CHARACTERIZATION OF TOXIGENIC *VIBRIO CHOLERAE* ISOLATES FROM KENYA, 2007-2010

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A thesis submitted in partial fulfillment of the requirements for the award of the degree of Master of Science (Biotechnology) in the School of Pure and Applied Sciences of Kenyatta University

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other University or for any other award.

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DEDICATION

Dear Dad and Mom, you have molded me to live well, believe in myself and work hard. For all I am and aspire to be it is because of what you have taught me and because of your belief in me.

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ABBREVIATIONS AND ACRONYMS

5KG	5-ketogluconate
ADH	Arginine dihydrolase
ATCC	American Type Culture Collection
BTB	Bromothymol blue agar
CDC	Centre for Disease Control
CFU	Colony forming unit
ctxA	Cholera toxin Subunit A gene
DDSR	Division of Disease Surveillance and Response
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
ICE	Integrating conjugative element
IND	Indole production
InDS	Class 1 integron
int	Integrase
KCl	Potassium chloride
KEMRI	Kenya Medical Research Institute
LAR	L-arabinose fermentation
LDH	Lysine decarboxylase
MDR	Multiple drug resistance
$MgCl_2$	Magnesium chloride
MH	Mueller Hinton agar
MIC	Minimum Inhibitory Concentration
Mini-API	Device used to identify microorganisms biochemically
MoH	Ministry of Health
NUITM	Nagasaki University Institute of Tropical Medicine
ODH	Ornithine dehydrogenase
PCR	Polymerase Chain Reaction
PFGE	Pulsed-field gel electrophoresis
strA	Streptomycin resistance gene
sul2	Sulfamethoxazole resistance gene
SXT	Trimethoprim-Sulphamethoxazole
TBE	Tris-Borate EDTA
TBE	Tris-Borate EDTA
TCBS	Thiosulphate citrate bile salt sucrose
TetA-2000	Tetracycline resistance gene
toxR	Cholera toxin regulatory gene
UNDP	United Nations Development program
UNHCR	United Nations High Commissioner for Refugees
URE	Urea test
WHO	World Health Organization

ABSTRACT

Over the last decade cholera outbreaks have become common in some parts of Kenya. A number of recent studies have described the epidemiology of cholera in Kenya but there has been little information regarding the phenotypic and molecular characteristics of *Vibrio cholerae* in these outbreaks. The aim of this study was therefore to characterize *Vibrio cholerae* isolates from Kenya. A total of 168 *Vibrio cholerae* O1, biotype El Tor isolates collected between 2007 and 2010 from various cholera outbreaks in Kenya were studied. Their serotypes, antimicrobial susceptibility, genetic mechanisms of resistance, and their genetic relatedness were determined. Biochemical identification was performed using the API 20E identification system. Serologic identification was done by slide agglutination. Antimicrobial susceptibility testing was performed using the E-test method and disk diffusion. Polymerase Chain Reaction (PCR) was used to detect pathogenic and antibiotic resistant genes whereas Pulsed-field Gel Electrophoresis (PFGE) was used to determine their genetic diversity. Biochemical characterization confirmed all isolates as *Vibrio cholerae* belonging to serotypes Inaba and Ogawa, with Inaba being the predominant one. The isolates displayed reduced susceptibity towards ceftriaxone, chloramphenicol, doxycycline and streptomycin antimicrobials. Majority were resistant to amoxicillin, cotrimoxazole, erythromycin and nalidixic acid. Notably, 45% of the isolates were resistant to three or more of the antimicrobials recommended for cholera treatment. All were susceptible to ciprofloxacin, gentamicin and ofloxacin. Majority of the isolates were also resistant to O/129 *Vibriostatic* agent that is used to differentiate *Vibrio* species from closely related organisms. PCR results for detection of virulence genes revealed that the isolates harbored the cholera toxin, *ctxA* and/or the cholera toxin regulatory gene, *toxR*. They were found to harbor genetic determinants for antimicrobial resistance namely the SXT integrating conjugative element and the class one intergron. Genes that code for antimicrobial resistance were detected in these elements. Pulsed-field Gel Electrophoresis results suggest that two clones of *Vibrio cholerae* strains were circulating within Kenya. The finding that a majority of the Kenyan isolates were resistant towards cotrimoxazole and erythromycin suggest that these antimicrobials should not be used to treat cholera caused by current *Vibrio cholerae* O1 strains. Surveillance for antimicrobial drug resistance should continue among *Vibrio cholerae* isolates. The finding that most isolates were resistant towards the O/129 *Vibriostatic* agent suggests that the agent should not be used to characterize Kenyan isolates. Whole genome sequencing should be performed to better understand the genetic mechanisms of resistance and genetic relatedness of these isolates. The findings from this study will be presented to the Kenyan Ministry of Health and hopefully be used in policy making.

CHAPTER ONE

INTRODUCTION

1.1 Introduction

Cholera, caused by infection with the toxigenic bacteria *Vibrio cholerae* O1 or O139, continues to cause severe outbreaks of dehydrating diarrhea in much of the developing world. Cholera is essentially a disease of poor sanitation and is transmitted by consumption of food and water contaminated with this bacterium (Birmingham et al., 1997). Since 1817, *Vibrio cholerae* O1 has emerged from the Indian subcontinent in seven pandemics of acute diarrheal disease, the most recent beginning in 1961 and continuing to the present day in Asia, Africa, the Middle East, and South and Central America.

Africa contributes more than 80% of the total cholera cases reported worldwide (Naidoo and Patric, 2002). According to the World Health Organization (WHO, 2010), from 1974 to 1989, Kenya reported cases every year with an average case fatality rate of 3.57%. Between 1997 and 1999, 33,400 cases were reported, representing 10% of all cholera cases reported from the Africa continent in the same 3 years. The influx in cases may have been as a result of global climatic changes like the effects of *El Niño* (David et al., 2001). Between 2000 and May 2006, a range of 291 to 1,243 cases were reported each year. Most outbreaks occur in the beginning of the rainy season in areas with poor sanitation probably due to

faecally contaminated surface runoffs which drain into water reservoirs such as lakes, rivers or boreholes.

Rehydration with intravenous or oral rehydration fluids is the mainstay of cholera treatment. However, antibiotics are an important adjunct because they reduce by about 50% the duration of illness, the diarrhea volume, and the rehydration requirements. Antimicrobial resistance to the first line drugs used for cholera treatment has been reported (Ichinose et al., 1986). Where first line antimicrobial resistance is evident, the recourse is the more expensive drugs such as fluoroquinolones, which are unavailable in local health centers. Isolates resistant to the *Vibriostatic* agent and polymixin-B have also been reported (Sundaram and Murthy, 1983).

Bacteria become resistant to antibiotics by mutation and/or by the acquisition of foreign DNA in the form of plasmids and transposons. Integrons have been recognized as a mechanism for the acquisition and spread of drug-resistance. These elements are able to capture, integrate, and express resistance cassettes in their variable regions (Rowe-Magnus et al., 2001).

The Pulsed-field gel electrophoresis (PFGE) technique is useful to establish the degree of relatedness among different isolates of the same species and has been termed as the ‘gold standard’ for molecular characterization (Correia et al., 1994).

The Polymerase chain reaction (PCR) has been widely used to amplify target deoxyribonucleotide (DNA) regions by the use of complementary short DNA sequences (primers). It has been applied in the detection of various genes in genomic DNA *in vitro.*

In this study, phenotypic and molecular techniques were used to analyse *Vibrio cholerae* O1 isolates collected between 2007 and 2010 in Kenya from clinical samples. It included studying their serotypes, antimicrobial susceptibility, virulence, genetic determinants of antimicrobial resistance and genetic diversity.

1.1.1 Problem Statement and Justification

Periodic and often devastating cholera outbreaks continue to occur in Asia, Africa, and South America. It is estimated that 120 000 people die of cholera each year, with probably 100 times more cases than are officially reported (WHO, 2003). Cholera is a major health concern in Kenya as evidenced from the sporadic outbreaks of cholera throughout the country. Of concern is resistance to antibiotics used in cholera management and resistance to commonly used biocides which may induce antibiotic resistance. In Kenya, first line antimicrobials used for cholera treatment include doxycycline or tetracycline for adults and erythromycin or chloramphenicol for children and pregnant women. Where resistance is confirmed or suspected, the recourse are the more expensive fluoroquinolones (MoH, 2002). However, resistance to these agents has been reported elsewhere. In Kenya,

reliable data concerning resistance to the frequently used antibiotics is limited. Subsequently, the genetic bases of resistance to antibiotics in *Vibrio cholerae* have not been extensively characterized.

Findings from this study will contribute to the knowledge of the epidemiology of *Vibrio cholerae* O1 and will help health professionals curb the occurrence and spread of cholera outbreaks as this bacterium goes on adapting to the existent climatic conditions in Kenya.

1.1.2 Research Question

Are the phenotypic and molecular characteristics of the *Vibrio cholerae* O1 isolates from cholera outbreaks in Kenya between 2007 and 2010 different from those of previous outbreaks?

1.1.3 Research Hypothesis

The phenotypic and molecular characteristics of *Vibrio cholerae* O1 isolates from cholera outbreaks in Kenya between 2007 and 2010 are not different from those of previous outbreaks.

1.1.4 Objectives

1.1.4.1 General objective

The aim of this study was to determine the phenotypic and molecular characteristics of *Vibrio cholerae* O1 isolates from cholera outbreaks in Kenya between 2007 and 2010.

1.1.4.2 Specific objectives

i) To characterize the *Vibrio cholerae* O1 isolates biochemically and serologically.

ii) To determine the susceptibility patterns of the *Vibrio cholerae* O1 isolates towards antimicrobials and the O/129 *Vibriostatic* compound using E-test and disk diffusion.

iii) To screen the *Vibrio cholerae* O1 isolates for the presence of genes encoding virulence determinants using polymerase chain reaction (PCR).

iv) To screen the *Vibrio cholerae* O1 isolates for genes encoding for antimicrobial resistance using polymerase chain reaction (PCR).

v) To determine the genetic diversity of the *Vibrio cholerae* O1 isolates using pulsed-field gel electrophoresis (PFGE).

CHAPTER TWO

LITERATURE REVIEW

2.1 Taxonomy and serological classification

Vibrio cholerae, a member of the family *Vibrio*naceae, is a facultative anaerobic, gram-negative, non-spore-forming motile curved rod, about 1.4–2.6µm long. It is oxidase positive, ferments glucose and grows best on nutrient agar or broth in the presence of sodium chloride. On thiosulphate citrate bile salts sucrose (TCBS) selective media, *Vibrio cholerae* grow as yellow colonies because it ferments sucrose whereas other *Vibrio* species causing gastroenteritis are sucrose negative and appear as green colonies. The string test and susceptibility towards the 0/129 *Vibriostatic* agent differentiates *Vibrio species* from closely related organisms such as *Aeromonas* and *Plesiomonas* species. Ninety nine percent of isolates of *Vibrio cholerae* O1 are reported sensitive towards the agent. Resistance to this agent has been reported (Huq et al., 1992).

Differences in the sugar composition of the heat-stable surface somatic “O” antigen are the basis of the serological classification of *Vibrio cholerae.* The bacterium is classified into 206 “O” serogroups (Shimada et al., 1994; Yamai et al., 1997). *Vibrio cholerae* serogroups O1 and O139 cause epidemic cholera while the remaining serogroups rarely cause epidemics and are classified as *Vibrio cholerae* non-O1 and non-O139 and do not produce the cholera toxin (CT) (Nair et

al., 1995). They may produce other toxins causing a self-limiting gastroenteritis, sepsis and wound infections. The O139 serogroup emerged in Bangladesh in 1993 (Albert, 1994).

The O1 serogroup exists as two biotypes, classical and El Tor. Antigenic factors allow further differentiation into two major serotypes, Ogawa and Inaba. Strains of the Ogawa serotype express the A and B antigens and a small amount of C antigen, whereas Inaba strains express only the A and C antigens. Serotype Hikojima expresses all three antigens but is rare and unstable. Classical biotypes are haemolysis of sheep red blood cells (-), haemagglutination of chicken cells (-), voges-proskauer (-), susceptibility to polymyxin-B 300U (+), classic phage IV (+), EI Tor phage 5 (-), while El Tor biotypes have opposite reactions (Amit et al., 2008).

2.2 Identification of Enterobactericiae using ID 32E Identification system

The ID 32 E (Biomeriux SA, France) is a standardized system for the identification of Enterobactericiae and other non-fastidious gram-negative rods which uses 32 miniaturised biochemical tests, as well as a specific database. Reading and interpretation are carried out automatically or manually. The ID 32 E strips consist of 32 test cupules which contain dehydrated test substrates. After an incubation period of 24 hours, the reactions are read either using the ATB

Expression or *mini API* instruments, or visually. Identification is obtained using the identification software.

The system is not for use directly with clinical or other specimens. The microorganisms to be identified must first be isolated on a suitable culture medium according to standard microbiological techniques.

2.3 Epidemiology

Cholera continues to cause severe outbreaks of dehydrating diarrhea in much of the developing world. Globally the burden of cholera is highest in sub-Saharan Africa (WHO, 1997). Between 1817 and 1961, six pandemics of cholera were recorded. The classical biotype was responsible for the first six pandemics whereas the El Tor biotype is the causative agent of the seventh cholera pandemic which began in 1961. The classical biotype has been completely displaced worldwide, except in Bangladesh where it reappeared in epidemic proportions in 1982 then became extinct again (Siddique et al., 1992).

Cholera is often transmitted by faecal contamination of water and food. A high infectious dose (10^8 bacteria) or a lower dose (10^5) if given with antacids to neutralize stomach acid is sufficient to cause severe cholera in healthy persons (Hornick et al., 1971; Sack et al., 1998). In cholera-endemic areas, the highest attack rates are in children aged 2–4 years (Glass et al., 1980). Secondary cases

sometimes occur during funeral feasts as a result of traditional but unhygienic funeral practices in some parts of the world (Gunnlaugsson et al., 1998).

2.3.1 Recent cholera outbreaks in Kenya

There were several cholera outbreaks in Kenya between 2007 and 2009. The World Health Organization (WHO) reported 625, 1243 and 11,769 cases in the years 2007, 2008 and 2009, respectively (WHO, 2010). The cases were reported from 76 districts. Cumulatively, 373 deaths were documented (average case fatality ratio = 2.74%). The outbreaks were fairly widely dispersed (geographically) within each location.

According to the WHO report on cholera outbreaks in Kenya (WHO, 2010), in 2007 the cholera outbreak affected 4 provinces: Rift Valley (West Pokot, Turkana), Coast (Kwale), North Eastern (Garissa, Wajir, Mandera) and Nyanza (Kisumu, Bondo and Siaya). In 2008, cholera outbreaks were reported from 4 provinces: Nyanza (Suba, Migori, Homabay, Rongo, Siaya, Kisumu, Bondo, Nyando, Kisii South), North Eastern (Mandera East, Mandera Central, Wajir), Western (Bunyala) and Rift Valley (Naivasha, Nakuru). Nyanza was the most affected province with 771 cases and 53 deaths being reported from 10 districts as of 31 March. The outbreak started in Suba district in November 2007 and further spread to cover all districts in the lake basin. In 2009, Kenya reported 11,769 cases including 274 deaths throughout 2009 (CFR 2.33%). The country had not

experienced such a high number of cases in the previous 10 years. The peaks occurred during March-April, June and October-November 2009.

2.4 Clinical features

After an incubation period of about 1 to 5 days, the clinical presentation of cholera ranges from an asymptomatic infection to a severe form. This results in watery diarrhea known as “rice water” stools, with a fluid loss of 500-1000ml per hour, leading to severe dehydration. Symptoms may also include vomiting. Signs of severe dehydration include absent or low-volume peripheral pulse, undetectable blood pressure, poor skin turgor, sunken eyes, and wrinkled hands and feet. Most deaths occur during the first day due to rapid fluid loss (Kaper et al., 1995).

2.5 Treatment and control

Rehydration with intravenous or oral rehydration fluids is the mainstay of cholera treatment. Replacement fluids should have a similar electrolyte composition to the fluids being lost. However, antibiotics are an important adjunct because they reduce by about 50% the duration of illness, the diarrhea volume, and the rehydration requirements. Antimicrobial agents recommended by WHO for treating cholera patients include tetracycline, doxycycline, furazolidone, trimethoprim-sulfamethoxazole, erythromycin, chloramphenicol, ciprofloxacin and norfloxacin (CDC, 1994). Because antimicrobial resistance has been a growing problem in many parts of the world, the susceptibility of *Vibrio cholerae*

O1 strains to antimicrobial agents should be determined at the beginning of an epidemic and be monitored periodically.

In Kenya, doxycycline is the antibiotic of choice (300 mg given as a single dose to adults). Tetracycline (500mg) is also recommended but should be taken four times a day for three days. Erythromycin (30-50mg/kg syrup) and chloramphenicol (50mg/kg) are given to children and pregnant women. The dose is taken four times a day for three days. For adults the two drugs are given at a dosage of 500mg. They may be used when doxycycline or tetracycline antibiotics are not available or when *Vibrio cholerae* O1 is resistant to them (MoH, 2002). Antibiotics should not be given to asymptomatic contacts as this greatly increases the risk of the development of resistance and is not cost effective (Sack, 1979).

Measures that can control the transmission of *Vibrio cholerae* include ensuring a safe water supply, improving sanitation and health education through mass media. Two kinds of vaccines are currently available. A killed oral vaccine (Dukoral) consisting of killed *Vibrio cholerae* organisms along with the cholera B subunit. The vaccine stimulates both antibacterial and antitoxic immunity. Two doses are given 1–6 weeks apart (Holmgren et al., 1989). The other vaccine (Orochol) is an avirulent mutant of *Vibrio cholerae*, strain CVD103HgR, given as a single-dose, lyophilized oral vaccine (Tacket et al., 1999).

2.5.1 Antimicrobial mode of action and bacterial resistance mechanisms

The tetracyclines include doxycycline, and tetracycline among others. They act by preventing protein synthesis at the level of binding of transfer RNA-amino acid complexes to the ribosomes and by being bacteriostatic. *Escherichia coli* and *Shigella* are increasingly becoming resistant (Meyers, 2002). Chloramphenicol acts by inhibiting protein synthesis at the 50S ribosomal site. It is also bacteriostatic and is a broad spectrum antibiotic. Cotrimoxazole (Trimethoprim/sulfamethoxazole) acts via a sequential double-block of folate synthesis. Trimethoprim blocks conversion of dihydrofolate to tetrahydrofolate, while sulfonamides inhibit formation of dihydrofolate. It is administered intravenously. Quinolones include the first generation quinolones like nalidixic acid and fluoroquinolones like ciprofloxacin, ofloxacin and norfloxacin. Quinolones act on DNA gyrase inhibiting nucleic acid synthesis (Meyers, 2002).

The β-Lactam antibiotics include the penicillins, cephalosporins and carbacephems. They interfere with cell wall synthesis by acting on penicillin-sensitive enzymes like carboxypeptidases, endopeptidases and transpeptidases, which are found on bacterial cell membranes. Bacterial resistance to β-lactam antibiotics is by production of β-lactamases, amylases or esterases. It may also be related to decreased affinities of these molecules to the penicillin binding proteins (PBPs) or to their inability to penetrate the bacterial cell (Meyers, 2002). Examples include amoxicillin and ampicillin.

The cephalosporins act by inhibiting cell wall synthesis, and being bactericidal. They include the drug ceftriaxone which is effective against gram positive and gram negative organisms. The aminoglycosides act by inhibiting protein biosynthesis at the 50S ribosomal site and by being bactericidal. They include erythromycin, gentamicin, and streptomycin among others. Bacterial resistance to aminoglycoside antibiotics is by inactivation of aminoglycosides and by failure to penetrate bacterial cells (Meyers, 2002).

2.6 Distribution of virulence-associated genes in *Vibrio cholerae*

The most important virulence factor produced by *Vibrio cholerae* is cholera toxin (CT), an A-B subunit toxin encoded by the *ctx* gene that catalyzes an ADP ribosylation reaction in epithelial cells (Pearson et al., 1993; Kaper et al., 1995). The resulting activation of adenylate cyclase leads to increased intracellular levels of cAMP, alteration of ion transport, and ultimately to secretory diarrhea. The major virulence-associated factors are present in clusters with at least three gene clusters in the *Vibrio cholerae* chromosome (Hacker et al., 1997). These regions are the CTX genetic element (Mekalanos, 1985), the *Vibrio cholerae* pathogenicity island (VPI) (Karaolis et al., 1998) and the RTX toxin gene cluster (Lin et al., 1999).

The CTX genetic element is comprised of a 4.5-kilobase (kb) dynamic region termed as the Core region or virulence cassette which is flanked by one or more

copies of a 2.7-kb repetitive sequence that encodes functions required for regulation, replication and integration of CTX Phage (CTXΦ). The core region harbors the *ctx* gene that codes for the cholera toxin (CT). Also of importance is regulon *toxR* which regulates and expresses genes for growth and survival. The *toxR* gene encodes a transcriptional activator controlling cholera toxin gene expression (*ctxA*) (Baudry et al., 1992; Karasawa et al., 1993; Trucksis et al., 1993; Kurazono et al., 1995).

2.7 Antimicrobial resistance

The greatest challenge towards antimicrobial therapy is the development of antimicrobial drug resistance. Multidrug resistance in *Vibrio cholerae* is on the rise and several studies have reported tetracycline and fluoroquinolone resistant *Vibrio cholerae* (Kitaoka et al., 2011). *Vibrio cholerae* can develop antimicrobial drug resistance through mutation or through acquisition of resistant genes on mobile genetic elements, such as plasmids, conjugative transposons, integrons, and integrating conjugative elements (ICEs) (Sjölund-Karlsson et al., 2011).

Integrons are DNA elements capable of mobilizing individual antimicrobial resistance gene cassettes into bacterial chromosomes by site-specific recombination (Amita et al., 2003). They have an integrase gene (*intI*), an attachment site (*attI*) into which genes conferring antimicrobial resistance are inserted, and a promoter sequence allowing expression of resistance genes

(cassette-associated genes). The *intI-attI* fragment is highly conserved in all integrons and is called a 5' conserved sequence (CS). Integrons have been categorized into nine different classes according to the sequences of their integrases. Class I integrons have frequently been detected in clinical isolates (Iwanaga et al., 2004).

Integrating conjugative elements integrate and replicate with the host chromosome and can excise themselves and transfer between bacteria by conjugation (Wozniak et al., 2009). The ICEs commonly carry several antimicrobial drug resistance genes and play a major role in the spread of antimicrobial drug resistance in *Vibrio cholerae* (Burrus et al., 2006). The first *Vibrio cholerae* ICE described was in an 0139 isolate in Madras, India, in 1992 and was named SXT after the resistance phenotype it conferred (trimethoprim/sulfamethoxazole) (Waldor et al., 1996). Other isolates of O1 and O139 have since acquired SXT or a closely related ICE (Wozniak et al., 2009).

Testing *Vibrio cholerae* against certain drugs may yield misleading results when *in vitro* results do not correlate with *in vivo* activity. The reliability of disk diffusion results for ciprofloxacin, furazolidone and nalidixic acid, have not been validated. Until interpretive criteria have been established for *Vibrio cholerae*, disk diffusion may be used to screen for resistance to ciprofloxacin, using interpretive criteria for the *Enterobacteriaceae* as tentative zone size standards. If zone sizes for these

drugs fall within the intermediate range, the organism should be considered possibly resistant (CDC, 1994).

2.7.1 Determination of antibiotic susceptibility using E-test

The E-test (AB Biodisk Co., Solna, Sweden) is a method based on the diffusion of a continuous concentration of an antimicrobial to determine the minimum inhibitory concentration (MIC in mg/ml) of individual antimicrobial agents on an agar medium. This test consists of an impenetrable, inert, thin reagent carrier strip, one side of which contains a pre-defined continuous concentration gradient of dried and stabilized drug. The gradient covers a wide range corresponding to 15 log2 dilutions of the conventional MIC procedure. The E-test method for antimicrobial susceptibility is performed in a similar way as the disk diffusion method (Aidara et al., 1998). Inhibitory concentrations are seen as a formation of an elliptical zone of inhibited growth, whose intersection between the values printed on the strip edge and the zone of inhibition is the MIC.

2.8 Molecular characterization

Due to the low discriminatory power of conventional epidemic tools such as serologic identification, biochemical properties, and antibiotic susceptibility tests, newer methods of molecular typing have been developed. They include ribotyping (Koblavi et al., 1990; Popovic et al., 1993), polymerase chain reaction (PCR), arbitrary primer PCR (Coelho et al., 1995) and pulsed-field gel Electrophoresis

(Cameron et al., 1994). Fields described a PCR for the amplification of a 564 basepair (bp) fragment of the cholera toxin subunit A gene (*ctxA*) (Fields et al., 1992). In addition, primers have been developed for the amplification of a 739bp fragment of the *toxR* gene, 592bp fragment of the SXT element (*int*), 380bp fragment of class 1 integron (*inDS*), 383bp fragment of the streptomycin resistance gene (*strA*), 625bp fragment of the sulfamethoxazole resistance gene (*sul2*) and the 950bp fragment of the tetracycline resistance gene (*tetA*).

2.8.1 Polymerase chain reaction (PCR)

The polymerase chain reaction (PCR is a technique to amplify a single or few copies of a piece of deoxynucleic acid (DNA) across several orders of magnitude, generating millions or more copies of a particular DNA sequence. Primers (short DNA fragments) containing sequences complementary to the target region along with a DNA polymerase are key components to enable selective and repeated amplification. As PCR progresses, the DNA generated is itself used as a template for replication, setting in motion a chain reaction in which the DNA template is exponentially amplified.

2.8.2 Pulsed-field gel electrophoresis (PFGE)

This is a molecular technique used to determine the genetic relatedness of strains of the same species. Bacterial cells are embedded into agarose prior to DNA extraction. This keeps the DNA intact. Proteins and carbohydrates are removed

from the small agarose plugs through a series of lysis buffers and enzyme treatments. The isolated, non-sheared DNA is treated with a restriction enzyme to cut the DNA at specific sequences. The amount and size of the resulting fragments is therefore dependent on the genetic sequence. The small agarose plugs are loaded into a larger agarose and current is applied to allow the fragments to migrate through the gel. The direction in which the current runs is periodically switched to enable large fragments to move through the gel.

The result is a “fingerprint” based on fragment size, with the larger fragments at the top of the gel and the smaller fragments closer to the bottom. The PFGE has been used to analyze *Pseudomonas species* (Grothues and Tummler, 1991), *Escherichia coli* (Arbeit et al., 1990), *Mycobacterium species* (Zhang et al., 1992), and *Camphylobacter species* (Yan et al., 1991). Cameron described PFGE as the most discriminating of several molecular subtyping methods they studied. It is also reproducible and relatively stable over time (Cameron et al., 1994).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study site

Cholera suspected outbreaks occurred in Kenya between 2007 and 2010. Three hundred and fifty eight diarrheal samples were received at the Institute of Tropical Medicine, Nagasaki University - Kenya Medical Research Institute, Nairobi, Kenya (NUITM-KEMRI) and microbiologically processed for the isolation of enteric pathogens using standard bacteriological methods (CDC, 1999). The outbreaks had occurred in Bungoma, Isiolo, Kisumu, Marsabit, Msambweni, Nairobi, Naivasha, Pokot, Suba and Turkana locations (Figure 1). Isolated *Vibrio cholerae* O1 strains were stocked in nutrient agar broth containing 15% glycerol at -80˚C.

3.2 Study design

This was a descriptive cross-sectional study in which *Vibrio cholerae* O1 isolates had been previously isolated from diarrheal stool specimens. The specimens had been obtained from subjects presenting with passage of three or more watery diarrhea with or without vomiting during suspected cholera outbreaks.

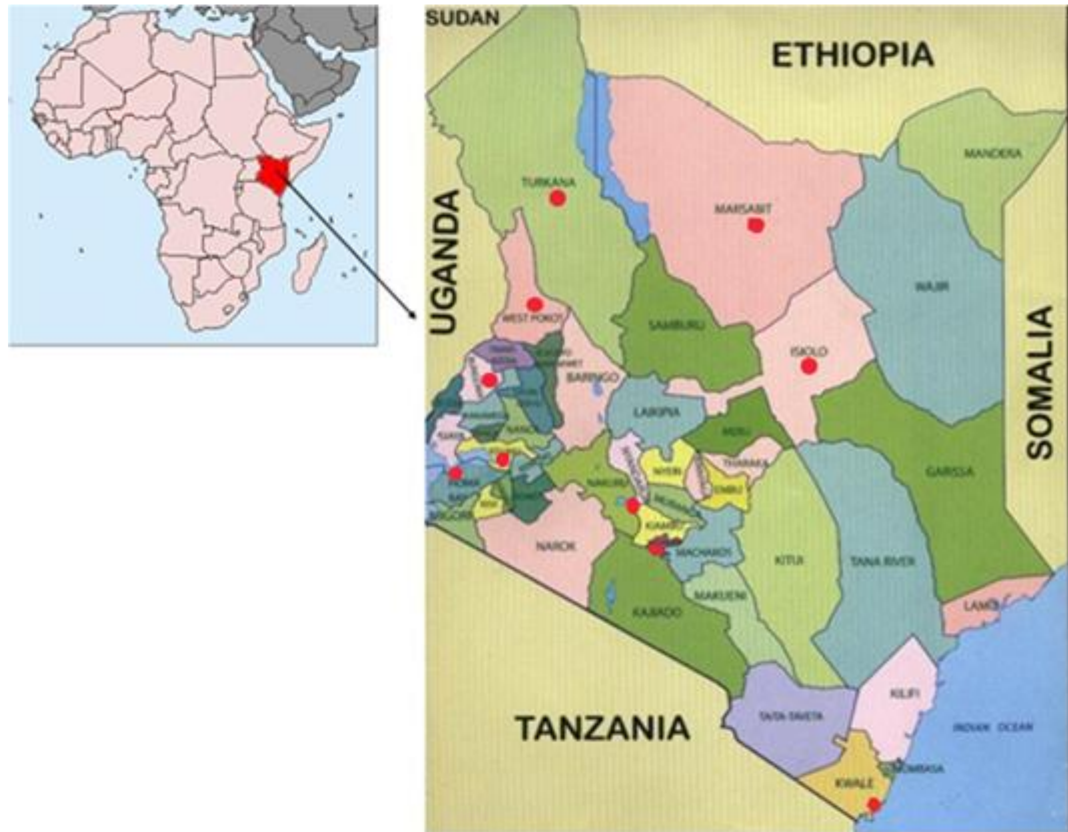

Figure 1: Map of Kenya with sites of the 5 outbreaks where samples were collected (indicated by red dots).
Source: Edited from WHO, Global task force on cholera control, Kenya profile (WHO, 2010) and flickr © Albert Kenyani Inima

3.3 Sample size

The sample size was calculated based on the prevalence of laboratory confirmed cholera cases during a cholera outbreak in Kenya at 18.8% (Isaac et al., 2008), using the formulae by Harris et al. (1991): $N = z^2 \times p(1-p) / d^2$

Where;N = minimum sample size

z = 1.96 (standard error)

p = 18.8% (prevalence of laboratory confirmed cholera cases during a cholera outbreak in Kenya

$d^2 = 0.07$ (absolute precision)

$N = z^2 \times p\,(1-p) / d^2 = 1.96^2 \times 0.188(1-0.188)/0.07^2$.

N=120; Therefore a minimum of 120 *Vibrio cholerae* O1 isolates were required for the study.

A total of 168 isolates were analysed (Table 1). The *Vibrio cholerae* O1 biotype classical strains Bgd17 and H218, and *Vibrio cholerae* 01 biotype El Tor strains isolated in Kenya in 1980 and the Philippines in 1982 were included in this study and used as positive controls. *Escherichia coli* ATCC 25922 standard strain was used as a negative control and for the antimicrobial susceptibility tests.

Table 1: Sources of *Vibrio cholerae* O1 isolates from Kenyan cholera outbreaks, 2007-2010

Location	No. of isolates	Year(s) collected
Bungoma	21	2008-2009
Isiolo	20	2009
Kisumu	2	2009
Marsabit	3	2009
Msambweni	15	2010
Nairobi	12	2009
Naivasha	5	2009
Pokot	14	2009-2010
Suba	72	2007-2009
Turkana	4	2009
Total	168	

3.4 Revival of stocked isolates

Vials of the stocked *Vibrio cholerae* isolates were removed from the -80˚C freezer and placed at room temperature to facilitate thawing. A loop full of the culture was inoculated onto TCBS (selective media for *Vibrio cholerae*) aseptically and incubated at 37˚C for 18-24 hours. A single yellow colony on the TCBS agar characteristic of *Vibrio cholerae* was subcultured onto BTB agar (differential media) and incubated at 37˚C for 18-24 hours. A well isolated green colony (non-lactose fermentor) characteristic of *Vibrio cholerae* was inoculated onto Nutrient agar and incubated at 37˚C for 18-24 hours. Biochemical identification and drug susceptibility tests were performed on the isolates that grew on Nutrient agar.

3.5 Phenotypic characterization

3.5.1 Biochemical identification

All isolates were confirmed as *Vibrio cholerae* by means of biochemical identification (API 20 E; BioMerieux, Charbonnieres-Les- Bains, France). The procedure was carried out according to the manufacturer's instructions. Two milliliters of API 0.85% NaCl was inoculated with one or several colonies of young cultures (18-24 hours old) to make a 0.5 McFarland suspension, measured with the ATB densitometer. Fifty-six microlitre of the suspension was dispensed into each cupule of the strip using the ATB electronic pipette. The ODC, ADH, LDH, URE, LARL, GAT and 5KG tests were covered with 2 drops of mineral oil. The lid was placed on the strip and incubated at 36±2˚C for 24 hours (±2 hours) in

aerobic conditions. One drop of JAMES reagent was added in the IND reaction and the strips were read using the mini API machine (BioMerieux, Charbonnieres-Les- Bains, France). The results were read via a computer and interpreted using the mini API identification software (BioMerieux, France). The biochemical characteristics of *Vibrio cholerae* O1 strains are shown in Appendix 3. The El tor biotype strains of *Vibrio cholerae* O1 strains K14, K3, 82P-17 and 82P-11 isolated in Kenya and the Philippines were used as *Vibrio cholerae* O1 positive controls.

3.5.2 Serological identification

The serologic identification was done by the slide agglutination technique with polyvalent antisera for *Vibrio cholerae* O1 and O139; and monovalents for serotypes Inaba and Ogawa (Denka Seiken Co., LTD, Japan). The confirmed *Vibrio cholerae* isolates were subcultured onto Nutrient agar and incubated overnight 37˚C for 18-24 hours in aerobic conditions. A well isolated colony was picked aseptically and mixed in a drop of sterile normal saline on a glass slide, making a milky suspension. A drop of the antiserum was added onto the drop and mixed to observe for agglutination. Positive agglutination confirmed the serotype and subtype of the isolates. In this test, K3 and K14 strains belonging to *Vibrio cholerae* O1 biotype El Tor, subtype Ogawa were used as positive controls while *Escherichia coli* strain ATCC 25922 was used as a negative control.

3.5.3 Antimicrobial susceptibility testing

Fourteen antimicrobials were used in this study. They included amoxicillin, ampicillin, ceftriaxone, chloramphenicol, ciprofloxacin, cotrimoxazole, doxycycline, erythromycin, gentamicin, nalidixic acid, ofloxacin, streptomycin, tetracycline and the O/129 *Vibriostatic* agent. Susceptibility to antimicrobial agents was assayed by the diffusion method. Drug incorporated disks were used for the O/129 *Vibriostatic* agent whereas for the rest of the antimicrobials, their MIC was determined by the E-test method. *Escherichia coli* standard strain ATCC 25922 was used for quality control. The procedure for antimicrobial susceptibility testing was performed in accordance with the CLSI guidelines (CLSI, 2010).

3.5.3.1 Susceptibility to 0/129 *Vibriostatic* agent (150µg)

A well isolated *Vibrio cholerae* colony was subcultured onto Nutrient agar and incubated overnight at 37˚C for 18-24 hours in aerobic conditions. Colonies from the plate were aseptically picked and suspended in 3ml sterile normal saline to make a 0.5 McFarland suspension (10^8 CFU/ml). A sterile cotton swab was dipped into the suspension and gently pressed on the tubes side to eliminate excess liquid. The suspension was then spread evenly on the surface of moisture-free Mueller Hinton agar plates and let to dry at room temperature. Disks containing 150µg of the O/129 *Vibriostatic* agent were placed on the media surface and incubated in an inverted position overnight at 37˚C in aerobic conditions. Inhibition zones of <15mm were interpreted as resistant and those >15mm as sensitive.

3.5.3.2 Susceptibility to various antimicrobials using E-test strips

Susceptibility to β-lactam antimicrobials was tested using amoxicillin (0.016-256µg/ml) and ampicillin (0.016-256µg/ml), while susceptibility to Cephalosporins was determined using ceftriaxone (0.016-256µg/ml). Nalidixic acid (0.016-256µg/ml) and ofloxacin (0.002-32µg/ml) were used for testing susceptibility to the Quinolones. Aminoglycosides included gentamicin (0.016-256µg/ml) and streptomycin (0.064-1024µg/ml). Tetracycline antibiotics included doxycycline (0.016-256µg/ml) and tetracycline (0.016-256µg/ml). Other antimicrobials included, erythromycin (0.016-256µg/ml), chloramphenicol (0.016-256µg/ml), and cotrimoxazole (trimethoprim / sulfamethoxazole) (0.002-32µg/ml).

The E-test strips were removed from the -30°C freezer and placed on the bench to attain room temperature. Well isolated morphologically similar colonies sub-cultured on nutrient agar were suspended in 3ml normal saline making a 0.5 McFarland suspension (10^8 CFU/ml). A sterile cotton swab was dipped into the suspension and gently pressed on the tubes side to eliminate excess liquid. The suspension was then spread evenly on the surface of moisture-free Mueller Hinton agar plates and let to dry at room temperature.

The E-test antimicrobial strips were aseptically placed on the agar surface, with the MIC scale facing upwards and the strips code to the outside of plate. Plates

were then incubated in an inverted position overnight at 37˚C in aerobic conditions. The MIC value where the edge of the inhibition ellipse intersected the strip was read. Intersection falling between two scale segments was rounded up to the higher value. Isolates were classified as sensitive or resistant based on the MIC interpretation scheme provided by the manufacturer and in accordance to the performance standards for antimicrobial susceptibility testing (CLSI, 2010). The interpretative criterion is described in Appendix 4. Isolates showing intermediate zones of inhibition were interpreted as resistant on the basis of previous MIC studies conducted with *Vibrio cholerae* (Yamamoto et al., 1995). The concentrations that inhibited 50% (MIC_{50}) and 90% (MIC_{90}) of the isolates were calculated for each of the antimicrobials according to the method described by Smith et al. (Smith et al., 1986). Briefly, the formula of geometric means was used as follows:

$$MIC_{50} = (M < 50) + \frac{(n - x) * [(M > 50) - (M < 50)]}{y}$$

Where:

$M < 50$ = MIC of the highest cumulative percentage below 50%

$M > 50$ = MIC of the lowest cumulative percentage above 50%

n = 50% of the number of organisms tested

x = number of organisms in the group at $M < 50$

y = the number of organisms in the group at M > 50

For MIC_{90}, the values of 50% in the above formula were replaced with 90% accordingly.

3.6 Molecular characterization

3.6.1 Polymerase chain reaction (PCR) for pathogenic and antibiotic resistant genes

In order to understand the virulence and drug susceptibility patterns of the isolates, the genetic determinants encoding virulence and antibiotic resistance were analysed. The SXT intergrating conjugative element and the class one intergron that are known to harbor antimicrobial resistance genes were targeted. Specific genes coding for streptomycin, sulfamethoxazole and tetracycline resistance were also targeted. Tetracycline resistance was considered since doxycycline (belonging to the tetracycline class of antibiotics) is empirically used for cholera treatment in Kenya.

The isolates were examined by PCR for the presence of *ctxA* (a gene encoding the A subunit of cholera toxin), *toxR* regulatory gene for the cholera toxin prophage, *inDS* (encoding class 1 integrons) (Dalsgaard et al., 1999), *int* (encoding for SXT integrase) (Hochhut et al., 2001), *strA, sul2* and *tetA* (encoding for genes conferring resistance to streptomycin, sulfamethoxazole and tetracycline respectively) (Hochhut et al., 2001; Yamai et al., 1997). Oligonucleotide primers

were used in the PCR assays, their sequences and the amplicon sizes are described in Appendix 5.

DNA was extracted from the isolates as described by (Yokoyama et al., 2005) with some slight modifications. A single colony of well isolated *Vibrio cholerae* O1 colonies grown on TCBS agar was inoculated in 2ml of Luria-bertani broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl) and incubated in a shaking incubator at 37˚C for 18-24 hours. The culture fluid was transferred into a sterile 2ml eppendorf tube and centrifuged at 10,000 X *g* for 10 minutes. The supernatant was discarded and 200 µl lysis buffer (0.1 M NaCl, 50 mM disodium EDTA, 0.1 M Tris-HCl, pH 8) containing 0.5% (w/v) sodium dodecyl sulfate 0.5% (w/v), proteinase K (0.5 mg/ml), and RNase (0.8 mg/ml) added to the pellet. After resuspension, the tube was placed in a water bath at 60°C for 5 minutes. Two hundred microlitres of the supernatant was placed in a new eppendorf tube and overlayed with 30 µl of 6 M NaCl and 2 volumes of room temperature 70% ethanol. The tube was kept at room temperature for five minutes, centrifuged at 1000 X g for three minutes. The supernatant was discarded and crude DNA rinsed with 70% ethanol and dried out by leaving the eppendorf tubes open for a few minutes in the clean bench. Finally the DNA was suspended in 200 µl of sterile water. The DNA was characterized by evaluating its concentration (an OD of 1 at 260 nm = 50 µg/ml of DNA) and its purity (OD at 260 nm/OD at 280 nm = 1.7-2.0).

Two hundred microlitre (200µl) tubes containing PuReTaq ready-to-go PCR beads (GE Healthcare UK limited, UK) were used to set the polymerase chain reaction. Each reaction contained 2.5 units of PuReTaq DNA polymerase, 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM dATP, dCTP, dGTP and dTTP, stabilizers, BSA, 1µl each of forward and reverse primers (6 pmol/µl), 2µl of template DNA, and Milli-Q water, added to a final volume of 25µl. The PCR amplifications of the target regions were carried out in a thermal cycler (Bio-Rad).

PCR conditions included an initial melting temperature of 95°C for 3 minutes (step one), melting at 95°C for 1 minute, annealing of 58˚C for *InDS* and *TetA-2000* primers and 54˚C for the rest of the primers for 1 minute (step 2), an extension at 72°C for 1 minute (step 3) and a final extension of 72°C for 10 minutes. Step two was repeated for 30 cycles. The PCR products were analyzed by electrophoresis in 2% agarose gels, stained with ethidium bromide (2µg/ml in 1% TBE buffer), visualized under UV light and recorded with the aid of a gel documentation system (Bio-Rad). Genomic DNA from *Vibrio cholerae* O1 biotype classical strain H218 was used as a positive control in screening for *ctxA* and *toxR* virulence genes. The DNA of isolates from culture collection known to harbor various genes of interest were used as appropriate positive controls in PCR experiments detecting for drug resistance genes.

3.6.2 Pulsed-field gel electrophoresis (PFGE)

PFGE was performed using the method described by Cameron et al. (1994) with some slight changes. A two-block block program with a first block ramp time of 2sec to 10 sec for 13 hrs at 6V/cm (for separation of smaller fragments) and second block with ramp time of 20 and 25 sec for 6hrs (for separation of larger fragments) at 6V/cm was used. The *NotI* restriction enzyme was used to digest the chromosomal DNA. Restriction fragments were separated in a 1% pulsed-field certified agarose gel in 1× TBE (8.9 mM Tris base, 8.9 mM boric acid, and 0.25 mM disodium EDTA) by using a CHEF-DR II system (Bio-Rad). CHEF DNA size standard of *Saccharomyces cerevisiae* (Bio-Rad Laboratories, Inc, CA) was used as a molecular mass standard. Following electrophoresis, gels were stained for 20 min with ethidium bromide (2µg/ml in 1% TBE buffer), destained, and visualized on a UV light box. To better explore the genetic ancestry of the current isolates, *Vibrio cholerae* O1 biotype El Tor archive isolates obtained from southern Asia (the Philippines) and Kenya were included. *Vibrio cholerae* O1 biotype classical strain H218 and Bgd-17 were also included.

The DARwin software version 5.0.158 (Perrier and Jacquemoud-Collet, 2006) was used to generate a phylogenetic tree so as to understand the genetic relation of the isolates. Isolates restriction patterns differing from each other in two or more band positions were considered different (Barrett et al., 2006). The analysis aimed to

determine the genetic relation of the isolates based by the location and secondly by year of isolation.

3.7 Data management and analysis

Results were reported in form of Figures and Tables. Biochemical data, serological data, antimicrobial susceptibility data and data on the presence or absence of a gene by PCR was recorded in the Laboratory Notebook, entered in the Microsoft Excel Spreadsheet, cleaned and exported to SPSS version 17.0 (SPSS Inc. Chicago, IL) Statistical Software for analysis.

Epidemiological details of the cholera patients were obtained from laboratory data of the Institute of Tropical Medicine, Nagasaki University, Kenya Medical Research Institute, Nairobi, Kenya (NUITM-KEMRI).

To investigate for a possible link between cholera and rainfall, the rainfall time series from January 2008 through December 2010 for four metrological sites representing the cholera hotspots was analysed. The sites included Lodwar, Marsabit, Nairobi and Kisumu. The monthly average precipitation was aggregated for each year and then checked for correlation with cholera data as explained by Venables and Ripley (Venables and Ripley, 2002). Rainfall data was obtained from the Kenya Metrological Department, Dagoretti corner, Ngong road.

Categorical data such as comparison of antibiotic resistance to outbreak period was analysed by Chi-square test. For consistency, a distinct outbreak was defined as a gap of at least 2 months between the last known cholera case and a report of a new case in the same location. Dendogram analysis generated from restriction fragments of Pulsed-field gel electrophoresis was used to cluster the isolates using DARwin software version 5.0.158 (Perrier and Jacquemoud-Collet, 2006). A difference in the presence, absence, or intensity of a band among isolates was given equal weights. Isolates that differed by two or more bands were assigned different pattern numbers (Barrett et al., 2006). A p value less than 0.05 was considered statistically significant.

CHAPTER FOUR

RESULTS

4.1 Epidemiological data of the *Vibrio cholerae* cases in the 2007-2010 cholera outbreaks in Kenya

Table 2 describes the epidemiological details of the *Vibrio cholerae* cases in the 2007-2010 cholera outbreaks in Kenya. The patients had a mean age of 23.07 years and a range of range 0.75 to 80 years. The most affected population was 5-14 years (23.8%) (χ^2 = 35.634; df = 7; p = <0.001). Generally, those above the age of 5 years were most affected and the least affected were in the age range above 65 years. For those whose gender was established, 53% were males while 47% were females. This distribution was however not significantly different (χ^2 = 0.426; df = 1; p = 0.514).

Majority (42.9%) of the infected persons were from Suba district followed by 12.5% from Bungoma, 11.9% from Isiolo while the least (1.2%) were from Kisumu (χ^2 = 227.476; df = 9; $p < 0.001$).

Majority (32.1%) of the outbreaks occurred during the month of September 2009-May 2010 followed by 25.6% in the month of December 2007. The least (6.5%) occurred during the months of March and June 2008 (χ^2 = 35.81; df = 4; $p < 0.001$).

Table 2: Epidemiological data of the *Vibrio cholerae* cases in the 2007-2010 cholera outbreaks in Kenya.

Characteristics	Frequency	Percentage	Chi-square	df	P value
Gender*					
Male	61	53.0	0.426	1	0.514
Female	54	47.0			
Age group, y†					
<5	16	15.8			
5-14	24	23.8			
15-24	23	22.8			
25-34	15	14.9	35.634	7	<0.001
35-44	7	6.9			
45-54	7	6.9			
55-64	5	5.0			
>65	4	4.0			
Location					
Bungoma	21	12.5			
Isiolo	20	11.9			
Kisumu	2	1.2			
Marsabit	3	1.8			
Msambweni	15	8.9	227.476	9	<0.001
Nairobi	12	7.1			
Naivasha	5	3.0			
Pokot	14	8.3			
Suba	72	42.9			
Turkana	4	2.4			
Outbreak season					
December 2007	43	25.6			
March-June 2008	11	6.5			
Sept 2008-Jan 2009	21	12.5	35.81	4	<0.001
April-June 2009	39	23.2			
Sept 2009-May 2010	54	32.1			

p value, by Chi-square test ; *n=115 for those whose gender was determined; †n=101 for those whose age was determined; n=168 for location and outbreak season

4.2 Seasonal patterns of cholera outbreaks

Figure 2 describes the seasonal patterns of the cholera outbreaks. Two rainfall periods were observed for each year. The first occurred between March and May, and the other between October and December. There was a clear trend between *Vibrio cholerae* isolation and the rainfall season. Peaks in isolation occurred during the first month of the rainy season for all years except in 2008 where peaks were observed in the months of June and September.

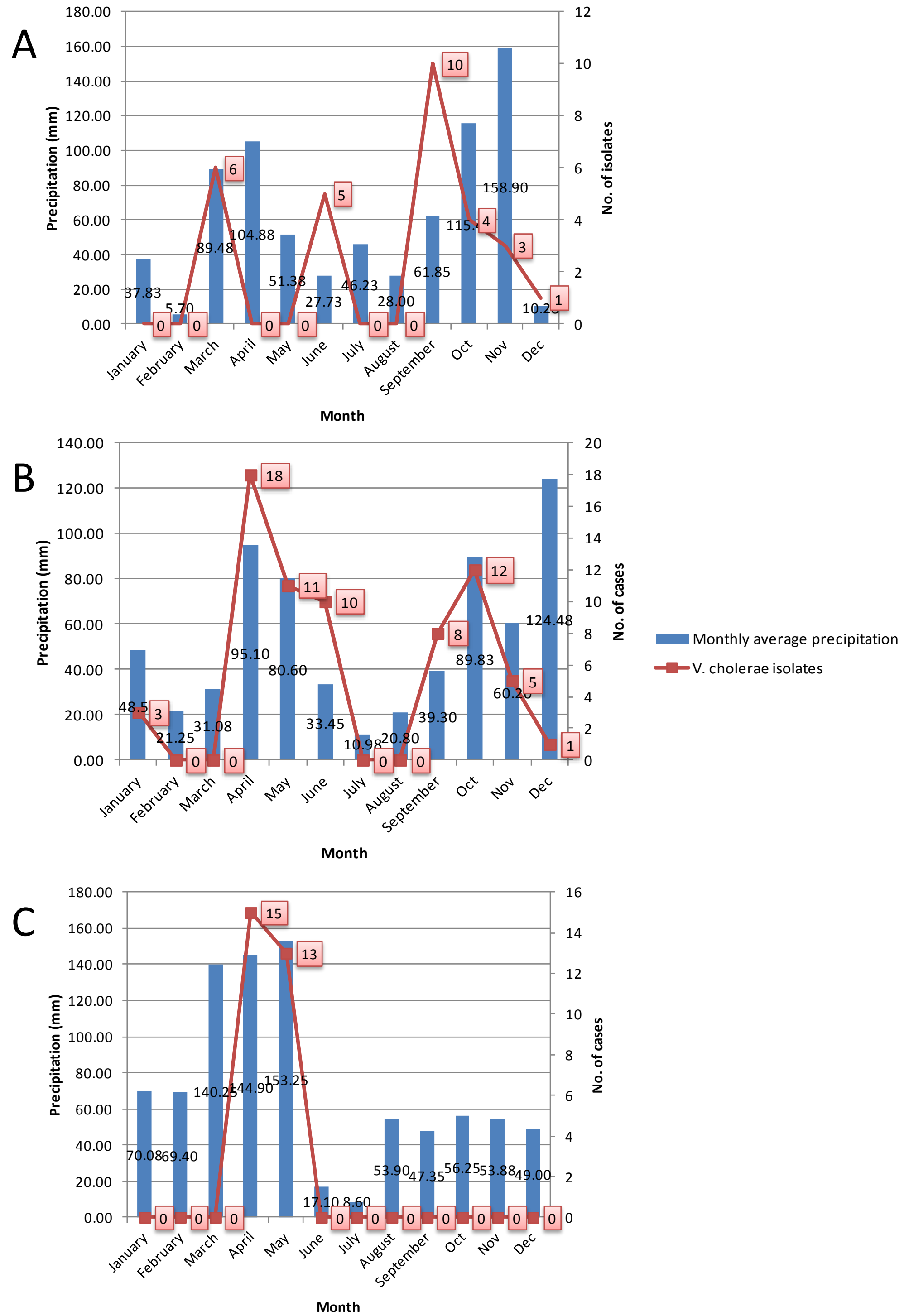

Figure 2: Seasonal patterns of cholera outbreaks in Kenya for the years 2008 (**A**), 2009 (**B**) and 2010 (**C**). Blue indicates the monthly average precipitation (in mm); red indicates the number of *Vibrio cholerae* isolates during that period.

4.3 Characterization of the *Vibrio cholerae* isolates

4.3.1 Biochemical characterization

Biochemical identification using the mini-API ID 20E (Biomeriux SA, France) confirmed all the isolates as *Vibrio cholerae.*

4.3.2 Serological characterization

Serological identification revealed that 152 (90.5%) isolates were of *Vibrio cholerae* O1 serotype Inaba and 16 (9.5%) as serotype Ogawa (Table 3).

Table 3: Serotype distribution among *Vibrio cholerae* isolates collected from outbreaks in Kenya, 2007-2010 (n=168)

Location	No. of strains typed	Serotype	
		Ogawa	Inaba
Bungoma	21		21
Isiolo	20		20
Kisumu	2		2
Marsabit	3	1	2
Msambweni	15	15	0
Nairobi	12		12
Naivasha	5		5
Pokot	14		14
Suba	72		72
Turkana	4		4
Total	168	16 (9.5%)	152 (90.5%)

4.3.3 Antimicrobial typing

4.3.3.1 Antimicrobial susceptibility patterns

Table 4 shows the overall susceptibility patterns of the 168 *Vibrio cholerae* isolates. All isolates were susceptible to ciprofloxacin, gentamicin and ofloxacin.

However, there was a reduced susceptibility to chloramphenicol, with 54.2% of the isolates being susceptible and 45.8% being resistant; MIC 0.25-96 μg/ml ($P<0.001$).

In total, 95.2% of the *Vibrio cholerae* isolates were susceptible to ampicillin ($p < 0.001$), 99.4% to ceftriaxone ($p < 0.001$), 97% to doxycycline ($p < 0.001$), 97.6% to streptomycin ($p < 0.001$), and 96.4% to tetracycline ($p < 0.001$). The MIC_{50} of ampicillin, ceftriaxone, doxycycline, streptomycin and tetracycline were all in the susceptible zone. The MIC_{90} of ceftriaxone, streptomycin and tetracycline were in the sensitive zone, that for ampicillin was in the intermediate zone, and that for doxycycline was in the resistant zone.

It was observed that 65.5% of the *Vibrio cholerae* isolates were resistant to amoxicillin (MIC 0.75->256μg/ml, $p < 0.001$), 94.6% to cotrimoxazole (MIC 0.023->32μg/ml, $p < 0.001$), 91.7% to erythromycin (MIC 0.125-48μg/ml, $p < 0.001$), and 81% to nalidixic acid (MIC 0.125->256μg/ml, $p < 0.001$). The MIC_{50} and MIC_{90} of cotrimoxazole and nalidixic acid were in the resistant zone. For amoxicillin and erythromycin, their MIC_{50} were in the sensitive zone whereas their MIC_{90} were in the resistant zone.

Table 5 describes the antimicrobial resistance of isolates by location. There was an association between antimicrobial resistance and the regions of *Vibrio cholerae*

outbreak for amoxicillin ($\chi^2 = 33.061$, $p < 0.001$), chloramphenicol $\chi^2 = 53.887$, $p < 0.001$) and nalidixic acid ($\chi^2 = 41.897$, $p < 0.001$).

Representative images of the *Vibrio cholerae* antimicrobial susceptibility test performed by the E-test method are highlighted in Figure 3.

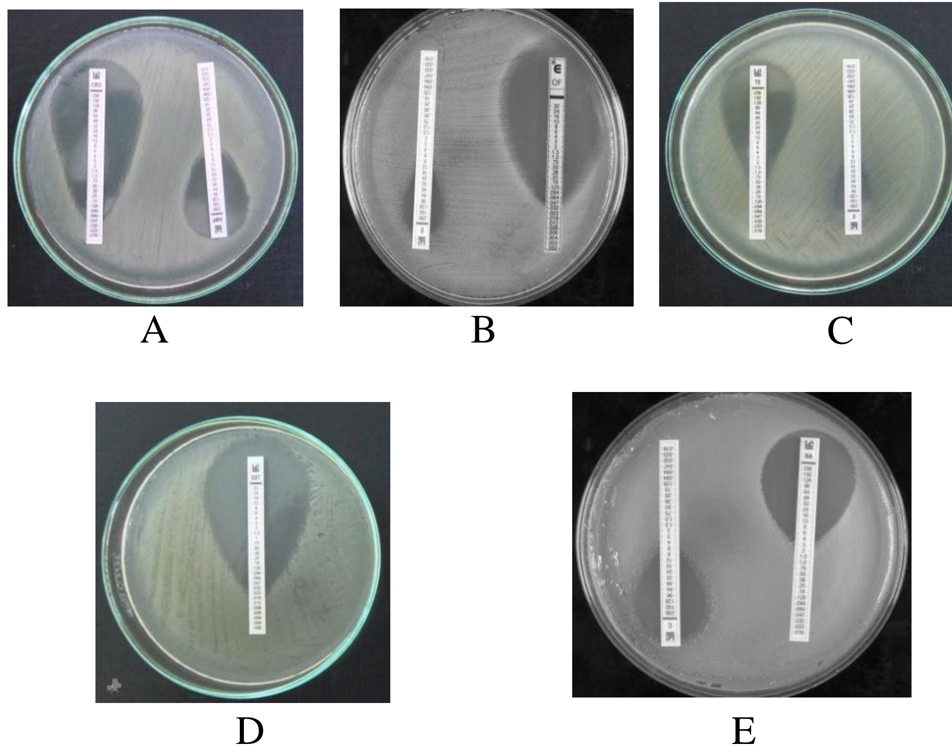

Figure 3: Representative images of antimicrobial susceptibility testing on agar plate by E-test
A: Ampilicin (AMP) and Ceftriaxone (C), **B:** Erythromycin (E) and Ofloxacin (OF), **C:** Tetracycline (TE) and Erythromycin (E), **D:** Cotrimoxazole (SXT) and **E:** Chloramphenicol (C) and nalidixic acid (NA)

Table 4: Antimicrobial susceptibility of *Vibrio cholerae* isolates from 2007-2010 Kenyan cholera outbreaks (n=168)

					Susceptibility patterns				
Antimicrobial agent	Concentration	MIC_{50}	MIC_{90}	MIC range (µg/mL)	Susceptible n(%)	Resistant n(%)	Chi-square	df	P Value
AMX	0.016-256 µg/mL	3.661	11.756	0.75->256	58 (34.5)	110 (65.5)	16.095	1	<0.001
AMP	0.016-256 µg/mL	1.867	27.64	0.5->256	160 (95.2)	8 (4.8)	137.524	1	<0.001
CRO	0.016-256 µg/mL	<0.016	<0.016	0.016-2	167 (99.4)	1 (0.6)	164.024	1	<0.001
CHL	0.016-256 µg/mL	8.696	32.04	0.25-96	91 (54.2)	77 (45.8)	1.167	1	<0.001
CIP	0.002-32 µg/mL	0.029	0.60	0.002-1	168 (100)	0	NA	NA	NA
SXT	0.002-32 µg/mL	>32	>32	0.023->32	9 (5.4)	159 (94.6)	133.929	1	<0.001
DOX	0.016-256 µg/mL	0.606	17.69	0.064-32	163 (97)	5 (3)	148.595	1	<0.001
ERY	0.016-256 µg/mL	1.543	10.09	0.125-48	14 (8.3)	154 (91.7)	116.667	1	<0.001
GEN	0.016-256 µg/mL	0.500	2.73	0.125-8	168 (100)	0	NA	NA	NA
NAL	0.016-256 µg/mL	39.466	>256	0.125->256	32 (19)	136 (81)	64.381	1	<0.001
OFX	0.002-32 µg/mL	0.144	1.47	0.004-2	168 (100)	0	NA	NA	NA
STR	0.064-1024 µg/mL	116.000	598.19	0.75->1024	164 (97.6)	4 (2.4)	152.381	1	<0.001
TET	0.016-256 µg/mL	0.858	3.68	0.19-32	162 (96.4)	6 (3.6)	144.857	1	<0.001
O129	150 µg				26 (15.5)	142 (84.5)	80.095	1	<0.001

p value, by Chi-square test, for comparisons of susceptible vs. resistant isolates within each category; NA, not analysed
AMX, Amoxicillin; AMP, Ampicillin; CRO, Ceftriaxone; CHL, Chloramphenicol; CIP, Ciprofloxacin; SXT, Cotrimoxazole; DOX, Doxycycline; ERY, Erythromycin; GEN, Gentamycin; NAL, Nalidixic acid; OFX, Ofloxacin; STR, Streptomycin; TET, Tetracycline; O/129, Vibriostatic agent

Table 5: Comparison of antimicrobial resistance by location of isolation during the 2007-2010 Kenyan cholera outbreaks (n=168)

Antimicrobial agent	Bungoma (n=21)	Isiolo (n=20)	Kisumu (n=2)	Marsabit (n=3)	Nairobi (n=12)	Naivasha (n=5)	Pokot (n=14)	Suba (n=72)	Turkana (n=4)	Msambweni (n=15)	Chi-square	P-value
	R (%)	R (%)	R (%)	R (%)	R (%)	R (%)	R (%)	R (%)	R (%)	R (%)		
AMX	17 (81)	8 (40)	1 (50)	2 (66.7)	3 (25)	2 (40)	13 (92.9)	53 (73.6)	0	11 (73.3)	33.061	<0.001
AMP	0	0	0	0	2 (16.7)	0	0	6 (8.3)	0	0	9.975	0.329
CRO	0	1 (5)	0	0	0	0	0	0	0	0	7.444	0.446
CHL	18 (85.7)	16 (80)	2 (100)	1 (33.3)	1 (8.3)	4 (80)	9 (64.3)	22 (30.6)	3 (75)	1 (6.7)	53.887	<0.001
CIP	0	0	0	0	0	0	0	0	0	0	NA	NA
SXT	21 (100)	17 (85)	2 (100)	2 (66.7)	12 (100)	5 (100)	14 (100)	67 (93.1)	4 (100)	15 (100)	12.789	0.188
DOX	0	0	0	0	1 (8.3)	0	0	4 (5.6)	0	0	5.428	0.634
ERY	21 (100)	19 (95)	2 (100)	3 (100)	12 (100)	5 (100)	12 (85.7)	64 (88.9)	2 (50)	14 (93.3)	14.722	0.108
GEN	0	0	0	0	0	0	0	0	0	0	NA	NA
NAL	21 (100)	15 (75)	2 (100)	3 (100)	3 (25)	5 (100)	14 (100)	56 (77.8)	2 (50)	15 (100)	41.897	<0.001
OFX	0	0	0	0	0	0	0	0	0	0	NA	NA
STR	0	0	0	0	0	0	1 (7.1)	3 (4.2)	0	0	4.354	0.672
TET	0	1 (5)	0	0	1 (8.3)	0	0	4 (5.6)	0	0	4.102	0.811
O/129	20 (95.2)	14 (70)	2 (100)	2 (66.7)	10 (83.3)	5 (100)	12 (85.7)	60 (83.3)	4 (100)	13 (86.7)	6.943	0.581

p value, by Chi-square test, for comparisons of individual antibiotic resistance by location
AMX, Amoxicillin; AMP, Ampicillin; CRO, Ceftriaxone; CHL, Chloramphenicol; CIP, Ciprofloxacin; SXT, Cotrimoxazole; DOX, Doxycycline; ERY, Erythromycin; GEN, Gentamycin; NAL, Nalidixic acid; OFX, Ofloxacin; STR, Streptomycin; TET, Tetracycline; R, Resistant; NA, not analysed; *percentage calculated from isolates within the respective location

4.3.3.2 Antimicrobial resistance patterns

Table 6 describes the antimicrobial resistance patterns of the isolates. Notably, 45% of the isolates were resistant to three or more antimicrobials used for cholera treatment in Kenya, namely chloramphenicol, cotrimoxazole, erythromycin, or tetracycline ($\chi^2 = 564.952$, df =25, $p < 0.001$) (Table 7).

Table 6: Antimicrobial resistance patterns of *Vibrio cholerae* isolates from cholera outbreaks in Kenya, 2007-2010 (n=168)

Antimicrobial pattern	Frequency, n (%)	Chi-square	df	P value
AMX-CHL-ERY-NAL-SXT	46 (27.4)			
AMX-CHL-NAL-SXT	45 (26.8)			
ERY-NAL-SXT	16 (9.5)			
CHL-ERY-NAL-SXT	13 (7.7)			
ERY-SXT	7 (4.2)			
CHL-ERY-SXT	6 (3.6)			
AMX-ERY-SXT	5 (3)			
ERY	4 (2.4)			
AMP-AMX-ERY-SXT	3 (1.8)			
AMP-AMX-ERY-TET-SXT	2 (1.2)			
AMX-CHL-ERY-NAL-STR-SXT	2 (1.2)			
CHL-SXT	2 (1.2)			
ERY-NAL	2 (1.2)	564.952	25	<0.001
ERY-NAL-STR-SXT	2 (1.2)			
SXT	2 (1.2)			
AMP-AMX-CHL-ERY-NAL-TET	1 (0.6)			
AMP-AMX-ERY-NAL-TET-SXT	1 (0.6)			
AMP-ERY	1 (0.6)			
AMP-NAL	1 (0.6)			
AMP-NAL-SXT	1 (0.6)			
AMX-CHL-ERY-NAL-TET-SXT	1 (0.6)			
AMX-CHL-ERY-OFX-SXT	1 (0.6)			
AMX-CHL-ERY-TET-SXT	1 (0.6)			
CHL-CRO-ERY-NAL-SXT	1 (0.6)			
CHL-ERY-NAL	1 (0.6)			
SXT-NAL	1 (0.6)			

p value, by Chi-square test for analysis of antimicromial resistance patterns
AMX, Amoxicillin; AMP, Ampicillin; CRO, Ceftriaxone; CHL, Chloramphenicol; SXT, Cotrimoxazole; DOX, Doxycycline; ERY, Erythromycin; NAL, Nalidixic acid; STR, Streptomycin; TET, Tetracycline

Table 7: Antimicrobial susceptibility patterns of the isolates towards antimicrobials used in cholera management in Kenya (n=168)

Antimicrobial pattern	Frequency (%)
CHL-ERY-SXT	70 (41.7)
ERY-TET-SXT	3 (1.8)
CHL-ERY-TET-SXT	2 (1.2)
CHL-ERY-TET	1 (0.6)
	76 (45%)

CHL, Chloramphenicol; SXT, Cotrimoxazole; ERY, Erythromycin; TET, Tetracycline

4.3.3.3 Susceptibility to O/129 *Vibriostatic* agent

The evaluation of susceptibility towards the O/129 *Vibriostatic* agent (Table 4) showed that majority (84.5%) (142 out of 168) of the *Vibrio cholerae* isolates were resistant to this agent at a concentration of 150µg with only 26 (15.5%) being sensitive (χ^2 = 80.095; df = 1; $p < 0.001$). Figure 4 shows a representative image of susceptibility of *Vibrio cholerae* against O/129 *Vibriostatic* agent.

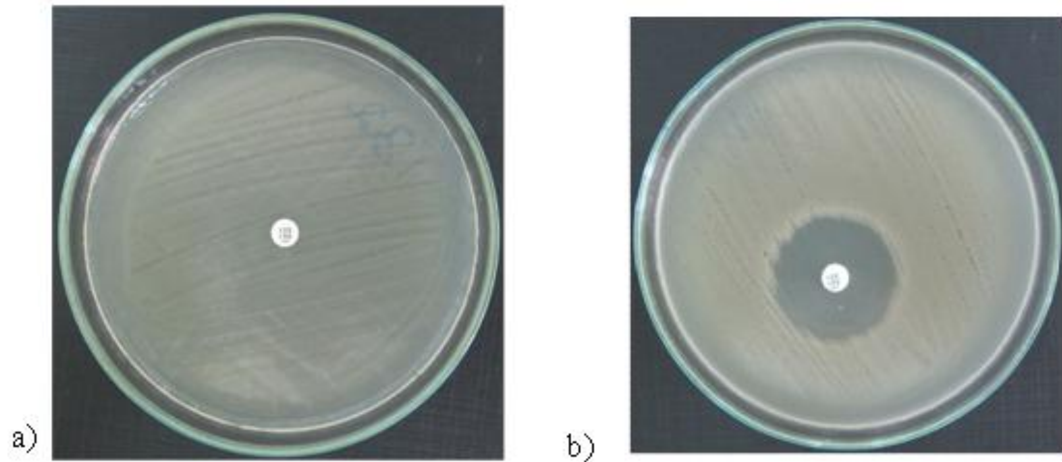

Figure 4: Representative image of susceptibility of *Vibrio cholerae* against O/129 *Vibriostatic* agent.

Representative images of resistant (a) and sensitive (b) *Vibrio cholerae* O1 isolates towards the O/129 *Vibriostatic* agent analysed in this study

4.3.4 *Vibrio cholerae* O1 virulence genes

One hundred and thirty-two (82.1%) isolates possessed the cholera toxin gene (*ctxA*) whereas 13 (17.9%) did not ($\chi^2 = 69.429$; df = 1; $p < 0.001$). Analysis of the *toxR* gene revealed that 166 (98.8%) harbored the *toxR* gene and only 2 (1.2%) did not ($\chi^2 = 160.095$; df = 1; $p < 0.001$). Table 8 describes the PCR results in regard to detection of pathogenic and antimicrobial resistance genes.

Table 8: Analysis of pathogenic and antimicrobial resistance genes by Polymerase Chain Reaction in *Vibrio cholerae* isolates from cholera outbreaks in Kenya, 2007-2010 (n=168)

Primer	Target gene	Positive n (%)	Negative n (%)	Chi-square	df	P Value
ctxA	cholera toxin	138 (82.1)	30 (17.9)	69.429	1	<0.001
toxR	regulatory gene	166 (98.8)	2 (1.2)	160.095	1	<0.001
inDS	class one intergron	149 (88.7)	19 (11.3)	100.595	1	<0.001
int	SXT intergrase	157 (93.5)	11 (6.5)	126.881	1	<0.001
strA	streptomycin resistance	146 (86.9)	22 (13.1)	91.524	1	<0.001
sul2	sulfamethoxazole resistance	156 (92.9)	12 (7.1)	123.429	1	<0.001
tetA	tetracycline resistance	3 (1.8)	165 (98.2)	156.214	1	<0.001

p value, by Chi-square test, for comparisons of presence and absence of target genes within each category; df, degree of freedom

4.3.5 Genes associated with antimicrobial resistance in *Vibrio cholerae*

The molecular characteristics of the isolates are described in Table 8. Analysis of these traits revealed that 149 (88.7%) of the isolates harbored the class I integron (encoded by *inDS* gene) while 19 (11.3%) did not ($\chi^2 = 100.595$; df = 1; $p < 0.001$). Majority, 157 (93.5%) were confirmed to possess the SXT integrating conjugative element (ICE) while 12 (7.1%) did not ($\chi^2 = 126.881$; df = 1; $p < 0.001$). The streptomycin resistance gene was present in 146 (86.9%) of the

isolates (χ^2 = 91.524; df = 1; p < 0.001), the sulfamethoxazole resistance gene in 156 (92.9%) (χ^2 = 123.429; df = 1; p < 0.001), and the tetracycline resistance gene in only 3 (1.8%) of the isolates (χ^2 = 156.214; df = 1; p < 0.001).

The association between the class I integron gene and antimicrobial resistance was analysed by the Chi-square test (Table 9). Significant associations between the class I integron gene and antimicrobial resistance were observed in amoxicillin (χ^2 = 80.327; df = 1; p < 0.001), chloramphenicol (χ^2 = 48.325; df = 1; p < 0.001), erythromycin (χ^2 = 90.416; df = 1; p < 0.001), nalidixic acid (χ^2 = 88.971; df = 1; p < 0.001), and O129 *Vibriostatic* agent (χ^2 = 94.761; df = 1; p < 0.001).

Table 9: Association between the class I integron gene and antimicrobial resistance (n=168)

Antimicrobial agent	Resistant isolates n(%)*	Class I intergron†		Chi-square	df	P value
		positive n(%)	negative n(%)			
Amoxicillin (AMX)	110 (65.5)	102 (92.7)	8 (7.3)	80.327	1	<0.001
Ampicillin (AMP)	8 (4.8)	8 (100)	0	NA	NA	NA
Ceftriaxone (CRO)	1 (0.6)	1 (100)	0	NA	NA	NA
Chloramphenicol (CHL)	77 (45.8)	69 (89.6)	8 (10.4)	48.325	1	<0.001
Ciprofloxacin (CIP)	0	0	0	NA	NA	NA
Cotrimoxazole (SXT)	159 (94.6)	141 (88.7)	18 (11.3)	95.151	1	<0.001
Doxycycline (DOX)	5 (3)	5 (100)	0	NA	NA	NA
Erythromycin (ERY)	154 (91.7)	136 (88.3)	18 (11.7)	90.416	1	<0.001
Gentamycin (GEN)	0	0	0	NA	NA	NA
Nalidixic acid (NAL)	136 (81)	123 (90.4)	13 (9.6)	88.971	1	<0.001
Ofloxacin (OFX)	0	0	0	NA	NA	NA
Streptomycin (STR)	4 (2.4)	4 (100)	0	NA	NA	NA
Tetracycline (TET)	6 (3.6)	6 (100)	0	NA	NA	NA
O129 vibriostatic agent	142 (84.5)	129 (90.8)	13 (9.2)	94.761	1	<0.001

p value, by Chi-square test, for comparisons of resistant isolates vs. presence of class I intergron gene.
ND, not done
*n=168, total isolates
†n within resistant isolates

Table 10 highlights the association of antimicrobial resistance to their respective antimicrobial resistance genes. Isolates that harbored the SXT integrating conjugative element (ICE) were screened for the presence of genes encoding for resistance to cotrimoxazole (trimethoprim/sulfamethoxazole). Of the 159 isolates resistant to cotrimoxazole, 148 (93.1%) harbored the SXT integrase ($\chi^2 = 118.044$; df = 1; $p < 0.001$). Of the 159 isolates resistant to cotrimoxazole, 147 (92.5%) harbored the sulfamethoxazole resistance gene ($\chi^2 = 114.623$; df = 1; $p < 0.001$). All isolates that were resistant to tetracycline harbored the tetracycline resistant gene.

Table 10: Association of antimicrobial resistance to their respective antimicrobial resistance genes (n=168)

Antimicrobial agent	Resistant isolates n*	Antimicrobial resistance gene†		Chi-square	df	P value
		SXT intergrase				
		positive, n(%)	negative, n(%)			
Cotrimoxazole (SXT)	159	148 (93.1)	11 (6.9)	118.044	1	<0.001
		Streptomycin resistance gene				
		positive, n(%)	negative, n(%)			
Streptomycin (STR)	4	4 (100)	0	114.623	1	<0.001
		Sulfamethoxazole resistance gene				
		positive, n(%)	negative, n(%)			
Cotrimoxazole (SXT)	159	147 (92.5)	12 (7.5)	114.623	1	<0.001
		Tetracycline resistance gene				
		positive, n(%)	negative, n(%)			
Tetracycline (TET)	6	6 (100)	0	NA	NA	NA

p value, by Chi-square test, for comparisons of resistant isolates vs. presence of class I intergron gene.
ND, not done
*total isolates=168
†n, within resistant isolates

4.3.6 Genetic diversity of the *Vibrio cholerae* isolates

Following digestion with *NotI* restriction enzyme, a total of 119 isolates that had identifiable PFGE restriction patterns were used to generate a phylogenetic tree using the Darwin software. Two major clusters of the *Vibrio cholerae* isolates were identified based on the location of isolation. The first cluster was a mono-cluster having 45 isolates obtained from different locations. Most of the isolates from the Philippines were clustered in this branch. The second cluster was highly divergent forming other minor clusters. The isolates restriction patterns were highly diverse even when considering those from the same location. In addition, some of the isolates from different locations formed mono-clusters at different levels (Figure 5).

Similar patterns were observed when analysed by the year of isolation. Two major clusters of the isolates were identified. The first cluster was a mono-cluster having isolates obtained in different years while the second was highly diverse forming other minor clusters of isolates collected in different years (Figure 6).

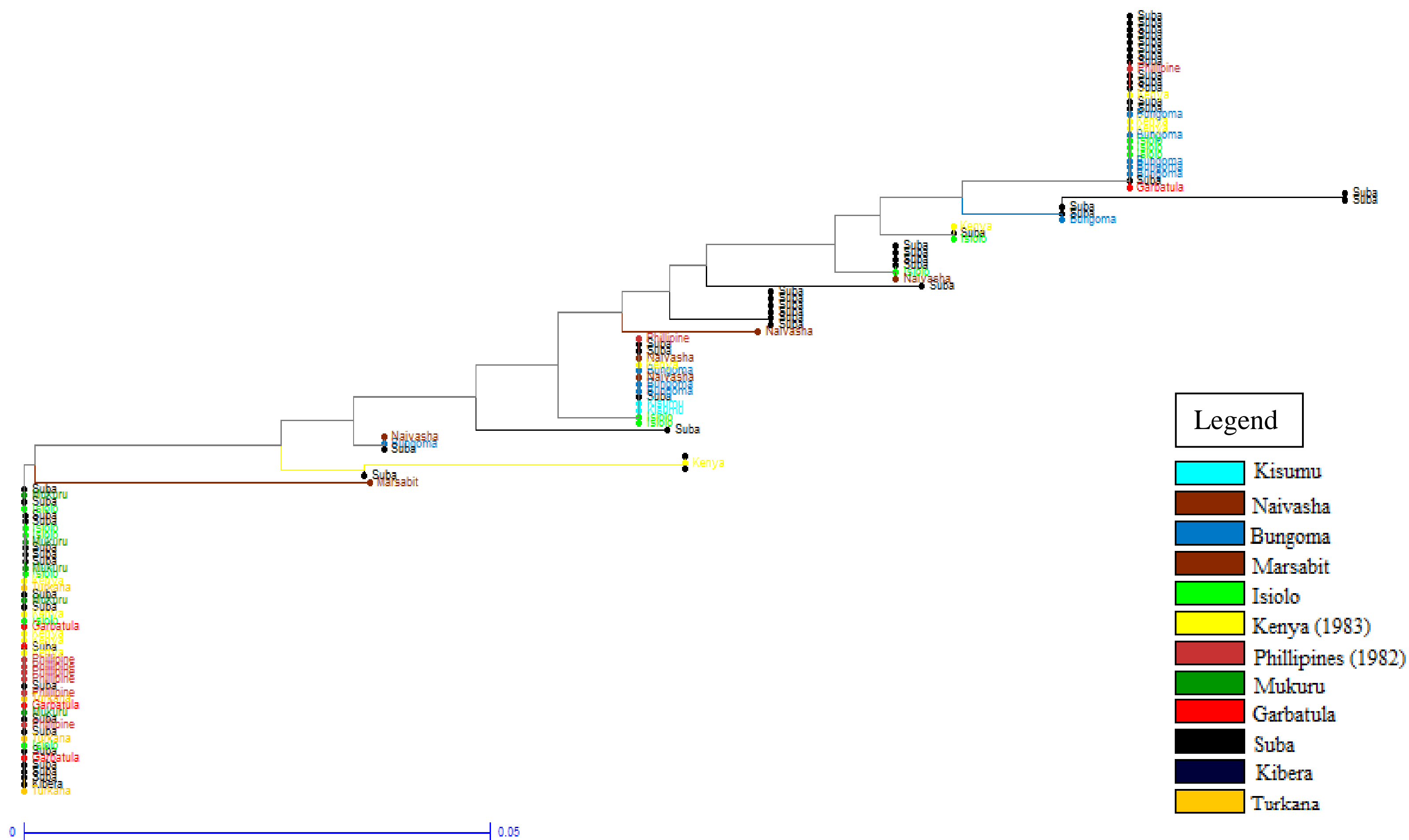

Figure 5: The Neighbor joining tree generated by 500 bootstraps showing the phylogenetic relationship of the Vibrio cholerae isolates based on the location of isolation

Figure 6: The Neighbor joining tree generated by 500 bootstraps showing the phylogenetic relationship of the Vibrio cholerae isolates based on the year of isolation

CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 Discussion

In this study, *Vibrio cholerae* isolates from cholera outbreaks from 2007 to 2010 were characterized phenotypically and by molecular means. Phenotypic characterization included serology and antimicrobial susceptibility testing. Molecular characterization entailed detection of pathogenic genes and antimicrobial resistance genes by polymerase chain reaction (PCR), and genetic diversity by pulsed-field gel electrophoresis (PFGE).

Serological characterization revealed that majority of the isolates belonged to serotype Inaba. This finding collaborates to a previous study done on *Vibrio cholerae* O1 strains isolated in Kenya between 1994 and 2007 where serotype Inaba emerged as the main cause of the epidemics (Kiiru et al., 2009). Isolates belonging to serotype Ogawa were frequently isolated from cholera outbreaks in Kenya in the 1980s (Iwanaga et al., 1982; Ichinose et al., 1986).

Data from the Kenya Metrological Department revealed that there were two rainfall periods for each year; the long rains occurring between March and May, and the short rains occurring between October and December. There was a clear trend between *Vibrio cholerae* isolation and the rainfall season. Peaks in isolation occurred during the first month of the rainy season for all years except in 2008 where peaks were observed in the months of June and

September. Water could therefore have played an important role in cholera transmission. Being a water borne infection, cholera is transmitted by ingesting food or water contaminated with the bacterium (WHO, 2011). If infected fecal material is dispossed into the environment, rainfall may be important in washing such material and contaminating water bodies like lakes or uncovered boreholes. Results from this study collaborate the findings from a study performed in the great lakes region of Africa between 1978 and 2008 (Nkoko et al., 2011) and another study performed in Zambia (Luque et al., 2009). They reported that the risk of cholera epidemics increased in the beginning of the rainy season and was preceeded six weeks earlier by a period of warm temperature.

In 2008, most of the cases were from Suba region, which is endemic to cholera infections. The occurrence of oubreaks in the region even in the dry season may be attributed to its location next to lake Victoria. A study done in rural western Kenya between 1997 and 1998 reported that cholera was more common among persons living in villages bordering Lake Victoria (Shapiro et al., 1999). They identified drinking water from the lake as an important risk factor for cholera infection.

A high percentage (44.64%) of the isolates were resistant to three or more of the following antimicrobials; chloramphenicol, ciprofloxacin, cotrimoxazole (trimethoprim/sulfamethoxazole), erythromycin and tetracycline. In Kenya, doxycycline and tetracycline are the first-line drugs used for cholera treatment

in adults. Erythromycin and chloramphenicol are used for treatment in children and pregnant women (MoH, 2002). A high resistance (91.7%) towards erythromycin was observed in this study. These findings suggest that erythromycin should not be used for treatment of cholera infections caused by current *Vibrio cholerae* O1 strains.

Tetracycline has been used extensively in this country for many years for cholera management. A previous study done in Kenya in 1982 to determine antimicrobial response of *Vibrio cholerae* isolates reported that all isolates were susceptible to tetracycline (Iwanaga et al., 1982). However, emergence of isolates resistant to tetracycline was reported the following year and was attributed to its prophylactic use in patients and their close contacts irrespective of whether or not the individual was infected (Ichinose et al., 1986). In this study, although there was resistance to tetracycline, it was only in 4.3% of the isolates suggesting that the antibiotic is effective in managing cholera in Kenya. However, close attention should be paid to the emergence of these resistant strains. In other countries, cholera epidemics caused by tetracycline resistant *Vibrio cholerae* have also been reported. A study on antimicrobial resistance of *Vibrio cholerae* O1 isolates done in Mozambique in 2007 reported high incidences of resistance to tetracycline (97.3%) which was used as a first-line drug for cholera treatment (Mandomando et al., 2007). Similar epidemics have also been reported in Bangladesh (Glass et al., 1980) and Tanzania (Towner et al., 1980).

Tetracycline is one of the oldest and extensively used antibiotics in sectors other than health. It was discovered in 1940s, and is relatively cheap. It has been extensively used in the prophylaxis and therapy of human and animal infections (Chopra and Roberts, 2001). In Kenya it is the leading antibiotic used in food producing animals and is often supplemented into animal feeds (Saidi, 2004). In a study done in Kenya in 2001 to assess the antimicrobial consumption in food producing animals, tetracycline was reported to contribute to approximately fifty five percent of the total consumption of antibiotics in animals (Mitema et al., 2001).

A high incidence of resistance to cotrimoxazole (trimethoprim-sulfamethoxazole) (94.6%) was observed in this study. This finding is in agreement with previous studies in Kenya (Kiiru et al., 2009) and Mozambique (Folgosa et al., 2001; Mandomando et al., 2007). Resistance to this agent among other *Enterobacteriaceae* has also been reported (Leverstein-van Hall et al., 2003). This may reflect the wide spread use of the antibiotic in Kenya. Cotrimoxazole is a first-line treatment antibiotic for diarrhea infections in Kenya. Its brand name is Septra or Septrin (GSK). It is prescribed for the treatment of respiratory tract infections, urinary tract infections, gastrointestinal tract infections including diarrhea, and skin infections. It is often prescribed to immunocompromised patients like those suffering from HIV (Chintu et al., 2004). Such vast usage of the antibiotic and the fact that it is relatively cheap and can be acquired over the counter even without a

prescription may have contributed to the emergence of resistance towards cotrimoxazole observed in this study.

A high incidence of chloramphenicol-resistant isolates (45.8%) was also observed. Chloramphenicol is a bacteriostatic and broad-spectrumed antibiotic. It is effective against a wide variety of Gram-positive and Gram-negative bacteria. Up to the late 1990s it was used as a first line antibiotic of choice for treatment of typhoid and other *Salmonella* infections (Hart and Kariuki, 1998). However due to resistance and safety concerns, it is no longer a first line treatment in enteritis. In low income countries, it is still widely used since it is inexpensive and readily available (Falagas et al., 2008). Chloramphenicol is recommended by the WHO for treatment of cholera in children and pregnant women. The high resistance observed may be explained by its frequent usage for the treatment of severe diarrhea and other infectious diseases. Other reports also found *Vibrio cholerae* O1 isolates resistant to chloramphenicol (Kiiru et al., 2009), and a similar incidence was observed in other pathogens causing diarrhea in a previous study conducted in the area (Oundo et al., 2000).

Resistance to β-lactam antibiotic was observed. A higher resistance rate to amoxicillin (65.5%) was observed compared to that towards Ampicillin (4.8%). Isolates from previous outbreaks in Kenya were known to exhibit resistance to Ampicillin (Pugliese et al., 2009), doxycycline and streptomycin (Scrascia et al., 2006). Kariuki et al. (1997) reported higher resistance rates in

Escherichia coli isolated from children in Kenya to amoxicillin (74%) compared to augmentin (amoxicillin + clauvalinic acid) (22%).

A majority (99.4%) of the isolates were sensitive to ceftriaxone which was in agreement with a study aimed at characterizing *Vibrio cholerae* O1 isolates from Kenyan cholera outbreaks in the period 1994 to 2007. In that study, all isolates were sensitive towards furazolidone, ceftriaxone, cefotaxime, nalidixic acid, amikacin and gentamicin (Kiiru et al., 2009). There was an emergence of isolates resistant to nalidixic acid, with 81% being resistant to the antimicrobial. All isolates were sensitive towards ciprofloxacin, gentamicin and ofloxacin confirming the higher efficiency of these agents against Kenyan *Vibrio cholerae* isolates. Ciprofloxacin and ofloxacin are second generation fluoroquinolones used in the treatment of gastroenteritis with severe diarrhea. Ciprofloxacin has previously been found to be the most superior in the treatment of *Vibrio cholerae* infection (Oliphant and Green, 2002).

Surprisingly, 44.64% of the isolates were resistant to three or more of the antimicrobials recommended for cholera treatment. Genetic mechanisms responsible for resistance were identified. Antimicrobial drug resistance in *Vibrio* species may arise through mutation or through acquisition of resistance genes on mobile genetic elements like plasmids, transposons integrons, and integrating conjugative elements (Sjölund-Karlsson et al., 2011). Isolates analysed in this study possessed the class I integron and the SXT integrating conjugative element. Genetic elements like the class I integron (*inDS*) and the

integrating conjugative elements such as SXT have been associated with the spread of genetic determinants, encoding for antimicrobial resistance in *Vibrio cholerae* (Dalsgaard et al., 2001). The SXT element has been reported to harbor genes encoding for resistance to chloramphenicol (encoded by *floR*), streptomycin (encoded by *strA and strB*), trimethoprim (encoded by *dfrA18*) and sulfamethoxazole (encoded by *sul2*) (Beaber et al., 2002). The class 1 integron has been reported to harbor aminoglycoside resistant gene cassettes in *Vibrio cholerae* O1 isolates (Dalsgaard et al., 1999). Resistance to erythromycin in the analysed isolates could be attributed to the presence of the class 1 integron gene.

The finding of susceptible isolates towards streptomycin but still amplifying a 383 bp fragment of the *strA* gene suggests that this gene is not an intrinsic feature of this family of integrase, but rather appears to have been inserted into these elements, becoming transmissible in bacterial populations, as reported by other investigators (Hochhut et al., 2001). Nalidixic acid resistance observed in this study could be attributed to mutations in the *gyrA* gene. Many investigators have reported *gyrA* gene mutations in fluoroquinolone resistant clinical isolates of *Vibrio cholerae* (Baranwal et al., 2002). However, more study needs to be done to confirm this.

There was a direct relationship between resistance towards the O/129 *Vibriostatic* agent and cotrimoxazole. The results obtained in this study showed that *Vibrio cholerae* O1 El Tor isolates from Kenya were resistant to

both of these agents and were positive by PCR for the *int* gene, indicating that the genes encoding resistance to cotrimoxazole and O/129 are linked, a finding further supported by the fact that *Vibrio cholerae* O1 strains isolated in Kenya between 1994 and 2007 possessed the integrating conjugative element (SXT integron) (Kiiru et al., 2009). Other researchers have also reported that resistance to O/129 *Vibriostatic* agent is invariably linked with resistance to cotrimoxazole (Ramamurthy et al., 1992). However, further studies are needed to confirm the presence of cotrimoxazole and 0/129 *Vibriostatic* agent resistance genes in the SXT integron. The occurrence of isolates resistant towards the O/129 *Vibriostatic* agent suggests the need to stop using the agent as a taxonomic tool for *Vibrio cholerae* classification (Ramamurthy et al., 1992).

Pulsed-field gel electrophoresis (PFGE) restriction patterns revealed that the isolates analysed in this study existed in two clusters with one having monoclusters suggesting that the outbreaks may have been caused by different *Vibrio cholerae* strains rather than a single strain. The outbreaks could therefore have originated from multiple sources and probably transmitted from more than one source of contamination. Comparison of the current isolates to those isolated previously in Kenya and the Philippines revealed that some had a similar restriction pattern. This suggests that the current isolates might have originated from Africa and southern Asia. The primary PFGE patterns continue to diverge as the infections continue and researchers have associated this to

complex interactions between the host immune system, environment and the epidemic populations of bacteria (Talkington et al., 2011).

Pulsed-field gel electrophoresis is therefore a key epidemiological tool for analysis of cholera outbreaks and other bacterial related outbreaks as reported by other researchers (Cameron et al., 1994).

5.2 Conclusion and recommendations

5.2.1 Conclusion

Biochemical characterization confirmed all isolates as *Vibrio cholerae*. Serological characterization revealed that *Vibrio cholerae* O1 serotype Inaba was predominant and occurred in different years and regions. All but one of *Vibrio cholerae* O1 serotype Ogawa occurred in the coastal region and in the year 2010.

The isolates displayed reduced susceptibity towards ceftriaxone, chloramphenicol, doxycycline and streptomycin. Majority were resistant to amoxicillin, cotrimoxazole, erythromycin and nalidixic acid. Notably, 44.6% of the isolates were resistant to three or more of the antimicrobials recommended for cholera treatment. All were susceptible to ciprofloxacin, gentamicin and ofloxacin. Majority of the isolates were also resistant to O/129 *Vibriostatic* agent that is used to differentiate *Vibrio* species from closely related organisms.

PCR results for detection of virulence genes revealed that the isolates were virulent harboring the cholera toxin (*ctxA*) and or the cholera toxin regulatory gene, *toxR*.

PCR screening for genetic determinants of antimicrobial resistance revealed that most of the resistance was caused by acquired genes. These genes were located in the SXT integrating conjugative element and the class one intergron. Pulsed-field gel electrophoresis results strongly suggest that the *Vibrio cholerae* strains circulating within Kenya were different with some being similar to those previously isolated in Kenya and the Philippines.

5.2.2 Recommendations

- To limit the development and spread of antimicrobial resistance among *Vibrio cholerae*, more care should be taken when prescribing antimicrobials. Such prescription should be restricted to severe cases and those truly warranting their use. Oral rehydration solution (ORS) usage for diarrhea management should be encouraged.

- The finding that most isolates were resistant towards the O/129 vibriostatic agent suggests that the agent should not be used to characterize *Vibrio cholerae* isolates from Kenya.

- The findings that a majority of the isolates were resistant towards cotrimoxazole and erythromycin suggest that these antimicrobials should not be used to treat cholera caused by current *Vibrio cholerae* O1 strains in Kenya. Surveillance for antimicrobial drug resistance should continue among *Vibrio cholerae* isolates from Kenya.

- Whole genome sequencing of the isolates should be performed to better understand the genetic mechanisms of resistance in the Kenyan *Vibrio cholerae* isolates.

- This study recommends the use of PFGE analysis for identifying the kinds of *Vibrio cholerae* clones circulating within various geographic regions, thereby providing useful genetic ancestry information for interpreting cholera outbreaks.

- Large-scale epidemiologic studies are recommended to identify the geographic source and mode of entry of outbreak strains in Kenya.

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APPENDICES

Appendix 1: Preparation of culture media

i) **Preparation of Thiosulfate Citrate Bile Salts Sucrose Agar (TCBS)**

Dissolve 86g of the TCBS powder (Eiken chemical Co., LTD, Tokyo, Japan) in 1 liter of distilled water and heat to dissolve for 5-10 minutes. Cool the media to 50-55˚C and dispense aseptically.

ii) **Preparation of Bromo Thymol Blue Lactose Agar (BTB)**

Dissolve 40g of the BTB powder (Becton, Dickinson and Company, Sparks, MD 21152 USA) in 1 liter of distilled water and heat to dissolve for 5-10 minutes. Cool the media to 50-55˚C and dispense aseptically.

iii) **Preparation of Nutrient Agar (NA)**

Dissolve 28g of the NA powder (Liofilchem s.r.l. Bacteriology Products, Italy) in 1 liter of distilled water and heat to dissolve for 5-10 minutes. Cool the media to 50-55˚C and dispense aseptically.

iv) **Preparation of Mueller Hinton Agar (MH)**

Dissolve 38g of the MH powder (Liofilchem s.r.l. Bacteriology Products, Italy) in 1 liter of distilled water and heat to dissolve for 5-10 minutes. Cool the media to 50-55˚C and dispense aseptically.

Appendix 2: Biochemical reactions used in identifying enterobacteria

i) **O-Nitrophenyl-B-D-galactosidase (ONPD)**

The substrate Ortho-Nitro-phenyl galactose (ONPG) is hydrolyzed by beta-galactosidase to release yellow ortho-nitro phenol from the colourless ONPG solution. Yellow color is positive for the test.

ii) **Arginine dihydrolase (ADH)**

Bacteria with the enzymes arginine dehydrolase transform arginine into ornithine, ammonia and carbon dioxide. An increase in PH changes the indicator phenol red from yellow to red.

iii) **Lysine decarboxylase (LDC)**

The enzyme metabolises lysine into a basic primary amine cadaverine. The amine causes an alkaline condition changing the phenol red indicator from yellow to red.

iv) **Ornithine decarboxylase (ODC)**

Ornithine is decarboxylated by the enzyme resulting in the production of putrescine, a basic amine which causes a pH rise in the buffered broth. This in turn changes the pH of phenol red from yellow to red.

v) **Citrate utilization (CIT)**

Bacteria are provided with citrate as a sole source of carbon. Utilization of citrate results in a pH rise and the indicator bromophenol blue changes from green to blue.

vi) **Hydrogen sulfide production (H_2S)**

Bacteria which produce hydrogen sulfide reduce thiosulfate in the media. The hydrogen sulfide reacts with ferric chloride to produce ferrous sulfide, which is observed as a black precipitate.

vii) **Indole production (IND)**

Metabolism of troptophane results in the production of Indole. Kovac's reagent reacts with Indole to give a pink or red coloured complex.

viii) **Urease test (URE)**

Urea is broken down by bacteria which produce Urease. The end products are ammonia and carbon dioxide. Ammonia causes the pH to rise, which causes the phenol red to change from yellow to red.

ix) **Tyrptophan deaminase (TDA)**

Bacteria with tryptophane transform tryptophane to indolepyruvic acid. The acid is detected by the presence of a dark brown color on adding ferric chloride.

x) **Voges Proskeur test (VP)**

Some bacteria produce acetoin following metabolism of glucose or pyruvate. Acetoin is detected by adding sodium hydroxide and a α-napthole forming a pink complex.

xi) **Gelatin liquefaction (GEL)**

Bacteria producing the proteolytic enzyme, gelatinase are able to break down gelatin releasing visible black pigments which diffuse in the media.

xii) **Carbohydrate fermentation**

Utilization of the carbohydrate results in acid production and a subsequent drop in pH. The indicator bromothymol blue changes from blue to yellow.

Appendix 3: Biochemical characteristics of typical *Vibrio cholerae* O1 strains

Test	% positive
Oxidase	100
String test	100
Kligler's iron agar	K/A, no gas, no H_2S
Triple sugar iron agar	A/A, no gas, no H_2S
Glucose[a] (acid production)	100
Glucose (gas production)	0
Sucrose (acid production)	100
Lysine[a]	99
Arginine[a]	0
Ornithine[a]	99
Growth in O% NaCl[b]	100
Growth in 1% NaCl[b]	100
Voges-Proskauer[a]	75[c]

[a] Modified by the addition of 1% NaCl.
[b] Nutrient broth base (Difco Laboratories)
[c] Most isolates of *Vibrio cholerae* serotype O1 biotype El Tor are positive in the VP test, whereas biotype classical strains are negative.

Appendix 4: Interpretative criteria for antimicrobials towards *Vibrio cholerae*

Antimicrobial agent	Strip antibiotic concentration (µg/ml)	S≤	I	R≥	*E. coli* 25922 MIC (µg/ml)
Amoxicillin	0.016-256	≤2	4	≥8	
Ampicillin	0.016-256	8	16	32	2-8
Ceftriaxone	0.016-256	≤1	2	≥4	0.032-0.125
Ciprofloxacin	0.002-32	≤1	2	≥4	
Chloramphenicol	0.016-256	8	16	32	
Doxycycline	0.016-256	≤4	8	≥16	0.5-2
Gentamycin	0.016-256	≤4	8	≥16	0.25-1
Nalidixic acid	0.016-256	≤8	16	≥32	1-4
Ofloxacin	0.002-32	≤2	4	≥8	0.016-0.125
Streptomycin	0.064-1024	≤2000		≥2000	2-8
Tetracycline	0.016-256	≤4	8	≥16	0.5-2
Cotrimoxazole	0.002-32	≤2		≥4	0.064-0.25
Erythromycin	0.016-256	≤0.5	1-4	≥8	

S-Sensitive, I-Intermediate, R-Resistant, MIC-Minimum Inhibitory Concentration

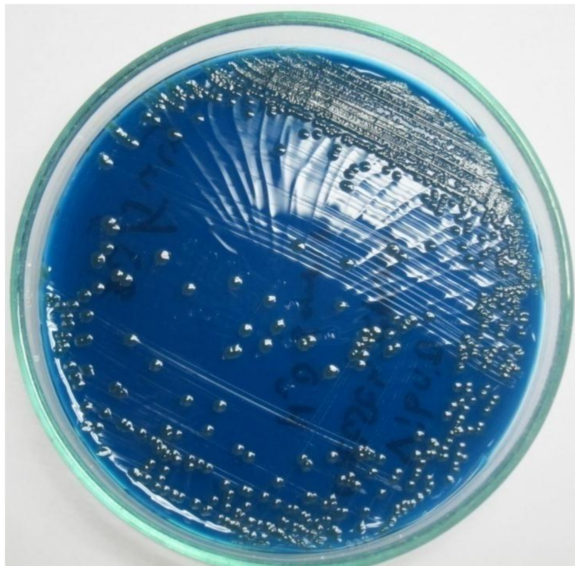
Source: CLSI guidelines on performance standards for antimicrobial sensitivity testing (CLSI, 2010).

Appendix 5: Oligonucleotide primers, sequences, amplicons and conditions used in PCR assays in this study

Primer, genes, and Sequence (5'→3')	Amplicon size (bp)	PCR conditions			Reference
		Melting	Annealing	Extension	
inDS **,** Class 1 integron F: CGGAATGGCCGAGATC R: CAAGGTTCTGGACCAGTTGCG	380	95˚C, 1min	58˚C, 1min	72˚C, 1min	Dalsgaard *et al* . (1999)
int, SXT element F: GCTGGATAGGTTAAGGGCGG R: CTCTATGGGCACTGTCCACATTG	592	95˚C, 1min	54˚C, 1min	72˚C, 1min	Hochhut et al. (2001)
StrA, Streptomycin resistance F: TTGATGTGGTGTCCCGCAATG R: CCAATCGCAGATAGAAGGCAA	383	95˚C, 1min	54˚C, 1min	72˚C, 1min	Hochhut et al. (2001)
Sul2, Sulfamethoxazole resistance F: AGGGGGCAGATGTGATCGAC R: TGTGCGGATGAAGTCAGCTCC	625	95˚C, 1min	54˚C, 1min	72˚C, 1min	Hochhut et al. (2001)
TetA-2000 **,** tetracycline resistance F: GTAATTCTGAGCACTGTCGC R: CTGCCTGGACAACATTGCTT	950	95˚C, 1min	58˚C, 1min	72˚C, 1min	Yamai et al. (1997)
ctxA, CT Subunit A F: TCAATTAGTTTGAGAAGTGC R: TCAGATTGATAGCCTGAAAA	564	95˚C, 1min	54˚C, 1min	72˚C, 1min	Unpublished
toxR, toxR operon F: TTAACGCTGAATTACATTCA R: TTAAGATTTACTGAACAGTA	739	95˚C, 1min	54˚C, 1min	72˚C, 1min	Unpublished

Appendix 6: Images of *Vibrio cholerae* culture

a) *Escherichia coli* ATCC 25922 on deoxycholate lactose (DHL) agar medium



b) *Vibrio cholerae* O1 on bromothymol blue lactose (BTB) agar medium