<table>
<thead>
<tr>
<th><strong>Title</strong></th>
<th>Dynamics of cholera outbreaks in endemic areas of Kenya</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Author(s)</strong></td>
<td>Saidi, Suleiman Mzee</td>
</tr>
<tr>
<td><strong>Editor(s)</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Citation</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Issue Date</strong></td>
<td>2014</td>
</tr>
<tr>
<td><strong>URL</strong></td>
<td><a href="http://hdl.handle.net/10466/13864">http://hdl.handle.net/10466/13864</a></td>
</tr>
<tr>
<td><strong>Rights</strong></td>
<td></td>
</tr>
</tbody>
</table>
Dynamics of cholera outbreaks in endemic areas of Kenya

（ケニアにおけるコレラアウトブレークの発生動態）

Suleiman Mzee Saidi

2014年
CONTENTS

GENERAL INTRODUCTION.........................................................................................1

CHAPTER 1. Epidemiology of cholera in endemic areas of Kenya

1.1. INTRODUCTION..............................................................................................13
1.2. MATERIALS AND METHODS.........................................................................15
1.3. RESULTS..........................................................................................................19
1.4. DISCUSSION....................................................................................................20
   TABLES AND FIGURES.........................................................................................26

CHAPTER 2. Prevalence of antimicrobial resistance in Vibrio cholerae isolates

2.1. INTRODUCTION..............................................................................................31
2.2. MATERIALS AND METHODS.........................................................................32
2.3. RESULTS..........................................................................................................34
2.4. DISCUSSION....................................................................................................35
   TABLES AND FIGURES.........................................................................................39

CHAPTER 3. Molecular characterization of Vibrio cholerae strains associated with cholera outbreaks

3.1. INTRODUCTION..............................................................................................44
3.2. MATERIALS AND METHODS.........................................................................46
3.3. RESULTS..........................................................................................................51
3.4. DISCUSSION....................................................................................................52
   TABLES AND FIGURES.........................................................................................57

GENERAL DISCUSSION........................................................................................65
ACKNOWLEDGEMENT..........................................................................................69
REFERENCES.........................................................................................................72
GENERAL INTRODUCTION

Cholera is an acute secretory diarrhea, which is characterized by voluminous excretion of rice water stool (Sack et al., 2004; Fig. I), vomiting, severe dehydration, sunken eyes and poor skin turgor (Chowdhury et al., 2010; Fig. II). It is the loss of body fluids that is associated with cholera deaths especially when the disease is not managed or treated. Cholera is one of the most ancient scourges that may have been recorded. Symptoms of a disease resembling cholera were described in the Indian Sanskrit scriptures in the 5th century (Pollitzer, 1954). However, it was not until the 19th century that records related to cholera started to appear. The causative agent of cholera was first described and named *Vibrio cholera* by Filippo Pacini in 1854, who also firmly believed that it was contagious, a concept which was vehemently opposed by his contemporaries (Bentivoglio & Pacini, 1995). Thirty years later in 1884, a German scientist Robert Koch isolated *Vibrio cholerae* in pure culture (Fig. III) and called it *kommabazillen*. The name *V. cholerae* was adopted in honor of Fillipo Pacini who had died in 1883 (Bentivoglio & Pacini, 1995).

Cholera is the only waterborne disease which occurs in the form of true pandemic. Since 1817, the world has experienced seven cholera pandemics (Fig. IV). The first pandemic started in the Bengal area and spread to India, China, Indonesia and reached Caspian Sea in Europe (Sack et al., 2004; Kaper et al., 1995). All pandemics were linked with the Bay of Bengal, which was traditionally referred to as the home of cholera except the current seventh pandemic which originated from the Celebes islands of Indonesia in 1961 (Kaper et al., 1995). The Seventh pandemic spread to other parts of the world including India in 1964, Africa and Europe in 1970 and South America in 1991 (Sack et al., 2004).

Since the beginning of the millennium cholera outbreaks in Africa has escalated and surpassed endemic countries in South Asia including Bengal which has all along been documented as the home of cholera (Mandal et al., 2011). More than 90% of all cases
reported worldwide between 1997 and 2009 originated from Africa (WHO 2010; Figs. V and VI). The magnitude of these cholera outbreaks were also associated with high fatality rates reaching up to 15% for some of the African countries (WHO, 2010). The shift of cholera outbreak from traditionally endemic areas to Africa has necessitated a need to carry out holistic research on all aspects of cholera since most African countries rely heavily on information originating from cholera endemic countries in Asia (Rebaudet et al., 2013). Information on the dynamics of cholera and *V. cholerae* in Africa does not correspond with global burden that the continent has reported.

Cholera is basically a waterborne disease and can only be contracted by drinking water or consumption of food contaminated with *V. cholerae* (Kaper et al., 1995). The epidemiology of cholera may have started with John Snow in the 1850’s, even before the discovery of *V. cholerae* (Dawson & Sartory, 2000). John Snow demonstrated that cholera was transmitted through one of the water supplies in London which sourced water from a contaminated area of river Thames. Since then it has been recognized that fecal contamination of drinking water was a key factor in the spread of cholera. Improvement of sanitation and provision of clean water has led to the eradication of cholera and other water borne diseases especially in developed countries. In contrast to the developed world, developing countries, especially Africa are yet to realize adequate supply of clean water and provision of good sanitation. The situation is further complicated by several other factors which allow the spread of cholera or promote endemicity in areas which were not prone to cholera. Such factors include political instability in many African countries (Hatch et al., 1994; Rebaudet et al., 2013) and natural disasters such as floods (de Magny et al., 2012). Africa will therefore have to deal with the reality of immediate management of cholera, as it continues to carry the biggest burden of the scourge.
The cornerstone of cholera management is provision of quality public health education, clean water and sanitation (Mandal et al., 2011). Although rehydration using oral rehydration solution (ORS) and intravenous fluid is recommended for management of cholera patients, antibiotics may be used to reduce duration of illness, minimize shedding of *V. cholerae* and prevent transmission of cholera. The use of antimicrobial agents started in the 1930’s, after the discovery of Penicillin by Alexander Fleming and colleagues (Cohen, 1992). The antimicrobial agents are classified according to their diverse mode of actions, but microorganisms have developed resistance to almost every antimicrobial drug that is available (Kitaoka et al., 2011; Ghosh and Ramamurthy, 2011). Guidelines on the use of antibiotics for cholera patients may vary, for example the WHO recommends use of antibiotics for managing severely dehydrated patients (≥10% dehydration), while the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR, B) advocates giving antibiotics to patients even with ‘some dehydration’ (5 to 10%) if confirmation is made within 24 hours of the onset of acute watery diarrhea that the patient has cholera and the diarrhea is not due to other pathogens (Nelson et al., 2011). Lack of clear guidelines and the imprudent use of antimicrobials have heralded the development of multidrug resistant (MDR) strains of toxigenic *V. cholerae* (Kaper et al., 1995; Sack et al., 2004). The guideline by ICDDR, B on management of cholera is based on comprehensive and systematic studies carried out by the Institute and similar establishments in South Asia. Such kinds of studies need to be adopted for Africa, as the continent may be a new homeland for cholera (Gaffga et al., 2007). It is important to note that one of the earliest report of tetracycline resistance, the antibiotic of choice for cholera treatment, emanated from Africa (Mhalu et al., 1979). Similarly, massive death from cholera caused by a tetracycline resistant strain of *V. cholerae* was reported from Goma, Zaire (Siddique et al., 1995). The responding international community may have lacked the information on tetracycline resistance in that particular strain. There is need to
monitor circulating strains of *V. cholerae* for prevailing antimicrobial resistance and genotypic characteristics. *V. cholerae* is one of the most studied bacteria and has evolved considerably since it was first recognized.

The genus *Vibrio*, a member of the family *Vibrionaceae* consists of Gram-negative motile, curved (comma) shaped or straight bacteria (Fig. III). The family *Vibrionaceae* is classified with *Enterobacteriacae* and *Pseudomonadaceae* under the class γ-proteobacteria based on phylogenetic analysis. However, *Vibrio* can be distinguished from enteric bacteria by being oxidase positive and from pseudomonads by facultative metabolism. *Vibrio* bacteria are aquatic and can thrive in fresh and salty water as free cells or in attachment with other aquatic cells (Faruque *et al*., 1998). *Vibrio* comprises of many species but of medical importance include *V. cholerae*, *V. parahaemolyticus*, *V. vulnificus* and *V. mimicus* (Faruque *et al*., 1998). *V. cholerae* is the most severe of all these species since some of the strains cause cholera disease (Sack *et al*., 2004). There are more than 200 ‘O’ serogroups of *V. cholerae* but only O1 was responsible for cholera epidemics and pandemics (Sack *et al*., 2004). Based on biotyping *V. cholerae* O1 is divided into two groups, classical and El Tor biotype. Furthermore, based on serotyping, *V. cholerae* O1 can be classified into Ogawa, Inaba or Hikojima. In 1992, however, *V. cholerae* non-O1 was reported to cause a large outbreak of cholera-like diarrhea in India and Bangladesh (Albert *et al*., 1993). This *V. cholerae* non-O1 strain was termed as *V. cholerae* O139 because it was not reactive to the 138 known antisera (Kaper *et al*., 1995). Later on the *V. cholerae* O139 strain was also recognized as the causative agent of cholera. Recently, there has been an emergence of new *V. cholerae* O1 pathogenic variants which possess traits of both classical and El Tor biotypes (Nair *et al*., 2002). These variants can be differentiated by genetic comparative analysis of several genes such as *ctxB*, *tcpA*, *rstC*, *rtxC*, etc. (Raychoudhuri *et al*., 2008; Table I). The O1 El Tor variant has been shown to be more virulent than the El Tor prototype and has been reported
from Asian and African countries (Safa et al., 2010). Pathogenicity of V. cholerae O1 is associated with the production of cholera toxin (CT) and toxin co-regulated pilus (TCP) encoded by the ctx and tcpA genes, respectively. On the other hand, V. cholerae non-O1/non-O139 normally lack ctx and tcpA genes but are associated with sporadic cases of diarrhea and extra-intestinal infections. Virulence in V. cholerae non-O1/non-O139 is varying and attributed to type three secretion system (T3SS), heat-stable enterotoxin (NAG-ST), cholix toxin (ChxA), etc.

In this study, epidemiological survey of infectious diarrhea including cholera was carried out in cholera endemic region located in the Kenyan coastal area (between 1991 and 1993 and from 2007 to 2009) and an inland lake area (from 2007 to 2009). Prevalence of V. cholerae was compared with that of Salmonella spp. and Shigella spp. in patients with diarrhea, food handlers and cholera index cases. By adopting new typing schemes, V. cholerae isolates were identified to their atypical levels. Prevalence of antimicrobial resistance in V. cholerae isolates was determined. Molecular traits, presence of virulence factors and clonal relatedness of V. cholerae isolates were assessed. The findings of this study illustrates that cholera in Kenya have not reached endemicity levels like those found in Asian countries, however, serotype switching, extended multidrug resistance and replacement of V. cholerae O1 El Tor prototype by the V. cholerae O1 El Tor variant have been observed. The phenotypic analysis of V. cholerae isolated in different years from the coast and lake regions of Kenya was included in Chapter 1. In Chapter 2, the prevalence of multidrug resistance in V. cholerae O1 and non-O1/non-O139 was determined. Genotypic characterization of isolates, determination of virulence factors and clonal relatedness of V. cholerae strains was carried out in Chapter 3.
Fig. I. Rice water stool typical of cholera patient (Chowdhury et al., 2010).
Fig. II. (A) Dehydrated cholera patient; (B) Cholera patient post recovery (Chowdhury et al., 2010).
Fig. III. (A) *Vibrio cholerae* growing on thiosulphate citrate bile salt sucrose (TCBS) agar plates; (B) *V. cholerae* as seen under electron microscope. Magnification 10,000x.

http://www.nature.com/nature/journal/v406/n6795/fig_tab/406469a0_F1.html
Fig. IV. Key evolutionary events in cholera epidemiology since 1817.
The first six pandemics are thought to be caused by the classical biotype. Between
the sixth and seventh pandemics, there was a pandemic free period. The ongoing
seventh pandemic, which was initiated from Indonesia in 1961, is caused by O1 El
Tor biotype. (Safa et al., 2010)
Fig. V. Cholera cases reported to World Health Organization by year and continent (1989-2012).

http://www.who.int/gho/epidemic_diseases/cholera/cholera_005.jpg
Fig. VI. Cholera, areas reporting outbreaks, 2007–2009. Sub-Saharan Africa including Kenya in yellow color indicates high cholera burden.

http://www.who.int/topics/cholera/en/
Table I. New scheme for biotyping of *V. cholerae* O1

<table>
<thead>
<tr>
<th>Traits</th>
<th>Biotype</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>El Tor</td>
</tr>
<tr>
<td>VP test</td>
<td>+</td>
</tr>
<tr>
<td>Susceptibility to polymyxin B (50U)</td>
<td>-</td>
</tr>
<tr>
<td>Chicken cell agglutination</td>
<td>+</td>
</tr>
<tr>
<td>Lysis by classical IV phage</td>
<td>-</td>
</tr>
<tr>
<td>Lysis by El Tor phage</td>
<td>+</td>
</tr>
<tr>
<td>CT epitype</td>
<td>CT2</td>
</tr>
<tr>
<td>ctxB genotype</td>
<td>El Tor</td>
</tr>
<tr>
<td>rtxC</td>
<td>+</td>
</tr>
<tr>
<td>tlc</td>
<td>+</td>
</tr>
<tr>
<td>tcpA</td>
<td>El Tor</td>
</tr>
<tr>
<td>RS element</td>
<td>RS1, RS2</td>
</tr>
</tbody>
</table>

VP: Voges-Proskauer; CT: cholera toxin; *ctxB*: cholera toxin B subunit; *rtxC*: repeat-in-toxin, *tlc*: toxin-linked cryptic phage gene; *tcpA*: toxin co-regulated pilus (Raychoudhuri *et al.*, 2008)
CHAPTER 1. Epidemiology of cholera in endemic areas of Kenya

1.1 INTRODUCTION

Cholera is one of the most devastating diseases encountered by human. The disease which is characterized by acute secretory diarrhea and loss of body fluids is caused by the Gram-negative bacterium, *Vibrio cholerae* (Bentivoglio and Pacini, 1995; Sack *et al.*, 2004). There are more than 200 serogroups of *V. cholerae* but only two, O1 and O139 are known to cause pandemic cholera (Kaper *et al.*, 1995). Based on the combination of biochemical and serological tests and more recently genotypic analysis, *V. cholerae* O1 is further classified into classical or El Tor biotype. Cholera is the only waterborne disease which can spread to various parts of the world simultaneously, in a form referred to as cholera pandemic. The history of cholera is older than the documented pandemics as reported in Indian Sanskrit scriptures back in the 5th century, which describes a disease that resembles cholera (Sack *et al.*, 2004). Since 1817, the world has experienced seven cholera pandemics. The 5th and 6th cholera pandemics were caused by the O1 classical biotype (Kaper *et al.*, 1995). The O1 El Tor biotype is associated with the 7th pandemic which originated from the Celebes islands of Indonesia in 1961 (Kaper *et al.*, 1995) and spread to other parts of the world including India in 1964, Africa and Europe in 1970 and South America in 1991 (Sack *et al.*, 2004).

What started as sporadic cholera outbreaks in Africa has escalated and surpassed some countries in South Asia which has long been documented as cholera endemic areas (Mandal *et al.*, 2011). An upsurge of cholera outbreaks in Africa was noted to supersede other continents. For example, between 1995 and 2005, Africa had 417 outbreaks out of a total global report of 632 outbreaks. The total numbers of cases were 423,904 that made up 87.6% of the global total of 484,246 cases (Griffith, 2006). More than 90% of all cases reported worldwide between 2006 and 2009 originated from Africa (WHO, 2010). The magnitude of these cholera outbreaks led to the speculation that cholera may have found a new homeland in
Africa (Gaffga et al., 2007). The speculation was augmented by effects of global weather change, natural disasters such as floods (de Magny et al., 2012; Bompangue et al., 2011; Reyburn et al., 2011; Emch et al., 2008; Alajo et al., 2006) and political instability which led to the mushrooming of refugee camps in various parts of Africa (Hatch et al., 1994; Rebaudet et al., 2013). The coastal and lake regions provide favorable ecological environments for *V. cholerae* to thrive as reported from aquatic studies from Asian countries (Sedas, 2007). Africa was also affected by global climate changes and may experience the cholera paradigm (Colwell, 1996). The role of human carriers and transmission of *V. cholerae* was reviewed following the Haiti cholera outbreak (Piarroux & Faucher, 2012). The role of cholera index cases have been reported elsewhere (Weil et al., 2009; Mukherjee et al., 2011) as well as the significance of food handlers in the dissemination of cholera outbreaks (Swaddiwudhipong et al., 2008).

Along with the global increase in the number of cholera outbreaks since 2000, the highest numbers of cases in Kenya were reported in 2007 and 2009 (WHO 2008; WHO 2010). In 2007, Kenya reported 1,206 cases with a case fatality rate of 5.56%, whereas 11,425 cases were reported in 2009 with a case fatality rate of 2.31% (WHO 2008; WHO 2010). Studies carried out in Kenya on cholera were mainly based on the following studies. (1) Analysis of *V. cholerae* O1 isolates collected from various outbreaks or/and different regions of the country (Kiiru et al., 2013; Mohamed et al., 2012). (2) Response to a given cholera outbreak (Shapiro et al., 1999; Iijima et al., 1995) and (3) Retrospective report or review of past findings (Mutonga et al., 2013). Similarly, in the routine analysis of diarrheal samples, *V. cholerae* is not considered compared to other enteropathogens (Sang et al., 2012). Examination of food handlers working in hotels and hospitality industry is carried out every six months and is confined to the detection of intestinal parasites and *Salmonella Typhi* antigen (Kamau et al., 2012). If Africa had a potential of new homeland for *V. cholerae*
(Gaffga et al., 2007), then there is need for holistic approach in the study of cholera outbreaks in Africa. Such an approach has been adopted in responding to the recent cholera outbreak in Haiti (Barzilay et al., 2013). The author identified the coastal area of Kenya for cholera investigation since the region has experienced almost every cholera outbreak that has occurred in the country (Kiiru et al., 2013; Mutonga et al., 2013). An epidemiological study on infectious diarrhea including cholera was carried out in Malindi between 1991 and 1993 and in Kwale from 2007 to 2009. The role of polymicrobial infections, food handlers and index cases was studied in relation to cholera outbreaks. The author also analyzed *V. cholerae* isolates from an outbreak which occurred in Nyanza, a lake region of Kenya in 2008. An epidemiological follow up study could not be carried out in Nyanza because of insecurity caused by a disputed presidential election in Kenya in 2007.

1.2 MATERIALS AND METHODS

1.2.1 Selection of study area and subjects

The main study areas were Malindi and Kwale, which are both located in coastal areas of Kenya (Fig. 1.1). These counties were selected, since they are among the cholera hot spots in Kenya. The author had access to laboratory units at Malindi and Kwale which were operated by the Centre for Microbiology Research (CMR), one of the centers of the Kenya Medical Research Institute (KEMRI). Stool samples were collected from patients attending Kwale and Malindi hospitals. In addition, food handlers working in various food establishments in Kwale were included in the study. Malindi and Kwale counties are situated in the northern and southern coast of Kenya respectively. Both Counties share similar weather pattern and environmental challenges and are the leading destination for tourists visiting Kenya. In this study, the Kwale County captured the cholera outbreak surge experienced in Kenya between 2007 and 2009. County is made up of four proposed sub-counties which include, Msambweni, Kinango, Lunga Lunga and Matuga. Although Kwale is mainly an
inland County it boosts one of the most popular coastlines south of Mombasa, the Diani beach. Several tourist resorts are found along the Diani beach. Kwale also has famous tourist attractions such as the Shimba Hills National Reserve and the Mwaluganje elephant sanctuary. The county has a population of approximately 496,000. The other area that was included in the study is Nyanza Province of Kenya which, like the coast province is one of Kenya's eight administrative provinces. It is located in the southwest part of Kenya around Lake Victoria. Specimens were collected from one of the districts in Nyanza called Suba which has a population of approximately 155,666 people (Fig. 1.1).

The study subjects were persons suffering from gastroenteritis with or without diarrhea and attending the Malindi and Kwale hospitals. Food handlers going for medical examination were recruited for the study after informed consent. Based on anecdotal clinical data from hospitals in the study area, it was estimated that, in the absence of diarrhea outbreaks the isolation rate of the study organisms is 1.0% in the area. A minimum of 400 clinical samples were statistically significant at confidence level of 95%. Cholera index cases and all other cholera patients reporting to the above medical facilities were investigated. Stool specimens were uniformly analyzed for *Salmonella* spp., *Shigella* spp. and *V. cholerae*. Due to ethical considerations, any other pathogens, such as parasites, rotavirus and other enteropathogens, if detected, were reported to the medical officers or clinicians for management of the patients.

1.2.2 Sample collection

Stool specimens were collected in wide mouthed containers and transported to the laboratory within two hours of collection. Rectal swabs were transported in Cary Blair medium. Similarly, if delay was anticipated the specimens were transferred into the Cary Blair transport media using a cotton swab for onward transmission to the laboratory.
1.2.3 Media and reagents

The following media were obtained from Oxoid Co. Ltd. (Basingstoke, England); Cary Blair, alkaline peptone water (APW), selenite broth base, Salmonella Shigella agar (SS), MacConkey agar, nutrient agar, Simmons citrate agar, triple sugar iron (TSI) agar, blood agar base, thiosulfate-citrate-bile salts-sucrose (TCBS) agar was purchased from Eiken chemical Co. Ltd. (Tokyo, Japan). The following were purchased from Nissui Pharmaceutical Co. Ltd. (Tokyo, Japan); xylose lysine deoxycholate (XLD) agar, heart infusion agar (HIA), lysine indole motility (LIM) medium, methyl red-Vogues Proskeur (MR-VP) medium and urea base broth medium. All reagents including oxidase strips, sodium chloride, sodium biselenite, etc. were obtained from Nissui Pharmaceutical Co. Ltd. (Tokyo, Japan). API 20E strips and relevant reagents were obtained from bioMeriux, France. Poly and monoclonal antisera for various serogroups of \( V. \) cholerae, Salmonella spp. and Shigella spp. were purchased from Denka Seiken Co. Ltd. (Tokyo, Japan). Whole sheep blood was obtained from the Faculty of Veterinary Medicine, University of Nairobi, Kenya.

1.2.4 Processing of specimens

Enrichment and direct plate inoculation was done for the stool samples. Enrichment for \( V. \) cholerae was done by inoculating stool samples in APW followed by incubation at 37°C for 6 h. The samples were inoculated in selenite fecal broth for enrichment of Salmonella spp. and incubated at 37°C for 18 h. The specimens were also inoculated on SS, XLD, MacConkey and TCBS agar, respectively. All the inoculated culture media were incubated at 37°C for 18 h. The APW enriched culture was sub-cultured onto TCBS agar plates while selenite broth was sub-cultured onto SS and XLD plates, respectively. These plates were incubated as described above. The following colonies were subjected to biochemical screening and identification; Yellow colonies from TCBS, pale or pale black colonies from SS agar and red or red black from XLD plates.
1.2.5 Biochemical screening for pathogens

Biochemical screening of *Enterobacteriaceae* was done by using TSI agar, LIM medium, Simmons citrate agar, MR-VP medium and urea medium. The tests were performed as described elsewhere (WHO, 2003; Sakazaki and Shimada, 1986). Suspected pathogens were further identified using the API-20 E test system as instructed by the manufactures (Appareils et Procedes d’Identification Montalieu Vercieu, France). Briefly, the test organism was inoculated on nutrient agar and incubated overnight at 37°C for 18 h. One to two colonies were picked from the nutrient agar plates and emulsified in sterile normal saline to match a MacFarland 0.5 standard tube. The API 20E cupules were inoculated as instructed by the manufacturer (API system, France). The results were read with the aid of a profile recognition system (P.R.S). Sucrose positive colonies growing on TCBS agar were sub-cultured on HIA plates for further screening including tests for hemolysis, oxidase, lysine decarboxylase and reaction in TSI. All these tests were carried out as described by CDC and WHO (www.cdc.gov/cholera/laboratory.html; WHO, 2003).

1.2.6 Serogrouping

*Salmonella* spp. and *Shigella* spp. were serotyped as described (WHO, 2003). Briefly, pure cultures from TSI slants were used. A drop of about 10 µl of the antiserum was placed on a clean dry glass slide. A sterile straight wire was used to pick bacteria from the TSI slant and mix with the antiserum. Agglutination was observed by tilting the slide up and down. Serogrouping was confined to somatic O-antigens.

Suspected *V. cholerae* strains were serotyped from HIA plates by using polyvalent O1 and O139 antisera and mono-specific Ogawa and Inaba antisera, respectively. The colonies were first screened using *V. cholerae* polyvalent O1 antisera. Isolates which were identified as
V. cholerae O1 were further serotyped using Ogawa and Inaba antisera. Strains which failed to agglutinate with the O1 antiserum were serogrouped with O139 antiserum.

1.3 RESULTS

1.3.1 Prevalence of V. cholerae, Salmonella spp. and Shigella spp. in patients

A total of 862 stool specimens were collected from patients with diarrhea in Malindi between 1991 and 1993, whereas 1,604 stool samples were collected from patients reporting gastroenteritis with or without diarrhea in Kwale between 2007 and 2009. Two hundred and thirty nine (27.7%) of the Malindi specimens yielded enteropathogenic organisms, with the distribution of Salmonella spp. (n=63; 7.3%) and Shigella spp. (n=56; 6.5%) but not V. cholerae (Table 1.1). In the case of Kwale, enteric pathogens were isolated from 347 (21.6%) of all the specimens including Shigella spp. (n=175; 11%), Salmonella spp. (n=110; 6.9%), V. cholerae O1 (n=59; 2.8%), V. cholerae non-O1/non-O139 (n=13; 0.8 %) (Table 1.2). The investigated pathogens were not detected in 1,247 (79%) of the samples (Table 1.2).

1.3.2 Carriage of V. cholerae, Salmonella spp. and Shigella spp. by food handlers

A total of 1,401 stool specimens were collected from food handlers for bacteriological analysis. Enteric pathogens were not detected from 1,369 (97.7%) of the specimens (Table 1.3). Salmonella spp. was isolated from 22 (1.6%) of the food handlers while Shigella spp. was isolated from 10 (0.7%) of the test group as shown in Table 1.3. V. cholerae O1 and V. cholerae non-O1/non-O139 were not isolated from any of the food handlers (Table 1.3).

1.3.3 V. cholerae in Index cases

Stool samples from 9 patients referred to as cholera index cases were analyzed for pathogenic bacteria. Six patients were investigated in the 2007 cholera outbreak and 3 during the 2009 outbreak. V. cholerae O1 was detected from 4 and 2 of the 2007 and 2009 cases,
respectively. *Aeromonas* spp. was isolated from two of the 2007 index cases, while non-typhoid *Salmonella* was isolated from a 2009 index case (Fig. 1.2).

### 1.3.4 Cholera outbreak cases

In 2007, *V. cholerae* were isolated from 40 (30%) out of 134 samples which were sent for cholera investigations in Kwale. Twenty five strains were *V. cholerae* O1 serotype Ogawa and 15 were *V. cholerae* non-O1/non-O139. In the 2009 Kwale cholera outbreak, *V. cholerae* O1 Inaba was detected from 19 (70%) of the 27 samples, which were investigated (Table 1.4). Eighty-one strains from the 2007 cholera outbreak in Nyanza were all identified as *V. cholerae* O1 Inaba strains except one strain, which was identified to be *V. cholerae* non-O1/non-O139. The adult population (>15 years) was mostly affected by the cholera outbreaks (Table 1.4).

### 1.4 DISCUSSION

Infectious diarrhea is a major cause of morbidity and mortality in developing countries (Haque *et al.*, 2003). In most of the African countries it is the 3rd cause of death in children under five years after malaria and pneumonia (WHO, 1995). The study suggests that diarrhea is still a major cause of concern especially among children in the coastal region of Kenya (Saidi *et al.*, 1997). Therefore, an increase in the case of cholera outbreaks in Africa (WHO, 2010) since the beginning of the millennium may have added to the burden of diarrheal diseases in the continent. There was concern that cholera may have found a new homeland in Africa (Gaffga *et al.*, 2007) now that the continent is reporting more cholera cases and cholera related deaths than Asian countries where cholera is established (Griffith *et al.*, 2006). This study shows that diarrheic disease including cholera is still a major health problem in the Kwale area of Kenya, however most of the results are different from what is reported in endemic Asian countries where cholera is established. In addition to *V. cholerae*, *Salmonella*
spp. and *Shigella* spp. were investigated in this study since the two organisms are of crucial public health concern (WHO, 2003). The frequency with which *Salmonella* spp. and *Shigella* spp. were isolated from patients with gastroenteritis with or without diarrhea is in consistent with reports from other studies, which were carried out in Kenya (Sang *et al*., 2012). However, this is the first report of microbiological investigation of diarrheal patients, food handlers and diarrheal outbreak which was carried out for a period spanning two cholera outbreaks in the same location in Kenya. In a systematic surveillance study on prevalence of enteropathogens among hospitalized patients in Orissa, India, Samal *et al*. (2008) were able to detect *V. cholerae* in periods which were not within the outbreak seasons between 2005 and 2006. They isolated *V. cholerae* O1 in at least nine months of each year. Studies on polymicrobial enteric infections carried out in Bangladesh, showed that *V. cholerae* was significantly isolated throughout the study and not limited to cholera outbreak periods (Nair *et al*., 2010; Lindsay *et al*., 2011; Alam *et al*., 2011). In this study, *Salmonella* spp. and *Shigella* spp. were isolated in several months in a given year. Institutional based outbreaks of shigellosis, especially in high schools, were observed in Kwale. However, only *V. cholerae* O1 was isolated during the cholera outbreaks of 2007 and 2009. Similar studies in Kolkata, India have shown that *V. cholerae* O1 can be detected in polymicrobial infections in routine pathological diagnosis in hospital settings (Nair *et al*., 2010; Lindsay *et al*., 2011). A recent study by Sang *et al*. (2012) reported isolation of *V. cholerae* O1 from four children with diarrhea. The study covered four out of seven provinces in Kenya and the isolation of *V. cholerae* was realized from two provinces namely Nairobi and Nyanza. However, it is important to note that Sang *et al*. (2012) carried their study in a period when those areas were experiencing cholera outbreaks (Mohamed *et al*., 2012). O’Reilly *et al*. (2012) reported a plethora of microorganisms excluding *V. cholerae* O1 as the cause of death among children hospitalized with diarrhea in cholera prone rural western Kenya between 2005 to 2007. Okeke *et al*.
(2003) did not isolate *V. cholerae* O1 in their study involving adults in south-western Nigeria who were suffering from acute diarrhea. Experiences elsewhere in Africa has shown that *V. cholerae* can only be isolated during cholera outbreaks (Adagbada *et al*., 2012; Alajo *et al*., 2006; Ansaruzzaman *et al*., 2004; Keddy *et al*., 2007) with the exception of the Democratic Republic of Congo (DRC). Historically, DRC was reported to bear the biggest burden of cholera in Africa (Bompangue *et al*., 2011). Massive displacement of people in DRC because of civil wars is associated with high incidence of cholera outbreaks. Inter-epidemic incidence of cholera cases were also reported from the same area (Bompangue *et al*., 2012) However, a systematic surveillance backed by microbiological investigations is needed to confirm such reports.

Food handlers play a key role in the transmission of enteric infection (Beatty *et al*., 2009). Studies carried on food handlers in Kenya have reported presence of intestinal pathogenic parasites and pathogenic *Escherichia coli* (Oundo *et al*., 2008; Kamau *et al*., 2012). Although *Salmonella* spp. and *Shigella* spp. were isolated from the study subjects who were investigated, the author did not isolate *V. cholerae* O1 from the food handlers. This is a significant finding as the study was carried out within the 2007-2009 cholera outbreaks and none of the food handlers contracted cholera. The author concludes that food handlers did not have any role in the dissemination of the disease. Food handlers have been reported to cause cholera outbreaks in Thailand (Swaddiwudhipong *et al*., 2008; Swaddiwudhipong *et al*., 2012). The situation is more complicated when food handlers are asymptomatic carriers of toxigenic *V. cholerae*, as reviewed in cholera cases in USA (Steinberg *et al*., 2001). Tilak *et al.* (1997) reported the control of a cholera outbreak in a Delhi cantonment in India after identifying and isolating two food handlers who were positive for *V. cholerae* O1 El Tor. Unlike records from other endemic regions, most of the reported outbreaks associated with food consumption in Africa, including Kenya are related to feasts especially following the
burial of cholera victims (St Louis et al., 1990; Shapiro et al., 1999). Stringent public health measures are advocated during cholera outbreaks in Kenya, such as closing of unlicensed food establishments, inspection of food premises and markets and microbiological screening of food handlers for carriage of *V. cholerae*. Results of microbiological analysis of food handlers from the public health offices in Kwale was not available, which is also a reflection of the dismal performance of The National Public Health Laboratory Services (NPHLS), Center for Microbiology Reference Laboratory in Kenya (Mutonga et al., 2013). The study is unique because it systematically examined food handlers for *V. cholerae* before, during and after cholera outbreaks.

Cholera index cases are pivotal not only in epidemiological studies but also in minimizing spread of diseases as soon as they are identified and treated. A study in Bangladesh reported that the identification of the index case was followed by illness among family or contacts within minimum of 2 days (Weil et al., 2009). Mukherjee et al. (2011) reported that the contamination of water ponds by an index case was able to culminate to a cholera outbreak. The author received 9 specimens for confirmation of possible index case (Fig. 1.2). *Aeromonas* spp. was isolated from two cases and *Salmonella* spp. from one case. *Aeromonas* spp. is rarely isolated in Kenya but can be easily misidentified as *V. cholerae* (Abbott et al., 1998). The patient with *Salmonella* infection had travelled to Kwale from Nairobi, Kenya where a cholera outbreak had been reported. He developed profuse watery diarrhea and was admitted in a cholera ward at the Kwale hospital. He was recorded as an index case and a cholera alert was initiated. However, stool culture yielded *Salmonella* spp. upon which a case of cholera-like diarrhea caused by *Salmonella* spp. was reported (Saidi et al., 2011). Such cases of misdiagnosis contribute to unnecessary dosing of patients and contacts with tetracycline or doxycycline, which adds up to antibiotic resistance in circulating *V. cholerae* strains (Sack et al., 2004). There is need to involve microbiologists right from the
initial stages of cholera outbreaks to avoid misdiagnosis and false alert for cholera outbreaks. There is need to investigate the role of index cases in spread or initiation of cholera outbreaks in the African setting.

During the study period, the author responded to two consecutive cholera outbreaks in the Kwale region, one in 2007 and the other in 2009. The 2007 outbreak was associated with *V. cholerae* O1 serotype Ogawa and *V. cholerae* non-O1/non-O139 strains. The 2009 outbreak was solely caused by *V. cholerae* O1 serotype Inaba. *V. cholerae* O139 was not detected. The Ogawa and Inaba strains were El tor biotypes as confirmed by phenotypic analysis. *V. cholerae* strains isolated in the 2007 outbreak differed from the 2009 isolates in serotype. This is the first report to document the replacement of *V. cholerae* O1 serotype Ogawa by *V. cholerae* O1 serotype Inaba in two consecutive outbreaks in an African setting. The epidemiological shift from Ogawa to Inaba has also been reported in Asian countries (Garg *et al*., 2000b; Longini *et al*., 2002; Khuntia *et al*., 2010). Serotype switching in *V. cholerae* O1 has been shown to originate from mutation of genes responsible for biosynthesis of the O-antigenic component of the lipopolysaccharides (Yamasaki *et al*., 1999), which can occur naturally (Blokesch and Schoolnik, 2007). Studies carried out in Kenya reported the detection of either serotype, or presence of both Ogawa and Inaba (Iijima *et al*., 1995; Mohamed *et al*., 2012; Kiiru *et al*., 2013). To the best of the author’s knowledge, this is the first study to report a serotype switch in consecutive cholera outbreaks in Africa where an Ogawa strain is completely replaced by an Inaba strain. This study also differs from the results of the current Haiti outbreak, in which Inaba strains evolved as the outbreak was ongoing (CDC, 2012). This study reported the shift after a lull of one year period *i.e.*, 2008.

Patients recovering from an infection by *V. cholerae* O1 Ogawa serotype may not be protected by subsequent exposure to infection by Inaba serotype (Ali *et al*., 2011). This may explain the magnitude of the 2009 outbreak in Kenya, which turned out to be one of the
largest outbreaks the country experienced in two decades (Mohamed et al., 2012). On the contrary infection by Inaba strains may confers immunity against both to Inaba and Ogawa serotypes (Ali et al., 2011). With increased incidence of cholera outbreak in Africa and slowed progress in improvement of sanitary and public health facilities, cholera vaccines may be included in the fight against cholera. It is important to map out the circulating serotypes and also to include the Inaba strain in vaccines adopted for Africa including Kenya.

Conclusions:

✓ Diarrhea is a major cause of concern in the coastal area of Kenya
✓ Cholera outbreaks have increased in Africa but have not reached endemicity levels found in Asia
✓ V. cholerae O1 and non-O1/non-O139 can be detected in Kenya only during the cholera outbreak period
✓ Multiple sources of cholera transmission in Africa including Kwale are different from the mechanisms in endemic Asian regions
✓ Food handlers do not play an important role in Kwale in the transmission of cholera
✓ Misdiagnosis of index cases is common in Kwale
✓ Serotype switch may influence magnitude of cholera outbreaks in Africa
Fig. 1.1. Malindi, Kwale and Nyanza in Kenya are the geographic source of isolates analyzed in this study. Kwale and Nyanza areas experienced serious cholera outbreaks during 2007-2009.
Fig. 1.2. Misdiagnosis of index cases in the 2007 and 2009 outbreaks

<table>
<thead>
<tr>
<th>Year</th>
<th>Total Index Cases</th>
<th>V. cholerae</th>
<th>Others (Salmonella spp. &amp; Aeromonas spp.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2007</td>
<td>27</td>
<td>12</td>
<td>15</td>
</tr>
<tr>
<td>2008</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2009</td>
<td>3</td>
<td>2</td>
<td>1</td>
</tr>
</tbody>
</table>
### Table 1.1. Enteropathogens isolated from diarrheal patients in Malindi (1991-1993)

<table>
<thead>
<tr>
<th>Pathogen detected</th>
<th>No. of cases</th>
<th>(%) Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Salmonella</em> spp.</td>
<td>63</td>
<td>7.3</td>
</tr>
<tr>
<td><em>Shigella</em> spp.</td>
<td>56</td>
<td>6.5</td>
</tr>
<tr>
<td><em>Vibrio cholerae</em> O1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>V. cholerae</em> non-O1/non-O139</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>None of the above pathogens</td>
<td>743</td>
<td>86.2</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>862</strong></td>
<td><strong>NA</strong></td>
</tr>
</tbody>
</table>
Table 1.2. Enteropathogens isolated from diarrheal patients in Kwale (2007-2009)

<table>
<thead>
<tr>
<th>Pathogen detected</th>
<th>No. of cases</th>
<th>(%) Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salmonella spp.</td>
<td>110</td>
<td>6.9</td>
</tr>
<tr>
<td>Shigella spp.</td>
<td>175</td>
<td>11.0</td>
</tr>
<tr>
<td>Vibrio cholerae O1</td>
<td>38</td>
<td>2.3</td>
</tr>
<tr>
<td>V. cholerae non-O1/non-O139</td>
<td>13</td>
<td>0.8</td>
</tr>
<tr>
<td>None of the above pathogens</td>
<td>1268</td>
<td>79.0</td>
</tr>
<tr>
<td>Total</td>
<td>1604</td>
<td>100</td>
</tr>
</tbody>
</table>

Table 1.3. Enteropathogens isolated from food handlers in Kwale (2007-2009)

<table>
<thead>
<tr>
<th>Pathogen detected</th>
<th>No. of cases</th>
<th>(%) Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salmonella spp.</td>
<td>22</td>
<td>1.6</td>
</tr>
<tr>
<td>Shigella spp.</td>
<td>10</td>
<td>0.7</td>
</tr>
<tr>
<td>Vibrio cholerae O1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>V. cholerae non-O1/non-O139</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>None of the above pathogens</td>
<td>1369</td>
<td>97.7</td>
</tr>
<tr>
<td>Total</td>
<td>1401</td>
<td>100</td>
</tr>
</tbody>
</table>
Table 1.4. Age distribution of observed cholera cases between 2007 and 2009

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>V. cholerae O1 (Kwale, 2007)</th>
<th>V. cholerae O1 (Kwale, 2008)</th>
<th>V. cholerae O1 (Kwale, 2009)</th>
<th>V. cholerae O1 (Nyanza, 2008)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>%</td>
<td>No.</td>
<td>%</td>
</tr>
<tr>
<td>0-5</td>
<td>4</td>
<td>20.0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>6—15</td>
<td>6</td>
<td>30.0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>15&lt;</td>
<td>10</td>
<td>50.0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>NA</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Total</td>
<td>20</td>
<td>-</td>
<td>0</td>
<td>-</td>
</tr>
</tbody>
</table>

NA: data not available
CHAPTER 2. Prevalence of antimicrobial resistance in \textit{V. cholerae} isolates

2.1 INTRODUCTION

The antibiotic era began with the discovery of penicillin by Alexander Fleming in 1929, and its subsequent crystallization by Ernest Chain and Howard Florey in 1938. This period saw the discovery of penicillin, tyrothricin and actinomycin, of fungal, bacterial and actinomycete origins, respectively (Swartz, 2000). Introduction of antibiotics in the mid-1930 heralded the opening of an era, which literally spared millions of people from death due to bacterial infectious diseases (Cohen, 1992). The antimicrobial agents were classified according to their mode of actions including those which interfere or inhibit molecular development of bacterial cell wall ($\beta$-lactams), membranes (polymyxins), proteins (aminoglycosides, macrolides and tetracyclines), DNA (flouroquinolones) and metabolic pathways (sulfamethoxazole and trimethoprim). However, bacteria have demonstrated the remarkable ability to develop and share resistance to almost every available antimicrobial that has been developed (Kitaoka \textit{et al.}, 2011). Mechanisms of antibiotic resistance from acquired to intrinsic have been extensively reviewed (Kitaoka \textit{et al.}, 2011; Ghosh and Ramamurthy, 2011).

One of the biggest challenges in combating cholera is the development of antimicrobial resistance among the causative agent \textit{V. cholerae} O1 or O139. These challenges are augmented by the pandemic nature of cholera, clinical presentation, excessive and imprudent use of antibiotics in treating diarrhea, appearance of cholera outbreak in new land like Haiti (Piarroux \textit{et al.}, 2011; Barzilay \textit{et al.}, 2013) and increase of cholera outbreaks in Africa (WHO, 2010). Guidelines on the use of antibiotics may vary, for example, the WHO recommends use of antibiotics for managing severely dehydrated patients ($\geq 10\%$ dehydration), while the ICDDR,B advocates giving antibiotics to patients with severe dehydration as well as for those with ‘some dehydration’ (5 to 10\%). Confirmation should be
made within 24 hours of the onset of acute watery diarrhea that the patient has cholera and the diarrhea is not due to other pathogens (Nelson et al., 2011). The mainstay of cholera treatment is rehydration therapy whereas antibiotics are used to reduce transmission and lessen duration of illness. Although the number of outbreaks of cholera in Africa has steadily increased since the beginning of the millennium, most African countries, especially within coastal areas rely on information and data generated from Asian countries mainly within the Bay of Bengal, in dealing with cholera outbreaks (Rebaudet et al., 2013). Therefore, lack of systematic data on circulating V. cholerae strains may lead to misdiagnosis and imprudent use of antibiotics. There is paucity of systematic research on multidrug resistance (MDR) within the coastal area of Kenya.

This study analyzed MDR in clinical V. cholerae isolated from a coastal area in Kenya (Kwale) in two consecutive cholera outbreaks in 2007 and 2009. Multidrug resistance among the strains was compared with isolates from the outbreak occurred in inland lake region of Kenya (Nyanza) in 2008.

2.2 MATERIALS AND METHODS

2.2.1 Chemicals and reagents

Mueller Hinton (MH) and Luria-Bertani (LB) broth were purchased from Difco laboratories (Detroit, Mich., USA). Tryptic soya agar (TSA) and bacteriological agar were purchased from Nissui Pharmaceutical Co. Ltd. (Tokyo, Japan), TCBS agar was from Eiken Chemical Co. Ltd. (Tokyo, Japan). Chemicals were purchased from Nacalai Tesque (Kyoto, Japan), Wako Pure Chemical Industries (Tokyo, Japan), or Sigma-Aldrich (St. Louis, Mo. USA). All the antimicrobials were obtained from Becton Dickinson (USA). E-Test strips were purchased from bioMeriux SA (France).
2.2.2 Antimicrobial susceptibility test

Antimicrobial susceptibility test was carried out according to the guidelines provided by the Clinical and Laboratory Standards Institute (Cockerill, 2011). Briefly, 6 h shaking culture of V. cholerae in LB media was adjusted to 1x10^8 cfu/ml by diluting the cells in normal saline and measurement of the absorbance at OD_{625}. The bacteria were inoculated on MH agar with sterile cotton swabs. Antibiotic discs were then placed on the bacterial lawn. The following antimicrobials, which were obtained from Becton Dickinson (USA) were used; sulfamethoxazole/trimethoprim (SXT) (23.75/1.25 µg), fosfomycin (FOF) (50 µg), ampicillin (AMP) (10 µg), imipenem (IPM) (10 µg), chloramphenicol (CHL) (30 µg), tetracycline (TET) (30 µg), norfloxacin (NOR) (10 µg), nalidixic acid (NAL) (30 µg), streptomycin (STR) (10 µg), kanamycin (KAN) (30 µg), cefotaxime (CTX) (30 µg) and ciprofloxacin (CIP) (5 µg). If available, CLSI criteria specific for V. cholerae were used for the interpretation of zone of inhibition size. If such criteria were unavailable the antimicrobial susceptibility for bacterial cells belonging to the family Enterobacteriaceae was used. Escherichia coli ATCC 25922 was used for quality control testing.

2.2.3 Determination of antimicrobial minimum inhibitory concentration (MIC)

The MIC of antimicrobials to which the bacteria were either resistant or showed intermediate resistance was determined by the E-Test method (WHO, 2003). Briefly, tube cultures of V. cholerae isolates and E. coli ATCC 25922 were prepared as described above. All the cultures were adjusted to a concentration of 1x10^8 cfu/ml. A sterile cotton swab was used to inoculate moisture free MH agar plates containing 1.7% agar. The swab was dipped in the culture and carefully drained on the edge of the tubes before spreading on the surface of the plates. The same swab was passed along the edge of the media which was then allowed to dry. E-Test strips were then placed on the surface of the agar and incubated at 37°C for 18 h.
The following E-Test strips were used; AMP (0.016-256 μg/ml), NAL (0.016-256 μg/ml), CHL (0.016-256 μg/ml) and FOF (0.064-1024 μg/ml).

2.3 RESULTS

2.3.1 Antimicrobial susceptibility of *V. cholerae* from inland lake region, Nyanza

Eighty-one strains, *V. cholerae* O1 (*n*=80) and one non-O1/non-O139 from the Nyanza 2008 cholera outbreak were tested for antimicrobial susceptibility. The results of antimicrobial susceptibility of *V. cholerae* O1 strains are presented as resistant and susceptible in Table 2.1. All the strains were susceptible to TET, CIP, FOF, KAN and NOR. A few resistant strains were observed for AMP (*n*=2) and CTX (*n*=1). On the other hand, 100% of these strains were resistant to SXT and NAL whereas 97.5%, 93.8% and 62.5% of the strains tested were resistant to CHL, STR and IPM respectively (Table 2.1). In contrast, the *V. cholerae* non-O1/non-O139 strain was susceptible to all these antimicrobials except AMP. Majority of the *V. cholerae* O1 strains (*n*=74, 92.5%) shared a multidrug resistant (MDR) patterns for SXT, NAL, STR and CHL (Table 2.2).

2.3.2 Antimicrobial susceptibility of *V. cholerae* from coastal region, Kwale

The results of antimicrobial susceptibility of *V. cholerae* O1 Ogawa strains isolated from 2007 outbreak are shown in Table 2.3. All these isolates were susceptible to FOF, AMP, IPM, CHL, TET, NOR, KAN, CTX and CIP. On the other hand, 100% of these strains were resistant to STR and NAL whereas 78% of the isolates were resistant to SXT (Table 2.3). The three multidrug resistant patterns were observed as shown in Table 2.5. The *V. cholerae* non-O1/non-O139 isolates were sensitive to all antimicrobials except NAL and SXT, both of which had resistance rates of 100% and 84% respectively. The *V. cholerae* O1 Inaba strains isolated in 2009 were resistant to nine of the twelve antimicrobials tested including SXT, FOF, AMP, IPM, CHL, STR, KAN, CTX and NAL. The strains were susceptible to NOR,
TET and CIP (Table 2.4). The multidrug resistance profile was common for all the Inaba strains from Kwale (Table 2.5).

2.4 DISCUSSION

Generally MDR strains were isolated from all the three areas targeted in this study. The definition of MDR is adopted from Magiorakos et al. (2012). Multidrug resistance observed in V. cholerae O1 strains from Nyanza may not be as severe as resistance profiles reported elsewhere (Mukhopadhyay et al., 1995; Garg et al., 2000a). Unlike previous studies conducted with the V. cholerae O1 strains from the same area which reported TET resistant strains (Scrascia et al., 2006; Shapiro et al., 1999), 100% of Nyanza strains were susceptible to TET (Table 2.1).

The antimicrobial susceptibility of the V. cholerae strains from Kwale differed from the Nyanza isolates remarkably although some similarities with the 2007 Ogawa strains were observed. There were no significant differences in the antimicrobial susceptibility with respect to the other tested antimicrobials. The V. cholerae O1 Inaba strains which were isolated in 2009 from Kwale cholera outbreak demonstrated a more resistant pattern compared to those isolated in 2008 in Nyanza and in 2007 in Kwale. V. cholerae O1 Inaba strains isolated in 2009 in Kwale were resistant to 9 out of the 12 antimicrobial tested in this study. To the best of the author’s knowledge this level of extended drug resistance (XDR) in V. cholerae O1 has not been previously observed in Kenya and has not been associated with initiating cholera outbreaks in Africa. Studies carried out in Nyanza area revealed presence of MDR V. cholerae O1 especially to SXT, NAL and STR (Shapiro et al., 1999). Similar observations were also reported by Kiiru et al. (2009 and 2013). These studies by Kiiru et al. (2009 and 2013) were carried out during the same period as this study and involved isolates obtained in 2007 and 2009 from Nyanza and Kwale. However, the findings of this study are different. Kiiru et al. did not report V. cholerae O1 Ogawa in 2007 and the MDR Inaba strains
presented in this work. Kiiru et al. (2009) also reported that all V. cholerae O1 Inaba strains isolated in 2009 were fully susceptible to AMP, cefuroxime and CHL which contradicts with the findings of this study. Similarly none of the other studies from Kenya during this period reported any unusually high multidrug resistant V. cholerae O1 strains (Mahamud et al., 2012; Mugoya et al., 2008; Pugliese et al., 2009). A study in Tanzania reported that there was an increase in antimicrobial resistance among V. cholerae O1 strains isolated in 1999 compared to those isolated in 1997. However, the difference was noted in commonly used antimicrobials such as TET, AMP and erythromycin (Urassa et al., 2000). Tanzania was one of the first countries to report the isolation of TET resistant V. cholerae O1 (Mhalu et al., 1979). Although V. cholerae O1 Inaba strains isolated in 2009 in Kwale were resistant to 75% of the antibiotics tested, the strains were susceptible to TET. This is important since TET is the drug of choice when use of antibiotic intervention is required in the management of cholera (Mandal et al., 2011; Sack et al., 2004). Failure to prevent cholera deaths among Rwandan refugees in Goma, Zaire was attributed to the use of TET against a TET resistant V. cholerae O1 strain (Siddique et al., 1995).

Ismail et al. (2013) reported the isolation of an MDR V. cholerae O1 Ogawa strain from a South African cholera outbreak between November 2008 and April 2009. The strains were resistant to AMP, AMP-clavulanic acid, SXT, NAL, CHL, KAN, TET, STR and ceftazidime. However these strains, which only formed 1.0% of the total isolates, were isolated in January 2009. V. cholerae O1 Inaba strains isolated in 2009 in Kwale were the only isolates obtained in the 2009 Kwale outbreak and 100% of the strains demonstrated a single pattern of extended resistance to the 9 antimicrobials. None of the studies in Africa reported such findings where a distinct MDR phenotype replaces another less resistant strain in consecutive cholera outbreaks. Multiple antimicrobial resistances among V. cholerae O1 strains have been extensively reviewed (Kitaoka et al., 2011; Ghosh & Ramamurthy, 2011).
The antimicrobial resistance of the Ogawa isolates to SXT, NAL and STR has also been reported elsewhere (Taneja et al., 2009). Mutreja et al. (2011) reported the acquisition of SXT resistance element by *V. cholerae* O1 between 1978 and 1984. The circulating Kenyan *V. cholerae* O1 strains have been shown to possess genes encoding for multiple antimicrobial resistance (Kiiru et al., 2009). It is possible that the *V. cholerae* O1 Inaba strains isolated in 2009 in Kwale harbors the SXT element in addition to the extended-spectrum β-lactamase (ESBL) genes. This study also reports for the first time in Kenya, the isolation of *V. cholerae* O1 strains which are resistant to a 3rd generation cephalosporin. Similar observations were reported in *V. cholerae* O1 strains, which were isolated from illegal miners in South Africa (Ismail et al., 2013). Similarly, other African countries have reported outbreaks of cholera due to MDR *V. cholerae* (Rakoto et al., 2001; Mwansa et al., 2007; Abera et al., 2010; Marin et al., 2013). Reduced susceptibility to NAL and CIP has also been reported elsewhere (Faruque et al., 2003; Quilici et al., 2010; Tran et al., 2012). All the *V. cholerae* O1 strains analyzed in this study were resistant to NAL but were susceptible to CIP. SXT is commonly used antimicrobial in Kenya to treat children suffering from infectious diseases (Saidi et al., 1997) and considering our results SXT should not be recommended to use for treatment of cholera in children.

*V. cholerae* non-O1/non-O139 isolated in 2007 in Kwale were only resistant to SXT (85%) and NAL (100%) (Table 2.3), unlike other *V. cholerae* non-O1/non-O139 reported elsewhere (Dalsgaard et al., 1999; Dutta et al., 2013; Luo et al., 2013) which displayed more diverse antimicrobial resistance. Antimicrobial susceptibility test for *V. cholerae* non-O1/non-O139 is rarely carried out in Kenya and clinical significance in the outbreaks is not considered as that in Asian countries. The *V. cholerae* non-O1/non-O139 strains can serve as reservoir for antimicrobial resistant genes in the environment and may cause cholera-like
infections. It is thus important to determine the virulence genes and clonal relatedness of \textit{V. cholerae} isolates as described in this study.

Conclusions:

- Replacement of a less resistant \textit{V. cholerae} O1 strain by an extended antibiotic resistant strain in a subsequent outbreak within the same locale can occur in Africa
- Resistance to extended generation cephalosporin may be common in Kenya
- Tetracycline resistant strains has diminished in the coastal area of Kenya
- \textit{V. cholerae} non-O1/non-O139 is resistant to most commonly used antimicrobials in Kenya and can be considered to be a potential health risk
- There is significant differences in multidrug resistant \textit{V. cholerae} O1 isolated in Kenya when compared to other reports
- The coastal area could be origin of a new strain which can be imported to mainland and vice versa in a mobile community
Table 2.1. Antimicrobial susceptibility of *V. cholerae* O1 strains (*n*=80) from Nyanza outbreak (2008)

<table>
<thead>
<tr>
<th>Antimicrobials</th>
<th>No. of Resistant (%)</th>
<th>No. of Susceptible (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SXT</td>
<td>80 (100)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Fosfomycin</td>
<td>0 (0)</td>
<td>80 (100)</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>2 (2.5)</td>
<td>78 (97.5)</td>
</tr>
<tr>
<td>Imipenem</td>
<td>50 (62.5)</td>
<td>30 (37.5)</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>78 (97.5)</td>
<td>2 (2.5)</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>0 (0)</td>
<td>80 (100)</td>
</tr>
<tr>
<td>Norfloxacin</td>
<td>0 (0)</td>
<td>80 (100)</td>
</tr>
<tr>
<td>Nalidixic acid</td>
<td>80 (100)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>75 (93.8)</td>
<td>5 (6.2)</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>0 (0)</td>
<td>80 (100)</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>1 (1.2)</td>
<td>79 (98.8)</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>0 (0)</td>
<td>80 (100)</td>
</tr>
</tbody>
</table>

SXT: sulfamethoxazole/trimethoprim
Table 2.2. Drug resistance profiles of *V. cholerae* O1 strains from Nyanza outbreak (2008)

<table>
<thead>
<tr>
<th>Resistance Type</th>
<th>Antimicrobial resistance pattern</th>
<th>Number of isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>SXT    AMP    STR    NAL</td>
<td>1</td>
</tr>
<tr>
<td>II</td>
<td>SXT    STR    NAL    CHL</td>
<td>74</td>
</tr>
<tr>
<td>III</td>
<td>SXT    STR    NAL</td>
<td>3</td>
</tr>
<tr>
<td>IV</td>
<td>SXT    AMP    NAL    CTX</td>
<td>1</td>
</tr>
<tr>
<td>V</td>
<td>SXT    NAL    STR    NAL</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>-      -      -      -</td>
<td>80</td>
</tr>
</tbody>
</table>

SXT: sulfamethoxazole/trimethoprim; NAL: nalidixic acid; AMP: ampicillin; STR: streptomycin; CTX: cefotaxime; CHL: chloramphenicol

Abbreviations for antimicrobials are from American Society of Microbiology (ASM)
Table 2.3. Antimicrobial susceptibility pattern of *V. cholerae* O1 and *V. cholerae* non-O1/non-O139 from Kwale outbreak (2007)

<table>
<thead>
<tr>
<th>Antimicrobials</th>
<th><em>V. cholerae</em> O1 (n=20) (Kwale, 2007)</th>
<th><em>V. cholerae</em> non-O1/non-O139 (n=13) (Kwale, 2007)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Resistant (%)</td>
<td>Susceptible (%)</td>
</tr>
<tr>
<td>SXT</td>
<td>16 (80.0)</td>
<td>4 (20.0)</td>
</tr>
<tr>
<td>Fosfomycin</td>
<td>4 (20.0)</td>
<td>16 (80.0)</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>0 (0)</td>
<td>20 (100)</td>
</tr>
<tr>
<td>Imipenem</td>
<td>10 (50.0)</td>
<td>10 (50.0)</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>0 (0)</td>
<td>20 (100)</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>0 (0)</td>
<td>20 (100)</td>
</tr>
<tr>
<td>Norfloxacin</td>
<td>0 (0)</td>
<td>20 (100)</td>
</tr>
<tr>
<td>Nalidixic acid</td>
<td>20 (100)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>20 (100)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>0 (0)</td>
<td>20 (100)</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>0 (0)</td>
<td>20 (100)</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>0 (0)</td>
<td>20 (100)</td>
</tr>
</tbody>
</table>

SXT: sulfamethoxazole/trimethoprim
Table 2.4. Antimicrobial susceptibility pattern of *V. cholerae* O1 from Kwale outbreak (2009)

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>V. cholerae O1 (n=18)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Resistant (%)</td>
</tr>
<tr>
<td>SXT</td>
<td>18 (100)</td>
</tr>
<tr>
<td>Fosfomycin</td>
<td>18 (100)</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>18 (100)</td>
</tr>
<tr>
<td>Imipenem</td>
<td>18 (100)</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>18 (100)</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Norfloxacin</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Nalidixic acid</td>
<td>18 (100)</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>18 (100)</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>18 (100)</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>18 (100)</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

SXT: sulfamethoxazole/trimethoprim
### Table 2.5. Drug resistance profiles of *V. cholerae* isolates from Kwale outbreaks (2007 and 2009)

<table>
<thead>
<tr>
<th>Isolates</th>
<th>Multidrug resistant pattern</th>
<th>No. of isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>V. cholerae</em> O1 (2007)</td>
<td>SXT  FOF  IPM  NAL  STR</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>SXT  IPM  NAL  STR</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>SXT  NAL  STR</td>
<td>7</td>
</tr>
<tr>
<td><em>V. cholerae</em> non-O1/O139 (2007)</td>
<td>SXT  NAL</td>
<td>11</td>
</tr>
<tr>
<td><em>V. cholerae</em> O1 (2009)</td>
<td>SXT  FOF  AMP  IPM  CHL  NAL  STR  KAN  CTX</td>
<td>18</td>
</tr>
</tbody>
</table>

SXT: sulfamethoxazole/trimethoprim; NAL: nalidixic acid; FOF: fosfomycin; AMP: ampicillin; STR: streptomycin; CTX: cefotaxime; CHL: chloramphenicol; IPM: Imipenem

Abbreviations for antimicrobials are from American Society of Microbiology (ASM)
CHAPTER 3. Molecular characterization of *V. cholerae* strains associated with cholera outbreak

3.1 INTRODUCTION

The genus *Vibrio* is a member of the family *Vibrionaceae* and consists of Gram-negative motile curved (comma shaped) or straight bacteria (Fig. III of general introduction). Vibrios are aquatic and can thrive in fresh and salty water as free cells or in attachment with other aquatic cells (Faruque *et al.*, 1998). Genus *Vibrio* comprises of many species but of medical importance including *V. cholerae*, *V. parahaemolyticus*, *V. vulnificus* and *V. mimicus* (Faruque *et al.*, 1998). *V. cholerae* can cause the most severe illness among these species since some of the strains can cause cholera (Sack *et al.*, 2004). Traditionally, identification of *V. cholerae* from the species to the subtype level was based on phenotypic tests involving biochemical, immunological and to some extent phage typing although the latter was confined to specialised laboratories (Kaper *et al.*, 1995). *V. cholerae* is classified into two biotypes, classical and El Tor, based on the following reactions; haemolysis of sheep erythrocytes, chicken erythrocytes agglutination (CEA), susceptibility to Polymyxin B, sensitivity to specific phages and Voges-Proskauer (VP) reaction (Table I in General Introduction) (Raychoudhuri *et al.*, 2008). *V. cholerae* was broadly classified into two groups, *V. cholerae* O1 and non-O1 or NAG (non-agglutinable), based on the somatic O-surface antigen until 1992. However a *V. cholerae* non-O1 initiated cholera outbreaks in West Bengal in 1992 and soon after spread to many other Asian countries. This *V. cholerae* non-O1 was termed as *V. cholerae* O139 synonym Bengal (Albert *et al.*, 1993). Now *V. cholerae* is serologically grouped into *V. cholerae* O1, O139 and non-O1/non-O139. *V. cholerae* O1 is further serotyped as Ogawa, Inaba and Hikojima (Kaper *et al.*, 1995). To date there are more than 200 *V. cholerae* serogroups but only *V. cholerae* O1 and O139 can cause cholera pandemics (Sack *et al.*, 2004). Cholera-causing *V. cholerae* O1 and O139 strains harbour cholera toxin.
phage (CTXΦ) that carries the ctxAB genes coding for the most important virulence factor, cholera toxin (CT). CT is associated with the explosive acute secretory diarrhea which symbolizes the main symptoms of cholera. The El Tor and classical biotype strains which harbour the CTXΦ can both cause cholera but differ in their disease manifestation. Classical biotype cause more severe form of cholera than the El Tor biotype does and is associated with more fatalities (Sack et al., 2004; Kaper et al., 1995). On the other hand, the El Tor biotype is associated with asymptomatic infections and less fatalities when compared to the classical biotype. V. cholerae non-O1/non-O139 generally lacks the ctx gene and generally has not been associated with cholera pandemic or major epidemics, although the strains have the potential to cause sporadic cases of cholera like diarrhea (WHO, 1969: Sharma et al., 1998; Dutta et al., 2013; Islam et al., 2013). V. cholerae non-O1/non-O139 can cause disease by mechanisms other than that found in toxigenic V. cholerae O1 strains, such as the type three secretion system (T3SS) (Shin et al., 2011). V. cholerae non-O1/non-O139 has been thought to be a possible progenitor of new variant of epidemic V. cholerae strain (Bik et al., 1995; Khuntia et al., 2010; Faruque et al., 2003). The extensive lateral transfer of genes between V. cholerae strains and evolution of the El Tor V. cholerae prototype has resulted in the ‘new variants’ of V. cholerae which cannot be reliably diagnosed by biotyping and serotyping only (Cho et al., 2010). These variants are genetic hybrids because they share mixed phenotypic and genotypic characteristics of classical and El Tor traits. To classify the hybrid types Raychoudhuri et al. (2008) proposed a new identification scheme, which embraces genotyping in addition to the phenotypic tests described above (Table I; Fig. 3.1). These markers include CT epitope CT1 (classical) and CT2 (El Tor); tcpA, toxin co-regulated pilin; ctxB, cholera toxin B; rtx, repeat in toxin including rtxA to rtxD; and RS elements. (Table I; Fig. 3.1). On the basis of genotyping the hybrids have been broadly classified as El Tor variants or hybrids. New variants of V. cholerae O1 were first reported in Bangladesh (Nair et
al., 2002). There is paucity of information on the epidemiology of cholera caused by *V. cholerae* variants in Africa as compared to reports emanating from Asian countries where cholera is endemic. Although African cholera burden may exceed the global burden by over 90% with case fatality rates above acceptable limits (WHO 2008) then El Tor variants would have featured in more countries than what is currently reported (Safa *et al*., 2008; Safa *et al*., 2010). Interestingly, one of the *V. cholerae* O1 El Tor variant was first isolated in Mozambique and is referred to as the Mozambique variant (Ansaruzzaman *et al*., 2004). There is a need to carry out more studies on molecular epidemiology of *V. cholerae* clinical strains in Africa to establish the spread of new variants in most cholera endemic countries instead of relying exclusively on research findings from Asian countries where cholera is endemic (Rebaudet *et al*., 2013). Kenya experienced an upsurge in cholera outbreaks between 2007 and 2009 (WHO, 2008; WHO, 2009; Mutonga *et al*., 2013). The case fatality rate was significantly high and even reached 11% in some cases (Shikanga *et al*., 2009).

This Chapter addresses the genetic characterization and clonal relatedness of *V. cholerae* O1 and *V. cholerae* non-O1/non-O139 strains, which were isolated from two endemic areas of Kenya between 2007 and 2009.

3.2 MATERIALS AND METHODS

3.2.1 Chemicals and enzymes

Luria-Bertani (LB) broth was purchased from Difco laboratories (Detroit, Mich., USA). Tryptic soya agar (TSA) was purchased from Nissui Pharmaceutical Co. Ltd. (Tokyo, Japan) and TCBS agar was from Eiken Chemical Co. Ltd. (Tokyo, Japan). Chemicals were purchased from Nacalai Tesque (Kyoto, Japan), Wako Pure Chemical Industries (Tokyo, Japan), or Sigma-Aldrich (St. Louis, Mo., USA). Proteinase K and RNaseA were also purchased from Sigma-Aldrich. Restriction enzymes, Takara *Taq* was purchased from
Takara Bio Inc. (Shiga, Japan). Seakem Gold agarose was from FMC bioproducts (Rockland, ME, USA) and LE agarose was from Lonza (Rockland, ME, USA). Pulsed-field certified agarose, low-melting agarose and molecular weight markers were purchased from Bio-Rad Laboratories (Hercules, CA, USA).

3.2.2 Preparation of DNA templates

DNA template of V. cholerae strains for PCR was prepared as described by Hoshino et al. (1998). In brief, a single yellow colony from TCBS agar plate was inoculated into 3.0 ml LB broth and incubated at 37°C overnight under shaking conditions at 180 rpm (New Brunswick Scientific, NJ, USA). The culture was diluted 10 times with TE buffer (10 mM Tris-HCl, 1.0 mM EDTA [pH 8.0]) and boiled for 10 min after which it was cooled on ice. The boiled culture was centrifuged at 8,900 g for 3 min. The supernatant containing target DNA was stored at -30°C until further use.

3.2.3 Primers used in this study

The specific PCR primers were used for detecting V. cholerae serogroups, virulence genes (ctxA, T3SS related genes, nag-ST, chxA) and typing of ctxB/tcpA/rstR/rstC genes. The primers used in this study are summarized in Table 3.1.

3.2.4 Specific multiplex PCR for rfb genes of V. cholerae O1, O139 and ctxA genes

Multiplex PCR was carried out according to Hoshino et al. (1998) with slight modification. Briefly, 1.0 μl of DNA template was used in a total of 30 μl PCR mixture with 2.5 U of Takara rTaq DNA polymerase and its buffer system (Takara Bio Inc.). The PCR was carried out in a TaKaRa PCR Thermal Cycler Dice (Takara Bio Inc.) or in a Veriti 96 well Thermal Cycler (Applied Biosystem Inc.). The reaction conditions were initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 min each, 1 min at 55°C, 1 min at 72°C and a final extension step at 72°C for 7 min. The controls were V. cholerae
strains N16961 for O1 and ctxA; VC 406 for O139, ctxA and Escherichia coli C600 as a negative control. The PCR products were subjected to 2.0% LE agarose gel electrophoresis in TAE buffer (40 mM Tris-acetate, 1.0 mM EDTA [pH 8.0]). The gels were stained in ethidium bromide solution (2 μg ml\(^{-1}\)) for 5-10 min and distained twice in distilled water each for 5-10 min with shaking. Then gel-pictures were captured with a Gel-Doc 2000 (Bio-Rad Laboratories, USA).

### 3.2.5 Typing of cholera toxin B subunit (ctxB) gene

A mismatch mutation amplification assay (MAMA) PCR was carried out as described by Morita et al. (2008) with slight modification. Briefly, 1.0 μl of boiled template was used in a total of 20 μl PCR mixture including 0.1 μl Taq polymerase (5 U/μl). The PCR conditions were as follows; initial denaturation at 96°C for 2 min, 25 cycles were run at 96°C for 10 sec, 55°C for 10 sec and an extension at 72°C for 30 sec. DNA templates of V. cholerae strains O395 (classical) and N16961 (El Tor) were used as controls while E. coli C600 was used as a negative control. The PCR product (3.0 μl) was loaded on 2.0 % LE agarose and subjected to electrophoresis in TAE buffer (40 mM Tris-acetate, 1.0 mM EDTA [pH 8.0]). The gels were stained in ethidium bromide solution (2 μg ml\(^{-1}\)) for 5-10 min and distained twice in distilled water each for 5-10 min with shaking. The gel-pictures were captured with a Gel-Doc 2000 (Bio-Rad Laboratories).

### 3.2.6 Determination of the type of CTXΦ

The type of CTXΦ was determined by an rstrR gene-based PCR (Faruque et al., 2003; Davis et al., 1999) using 0.5 μl of DNA template and 0.08 μl rTaq polymerase in a 15 μl of PCR mixture. The PCR conditions were set as follows, after initial denaturation at 94°C for 5 min, reaction was run for 30 cycles of 30 sec each at 94°C, 55°C and 72°C, followed by an extension of 72°C for 7 min. Control templates were prepared from V. cholerae O1 strain
As522 which carried \( \text{rstR} \) El Tor and \( \text{rstR} \) Calcutta, \( V. \text{cholerae} \) O1 strain O395 with \( \text{rstR} \) classical. The PCR products were subjected to 2% LE agarose gel electrophoresis in TAE buffer (40 mM Tris-acetate, 1.0 mM EDTA [pH 8.0]). The gels were stained in ethidium bromide solution (2 \( \mu \text{g ml}^{-1} \)) for 5-10 min and distained twice in distilled water each for 5-10 min with shaking. Then gel-pictures were captured with a Gel-Doc 2000 (Bio-Rad Laboratories).

### 3.2.7 Classification of \( \text{tcpA} \) genes

A multiplex PCR was employed to verify the \( \text{tcpA} \) genotype. DNA template in 15 \( \mu \text{l} \) PCR working mixture was subjected to the following PCR conditions: initial denaturation at 94°C, followed by 30 cycles, each at 94°C for 30 sec, 55°C for 30 sec, and 72°C for 45 sec followed by a 7 min extension at 72°C. The \( V. \text{cholerae} \) control standards which were used in this assay were strains N16961 for El Tor and O395 for the classical, respectively. \( E. \text{coli} \) 600 was used as a negative control. Electrophoresis of PCR products, staining and capturing of gel pictures was carried out as described above.

### 3.2.8 Screening for \( \text{rstC} \) gene

A total of 15 \( \mu \text{l} \) of PCR mixture was prepared. The PCR conditions which were set for screening for \( \text{rstC} \) after an initial denaturation of 94°C for 5 min were 30 cycles of 30 sec each at 94°C, 55°C and 72°C followed by an extension for 7 min at 72°C. \( V. \text{cholerae} \) O1 strain N16961 and \( E. \text{coli} \) strain C600 were used as a positive and negative control respectively. The PCR products were subjected to 2.0% LE agarose gel electrophoresis in TAE buffer (40 mM Tris-acetate, 1.0 mM EDTA [pH 8.0]). The gels were stained in ethidium bromide solution (2 \( \mu \text{g ml}^{-1} \)) for 5-10 min and distained twice in distilled water each for 5-10 min with shaking. Then gel-pictures were captured with a Gel-Doc 2000 (Bio-Rad Laboratories).
3.2.9 Detection of \( chxA, rtxC, nag-ST \) and T3SS related genes

Detection of \( chxA, rtxC, nag-ST \) and T3SS related genes (\( vcsC_2, vcsN_2 \) and \( vopF \)) was performed by colony hybridization as described by Awasthi et al. (2013). Briefly, \( V. \) cholerae strains were grown on nitrocellulose membrane (Schleicher & Schuell, Dassel Germany) overlaid on LB agar at 37°C for 4-6 h. The colonies were lysed and DNA was denatured by alkaline lysis method followed by UV cross-linking. The processed nitrocellulose membranes were hybridized with respective target probes in suitable buffer conditions and radioactivity was visualized by BAS FLA-3000 system (Fuji Film, Tokyo, Japan).

3.2.10 Pulsotyping

Pulsed-field gel electrophoresis (PFGE) was performed as described in Pulse Net USA protocol (www.cdc.gov/pulsenet/protocols.htm) with minor modifications. Briefly, fresh cells of \( V. \) cholerae were embedded into 1.0 % Seakem Gold agarose followed by lysis of the cells with 0.5 mg ml\(^{-1}\) Proteinase K (Sigma-Aldrich) and 1.0 % Sarcosine (Sigma-Aldrich) at 54°C for 1 h. Agarose blocks containing genomic DNA were equilibrated in 500 \( \mu \)l of 1× H-buffer (Takara Bio Inc.) at 37°C for 10-20 min and digested with 40 U of \( NotI \) in a 400 \( \mu \)l of 1× H-buffer at 37°C for 3 h. DNA fragments were separated on a CHEF MAPPER (Bio-Rad Laboratories) according to Yamasaki et al. (1997). Gels were stained for 30 min, destained twice for 15 min each and pictures were captured as described before. \( XbaI \)-digested fragments of \( Salmonella \) enterica serovar Braenderup DNA were used as molecular mass standard. PFGE fingerprints were analyzed using Fingerprinting II software (Bio-Rad Laboratories). After background subtraction and gel normalization, the fingerprint patterns were subjected to cluster analysis by the unweighted-pair group method using average (UPGMA) linkages. For similarity value calculation, band-based (Dice coefficient) option was applied.
3.3 RESULTS

3.3.1 Specific multiplex PCR for *rfb* genes of *V. cholerae* O1, O139 and *ctxA* genes

A total of 132 *V. cholerae* isolates were investigated by multiplex PCR targeting the specific somatic O1- and O139-antigene (*rfb*) genes. One hundred and eighteen isolates were confirmed to be *V. cholerae* O1. Eighty of these isolates were from Nyanza area and remaining 38 strains were from Kwale. The Kwale isolates included 20 from the 2007 outbreak and 18 from the 2009 outbreak. All the *V. cholerae* O1 strains carried the *ctxA* gene. Fourteen isolates were negative for *ctxA* gene, O1- and O139-*rfb* genes. None of the isolates were identified to be O139 (Table 3.2; Fig. 3.2).

3.3.2 Typing of cholera toxin B subunit (*ctxB*) gene

The *ctxB* gene of 118 *V. cholerae* O1 strains was characterized. Twenty strains were from the 2007 cholera outbreak in Kwale, 18 strains were from the 2009 cholera outbreak from the same region and 80 strains from Nyanza. The MAMA-PCR showed that all the isolates that were analyzed harbored the classical *ctxB* allele (Table 3.2; Fig. 3.3).

3.3.3 Determination of the type of CTXΦ

One hundred and eighteen *V. cholerae* isolates were investigated for El Tor, classical and Calcutta *rstR* gene by multiplex PCR. All the strains analyzed in this study harbored the El Tor type *rstR* gene (Table 3.2; Fig. 3.4).

3.3.4 Screening for *rstC* gene

*V. cholerae* O1 isolates that were screened with *rstC* specific PCR were found to harbor the *rstC* gene (Table 3.2; Fig. 3.5).
3.3.5 Classification of tcpA genes

Multiplex PCR for tcpA gene for El Tor and classical showed that all the 118 V. cholerae strains carried the El Tor tcpA gene (Table 3.2; Fig. 3.6).

3.3.6 Colony hybridization

All the V. cholerae O1 strains were negative for the heat-stable enterotoxin (nag-ST) gene, T3SS related genes (vcsC2, vcsN2 and vopF) and chxA toxin (chxA) gene. The V. cholerae non-O1/non-O139 isolate (n=1) from Nyanza was positive for chxA, but like the other V. cholerae non-O1/non-O139 strains from Kwale it was negative for nag-ST and T3SS related genes (vcsC2, vcsN2 and vopF).

3.3.7 Pulsotyping

Analysis of NotI restriction enzyme digested PFGE profile of V. cholerae O1 strains isolated from Nyanza province revealed that they were most likely of clonal origin although subtle differences were detectable (Fig. 3.7). The PFGE patterns exhibited the distribution of ~24 bands over a size range of 20 to 300 kbp. All the V. cholerae O1 Ogawa strains isolated in 2007 in Kwale demonstrated identical PFGE profiles except one strain showing subtle difference with an insertion of a single band (~90kb) (Fig. 3.7). V. cholerae O1 Inaba strains isolated in 2007 in Kwale showed same PFGE patterns. Interestingly, V. cholerae O1 Inaba strains isolated in 2007 in Kwale seem to be more closely related to those isolated from the 2008 outbreak in Nyanza (Fig. 3.7).

3.4 DISCUSSION

Phenotypic results obtained in this study led to presumptive identification of the isolates as V. cholerae O1 El Tor and V. cholerae non-O1/non-O139. In addition to commonly used phenotypic markers which include susceptibility to polymyxin B, production of 2, 3-butanediol (Voges-Proskauer test), chicken erythrocyte agglutination (CEA) and
hemolysis of sheep erythrocytes, Raychoudhuri et al. (2008) proposed the inclusion of molecular markers to classify *V. cholerae* O1 strains into classical, El Tor, hybrid and El Tor variant (Table I). These genetic markers, which were adopted in this study, are ctxB, tcpA, rstR and rstC. Phenotypic and genotypic results in our study confirmed that the Nyanza 2008 and Kwale 2007 and 2009 cholera outbreaks were caused by *V. cholerae* O1 El Tor variant strain. New variants of *V. cholerae* O1 were first reported in Bangladesh (Nair et al., 2002). Since then several studies have reported on the prevalence of *V. cholerae* O1 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 have emanated from Africa on *V. cholerae* O1 El Tor variants. These include countries from West Africa; Nigeria and Cameroon (Quilici et al., 2010), South Africa; Mozambique (Ansaruzzaman et al., 2004), Angola (Ceccarelli et al., 2011), Zimbabwe (Islam et al., 2011), and South Africa (Ismail et al., 2012). This report is the first systematic study to implicate *V. cholerae* O1 El Tor variant strains with the Nyanza and Kwale cholera outbreaks. A current report by Naha et al. (2013) showed that *V. cholerae* O1 El Tor variant which produces higher levels of cholera toxin than that of prototype *V. cholerae* O1 El Tor was responsible for a cholera outbreak in the Zanzibar archipelago in 2009. The *V. cholerae* O1 Inaba strains isolated in 2009 in Kwale were different from the Zanzibar strains, which were Ogawa serotype and also differed in the antimicrobial profile. However, it is important to appreciate the proximity of Kwale to Zanzibar. It takes 3 to 6 h by boat from Kwale to Pemba, which is one of the two main islands of the Zanzibar archipelago. There are also fishing activities between the two regions, and this is a potential route for transmission of microorganisms. It is important to monitor cross border spread of new *V. cholerae* O1 variants. Both the 2007 Kwale (Ogawa) and 2009 Kwale (Inaba) *V. cholerae* O1 strains were El Tor variant and this study establishes that the variant has replaced the El Tor *V. cholerae* prototype in the coastal area of Kenya.
According to Shikanga et al. (2009), the severity of the disease and high case fatality rate of 11% observed in the Nyanza cholera outbreak were mainly associated with the 2008 post-election violence in Kenya, which led to inadequate supplies and shortage of health personnel among other factors. Shikanga et al. did not report the virulence gene profile of V. cholerae strains isolated in that particular outbreak. This study suggests that the carriage of the classical ctxB gene in these strains may justify the severity (Siddique et al., 2010) of the Kenyan 2007-2008 outbreak, since these strains have potential to produce high levels of CT (Ghosh-Banajee et al., 2010). In this context, it is noteworthy that a previous study failed to detect any V. cholerae O1 El Tor variant although the study had analyzed V. cholerae O1 epidemic strains which were obtained from various parts of Kenya between 1994 to 2007 (Kiiru et al., 2009). Similarly, Mohamed et al. (2012) detected V. cholerae O1 strains with the classical ctxB gene from isolates obtained between 2009 and 2010 and concluded that the genotype did not exist prior to this period. To the best of the author’s knowledge this is the first systematic report on the prevalence of V. cholerae O1 El Tor variant in cholera endemic areas in Kenya.

This study identified 13 V. cholerae non-O1/non-O139 strains representing 42% of the V. cholerae isolates detected during the 2007 Kwale cholera outbreak. Although these strains lacked the nag-ST gene, T3SS related genes (vcsC2, vcsN2 and vopF) and chxA gene. It is possible that the V. cholerae non-O1/non-O139 strains may have played a role in causing diarrhea by unknown mechanism(s) (Sharma et al., 1998; Hasan et al., 2012; Islam et al., 2013). To emphasize the importance of these bacteria, Sharma et al. (1998) proposed the name enteropathogenic V. cholerae for non-O1/non-O139, which lack known virulence genes. Interestingly, Islam et al. (2013) reported pathogenicity in V. cholerae non-O1/non-O139 isolated from a non-cholera endemic area. In Haiti, V. cholerae non-O1/non-O139 strains were isolated from 21% of diarrhea samples from cholera suspected cases. Isolation of
the organism as the sole agent or in concert with *V. cholerae* O1 was treated as significant. Genomic analysis of the strain showed that the bacteria can act as reservoir for pathogenic genes (Hasan *et al.*, 2012). The molecular investigation of clinical *V. cholerae* non-O1/non-O139 had not been previously carried out in Kenya, although a *V. cholerae* non-O1 was reported to cause cholera-like disease in the neighboring country, Sudan (WHO 1969) and in India (Dutta *et al.*, 2013). Marin *et al.* (2013) have also recently reported that *V. cholerae* non-O1/non-O139 was also partly associated with the 2009 cholera outbreak in Nigeria.

Interestingly, one *V. cholerae* non-O1/non-O139 strain isolated in 2008 in Nyanza carried the *chxA* II gene, whose product (ChxA II) is more potent than prototype ChxA (ChxA I) in mice lethality assay (Awasthi *et al.*, 2013). To the best of the author’s knowledge this is the first report of detection of *chxA* gene in *V. cholerae* non-O1/non-139 in Africa. Although the ChxA was demonstrated to be associated with extra-intestinal infections and no enterotoxigenicity was detectable in animal experiments (Awasthi *et al.*, 2013), presence of the *chxA* gene in *V. cholerae* non-O1/non-O139 strain isolated from diarrheal patient may indicate its pathogenic potential particularly its enterotoxigenic activity in human. The role of *V. cholerae* non-O1/non-O139 in emergence of virulent genotypes is exemplified by the evolution of *V. cholerae* O139, which most likely evolved through genetic exchange between a *V. cholerae* non-O1 and a *V. cholerae* O1 El Tor isolate (Bik *et al.*, 1995; Faruque *et al.*, 2000).

Clonal diversity among *V. cholerae* O1 strains was not evident from the PFGE patterns. PFGE analysis of *V. cholerae* O1 strains isolated from 1994 to 2007 showed that regardless of the year of isolation, all the strains were clonally related (Kiiru *et al.*, 2009). Interestingly *V. cholerae* O1 strains isolated in 2008 in Nyanza showed close clonal similarities to *V. cholerae* O1 Inaba strains isolated in 2009 in Kwale (Figure 3.7). The 2007 Kwale (Ogawa) and the 2009 Kwale (Inaba) strains showed subtle differences. This agrees
with study of Mohamed et al. (2012), which reported minor clonal differences by multilocus-variable tandem repeat analysis (MLVA) among the Nyanza \textit{V. cholerae} O1 strains. Kiiru et al. (2013) reported genetic variations by a combination of whole genome sequences of clinical and environmental \textit{V. cholerae} O1 isolated from various part of Kenya including Nyanza and Kwale areas of Kenya.

Conclusions:

- \textit{V. cholerae} O1 El Tor variant has replaced the \textit{V. cholerae} O1 El Tor prototype in Kenya
- High case fatality rates in cholera outbreaks in Kenya can be associated with \textit{V. cholerae} O1 El Tor variant
- \textit{V. cholerae} non-O1/non-O139 may be associated with diarrheal disease in Kenya
- Cholera outbreaks in different endemic areas in Kenya can be caused by \textit{V. cholerae} strains which share close clonal relatedness
Fig. 3.1. Structure of CTXΦ and the RS1 element in different *V. cholerae* O1 variants. In the prototype El Tor (a) and atypical El Tor (c) strains, parentheses delineate the RS1 elements that flank the integrated CTXΦ genome. (b) CTXΦ in prototype classical strains, which lack the RS1 element. (d) A hypothetical hybrid CTXΦ. Polymorphic positions within *ctxB* are indicated for H (histidine), T (threonine), Y (tyrosine) or I (isoleucine). The different *rstREl* and *rstRCla* alleles are highlighted in different colors. (Safa *et al*., 2010)
Identification by toxR PCR

Identification of toxR negative strains by 16S rRNA sequencing

Fig. 3.2. Representative result of a multiplex PCR for O1-rfb, O139-rfb and ctxA genes (A), and a PCR for V. cholerae-specific toxR gene (B).

(A) Lane 1-21 are presumptive V. cholerae isolates from diarrheal patients; (B) Lane 1-16 are strains negative by a multiplex PCR for O1-rfb, O139-rfb and ctxA genes which were further characterized by a PCR for V. cholerae-specific toxR gene.
P: positive control (V. cholerae O1/ctxA+ strain N16961 and O139/ctxA+ strain VC 406); N: negative control (E. coli strain C600); M: 100 bp DNA ladder (Takara)
Fig. 3.3. Representative results of a *ctxB* classical (A) and *ctxB* El Tor (B) gene specific PCR for *V. cholerae* O1 isolates (Lane 1-20). P: positive control (*V. cholerae* O1/El Tor strain N16961 and O1/classical strain O395); N: negative control (*E. coli* strain C600); M: 100 bp DNA ladder (Takara)
Fig. 3.4. Representative results of a multiplex PCR for *rstR* El Tor and Calcutta genes (A) and uniplex PCR for *rstR* classical gene (B) from *V. cholerae* O1 isolates (Lane 1-20).

P: positive control (*V. cholerae* O1 strain As522 [*rstR* El Tor and *rstR* Calcutta] and *V. cholerae* O1 strain O395 [*rstR* classical]); N: negative control (*E. coli* strain C600); M: 100 bp DNA ladder (Takara); M’: pHY marker (Takara)
Fig. 3.5. Representative result of *rstC* gene PCR for *V. cholerae O1* isolates (Lane 1-20).
P: positive control (*V. cholerae* O1 strain N16961); N: negative control (*E. coli* strain C600); M: pHY marker (Takara)

Fig. 3.6. Representative results of multiplex PCR for *tcpA* Classical/El Tor gene from *V. cholerae O1* isolates (Lane 1-18).
P1: positive control (*V. cholerae* O1 strain N16961 [*tcpA* EL Tor]);
P2: positive control (*V. cholerae* O1 strain O395 [*tcpA* classical]);
N: negative control (*E. coli* strain C600); M: pHY marker (Takara)
Fig. 3.7. Representative PFGE profiles of *V. cholerae* O1 isolates from Nyanza, 2007 (lane 1-5), *V. cholerae* O1 isolates from Kwale, 2007 (lane 6-9) and *V. cholerae* O1 isolates from Kwale, 2009 (lane 10-13) analyzed in this study. M: *XbaI*-digested *Salmonella* serovar Braenderup DNA used as markers.
<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primer Identity</th>
<th>Primer sequence (5’-3’)</th>
<th>Size (bp)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>O1 rfb</strong></td>
<td>O1F2-1</td>
<td>GTTTCACTGAAACAGATGGG</td>
<td>192</td>
<td></td>
</tr>
<tr>
<td></td>
<td>O1R2-2</td>
<td>GGTACGTCTGTAAGTACAAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>O139 rfb</strong></td>
<td>O139F2</td>
<td>AGCCCTTTATTACGGGTGG</td>
<td>449</td>
<td>Hoshino et al., 1998</td>
</tr>
<tr>
<td></td>
<td>O139R2</td>
<td>GTAAACCCGATCGTAAGGG</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>ctxA</strong></td>
<td>VCT1</td>
<td>ACAGAGTGATGACTTTGACC</td>
<td>307</td>
<td></td>
</tr>
<tr>
<td></td>
<td>VCT2</td>
<td>ATACCACTCCATATATTGGGAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>ctxB</strong></td>
<td>ctx - cla3</td>
<td>CCTGTGATCTTTACTTGAAAC</td>
<td>366</td>
<td>Morita et al., 2008</td>
</tr>
<tr>
<td></td>
<td>ctx el3</td>
<td>CCTGGTACTCTACTTGAAACA</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>ctx - com5</td>
<td>CCATCGATGAGTAATACTTGC</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>tcpA</strong></td>
<td>tcpA- F (elt)</td>
<td>GAAAGAAGTTTGTTAAAGAAGAACAC</td>
<td>617</td>
<td>Mukhopadhy et al., 2001</td>
</tr>
<tr>
<td></td>
<td>tcpA-R(elt)</td>
<td>GAAAGACCTTCTTTTACGGTA</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>tcpA</strong></td>
<td>tcpA-F(cla)</td>
<td>CACGATAAAGAAAACCCGTCAAGAG</td>
<td>471</td>
<td>Awasthi et al., 2013</td>
</tr>
<tr>
<td></td>
<td>tcpA-R(cla)</td>
<td>ACCAAGTCAACGCCGAATGAGGC</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>chxA</strong></td>
<td>chxA1</td>
<td>GTCGAAGATGAGTTAACCATT</td>
<td>1904</td>
<td></td>
</tr>
<tr>
<td></td>
<td>chxA1</td>
<td>TTATTCAGTCATCTTCGTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>vcsN2</strong></td>
<td>vcsN2-F</td>
<td>CAACACCTTCAAAGCCTTG</td>
<td>848</td>
<td>Awasthi et al., 2013</td>
</tr>
<tr>
<td></td>
<td>vcsN2-R</td>
<td>GCGACCTCAATGGAAAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>vcsC2</strong></td>
<td>vcsC2-F</td>
<td>GGTCTCATAGACACTACG</td>
<td>589</td>
<td></td>
</tr>
<tr>
<td></td>
<td>vcsC2-R</td>
<td>ACGATGCTATGGGATATG</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>vopF</strong></td>
<td>vopF-U</td>
<td>GGAAATTCCGCAAGTGTAA</td>
<td>839</td>
<td></td>
</tr>
<tr>
<td></td>
<td>vopF-R</td>
<td>CAAAACCGTCATACAAAGC</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>stn</strong></td>
<td>stnF</td>
<td>GAAAGAACCTTATTCATG</td>
<td>216</td>
<td>Vicente et al., 1997</td>
</tr>
<tr>
<td></td>
<td>stR</td>
<td>GCAAGCTGGATTGCCAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>rtx</strong></td>
<td>rtxA-F</td>
<td>CTGAAATATGAGGTTGAGTCTAC</td>
<td>418</td>
<td>Awasthi et al., 2013</td>
</tr>
<tr>
<td></td>
<td>rtxA-R</td>
<td>GTTGTATTGTGTTGATTGACATGC</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>toxR</strong></td>
<td>VC toxR 403F</td>
<td>GAAAGCTGGCTCATGACATC</td>
<td>275</td>
<td>Neogi et al., 2010</td>
</tr>
<tr>
<td></td>
<td>VC toxR 678R</td>
<td>AAGATCAGGTTGATTATC</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>rstR</strong></td>
<td>rstR-classF</td>
<td>CTTCTCATCAGAAAGCCCTCATT</td>
<td>313</td>
<td>Faruque et al., 2003</td>
</tr>
<tr>
<td></td>
<td>rstR-ELT F</td>
<td>GCACCATGATTAAAGATGTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>rstR-cal F</td>
<td>CTGTAAATCTCTCTCAATCTAGG</td>
<td>617</td>
<td>Davis et al., 1999</td>
</tr>
<tr>
<td></td>
<td>rstR- com-R</td>
<td>TCGAGTTGTAATTTCTCAAGAGGC</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>rstC</strong></td>
<td>rstC-F</td>
<td>ATGAGTTGGAACACATCAACTTT</td>
<td>225</td>
<td>Faruque et al., 2002</td>
</tr>
<tr>
<td></td>
<td>rstC-R</td>
<td>TTACAGTGTGGATGACTCAAA</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>rtxC</strong></td>
<td>rtxC-F</td>
<td>CGACGAAGATCATTGACGAC</td>
<td>263</td>
<td>Awasthi et al., 2013</td>
</tr>
<tr>
<td></td>
<td>rtxC-R</td>
<td>CATCGTCGTTGATTGTACG</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Table 3.2. Genotypic and phenotypic characterization of *V. cholerae* isolates from Kenyan outbreaks

<table>
<thead>
<tr>
<th>Origin</th>
<th>Serogroup</th>
<th>Serotype</th>
<th>Outbreak year</th>
<th>Strains (n)</th>
<th>toxR</th>
<th>O1</th>
<th>O139</th>
<th>ctxA</th>
<th>Cla El Tor</th>
<th>rstC</th>
<th>Cla El Tor</th>
<th>Calc</th>
<th>Cla El Tor</th>
<th>Hemolysis</th>
<th>PxB</th>
<th>CCA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nyanza</td>
<td>O1</td>
<td>Inaba</td>
<td>2008</td>
<td>80</td>
<td>ND</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>β*</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>NA</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>nonO1/O139</td>
<td>ND</td>
<td>2008</td>
<td>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>NA</td>
<td></td>
</tr>
<tr>
<td>Kwale</td>
<td>O1</td>
<td>Ogawa</td>
<td>2007</td>
<td>20</td>
<td>ND</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>β</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>NA</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>nonO1/O139</td>
<td>ND</td>
<td>2007</td>
<td>13</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>NA</td>
<td></td>
</tr>
<tr>
<td>Kwale</td>
<td>O1</td>
<td>Inaba</td>
<td>2009</td>
<td>18</td>
<td>ND</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>β</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

*rfb*: gene responsible for *V. cholerae* O-antigen synthesis; *ctxA*: cholera toxin A subunit gene; *tcpA*: toxin co-regulated pilus gene; *rst*: CTX phage related genes; PxB: polymyxin B; CCA: chicken cell agglutination; Cla: classical; Calc: Calcutta; ND: not done; NA: not applicable; *One *V. cholerae* O1 strain was alpha hemolytic and could not agglutinate chicken erythrocytes.
GENERAL DISCUSSION

Cholera is a secretory acute infectious diarrhea, which occurs as pandemic and it is estimated to affect 3-5 million persons resulting in 100,000 to 120,000 deaths annually. The world has experienced seven pandemics all of which originated from the Bay of Bengal, except the 7th one that originated from the Celebes Island of Indonesia. The disease is caused by the Gram-negative bacterium *Vibrio cholerae* O1 of classical or El Tor biotype. There are more than 200 serogroups of *V. cholerae*, but until now cholera epidemics and pandemics are associated with only O1 and O139 serogroups. The other serogroup of *V. cholerae* are referred to as non-O1/non-O139, and may occasionally cause diarrhea. Cholera is water-borne disease and the main way of contracting infection is via the fecal oral route. Development of quality sanitation and health management systems has seen cholera eradicated in developed countries and only imported cases are reported from these regions. On the other hand, cholera outbreaks in developing countries in Asia, South America and Africa still persist. South Asia was considered the home of cholera until the beginning of the millennium. Since the year 2000, the number of cholera outbreaks reported from Africa including Kenya, has increased to surpass the total number of cholera cases reported worldwide by over 90% (WHO, 2010). African countries also reported unusually high case fatality rate (CFR) with some countries reporting as high as 15% when compared to the WHO acceptable CFR of >1%. The shift of high frequency of cholera outbreaks from Asian countries to Africa indicated that cholera may have found a new homeland in Africa (Gaffga, *et al.*, 2007). Yet, there is paucity of information in cholera research in Africa and African countries continue to rely heavily on information generated from the countries located near Bay of Bengal where cholera is endemic (Rebaudet *et al.*, 2013). There is an urgent need to carry out further study to understand the dynamics of cholera in Africa in order to contain the disease and eventually eradicate it all together.
In this study, the burden of diarrheal disease in the coastal region of Kenya was investigated. The dynamics of cholera outbreaks in the coastal area was carried out in Kwale, between two cholera outbreaks in 2007 and 2009. *V. cholerae* O1 isolates were also compared with those isolated from an inland lake region of Nyanza, which experienced a severe cholera outbreak in 2008. The study confirms WHO reports that diarrhea is still a major cause of public health concern in developing countries including Kenya. The study also confirms to the WHO reports on increase in the number of cholera outbreaks in Kenya since beginning of the new millennium. However, there were some discrepancies in the findings obtained in this study with similar studies carried out in cholera endemic areas in South Asia. *V. cholerae* O1 and non-O1/non-O139 were only isolated during cholera outbreaks. Unlike most of the studies in Asia where polymicrobial pathogens were isolated together with *V. cholerae* during lull periods when there was no cholera outbreak, the target enteropathogens in this study, *Salmonella* spp. and *Shigella* spp., were the only pathogens isolated during the lull periods in Kwale. Interestingly, no specimen yielded *V. cholerae* isolate during the lull period in the entire study period. The role of human transmission has been revisited especially now that cholera is shifting to new areas in Africa and Central America. The role of food handlers in cholera outbreak has been frequently reported from South Asian countries (Swaddiwudhipong *et al.*, 2012). Although *Salmonella* spp. and *Shigella* spp. were isolated in this study, *V. cholerae* was not detected in any of the food handlers. A study with a larger sample size of food handlers is necessary to establish their role in transmission of cholera. It is possible that cholera outbreaks may have increased in Africa but yet to reach endemic levels as observed in South Asia. Similarly, results obtained in this study on the role of cholera index cases suggest that more research is required in the African context as cross-referenced with work on index cases in the countries located near Bengal. This study shows that there is misdiagnosis,
inappropriate response to cholera outbreak and false alarm based on failure to recognize index cases in time.

In the present study, isolation of toxigenic *V. cholerae* O1 was attempted from 2007 to 2009 in Kwale. As described earlier *V. cholerae* O1 strains were isolated during the outbreak periods only in 2007 and 2009. However, *V. cholerae* O1 serotype Ogawa was isolated in 2007 whereas *V. cholerae* O1 serotype Inaba was isolated in 2009. The serotype shift from Ogawa to Inaba has also been reported in South Asian countries (Longini *et al*., 2002; Khuntia *et al*., 2010). However, this is the first study to report a serotype switch in consecutive cholera outbreaks in Africa where a *V. cholerae* O1 Ogawa strain is completely replaced by a *V. cholerae* O1 Inaba strain. This observation is crucial for a continent overwhelmed by increased numbers of cholera outbreaks and cholera related deaths.

This study also analyzed the prevalence of multidrug resistant (MDR) *V. cholerae* O1 in Kwale and Nyanza areas of Kenya. Multidrug resistance profile of *V. cholerae* O1 strains from Nyanza and Ogawa strains were similar to most profiles reported worldwide. In contrast, the 2009 Kwale Inaba strain displayed an extended spectrum by being resistant to 75% of the antimicrobials tested. This observation was interesting because the preceding 2007 cholera outbreak in the same area was caused by a less resistant strain. This level of resistance was never reported before from Kenya and it is suggested that unique *V. cholerae* O1 strains may emanate from the Kenyan coastal areas and spread inland. This study reiterates the need for continuous monitoring of antimicrobial resistance in circulating *V. cholerae* O1 whereas similar or more comprehensive studies on the same may assist in formulation of African guidelines for antimicrobial use in cholera outbreaks. Currently, the use of antimicrobial in management of cholera in Africa is adopted from WHO or ICDDR, B.

Genotypic results from this study suggest that *V. cholerae* O1 El Tor variant possessing the classical *ctxB* gene has replaced *V. cholerae* O1 El Tor prototype in Kenya,
and may be responsible for the high CFR in cholera outbreaks. This study also demonstrated for the first time in Africa the presence of *V. cholerae* non-O1/non-O139 possessing *chxA* gene. It is therefore suggested that *V. cholerae* non-O1/non-O139 may serve as a reservoir for pathogenic genes in Kenya and the strains are a potential health hazard.

In conclusion, cholera has reemerged with unprecedented force in Africa and more recently in Central America. There is paucity of information from Africa, in particular Kenya on dynamics of cholera. If cholera is to establish a new home in Africa, then more studies are required to contain outbreaks, prevent transmission and cholera related deaths and eventually to eradicate the disease. *V. cholerae* O1 El tor variant has replaced the *V. cholerae* O1 El tor prototype in Kenya and may be associated with severity of the disease. This study reports for the first time in Kenya, serotype switch and replacement of less resistant strains by multidrug resistant strains in the same area of Kenya during consecutive outbreaks. Unlike endemic areas in South Asia toxigenic *V. cholerae* O1 is isolated only during outbreak periods and food handler may not play a key role in transmission of *V. cholerae*. Cholera outbreaks in different endemic areas in Kenya are caused by *V. cholerae* O1 El Tor variants, which share close clonal relatedness. Cholera outbreak has increased in Africa but it has not reached to endemicity levels found in South Asia. There is a need to boost laboratory facilities and coordinate cholera research in Kenya and in shared boundary areas with neighboring countries.
ACKNOWLEDGEMENT

I take this opportunity to express my profound gratitude and deep regards to my mentor and supervisor, **Prof. Shinji Yamasaki**, Laboratory of International Prevention of Epidemics, Graduate School of Life and Environment Sciences, Osaka Prefecture University, Osaka, Japan for his tremendous intellectual and administrative goodwill which culminated to my success in this PhD program. I am deeply indebted to him for his constructive criticism, suggestions and continuous review of my work throughout the program. I envy and appreciate the pace that Prof. Yamasaki set for me in this program, so challenging yet so rewarding.

I am grateful to **Prof. Kazumi Sasai**, Laboratory of Veterinary Internal Medicine, and **Prof. Masafumi Mukamoto**, Laboratory of Veterinary Epidemiology, Graduate School of Life and Environment Sciences, Osaka Prefecture University, Osaka, Japan for critically reviewing my thesis and providing valuable scientific inputs and suggestions.

In equal measure I am greatly indebted to **Dr. Yoshio Iijima**, Director, Kobe Institute of Health, Kobe, Japan, whose support towards my scientific career spans over a decade and I wish to express a deep sense of gratitude to him for his critical review of my work and enabling me to carry out most of the fieldwork.

Thanks to **Assoc. Prof. Yoshihiro Ohnishi** for his moral support and encouragement. I would like to show my greatest appreciation to **Asst. Prof. Atsushi Hinenoya** who assisted me during the course of my program and special thanks also to **Dr. Masahiro Asakura** who was the first to set my early experiments in molecular work. I am grateful to them for their kind help and important suggestions during the laboratory experiments.

I would like to offer my special thanks to **Dr. Rupak K. Bhadra**, Senior Principal Scientist, Infectious Diseases and Immunology Division, CSIR-Indian Institute of Chemical Biology, Kolkata, India for critically reviewing my work and publications as well as the
valuable suggestions and recommendations shared with me towards the improvement of the study.

I express my gratitude to Dr. Nityananda Chowdhury for his technical and bench support without which my project would have been constrained. At this juncture, I express my heartfelt gratitude to Dr. Sharda Awasthi, whose intellect and technical expertise was a valuable asset towards my work.

I express my heartfelt gratitude to my colleagues of the Laboratory of International Prevention of Epidemics. Without their friendly support and inspiration the work would have been impossible. I would like to express my sincere thanks to Ph.D candidates, Mr. Shamim Zahid Hassan, Ms. Srinuan Somroop, Mr. Sikandar Sheikh, Mr. Noritomo Yasuda, Mr. Noritoshi Hatanaka, Ms. Hoang Hoai Phuong, Research student, Mr. Rabee Alhossiny, and undergraduate students, Mr. Kentaro Okuno, Ms. Azimun Nahar, Mr. Hidetoshi Ichimura, Ms. Yuko Hiratou, Ms. Mao Okujima, Mr. Nobuo Arai, Mr. Youhei Saburi, Ms. Yumika Matsui and many other students whom I met during my stay at the department.

I would like to express my gratitude to Prof. J.K.Z Mwatela, Vice Chancellor, Technical University of Mombasa (TUM), for permission to complete my study, Dr. J. Ochieng Odalo, Dean, Faculty of Applied Health and Sciences (TUM) and Mr. Mohamed Mbogah, Chairman, Department of Medical Sciences (TUM), for their administrative support.

I also wish to thank my former colleagues at the Kenya Medical Research Institute (KEMRI), particularly Dr. Sam Kariuki, Prof. Mohamad Karama, Dr. George Micuki, Mr. Sora Guyo and Mr. John Mwaniki among others for the various fruitful discussions.

I recognize with appreciation the technical support received from my students who completed their training at the Centre for Microbiology Research, KEMRI, Kwale: Ruth
Fatima Odipo, Simon Mutua, Susan Chao Mutua, Matthew Mutinda, Margaret Shigare and Asha Matano. I am grateful to Mr. Juma Changoma for facilitating the field visits. I acknowledge the moral support I got from other CMR, Kwale staff including, Mr. Justus Makau, Mr. Masoudi, Mr. Amos Mwavita, Mr. Muema and Mr. Ngala.

I am grateful for the cooperation that I got from the Coast Provincial Medical Officers, Dr. Kahindi, Dr. Anisa, Dr. Chidagaya and Public Health Officers based in Kwale particularly Ms. Redemta, Ms. Kaache, Mr. Juma, Mr. Mohamed among many others. I appreciate the cooperation from members of the Kwale district hospital, particularly Mr. Shambe, Mr. Musa and all the clinical officers.

I am humbled by the generosity of the supporting grant which enabled me to complete my study at the Osaka Prefecture University.

I also received generous support from Ms Izzat, Mr. Amir Swaleh, Sheikh Abu Hamza and Mr Hafidh Muhsin.

Finally, I pay tribute to my family, my mother, my wife Mwanaisha Suleiman, my children Rama, Shumi, Nasra, Husna and to all my grandchildren.

I thank the Almighty for a long journey well completed.
REFERENCES


Haque R, Mondal D, Kirkpatrick BD, Akther S, Farr BM, Sack RB, Petri WA Jr. 2003. Epidemiologic and clinical characteristics of acute diarrhea with emphasis on


Marin MA, Thompson CC, Freitas FS, Fonseca EL, Aboderin AO, Zailani SB, Quartey NKE, Okeke IN, Vicente ACP. 2013. Cholera outbreaks in Nigeria are associated with


Shin OS, Tam VC, Suzuki M, Ritchie JM, Bronson RT, Waldor MK, Mekalanos JJ. 2011. Type III secretion is essential for the rapidly fatal diarrheal disease caused by non-O1, non-O139 *Vibrio cholerae*. mBio. 2:e00106-11.


