All *V. parahaemolyticus* strains were grown in Luria–Bertani broth with 1 % NaCl.

O : K serotyping. The O (somatic) and K (capsular) antigenic formula of confirmed *V. parahaemolyticus* isolates was determined using commercial antisera (Toshiba Kagaku Kogyo) in accordance with manufacturer's instructions.

PCR methods. PCR assays testing for *toxR*, *tdh* and *trh* genes and GS-PCR and ORF8 PCR for pandemic markers were performed as described previously (Ansaruzzaman *et al.*, 2005). AP-PCR was performed as described previously by Matsumoto *et al.* (2000). Briefly, 25 ng purified total DNA, 25 pmol primer 4, 2.5 U *Taq* polymerase (ExTaq; Takara), 10 × buffer containing 20 mM $MgCl_2$, and 0.125 mM each dNTP were used for each amplification reaction. The amplification conditions were one cycle of 95 °C for 4 min, followed by 45 cycles of denaturation at 95 °C for 1 min, annealing at 36 °C for 1 min and extension at 72 °C for 2 min, followed by a final extension at 72 °C for 7 min. The PCR product was visualized by electrophoresis in a 1.2 % agarose gel and documented using a GelDoc system (Bio-Rad Laboratories).

PFGE. Intact agarose-embedded chromosomal DNA from clinical isolates of *V. parahaemolyticus* was prepared and PFGE was performed with the *Not*I restriction enzyme using a contour-clamped homogeneous electric field (CHEF-DRII) apparatus (Bio-Rad Laboratories) according to procedures described recently (Cooper *et al.*, 2006). Band patterns were established by the criteria described previously (Tenover *et al.*, 1995). A *Salmonella* Braenderup reference standard strain, H9812, was used as a molecular size marker to produce the dendrogram. The clonal relatedness among the Mozambique strains was determined using the Dice coefficient, and cluster analysis was carried out using BioNumerics software (Applied Maths) using unweighted-pair grouping with mathematical averaging (UPGMA).

MLST. MLST analysis of *V. parahaemolyticus* isolates was performed as described by González-Escalona *et al.* (2008). The DNA fragments of seven loci (*dnaE*, *gyrB*, *recA*, *dtdS*, *pntA*, *pyrC* and *tnaA*) were amplified and sequenced. The DNA sequence of each allele was compared with the corresponding allele in the public MLST database (http://pubmlst.org), with allele type numbers consequently being assigned. Newly identified sequences were deposited in GenBank and given a consecutive allele type number. A new sequence type (ST) number was assigned to an allele profile containing a new allele type and/or a new combination of allele profile.

RESULTS AND DISCUSSION

Numerous *V. parahaemolyticus* infections were identified during a diarrhoea surveillance study in Beira, Mozambique, during 2004–2005, revealing that the pandemic strains of *V. parahaemolyticus* have spread to the African continent (Ansaruzzaman *et al.*, 2005). In this study, we analysed 38 isolates of serotype O3 : K6, four isolates of serotype O4 : K68 and 16 isolates of non-pandemic serotypes from diarrhoeal patients in Beira in 2004 and 2005. The majority (72 %) of the isolates collected in the study belonged to the pandemic serotypes O3 : K6 and O4 : K68, a finding similar to results from other studies (Gil *et al.*, 2007; Nair *et al.*, 2007).

Identification of pandemic strains of *V. parahaemolyticus*

In 2004, 32 isolates of O3 : K6, two isolates of O4 : K68, six isolates of O3 : K58, one isolate of O3 : KUT and one isolate of O4 : K13 serotypes of *V. parahaemolyticus* were collected (Ansaruzzaman *et al.*, 2005). Of the 16 isolates collected in 2005, six were serotype O3 : K6, two were O4 : K68, five

were O4 : K13, two were O3 : K58 and one was O8 : K41. All isolates of O3 : K6 and O4 : K68 were positive for *toxR* and *tdh*, GS-PCR and ORF8 PCR, but negative for *trh*, which are the characteristics of the pandemic clones (Table 1). All other serotypes were negative for GS-PCR and ORF8 PCR.

The AP-PCR patterns of representative strains of different serotypes are shown in Fig. 1. All isolates of serotypes O3 : K6 and O4 : K68 isolated in two consecutive years, 2004 and 2005, showed an identical AP-PCR pattern designated 'a' (Fig. 1). Reference strains of pandemic serotype O3 : K6 (ATCC BAA-239 and AN7410) and serotype O4 : K68 (ATCC BAA-241 and AN11790) showed an identical 'a' AP-PCR pattern. Isolates belonging to the non-pandemic serotypes O3 : K58 (n=8), O4 : K13 (n=6) and O8 : K41 (n=1) collected in the same period showed three different AP-PCR patterns, designated 'b', 'c' and 'd', unrelated to AP-PCR pattern 'a' (Fig. 1). This result indicated that serotype O3 : K6 and O4 : K68 isolates collected in this area belong to the pandemic clone of *V. parahaemolyticus*, whilst other serotype isolates are non-pandemic strains.

PFGE

The electrophoresis migration pattern of *Not*I-digested fragments of the chromosomal DNA of each of the 58 strains of *V. parahaemolyticus* was obtained by PFGE. Analysis of PFGE patterns revealed that the outbreak strains from Mozambique could be divided into five types, A, B, C, D and E, with few subtypes (Table 1). Different PFGE types were denoted by capital letters and those non-identical types (one to three band differences) belonging to the same type were denoted by the same capital letter followed by different numbers.

All 38 strains of serotype O3 : K6 isolated in 2004 (n=32) and 2005 (n=6) belonged to PFGE type A2 and were identical to the PFGE pulsotype of reference strain AN7410 (Fig. 2). PFGE type A2 of Mozambique O3 : K6 and strain AN7410 differed in PFGE band pattern from PFGE type A1 of reference strain ATCC BAA-239 by two bands (Fig. 2). All of the strains of serotype O4 : K68 belonged to PFGE type C1. All O4 : K68 isolates from Mozambique showed an identical PFGE pattern C1 to reference strains of serotype O4 : K68, ATCC BAA-241 and AN11790 (Fig. 2). PFGE pulsotype C1 and PFGE pulsotype A1 are closely related and could be clustered in the same group along with pulsotype A2 as shown in Fig. 3. Isolates of serotype O3 : K58 (n=8) produced either PFGE types B1 or B2 subtypes, differing by only a few bands from each other, but differed considerably from the PFGE pattern of serotypes O3 : K6 and O4 : K68. All strains of serotype O4 : K13 (n=6) and O8 : K41 (n=1) belonged to PFGE types E and D, respectively, which are not related to either PFGE type A of serotype O3 : K6 or PFGE type C of serotype O4 : K68 (Fig. 2).

The degree of similarity was calculated using the Dice coefficient. The similarity matrix was converted into a dendrogram by BioNumerics software using UPGMA. The isolates of serotypes O3 : K6 and O4 : K68 including reference strains grouped into a single cluster denoted as cluster 1 (Fig. 3). The serotypes O3 : K58 and O4 : K13 belonged to clusters 2 and 3, respectively, whereas the serotype O8 : K41 was grouped into cluster 3, but the degree of similarity was different from serotype O4 : K13. On a scale of similarity ranging from 0 to 1, cluster 1 belonging to the pandemic serotypes showed >0.82 similarity among all strains. The majority of these strains were identical to each other, with 0 standing for total

Table 1. Serotype and genetic traits of *V. parahaemolyticus* isolated during 2004–2005 in Beira, Mozambique, compared with those of reference strains

O : K serotype	Year of isolation	No. of strains	Presence of genes			Pandemic marker		PFGE/AP-PCR type
			toxR	*tdh*	*trh*	GS-PCR	ORF8 PCR	
O3 : K6	2004	32	+	+	−	+	+	A2/a
O3 : K6	2005	6	+	+	−	+	+	A2/a
O3 : K58	2004	6	+	+	−	−	−	B1, B2/b
O3 : K58	2005	2	+	+	−	−	−	B1, B2/b
O4 : K68	2004	2	+	+	−	+	+	C1/a
O4 : K68	2005	2	+	+	−	+	+	C1/a
O4 : K13	2004	1	+	+	−	−	−	E/d
O4 : K13	2005	5	+	+	−	−	−	E/d
O8 : K41	2005	1	+	+	−	−	−	D/c
O3 : KUT	2004	1	+	+	−	−	−	ND*
O3 : K6 ATCC BAA-239	1996	1	+	+	−	+	+	A1/a
O3 : K6 AN7410	1998	1	+	+	−	+	+	A2/a
O4 : K68 ATCC BAA-241	1998	1	+	+	−	+	+	C1/a
O4 : K68 AN11790	1998	1	+	+	−	+	+	C1/a

*ND, Not determined.

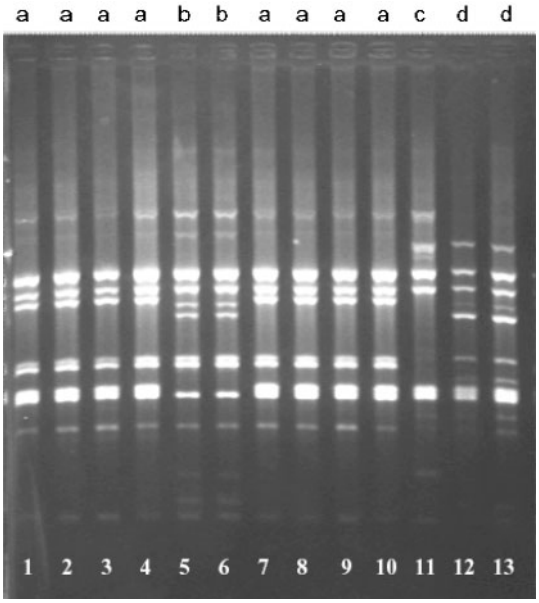

Fig. 1. AP-PCR pattern of *V. parahaemolyticus* isolated in Mozambique. Lanes: 1, O3 : K6 (ATCC BAA-239); 2, O3 : K6 (AN7410); 3, O3 : K6 (Mozambique, 2004); 4, O3 : K6 (Mozambique, 2005); 5, O3 : K58 (Mozambique, 2004); 6, O3 : K58 (Mozambique, 2005); 7, O4 : K68 (ATCC BAA-241); 8, O4 : K68 (AN11790); 9, O4 : K68 (Mozambique, 2004); 10, O4 : K68 (Mozambique, 2005); 11, O8 : K41 (Mozambique, 2005); 12 and 13, O4 : K13 (Mozambique, 2004–2005).

dissimilarity and 1 standing for complete uniformity. The other two clusters, cluster 2 of serotype O3 : K58 and cluster 3 of serotype O4 : K13, showed <0.45 similarity, indicating a different lineage from that of the major cluster 1 (Fig. 3). Notably, the strains of serotype O4 : K13 in cluster 3 were identical but differed markedly from serotype O8 : K41, whereas the strains of cluster 2 belonging to serotype O3 : K58 were very closely related to each other.

MLST analysis

Eight clinical isolates and the reference strains (ATCC BAA-239 and AN7410) of serotype O3 : K6 were sequenced for all seven loci of the MLST analysis, and the remaining 30 O3 : K6 isolates were sequenced for two loci (*recA* and *tnaA*). Two reference strains of O4 : K68 (ATCC BAA-241 and AN11790) and three clinical isolates of O4 : K68, and all of the clinical isolates of O3 : K58 ($n=8$), O4 : K13 ($n=6$) and O8 : K41 ($n=1$) were analysed for all seven loci. Two more reference strains, ATCC BAA-238 of serotype O3 : K6 and ATCC BAA-242 of serotype O1 : KUT (Chowdhury *et al.*, 2000), belonging to the pandemic clone were also analysed by MLST. One clinical isolate of serotype O3 : KUT, which was not analysed by AP-PCR or PFGE, was also subjected to MLST analysis. The results of the MLST analysis are shown in Table 2.

All of the O3 : K6 and O4 : K68 clinical isolates and reference strains possessed the same MLST sequence type (ST3, allele profile of 3, 4, 19, 4, 29, 4 and 22 in the order *dnaE*, *gyrB*, *recA*, *dtdS*, *pntA*, *pyrC* and *tnaA*), which is the most common pandemic clonal ST (González-Escalona *et al.*, 2008). All 30 clinical isolates of serotype O3 : K6 sequenced for *recA* and *tnaA* loci contained the same allele type of *recA19* and *tnaA22* as the pandemic clonal ST3, which indicates that they also belong to the pandemic clonal ST. One reference strain of the O1 : KUT serotype (ATCC BAA-242) also contained the same ST as the other pandemic clonal strains. Although the PFGE patterns of these serotypes varied slightly, AP-PCR patterns and ST were identical among the O3 : K6 and O4 : K68 strains. This

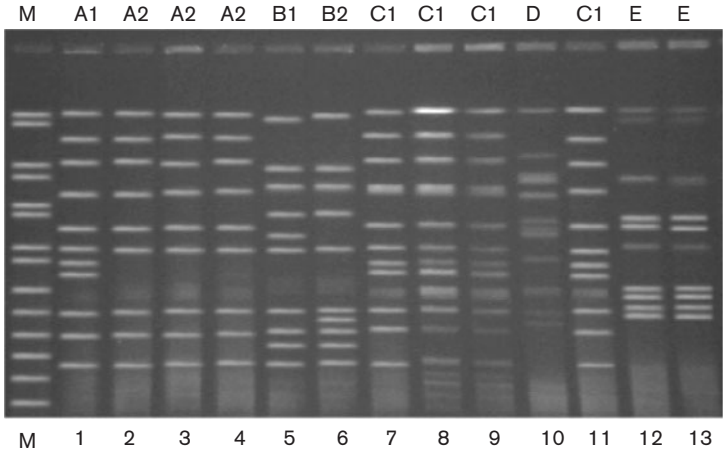

Fig. 2. PFGE profile of the genomic DNA of *V. parahaemolyticus* isolates from Mozambique and reference strains belonging to different pulsotypes digested with *Not*I. Lanes: M, molecular marker, *Salmonella* Braenderup; 1, O3 : K6 (ATCC BAA-239) of pulsotype A1; 2, O3 : K6 (AN7410) of pulsotype A2; 3, O3 : K6 (Mozambique, 2004) of pulsotype A2; 4, O3 : K6 (Mozambique, 2005) of pulsotype A2; 5, O3 : K58 (Mozambique, 2004) of pulsotype B1; 6, O3 : K58 (Mozambique, 2005) of pulsotype B2; 7, O4 : K68 (ATCC BAA-241) of pulsotype C1; 8, O4 : K68 (AN11790) of pulsotype C1; 9, O4 : K68 (Mozambique, 2004) of pulsotype C1; 10, O8 : K41 (Mozambique, 2005) of pulsotype D; 11, O4 : K68 (Mozambique, 2005) of pulsotype C1; 12 and 13, O4 : K13 (Mozambique, 2004–2005) of pulsotype E.

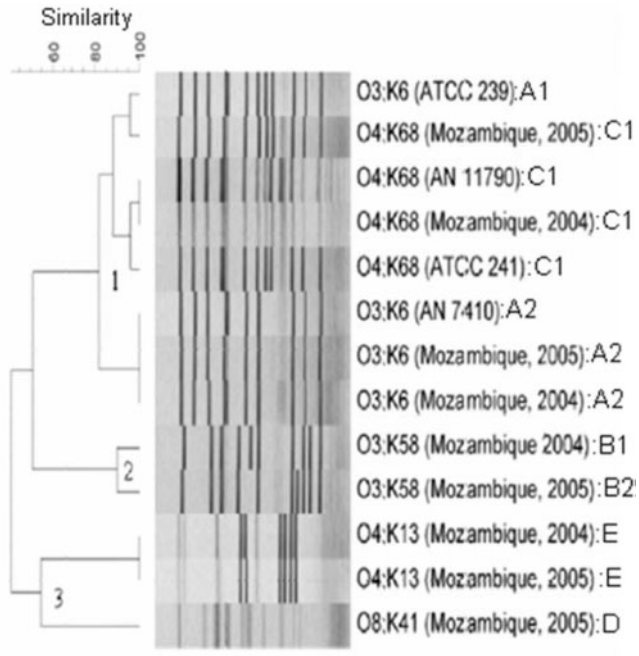

Fig. 3. Dendrogram derived from PFGE showing representative *V. parahaemolyticus* strains of different serotypes using *Salmonella* Braenderup as the standard. The dendrogram was constructed using BioNumerics software. The year and place of isolation, O : K serotype, pulsotype and designation of each cluster are shown in the figure.

result confirmed that the various serotypes of the pandemic *V. parahaemolyticus* originate from a common ancestor.

Eight isolates of the O3 : K58 serotype contained the allele profile 42, 25, 3, 40, 35, 38 and 31, which is the first identification of this sequence type, designated ST66. Two PFGE types were shown in this serotype, but their AP-PCR type and MLST type were the same. All of the O4 : K13 serotype isolates had the same allele profile of 41, 40, 36, 41, 36, 39 and 32 to which the designation ST68 was given. The PFGE pattern and AP-PCR type of O4 : K13 were also the same and showed identity in cluster analysis (Fig. 3). The O8 : K41 serotype isolate had an allele profile of 43, 41, 31, 42, 37, 40 and 33 and the O3 : KUT serotype isolate possessed 44, 42, 35, 29, 26, 41 and 13. ST69 and ST67 were designated to these allele profiles, respectively. It was evident that these isolates did not belong to the pandemic clone. Non-pandemic strains contained STs distinguishable from those of the pandemic clonal strains and these results coincide with the results of other studies (González-Escalona *et al.*, 2008; Nair *et al.*, 2007). The four new STs (ST66–ST69) have been deposited in the *V. parahaemolyticus* MLST database (http://pubmlst.org/vparahaemolyticus).

Our investigation demonstrates the spread of the pandemic clones O3 : K6 and O4 : K68 of *V. parahaemolyticus* in Beira, Mozambique. The results also showed that the pandemic clonal strains isolated in Mozambique are closely related to pandemic reference strains O3 : K6 and O4 : K68, which first emerged in Asian countries in 1996. Most of the non-pandemic strains (O3 : K58 and O4 : K13), however, showed significantly different genetic characteristics (AP-PCR, PFGE type and MLST type) from those of the universal clone of the pandemic serotype. One isolate of each of serotype O8 : K41 and O3 : KUT found in this study was different in ST by MLST analysis from the pandemic clone and should be considered as non-pandemic strains. How and when the pandemic strains arrived in this remote area of Mozambique remains uncertain. We suggest that, as the emergence of such pandemic strains of *V. parahaemolyticus* may influence future strategies for controlling diarrhoea in Mozambique, continuous monitoring of the epidemic strains will be crucial.

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Table 2. MLST allelic profile of *V. parahaemolyticus* analysed in this study

Serotype (no. of isolates analysed)	*dnaE*	*gyrB*	*recA*	*dtdS*	*pntA*	*pyrC*	*tnaA*	ST
O3 : K6 (8)	3	4	19	4	29	4	22	3
O4 : K68 (3)	3	4	19	4	29	4	22	3
O3 : K58 (8)	42	25	3	40	35	38	31	66
O4 : K13 (6)	41	40	36	41	36	39	32	68
O8 : K41 (1)	43	41	31	42	37	40	33	69
O3 : KUT (1)	44	42	35	29	26	41	13	67
O3 : K6 (ATCC BAA-239)	3	4	19	4	29	4	22	3
O3 : K6 (AN7410)	3	4	19	4	29	4	22	3
O3 : K6 (ATCC BAA-238)	3	4	19	4	29	4	22	3
O4 : K68 (ATCC BAA-241)	3	4	19	4	29	4	22	3
O4 : K68 (AN11790)	3	4	19	4	29	4	22	3
O1 : KUT (ATCC BAA-242)	3	4	19	4	29	4	22	3

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