Phenotypic and molecular characterization of *Vibrio cholerae* isolated from cholera outbreaks in western Kenya

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other university

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This thesis has been submitted for examination with our approval as University Supervisors

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DEDICATION

This research work is dedicated to my whole family who gave me enormous support throughout the entire period of study. Let the Almighty God bless them abundantly.

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LIST OF ABBREVIATIONS

AC	Amoxicillin
ace	accessory cholera enterotoxin
ADP	Adenosine diphosphate
AFLP	Amplification fragment length polymorphism
AM	Ampicillin
ATCC	American type culture collection
bp	base pair
BTB	Bromothymol blue
cAMP	cyclic adenosine monophosphate
CHEF	Contour-Clamped Homogeneous Electric Fields
CI	Ciprofloxacin
CL	Chloramphenicol
CT	Cholera toxin
DC	Doxycycline
DNA	Deoxyribonucleic acid
EHEC	Enterohemorrhagic *Escherichia coli*
EM	Erythromycin
FIGE	Field-Inversion Gel Electrophoresis
Fig	Figure
GM1	Ganglioside

GM	Gentamycin
GTP	Guanosine triphosphste
IBR	Institute for Biotechnology Research
IgA	Immunoglobulin A
IgG	Immunoglobulin G
JKUAT	Jomo Kenyatta University of Agriculture and Technology
kb	Kilobase
KEMRI	Kenya Medical Research Institute
KM	Kanamycin
LAMP	Loop-mediated isothermal amplification
LPS	Lipopolysaccharide
LT	Heat labile toxin of *Escherichia coli*
mb	mega base pairs
MIC	Minimum Inhibitory Concentrations
ml	milliliter
mm	millimeter
mRNA	messenger ribonucleic acid
NAD	Nicotinamide adenine diphosphate
NA	Nalidixic acid
NUITM	Nagasaki University Institute of Tropical Medicine
OFAGE	Orthogonal-Field Alternation Gel Electrophoresis
OF	Ofloxacin
PACE	Programmable Autonomously-Controlled Electrodes

PCR	polymerase chain reaction
PFGE	Pulsed field gel electrophoresis
PMSR	Phenylmethylsulfonyl
Prof	Professor
RITTARD	removable ileal loop tied adult rabbit diarrhea test
RGE	Rotating Gel Electrophoresis
RPLA	Reverse passive latex agglutination
SDS-PAGE	sodium dodecyl sulphate polyacralymide gel electrophoresis
SM	Streptomycin
Spp	Species
TAFE	Transverse-Alternating Field Gel Electrophoresis
TCBS	Thiosulphate citrate bile salt
TC	Tetracycline
ToxR	transmembrane protein R
TS	Trimethoprim/Sulphamethaxazol
TX	Ceftriaxone
VC	*Vibrio cholerae*
VP	Voges Proskauer Test
Zot	Zonula occludans toxin

ABSTRACT

Cholera disease is a major public health problem in Kenya and is caused by toxigenic *Vibrio cholerae*. *V. cholerae* are curved or straight Gram-negative bacilli. Epidemic cholera occurs in areas with water contamination and poor sanitation. This research investigated strains of *Vibrio cholerae* isolated from western Kenya during the 2007 to 2009 outbreaks. Stocked isolates were sub-cultured onto BTB agar and 24hr-colonies were used to perform specific biochemical tests on the isolates to determine their biotypes, serotypes and sub- serotypes. Presence of cholera toxin (*ctx*) and antibiotic resistance genes in the genome of the isolates was detected by amplification using PCR. Pulsed field gel electrophoresis (PFGE) was done to determine the clonal relatedness of the strains. The collected data were coded and analysed using statistical package for social sciences (SPSS) version 11 to determine chi square distributions for qualitative data. All the isolates were confirmed to be *V. cholerae* O1 El Tor biotype. Majority (97%) of them were Inaba serotype while 3% were Ogawa serotype and none was Hikojima serotype. PCR assays showed that 97% of the isolates were toxigenic and contained the *ctx* gene. The isolates showed varied antibiotic resistance patterns while the presence of resistant genes to commonly used antibiotics was not uniform but random in their genomes. Analyses of the PFGE bands to infer population relationships using the unweighted Neighbor-joining method viewed using the program DARwin software did not show any significant genetic diversity. Further

investigation on the Western Kenya isolates using the *in-vivo* as well as *in-vitro* bioassays methods and comparison to other isolates from endemic regions in Kenya is required

CHAPTER ONE

1.0. INTRODUCTION

1.1. Background

Vibrio species are curved or straight Gram-negative bacilli, oxidase positive bacteria classified in the family *Vibrionaceae*. Nine species including *Vibrio cholerae* have been isolated from clinical specimens. There are two serogroups of *V. cholerae* associated with cholera epidemics, *V. cholerae* O1 and *V. cholerae* O139. The remaining more than 150 serogroups rarely cause epidemics and are classified as *V. cholerae* non-O1. Cholera is essentially a disease of poor sanitation, and epidemics in Africa have been linked to consumption of food and water from unsafe sources like drinking or bathing in lakes (Birmingham *et al*.,1997), drinking river water (Swerdlow *et al.*, 1997), eating at large funeral feasts (Gunnlaugsson *et al.*, 1998), and eating cold leftover foods (Swerdlow *et al* .,1997). Since 1817, *Vibrio cholerae* emerged from the Indian subcontinent in seven pandemics of acute diarrheal disease and the most recent began in 1961 and continues to the present day in Asia, Africa, the Middle East, and the South and Central America. In many areas cholera has become an endemic disease (WHO, 1997).

The burden of cholera is highest in sub-Saharan Africa. In 1996, Africa reported the largest number of cholera cases in the world, with 108,535 cases, more than three times the number in the rest of the world combined. In addition, 6,216 deaths were reported, representing a case fatality rate of 5.7% and accounting for 93% of all

reported cholera deaths worldwide (WHO, 1997). In 2007, fifty three countries reported 177,993 cholera cases and 4,031 cholera deaths to the World Health Organization (Julie *et al.,* 2009). Countries in sub-Saharan Africa reported more than 93% of the cases and 99% of the deaths (WHO, 2008) Cholera is an acute diarrheal disease caused by *V. cholerae* O1 or O139. Cholera epidemics spread rapidly and, without intervention, can lead to death due to dehydration. Antimicrobials shorten the duration and reduce the severity of the disease, curbing transmission. Thus, antimicrobial resistance can increase the outbreak size, duration and case fatality rates. Tetracycline was originally the antimicrobial of choice for cholera and has been used widely even in Africa. Trimethoprim/sulphamethaxazol, ampicillin and quinolones have been used for resistant strains to tetracycline; however resistance to these and other drugs have been reported (Okeke *et al*., 2003).

1.1.1. The history of cholera in Kenya

Since 1971 Kenya has experienced several cholera outbreaks. From 1974 to 1989, Kenya reported cholera cases every year with an average case fatality rate of 3.57%. The largest epidemic started in 1997 and lasted until 1999, with more than 33,400 notified cases, representing 10% of all cholera cases reported from the African continent in the same 3 years (WHO, 2007). In 1997, the outbreaks started in June in Nyanza province near the Tanzanian border, along Lake Victoria. In mid-October, the epidemic reached Kisumu, the third largest city in Kenya and by early November, cholera had spread northwest of Kisumu into Siaya district. From 2000 to 2006, cases

were reported each year ranging from 1,157 to 816 except for 2002, with 291 cases (Julie *et al.,* 2009).

In 2007, the cholera outbreak affected 9 districts: West Pokot, Turkana, Kwale, Garissa, Wajir, Mandera, Kisumu, Bondo and Siaya. The cases in Kwale were reported since mid February, shortly after the end of the floods, whereas the rest of the districts reported cases from late March, 2007. As of 15th May, a cumulative total of 625 cases have been reported with 35 deaths leading to a case fatality rate of 5.6%. Stool analyses from initial cases indicated the presence of *Vibrio cholerae* O1 Inaba and Ogawa. The intervention measures undertaken in all districts included case management, public health education, water chlorination and enhanced surveillance. The outbreak was first controlled in Kwale, West Pokot and Turkana and later in the rest of the districts (WHO, 2007). In 2008, Cholera outbreaks were reported from 4 provinces: Nyanza, North Eastern, Western and Rift Valley. Western Kenya was the most affected region with 771 cases and 53 deaths being reported from 10 districts. The outbreak started in Suba in November 2007 and further spread to cover all districts in the lake basin (Julie *et al.,* 2009).

Vibrio cholerae produces oxidase and thus the oxidase test differentiates it from other *enterobacteriacae*. On the basis of their carbohydrate determinants of their lipopolysaccharide (LPS) *Vibrio* have been classified into two groups namely O1

group and Non- O1 group (Fig.1). Other *vibrios* that can cause the disease include *V. mimicus, V. fluvialis, V.parahaemolyticus, V. alginolyticus* and *V. vulnificus.*

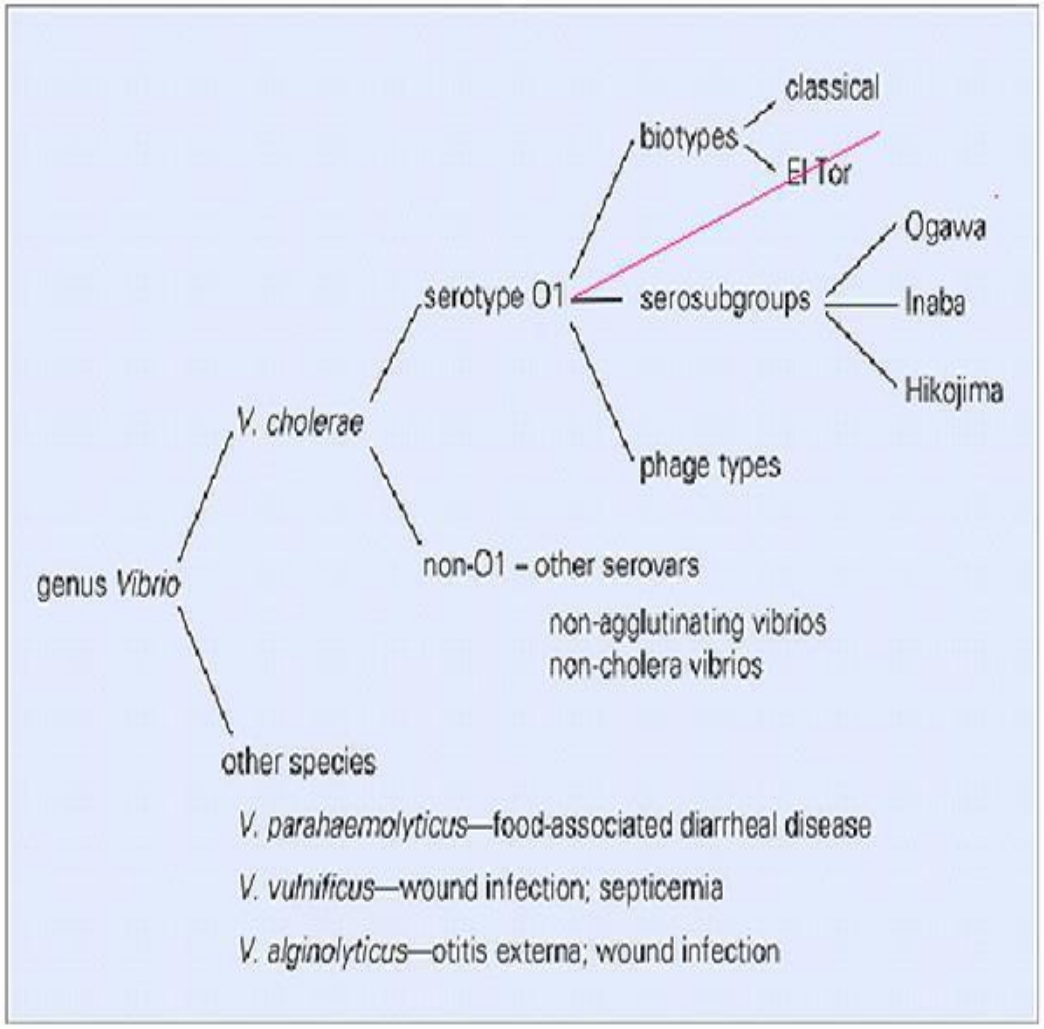


Figure 1. Classification of the *Vibrio* bacteria (Source: Elsevier, 2004)

Diseases caused by these other *Vibrio species* are as follows: *V. mimicus*-enteritis following ingestion of contaminated food, *V. Fluvialis* causes enteritis and dysentery in some countries, *V. parahaemolyticus* causes diarrhea and dysentery associated with

ingestion of contaminated food and *V.alginolyticus* being an opportunistic pathogen that causes wound, ear and eye infections. The aim of this research was to characterize the various *V. cholerae* isolated from diarrheal-diseases outbreaks in Western Kenya biochemically, immunologically and through molecular typing in order to place them in both their clinical and epidemiological contexts.

1.2. Statement of the Problem

The cholera is prevalent throughout third world countries and outbreaks occur more in areas with poor sanitation. Evidently cholera is a major health concern in Kenya as indicated from the sporadic outbreaks of cholera throughout the country. The ability to produce cholera toxin (CT) by the pathogenic bacteria is a major determinant of virulence in *V. cholera*. In general, isolates of *V. cholerae* O1 or O139 that produce cholera toxins are considered fully virulent and capable of causing epidemic cholera (Kurazono *et al.,* 1995). Most *V. cholerae* isolated during cholera outbreaks are toxigenic serogroup O1 or O139. However, some isolates of *V. cholerae* O1 do not produce CT and cannot cause epidemic cholera. Therefore when these isolates are encountered, they must be considered within their clinical and epidemiologic context. Non-toxigenic isolates may be associated with sporadic diarrheal disease. Furthermore the phenotypic and molecular characteristics of the *V. cholerae* isolates in Kenya seems not to have been reported and/or thoroughly documented recently (especially the biotype, serotype and toxigenicity of local strains).

1.3. Justification

Frequent diarrheal outbreaks all over Kenya caused by the bacterium *V. cholerae* are a steady reminder of the immense importance of cholera as a national threat and a major public health problem in Kenya. The disease may become life-threatening if appropriate therapy is not undertaken quickly; hence, fast, accurate, and sensitive detection of this organism is of foremost importance.

Infact, the cholera outbreaks have continued since they occurred in Suba and south Nyanza in November 2007. In addition, the case-fatality rate is fairly high (Ichinose *et al.,* 2008). Therefore, more efficient counter measures need to be taken to confine the outbreaks perfectly and improve case-fatality of cholera as well as develop effective parameter of case mortality. To provide new insights into the epidemiology of the disease, during an outbreak or epidemic, it is worth documenting and characterizing the biotype, serotype and antibiotic susceptibility/resistance patterns of the isolates as epidemiological parameters as well as the virulence of this isolates in Kenya. Therefore research on characterization of epidemic strains is essential to monitor their endemicity in order to combat the enteric disease.

1.4. Objectives

1.4.1. General Objective

To characterize strains of *Vibrio cholera*e phenotypically using biochemical and immunological tests and molecular typing by polymerase chain reaction and pulsed field gel electrophoresis.

1.4.2. Specific Objectives

1. To characterize the pathogenic *V.cholerae* isolates using biochemical tests

   (Confirmatory identification using API32E strips, biotyping and serotyping)

2. To characterize the *V.cholerae* isolates by their susceptibility/resistance to various antibiotics using the Etest method.

3. To amplify the cholera toxin (CT) gene and the antibiotic resistance genes for commonly used antibiotics and to establish clonal relatedness of the isolates using the pulsed field gel electrophoresis (PFGE) by the CHEF-DR II system (BioRad).

CHAPTER TWO

2.0. LITERATURE REVIEW

2.1. Taxonomy and serological classification of *Vibrio cholerae*

Vibrio cholerae is a member of the family *vibrionaceae, a* facultative anaerobic, gram-negative, non-spore forming bacteria capable of respiratory and fermentative metabolism. It is a slightly curved- rod shaped bacteria (plate1) about 1.4-2.6mm long (Barua, 1992). The bacterium is well defined on the basis of biochemical tests and DNA homology and is oxidase positive, reduces nitrate and is motile by means of a single sheathed polar flagellum (Baumann *et al*; 1984). Growth of *V. cholerae* is stimulated by addition of 1% sodium chloride. However, an important distinction from other *Vibrio* species is the ability of *V. cholerae* to grow in nutrient broth without addition of sodium chloride. Freshly isolated agar-grown *V. cholerae* of the El Tor biotype, in contrast to classical *V. cholerae,* produce a cell-associated mannose-sensitive hemagglutinin active on chicken erythrocytes (Karasawa *et al.*, 1993).

Differences in the sugar composition of the heat-stable surface somatic antigen are the basis of the serological classification of *V. cholerae* first described by Gardner and Venkatraman (1935) but currently the bacteria is classified into 206 "O" serogroups (Shimada *et al*., 1994, Yamai *et al*., 1997). Until recently, epidemic cholera was exclusively associated with *V. cholerae* strains of the O1 serogroup. All strains that were identified as *V. cholerae* on the basis of biochemical tests but that did not

agglutinate with "O" antiserum were collectively referred to as non-O1 *V. cholerae.* The non-O1 strains are occasionally isolated from cases of diarrhea (Ramamurthy *et al.*, 1993) and from a variety of extra-intestinal infections, from wounds and from the ear, sputum, urine and cerebrospinal fluid (Morris and Black, 1985). Some strains are ubiquitous in estuarine environments, and infections due to these strains are commonly of environmental origin (Morris *et al*., 1990).

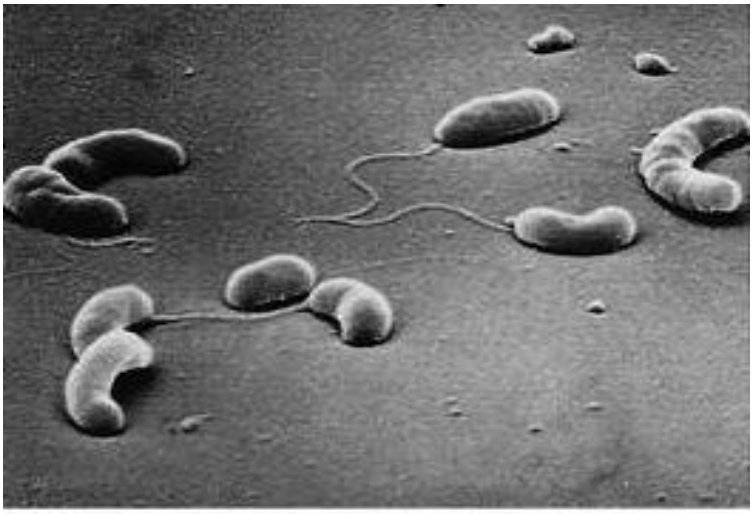


Plate 1: Scanning electron micrograph of *Vibrio cholerae* (Source: Elsevier, 2004.)

The O1 serogroup exists as two major biotypes, classical and El Tor (Fig. 2). 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 the Inaba strains express only the A and C antigens. A third serotype called Hikojima expresses all the three antigens but is rare and unstable (Morris *et al*., 1990).

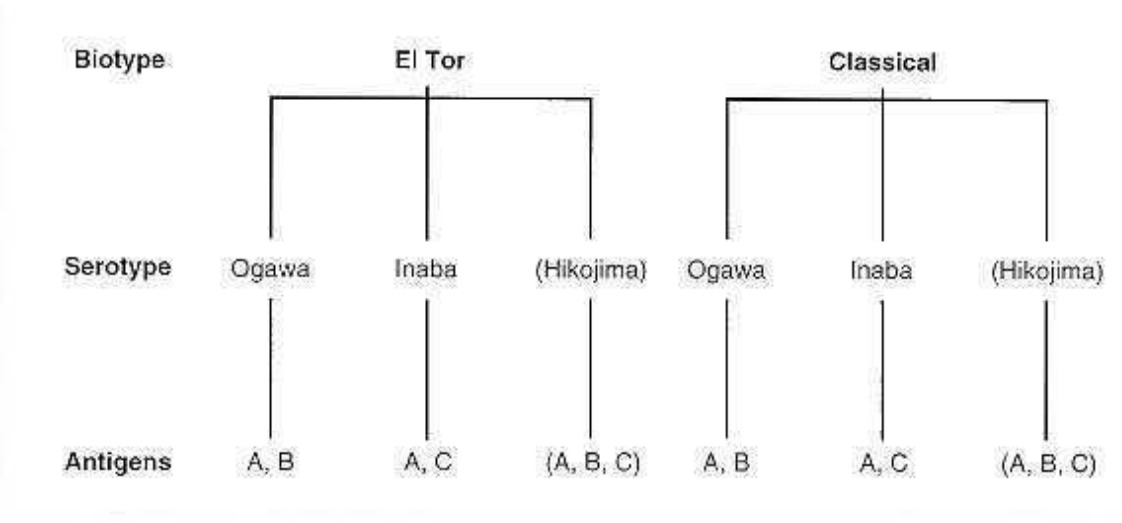


Figure 2. *Vibrio cholerae* O1 Biotypes and serotypes

Cholera is transmitted by the fecal-oral route. *Vibrio cholerae* are sensitive to acid, and most die in the stomach. Surviving virulent organisms may adhere to and colonize the small bowel, where they secrete the potent cholera enterotoxin (CT, also called "choleragen"). This toxin binds to the plasma membrane of intestinal epithelial cells and releases an enzymatically active subunit A that causes a rise in the production of cyclic adenosine 51-monophosphate (cAMP). The resulting high intracellular cAMP level causes massive secretion of electrolytes and water into the intestinal lumen (Fields *et al.*, 1992). Perhaps by production of CT, the *V. cholerae* thus ensures their survival by increasing the likelihood of finding another human host. Recent evidence suggests that prostaglandins may also play a role in the secretory effects of cholera enterotoxin. Recent studies in volunteers using genetically-engineered strains of *V. cholerae* have revealed that the bacteria have putative mechanisms in addition to

cholera toxin (CT) for causing (milder) diarrheal disease. These include Zonula occludes toxin (*zot)* and Accessory cholera enterotoxin (*ace*), and perhaps others, but their role has not been established conclusively (Fields *et al.*, 1992). Certainly cholera toxin is the major virulence factor and the act of colonization of the small bowel may itself elicit an altered host response (e.g., mild diarrhea), perhaps by a trans-membrane signaling mechanism. Cholera toxin approaches target cell surface. The B subunits bind to oligosaccharide of GM1 ganglioside (Fishman, 1990). Conformational alteration of holotoxin occurs, allowing the presentation of the A subunit to cell surface. The A subunit enters the cell whereby its disulfide bond is reduced by intracellular glutathione, freeing A1 and A2. NAD is hydrolyzed by A1, yielding ADP-ribose and nicotinamide. One of the G proteins of adenylate cyclase is ADP-ribosylated, inhibiting the action of GTPase and locking adenylate cyclase in the "on" mode (Fig. 3).

Thus gastric acid, mucus secretion, and intestinal motility are the prime nonspecific defenses against *V. cholerae*. Breastfeeding in endemic areas is important in protecting infants from the disease. The disease results in effective specific immunity, involving primarily secretory immunoglobulin (IgA), as well as IgG antibodies, against *Vibrio spp*, somatic antigen, outer membrane protein, and/or the enterotoxin and other products (Fishman, 1990).

Other serogroups of *V. cholerae* may cause diarrheal disease and other infections but are not associated with epidemic cholera. *V. parahaemolyticus* is an important cause of enteritis associated with the ingestion of raw or improperly prepared seafood. Other *Vibrio* species, including *V. vulnificus*, can cause infections of humans and other animals including fish.

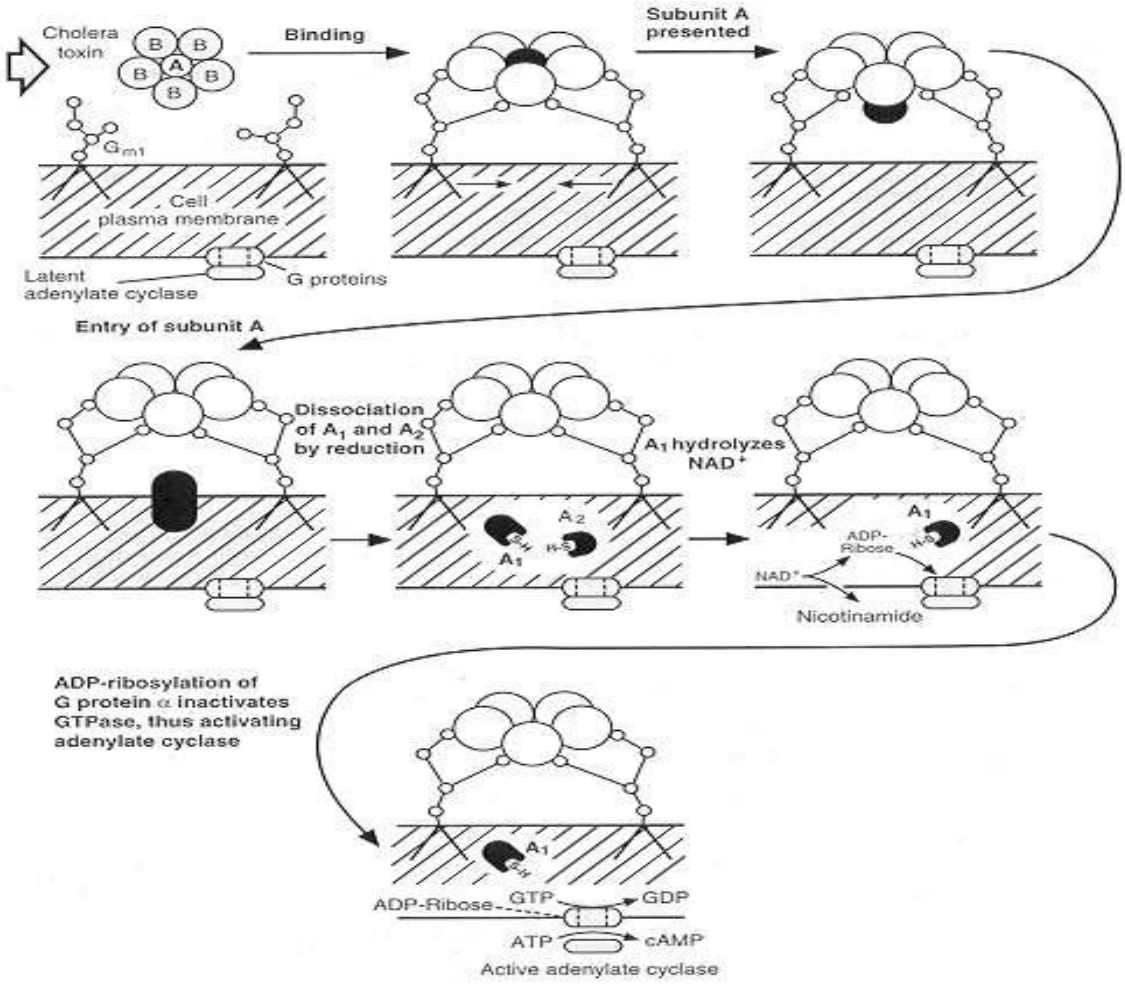


Figure 3: Mechanism of action of cholera enterotoxin (Modified from Fishman, 1990)

2.2. Diagnosis, Epidemiology, Treatment and Control of cholera

The diagnosis is suggested by strikingly severe, watery diarrhea. For rapid diagnosis, a wet mount of liquid stool is examined microscopically. The characteristic motility of *vibrios* is stopped by specific antisomatic antibody. Other methods are culture of stool or rectal swab samples on thiosulphate citrate bile salt (TCBS) agar and other selective and nonselective media; the slide agglutination test of colonies with specific antiserum; fermentation and oxidase tests and enrichment in peptone broth followed by fluorescent antibody tests, culture, or retrospective serologic diagnosis (Kaufmann and Pitt, 1994). More recently the polymerase chain reaction (PCR) and additional genetically-based rapid techniques have been recommended for use in specialized laboratories.

Cholera appears to exhibit three major epidemiologic patterns: heavily endemic, neoepidemic (newly invaded, cholera-receptive areas) and in developed countries with good sanitation, occasional limited outbreaks (Fishman, 1990). These patterns probably depend largely on environmental factors (including sanitary and cultural aspects), the prior immune status or antigenic experience of the population at risk, and the inherent properties of the *V. cholerae* themselves, such as their resistance to gastric acidity, ability to colonize, and toxigenicity. There are several characteristics of the El Tor strain that confer upon it a high degree of "epidemic virulence" allowing it to

spread across the world as previous strains have done (Fields *et al.*, 1992. First, the ratio of cases to carriers is much less than in cholera due to classic biotypes (1: 30-100 for El Tor vs. 1: 2 - 4 for "classic" biotypes). Second, the duration of carriage after infection is longer for the El Tor strain than the classic strains. Third, the El Tor strain survives for longer periods in the extra-intestinal environment.

Because humans are the only reservoirs, survival of the *V. cholera* during inter-epidemic periods probably depends on a relatively constant availability of low-level undiagnosed cases and transiently infected, asymptomatic individuals. Long-term carriers have been reported but are extremely rare (Kaufmann and Pitt, 1994). During epidemic periods, the incidence of infection in communities with poor sanitation is high enough to frustrate the most vigorous epidemiologic control efforts. Although transmission occurs primarily through water contaminated with human feaces, infection also may be spread within households and by contaminated foods. Thus, in heavily endemic regions, adequate supplies of pure water may reduce but not eliminate the threat of cholera (Swerdlow *et al*., 1997).

Treatment of cholera consists of essentially replacing fluids and electrolytes. Formerly, this was accomplished intravenously, using costly sterile pyrogen-free intravenous solutions (Kaufmann and Pitt, 1994). Antibiotics such as tetracycline, to which the *V. cholerae* are generally sensitive, are useful adjuncts in treatment. They shorten the period of infection with the *V. cholerae* thus reducing the continuous

source of cholera enterotoxin; this results in a substantial saving of replacement fluids and a markedly briefer hospitalization. Note, however, that fluid and electrolyte replacement is all-important; patients who are adequately rehydrated and maintained will virtually always survive, and antibiotic treatment alone is not sufficient.

Cholera is essentially a disease associated with poor sanitation. The simple application of sanitary principles protecting drinking water and food from contamination with human feaces would go a long way towards controlling the disease (Gunnlaugsson *et al.,* 1998). However, at present, this is not feasible in the underdeveloped areas that are afflicted with epidemic cholera or are considered to be cholera receptive. Meanwhile, development of a vaccine that would effectively prevent colonization and manifestations of cholera would be extremely helpful. Antibiotic or chemotherapeutic prophylaxis is feasible and may be indicated under certain circumstances. It was noted that the incidence of cholera is significantly higher in formula-fed than in breast-fed babies (Kaufmann and Pitt, 1994).

2.3. Genetic Organization and Regulation of Virulence Factors in *V.cholerae*

In *V.cholerae*, the production of virulence factors is regulated at several levels (Dalsgaard *et al.,* 1996). Regulation of genes at the transcriptional level, especially the genes for toxin production and fimbrial synthesis, has been studied in the greatest detail. *V. cholerae* enterotoxin is a product of *ctx* genes. *CtxA* encodes the A subunit of the toxin, and *ctx*B encodes the B subunit. The genes are part of the same operon. The

transcript messenger ribonucleic acid (mRNA) of the *ctx* operon has two ribosome binding sites (rbs), one upstream of the A coding region and another upstream of the B coding region. The rbs upstream of the B coding region is at least seven-times stronger than the rbs of the A coding region. In this way the organism is able to translate more B proteins than A proteins, which is required to assemble the toxin in the appropriate 1A: 5B proportion (Kaufmann and Pitt, 1994). The components are assembled in the periplasm after translation. Any extra B subunits can be excreted by the cell, but A must be attached to 5B in order to exit the cell. Intact A subunit is not enzymatically active, but must be nicked to produce fragments A1 and A2 which are linked by a disulfide bond. Once the cholera toxin has bound to the GM1 receptor on host cells, the A1 subunit is released from the toxin by reduction of the disulfide bond that links it to A2, and enters the cell by an unknown translocation mechanism (Fishman, 1990). One hypothesis is that the 5 B subunits form a pore in the host cell membrane through which the A1 unit passes.

Transcription of the *ctx*AB operon is regulated by a number of environmental signals, including temperature, pH, osmolarity, and certain amino acids. Several others *V. cholerae* genes are co-regulated in the same manner including the *tcp* operon, which is concerned with fimbrial synthesis and assembly (Dalsgaard *et al*., 1996). Thus the *ctx* operon and the *tcp* operon are part of a regulon, the expression of which is controlled by the same environmental signals. The proteins involved in control of this regulon

expression have been identified as ToxR, ToxS and ToxT (Kurazono *et al.,* 1995). ToxR is a transmembranous protein with about two-thirds of its amino terminal part exposed to the cytoplasm. ToxR dimers, but not ToxR monomers, will bind to the operator region of *ctx*AB operon and activate its transcription. ToxS is a periplasmic protein. It is thought that ToxS can respond to environmental signals, change conformation, and somehow influence dimerization of ToxR which activities transcription of the operon.

ToxR and ToxS appear to form a standard two-component regulatory system with ToxS functioning as a sensor protein that phosphorylates and thus converts ToxR to its active DNA binding form. ToxT is a cytoplasmic protein that is a transcriptional activator of the tcp operon. Expression of ToxT is activated by ToxR, while ToxT, in turn, activates transcription of tcp genes for synthesis of tcp pili. Thus, Kurazono *et al.,* 1995, postulates that the ToxR protein is a regulatory protein which functions as an inducer in a system of positive control. ToxR is thought to interact with ToxS in order to sense some change in the environment and transmit a molecular signal to the chromosome which induces the transcription of genes for attachment (pili formation) and toxin production (Fishman, 1990)..

It is reasonable to expect that the environmental conditions that exist in the gastrointestinal (GI) tract (37°C temperature, low pH, high osmolarity, etc.), as

opposed to conditions in the extraintestinal (aquatic) environment of the vibrios, are those that are necessary to induce formation of the virulence factors necessary to infect. However, there is conflicting experimental evidence in this regard, which leads to speculation of the ecological function of the toxin during human infection (Kurazono *et al.,* 1995).

Detection of cholera toxin (CT)-producing *V. cholerae* using conventional culture, biochemical and immunological-based assays is time-consuming and laborious, requiring more than three days. Commercially available kits cannot distinguish between the heat-labile enterotoxin (LT) of *Escherichia coli* and cholera toxin (CT). A rapid, reliable and practical assay for the detection of CT-producing *V. cholerae* has thus been sought.

Several PCR assays offer a more sophisticated approach to the identification of *Vibrio cholerae* (Hoshino et *al.*, 1998). Although PCR assays provide more rapid identification of *Vibrio cholerae* than conventional assays, they require the use of electrophoresis to detect amplified products, which is time-consuming and tedious. Real time PCR assays recently developed for the rapid identification of *Vibrio cholerae* (Lyon, 2001), are not routinely used due to their requirement for an expensive thermal cycler with a fluorescence detector. Among other techniques, however, one promising candidate is a novel nucleic acid amplification method termed

loop-mediated isothermal amplification (LAMP) (Lyon, 2001). LAMP is based on the principle of autocycling strand displacement DNA synthesis performed by the *Bst* DNA polymerase large fragment for the detection of a specific DNA sequence with specific characteristics (Blackstone *et al*., 2007).

2.4. Phenotyping Methods

Phenotypic methods detect the presence or absence of metabolic or biological activities as expressed by microorganisms. These methods include for example biotyping, serotyping, phage typing, antimicrobial sensitivity testing, immunoblotting and multilocus enzyme electrophoresis (MLEE). Many phenotyping methods are widely available, easily performed and relatively inexpensive (Okeke *et al*., 2003).

2.4.1. Biotyping

Biotyping includes metabolic activities expressed by an isolate like specific biochemical reactions, colonial morphology and environmental tolerances (Johnson J.R., 2000). Biotyping has only a limited ability to differentiate among strains within species and has relatively poor discrimination power in epidemiological studies.

2.4.2. Serotyping

Serotyping is based on different variations of antigenic determinants expressed on the cell surface, including lipopolysaccharides, capsular polysaccharides, membrane proteins and extra cellular organelles (Okeke *et al*., 2003).

2.4.3. Antimicrobial susceptibility tests

The determination of antimicrobial resistance patterns is a routine test in most clinical microbiology laboratories. However the method has a limitation in its discriminatory ability because strains under selective pressure can get or loose specific resistance genes via plasmids and transposons from other strains or species (Okeke *et al*., 2003).

2.4.4. Detection of proteins/ toxins

Detection of toxin production uses various enzyme immune assays (Johnson J.R., 2000). At present; the reversed passive latex agglutination assay (RPLA) is commercially available for some bacteria like enterohemorrhagic *Escherichia coli* (EHEC). In the reversed agglutination assay, the antibody attached to the latex particles reacts with the soluble antigen (Okeke *et al*., 2003).

2.5. Genotyping Methods

The genotyping methods account for DNA-based analyses of chromosomal or extra chromosomal genetic elements. These include plasmid analysis, ribotyping, polymerase chain reaction (PCR), pulsed–field gel electrophoresis (PFGE) and amplification fragment length polymorphism (AFLP). The selection of the suitable and most applicable typing technique depends on the purpose of analysis (Okeke *et al.*, 2003).

Plasmid analysis can include typing of plasmid profile, plasmid fingerprinting and identification of plasmid-mediated virulence genes. Plasmid profiling is based on the

number and characteristic molecular weight of plasmids. In plasmid finger-printing, the plasmid DNA is digested with restriction enzymes (Okeke *et al*., 2003). Many pathogens (like *E. coli*) have plasmids associated with virulence and are characteristic of these pathogens. Therefore the determination of molecular weights of these plasmids can be useful marker

2.5.1. Polymerase Chain Reaction (PCR) and Restriction Fragment length polymorphism

PCR is an *in vitro* method for enzymatically synthesizing DNA from selected regions of a genome to be amplified. This particular DNA sequence is called a template and at least part of its nucleotide sequence is already known. The template should represent a relatively small fragment of DNA, typically 0.2 to 2.0 kb, because larger target sequences are difficult to amplify effectively, (Okeke *et al*., 2003). Many different applications of PCR have been described by Rappelli *et al.*, 2001. In polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP), a known sequence is amplified, cut with restriction enzyme and the restriction fragments are compared between different strains. Amplified fragment length polymorphism (AFLP) is the most recently adopted PCR-based typing technique (Galane and Le Roux, 2001). In this method, the genomic DNA is digested with two enzymes, a frequently cutting enzyme and a less frequently cutting enzyme. Then the resulting fragments are ligated to specific adaptors and amplified using primers recognizing the adaptors. Finally, the labeled products are separated by electrophoresis. Similarly, with PCR it is

simple to accumulate a large amount of material that can be sequenced directly after purification.

2.5.2. Pulsed-Field Gel Electrophoresis (PFGE)

DNA fragments from 100 base pairs (bp) up to 50 kilobase pairs (kbp) are routinely separated by conventional gel electrophoresis techniques. Above 50 kbp, because of the size of the molecules, the sieving action of the gel is lost, and fragments run as a broad, unresolved band with anomalously high mobility. In 1982, Schwartz *et al.*, introduced the concept that DNA molecules larger than 50 kb can be separated by using two alternating electric fields (i.e. Pulsed-Field Gel Electrophoresis**)**.

Since that time, a number of instruments based on this principle have been developed, and the value of using pulsed fields has been demonstrated for separating DNAs from a few kilobases to over 10 mega base pairs (mb). The basic components of a PFGE system consist of a gel box with some means of temperature regulation, a switching unit for controlling the electric fields, a cooler and a power supply (Schwartz *et al*., 1982). The development of PFGE has increased by two orders of magnitude the size of DNA molecules that can be routinely fractionated and analyzed. This increase is of major importance in molecular biology because it simplifies many previously laborious investigations and makes possible many new ones. Its range of application

spans all organisms (Southern and Elder, 1995) from bacteria and viruses to mammals (Kaufmann and Pitt, 1994).

PFGE has shown excellent ability to separate small, natural linear chromosomal DNAs ranging in size from 50kb parasite micro chromosomes to multimillion-bp yeast chromosomes. However, intact human chromosomes range in size from 50 million to 250 million bp (Mb), too large for direct pulsed-field gel electrophoresis separations (Southern and Elder, 1995). Pulsed-field gel electrophoresis provides the means for the routine separation of fragments exceeding 6,000 kb. Different types of pulsed-field gel electrophoresis are described (HacÝoÛlu *et al*., 1996). The Pulsed-Field Gel Electrophoresis of DNA fragments obtained by using endonucleases produce a discrete pattern of bands useful for the fingerprinting and physical mapping of the chromosome (Correla *et al*., 1991). The ability to separate, isolate and analyze mega base size fragments of DNA is already providing insights into the genome organization of organisms as diverse as bacteria and humans (Dempsey *et al.*, 1991).

CHAPTER THREE

3.0. MATERIALS AND METHODS

3.1. Sample processing

Seventy five (75) isolates from recent diarrheal disease outbreaks of *Vibrio cholerae* in Western Kenya (2007-2009) were used in this study. The isolates were collected by the team members of Nagasaki University, Institute of Tropical Medicine (NUITM). The isolates were stocked in nutrient agar and glycerol and stored at -80°C. The isolated were revived on TCBS and BTB and all the analysis was done at the NUITM laboratories collaborating with the Kenya medical research institute (KEMRI), in Nairobi. *V. cholerae* isolates were sub- cultured on to bromothymol blue (BTB) media from stocked *V. cholera*e stored in nutrient agar broth or glycerine. The top-most portion of the stocked isolates was scraped using a sterile loop and streaked onto BTB growth medium in the safety cabinet and incubated overnight at 37°C.

3.2. Phenotypic characterization

3.2.1. Biochemical tests

The automated microbiological analyser (mini-Api machine) was used for confirmatory identification of the isolates as *V.cholerae*. All strains were identified as *V. cholerae* by means of biochemical identification (API32 E test strips, Biomereux, France)

3.2.2. Biotyping

The *V. cholerae* were typed using three main tests namely hemolysis of 5% sheep red blood cells, hemaglutination of 2.5% chicken red blood cells and the sensitivity of the isolates to Polymixin B .

3.2.3. Hemolysin test

A single colony of *V.cholerae* growing on BTB was picked from nutrient agar plate and inoculated into 1ml of brain heart infusion broth and vortexed. Two milliters of 5% sheep red blood cells (RBC) were added into the inoculum and incubated at 37^{o}c for two hours then placed at 4^{o}C overnight. The positive results were read as red colorations in the tube while negative results were read as a collection of RBCs in the bottom of the tube.

3.2.4. Hemaglutination test

A drop of 2.5% chicken RBC was placed onto a clean microscope slide and emulsified with a single colony of *V. cholerae*. A positive test showed an aggregation of the RBCs together on the slide while for a negative test there was no aggregation of RBC

3.2.5. Voges Proskauer (VP) test

From a stock solution of MR-VP Broth, 0.2-mL aliquots were aseptically pipetted into sterile test tube and a loopful of test *V. cholerae* colony from an overnight BTB culture was inoculated into the broth and incubated at 37°C (water bath) for 4 hours.

Two drops of creatine reagent were added and mixed gently followed by the addition of 3 drops of α-Naphthol reagent then 3 drops of 40% potassium hydroxide and gently mixed for 10 seconds. The tube was allowed to stand for 15 minutes before interpreting the color change in the tube. A pink or red color at the surface of the medium indicated a positive VP test while yellow or copper color indicated a VP negative test.

3.2.6. Polymixin B test

V. cholerae isolates were sub-cultured onto nutrient agar (NA) plates. A single disc of Polymix B was placed onto each plate and the plates incubated at 37^{o}C overnight. The zone of growth inhibition was measured to determine sensitivity or resistance to polymixin B.

3.2.7. Serological identification

The serological identification was done using commercial polyvalent O1, anti-Ogawa, and anti-Inaba antisera. A clean dry slide was marked into three regions using a blue/black marker pen. A drop of each of the antisera was placed into the individual region of the slide. Each antiserum was emulsified with a loopful of the test organism and observed for the immunological reaction (agglutination).

3.2.8. Antibiotic susceptibility test

Minimum Inhibitory Concentrations (MICs) to antimicrobial agents was determined by the E-test method. The procedure for E-test method was performed as follows.

Mueller-Hinton agar medium was inoculated with a broth suspension equivalent to 0.5 MacFarland standards prepared by directly inoculating organisms from 24-hr-old nutrient agar medium. A cotton swab was used to apply the suspension onto the Mueller-Hinton agar in a confluent manner. The E-test strips were applied onto the plate and incubated overnight at 37^{o}C. After incubation inhibitory concentrations were read 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 formed the minimal inhibitory concentration (MIC). A rigorous standardization of operator conditions was established and *Escherichia coli* strain ATCC 25218 was used as a control. Standard strips for the following antibiotics were used: ciprofloxacin, ceftriaxone, tetracycline, trimethoprim/ sulphamethaxazol, kanamycin, ampicillin, amoxicillin, nalidixic acid, erythromycin, doxycycline, ofloxacin, streptomycin, gentamycin (low and high) and chloramphenicol.

3.3. DNA extraction

DNA was extracted from strains by the method described by Yokoyama *et al.,* 2003. Test strains were cultured in 2 ml of Luria-Bertani broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl) and incubated overnight at 37°C with shaking. Thirty-six micro liters of broth culture was added to 4 µl of 10× Tris-EDTA buffer (100 mM Tris-HCl, 10mM EDTA, pH 8.3), and 60 µl of 2× proteinase K buffer (100 mM KCl, 20 mM Tris-HCl, 5 mM MgCl2, 1% Tween 20, 800µg of proteinase K/ml, pH 8.3) was added. After incubation for 90 min at 56°C and 10 min at 95°C, the sample were centrifuged

at 10,000 × *g* for 1 min, and the supernatant was stored at -20˚C and used as the DNA template.

3.3.1. Polymerase chain reaction (PCR) assays

The primer set *int* was used to detect the SXT intergrase. *StrA, sul2* and *tetA* primer sets were used to detect genes conferring resistance to streptomycin, sulphamethaxazol and tetracycline respectively. Primer sets *ctxAB* was used to detect the CTX genetic element for cholera toxin. PCR was carried out in 0.2 ml tubes containing PuReTaq ready-to-go PCR beads (GE Healthcare UK limited, UK). When brought to a final volume of 25µl, each reaction tube contained 2µl template DNA, 2µl (6 pmol/µl) each of appropriate primers, ~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 and bovine serum albumin (BSA). The PCR primers used are given in Table 1**.** All PCR assays were performed using an automated thermal cycler (GeneAmp PCR System 2400; Applied Biosystems). The PCR products were analysed by electrophoresis in 2.5% agarose gels, stained with ethidium bromide, visualized under UV light and recorded with the aid of a gel documentation system (Bio-Rad systems).

3.3.2. The pulsed-field gel electrophoresis (PFGE)

PFGE was carried out by the method described by Kurazono *et al.*, (1995) using the genomic DNA of the *V. cholerae* strains prepared in agarose plugs. A single colony of

V. cholerae was inoculated into 2ml of Luria-Bertani broth (1% tryptone, 0.5% yeast extract, 0.5% Nacl) and cultured at 37°C for 16-24hrs with shaking (about 200 rpm). Two hundred microliters of the culture broth was pipetted into a sterile eppendoff tube. One point two percentage InCert agarose (BioWhittaker Molecular Applications, USA) was prepared in 1×TE buffer (10mM Tris, 1 mM EDTA, pH 8.0) and the melted agarose placed in a 55-60°C water bath until ready to use. Zero point one milliliter of the melted 1.2% InCert agarose was added to the culture broth directly, and mixed by gently pipetting the mixture up and down a few times. Part of the mixture was then dispensed immediately into CHEF Plug Caster 0.7mm without allowing bubbles to form. Plugs were allowed to solidify at room temperature for 10-15 min (or at 4°C for 5min).

Plugs were placed in clean tubes containing 3 ml of lysis buffer (0.45M EDTA, pH 8.5 + 1% sarcosin + 1mg/ml proteinase K). Bacteria DNA were lysed in the agarose plugs for 48 hrs at 37 °C. The plugs were rinsed briefly with 0.2M EDTA and incubated in Phenylmethylsulfonyl fluoride (PMSF) (0.8 g in 2ml 0.2M EDTA) for 1 hr so as to deactivate the lysis buffer. The PMSF was rinsed off using sterile water and the plugs placed in digesting buffer (35µl of ×1 high conc. buffer, 35 µl of 0.1% BSA, 35 µl of ×1 triton buffer, 0.5 µl of *Not1* restriction enzyme and 276µl of sterile water) for 5 hrs at 37°C. A small portion of the plug (2 by 7 mm) was sliced off and placed in the well of 1% pulsed field certified agarose gel (Bio-Rad Laboratories, Inc, CA).

Restriction fragments were separated in a 1% pulsed field certified agarose gel in 1× TBE (10× TBE is 89 mM Tris base, 89 mM boric acid, and 2.5 mM disodium EDTA) by using a CHEF-DR II system (Bio-Rad). CHEF DNA size standard 5 blocks ladder (*S. cerevisiae*) (Bio-Rad Laboratories, Inc, CA) was used as molecular mass standards. A model 1000 mini chiller (Bio-Rad) was used to maintain the temperature of the buffer at 14°C. A two-block block programme was used with a first block ramp time of 2 sec to 10 sec for 13 hrs at 6 V/cm (for separation of smaller fragments) and second block with ramp time of 20 s and 25 s for 6 hrs (for separation of larger fragments) at 6 V/cm. Following electrophoresis, gels were stained for 20 min with ethidium bromide (2µg/ml in water), destained, and visualized on a UV light box. *V. cholerae* O1 isolates were separated into patterns on the basis of differences in band arrangements. Differences in the presence, absence, or intensity of a band among strains were given equal weights. Strains that differ by one band were assigned different pattern numbers.

3.3.5. Data Analyses

The collected and recorded data were coded and analysed using Statistical Package for Social Sciences (SPSS) version 11 to determine chi square distributions for qualitative data such as the distribution of *V.cholerae* by the year, location and sources of

isolation etc. The charts and tables were used to represent these data. The mean and the range were calculated for quantitative data such as minimum inhibition concentration. Levels of genetic variation were calculated for individual populations and then averaged across all populations as well as for the total population. Chi-square tests for conformance to Hardy-Weinberg equilibrium at each locus were computed from expected genotypic frequencies under random mating assumption using the algorithm by Levene (1949) using POPGENE version 1.3 (Yeh and weir, 1999).

Out of the well resolved PFGE analysis, a total of 18 putative loci were identified and were thus used for analysis in this study. The loci were based on their sizes and were denoted as No.1 to No. 18. The alleles at each of the 18 restriction loci were scored as either 1 or 0 depending on the presence or the absence of the band at that locus.

Population relationships were inferred using the unweighted Neighbor-joining method viewed using the program DARwin software http://darwin.cirad.fr/darwin (Perrier and Jacquemoud-Collet, 2006). For all the statistical analyses, a P-value of < 0.05 was considered to be significant.

CHAPTER FOUR

4.0. RESULTS

4.1. Characteristics of the specimen collected for isolation *V. cholerae*

A total of seventy five (75) *V. cholerae* were isolated from two different sources majority 67/75 (89.3%) being from rectal swab and only 8/75 (10.7%) from different drinking water sources (either stored, borehole or river) as shown in Figure 4.

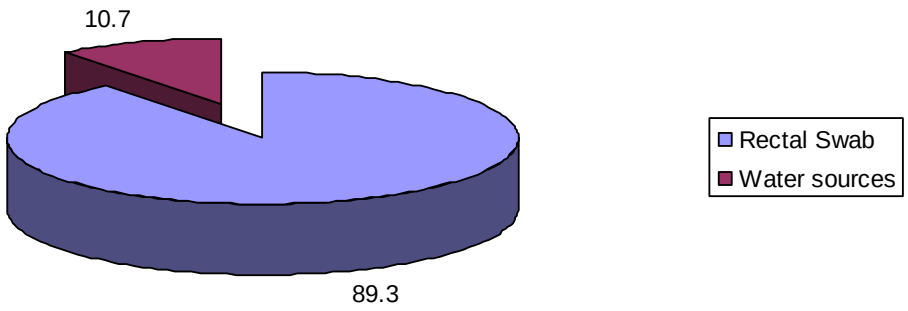


Fig. 4: Proportion of specimen types collected

The majority 42/75 (56%) of these specimens were obtained in the year 2007 while 24/75 (33.3%) in the year 2008 and only 8/75 (10.7%) in the year 2009. The specimen collection differed significantly per year with majority of the specimen turning positive for *V. cholerae* in the year 2007 as shown in figure 5.

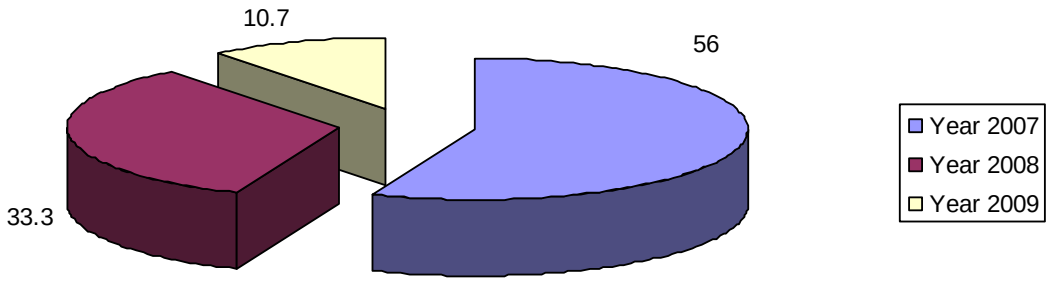


Fig. 5: The percent distribution of specimen collection per year

These samples were collected from different locations of Western and Nyanza parts of Kenya (table 1), majority 25.3% being from Mbita followed by 18.7% Sindo, 13.3% Bungoma, 10.7% Rusinga, 8% Kisumu, 6.7% Tom Mboya, 4% Suba. Others included 2.7% each from Kongata, St. Camilus, Magunga and Lwanda and 1.3% each from Sena and Siaya. The source of isolation was significantly different (table 1).

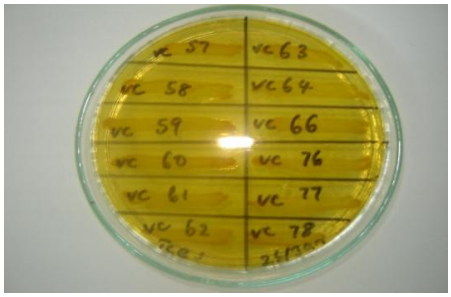
Table 1: The distribution of *V. cholerae* by location of isolation

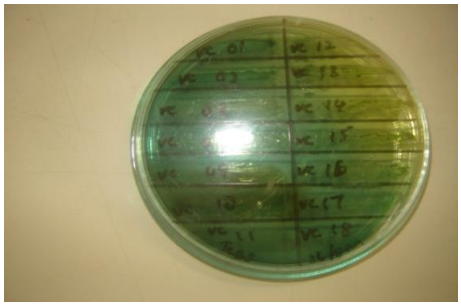
Location	Freq	%	$\chi 2$	P Value
Bungoma	10	13.3		
Kisumu	6	8		
Kongata	2	2.7		
Lwanda	2	2.7		
Magunga	2	2.7		
Mbita	19	25.3	65.222	0.0001
Rusinga	8	10.7		
Sena	1	1.3		
Siaya	1	1.3		
Sindo	14	18.7		
St. Camilus	2	2.7		
Suba	3	4		
Tom Mboya	5	6.7		
Total	75	100		

Freq - Frequency; % - Percentage

4.2. Biochemical and serological characteristics of isolated *V. cholerae*

The isolated organisms were initially cultured onto thiosulphate citrate bile salt (TCBS) agar as shown on plate 2 and plate 3, before and after pre-enrichment in alkaline peptone water (pH 8.6).

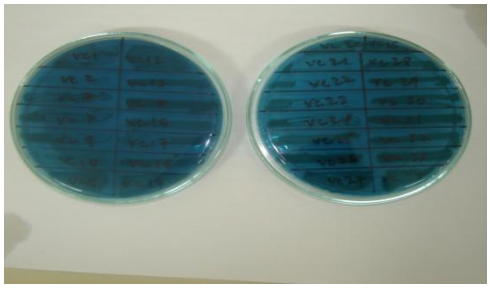


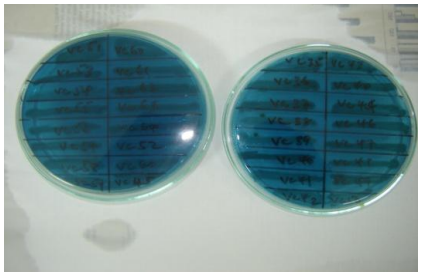


(2) (3)

Plate 2 and plate 3 showing pure (yellow) and contaminated (green, by other enteric bacteria) *V. cholerae* colonies respectively on thiosulphate citrate bile salts (TCBS) media.

All the isolates were then sub-cultured onto bromothymol blue (BTB, Plate 4 and plate 5) and nutrient agar media for identification because they exhibited colony morphological features typical to *V. cholerae* i.e. yellow sucrose fermenting colonies. In this case 75 organisms were identified as *V. cholerae* based on this criterion.

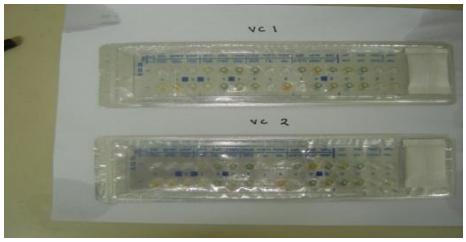




(4) (5)

Plates 4 and plate 5 showing pure *Vibrio cholerae* colonies growing on Bromothymol blue (BTB) media.

In this study, 75 organisms were identified as *V. cholerae* based on this criterion and tallied with the *confirmatory* test using the API32E strips (plate 6 and plate 7).



(6)

(7)

Plate 6 and plate 7 showing unused and used (inoculated with *V.cholerae*) mini API 32E strips respectively for the automated identification of *V. cholerae* isolates.

All the 75 isolates hemaagglutinated with 2.5% chicken red blood cells as well as lysed with 5% sheep red blood cells which are properties and confirmation of *V. cholerae* ET Tor (table 2). The sensitivity to polymixin B compound showed an irregular pattern since some isolates were ether slightly sensitive or resistant as shown in table 2. All the V. cholerae isolates agglutinated with O1 antisera and none agglutinated with the O139 antisera. Seventy three (97%) of the *V. cholerae* isolates agglutinated with the Inaba antisera, two isolates (3%) with Ogawa antisera but there was no agglutination of the *V. cholerae* isolates with the Hikojima antisera.

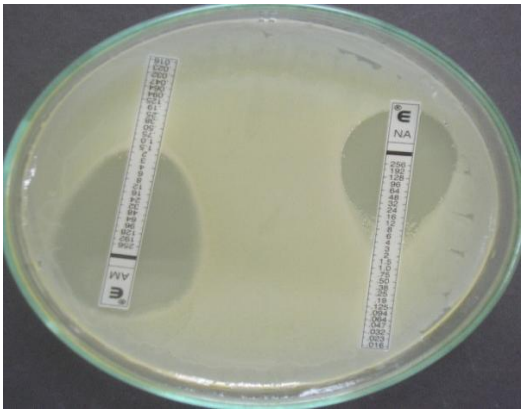
Table 2: The biochemical properties of *V. cholerae* isolates

Location of Isolation	Sample size	2.5%chicken Agglutination Test	Polymix B Sensitivity	5% Sheep Blood Cells	Voges Proskauer (VP)Test	Biotype
Mbita	19 (25.3)	100% S	52.6% S 47.4% R	100% S	100% S	100% EL Tor
Sindo	14 (18.7)	100% S	71.4% S 28.6% R	100% S	100% S	100% EL Tor
Bungoma	10 (13.3)	100% S	60% S 40% R	100% S	100% S	100% EL Tor
Rusinga	8 (10.7)	100% S	62.5% S 37.5% R	100% S	100% S	100% EL Tor
Kisumu	6 (8)	100% S	66.7% S 33.3% R	100% S	100% S	100% EL Tor
Tom Mboya	5 (6.7)	100% S	60% S 40% R	100% S	100% S	100% EL Tor
Suba	3 (4)	100% S	66.7% S 33.3% R	100% S	100% S	100% EL Tor
Kongata	2 (2.7)	100% S	50% S 50% R	100% S	100% S	100% EL Tor
Lwanda	2 (2.7)	100% S	50% S 50% R	100% S	100% S	100% EL Tor
St. Camilus	2 (2.7)	100% S	50% S 50% R	100% S	100% S	100% EL Tor
Magunga	2 (2.7)	100% S	50% S 50% R	100% S	100% S	100% EL Tor
Sena	1 (1.3)	100% S	100% S	100% S	100% S	100% EL Tor
Siaya	1 (1.3)	100% S	100% R	100% S	100% S	100% EL Tor

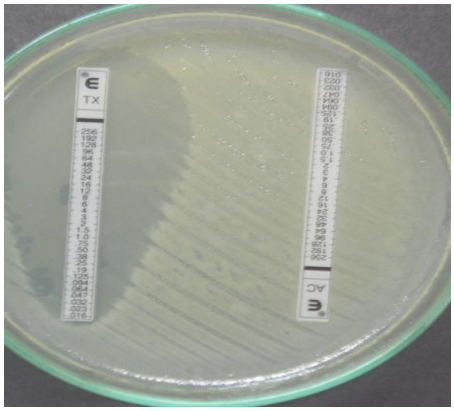
R - Resistant, S - Sensitive, Figures in brackets represents frequencies and percentages

4.3. The distribution of antibiotic-resistant types among *V. cholerae* isolates

The MIC of the fifteen antibiotics was tested using the *E*-test method for all the 75 *V. cholerae* (plate 8 and plate 9). Very high concentrations were required to achieve MIC for tetracycline and nalidixic acid (>192 µg/mL), Sulphamethaxazol/trimethroprim (256 µg/mL) and streptomycin (512 µg/mL) (Table 3). The *V. cholerae* isolates had varied antibiotic resistance levels ranging from erythromycin 2.7%, tetracycline 2.7%, amoxicillin 5.3%, nalidixic acid 38.7%. All the isolates were almost resistant to sulphamethaxazol/trimethroprim 93.3% and were fully resistant to amplicilin 100% (Table 3).



(8)



(9)

Plate 8 showing the minimal inhibitory concentrations (MIC) for Ampicillin and Nalidixic acid while plate 9 indicates MIC for Ceftriaxone and Amoxicillin.

Table 3: MIC resistance range of the *V. cholerae* isolates

Antibiotics	No. (%) resistant	MIC range (µg/mL)	Mean MIC (µg/mL)
Nalidixic acid	29 (38.7)	0.13 to 192	27.97
Streptomycin	0	1.5 to 512	156.89
Ceftriaxone	0	0.002 to 0.094	0.0072
Gentamicin low	0	0.15 to 1	0.548
Gentamicin High	0	0.125 to 16	0.578
Amoxicillin	4 (5.3)	1 to 256	7.57
Erythromycin	2 (2.7)	0.5 to 4	1.716
Ciprofloxacin	0	0.003 to 0.64	0.0668
Doxycycline	0	0.13 to 8	0.698
Kanamycin	0	3 to 6	4.16
Tetracycline	2 (2.7)	0.25 to 192	4.425
Sulphamethaxazol/trimethroprim	70 (93.3)	0.047 to 32	29.472
Ofloxacin	0	0.004 to 1.9	0.253
Ampicillin	75 (100)	0.5 to 256	12.5
Chlororamphenicol	0	0.38 to 16	7.512

MIC - Minimum inhibitory concentration, No - Frequency; % - Percent

Different multi-drug resistance (resistance to two or more antibiotics) was seen in different isolates including; the most common double resistance patterns was seen in sulphamethaxazol/trimethroprim and amoxicillin (93.3%), followed by resistance to

sulphamethaxazol/trimethroprim / nalidixic acid and nalidixic acid / amoxicillin accounting for 38.7% each (table 4). Resistance to nalidixic acid and erythromycin was seen among two isolates (2.6%) while that of nalidixic acid and amoxicillin was seen among one isolate (1.3%). Triple drug resistance included; two (2.6%) isolates resistant to three antibiotics including; tetracycline / sulphamethaxazol/trimethroprim / ampicillin and erythromycin, sulphamethaxazol/trimethroprim and ampicillin. Four (5.3%) isolates were resistant to AC/TS/AM and AC/TS/CL yet other 29 (38.7%) isolates were resistant to NA/TS/CL. Three isolates were resistant to four different antibiotics; one (1.3%) to NA/AC/TS/AM and two others (2.6%) to NA/EM/TS/AM as shown in table 4.

Table 4: The multi-drug resistant types among the *V. cholerae* isolates

Multi-drug resistant types	Frequency	
	No.	%
NA, AC	1	1.3%
NA, EM	2	2.6%
NA, AM	29	38.7%
NA, TS	29	38.7%
TS, AM	70	93.3%
TC, TS, AM	2	2.6%
EM, TS, AM	2	2.6%
AC, TS, AM	4	5.3%
AC, TS, CL	4	5.3%
NA, TS, CL	29	38.7%
NA, AC, TS, AM	1	1.3%
NA, EM, TS, AM	2	2.6%

No - Number, % - Percent

NA-nalidixic acid, AC-Amoxicillin, EM-erythromycin. AM-ampicillin, TS-trimethoprim, sulphamethaxazol, TC-tetracycline, CL-chloramphenicol

4.4. Amplification products for the target genes from PCR assays

Majority (74/75) 99% of the *V. cholerae* isolates carried the cholera toxin gene (ctx*A*) in their genome (table 5). The presence of the target antibiotic resistance genes for sulphonamides, trimethoprim and streptomycin (SXT), sulphonamides (SULii), streptomycin (StraA) and tetracycline (TetA) are shown in Table 5.

Table 5: Presence of cholera toxin and antibiotic resistance genes

Toxin type	Sample size	Proportion of *V. cholerae*		$\chi 2$	P Value
		No	%		
Cholera toxin gene (CTXAB)	74	72	97.3	66.216	0.001
Resistance gene for sulphonamides, trimethoprim and streptomycin (SXT)	74	70	94.6	58.865	0.001
Resistance gene for sulphonamides (SUL ii)	74	68	91.9	51.946	0.001
Resistance gene for streptomycin (StraA)	74	73	98.6	70.054	0.001
Resistance gene for tetracycline (Tet A)	74	73	98.6	70.054	0.001

Number; % - Percentage; $\chi 2$ - Chi square; P value - Level of significance

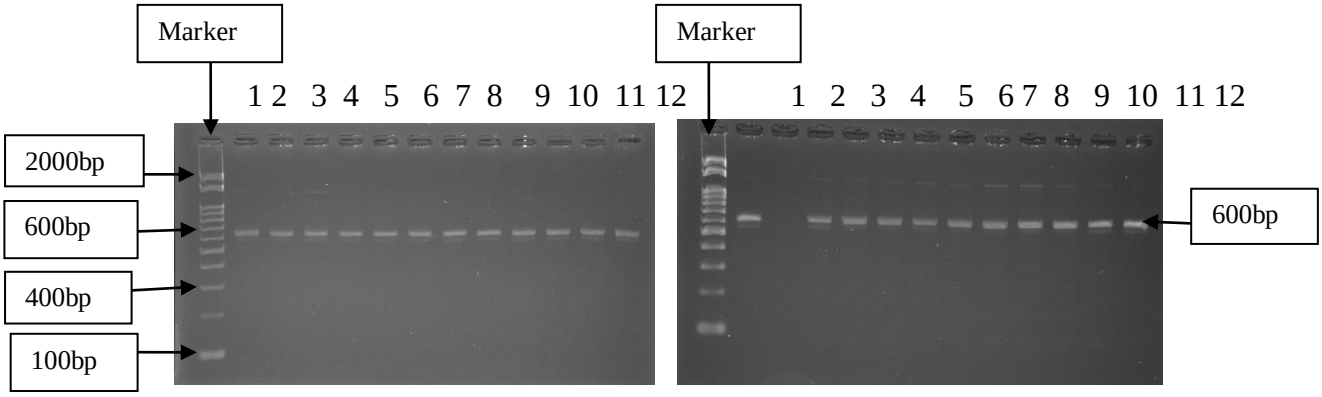


Plate 10 Plate 11

Plate 10 and plate 11 indicating examples of PCR products of *V. cholerae* isolates (VC1 to VC12) using the primers sets *ctx* for cholera toxin gene and *SULII* for sulphonamide resistant gene respectively. The size of the PCR amplicon products was 600bp while the range of the Gene ladder was 0.1-2 kbp.

4.5. Genetic analysis

4.5.1. Allele frequencies

Allele frequencies for the polymorphic loci are presented in table 6. Overall, the allelic variation among these 18 loci was not partitioned equally, in as much as the locus No. 3 and No.6 - 18 were monomorphic, and with all the individuals *V. cholerae* isolates scored possessing a restriction site with identical mobility at each of these loci, and all the other loci (No.1, 2, 4 and No.5) were polymorphic in their restriction sites.

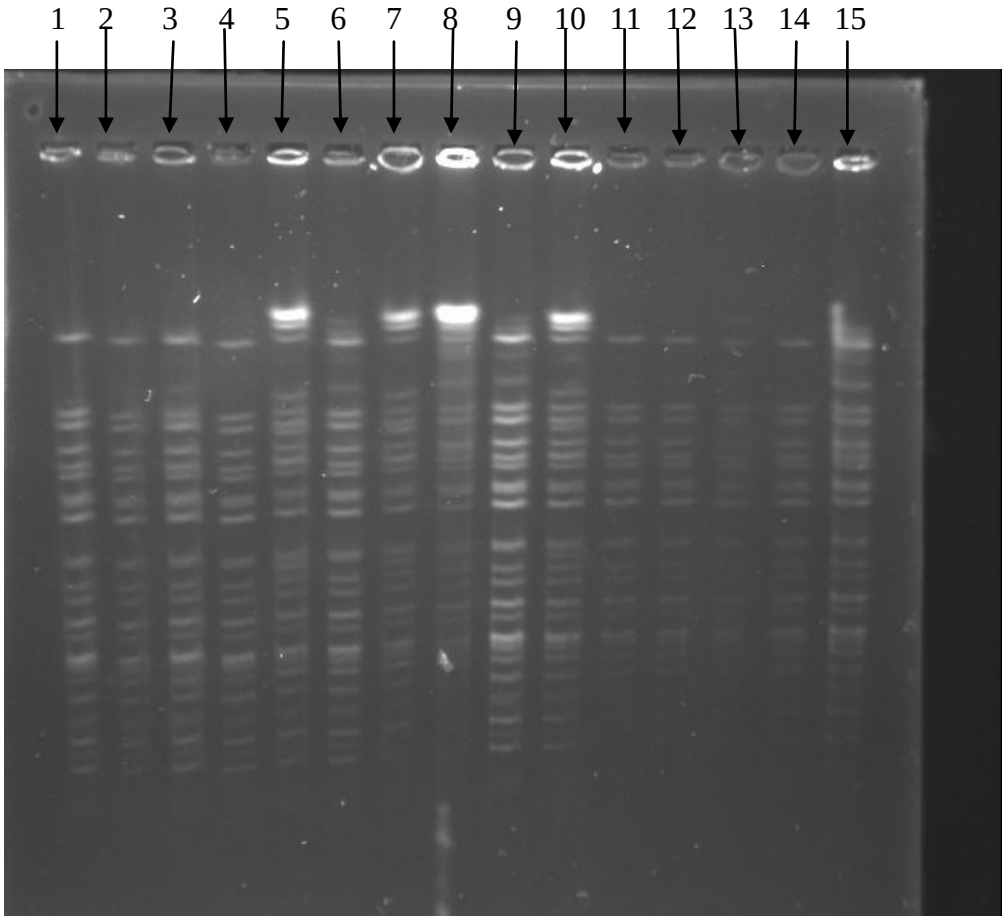


Plate 12

Plate 12 shows the PFGE patterns of *Not1*-digested chromosomal DNA from *V. cholerae* isolates Vc1, Vc2, Vc3, Vc4, Vc5, Vc6, Vc7, Vc8, Vc9, Vc10, Vc11, Vc12, Vc13, Vc14 and Vc15 respectively. Generally the V.cholerae isolates showed a similar PFGE pattern thus indicating that the isolates from Western Kenya were genetically similar strains.

Table 6: The Allelic frequencies estimated at 18 loci for the isolated *V. cholera*.

Locus	**Alleles**	Freq	95% CI	
No. 1	1	0.2	82.70	88.18
	0	0.8	11.52	16.95
No. 2	1	0.36	60.55	68.17
	0	0.64	31.83	39.46
No. 3	1	1		
	0	0		
No. 4	1	0.253	82.70	88.18
	0	0.746	11.52	16.95
No. 5	1	0.48	82.70	88.18
	0	0.52	11.52	16.95
No. 6	1	1		
	0	0	-	
No. 7	1	1		
	0	0	-	
No. 8	1	1		
	0	0		
No. 9	1	1		
	0	0		
No. 10	1	1		
	0	0		
No. 11	1	1		
	0	0		
No. 12	1	1		
	0	0		
No. 13	1	1		
	0	0		
No. 14	1	1		
	0	0		
No. 15	1	1		
	0	0		
No. 16	1	1		
	0	0		
No. 17	1	1		
	0	0		
No. 18	1	1		
	0	0		

1- Present; 0- Absent; Freq - Frequency; CI - Confidence interval

4.5.2. Phenetic relationships

Nei's (1978) unbiased pair-wise genetic distances estimated among the pairs of the 75 *V. cholerae* populations studied ranged from 0.2513 between isolates No. 1 and 55; No.1 and 59 to near zero between some isolates such as N0.1 and 11, 12, 13and 14 . Cluster analysis (UPGMA) was used to produce a phenogram and to show the genetic relationships of the populations studied (Fig.6 and Fig.7).

4.5.3. Phenetic relationship based on location of *V. cholerae* isolation

Generally three major clusters of the *V. cholera* isolates were identified based on the location of isolation. There was no unique clustering identified based on the sources or location of isolation. Generally *V. cholera* were unique and no clustering together were identified based on the location of isolation (Fig. 6)

4.5.4. Phenetic relationship based on the year of *V. cholerae* isolation

Similarly here three major clusters of the *V. cholera* isolates were identified based on the year of isolation. No unique clustering was identified based on the year of *V. cholearae* (Fig.7).

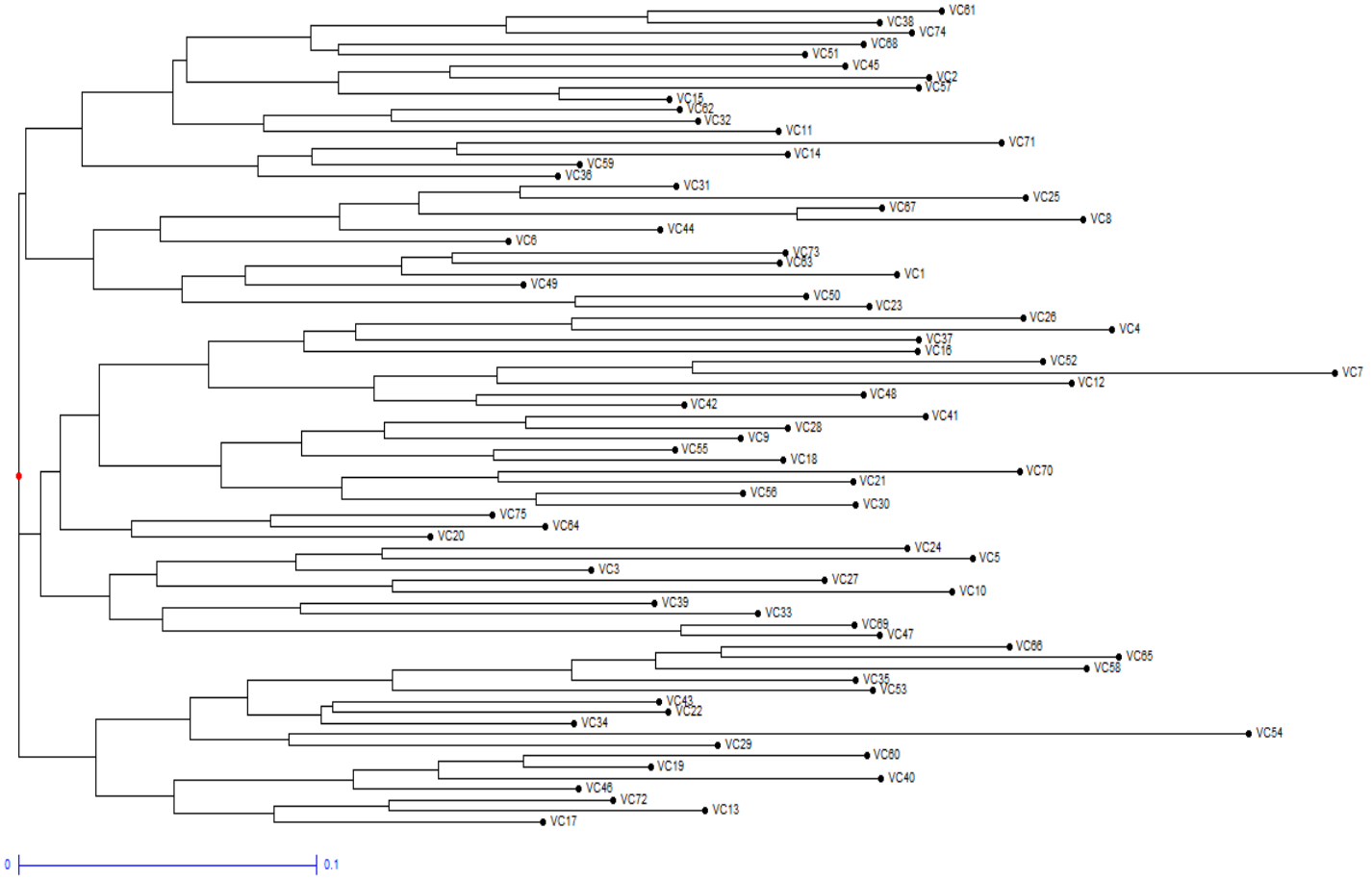


Fig. 6: The Neighbor Joining tree showing the phenetic relationship of the *V. cholera* isolates based on the location of isolation.

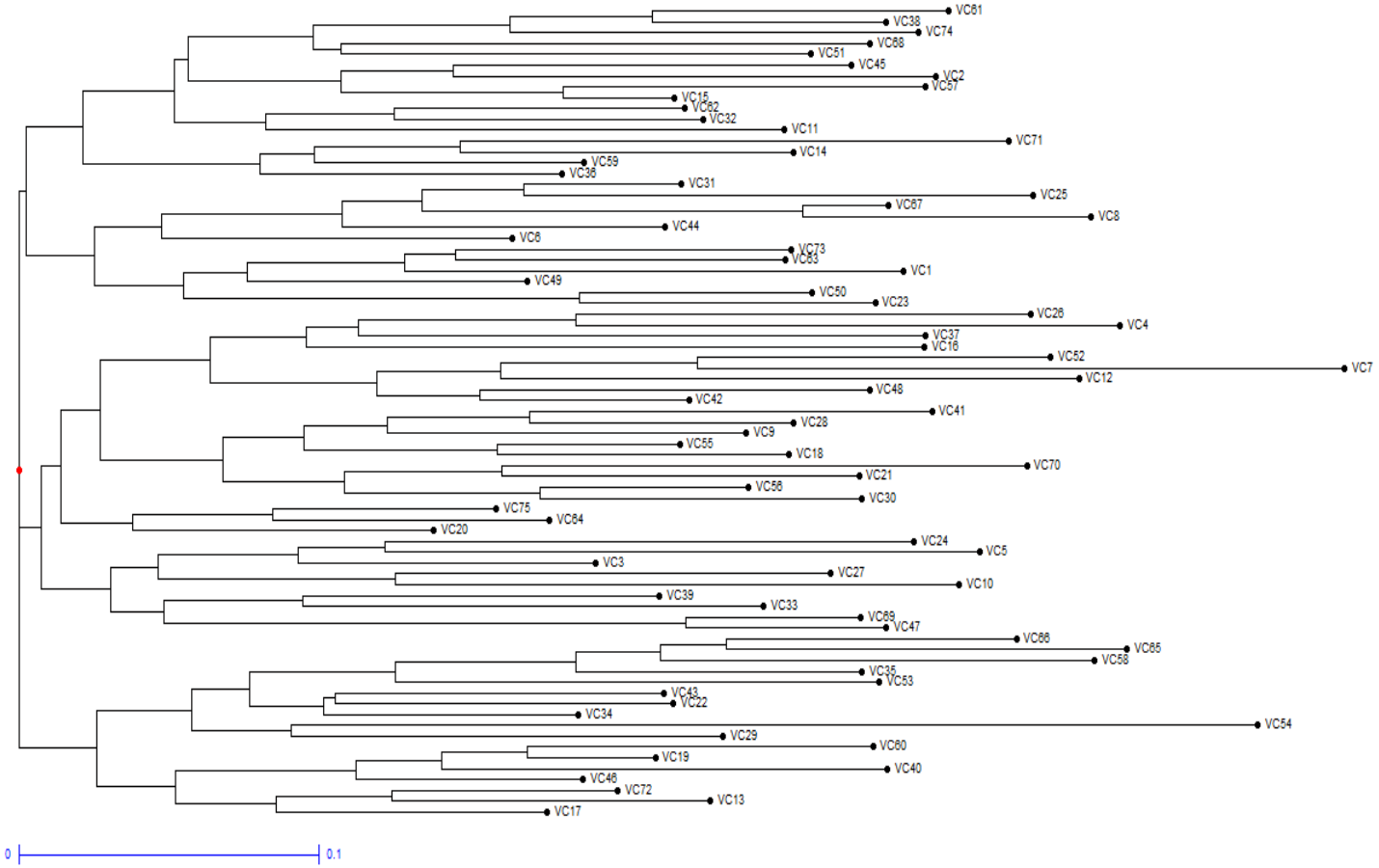


Fig. 7: The Neighbor Joining tree showing the phenetic relationship of the *V. cholera* isolates based on the year of isolation.

CHAPTER FIVE

5.0. DISCUSSION

Majority of isolates were obtained in the year 2007 because the outbreaks started in Suba in November of the same year and spread further to cover all the lake basin districts and the other Western Kenya regions. This further explains why Mbita and Suba contributed the highest number of *Vibrio cholerae* isolates. As suggested by Kaufmann *et al*., 1994, the unique characteristics of the *V. cholerae* El Tor strain confer upon it a high degree of "epidemic virulence" that allowed it to spread across Western Kenya region. In 2008, a cholera outbreak with unusually high mortality occurred in western Kenya during civil unrest after disputed presidential elections. Through active case finding, it was found there was a 200% increase in fatal cases and a 37% increase in surviving cases over passively reported cases; the case-fatality ratio increased from 5.5% to 11.4% (Shikanga et al., 2009). The mass movement of people during the post-election crisis may also have contributed to the outbreak and spread of the disease as people found themselves in areas with inadequate water and sanitation facilities.

The detection and identification of *V. cholerae* requires three successive phases: (i) enrichment in selective medium, (ii) plating onto isolation agars and presumptive identification, and (iii) confirmation with biochemical, serological, and toxigenicity tests. This method is based on a procedure published in the Bacteriological Analytical Manual (BAM, 1998). In this research, all the seventy five (75) *V. cholerae* isolates

were subjected to this principle and they were therefore conclusively confirmed to be pathogenic *V. cholerae* O1 El Tor biotypes. The stored samples were individually enriched in sterile alkaline peptone water (APW) before being plated onto thiosulphate citrate bile salts agar (TCBS) agar. After 18-24 hour incubation on TCBS agar, all the 75 samples formed medium-sized, smooth, yellow colonies with opaque centers and translucent peripheries. The colonies appeared yellow because the *V. Cholerae* samples fermented sucrose. Other *Vibrio spp*. that doesn't ferment sucrose appears green on TCBS. Another selective medium that can be used for *V. cholerae* is modified cellobiose-polymixin B-colistin (mCPC) agar on which colonies of *V. cholerae* El Tor are green to purple because they don't ferment cellobiose. Most other *Vibrio spp*. do not grow readily on mCPC agar. The reactions of samples on triple sugar iron (TSI) agar or Klingler iron sugar (KIA) agar slants and butts allowed for the early presumptive differentiation of *Vibrio spp*. from other enteric bacteria like *Aeromonas, Plesiomonas,* and *shigella BAM*. This was inferred from the acid slant, acid butt and negative for both gas and hydrogen sulphide.

Determination of classical and El Tor biotypes was done based on two major tests namely the hemolysin test and hemaglutination tests. All the isolates were 100% positive for the two tests (table 1) and were thus concluded to be El Tor biotypes. The sensitivity of the isolates to polymixin B and their Voges-Proskauer tests were varied and divergent (table 1) but this did not hinder the differentiation of the biotypes because basically they are supplementary tests. Usually the El Tor strains will either grow to the

edge of the polymixin B disk or will be inhibited slightly. It is recommends by BAM 1998, that the use of more than one test is necessary to differentiate biotypes. The bacteriophage susceptibility of the bacterial isolates can also be a useful test to distinguish the two biotypes of *V. cholerae* although it tedious and expensive to perform.

The serological characterization was done after all the biochemical tests were complete because antigens in different species of *Vibrio* bacteria may be related therefore can cross-react. All the isolates agglutinated in group O1 antiserum, and thus were taken to belong to *V. cholerae* group O1 because the biochemical tests had confirmed them to be *V. cholerae.* Since there were no isolates that did not agglutinate in group O1 antiserum, then it is correct to say that there was no any non-O1 *V. cholerae*. Therefore there was no need to test the isolates with O139 antiserum. On further sub- typing with Inaba and Ogawa antibodies, 97% of the isolates agglutinatinated in Inaba while only 3% agglutinated in Ogawa. This relates with the findings of Kariuki *et al*., (2005), in which the cholera outbreaks in Kenya and Africa in general are caused by *V. cholerae* O1 El Tor Inaba and Ogawa but rarely has the Hikojima serotype been encountered. Actually we can say that the pathogenic *V. cholerae* has remained stable in terms of its biotype, serotype and sub-serotypes. A possible explanation why the Hikojima subtype was not encountered in this research is that this particular subtype is very unstable and sometimes its very difficult to type it because it agglutinates both the Inaba and Ogawa antisera indicating that it carries all the three factors (A, B and C). It's also confirmed

that isolates that agglutinate in poly (group O1) antisera but not in Inaba and/or Ogawa antisera cannot be typed using these antisera. Thus the *V. cholerae* O1 El Tor biotype continues to be the etiological agent responsible for virtually all cholera cases throughout Western Kenya during the entire period this research (2007 to 2009). The climatic changes of the year 2007 as postulated by Shikanga *et al.,* 2009 may have had a direct importance in the dissemination and the dynamics of transmission of infectious diarrheal diseases especially cholera. In Western Kenya, the occurrence of diarrheic illness of bacterial etiology and the temperature have always shown a direct relationship as has been reported in previous research finding by Julie *et al*., 2009.

From the Etest results, the *V. cholerae* isolates exhibited both susceptibility and resistance to the fifteen (15) antibiotic agents tested in this research (table 3). All the *V.cholerae* isolates were 100% susceptible to ceftriaxone. The increase in antimicrobial resistance is frequently reported in areas where cholera is endemic as indicated by Kariuki *et al*., 2005, for example in Western Kenya. This is due to the constant treatment with certain antibiotics like from this research, the antibiotic ampicillin, hence the antibiotics are known to quickly reduce the fecal excretion of *V. cholerae* and they can cover a wide spectrum of enteropathogens. However this has been shown to increase antibiotic resistance to these frequently used brands as suggested by Pan *et al* (2008). This explains why in this research, all isolates were 100% resistant to ampicillin, 93% resistant to sulphamethaxazol/trimethoprim and 38% resistant to nalidixic acid (table 3). Furthermore, the costs of the antibiotics can be an important

factor in the use of these antibiotics. Kariuki *et al.,* 2005 suggested that to increase antibiotic susceptibility, the utilization of antimicrobial in treatment should be reorganized and reevaluated. For the reduction or prevention of development of antibiotic-resistant pathogens (*V. cholerae*), both the prescribers and patients should follow the guidelines for limited and rationale exploitation of antibiotics.

Some *V. cholerae* isolates showed multiple resistances to some commonly prescribed/used antibiotics (table 4).This indicates the possibility of such isolates carrying plasmid(s) encoding for multi-drug resistance. The drug resistance conjugative plasmid PMRV150 has been reported from china in *V. cholerae* O139 by Pan *et al,* (2008) and this plasmid mediates multiple-drug resistance (MDR) to atleast six antibiotics including ampicillin, streptomycin, Gentamycin, tetracycline, chloramphenicol and trimethoprim/sulphamethaxazol. In this research, it is also evident that the MDR involved these particular antibiotics as indicated in table 5. Therefore more research is required to confirm this or explain this phenomenon of MDR in the *V. cholerae* isolate from western Kenya.

The PCR identification of the target genes indicated the presence of the cholera enterotoxin (ctxA) gene in 74 out of the 75 *V. cholerae* isolates (table 5). The presence of this gene in the genome of the bacteria qualifies a *V. cholerae* isolate to be classified as a toxigenic strain and this gene is unique only to these strains and is basically absent in non-toxigenic strains of V .cholerae. The ctxA gene is responsible for the production of the cholera toxin (CTX) which is a major virulent factor for the cholera disease. The

coordinate expression of the network of pathogenecity genes enables *V. cholerae* organisms to colonize the small intestines and produce the cholera toxin which leads to a secretory diarrhea. These correlates well with the patient rectal swaps from which the seventy five *V. cholerae* isolates were isolated from. Since the PCR results that showed the of presence of the ctxA could not used to conclusive determine complete enterotoxigenic, its therefore necessary to check for cholera toxin production using either bioassays like the rabbit illeal loop test or in-vitro tests like reverse passive latex agglutination (RPLA) test.

The presence of target antimicrobial resistance genes in the genome of the *V. cholerae* isolates as indicated by the PCR results (table 5) did not tally with the phenotypic antibiotic characteristics (resistance). These results agree with the findings of both Shikanga *et al* (2009) and Kariuki *et al* (2005).Some isolates were sensitive to a specific antibiotic like tetracycline yet they did not contain the tetA gene in the genome. These points to a possibility that the tetA gene was not expressed in that particular *V. cholerae* isolate. The presence of antimicrobial resistance genes in the genome *V. cholerae* isolates could have determined the increment from resistance to different antimicrobials of clinical use in strains isolated during the 2007 to 2009 outbreaks compared to the previous year's outbreaks.

The pulsed field gel electrophoresis of the *NotI*-digested chromosomal DNA showed that all the seventy five (75) *V. cholerae* isolates causing cholera in Western Kenya were generally genetically similar strains. This is because there no identifiable unique clustering based on the sources or location of isolation (Fig6 and Fig 7).

Similarly here three major clusters of the *V. cholera* isolates were identified based on the year of isolation. No unique clustering was identified based on the year of *V. cholerae*. Basically all bacteria isolated from both Nyanza and Western provinces were genetically similar. A likely explanation for this finding is that the cholera outbreaks spread from Suba, the epicenter of the cholera outbreak to other districts in Western Kenya. Hence there was no specific clustering of the isolates. Therefore this genetic similarity together with temporal clustering combined with transport connections between the locations of isolation suggest that some or all the cholera outbreaks might be linked epidemiologically. However this genetic similarity of the *V. cholera* isolates from Western Kenya does not necessarily prove an epidemiological link but it rather indicates a predominant clonal strain in circulation during that period of 2007 to 2009.

CHAPTER SIX

6.0. CONCLUSIONS AND RECOMMENDATIONS

- The cholera outbreaks during the period 2007 to 2009 in Western Kenya were mainly caused by the *Vibrio cholerae* O1 El Tor biotype. The predominant sub serotype was Inaba (97%), followed by the Ogawa (3%) sub serotype and there was no Hikojima sub serotype.
- For the entire period of this research study, the circulating pathogenic *V. cholerae* remained stable in terms of its biotype, serotype as well as the sub serotypes.
- All the *V. cholerae* isolates from western Kenya were pathogenic (100%). Of these (97%) of isolates were toxigenic since their PCR amplicon contained the cholera toxin (ctxA) gene.
- The *V. cholerae* isolates showed varied sensitivity and resistance to the fifteen test antibiotics. All the isolates showed 100% sensitivity to ceftriaxone and 100% resistance to ampicillin.
- The phenetic analyses of the isolates did not show any significant genetic diversity in terms of the location and the year of isolation. Similarly there was no unique clustering of the *V. cholerae* isolates.

RECOMMENDATIONS

The following recommendations are put forward for further investigation of the pathogenic *V. cholerae* isolates from Western Kenya

1. The use of *in vitro* assays like reverse passive agglutination to detect and quantify the amount of virulence factors (cholera toxin, hemolysin and pili) produced by the isolates.

2. A follow-up study on the current state of antibiotic sensitivity/resistance patterns of the isolates to commonly prescribed antibiotic drugs.

3. Enumeration of the pathological potential of the *V. cholerae* isolates using the *in vivo* assays including the suckling mice test and the RITTARD.

4. The isolation and characterization of the bacterial proteins expressed by the *V. cholerae* isolates using SDS-PAGE, western blotting and size exclusion chromatography

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APPENDICES

Appendix 1: Summary of Etest performance, interpretive criteria and quality control ranges for *V.cholerae* results

Drug	**MIC**	**S≤**	**I**	**R≥**	**Limit**
CI-Ciprofloxacin	0.002-32µg/mL	1	2	4	32
TXL-Ceftriaxone	0.002-32 µg/mL	8	16.32	64	32
TC-Tetracycline	0.016-256 µg/mL	4	8	16	256
TS-Trim/Sulf	0.002-32 µg/mL	2	-	4	32
KM-Kanamycin	0.016-256 µg/mL	16	32	64	256
NA-Nalidixic acid	0.016-256 µg/mL	16	-	32	256
SM-Streptomycin	0.016-256 µg/mL	-	-	1000	256
TX-Ceftriaxone	0.002-32 µg/mL	8	16	64	256
AC-Amoxicillin	0.016-256 µg/mL	2	4	8	256
EM-Erythromycin	0.016-256 µg/mL	1	2	4	256
CL-Chlororamphenicol	0.016-256 µg/mL	8	16	32	256
DC- Doxycycline	0.016-256 µg/mL	4	8	16	256
OF-Ofloxacin	0.002-32 µg/mL	2	4	8	32
AM-Ampicillin	0.016-256 µg/mL	0.25	-	0.5	256
GM-Gentamycin (Low)	0.016-256 µg/mL	6	8	16	256
GM-Gentamycin (High)	0.064-1024 µg/mL	4	8	16	1024

Appendix 2: PCR Primers Sequences used for detection of target genes

Primer, genes, and Sequence (5'→3')	**Amplicon size (bp)**	**PCR conditions** Denaturation	Annealing	Extension
Int, SXT element F: GCTGGATAGGTTAAGGGCGG R: CTCTATGGGCACTGTCCACATTG	592	95˚C, 1m	54˚C, I m	72˚C, I m
StrA, Streptomycin resistance F: TTGATGTGGTGTCCCGCAATG R: CCAATCGCAGATAGAAGGCAA	383	95˚C, 1m	54˚C, I m	72˚C, I m
Sul2, Sulphamethaxazol resistance F: AGGGGGCAGATGTGATCGAC R: TGTGCGGATGAAGTCAGCTCC	625	95˚C, 1m	54˚C, I m	72˚C, I m
TetA-2000, tetracycline resistance F: GTAATTCTGAGCACTGTCGC R: CTGCCTGGACAACATTGCTT	950	95˚C, 1m	58˚C, I m	72˚C, I m
CtxA, CT Subunit A F: TCAATTAGTTTGAGAAGTGC R: TCAGATTGATAGCCTGAAAA	564	95˚C, 1m	54˚C, I m	72˚C, I m

Appendix 3: **Preparation of stock solutions**

Preparation of stock solution (×20) Tris Borate EDTA (TBE)

Weigh (i) 216g Tris base

(ii) 110g Boric acid

Dissolve both in approximately 900ml of deionised water and then add 20ml of 0.5M EDTA (pH 8.0).adjust the solution to a final volume of 1 liter. Autoclave and store at room temperature in a glass bottle.

Preparation of phosphate buffered solution (PBS)

Dissolve one tablet of PBS in 200ml of deionised water, then autoclave and store at room temperature.

Preparation of Proteinase K buffer

Measure 1 ml of 1M potassium chloride into a coning tube and add 2ml of 100mM tris hydrochloric acid followed by 1ml of 50mM magnesium chloride and 1 ml of 10% tween 20. Add 8 mg of proteinase K and mix on ice