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 been...
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 Kliger’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 (O1/classical/ctxA+), VC406 (O139/ctxA+), Ax522 (rstR−/ElTor+, calcite+), Vc129 (ctxA+/T3SS+/rtxA+/lhb−) 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-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 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 *ttrX* gene (Neogi et al., 2010). Mismatch amplification mutation assay (MAMA)-PCR was performed to distinguish El Tor and classical *ctxX* 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 *ctxA*, *rtxC*, nag-ST and T3SS-related genes (*vcsC*, *vcsN2* 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+), Ax522 (rstR−/ElTor+, calcite+), Vc129 (ctxA+/T3SS+/rtxA+/lhb−) 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).
**Table 1.** Phenotypic and genotypic characteristics of *V. cholerae* isolates collected in Nyanza, Kenya

<table>
<thead>
<tr>
<th>Serotype</th>
<th>rfb</th>
<th>O1</th>
<th>O139</th>
<th>ctxA</th>
<th>ctxB</th>
<th>rstR</th>
<th>tcpA</th>
<th>T3SS</th>
<th>nag-ST</th>
<th>chxA</th>
<th>HLY</th>
<th>PxB</th>
<th>CCA</th>
</tr>
</thead>
<tbody>
<tr>
<td>O1 (<em>n</em> = 80)</td>
<td>ND</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>β</td>
</tr>
<tr>
<td>Non-O1/O139 (<em>n</em> = 1)</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>β</td>
</tr>
</tbody>
</table>

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

The *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, intermediate 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.

**Pulsotyping**

Analysis of the NotI 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...
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 study associated 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 diarrheal patient may indicate its pathogenic potential, particularly its enterotoxigenic 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 closely 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; Scrasca 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 antimicrobic 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.

ACKNOWLEDGEMENTS

We thank Rupak K. Bhadra (Indian Institute of Chemical Biology) for critically reading this manuscript. This study was performed in partial fulfilment of the requirements of a PhD thesis for S.M.S. from the Graduate School of Life and Environmental Sciences, Osaka Prefecture University, Osaka, Japan. This work was supported in part by a Grant-in-Aid for Scientific Research from JSPS.

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