Prevalence of *Vibrio cholerae* O1 El Tor variant in a cholera-endemic zone of Kenya

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Since 2007, Kenya has experienced an increase in cholera outbreaks characterized by a high fatality rate. In this study, we characterized 81 *Vibrio cholerae* isolates from diarrhoeal stool samples in Nyanza, a cholera-endemic lake region of Kenya, for virulence properties, clonality and antibiotic susceptibility. Eighty of these isolates were *V. cholerae* O1 El Tor variants carrying the classical *ctxB* gene sequence, while one isolate was *V. cholerae* non-O1/O139. All of the El Tor variants were of clonal origin, as revealed by PFGE, and were susceptible to ampicillin, tetracycline, ciprofloxacin, fosfomycin, kanamycin and norfloxacin. However, the isolates showed resistance to sulfamethoxazole/trimethoprim and streptomycin, and intermediate resistance to nalidixic acid, chloramphenicol and imipenem. The non-O1/O139 isolate carried the cholix toxin II gene (*chxA* II) and was susceptible to all antimicrobials tested except ampicillin. We propose that an El Tor variant clone caused the Nyanza cholera outbreak of 2007–2008.

INTRODUCTION

Cholera is one of the most devastating diseases encountered by humans. The disease, which is characterized by severe watery diarrhoea and loss of body fluids, is caused by a Gram-negative bacterium, *Vibrio cholerae* (Bentivoglio & Pacini, 1995). There are more than 200 serogroups of *V. cholerae*, but only O1 and O139 are known to cause cholera pandemics (Kaper *et al.*, 1995). *V. cholerae* O1 has been classified into two biotypes, El Tor and classical. At least the fifth and sixth cholera pandemics were caused by the classical biotype, with the seventh pandemic caused by the El Tor biotype, which originated on the Celebes islands of Indonesia in 1961 and gradually replaced classical strains. The seventh pandemic initially spread to other parts of Asia and reached Africa in the 1970s (Kaper *et al.*, 1995).

Africa was noted to have a greater upsurge in cholera outbreaks than other continents. For example, between 1995 and 2005, 417 out of a total global report of 632 outbreaks occurred in Africa (Griffith *et al.*, 2006). The total number of cases in Africa was 423 904, which made up 87.6 % of the global total of 484 246 cases (Griffith *et al.*, 2006). Another upsurge in cholera outbreaks occurred between 2006 and 2010 (WHO, 2006, 2010). Again, more cases were reported in Africa than other parts of the globe. The magnitude of these outbreaks led to speculation that cholera may have found a new homeland in Africa (Gaffga *et al.*, 2007). The impact of cholera was augmented by the effects of global weather change (Emch *et al.*, 2008), natural disasters such as floods (de Magny *et al.*, 2012) and political instability, which led to the mushrooming of refugee camps in various part of Africa (Hatch *et al.*, 1994; Iijima *et al.*, 1995; Shultz *et al.*, 2009). In additional to the increased rate of outbreaks, the case fatality rate in Africa remained above 1.0 %, with some African countries experiencing case fatality rates as high as 12 % (WHO, 2009). The severity of cholera can also be attributed to more virulent *V. cholerae* O1 genotypes (Ghosh-Banerjee *et al.*, 2010).

Since 2007, Kenya has experienced cholera outbreaks characterized not only by the size of the epidemics but also by increased mortality rates (WHO, 2008). A cholera outbreak that started in Nyanza in November 2007 had

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Abbreviations: MAMA, mismatch amplification mutation assay; SXT, sulfamethoxazole/trimethoprim; T3SS, type III secretion system.

The GenBank/EMBL/DDBJ accession number for the *chxA* gene sequence of non-O1/O139 is AB856482.

One supplementary table and one supplementary figure are available with the online version of this paper.

claimed 67 lives out of 1243 cases by April 2008 (WHO, 2010). Previous cholera outbreaks in Nyanza were not as severe as the 2007 outbreak (Shapiro *et al.*, 1999; Mugoya *et al.*, 2008). Shikanga *et al.* (2009) have also reported on the severity of the 2007 cholera outbreak in Kenya. In addition to antimicrobial susceptibility testing and clonal analysis by PFGE, this study also characterized *V. cholerae* isolates for pathogenicity-determinant genes including those encoding cholera toxin (*ctx*), toxin co-regulated pilus (*tcpA*), heat-stable enterotoxin (*nag*-ST), type III secretion system (T3SS), cholix toxin (*chxA*) and hybrid or variant markers.

METHODS

***V. cholerae* isolates.** *V. cholerae* isolates characterized in this study were obtained from a cholera outbreak that occurred from November 2007 to July 2008 in the former Nyanza province of Kenya (Shikanga *et al.*, 2009). Stool samples were randomly collected from 81 patients with severe diarrhoea who were suspected of having cholera and who were attending five different dispensaries in adjacent villages in a rural part of western Nyanza in May 2008. Diarrhoeal stool samples were cultured by enrichment in alkaline peptone water (Nissui) followed by 6 h incubation at 37 °C. The cultures were plated on thiosulfate citrate bile salts sucrose agar (Eiken). The plates were incubated at 37 °C for 18–24 h. Typical yellow colonies, which were presumed to be *V. cholerae*, were subjected to biochemical, serological and genotypic analyses.

Biochemical and phenotypic analysis. The yellow colonies growing on the agar plates were subcultured in Luria–Bertani agar for further tests, which included oxidase, lysine decarboxylase and arginine dehydrogenase tests, string test, haemolysis, reactions in Kligler's iron agar and triple-sugar iron agar (http://www.cdc.gov/cholera/laboratory.html) (WHO, 2003). Serogrouping of *V. cholerae* isolates was carried out using the slide agglutination method (WHO, 2003). Polyvalent sera for *V. cholerae* O1 and O139 surface antigens were used for serogrouping (antiserum for *V. cholerae* O1 was purchased from Denka Seiken). Haemolysin activity was detected by streaking part of a colony on tryptic soy agar (Nissui) supplemented with 7.0 % sheep blood followed by incubation of the plate at 37 °C for 24 h. A chicken erythrocyte agglutination test was performed by standard methods (Sakazaki & Shimada, 1986). *V. cholerae* O1 strains N16961 (El Tor) and O395 (classical) were used as controls in the above tests.

Susceptibility testing to polymyxin B (50 U) was carried out with the disc diffusion method (Bauer *et al.*, 1966) using Mueller–Hinton agar (Difco). The zone of inhibition was observed after overnight incubation at 35 °C. *Escherichia coli* ATCC 25922 was used as a control strain. For polymyxin B, an inhibition zone of $\geqslant$12 mm was interpreted as susceptible and $\leqslant$11 mm as resistant. Similarly, susceptibility testing was performed for other antimicrobials and the size of the inhibition zone was interpreted as susceptible, intermediate or resistant according to guidelines provided by the Clinical and Laboratory Standards Institute (CLSI, 2011). The following antimicrobials were used: sulfamethoxazole/trimethoprim (SXT) (1.25/23.75 μg), fosfomycin (50 μg), ampicillin (10 μg), imipenem (10 μg), chloramphenicol (30 μg), tetracycline (30 μg), norfloxacin (10 μg), nalidixic acid (30 μg), streptomycin (10 μg), kanamycin (30 μg), cefotaxime (30 μg) and ciprofloxacin (5 μg) (all Becton Dickinson). *E. coli* ATCC 25922 was used as a standard test organism. Interpretation of the zone of inhibition size was based on antimicrobial susceptibility for bacterial cells belonging to the family *Enterobacteriaceae*.

Genotyping by PCR. Multiplex PCR targeting the specific somatic O1 and O139 antigen (*rfb*) and the cholera toxin A subunit (*ctxA*) genes was carried out as described by Hoshino *et al.* (1998). Similarly, multiplex PCR was also performed for the El Tor and classical *tcpA* genes. Isolates, which were negative for *rfb*/*ctxA* on multiplex PCR, were analysed for the presence of the *V. cholerae*-specific *toxR* gene (Neogi *et al.*, 2010). Mismatch amplification mutation assay (MAMA)-PCR was performed to distinguish El Tor and classical *ctxB* genes, as described by Morita *et al.* (2008). The type of cholera toxin Φ was determined by *rstR* gene-based PCR (Faruque *et al.*, 2003). Detection of *chxA*, *rtxC*, *nag*-ST and T3SS-related genes ($vcsC_2$, $vcsN_2$ and *vopF*) was performed using a colony hybridization method, as described by Awasthi *et al.* (2013). *V. cholerae* strains N16961 (O1/El Tor/$ctxA^+$), O395 (O1/classical/$ctxA^+$), VC406 (O139/$ctxA^+$), As522 ($rstR^{ElTor+,\ Calcutta+}$), Vc129 ($chxA^+$/T3SS$^+$/$rtx^+$/$hly^+$) and GP156 (*nag*-ST$^+$) were used as positive controls in PCR and colony hybridization experiments. *E. coli* strain C600 was used as a negative control in all PCR assays. The primers used in this study are listed in Table S1 (available in the online Supplementary Material).

PFGE was performed by embedding freshly cultured *V. cholerae* cells into 0.5 % SeaKem gold agarose (Bio-Rad Laboratories). Bacterial cells in plugs were then lysed and processed following the PulseNet USA protocol (www.cdc.gov/pulsenet/pathogens). *Not*I-digested DNA fragments were separated by using CHEF Mapper (Bio-Rad Laboratories), essentially as described by Yamasaki *et al.* (1997). PFGE fingerprints were analysed using Fingerprinting II software (Bio-Rad Laboratories). The unweighted pair group method with arithmetic mean was applied during dendrogram analysis following the band-based (Dice coefficient) option.

RESULTS

Biotyping and serotyping

A total of 81 *V. cholerae* isolates were analysed. Eighty of these isolates were identified as *V. cholerae* O1 of the El Tor biotype, while one isolate failed to agglutinate with O1 or O139 antisera and thus belonged to a non-O1/O139 serogroup. Phenotypic characteristics of the isolates are shown in Table 1. All *V. cholerae* O1 isolates were β-haemolytic and could agglutinate chicken erythrocytes except one isolate, which was partially haemolytic and unable to agglutinate chicken erythrocytes. These phenotypic results further allowed a presumptive identification of *V. cholerae* O1 isolates from Nyanza as the El Tor biotype. The single non-O1/O139 isolate identified in this study also showed β-haemolytic activity.

Genotyping by PCR

Multiplex PCR targeting the specific somatic O1 and O139 antigenic (*rfb*) genes of the isolates confirmed that the 80 isolates were *V. cholerae* O1 and carried the *ctxA* gene (Table 1). The single isolate, which was non-agglutinable with anti-O1 or anti-O139 antiserum, as mentioned above, was identified as *V. cholerae* non-O1/non-O139. To further confirm this, we performed O1 and O139 *rfb* gene-specific PCR assays, which gave negative results (data not shown). The isolate was also negative for the *ctxA* gene, as assessed by PCR (data not shown). However, when the isolate was further tested for the presence of the *V. cholerae*-specific

Table 1. Phenotypic and genotypic characteristics of *V. cholerae* isolates collected in Nyanza, Kenya

		rfb			*ctxB*			*rstR*			*tcpA*							
Serotype	***toxR***	**O1**	**O139**	***ctxA***	**Cla**	**El Tor**	***rstC***	**Cla**	**El Tor**	**Calc**	**Cla**	**El Tor**	**T3SS**	***nag-ST***	***chxA***	**HLY**	**PxB**	**CCA**
O1 (n=80)	ND	+	−	+	+	−	+	−	+	−	−	+	−	−	−	β	R	+
Non-O1/ O139 (n=1)	+	−	−	−	NA	NA	NA	−	−	−	−	−	−	−	+	β	NA	NA

+, Positive; –, negative; Cla, classical; calc, Calcutta; HLY, haemolysis; NA, not applicable; ND, not determined; PxB, polymyxin B; R, resistant.

toxR gene by PCR, it gave the desired PCR amplicon, further confirming the isolate as *V. cholerae*. The Nyanza isolates harboured the classical *ctxB* allele, as revealed by MAMA-PCR (Table 1). Respective PCR-based genotyping of the *tcpA*, *rstR* and *rstC* genes showed that the isolates carried all these genes and were confirmed as the El Tor variant. On the other hand, *chxA* and T3SS genes were not detected in *V. cholerae* O1 El Tor variants. However, the *chxA* gene was detected in the genome of the *V. cholerae* non-O1/O139 isolate identified in this study. Sequencing of this *chxA* gene from the non-O1/O139 isolate revealed that it belonged to the *chxA* II variant category (data not shown).

Antimicrobial susceptibility profile

Antimicrobial susceptibility of the *V. cholerae* isolates is presented as resistant, intermediately resistant and susceptible in Table 2. All of the isolates were susceptible to ampicillin, tetracycline, ciprofloxacin, fosfomycin, kanamycin and norfloxacin. On the other hand, 100 % of isolates were resistant to SXT and showed high intermediate resistance to nalidixic acid, chloramphenicol and imipenem (Table 2). In sharp contrast, the non-O1/O139 isolate was susceptible to all of these antimicrobials, except ampicillin. Multidrug-resistance patterns of *V. cholerae* O1 isolates are shown in Table 3.

Table 2. Antimicrobial susceptibility patterns of *V. cholerae* O1 isolates (n=80) from Nyanza, Kenya

Values in parentheses indicate the zone diameters (in mm) used for interpreting susceptibility. SXT, sulfamethoxazole/trimethoprim.

Antibiotic	**Reaction of isolates (%)**		
	Resistant	**Intermediate**	**Sensitive**
SXT	100 ($\leqslant$10)	0 (11–15)	0 ($\geqslant$16)
Fosfomycin	0 ($\leqslant$12)	0 (13–15)	100 ($\geqslant$16)
Ampicillin	0 ($\leqslant$13)	0 (14–16)	100 ($\geqslant$17)
Imipenem	0 ($\leqslant$19)	62.5 (20–22)	37.5 ($\geqslant$23)
Chloramphenicol	0 ($\leqslant$12)	98.8 (13–17)	1.2 ($\geqslant$18)
Tetracycline	0 ($\leqslant$11)	0 (12–14)	100 ($\geqslant$15)
Norfloxacin	0 ($\leqslant$12)	0 (13–16)	100 ($\geqslant$17)
Nalidixic acid	3.7 ($\leqslant$13)	96.3 (14–18)	0 ($\geqslant$19)
Streptomycin	88.8 ($\leqslant$11)	5 (12–14)	6.3 ($\geqslant$15)
Kanamycin	0 ($\leqslant$13)	0 (14−17)	100 ($\geqslant$18)
Cefotaxime	1.2 ($\leqslant$22)	2.5 (23–25)	96.3 ($\geqslant$26)
Ciprofloxacin	0 ($\leqslant$15)	0 (16–20)	100 ($\geqslant$21)

Pulsotyping

Analysis of the *Not*I restriction enzyme digested PFGE profile of *V. cholerae* O1 isolates isolated from Nyanza province revealed that they were most likely of clonal origin, although subtle differences were detected in one isolate as compared with the rest of the *V.* cholerae O1 isolates analysed in this study (Fig. S1).

DISCUSSION

Phenotypic results obtained in this study led to presumptive identification of the Nyanza isolates as the *V. cholerae* O1 El Tor biotype, except for one isolate, which belonged to a non-O1/O139 serogroup. Further genotypic analysis confirmed that all of the isolates were *V. cholerae* O1 El Tor variants. New variants of *V. cholerae* were first reported in Bangladesh (Nair *et al.*, 2002). Since then, several studies have reported on the prevalence of *V. cholerae* variants in several Asian countries (Taneja *et al.*, 2009; Okada *et al.*, 2010; Teh *et al.*, 2012). On the other hand, only a few reports on *V. cholerae* O1 El Tor variants have emanated

Table 3. Multidrug-resistance profile of *V. cholerae* O1 isolates from Nyanza, Kenya

Intermediate resistance is not shown.

Resistant type	**Antimicrobial resistance pattern**				**Number of isolates**
I	SXT	AM	Sm		1
II	SXT	Sm			74
III	SXT	Sm	CTX		3
IV	SXT	AM	Sm	CTX	1
V	SXT	NA	Sm		1
Total	–	–	–	–	80

AM, Ampicillin; CTX, cefotaxime; NA, nalidixic acid; Sm, streptomycin.

from Africa. These include countries in West Africa, Nigeria, Cameroon (Quilici *et al.*, 2010), South Africa, Mozambique (Ansaruzzaman *et al.*, 2004), Angola (Ceccarelli *et al.*, 2011), Zimbabwe (Islam *et al.*, 2011), South Africa (Ismail *et al.*, 2012) and Zanzibar (Naha *et al.*, 2013). All of the *V. cholerae* O1 isolates in our study were identified as the El Tor variant genotype. In this context, it is noteworthy that we failed to detect any *V. cholerae* O1 El Tor variants in our earlier study among *V. cholerae* O1 epidemic strains isolated between 1994 and 2007 (Kiiru *et al.*, 2009). Similarly, Mohamed *et al.* (2012) detected the classical *ctxB* gene from *V. cholerae* O1 strains obtained between 2009 and 2010, and concluded that the genotype did not exist prior to this period.

Our study associates the *V. cholerae* O1 El Tor variant with the 2007–2008 cholera outbreak, which was reported to be a more severe form of cholera epidemic (Shikanga *et al.*, 2009). Africa, including Kenya, has experienced an upsurge in cholera outbreaks since the beginning of the millennium (WHO, 2009). The severity of infection and high case fatality rate of 11 % observed in the Nyanza cholera outbreak was mainly associated with 2008 post-election violence in Kenya, which led to inadequate supplies and a shortage of health personnel, among other factors (Shikanga *et al.*, 2009). We speculate that the O1 El Tor variant of *V. cholerae* may also have contributed to the clinical manifestations of the disease, due to the potential of strains to produce high levels of cholera toxin (Ghosh-Banerjee *et al.*, 2010).

Mohamed *et al.* (2012) recently reported that *V. cholerae* isolates from cholera patients across Kenya, including Nyanza, during 2009–2010 were of the El Tor variant. Our findings, which preceded this study, also associate the 2007–2008 Nyanza outbreak with the *V. cholerae* El Tor variant. On the other hand, *V. cholerae* non-O1/O139 strains are usually involved with sporadic cases of diarrhoea and cholera-like outbreaks (WHO, 1969; Dutta *et al.*, 2013; Marin *et al.*, 2013). Furthermore, Bik *et al.* (1996) have shown that a non-O1/non-O139 *V. cholerae* strain belonging to the serogroup O37 caused a suspected cholera outbreak in Sudan (WHO, 1969) and carried the classical-type *ctxB* gene. In the current study, however, the single *V. cholerae* non-O1/non-O139 isolate was devoid of *ctx* genes. The presence of the *chxA* gene in *V. cholerae* non-O1/O139 isolated from a diarrhoeal patient may indicate its pathogenic potential, particularly its enterotoxic activity. The only *V. cholerae* non-O1/O139 isolated in this study carried the *chxA* II gene, the product of which (ChxA II) has been found to be more potent than prototype ChxA (ChxA I) in mice lethality assays (Awasthi *et al.*, 2013). Clonal diversity among these *V. cholerae* O1 isolates was not evident from the PFGE patterns. PFGE analysis of *V. cholerae* O1 strains isolated during the period 1994–2007 showed that regardless of the year of isolation, all of the strains were clonally related (Kiiru *et al.*, 2009). However, the *V. cholerae* O1 isolates analysed in this study showed close clonal similarity to the isolates of the 2009 outbreak in the coastal area of Kenya (unpublished observation). Mohamed *et al.* (2012) reported minor clonal differences by multilocus variable tandem repeat analysis among the Nyanza *V. cholerae* O1 isolated during 2009–2010. Kiiru *et al.* (2013) reported genetic variations using a combination of whole-genome sequences of clinical and environmental *V. cholerae* isolated from various parts of Kenya, including the Nyanza area. However, the PFGE conditions used in the above-mentioned study differ from those in the current study. It is therefore difficult to draw any conclusions regarding the clonal relationship between our isolates and the strains identified by Kiiru *et al.* (2009).

The multidrug resistance observed in the *V. cholerae* O1 isolates analysed in this study might not be as severe as resistance profiles reported elsewhere (Mukhopadhyay *et al.*, 1995). Unlike previous studies conducted with *V. cholerae* O1 isolated from the same area, which reported tetracycline resistance (Shapiro *et al.*, 1999; Scrascia *et al.*, 2006), 100 % of our isolates were susceptible to tetracycline (Table 2). It is thus important to monitor the tetracycline resistance of *V. cholerae* strains, since this is the drug of choice during cholera outbreaks (Kumar *et al.*, 2012). Mutreja *et al.* (2011) reported on the acquisition of an SXT antibiotic-resistance element by *V. cholerae* between 1978 and 1984. All of the *V. cholerae* O1 isolates examined in this study showed resistance to SXT (Table 2). This confirms the results of an earlier study that reported SXT-resistant *V. cholerae* O1 strains in Kenya (Kiiru *et al.*, 2009). SXT is commonly used in Kenya to treat children suffering from infectious diseases (Saidi *et al.*, 1997) but, considering our results, SXT should not be recommended for the treatment of cholera in children. Reduced susceptibility to nalidixic acid and ciprofloxacin has also been reported elsewhere (Faruque *et al.*, 2003; Kim *et al.*, 2010; Quilici *et al.*, 2010; Tran *et al.*, 2012). Although intermediate resistance to nalidixic acid was observed in more than 90 % of *V. cholerae* O1 isolates analysed in the current study, all of the isolates were susceptible to ciprofloxacin (Table 2).

With genome-sequencing technologies and detailed statistical analysis it is now possible to identify patterns of variation, which are the result of recent evolutionary events. Mutreja *et al.* (2011) have attempted to trace the source and spread of the latest cholera pandemic by whole-genome sequencing. The seventh pandemic, which originated in Sulawesi in Indonesia, has spread from the Bay of Bengal in at least three independent but overlapping waves and several transcontinental transmission events. The study traced the origins of the pandemic strain to its roots 40 years ago in the Bay of Bengal. From this base, it has since infected people around the world, including in Africa, South Asia and South America. Kiiru *et al.* (2013) have performed a combination of phylogenetic and phenotypic analyses based on whole-genome sequences derived from 40 environmental and 57 clinical *V. cholerae* isolated from different regions of Kenya between 2005 and 2010. In their study, some environmental and all clinical isolates were mapped back onto wave three of the monophyletic seventh pandemic. Thus, it would be interesting and highly informative to identify to which branch of the cholera phylogenetic tree the isolates of the current study belong.

Lake Victoria is the second-largest fresh-water lake in the world. Communities living on its shores are frequently exposed to waterborne diseases, including cholera (Shapiro *et al.*, 1999). Studies on enteric infections in the environs of Lake Victoria indicate that cholera is endemic in the region (Shapiro *et al.*, 1999). As shown in this study, isolation of the *V. cholerae* O1 El Tor variant, which could be more virulent, may complicate the management of cholera outbreaks in the region. Provision of clean water, adequate sanitation and health education are the mainstays of cholera control. Meanwhile, an option for vaccination should be considered for cholera-prone populations. Nevertheless, there is a need to monitor *V. cholerae* genotypes in addition to the antimicrobial susceptibility of the organisms. Here, we report for the first time on the occurrence of a *chxA* II gene-positive clinical *V. cholerae* non-O1/O139 isolate in Kenya, which warrants further investigation related to the epidemiology *V. cholerae* of non-O1/O139 strains in cholera-endemic areas in Kenya. Together, our studies and similar investigations reported in recent times regarding the rapid emergence of different clones of cholera causing *V. cholerae*, along with differential antimicrobial resistance patterns, indicate that constant characterizations of *V. cholerae* isolates are required. Therefore, there is a need to strengthen and provide proper laboratory support in the near future in order to effectively combat cholera in Kenya.

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