Characterization of *V. cholerae* O1 isolates responsible for cholera outbreaks in Kenya between 1975 and 2017

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ABSTRACT

Kenya is endemic for cholera with different waves of outbreaks having been documented since 1971. In recent years, new variants of *V. cholerae* O1 have emerged and replaced most of the traditional El Tor biotype globally. These strains also appear to have increased virulence, and it is important to describe and document their phenotypic and genotypic traits. In this study, we characterized 146 *V. cholerae* O1 isolates from cholera outbreaks that occurred in Kenya between 1975 and 2017. Our study reports that the 1975-1984 strains had typical classical or El Tor biotype characters. New variants of *V. cholerae* O1 having traits of both classical and El Tor biotypes were observed from 2007 with all strains isolated between 2015 and 2017 being sensitive to polymyxin B and carrying both classical and El Tor type *ctxB*. All strains were resistant to Phage IV and harbored *rstR, rtxC, hlyA, rtxA* and *tcpA* genes specific for El Tor biotype indicating that the strains had an El Tor backbone. Pulsed Field Gel Electrophoresis (PFGE) genotyping differentiated the isolates into 14 pulsotypes. The clustering also corresponded with the year of isolation signifying that the cholera outbreaks occurred as separate waves of different genetic fingerprints exhibiting different genotypic and phenotypic characteristics. We report the emergence and prevalence of *V. cholerae* O1 strains carrying El Tor type and classical type of *ctxB* in Kenya. These strains have replaced the typical El Tor biotype in Kenya and are potentially more virulent and easily transmitted within the population.

Keywords: *Vibrio cholerae* O1, cholera outbreaks, biotyping, Pulsed-field gel electrophoresis

INTRODUCTION

Cholera is an infectious disease caused by *Vibrio cholerae* O1 and O139, and its pandemic continues to occur in much of the developing world with Africa being the most affected continent (1). The disease is endemic in Kenya, and several waves of outbreaks have been reported since 1971 (2). According to the World Health Organization (WHO), from 1974 to 1989, Kenya reported cases every year, with an average case fatality rate (CFR) of 3.57% (3). Between 1997 to 2010, a total of 68,522 clinically suspected cases of cholera and 2,641 deaths were reported (CFR=3.9%) (4). In 2005, a total of 990 cases and 25 deaths (CFR=2.5%) were reported in five distinct outbreaks, including 186 laboratory-confirmed cases (5). Between January 2015 to January 2016, a total of 11,033 cases were reported with 178 deaths (CFR=1.6%) in 22 of 47 counties (6).

V. cholerae is classified into approximately 206 serogroups of which, O1 and O139 produce cholera toxin associated with cholera pandemics (7). The O1 serogroup is further divided into three serotypes, namely Inaba, Ogawa, and Hikojima and two biotypes, namely classical and El Tor. *V. cholerae* O1 can be phenotypically characterized based on a number of phenotypic traits such as susceptibility to polymyxin B (50U), chicken erythrocyte agglutination, hemolysis of sheep erythrocytes, the Voges-Proskauer (VP) test which measures the production of acetylmethylcarbinol, and sensitivity of specific bacteriophages (8). The El Tor strains are resistant to polymyxin B (50U), VP test positive, agglutinate chicken erythrocytes and are resistant to the classical bacteriophage IV. Classical strains show reverse traits (8).

The two biotypes can also be distinguished using molecular biotyping methods (9). The cholera toxin (CT) is the primary toxin produced by *V. cholerae* O1 and O139 and is mostly responsible

for the manifestations of cholera. The *ctxAB* gene, which is located in the CTX prophage of the *V. cholerae* chromosome encodes the cholera toxin (9). The classical toxin (CT1) is produced by classical strains and US Gulf Coast strains, whereas the El Tor type toxin (CT2) is produced by the El Tor and O139 strains (10). The two toxins differ in the cholera toxin B subunit and a mismatch amplification mutation PCR assay (MAMA) PCR has been used to differentiate between the classical type *ctxB* and El Tor type *ctxB* (11). The Vibrio Pathogenicity Island-1 (VPI-1) DNA region of the large chromosome is composed of the toxin-coregulated pilus (TCP) and the accessory colonization factor (ACE). The *tcpA* gene of the TCP cluster has alleles that are specific to the classical and El Tor biotypes and can be determined using specific primers (12). The CTXΦ of El Tor and O139 strains are flanked by a genetic element designated RS1 (13, 14). RS1 differs from RS2 in that the *rstC* gene is contained in RS1 alone (13). *V. cholerae* also secretes several "accessory" toxins including the repeat toxin (RTX) which encodes for a cytotoxic activator protein and is composed of four open reading frames (ORFs) of which only the *rtxC* gene is found in the El Tor biotypes (9, 15). The *hlyA* gene encodes for the hemolysin encoding gene, which differs in nucleotide sequence between the El Tor and classical strains (12).

Over the last few decades, new variants of *V. cholerae* have emerged that cannot be typed using the current biotyping scheme. Nair *et al.* (16) identified strains of *V. cholerae* O1 El Tor with classical biotype traits from patients in Bangladesh. They termed the strains as "hybrid type" strain since they could not be biotyped. Due to the limitations in the currently used biotyping scheme, Raychoudhuri *et al*. proposed a revised biotyping scheme for *V. cholerae* O1 that incorporates hybrid biotypes and the El Tor variant strains (8). With the emergence of new variants of *V. cholerae* globally, it is important to characterize strains isolated from Kenyan outbreaks and

90 determine how they compare with these strains. A study by Kiiru *et al.* (17) characterized 65 *V.*
91 *cholerae* O1 strains isolated in Kenya between 1994 and 2007 and identified the strains as El Tor
92 biotype lacking any classical biotype markers. Another study reported that strains isolated between
93 2007 and 2010 were biotype El Tor variants possessing the cholera toxin B subunit gene and were
94 positive for the *ctxA, tcpA* of El Tor biotype and *rtxC* genes (18). However, their evolutionary
95 lineage and how they compare with other strains previously isolated from other countries where
96 cholera is endemic has not been described. This study aimed to describe the evolving phenotypic
97 and genotypic traits of *V. cholerae* O1 strains that were collected over the last four decades in
98 Kenya and other Asian countries where cholera outbreaks are prevalent.
99
100 **MATERIALS AND METHODS**
101 **Ethics statement**
102 The strains used in this study were isolated and stocked during previous studies. The study was
103 approved by the KEMRI Scientific and Ethics Review Unit (SERU) SSC No. 1323 and the ethical
104 committee of Nagasaki University Graduate School of Biomedical Sciences.
105
106 **Description of cholera strains**
107 A total of 146 *V. cholerae* O1 stocked strains isolated from patients during cholera outbreaks in
108 Kenya between 1975 and 2017 were analysed in this study (Table 1). The strains were collected
109 during surveillance programs by the Nagasaki University, Institute of Tropical Medicine, Kenya
110 Research Station (NUITM-KEMRI) following requests from the Kenya Ministry of Health and
111 other local health centers. These strains were grouped into three categories based on the period
112 when they were isolated with 1975-1984 (36 strains) representing the entry of cholera into Kenya,

2007-2010 (37 strains) representing the period when variants of *V. cholerae* were reported in the country, and 2015-2017 (66 strains) representing the most recent outbreaks in the country. Selected isolates from several Asian countries isolated between 1958 and 2010 (19 strains) were also included in this study for comparison (Table 2). The strains were donated by the institute's partners in the collaborating countries and stocked at NUITM, Nagasaki, Japan. The El Tor biotype K-23 strain and classical biotype 569B and H218 reference strains were used as controls. All strains were confirmed as *V. cholerae* O1 by standard biochemical tests and serology (19, 20).

Biotyping of cholera strains

Phenotypic tests for biotype determination included hemolysis of sheep red blood cells as described by Feeley and Pittman (21), sensitivity to polymyxin B by the E-test strip method with sensitive strains having a MIC of ≤2 μg/mL, sensitivity to classical phage IV as described by S. Mukerjee (22), the VP test which measures the production of acetylmethylcarbinol (23), and the hemagglutination of chicken erythrocytes (24).

Genotypic tests complemented the phenotypic tests and included Polymerase Chain Reaction (PCR) assays targeting various virulence and biotype determining genes. Primer sets *ctxA* (25), and *tcpA* (12) were used to detect the major virulence factors of pathogenic *V. cholerae*, cholera toxin (CT) and toxin coregulated pili (TCP). Other virulence genes assayed included *toxR* (26), *rstC* (27), *rtxA* (28) and *hlyA* (12). Primer sets *ctxB* (11), *hlyA* (12), *rstC* (27), *rstR* (29), *rtxC* (28), and *tcpA* (12) were used to detect classical and El Tor biotype specific alleles in the strains.

Results from at least three phenotypic tests and all genotypic tests were used to determine the strain's biotype as either "classical", "El Tor", "El Tor variant" or "hybrid" as described by Raychoudhuri *et al* (8). Strains that could not be biotyped according to this criteria were termed "unclassified".

Polymerase Chain Reaction (PCR)

Genomic DNA extraction was performed by the phenol-chloroform–isoamyl alcohol extraction method as described by Huq *et al.* (30) and PCR performed using PuReTaq ready-to-go PCR beads as per the manufacturer's instructions (GE Healthcare UK limited, UK). The PCR products were analysed by electrophoresis on 2.5% agarose gels, stained with ethidium bromide, visualized under UV light and recorded with the aid of a gel documentation system (E-BOX 1000, Vilber Lourmat, Marne-la-Vallee, France).

Pulsed-field Gel Electrophoresis (PFGE)

Twenty-six isolates randomly selected based on their identified biotype and year of isolation (Table 1) were analyzed using the PulseNet standardized pulsed-field gel electrophoresis (PFGE) protocol for *V. cholerae* (31) with slight modifications. The genomic DNA was restricted with 50U of *Not*I restriction enzyme (Takara Bio Inc., Shiga, Japan) at 37ºC for 4 hours and electrophoresis run using a CHEF-DRIII system (Bio-Rad, USA). Gel images were captured on a Gel documentation system (E-BOX 1000, Vilber Lourmat, Marne-la-Vallee, France) and the restriction patterns analysed using BioNumerics software V7.6 (Applied Maths, Inc., Belgium). The Dice similarity coefficient determined the banding similarity with a 1.0% band matching tolerance, and a dendrogram constructed using the unweighted-pair group method with arithmetic mean algorithm (UPGMA).

158

159 **RESULTS**

160 *Epidemiology:*

161 Different serotypes of *V. cholerae* O1 were isolated in each wave of cholera outbreaks. All strains
162 isolated between 1975 and 1984 were Ogawa type whereas those isolated between 2007 and 2009
163 were Inaba type. Between the years 2007 and 2010, the predominant serotype was Inaba. The 2010
164 outbreaks occurred in Kwale and Pokot Counties of Kenya. Serotype Ogawa emerged in 2010 in
165 Kwale county cholera outbreaks and prevailed from 2015 to 2017. Most strains carried pathogenic
166 genes for *V. cholerae* O1 with 90% (131/146) of the strains being positive for subunit A of cholera
167 toxin gene (*ctxA*), 89% (130/146) positive for toxin-coregulated pilus gene (*tcpA*) and the *rstC*
168 antirepressor. All strains carried the toxin-coregulated pilus (*toxR)*, repeat in toxin RTX (*rtxA)*, and
169 the hemolysin A (*hlyA)* virulence genes (Table 3).

170

171 *Phenotypic tests:*

172 Based on the phenotypic test results for biotype determination as described in Table 1, all strains
173 were resistant to phage IV, a trait exhibited by El Tor biotype strains. Chicken erythrocytes
174 agglutination test results showed variable results for the 1975-1984 strains. Ninety-five point five
175 percent (105/110) of strains isolated between 2007 and 2017 were positive for this test. Strains
176 isolated between 1975 and 1984 were resistant to polymyxin B and had a minimal inhibitory
177 concentration (MIC) of > 2µg/mL based on the E-test method. The results were similar to those of
178 typical El Tor biotype strains. Seventy-five percent of the 2007 strains, 93% of the 2015 strains
179 and all of the 2016 and 2017 strains were sensitive to polymyxin B, results similar to those for the

180 classical biotype strains. Variable results were observed for the VP and Sheep red blood cell
181 hemolysis tests (Table 1).
182
183 *Genotypic tests:*
184 According to the genotypic test results for biotype determination as described in Table 1, all of the
185 146 *V. cholerae* O1 strains carried the hemolysin encoding gene (*hlyA*) specific for the El Tor
186 biotype. Our study also analyzed 19 *V. cholerae* O1 strains isolated from Asian countries between
187 1961 and 2010 and classified these strains as either classical biotype, El Tor biotype or El Tor
188 variant biotype possessing classical *ctxB* (Table 2). Results of the other biotype determination PCR
189 assays showed that all strains possessed the El Tor biotype markers for the hemolysin encoding
190 gene (*hlyA*) while a majority possessed the repeat-sequence transcriptional regulator gene (*rstR*),
191 *rtxC* gene of the RTX toxin gene cluster, and the toxin-coregulated pilus (TCP) gene (*tcpA*) specific
192 for the El Tor biotype.
193
194 *Pulsed Field gel electrophoresis (PFGE):*
195 PFGE analysis of *Not*I digested genomic DNA differentiated the isolates into 14 pulsotypes (Figure
196 1). UPGMA differentiated the isolates into 2 clusters (I and II) at 82% similarity cut off and five
197 sub-groups (A-E) at 94% similarity cut off. A general association between the period of the
198 outbreaks and the cluster subgroups was observed. Strains isolated between 1975 and 1984 were
199 clustered in cluster I (sub-group A-C). Those isolated between 2007 and 2010 were clustered in
200 cluster II (sub-group D) except for one strain. Isolates from the most recent outbreaks in the country
201 (2015-2017) were clustered in cluster II (sub-group E). Another observed association was between
202 the biotypes. El Tor biotype strains isolated between 1975 and 1984 were grouped in cluster I (sub-

group A-C) whereas atypical El Tor strains occurring between 2007 and 2017 were grouped in cluster II.

In 2010, strains isolated in Kwale County were of serotype Ogawa while those of Pokot serotype Inaba. One strain from Pokot (Pulsotype D6) showed 98% similarity with two Kwale strains (Pulsotype D3). Another interesting observation was that one strain of serotype Inaba isolated in Pokot in 2010 that harbored both classical and El Tor type *ctxB* showed 100% similarity with two serotype Ogawa strains isolated in 2015 and 2016 and 99% similarity with the other 2015-2017 strains.

DISCUSSION

In this study, we characterized 146 *V. cholerae* O1 strains isolated during cholera outbreaks that occurred in Kenya between 1975 and 2017 by analyzing their phenotypic and genetic traits and comparing this data with genotypic data of representative strains of several Asian countries. Of the five phenotypic tests, the phage IV resistance test yielded the most stable results and classified the strains as El Tor biotype. Voges-Proskauer (VP) and chicken cell agglutination test result classified the 1975-1984 *V. cholerae* O1 isolates as typical El Tor biotype and the rest as having both classical and El Tor traits. Results of the Phage IV test were consistent with those previously reported in Kenya (32) demonstrating the stability of this test. In a study done by Safa *et al.* (33) where they analysed the phenotypic and genotypic traits of various *V. cholerae* O1 strains, the Phage IV test was able to discriminate between classical and El Tor biotypes. Our results of the sheep erythrocyte hemolysis test were consistent with those reported elsewhere where the test has been reported to be of limited use since the El Tor strains lost their hemolytic activity in the late 1960's yielding

variable results (19). Previous cholera studies in Kenya in 1983 (32) and 2007-2010 (18) outbreak strains as being reported as polymyxin B resistant. Results from this study demonstrate that polymyxin B sensitive strains emerged in Kenya in the mid-2000s and completely replaced polymyxin B resistant strains by the year 2016. Polymyxin B susceptible El Tor biotype strains have also been reported in other countries (16, 31). We found that phenotypic tests for biotype determination can classify the 1975-1984 isolates as El Tor biotype and the rest of the strains as having features of both classical or El Tor biotypes.

Besides of phenotypic test, genetic characterization including PCR analysis of biotyping genes can determine the isolates biotypes. The classical toxin (CT1) is produced by classical strains and US Gulf Coast strains, whereas the El Tor type toxin (CT2) is produced by the El Tor and O139 strains (10). The results of the mismatch amplification mutation PCR assay (MAMA) PCR (11) that differentiates between the classical type *ctxB* and El Tor type *ctxB* revealed the emergence of strains carrying both the classical and El Tor *ctxB* in 2007. All strains isolated between 1975-1984 had El Tor *ctxB,* 75% (33/44) of the 2007-2010 isolates and 94% (62/66) of 2015-2017 isolates carried both classical and El Tor *ctxB.* Probably these strains might have evolved from El Tor variants that acquired specific attributes from the classical genome. The other biotype determination PCR assays (*rstR, rtxC, hlyA, rtxA,* and *tcpA* genes specific for the classical or El Tor biotype) showed that all strains possessed the El Tor biotype markers and none was positive for the classical biotype indicating that the strains had an El Tor backbone. A previous study that characterized 65 *V. cholerae* O1 strains isolated in Kenya between 1994 and 2007 identified the strains as El Tor biotype lacking any classical biotype markers (17). However, several studies have reported El Tor variant strains producing cholera toxin of the classical biotype (10, 20, 31) with some studies

249 indicating that they produce higher levels of cholera toxin than the classical biotype strains (30). A
250 study conducted in India reported the emergence of strains producing both classical and El Tor
251 *ctxB* in 1991 and 1992, but these were replaced by classical type *ctxB* strains in subsequent years
252 (34).
253
254 Classical and El Tor biotypes differ in not only their phenotypic and genotypic properties, but also
255 their pathogenic potentials, survivability, and infection patterns in the hosts (35). We found that
256 71% of the isolates carried the *V. cholerae* virulence factors *ctxA, tcpA, toxR, rstC, rtxA, and hlyA*
257 confirming the toxin-producing capability and endemic potential of these strains. The *rtxA*, *toxR,*
258 and *hlyA* virulence genes were carried in all the strains suggesting their importance in pathogenicity
259 of *V. cholerae* more so in strains that lacked the *ctxA* gene.
260
261 Our study indicates that serotype Ogawa caused cholera outbreaks occurring between 1975 and
262 1984, and those from 2007-2009 were serotype, Inaba. This was in agreement with previous reports
263 in the country (4, 17, 32). Serotype Ogawa reemerged in 2010 and replaced serotype Inaba in the
264 2015-2017 cholera outbreaks. The serotype switching from O1 Ogawa to Inaba and vice versa is
265 common in many cholera-endemic areas (36). It has been attributed to a change in the genetic
266 make-up of the *wbtT* gene that determines the Ogawa specificity (37-38). Serotype conversion in
267 endemic strains has been linked to environmental factors and immune pressure within the
268 population (38-40).
269
270 The results of the phenotypic and genetic characterization reveal that earlier strains isolated in
271 Kenya from 1975-1984 had typical El Tor characters, which is in agreement with previous studies

done in Kenya (32). Cholera outbreaks occurring between 2007 and 2017 were caused by new variants of *V. cholerae* O1 that had traits of both the classical and El Tor biotypes and were classified as hybrid type, El Tor variants, or unclassified (for those that did not fit in any category). In order to understand the Kenyan isolates, we compared *V. cholerae* O1 strains with other countries and biotyped representative strains isolated from several Asian countries. Phenotypic and PCR analysis classified them as either classical type, typical El Tor, or El Tor variants justifying the occurrence of altered *V. cholerae* O1 strains in other parts of the World (8, 10, 16, 33). None of the strains carried both classical and El Tor *ctxB* (Table 2). We report the emergence of *V. cholerae* O1 strains carrying both the classical and El Tor *ctxB* in Kenya. Kenya has been reporting severe cholera outbreaks in different waves since 2007 which coincides with the emergence of these new variants. The acquisition of cholera toxin of the classical type in addition to that of El Tor type potentially makes them more virulent, persistent in the environment, and easily transmissible within the population.

A review of the study by Weill *et al.* (1) shows that cholera epidemics were introduced in Africa by a single expanded lineage since 1970. Specifically, the T5 event was introduced in 1970 and transmitted cholera within Kenya between 1980-1989 during the first wave of global cholera transmission. Wave 2 involved T6 event which was introduced in the continent between 1986 and 1989 and occurred in Kenya between 1990 and1999. Wave 3 was associated with T10 event which was introduced in Africa during 1991-1995 and transmitted in Kenya between 1990 and 2014. A study by Mutreja *et al.* (41) reported the transmission of the 7th pandemic from the Bay of Bengal in three independent waves with wave 1 reaching Kenya between 1973-1985 and wave 3 between 1989-1997. Our PFGE results differentiated the strains into two distinct clusters (cluster 1: 1975-

1984 strains; and cluster 2: 2007-2017 strains). The clustering also corresponded with the year of isolation signifying that the cholera outbreaks occurred as separate waves of different genetic fingerprints exhibiting different genotypic and phenotypic characteristics. There is a need to perform whole genome sequencing on *V. cholerae* outbreak strains isolated in Kenya to determine their location in the genomic history of African cholera.

In summary, we report the emergence of *V. cholerae* O1 strains sensitive to polymyxin B and harboring both classical and El Tor type *ctxB*. The strains had an El Tor biotype backbone as they possessed *rstR, rtxC, hlyA, rtxA,* and *tcpA* El Tor biotype markers. This change is a significant event in cholera epidemics in Kenya as few countries have reported the circulation of virulent *V. cholerae* O1 strains harboring both classical and El Tor type *ctxB* and sensitive to polymyxin B. The isolation of virulent atypical *V. cholerae* O1 strains and the frequent cholera outbreaks in the country calls for further investigation to determine their clinical and epidemiologic significance. This information could be useful in understanding the characterization and evolution of *V. cholerae* and for the management of cholera outbreaks associated with new variants of *V. cholerae* O1.

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DISCLOSURE

The authors have no conflict of interest to declare.

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439

440 **List of abbreviations:**

441 CFR, case fatality rate; CT, cholera toxin; *ctxA,* cholera toxin A subunit gene; *ctxB,* cholera toxin
442 B subunit gene; *hlyA,* Hemolysin A gene; MIC, minimal inhibitory concentration; PCR,
443 Polymerase Chain Reaction; PFGE, Pulsed-Field Gel Electrophoresis; RBC Red Blood Cells; RTX,
444 repeat toxin; *rtxA*, rtxA cytotoxin gene; TCP, toxin-coregulated pilus; *toxR*, cholera toxin
445 transcription activator gene; UPGMA, arithmetic mean algorithm; VP, Voges-Proskauer; VPI-1,
446 Vibrio Pathogenicity Island-1; WHO, World Health Organization

447

448 **Table 1.** Biotypes of the 146 clinical *V. cholerae* O1 isolated from cholera outbreaks in Kenya

Year of isolation (n)	*ctxA*	Genotype					Phenotype				Biotype
		ctxB	*rstR*	*rtxC*	*hlyA*	*rtxA*	Phage IV	CCA	PB	VP	
1975 (6)	+	E	E	E	E	E	-	+, 3 (50%)	R	+	El Tor
1980 (6)	+	E	E	E	E	E	-	+, 3 (50%)	R	+	El Tor
1980 (1)	-	-	E	E	E	E	-	-	R	+	El Tor
1981 (4)	+	E	E	E	E	E	-	+, 2 (50%)	R	+	El Tor
1981 (1)	-	-	E	E	E	E	-	-	R	+	El Tor
1982 (5)	+	E	E	E	E	E	-	+, 3 (60%)	R	+	El Tor
1982 (1)	-	-	-	E	E	E	-	-	R	+	El Tor
1983 (4)	+	E	E	E	E	E	-	+, 2 (50%)	R	+	El Tor
1983 (2)	-	-	E	E	E	E	-	+	R	+	El Tor
1984 (4)	+	E	E	E	E	E	-	+, 1 (25%)	R	+	El Tor
1984 (2)	-	-	E	E	E	E	-	+, 1 (50%)	R	+	El Tor
2007 (7)	+, 6 (86%)	E+C	E	E	E	E	-	+	R, 2 (29%)	+	Hybrid
2007 (4)	+	E+C	E	-	E	E	-	+	R, 1 (25%)	+	Hybrid
2007 (1)	-	-	-	E	E	E	-	+	S	+	Unclassified
2008 (1)	+	E+C	E	E	E	E	-	+	R	+	Hybrid
2008 (2)	+	E+C	E	-	E	E	-	+	R	+, 1 (50%)	Hybrid
2008 (1)	-	-	-	E	E	E	-	+	S	+	Unclassified
2008 (1)	-	-	-	-	E	E	-	+	S	+	Unclassified
2009 (7)	+, 4 (57%)	E+C	E	E	E	E	-	+	R, 6 (86%)	+, 3 (43%)	Hybrid
2009 (3)	+, 2 (67%)	E+C	-	E	E	E	-	+, 2 (67%)	R, 1 (33%)	+	Hybrid
2009 (7)	+	E+C	E	-	E	E	-	+	R, 5 (71%)	+, 5 (71%)	Hybrid
2009 (1)	+	E	E	E	E	E	-	-	S	+	Hybrid
2009 (1)	-	E	-	-	E	E	-	+	S	+	Unclassified
2009 (1)	+	C	E	-	E	E	-	+	S	+	Hybrid
2010 (1)	+	E+C	E	-	E	E	-	+	R	+	Hybrid
2010 (2)	+	-	E	-	E	E	-	+	R, 1 (50%)	+	Unclassified
2010 (3)	+	E	-	E	E	E	-	+	R, 1 (33%)	+	El Tor
2010 (1)	+	C	E	-	E	E	-	+	S	-	Hybrid
2015 (53)	+	E+C	E	E	E	E	-	+, 50 (93%)	R, 3 (6%)	+, 30 (56%)	Hybrid
2015 (1)	+	E+C	-	E	E	E	-	+	R	-	Hybrid
2015 (1)	+	E+C	E	-	E	E	-	+	R	-	Hybrid
2015 (1)	+	E	E	E	E	E	-	+	S	+	El Tor
2015 (3)	+	C	E	E	E	E	-	+	S	+	El Tor Variant
2016 (2)	+	E+C	E	E	E	E	-	+	S	-	Hybrid
2016 (1)	+	E+C	E	-	E	E	-	+	S	+	Hybrid
2017 (4)	+	E+C	E	E	E	E	-	+	S	+	Hybrid

449 n, total number of strain(s) of the indicated year; *ctxA*, cholera toxin subunit A; +, present, -, absent

450 *Genotype: ctxB*, *rtxC, rtxC*, *hlyA*, and *rtxA* genes specific for classical or El Tor biotype; +, present,
451 -, absent; C, classical; E, El Tor
452 *Phenotype:* Phage IV, lysis by classical IV phage; CCA, chicken red blood cell agglutination; PB,
453 susceptibility to 50 units of Polymyxin B; VP, Voges-Proskauer test; R, resistant, S, sensitive; +,
454 positive, -, negative
455

456 **Table 2.** Genotypic traits of *V. cholerae* O1 strains isolated from cholera outbreaks in Asian

457 countries

Test strain origin	Year of isolation	No. of isolates	Genotypic trait							Biotype
			***ctxB* type**	***hlyA* type**	***rstR* type**	***tcpA* type**	***rstC***	***rtxA***	***rtxC***	
Thailand	1958	1	C	C	C	-	-	+	-	classical
Philippines, Hong Kong, Indonesia	1961	4	E, 3 (75%)	E, 3 (75%)	E	E, 3 (75%)	+, 2 (50%)	+	+, 3 (75%)	El Tor
Taiwan	1962	1	E	E	E	E	+	+	+	El Tor
Philippines	1969	1	-	E	E	E	+	+	+	El Tor
Bangladesh	1980	1	E	E	E	E	+	+	+	El Tor
Philippines	1982	1	E	E	E	E	+	+	+	El Tor
Thailand	1996	1	E	E	-	E	+	+	+	El Tor
Bangladesh	1997	1	E	E	E	E	-	+	+	El Tor
Laos, Thailand	1999	2	C	E	E	E	+	+	+	El Tor variant
Bangladesh	2000	1	C	E	C	E	-	+	+	El Tor variant
Vietnam	2007	1	C	E	E	E	+	+	+	El Tor variant
Vietnam	2008	1	C	E	E	E	+	+	+	El Tor variant
Vietnam	2009	2	C	E	E	E	+	+	+	El Tor variant
Vietnam	2010	1	C	E	E	E	+	+	+	El Tor variant

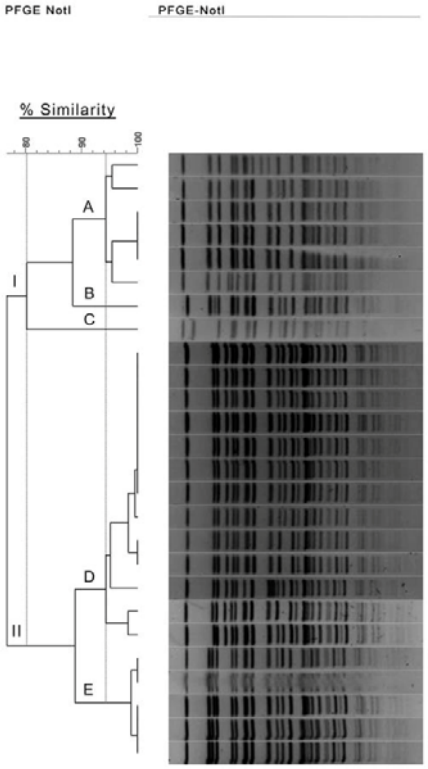
458 +, present, -, absent; C, classical; E, El Tor

459

460 **Table 3.** Analysis of *V. Cholerae* O1 virulence factors in strains isolated from cholera outbreaks
461 in Kenya

Year of isolation (n)	Virulence genes					
	ctxA	*tcpA*	*toxR*	*rstC*	*rtxA*	*hlyA*
1975-1984 (36)	+, 29 (81%)	+	+	+, 35 (97%)	+	+
2007-2010 (44)	+, 36 (82%)	+, 29 (66%)	+	+, 30 (68%)	+	+
2015-2017 (66)	+	+, 65 (98%)	+	+, 65 (98%)	+	+

462 n, total number of strain(s) of the indicated year period; ctxA, cholera toxin subunit A; *tcpA*, toxin-
463 coregulated pilus; *toxR*, cholera toxin transcriptional activator; *rstC*, an antirepressor located in the
464 RS1 element of pathogenic *V. cholerae*; *rtxA*, actin-cross-linking repeats-in-toxin (RTX) toxin
465 gene; *hlyA*, hemolysin; +, present, -, absent.
466



		Genotype			Phenotype							
Year	Location	ctxA	ctxB	rtxC	PhageIV	Chicken RBCs	Polymyxin B	VP test	Sheep RBCs	Biotype	Serotype	Pulsotype
1981	Mombasa	-	-	+	R	-	R	+	+	El Tor	Ogawa	A1
1984		+	E	+	R	-	R	+	+	El Tor	Ogawa	A2
1981		+	E	+	R	-	R	+	-	El Tor	Ogawa	A3
1982		-	-	+	R	-	R	+	+	El Tor	Ogawa	A3
1983		+	E	+	R	-	R	+	+	El Tor	Ogawa	A3
1980		-	-	+	R	-	R	+	+	El Tor	Ogawa	A4
1980		+	E	+	R	-	R	+	+	El Tor	Ogawa	B
1975		+	E	+	R	-	R	+	-	El Tor	Ogawa	C
2007	Homabay	+	E+C	+	R	+	R	+	+	Hybrid	Inaba	D1
2008	Bungoma	+	E+C	-	R	+	R	+	+	Hybrid	Inaba	D1
2008	Bungoma	+	E+C	-	R	+	R	+	+	Hybrid	Inaba	D1
2008	Homabay	-	-	+	R	+	S	+	+	Unclassified	Inaba	D1
2008	Homabay	-	-	-	R	+	S	+	+	Unclassified	Inaba	D1
2009	Turkana	+	C	-	R	+	S	+	-	Hybrid	Inaba	D1
2009	Isiolo	+	E+C	-	R	+	R	+	+	Hybrid	Inaba	D1
2009	Nairobi	+	E+C	+	R	+	R	+	+	Hybrid	Inaba	D2
2010	Kwale	+	-	-	R	+	R	+	+	Unclassified	Ogawa	D3
2010	Kwale	+	C	-	R	+	S	-	+	Hybrid	Ogawa	D3
2007	Homabay	+	E+C	+	R	+	R	+	+	Hybrid	Inaba	D4
2010	Kwale	+	E	+	R	+	R	+	+	El Tor	Ogawa	D5
2010	Pokot	+	E	+	R	+	R	+	+	El Tor	Inaba	D6
2015	Wajir	+	E+C	+	R	+	R	+	+	Hybrid	Ogawa	E1
2017	Nairobi	+	E+C	+	R	+	S	+	-	Hybrid	Ogawa	E1
2010	Pokot	+	E+C	-	R	+	R	+	+	Hybrid	Inaba	E2
2015	Homabay	+	C	+	R	+	S	+	-	El Tor Variant	Ogawa	E2
2016	Siaya	+	E+C	+	R	+	S	-	-	Hybrid	Ogawa	E2

Figure 1: Pulsed-field gel electrophoresis (PFGE) patterns among 26 Kenya *V. cholerae* O1 isolates (1975-2017). Unweighted Pair Group Method with Arithmetic (UPGMA) algorithm differentiated the isolates into 2 clusters (I and II) at 82% similarity cut off and 5 sub-groups (A-E) at 94% similarity cut off. Genotype: +, present; -, absent; C, classical; E, El Tor. Phenotype: +, positive; -, negative; R, resistant; S, sensitive.