RELATIONSHIP BETWEEN PHYSICOCHEMICAL FACTORS AND OCCURRENCE OF *VIBRIO* STRAINS IN THE ENVIRONMENT OF THE LAKE VICTORIA BASIN

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ABSTRACT

Cholera is a substantial health burden in the developing world and is endemic in Africa, Asia, South and Central America. The exact scale of the problem is uncertain because of limitations in existing surveillance systems, differences in reporting procedures, and failure to report the disease to the World Health Organization. Lake Victoria basin bares the greatest burden of cholera outbreaks in Kenya due to sporadic cases and seasonal epidemic associated with poverty and low hygienic standards. The polluted water in the region enhances prolonged survival of *V. cholerae* through phage formation and therefore transmission is hypothesized to radiate from these sources. Therefore, establishing the relationship between physicochemical factors, colony forming unit and occurrence of *Vibrio* strains in this region is essential in order to discern confounding factors that enhance the epidemiology of the respective strain in the regions as well as annotate sequences that would be useful in molecular diagnostic kits and possibly add to the existing vaccine candidate sequences with the goal of controlling the occurrence and spread of cholera. The objective of this study therefore was to determine the relationship between physicochemical factors, colony forming unit and occurrence of *Vibrio* strains in the environment of the Lake Victoria Basin.This was a cross-sectional study where environmental (water) and clinical samples (stools) were collected from Migori, Nyando, Sondu, Bondo and Yala regions in Western Kenya and transported to the Molecular Microbiology Laboratory at the department of Zoology, Maseno University for isolation and identification of *Vibrio* species using conventional microbiological methods. A total of 811 samples (596 water and 215 stool samples) were collected during the study periods of May to December 2013 and August to September 2014. The human stool samples were collected from Migori District Hospital (120), Nyando District Hospital (82) and Bondo District hospital (13) using study permit issued by the Maseno University Ethics Review Committee and the hospital authorities. Water samples were collected from rivers, viz:-. Migori (147), Sondu-Miriu (99) Nyando (109), Yala (151) and Bondo swamps (90). The average colony forming unit and physicochemical factors variability was calculated as the geometric mean and standard deviation respectively. Quantitative Polymerase Chain Reaction (PCR) technique was used for molecular identification of *Vibrio* strains. Species-specific primers for *Vibrio* strains (*V. cholera, V. parahaemolyticus* and *V. vulnificus*) were used where DNA extracts did not amplify with the intended primer sequences. Species-confirmed isolates were screened for virulence-associated genes. *Vibrio vulnificus* and *V. cholerae* were isolated in the study region. However, *V. parahaemolyticus* was not found in any of the isolates during the study period. The waters where *V. cholerae* was isolated had a pH range of between 7.7 - 8.2 ($P \leq 0.01$), temperature of 22-28°C ($P \leq 0.01$), water salinity of 17-161.2 µS•cm–1 (0.2 to 2.3% ($P \leq 0.01$). Serologically, the type of *V. cholerae* identified in these regions was inaba and ogawa. The PCR results for 16SrRNA, Vib 1, Vib 2 showed that there was polymorphism in the genes, an indication that there was high frequency recombination (Hfr) of genes from more than one strain in one isolate. The analysis showed the presence of species specific *ctxA* genes (564bp) responsible for cholera toxin. The study showed the presence *V. cholerae* (Ogawa and inaba) and Type B *V. vulnificus* in water and human stool in the study area. These results are crucial in controlling and managing unpredictable cholera outbreaks in this region. This can be done through ensuring that physicochemical parameters which enhance the growth of *Vibrio* strains in the region are monitored and constant survaillace undertaken to mitigate circulating strains of *Vibrio cholerae* and *V. vulnificus* in the region.

CHAPTER ONE

INTRODUCTION

The genus *Vibrio* is in the family Vibrionaceae, which also includes the genera *Aeromonas*, *Plesiomonas*, and *Photobacterium* (Atlas, 1997). *Vibrios* have been classified by scientists based on their respective environmental survival preferences which are suitable; salinity, pH, temperature, planktons. In this regard, the most pathogenic *Vibrio* strains to human are *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* (Neog *et al.*, 2010).

Cholera epidemics are caused by *Vibrio cholerae* 01 (El Tor and classical biotypes) and 0139 serogroups while some strains of serogroups (non-01/non-0139) are associated with sporadic gastroenteritis. Cholera continues to be an important cause of morbidity and mortality in many areas of Asia, Africa, and Latin America (WHO, 2010). It is important to note that the numbers of reported cholera cases to WHO have been on the increase both globally and regionally (WHO, 2010). However, the exact total number of cases affected by the outbreak of cholera is not known since not all the cases are reported (WHO, 2010). Therefore the actual cases of cholera on the ground are never refelected (WHO, 2010). The developing world is highly affected by cholera outbreak, Africa alone recorded over 2.4 million cases and 120,000 deaths from 1970 to 2005 (WHO, 2006). This accounts for over 90% of both worldwide cases and deaths (WHO, 2004; WHO, 2005; WHO, 2006). These statistics are on the increase and according to WHO (2009), between 2004 to 2008, a total of 838,315 cases were notified to WHO, compared with 676,651 cases between 2000 and 2004, representing a 24% increase in the number of cases (WHO, 2009). In 2009, 177,993 cases and 4,031 cholera deaths were reported to the WHO (WHO, 2007; Julie *et al.,* 2009). Kenya has not been spared in the cholera outbreak (Shapiro *et al.,* 1999). Waves of cholera recrudescence has between experienced in the country with the largest epidermic occuring in 1997 with 17,200 cases notified to the WHO (Shapiro *et al.,* 1999). Most of the epidemics in the Western region start in Migori at the boarder of Kenya and Tanzania and spread to Kisumu and then to Siaya as experienced in 1997 (Shapiro *et al.,* 1999). From 2000 to 2006, cases were reported each year ranging from 1,157 to 816 except for 2002, with 291 cases (WHO, 2010). In 2007, the cholera

outbreak affected 9 districts: West Pokot, Turkana, Kwale, Garissa, Wajir, Mandera, Kisumu, Bondo and Siaya (Shikanga *et al.*, 2009).

During epidemics of cholera, the control measures put in place include education of the public on the need to seek immediate treatment, provision of appropriate clinical treatment facilities, disinfection of drinking water, education on hygienic food preparation, and provision of adequate facilities for human waste disposal (Heymann, 2008). These are public health intervention initiatives that help reduce the spread of outbreaks in the respective regions. However, getting to know the causative factors of these outbreaks is always assumed since medics and paramedics react to situation analysis interventions. Thus it is imperative that the route cause of these outbreaks be determined and documented for future implenetations. Of late, there are two promising oral cholera vaccines which are available (WHO, 2011) and they are both administered to adults and children over two years in two doses spaced one to six weeks apart and confer immunity for up to 2 years (WHO, 2010). WHO recommends use of the oral cholera vaccine to complement other prevention and control measures in areas where cholera is endemic and in areas at risk for outbreaks (WHO, 2010). WHO has also developed a risk assessment tool and decision-making tool to assist health authorities in determining whether to use the oral cholera vaccine in complex emergencies (WHO, 2010). Vaccination is not considered to be effective after an outbreak has started unless the vaccination campaign is targeted at other well defined, high risk populations not yet affected by the outbreak (WHO, 2010). However, due to both financial, personnel and infrastructural constrains in our health facilities; enforcement of this intervention initiative is usually underscored thus leading to a vulnerable group of individuals within these regions. This requires risk assessment and exposure rate determination in order to galvanize and protect the suffering groups hence this study.

Kenya has a number of health-related laws, policies, and strategies with relevance to cholera prevention and control. These include National Environmental Sanitation and Hygiene Policy which is geared towards access to "hygienic, affordable, functional, and sustainable toilet and hand washing facilities" in "every school, institution, household, market, and other public place" by 2015 (MoH, 2007). In 2007 Plan Kenya introduced the Community Led Total Sanitation (CLTS) approach in selected rural

communities. This aimed to mobilize communities to eliminate open defecation and promotes hygiene practices like hand washing (MoH, 2007). In 2010 the Ministry of Public Health and Sanitation, in partnership with UNICEF and SNV, pilot tested CLTS in 6 districts in Western Kenya (MoPHS, 2012). The Ministry subsequently adopted CLTS as its strategy for eliminating open defecation and launched a campaign in 2011 to declare Kenya open defecation free (ODF) by 2013. The community strategy is another national strategy that may be relevant to cholera prevention and control. In 2007, the Ministry of Public Health and Sanitation adopted this strategy in the National Health Sector Strategic Plan II. The community strategy aims to enhance community access to health care and to strengthen household and community participation in health and health-related development issues (MoPHS, 2008). It is also worth noting that Kenya adopted the WHO African Regional Office (WHO AFRO) Integrated Disease Surveillance and Response (IDSR) strategy that was introduced in 1998 as a means of improving disease surveillance and response to epidemics. In 2007 WHO AFRO issued a resolution on the resurgence of cholera in Africa (WHO, 2007). This resolution acknowledged that the cholera situation in Africa had been worsening since the early1990s and urged member states to engage in activities to strengthen cholera prevention and control including development of multi-sectoral plans.

Vibrio parahaemolyticus is among the leading cause of seafood-borne gastroenteritis in developing and developed countries (Nair *et al.*, 2007). In the U.S.A. gastroenteritis due to consumption of *V. parahaemolyticus* contaminated seafood leads to estimated 35,000 cases each year (Scallan *et al.*, 2011). In a study conducted from 1998 to 2000 in United Nations, 62% of *V. parahaemolyticus* illnesses was due to contaminated oyster consumption and wound associated (Urakawa and Rivera, 2006). In South Korea, a total of 2,242 Koreans were infected due to the consumption of fish contaminated with pathogenic *V. parahaemolyticus* from 2003 to 2006 (Kaysner and DePaola, 1998). In addition, *Vibrio vulnificus* causes gastroenteritis, necrotizing fasciitis and wound infections with high fatality rate in immunocompromised patients (Jones and Oliver, 2009). Wound infection can occur as a result of exposure of open and/or breached skin surface to the contaminated water, and consumption or handling of marine infected fish (Howard and Lieb, 1988; Shapiro *et al.*, 1998). *V. vulnificus* is responsible for 95% of all seafood related mortalities, and carries a 50% to 75% mortality rate with primary septicemia. Patients suffer septicemic shocks (Rippey,

1994). Inflammation occurs around the wound site, and the disease can advance to lesions similar to those of primary septicemia, and finally to septicemic symptoms. Fatality ranges from 20 to 30%. According to CDC report in 2014, since 2006-2008, an average of over 600 cases of *V. vulnificus* infections have been reported annually through the Center for Disease Control's COVIS system (http://www.cdc.gov/nationalsurveillance/cholera Vibrio surveillance.html). Among these cases, approximately 80% of infections occurred when the level of *V. vulnificus* in the environmental reservoir and marine environment were high (Motes *et al.*, 1998) indicating seasonality of infection.

Vibrio cholerae, *V. parahaemolyticus* and *V. vulnificus* can all exist in aquatic environment and diseased animal (Mahmud *et al.*, 2008). Environmental factors, such as temperature and rainfall, which are broadly termed as "climate", play a decisive role, not only in cholera outbreaks, but in many infectious diseases caused by bacteria pathogens (Colwell, 1996; Pascual *et al.*, 2000). Temperature dependence in the environment is evident from the seasonal dominance exhibited by some of the *Vibrio* species (Hilton *et al.*, 2006). *Vibrio* presence is documented to coincide with warm temperatures in several studies and temperature variations have been recorded to influence virulence factor and survival of bacteria (Hilton *et al.*, 2006). The relationship between physicochemical parameters and existence of *Vibrio* strains in the study region has never been established but speculated especially in the era of climate change and zonation of micro-climate. In this regard, it is necessary to determine and document these factors.

In *in vitro* conditions, tolerance to temperature varies with *Vibrio* species. Effect of pH on molecular mechanisms of bacteria has been extensively studied (Hulsmann *et al.*, 2003; Park *et al.*, 2004). Most of the species of *Vibrio* can tolerate moderately alkaline condition and can grow at pH 9 (Park *et al.*, 2004). *Vibrios* are aerobic or anaerobic facultative organisms and have various survival patterns in various oxygen concentrations (Farmer, *et al.*, 2005). The survival pattern of *Vibrio* species in controlled oxygen levels have not been attempted so far in this study region and hence its inclusion as one of the physicochemical parameters in this study.

All *Vibrios* are ubiquitous in the marine environment and all species except *Vibrio cholerae* and *Vibrio mimicus* require sodium chloride supplementation of media for growth (Drake *et al.*, 2007). Salinity can play a critical role in *Vibrio* distribution. When salinity ranges are too high or too low, the number of *Vibrios* are expected to be low (Jackson *et al.*,1997). Salt water intrusion is expected to cause future pressure on the fresh water supplies and may alter salinity regime in estuarine and brackish habitats (Jackson *et al.*, 1997). These factors have in the past contributed heavily to cholera outbreak in the Western region especially after a long span of drought followed by heavy rainfalls (Feikin *et al.*, 2010). Hence, the need to establish the salinity of water in this study. Going by these observations, it is therefore important to study environmental factors (physico-chemical parameters) in a given region with intention of understanding their contribution to *Vibrio* disease causing dynamism in a community set-up.

Most species produce oxidase and catalase and ferment glucose without producing gas (McLaughlin, 1995). This is a key factor that is considered in determining this genus (McLaughlin, 1995). The world is experiencing the seventh pandemic of cholera which began in 1961 in Southern Celebes, spreading to Middle East and Europe and reaching Africa via Guinea in 1970 (Barua, 1992). This is caused by *Vibrio cholerae* 01 biotype E1 Tor (Butterton and Calderwood, 1995). However, in October 1992, a new variant of vibrio appeared in a suburban area north of Madras, India (Nair *et al.,* 1995). This was designated a new serotype, 0139 syn. Bengal, according to Sakazaki typing system (Albert, 1994). It is in view of this that phenotype of pathogenic *Vibrio* species circulating in the study region was determined to provide information which is important to epidemiology and public health in preventing or controlling their related diseases.

Bacterial viruses (phages) are known to play a critical role in the evolution of pathogenic bacterial species, and *V. cholerae* in particular (Faruque *et al.,* 2005). It has been reported that the presence of bacterial viruses acting on *V. cholerae* O1 or O139 (cholera phages or vibriophages) inversely correlates with the occurrence of viable *V. cholerae* in the aquatic environment and the number of locally reported cholera cases (Faruque *et al.,* 2005).

Vibrio goes to lytic stage and forms phage when environmental conditions for its survival are not attainable (Urakawa and Rivera, 2006). At this stage the *Vibrios* are viable but none culturable (VBNC) and can not therefore grow in any culture media (Childers and Klose, 2007). At this point they form biofilm in respective medium (food, sediments, industrial equipment etc) that are equally difficult to determine using conventional procedures like CFU analysis, next generation sequencing (NGS), flow cytometry, Propidium Monoazide quatitative PCR (PAMqPCR) among the others. When environmental conditionsd are favourable then the *Vibrios* become active and when it attains an infectious dose then it causes cholera to a healthy person (Childers and Klose, 2007). It was therefore important to establish the colony forming units of the isolated pathogenic *Vibrio* species in the study region to realate it to any would be outbreak of cholera.

The relationships among members of the genus *Vibrio* have been extensively studied by use of phenotypic and genotypic characterization methods hence grouping them into 65 *Vibrio* species (Iwamoto *et al.*, 2010). The overall genotypic diversities differ depending on the pathogenicity of strains: Pandemic strains show a high uniformity, whereas non-pandemic strains are highly diverse, leading to the observation that an analyzed geographically restricted subpopulation is genetically as diverse as the entire worldwide pubMLST (Multilocus data base) database (Chowdhury *et al.*, 2004; Ansaruzzaman *et al.*, 2008; Neog *et al.*, 2010; Ellis *et al.*, 2012; Theethakaew *et al.*, 2013). The *Vibrio* pathogenic species produce various virulence factors including enterotoxin, haemolysin, cytotoxin, protease, lipase, phospholipase, siderophore, adhesive factor and/ or haemagglutinins (Zhang and Austin, 2005). The virulence of *V. parahaemolyticus* strains is commonly associated with expression of thermostable direct hemolysin (TDH) and TDH-related hemolysin (TRH), which are encoded by the *tdh* and *trh* genes. Therefore, the *tdh* gene, marked by a β-type haemolysin (Nishibuchi and Kaper, 1995), and the *trh* gene, correlates to a positive urease tests (Okuda *et al.* 1997) and serve as markers for pathogenic strains. Similarly, the major virulence-associated genes which encode colonization factors and cholera toxin (CT) in the *V. cholerae* are part of larger genetic elements composed of clusters of genes (Pearson *et al.*, 1993). The CT is encoded by cholera toxin (*ctx*) gene (Pearson *et al.*, 1993). According to Aidara *et al.*, 1998, the toxin increases an ADP ribosylation reaction in

epithelial cells. This results in activation of adenylate cyclase that leads to increased intracellular levels of cyclic Adenosine monophosphate (cAMP), alteration of ion transport and finally to secretory diarrhea (Aidara *et al.*,1998). Both clinical and environmental strains of *V. vulnificus* expresses secreted factors such as cytolysin/hemolysin (*vvhA* gene) and metalloprotease (*vvpE*), which were initially thought to contribute to pathogenicity in mammalian models (Aidara *et al.*, 1998). Establishment of the genotype of the *V. cholerae, V. parahaemolyticus* and *V. vulnificus* in a given region is important in order to avoid new infections and reduce fatalities given the fact that these strains produce various virulent factors (Zhang and Austin, 2005). It was therefore deemed important to establish the virulent gene in the pathogenic *Vibrio* strains isolated from the study region.

Based on the above background, this study was therefore set to determine the relationship between physicochemical factors, colony forming unit and identification (Phenotype and Genotype) of *Vibrio* strains isolated from environmental and clinical samples from Lake Victoria Basin in Western Kenya with the aim of developing a model for predicting cholera to allow intervention in preventing cholera epidermic and any other disease caused by the circulating pathogenic *Vibrio* strains in the region.

1.1 Problem statement

It is beyond doubt that the effect of climate change is currently being felt in health sector where the health risks of climate-related thermal stress, floods, and infectious diseases have been the most amenable to conventional epidemiological studies. For instance cholera proliferates more rapidly at high temperatures in water. In regions where low temperature, low rainfall, or absence of vector habitat restricts transmission of vector-borne disease, climatic changes have been found to tip the ecological balance and trigger epidemics. Therefore, determination of the physicochemical parameters in the study region which increase the growth of Vibrio strains was necessary.

Environmental water is an important reservoir for *Vibrio cholerae*, and effective surveillance of the pathogen can help to warn of and prevent its infection (Duochun *et al.*, 2015). In the study area, the water is utilized for domestic use. This water may contain various microorganisms that may be associated with diarrhea and therefore isolation of the microorganisms of interest is essential in any given study.

Determination of the phenotype of *Vibrio* strains was a necessity in this study in order to isolate them among other microorganisms.

The pattern shown by the outbreak of cholera indicates that it is emerging and re-emerging disease that requires regular survaillance. Therefore, there is need to determine the type of *V. cholerae* in a given region inorder to establish if there are new strains of cholera in the study region and prectability of disease outbreak for early warning. This will help in controlling and have mitigation measures in place.

Colony forming unit plays a great role in the outbreak of cholera. Certain infectious dose (10^6 to 10^7 colony forming unit) is required to cause cholera to a healthy person (Childers and Klose, 2007). However, those with immune suppression in addition to those who are malnourished require lower infectious doses (Childers and Klose, 2007). Vibrio will acquire inactive/quasi state when conditions are not favourable and form biofilm on substances (Li *et al.*, 2014). In this regard, it is noted that in order to determine its presence, there is need to undertake CFU since it is widely used for determining the suitability of most new biofilm cells quantification techniques (Cerca *et al.*, 2005; Freitas *et al.*, 2014). Moreover, biofilms harbour viable but non-culturable cells (VBNC) (Li *et al.*, 2014). The VBNC are living cells that have temporarily lost ability to grow on routine media (Oliver, 2000). VBNC are cells in latency state and can lead to disease recurrence (Rivers and Steck, 2001) therefore the existence of unfavourable microenvironment within the biofilm matrix or exposure to antibiotics predisposes biofilm cells to VBNC state (Stewart and Franklin, 2008; Pasquaroli *et al.*, 2013) and hence need to be studied. The CFU was determined in this study to inform of any would be cholera outbreak and therefore have measures in place from reaching infectious doses required to cause infection to a healthy individual.

The microorganisms can alter their gene expression profiles when transitioning between environments for their survival (Almagro-Moreno and Taylor, 2013). Incase of *V. cholerae*, upon its entry into the human host, the expression of its two major virulence factors namely toxin co-regulated pilus (TCP) and cholera toxin (CT) are induced (Anchez and Holmgren, 2008). Dermination of the virulent genes in circulating Pathogenic *Vibrio* strains in the study region was therefore necessary.

1.2 Justification and significance of the study

Although many bacteria in the genus *Vibrio* (referred to generically as "*Vibrios*") are not harmful to humans, there are a few strains that are especially harmful (Finkelstein, 1996). Of the more than 65 species of *Vibrio*, at least 14 are pathogenic to humans and have been associated with food-borne disease (Janda *et al.,* 1998; Chakraborty *et al.*, 2000 and Iwamoto *et al.*, 2010). All these species are either associated with human infection by consumption of contaminated food, untreated water or by contact directly with these species through skin wounds (West, 1989; Austin and Zhang, 2006). Gastroenteritis is the most common presentation of *Vibrio* infection from consumption of contaminated food (Altekruse *et al.,* 2000) with an onset of symptoms (nausea, vomiting, diarrhea, abdominal cramps, fever, and bloody diarrhea) occurring seven days after infection. However comparably, persons infected with *V. parahaemolyticus* are more likely to have gastroenteritis than persons infected with other species (Altekruse *et al.,* 2000; Daniels *et al.,* 2000). Cholera, caused by *Vibrio cholerae*, is rare in industrialized nations. Cholera cases have increased steadily since 2005 and the disease still occurs in many places including Africa, Southeast Asia and Haiti (WHO, 2010). The disease is enhanced by influences of climate change (Emch *et al.,* 2008). The prolonged bimodal causes of the cholera epidemic suggest the possible establishment of at least a transient environmental reservoir for cholera in the Western region (Shapiro *et al.,* 1999). Therefore, understanding how *Vibrio* strains interact with the physicochemical factors in the study region is important aspect. This information could lead to better understanding and prediction of the timing and location of increased threats to public health and efforts to establish preventative measures in the study region.

The geography of morbidity and disease has long been a topic of interest because bacteria are a dynamic subject and continuously evolving (Huq *et al.*, 2005). Identifying *Vibrio* strains in the study area is important to provide information which can be used by the epidemiologists in controlling cholera in the region.

Vibrio strains can get into viable but non cultural state for their survival when environmental conditions are not favourable (Alam *et al.,* 2007). At this stage they form Vibriopahages (Alam *et al.,* 2007). To establish early signs of possible out break of cholera, it is important to determine the colony forming units of the *Vibrio* strains in

the study region. This information can be used by the epidemiologists in preventing the outbreak of cholera in the region.

There are early indications that cholera may become established as an endemic or recurrent pathogen in western Kenya, as it has in parts of South America since first appearing in 1991 (Shapiro *et al.,* 1999). The prolonged bimodal causes of the epidemic suggest the possible establishment of at least a transient environmental reservoir for cholera in the region (Shapiro *et al.,* 1999). Nyanza represented a disproportionate burden of Kenya's cholera cases, and both the large cholera outbreak in 1997–1998 and the smaller outbreak in 2008 occurred in Nyanza (Shapiro *et al.,* 1999). After people bordering the lake become infected with cholera, the outbreak likely spreads through the established transmission routes of contaminated water and food, and it can disseminate rapidly around a country with a developed transportation system, like Kenya (Mugoya *et al.,* 2008). Direct association of *V. cholerae* cases with positive *V. cholerae* water testing for the relationship between water quality at the point-of-use and incidences have been documented (Gundry *et al.,* 2006). Thus the need for *Vibrio* spps surveillance in this study, has been prompted by the fact that fresh water *V. cholerae* have been reported to carry both virulence factors emphasizing the need for continuous and active surveillance especially in rural communities where environmental water sources from river, lakes and boreholes are used untreated for domestic purpose (Fraga *et al.,* 2007). Analysis of isolated local *Vibrio* strains is the study region was deemed necessary in providing genetic diversity data for managing the disease through streghtening the existing programs or developing policies. Genetic engineering can also be applied once the genotype of the *Vibrio* strains is established where for instance expression of two major virulence factors of *V. cholerae* namely toxin co-regulated pilus (TCP) and cholera toxin (CT) genes can be prevented. The genetic data of *V. cholerae* can also be used by clinicians to keep abreast on treatment options of cholera in the study areas.

1.3 Null Hypothesis

1. There is no the relationship between physicochemical parameters and *Vibrio*
2. strains in R. Migori, R. Nyando, R. Sondu, Bondo Swamp and R. Yala region in Western Kenya

3. *Vibrio* strains isolated from human stool and water samples from Migori, Nyando, Sondu, Bondo and Yala regions in Western Kenya have the same phenotype and colony forming units

4. There is no difference in the genotype of *Vibrio* strains obtained from human stool and water samples from Migori, Nyando, Sondu, Bondo and Yala regions in Western Kenya

1.4 General Objective

To determine relationship between physicochemical factors, Colony Forming Unit and occurrence of *Vibrio* Strains in the environment of the Lake Victoria Basin

1.4.1 Specific Objectives

1. To determine the relationship between physicochemical factors and *Vibrio* strains in R. Migori, R. Nyando, R. Sondu, Bondo Swamp and R. Yala regions in Western Kenya

2. To determine phenotype and colony forming units of *Vibrio* strains isolated from human stool and water from Migori, Nyando, Sondu, Bondo and Yala regions in Western Kenya

3. To determine genotype of *Vibrio* strains obtained from human stool and water from Migori, Nyando, Sondu, Bondo and Yala regions in Western Kenya

CHAPTER TWO

LITERATURE REVIEW

2.1 General characteristics of *Vibrio* strains

Vibrio species are Gram-negative, facultative anaerobe, halophilic and curved-rod shape aquatic c-Proteobacteria that interacts with a variety of organisms in estuarine and coastal aquatic ecosystems (Farmer, *et al.*, 2005). They are heterotrophic bacteria that occur naturally in the estuarine environment worldwide (Jones *et al.,* 2012) and have the GC content of DNA of 38-63 base pairs (Barrow and Feltham, 1993). They may be found as free-living organisms or associated with zooplankton and phytoplankton (Oberbeckmann *et al.*, 2011). Among the free-living species are small groups that can form pathogenic or symbiotic relationship with humans Yildiz and Visick, 2009). Pathogenic strains are of worldwide concern in public health systems and seafood industries (Oliver *et al.*, 2005; Neogi *et al.,* 2010; Jones *et al.,* 2012).

Strains that are of major public health importance are *V. parahaemolyticus,* toxigenic strains of *V. cholerae* (O1 and O139 serotype), and *V. vulnificus* that cause disease to human (Thompson *et al.,* 2006; Neogi *et al.,* 2010). Morphologically, most *Vibrio* strains have a single polar flagellum and show motility when grown in liquid medium (Thompson *et al.,* 2006). They are chemo-organotroph and capable of fermentative and respiratory metabolism (Thompson *et al.,* 2006). *Vibrio* strains are mainly mesophilic and primarily aquatic (Thompson *et al.,* 2006).

Vibrio also play vital role in their ecosystems by processing and recycling nutrients through the degradation of chitin and other organic materials (Colwell, 1994; Li and Roseman, 2004). This is especially seen in the species *V. diazotrophicus* which is capable of nitrogen fixing (Tibbles and Rawlings, 1994). A few species of *Vibrio* are luminescent and form symbiosis with higher invertebrates. They include psychrophilic as well as barophilic strains (Lee and Ohawada, 1995). These divergent physiological capabilities made them to occupy various ecological niches of human interest.

Biochemically, majority of *Vibrios* produce oxidase and catalase, and ferment glucose without producing gas (Baumann, 1984). The *Vibrio* pathogenic species produce

various virulence factors including enterotoxin, haemolysin, cytotoxin, protease, lipase, phospholipase, siderophore, adhesive factor and/ or haemagglutinins (Zhang and Austin, 2005).

V. parahaemolyticus strains are classified based on the types and variants of their O antigen and flagellar antigen (K). There are 13 O-serogroups and 71 K antigens and various combinations of these give rise to a wide variety of serovars which have been recognized as the causative agents of the disease (Suma *et al.,* 2013). In terms of species pathogenicity, *Vibrio parahaemolyticus* is the causative agent of food-borne gastroenteritis. Most strains of *V. parahaemolyticus* isolated from the environment or food, in contrast to clinical strains, do not produce a thermostable direct hemolysin (TDH) and/or a TDH-related hemolysin (TRH) (Daniela *et al.*, 2013). *Vibrio parahaemolyticus* can exist in a free-swimming state or sessile attached to inert (particulate matter) or animate (zooplankton, fish and shell fish) surfaces (Daniela *et al.,* 2013). Based on environmental conditions, *V. parahaemolyticus* can produce a capsule with different somatic and capsular antigens which are basically used to classify the strains of this bacterium. Molecular epidemiological studies has revealed a strong correlation between the possession of particular hemolysin genes (*tdh*, *trh*, or both) and the ability to cause disease, supporting the fact that these are important virulence genes (Yung *et al.,* 2012). *V. parahaemolyticus* appear as round, opaque, green or bluish colonies with 2 to 3 mm in diameter on thiosulfate citrate bile salts sucrose (TCBS) agar. The genome of *V. parahaemolyticus* is organized in two circular chromosomes. The larger chromosome I contain about 3.3 Mbp and encodes most metabolic genes and constitutive flagellum (Makino *et al.*, 2003). The smaller chromosome II has about 1.8 Mbp of information, with an average G+C content of 45.4% and a separate lateral flagella system is encoded on this chromosome (Makino *et al.,* 2003). There are independent motility and virulence systems encoded on each chromosome as well (Makino *et al.,* 2003).

Vibrio vulnificus is a halophilic, gram-negative, opportunistic pathogen that is associated with plankton and shellfish (oysters, clams, and mussels) (Mahmud *et al.*, 2008). This bacterium exhibits distinct seasonality and is frequently isolated at temperatures greater than 20°C with water salinity between 1.6 and 2.3% (Kelly, 1982; Tamplin *et al.,* 1982). Adaptations in the surface structures of *V. vulnificus* may

influence the environmental reservoirs of disease. Additionally, survival of *V. vulnificus* may also be dependent on phase variation of these cell surface structures. *Vibrio vulnificus* can cause gastroenteritis, necrotizing fasciitis and wound infections with high fatality rate among immune-compromised patients (Gulig *et al.* 2005; Neogi *et al.*, 2010). Several different biotypes of *V. vulnificus* exist and they are divided into BT1, BT2 and BT3 based on the biochemical trait, host range, and epidemiological pattern (Tison *et al.,* 1982). All biotypes have been isolated from human cases, but only the BT2 strains are pathogenic to eels (Tison *et al.,* 1982). The specific biotype associated with human infection is biotype 3, which contains the gene for cytotoxin, making it critical for pathogenicity (Colodner and Meir, 2004). Most *V. vulnificus* cannot ferment sucrose and hence form green colonies on TBCS agar. However, a small percentage (3-15%) of *V. vulnificus* strains can ferment sucrose and form yellow colonies on TCBS agar (Kim and Jeong, 2001). In cold seasons, the bacteria enter into the viable but non culturable (VBNC) state in which they are unable to grow on routine bacteriological media (Oliver, 1993). During entry into the VBNC state, cells characteristically become smaller in size and drastically decrease production of macromolecules (Oliver, 2000).

In addition is *Vibrio cholerae* which are gram negative, rod shaped, non-invasive bacteria that cause the often fatal diarrheal disease cholera (Sechi *et al.,* 2000). *V. cholerae* appears as translucent, flat, yellow colonies with elevated centers on TCBS and colorless colonies on TTGA, with a characteristic dark centre after two days' growth, surrounded by a halo, which is as a result of hydrolysis of gelatin (Anwar *et al.,* 2012). Although there are over 200 known *V. cholerae* serogroups based on Lipopolysaccharides O antigen, only two are pathogenic (Kaper *et al.,* 1995). A characteristic feature of the novel atypical *V. cholerae* El Tor clones is their ability to produce cholera toxin of the classical type that is determined by substitution of the El Tor specific gene of the cholera toxin subunit B (*ctxB*) into the first genotype (Safa *et al.,* 2010). Population levels of *V. cholerae* in the environment appear to be self-regulated by serogroup specific lytic phage. Several authors have documented that as levels of serogroups O1 and O139 in the environment begin to increase rapidly during an outbreak, levels of their respective lytic vibriophages rise in response (Nelson *et al.,*

2009). Once lytic vibriophage concentrations are high enough, bacterial levels begin to drop rapidly and levels of lytic vibriophage eventually follows (Nelson *et al.*, 2009).

Table 1. Human pathogenic *Vibrio* species according to Janda *et al.*, (1998)

Species	Key features
1. *V. cholerae*	
a) (01/0139 serotypes)	Cause of classical cholera
b) (non 01/non 0139 serotypes)	Wound infection and gastroenteritits
2. Virulent strains of *V. parahaemolyticus*	Gastroenteritis and wound infection
3. *V. vulnificus*	Primary speticemia and wound infection
4. *V. alginolyticus*	Ear infection and wound infection
5. *V. mimicus*	Gastroenteritis
6. *V. fluvialis*	Gastroenteritis
7. *V. furnissii*	Gastroenteritis
8. *V. hollisae*	Gastroenteritis
9. *V. metschnikovii*	Gastroenteritis
10. *V. damsela*	Wound infection
11. *V. cincinnatiensis*	Septicemia
12. *V. carchariae*	Gastroenteritis

2.2 Epidemiology of *Vibrio parahaemolyticus*, *V. vulnificus* and *V. cholerae* in humans

Prevalence of cholera in the world over and then in the respective regions is currently on the increase and surveillance studies needs to be initiated if not re-

enforced (Saidi *et al.,* 2014). Therefore, there have been concerted efforts to try and get surveillance data from various regions. Numerous cases of *V. parahaemolyticus* infection have been reported in North America, South East Asia (Lee *et al.*, 2008), Japan (Kubota *et al.*, 2011), Taiwan (Pan *et al.*, 1997; Chang *et al.*, 2011) and Thailand (Yamamoto *et al.*, 2008; Jatapai *et al.*, 2010). In Japan, about 70% of gastroenteritis cases are due to *V. parahaemolyticus* (Kubota *et al.*, 2011). In Taiwan, it caused outbreaks of food borne disease between 1986 to 1995 (Pan *et al.*, 1997). In Iran, few studies on the incidence of this bacterium in seafood have been reported (Bauer *et al.*, 2006). For example, 37.7%. *V. parahaemolyticus* serotype 03:K6was isolated from different samples of shrimp (Rahimi *et al.*, 2010). This finding was consistent with the results reported from retail shrimp (Nishibuchi and Kaper, 1995; Okuda *et al.*, 1997; Nair *et al.*, 2007; Zarei *et al.*, 2012) giving the illness a pandemic status. In United States there has been two *V. parahaemolyticus* reported outbreaks in Texas (416 cases) and in Washington (43 cases) as a result of raw oysters consumption (Su and Liu, 2007). Several reports of *V. parahaemolyticus* infections in Europe exist e.g. there was an outbreak of 64 cases of *V. parahemolyticus* in Galicia, Northern Spain in 1999, due to consumption of raw oysters (Lozano-León *et al.*, 2003). Martinez-Urtaza *et al.*, (2005) also reported another outbreak of 80 cases in 2004 in Spain related with consumption of boiled crab. Italy, Denmark and France have not been left out by the infection according to several authors (Ottaviani *et al.*, 2010; Serracca *et al.*, 2011). Prevalence of potentially pathogenic *V. parahaemolyticus* in Chinese mitten crabs has been reported in England by Wagley *et al.*, (2009). In Norway, the occurrence of potentially pathogenic *V. parahaemolyticus* in blue mussels has also been reported (Bauer *et al.*, 2006). This makes the prevalence of pathogenic

Vibrios in seafood public health concern and is an open ended issue which needs to be continuously addressed (Bauer *et al.*, 2006).

The emergence of pathogenic strains is most likely due to rising global temperatures and increased seafood consumption (Nair *et al.,* 2007). In 1996, the pandemic *V. parahaemolyticus* 03:K6 serotype emerged in Asia and was identified as the predominant cause of numerous outbreaks throughout the world (Okuda *et al.*, 1997; Nair *et al.*, 2007). Since then, approximately 50% of all cases of food-borne gastroenteritis in Southeast Asia are reported to be due to *V. parahaemolyticus* more or less the same to that of *V. vunificus* in the United States. In addition, major out breaks as from the year 2000 have been associated with the 03:K6 serovar and observed in Asia (Matsumoto *et al.*, 2000), North America and Chile (Martinez-Urtaza *et al.*, 2005). This serovar has also been reported from outbreaks or sporadic cases in Europe, France and Spain (Martinez-Urtaza *et al.,* 2005), and Africa and Russia (Nair *et al.*, 2007). It is one of the major health and economic problems in this regions and the incidence of infection is rising throughout the United States, South America and Europe (Okuda *et al.*, 1997; Daniels *et al.*, 2000; Chowdhury *et al.*, 2004; Martinez-Urtaza *et al.*, 2005; Boyd *et al.*, 2008).

The reported incidence and case-fatality rates of *V. vulnificus* infections in subtropical seacoast regions or countries, such as the coastal regions of the United States and East Asia are approximated to be 0.001 to 1.237 per million people and 10% to 54% over the past three decades, respectively (Park *et al.*, 1991; Chuang *et al.,* 1992; Klontz *et al.*, 1998; Shapiro *et al.*, 1998; Chang *et al.*, 2005; Liu *et al.*, 2011). According to Oliver and Kaper (2001), *V.vulnificus* contributed more than

95% of all seafood-related infections in the United States. Every year in the U.S., approximately 50 people are hospitalized from a food-borne *V. vulnificus* infection, responsible for an astonishing ~95% of all shellfish related deaths in the U.S. This number increased by 78% from 1996-2006, with 121 cases confirmed in 2005 (Jones and Oliver, 2009). The CDC believes that these numbers are in fact underestimated due to non-reported or misdiagnosed infections (Hlavsa *et al.*, 2011). In Israel from 1996-97, there were 62 cases of wound infection and septicemia due to *V. vulnificus*. Approximately 66% of the patients underwent surgical debridement to eliminate infection, nearly 12% had finger amputations and one patient endured a total limb amputation (Bisharat *et al.*, 1999). These infections manifested in three different forms in humans: serious wound infections, a potentially fatal septicemia, and gastroenteritis (Oliver and Kaper, 2001). A wound infection due to *V. vulnificus* is believed to occur when a pre-existing or newly inflicted wound is exposed to estuarine water containing the bacterium. Thereafter severe necrosis of the tissue surrounding the wound occurs, often requiring debridement or amputation of the affected areas. Primary septicemias result in mortality rates exceeding 50%, increasing to more than 90% for patients in shock, despite aggressive treatment (Klontz *et al.*, 1988; Koenig *et al.*, 1991). Patients with elevated serum iron levels as a predisposing risk factor tend to suffer septicemia much more and this is mostly in male patients of forty years (Klontz *et al.*, 1988; Hor *et al.*, 1999; Oliver, 2006; Oliver and Kaper, 2007). Therefore there is need to establish if there is *V. vulnificus* in the study region that would lead to such like disease conditions observed in the region.Since the outbreak of cholera in early nineties, a dramatic increase in cases has been observed globally with climate change, poverty and human mobility being among its major drivers (Harris, 2010).

Prevalence of people suffering from Cholera by 2010 was estimated to be 3–5 million with more than 100,000 reported cholera deaths worldwide (CDC, 2014). According to WHO weekly report (2010), there was an increase of cholera cases by 50% from that recorded in 2009 with a total of 13,819 cholera cases being reported from 14 countries of Asia that year. In the weekly report in the same year, India accounted for 5,155 cholera cases (WHO, 2010). Sixty-eight outbreaks of cholera which were reported in India during 1997 to 2006 affected more than 200,000 cases and 823 deaths (Kanungo et al., 2010). In 2010, 192 cholera cases were reported in West Bengal (Government of West Bengal. Department of Health and Family Welfare, "Communicable Disease Report," 2010, http://www.wbhealth.gov.in/Health Statistics/1.asp?pass file id=25&stat main id=100. Accessed16 August 2014). In 2011, WHO reported 589,854 cholera cases from 58 countries with death rate increased from 7543 to 7816 (WHO, 2014). Of late, Haiti and sub-Saharan Africa are the most affected areas in the world with 93-98% of all cases reported from Africa during the years 2001-2009, and in 2010, 56% of all cases were reported from Haiti (WHO, 2011). Most recently, the epidemic in Haiti caused more than 438,000 reported cases and more than 6,000 deaths (from October 2010 to August 2011) (Ali et al., 2011), and an outbreak in Central Africa (i.e. Chad, Cameroon, Niger, Nigeria) in 2010 caused more than 62,000 reported cases (WHO, 2012). In Cameroon, the case-fatality rate of cholera in Douala between 2000 and 2005 was recorded at 10.2% of total Africa cases (Fraga et al., 2007). Outbreaks have usually started in the densely populated slums with inadequate sanitation facilities, potable water supply and poor hygiene practices. Mortality from cholera is high among patients of all ages. However, the estimated mortality from cholera reported to WHO by Member States may be

the latest being in Bauchi State where 54 people died from the outbreak in April 2013 (Malami, 2014). In East Africa, the worst cholera cases in Tanzania were reported in 2006 with a total of 3,169 cases and 254 related deaths (WHO, 2008). Uganda reported cholera epidemics in six districts across the country with 724 cases and 28 deaths between April and May 2012 (WHO, 2012).

It has been reported that since 1971, Kenya have been affected by waves of cholera occurrences (WHO, 2010). This was reported yearly with average case fatality of rate of 3.5% from 1974 to 1989. The greatest epidemic started in 1997 to 1999 with more than 33,400 notification case representing 10% of all reported cholera cases from Africa continent in these three years. The outbreak started in Nyanza near Tanzania border along Lake Victoria in 1997.This reached Kisumu and spread into Siaya district (Shapiro *et al.,* 1999).

From 2000 to 2006 reported cases ranged from 1,157 to 816 with 291 cases being reported in 2002. In 2007, the cholera outbek affected four provinces in Kenya including Nyanza (Kisumu, Bondo and Siaya) (Shapiro *et al.,* 1999). A total of 625 cases were reported with 35 deaths leading to fatality rate of 5.6%. In 2008, the areas affected in Nyanza province were Suba, Migori, Homa bay, Rongo, Siaya, Kisumu, Bondo Nyando and Kisii South. The outbreak started in Suba district and spread to the other regions. This was associated with movement from Ethiopia and Somalia where 325 cases were reported with 11 deaths (Shapiro *et al.,* 1999). In 2009, 11,769 cases were reported including 274 deaths. This was first reported in Nyanza followed by Western, Eastern, North Eastern, Rift Valley, Coast and finally Nairobi (WHO, 2010). While in 2010, 3,024 cases were reported with 53 deaths.

2.3 Environmental factors that influence the survival of *Vibrio* species in various geographical regions

Several environmental parameters have been shown to influence *Vibrio* communities and one of the several survival strategies of *Vibrio* spp. to unfavorable environmental conditions such as nutrient depletion or cold temperatures is by the formation of biofilms, attachment of bacteria to algae, crustaceans or other marine organisms, and utilizes the nutrients released by these organisms (Huq, *et al.,* 1983; Yildiz and Visick, 2009). In addition, they also survive entering into a viable but non-culturable (VBNC) state (Asakura, *et al.,* 2008). In VBNC state they do not form colonies on any media, but are metabolically active and very resistant to environmental stress (Oliver, 2005). The main driving factors seem to be temperature, salinity and phytoplankton occurrence (Drake, *et al.*, 2007; Hsieh *et al.*, 2008; Martinez-Urtaza, *et al.*, 2008; Vezzulli, *et al.,* 2009). Especially high water temperature has been reported to correlate with increased *Vibrio* abundance (Blackwell and Oliver, 2008). However, *V. anguillarum* and *V. aestuarianus*, are adapted to colder water temperatures (Eiler *et al.,* 2006). Previous studies have shown that *Vibrio cholerae* is triggered by temperature which results in cholera outbreak when its colony forming unit is between 10^6 to 10^7 (Childers and Klose, 2007).

Salinity is also an important parameter in the dynamics of vibrios in marine systems (Hsieh *et al.*, 2008). Many studies have shown a strong correlation between the presence of *V. cholerae, V. Vulnificus* and *V. parahaemolyticus* and salinity (Blackwell and Oliver, 2008; Colwell *et al.*, 1977; DePaola *et al.,* 2003; Jiang, 2001; Motes *et al.,* 1998; Pfeffer *et al.*, 2003; Randa *et al.*, 2004; Wright *et al.*, 1996). It has also been reported that a decrease in salinity favors *Vibrio* growth and proliferation, particularly in brackish waters of estuaries. Study by Cantet *et al.,* 2013 in French Mediterranean coastal lagoons showed that the highest concentrations of *V. parahaemolyticus*, *V. vulnificus* and *V. cholerae* occurred in the Prévost and Mauguio lagoons, both of which have lower salinities than the Thau lagoon. A higher abundance of *V. vulnificus* was observed in the Mauguio lagoon, where salinity ranges from 20 to 29‰, confirming that salinity is a strong determinant of *V. vulnificus* abundance and dynamics, as previously reported by Randa *et al.*, 2004.

Phytoplankton blooms may be another important biotic factor to fuelling the proliferation of Vibrio in marine environments (Hsieh *et al.*, 2007). Phytoplankton blooms can serve as substrates by providing attachment surfaces and can also sustain bacterial communities by providing dissolved and particulate organic matter through a variety of processes such as excretions, exudation and cell death (Karl, 2007).

Bacteria in the genus *Vibrio* are commonly found in brackish and marine waters of 0.2 to 2.0% and contribute significantly to the microbial ecology and biogeochemical process in these habitats (Farmer *et al.,* 2005; Thompson *et al.,* 2006). Members of some species in the genus are opportunistic pathogens of humans and have been shown to thrive in warm waters of 25°C with moderate salinity (Thompson *et al.,* 2006). Among the most preferred parameters for halophilic *Vibrio* spp are: - nutrients, sources of energy, water, temperature, pH, oxygen, and dissolved inorganic solids (Singleton, 1982). Effect of temperature and salinity on bacterial growth and seasonal abundance of bacteria has been extensively studied due to their direct association with overall water densities in any aquatic environment (Reilly, 2011). It is also noted that surface water temperature (s) in aquatic ecosystem are bound to fluctuate due to "Radiation income" and "prevailing winds" that are influential to the temperature in estuarine waters which eventually affects the general survival of *Vibrio* strains (Mutreja *et al.,* 2011).

Salinity is another vital factor to consider for the survival of *Vibrio*. It is a measure of inorganic salts dissolved in water (Urakawa and Rivera, 2006). Salinity is commonly expressed in the field of oceanography as parts per thousand (ppt or denoted by ‰). The average salinity of the surface marine water is approximately from 3.3% (33 ppt) to 3.7% (37 ppt) (Chang 2011). Many studies have shown lack of correlation between density of *Vibrio* spp. and salinity (Randa and Polz, 2004). However, slight negative correlation has been observed between bacteria density and salinity (Thompson *et al.,* 2004). *V. cholerae* alone has the ability to survive at a salinity of 0%, although it has an absolute growth requirement for sodium (Thompson *et al.,* 2004). However, salinity and survival of *V. cholerae* in various

environments has been found to be associated with the amount of nutrients in the environment (Borroto, 1997). Each species responds differently to seasonal fluctuations in temperature and salinity. For example *V. vulnificus* is known to exhibit a more pronounced temperature-driven seasonality compared to *V. parahaemolyticus* and *V. cholerae* (Lipp and Rose, 1997). Distribution of *V.parahaemolyticus* in the marine environment is related to water temperature and it is rarely isolated from sea water until the temperature raises to15°C and higher (Kaneko and Colwell, 1973). Both temperature and salinity play important and interrelated roles in the levels of *Vibrio* spp. Regardless of the role of salinity, temperature probably has the most important effect on the prevalence and levels of the pathogenic *Vibrios*. The average temperature for the recovery of *V. parahaemolyticus* is between 5 and 45°C with water salinity of 1.3% (Kelly and Stroh, 1988) while that of *V. vulnificus*, the temperature is between 13 and 37°C (Kelly, 1982; Kaspar and Tamplin, 1993) while salinity is between 0.5-2.5% (Kaspar and Tamplin, 1993; Motes *et al.,* 1998). This explains the seasonality of recovery from various environments and samples as well as geographical regions. Thus the outbreaks will also coincide with the seasonality of high recovery of colony forming units (CFU/g or /ml). The most preferred pH range for *V. parahaemolyticus* range from 4.8–11.0 in the presence of salt (1-8% NaCl); however, optimal growth of *V. parahaemolyticus* usually occurs at 30–35°C and pH 7.6–8.6 with a salt concentration in the growth medium of 2– 3% NaCl (Jay *et al.*, 2005). In pursuit of the above, it was demed important to establish the temperature, pH, salinity that affect the occurance of *Vibrio* strains in the study region in view of predicting the conditions which would lead to diseases caused by them and hence ensuring relevant prevention measures.

2.4 Phenotypic identification and colony forming unit of *V. parahaemolyticus*, *V. vulnificus* and *V. cholerae*

Vibrio parahaemolyticus, V.vulnificus and *V. cholera,* show different phenotypic characteristic in the thiosulfate-citrate-bile salts-sucrose (TCBS) and during biochemical and serological tests. Colonies suspected to be *V. parahaemolyticus* produce blue-green colour in the same medium. *Vibrio vulnificus* colonies produce green isolated shiny colonies or yellow colonies in cases where they ferment sucrose. The *V. cholerae* colonies appear on TCBS as yellow shiny colonies, 2 to 4 mm in diameter. The yellow colour is caused by fermentation of sucrose in the medium.

The above *Vibrio* strains are oxidase positive and upon biochemical test, produce purple colour within 10 seconds when smeared on wet paper with oxidase reagent (1% N, N, N, N'-tetramethyl-p-phenylenediamine.2HCl). The colonies of these *Vibrio* strains also develop string when crushed on a drop of 0.5% aqueous solution of sodium deoxycholate on a glass slide (Charles and Angelo, 2004).

Bacterial abundances are expressed as colony forming unit (CFU) per volume of water. The number of colony forming units (CFU) is multiplied by the dilution factor and expressed in CFU/mL of water (Djaouda *et al.,* 2013). The CFU gives an indication of numbers of *Vibrio* strains that would be adequate to cause illness in at least a percentage of the population (Franco *et al.,* 1997) and hence the need to include it in this study.

2.5 Genetic basis of *V. parahaemolyticus*, *V. vulnificus* and *V. cholerae* infections

The *Vibrio* pathogenic species produce various virulence factors including enterotoxin, haemolysin, cytotoxin, protease, lipase, phospholipase, siderophore,

adhesive factor and/ or haemagglutinins (Zhang and Austin, 2005). The virulence of *V. parahaemolyticus* is commonly associated with expression of thermostable direct hemolysin (TDH) and TDH-related hemolysin (TRH), which are encoded by the *tdh* and *trh* genes. Therefore, the *tdh* gene, marked by a β-type haemolysis (Nishibuchi and Kaper, 1995), and the *trh* gene, correlated to a positive urease tests (Okuda *et al.,* 1997), serve as markers for pathogenic strains. Most clinical isolates of *V. parahaemolyticus* are differentiable from environmental strains by their ability to produce a TDH (Charles, 2004). A thermostable related hemolysin (TRH), which shares 60% homology with TDH, has also been associated with strains causing gastroenteritis (Charles, 2004). The major virulence-associated genes which encode colonization factors and cholera toxin (CT) in the *V. cholerae* are part of larger genetic elements composed of clusters of genes (Pearson *et al.,* 1993). The CT is encoded by cholera toxin (*ctx*) gene. Although CT is responsible for severe dehydrating diarrhea associated with *V. cholerae,* the search for additional enterotoxins produced by *V. cholerae,* which has included volunteer studies of genetically engineered *ctx*-deleted *V. cholerae,* has led to the discovery of new toxins (Kaper *et al.,* 1995).

The studies on the molecular analysis of *V. cholerae* strains isolated during epidemics between 1961 and 1996 in Bangladesh (Faruque *et al.,* 1997) revealed clonal diversity among strains isolated during different epidemics. Another study was undertaken in India where a total of 26 strains of *Vibrio cholerae*, including members of the O1, O139, and non-O1, non-O139 serogroups from both clinical and environmental sources, were examined for the presence of genes encoding cholera toxin (*ctx*A), zonula occludens toxin (*zot*), accessory cholera enterotoxin

(*ace*), hemolysin (*hly*A), NAG-specific heat-stable toxin (*st*), toxin-coregulated pilus (*tcp*A), and outer membrane protein (*omp*U), for genomic organization, and for the presence of the regulatory protein genes toxin coagulated pilus (*tcp*I) and *tox*R in order to determine relationships between epidemic serotypes and sources of isolation. This study showed that the non-cholera-toxin-producing strains of *V. cholerae*, whether of clinical or environmental origin, possess the ability to produce a new secretogenic toxin that is entirely different from the toxin produced by toxigenic *V. cholerae* O1 and O139 strains (Singh *et al.,* 2001). Therefore, the need to study *V. cholerae* genotype in a given region in order to ascertain clonal diversification. Although pathogenic *Vibrio* strains seem to be spreading geographically and their caused infections are reported more frequently, no regulated monitoring for *Vibrio* spp. exists in Kenya and especially in Western Kenya.

The difference in a 17-bp nucleotide sequence of the 16S rRNA gene is one way of identifying *V. vulnificus*. By this criterion two major groups of *V. vulnificus* have been identified, designated types A and B. The variations in the sequences of the 16S rRNA gene of the virulence-correlated gene (*vcg*) and of the pilus Factor (*pilF)* gene encoding a protein required for pilus type IV assembly are used to distinguish clinical from environmental *V. vulnificus* (Nadja *et al.,* 2013). The genotype of *Vibrio* strains circulating in the study region has not been established to provide information which can make these strains non pathogenic most preferably through genetic engineering.

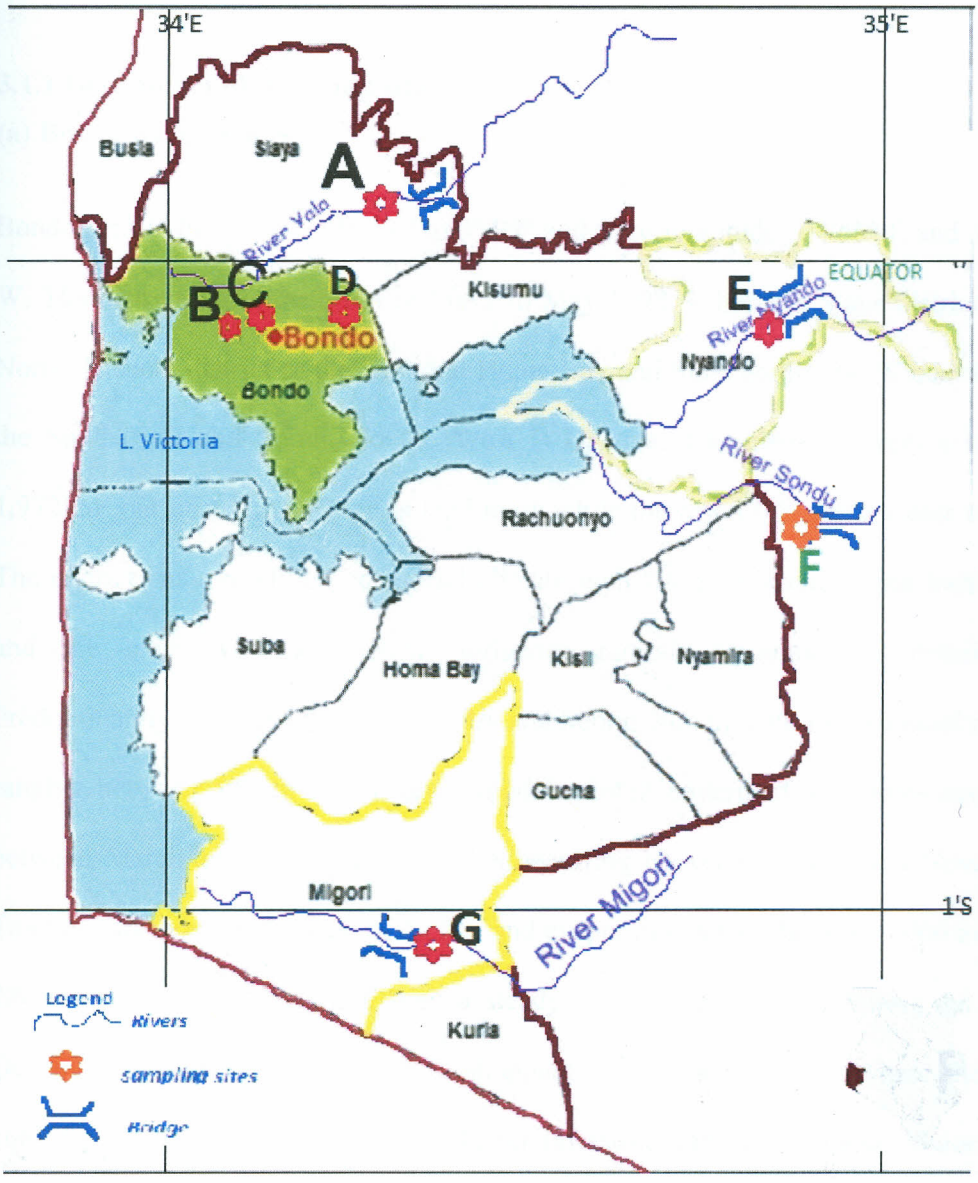
CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Sites

This study was carried out in Migori (1° 4′0S, 0°17′48′′S), Nyando (0 ° 14′19′′N) and Siaya (34° 28′ 0′′E, 34 ° 51′ 31′′ E, 34 ° 16′0′′E) (Fig. 1) counties. The three regions experience different micro-ecological climate (LVEMP, 2012) and the survival dynamics of infectious disease pathogens were expected to present differently thus their selection in this study. These areas are prone to seasonal cholera epidemics in western region and chances of isolating the pathogen are pre-summed to be high (Shapiro *et al.,* 1999). In addition, the sites, boarder the agro-ecological sugar belt regions where fertilizers that contribute to eutrophication are frequently used (LVEMP, 2012) thus forming enabling environment for cholera to attach and form biofilms making it ideal for sampling. In Bondo, change in the water levels and water use by humans and livestock as observed in this study provide conducive environment for the swamps to harbor pathogenic bacteria (Shapiro *et al.,* 1999). The four Kenya rivers that drain into Lake Victoria (R. Nyando, R. Sondu –Miriu, R. Nzoia and R. Yala) that flow from tea and sugar plantations and empty their products to Lake Victoria are deemed to collect refuses and contaminants on its flow to the lake (LVEMP, 2012). This makes rivers to have different turbidity levels with R. Nyando having the highest level (380NTUs), R. Sondu-Miriu the lowest levels (130NTUs), and R. Nzoia and R. Yala having equal intermediate levels. Water related diseases e.g. dysentery, diarrhoea and typhoid have been considered important diseases in these sites with the latest being during long rains in April-May 2012 (LVEMP, 2012). Depending on the water velocity and deposition of sediments (sand and gravel), the pathogenic bacteria

may form an attachment on the substrates forming biofilms that makes them available for new infections thus the need to consider them in the study.



Legend:

A-Next to Yala market,; B-Garage water pan; C- Rozana swamp; D- Miruka swamp; E – Next to rice mill; F- Next to car wash; G- water collection point after the bridge

Fig.1: Map showing the locations of the study area (Courtesy of Agriculture Livestock Sector Working Group of the Kenya Food Security Steering Group (KFSSG)), 2011

3.1.1 Description of the study sites

(a) Bondo water pan (s)

Bondo district lies between 0° 26° to 0° 90° and from longitude 33° 58° E and 34° 35° W. The district was curved out of Siaya in May 1998. It borders Siaya district to the North, Kisumu district to the East and Homa Bay and Suba across the Winam Gulf to the South East and South. To the West is Uganda. The district covers a total of 1,972km^2 out of which 972km^2 is land mass while the rest 1,000km^2 is water surface. The district has a modified equatorial climate with strong influence from local relief and the expansive lake, which influence rainfall amounts and distribution. Predominantly, the district has warm, dry and humid climate with mean annual rainfall ranging between 800-1600 mm on bi-modal rainfall pattern of long rains occurring between March and May and short rains occurring between October and November. Temperatures vary with mean of 22.5°C and evaporation varies between 2000 mm and 2200 mm annually. There has been a steady increase in population over the years. Densities are high in urban centres, shopping centres and beaches where there are considerable economic activities and better infrastructural development. Major water sources are Lake Victoria, pans and dams. Others are piped water, river Yala and boreholes that are very few. About 35% of the total population have access to portable and clean water, the rest depend on water from pans, dams or from the lake. The main sources of water pollution include agro-chemicals, open air defecation in bushes due to lack of pit latrines and waste water that end into water points during surface run off. Ground water reserves include boreholes, shallow wells and springs. This condition predisposes the community to higher risks of contracting waterborne diseases such as typhoid and cholera among others since majority of them do not observe basic hygiene

practices such as boiling water before drinking (Bondo District Environmental Action Plan, 2006-2011- NEMA).

(b) River Yala

River Yala boarders Bondo District towards the North and it empties its water through the Yala swamp and eventually into Lake Victoria. Its upper section reaches from 125 km – 190km. The river flows in a slightly meandering channel through a V shape valley with a longitudinal gradient of 1 in 120. On either side of the 20m wide channel the main land use is grazing with few human settlements. Lower down, in the next 100 Km stretch the channel width increases to 30m and the bed gradient becomes flatter to 1 in 250. With a few human settlements close to the river the main land use is subsistence farming and cattle grazing. In the last 25 Km reach up to its outfall into the lake, the bed gradient becomes very flat to a gentle 1 in 2700. While the width of the river channel increases to only 30m, the banks on either side become low, causing the flood waters to spill over a wide flood plain, a considerable part of which is the famous Yala Swamp with few human settlements. The remaining part of the plains is intensively used for agriculture (GOK, 2009).

(c) River Nyando

The greater Nyando District covered an area of 1,168 km² and was divided into three parliamentary constituencies namely Nyakach, Nyando and Muhoroni. The boundaries of Nyando and Muhoroni constituencies extend beyond the district boundary. The greater Nyando District is located along the shore of Lake Victoria. It borders Nandi District to the North and Kericho District to the East (both located in the Rift Valley Province), Rachuonyo to the South and Kisumu to the West. The district has only a small shoreline to the southwest where it touches Lake Victoria and comprises 71 km² of the lake water surface. The upper reaches of the Nyando river basin lies between 1800 and 3000 meters above sea level (Main Streaming environmental Planning in

Kenya-Final Report 2010). The rainfall regime is bimodal with the long rains in March/April/May and the short rains in September/October. Annual average rainfall ranges from 1200mm to 1600 mm. The annual average maximum temperatures range from 19°C to 27°C. While the annual average minimum temperatures range from 5°C to 12°C (Onyango *et al.*, 2005). The Nyando River is one of the major Rivers in the Lake Victoria Basin. It drains parts of Nandi, Kericho and Nyando districts. It has a catchment area of about 3,600km^2 and an average discharge of approximately 15 m^3 s^{-1}. The population of the basin is 746,515.

(d) River Migori

Migori covers an area of 2,597 Km^2 and was divided into five parliamentary constituencies namely Rongo, Migori, Uriri, Nyatike and Kuria. It has two rainy seasons and the highest rainfall is between March and May. The average rainfall is approximately 1200 mm and above. The temperature ranges between 21°C-35°C. The county has a population of 917,170. The main economic activities are Agriculture, fishing, manufacturing and mining.

3.2 Study Design

This was a cross-sectional study. This design was used because the study involved collecting human stool samples and water samples after every two weeks from Migori, Nyando, Bondo, Sondu and Yala regions. The study sites were selected based on the following criteria:-

i. Common bathing places by the inhabitants with low or almost neglegible stream flow was used since such places have sediments and copepodes that are used by *Vibrio* strains to form biofilm (Alam *et al.,* 2007). Fast flowing waters were avoided since it is documented that *Vibrios* do not thrive in such environments.

ii. Daily or frequent water collection point for both domestic and commercial use by the community. The ecological charateristic of the site was considered since this affects the growth of phytoplanktons where *Vibrio* strains can form biofilms. The site should be having papyrus reeds or over grown grasses to be used by the pathogen for anchoring.

iii. Temporally designated animal watering points as evidenced by the holves marks and physical presence of animal was also considered as a site of study. Animals are known to tranport/tranfer, microbes from one biome to another especially in transboundary transmissions.

iv. Estuarine points where inland flowing streams join main streams or rivers were also considered for the study since at this point, the nutrient levels and temperatures are significant due to slow flow of water and *Vibrio* strains will thrive in such an environment.

3.3 Samples size calculation and determination

Given the *Vibrio cholerae* prevalence of 31.7% in the region based on the previous epidemics (Kaneko *et al.,* 2014), the sample size was calculated using formula by Lwanga and Lelemshows (1991) that states that if the prevalence of any disease in a population is proportional to that of individuals in a population posing a given characteristic is equal to a particular value, then the test value of the population

proportion under the null hypothesis is needed. The anticipated value of the population proportion, level of significance, power of the test and alternative hypothesis "either" "or" are put in the consideration. This study therefore assumed that the number of infected individuals in health centers within study sites was directly proportional to rate of bacterial infection (Brooke *et al.*, 2013). The following formula by Lwanga and Lelemshows (1991) was used.

$N=Z^2$ 1-aP (1-P)/d^2

N=sample size

Z=standard normal deviation=1.96

a=level of significance of 5%

P=Prevalence of condition under study=0.317

d=Precision of study=0.05

$N=1.96^2$x1-0.05x0.317 (1-0.317)/0.05^2 = 316 samples.

Therefore, the study needed a minimum collection of 316 samples of water and stool. However, a total of 811 samples were collected during the study period starting from May to December 2013 to increase the chances of isolating *Vibrio* strains. Following the rainfall in August and September 2014, some samples were collected during that period in order to consider seasonality of transmission.

3.4 Criteria for inclusion and exclusion of patients

The patients who are residents of the area with unknown or unsuspected cause of diarrhea (not malaria related diarrhea usually associated with high fever) who were sent to the laboratory by clinical officer in Migori, Nyando and Bondo District Hospitals were included in the study. The remnants of their stool after routine laboratory analysis by technician were collected irrespective of the gender or age of the patients.

3.5 Ethical considerations

The study did not seek for ethical approval (consent) from patients. This is because the study considered the use of remnants of the stool samples from patients diagnosed with

diarrhea by the clinical officers before being discarded at the time of collection in the respective hospital laboratories. So the patients were not asked to sign consent forms since no pain was to be inflicted on them neither their confidentiality was at jeopardy. For the purpose of publication, the hospital authorities were asked for consent which they provided. In order to undertake the study, the proposal was submitted for Ethical approval by Maseno University Ethics Review Committee.

3.6 Study clearance

The study clearance was provided by Maseno Ethical Review Committee (Appendix 1) and the School of Graduate Studies in Maseno University.

3.7 Physicochemical parameters

(a) Depth of water

The depth of the water was measured by a graduated rod with a heavy iron ring attached to it (Appendix 2) and measurements determined and recorded.

(b) Water salinity

Salinity was determined by using salinity meter (HACH model CO150). The sensor electrode was immersed into the water, data determined and recorded. The data was taken in the morning hours (10.30am to 11.30am). Since at this time the water water temperatures are between 20-25°C that is condusive for bacteria activities.

(c) Disolved oxygen and pH

Dissolved oxygen and pH were measured with a portable HACH (model DO175) dissolved oxygen meter and Orion field pH meter (model 210A, Orion Laboratories, England) respectively.

(d) Water temperature

Water temperature was determined using potable meter (HACH model C0150 conductivity meter) and the data recorded.

3.8 phenotype and colony forming units of Vibrio strains isolated from human stool and water from Migori, Nyando, Sondu, Bondo and Yala regions in Western Kenya

3.8.1 Collection of human stool and water samples

(a) Diarrhea sample collection

In the respective hospital laboratories (Bondo, Nyando and Migori) remnant of fresh diarrhea samples (1x100g) from patients who presented with diarrhea and not malaria related were collected in labelled bijoux bottles with alkaline peptone water (1% NaCl pH 8.6) (HIMedia Laboratories Pvt. Ltd Mumbai, India). These samples were collected after every two weeks and were transported within 4-6 hours in iced cool box at 8°C to the Microbiology Laboratory at Maseno University for culture and isolation of *Vibrios*.

(b) Water sample collection and preparation

The water samples were collected from the surface (19 to 55-cm deep) at each sampling site in 50 ml, autoclaved universal bottles, between June and December 2013; January to February 2014 and August to September 2014. This was done by using graduated rod (Appendix 2). *In situ* measurements of temperature, salinity, pH and dissolved oxygen concentration were done at the period of water sample collection and data recorded as described in 3.7 (a-d). 5mL of water sample was added to 2.5 mL of alkaline peptone water (1% NaCl pH 8.6) (HIMedia Laboratories Pvt. Ltd Mumbai, India). The samples were collected after every two weeks and were transported within 4-6 h in cool box at 8°C to the Microbiology Laboratory at Maseno University for culture and isolation of *Vibrios*.

3.8.2 Culture and Isolation of *Vibrio* strains

The collected stool and water samples were processed within 4 to 9 h after collection on arrival to the laboratory. After the overnight culture in alkaline peptone water (APW) (E&O laboratories Ltd, UK), two loopfuls of the surface and top-most portion of the APW broth was obtained and then sub cultured by streaking on thiosulphate-citrate- bile salt sucrose agar (Oxoid Basingstoke, England) used for the selective isolation of *Vibrio* strains. After 18 to 24 h of incubation at 37°C, the cultures giving pure green colonies were randomly selected using a sterilized inoculating wire loop and sub cultured on tryptic soy agar (Fluka, Sigma-Aldrich, USA) supplemented with 1% NaCl according to Hara-Kudo *et al.* (2001). The isolated bacteria were frozen at −20°C with 20% (v/v) glycerol for further analysis.

3.8.3 Phenotypic identification of the *Vibrio* isolates

For the purpose of specific identification of *Vibrio* phenotypes, single colonies of the bacteria isolated from the specimen were streaked on plates of TCBS. The sub-culturing was done by streaking the colonies from the Nutrient Agar that had thawed to room temperature (25°C). The isolates were tentatively identified based on their color changes and size in the TCBS media. The isolates that retained the size of 2 to 3mm were further subjected to oxidation test by transferring the colony by an applicator stick to a filter paper saturated with oxidase reagent (1% N,N,N,N'-tetramethyl-p-phenylenediamine.2HCl). A dark purple color developing within 10 seconds implied a positive test growth. The isolates that were oxidase positive were subjected to string test (serology) that involved putting a colony on the slide and adding one drop of sodium deoxycholate solution and then using a plastic loop to break the colony. The samples that showed or did not show positive results were subjected to serological agglutination test by using polyvalent 01 antigen and antisera for Ogawa and Inaba. Positive polyvalent antisera isolates for any of the strains was then processed and stored at -20°C for future molecular work.

3.8.4 Colony Forming Units of *Vibrio* strains in water and stool samples

Preserved water and stool samples at -20°C were thawed and then cultured in TCBS at 37°C for 6h. About 1×10^6 isolates were then picked using inoculating wire loop and

diluted to 1ml of physiological saline. Subsequently, serial dilutions were made from this 1ml as follows; 10^{-2}, 10^{-4}, 10^{-6} and 10^{-8}. Out of these respective dilutions, cultures were made on TCBS and incubated at 42°C for 18 h. The isolates growth rate was then determined by the number of colonies count using a colony counter on the respective plates and data recorded. The colony forming unit (CFU) was recorded from those plates where the sample dilution resulted into colonies. The counts were expressed as $\log_{10}$ CFU/g of sample. With the help of a hand lens, colonies with green and yellow colors were handpicked from the plates with sterile plastic loops and stored in 15% glycerol (v/v) at −20°C for subsequent analysis.

3.9 Genotype of Vibrio strains obtained from clinical and water from Migori, Nyando, Sondu, Bondo and Yala regions in Western Kenya

3.9.1 Vibrio *strains* DNA Preparation and Extraction

Previously stored *Vibrio* strains (3.9) were allowed to thaw after which they were streaked on TCBS agar for isolation and incubated overnight at 37° C (EchoTherm™ heating dry baths, Model IC22, Torrey, Pines scientific Inc.). An isolated colony from the plate was transferred by heat sterilized inoculating wire loop to 1ml of Tryptic soy Broth (TSB), which was incubated overnight at 37°C (EchoTherm™ heating dry baths, Model IC22, Torrey, Pines scientific Inc.). 1ml of overnight growth was then transferred by pipette to a sterile 1.5ml Eppendorf tube (Sarstedt Ltd, Germany) and centrifuged using Thermo Scientific microcentrifuges (Thermo Scientific™) at 6000 rpm for 1 minute. The supernatant was then discarded and the bacteria pellets were re-suspended in 0.5ml sterile distilled water. After a brief vortexing the suspension (SA8 vortex mixer, Keison Products, ltd, UK), the tubes were then heated at 100°C water bath for 10 minutes to lyse the bacteria. The tubes were then centrifuged again at 6000 rpm (Eppendorf centrifuge 5415D, Germany) for 5 minutes at 4°C to pellet cell debris. The obtained supernatant was carefully aliquoted by micropipette into sterile 0.5ml and the tubes stored at -20°C for use as template DNA (Mazel *et al.*, 1998).

3.9.2 *Vibrio* strains DNA Amplification

Twenty microliters of PCR master Mix (Table 2) + 5 microliter DNA template were aliquoted into 0.2 ml eppendorf tubes. The mixture was thoroughly vortexed and then centrifuged at 10,000 rpm for 6sec (Eppendorf centrifuge 5415D, Germany). The

prepared mixture was then amplified using MJ Gradient Thermocycler (PTC -225, Peltier Thermocycler, BioEnymes, Germany) PCR with the following conditions (Table 3): building up of quantitative temperature at 95°C for 1min, denaturization temperature at 95°C for 30 sec, annealing temperature of 55°C for 1 minute, and extension temperature of 68°C for 1 minute and final extension at 68°C for 10 minutes.

Table 2: PCR master mix for DNA amplification reaction (according to Erin *et al.*, 2003)

Component	Quantity
10Xbuffer	1µl
dNTPs	2.5µl
Primers F from (100Ml working solution)	0.5µl
Primer R	0.5µl
Taq polymerase	0.2µl
PCR water	15.8µl
DNA Template	5µl

The amplified amplicons were then loaded on to a casted 1.5% agarose gel (2 grams agarose powder + 100ml 1 x TAE Buffer+0.2microlitre of ethidium bromide) with a gene marker of 100bp, and negative and positive controls. This was then let to run for 45 minutes at 135V after which the UV pictures were taken using the UV photo transmitter (Gel Logic 100 Imaging System, Kodak). (See primer sequence appendix 3).

3.10 Data Analysis

Generated data was entered in MS Excel Windows XP professional 2007 and analysed on GENSTAT Version 6. The data collected was combined for analysis over the study period based on sampling points and time of data collection. Descriptive analysis was carried out on physicochemical parameters. Analysis of physicochemical parameters data was done by using Analysis of variance (ANOVA) to establish the significant difference between the depth, salinity, oxygen concentration, pH, water, temperature and the time of sample collection within the rivers and dams and water pan. The correlations between the physicochemical parameters was done by using Pearson Product Moment Correlation at P value of <0.05.

Descriptive statistics were generated to assess the occurance and distribution of *Vibrio* strains among sampled source. Average concentrations of Colony Forming Units (CFU) for all collected environmental and human stool samples from each region was transformed to $\log_{10}$ before analysis.

CHAPTER FOUR

RESULTS

4.1 Physicochemical parameters of R. Migori, R. Nyando, R. Sondu, Bondo Swamp and R. Yala

Water depth, salinity, temperature, oxygen concentration, pH, air and water temperature were measured at the period of water sample collection (Huq *et al.,* 2005) and data recorded. The average physicochemical parameters (Table 3) in the study region were established during the study period (June to December 2013, January, February, August and September 2014).

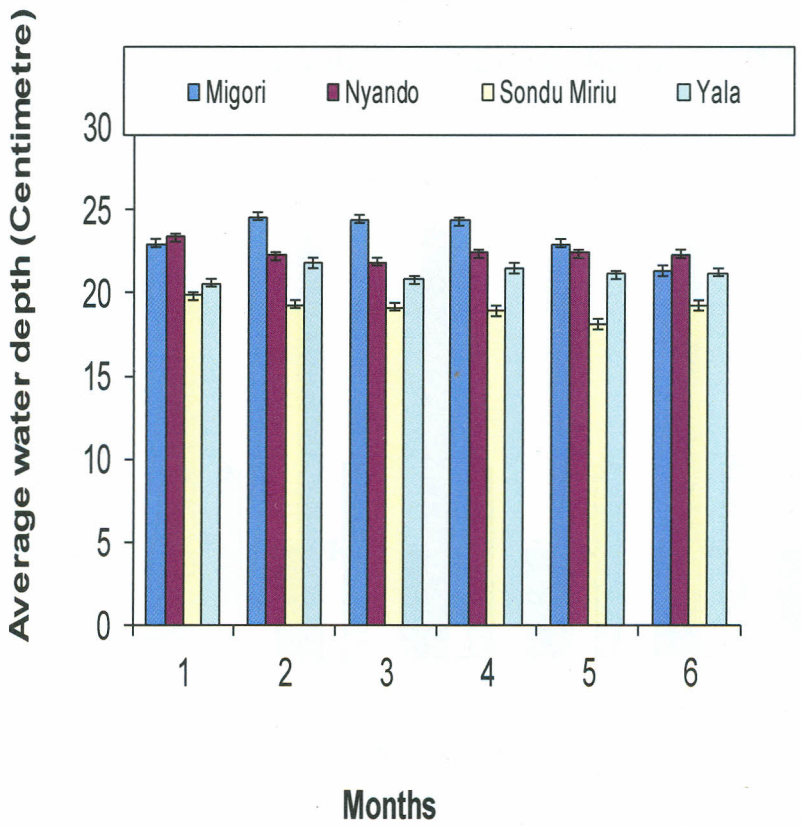
Table 3: Average physicochemical parameters in the study region

Sampled parameters		Study Sites				
	Mean/Std Dev	R. Migori	R. Sondu Miriu	R. Nyando	R. Yala	Bondo Swamp
Water Depth (m)	Mean	0.18	0.21	0.193	0.22	0.2
	Std Dev	0.16	0.17	0.17	0.18	0.17
Water Salinity (µm/cm)	Mean	42.28	41.44	161.92	73.65	17.01
	Std Dev	1.8	3.6	11.9	4.6	3.1
Dissolved Oxygen (mg/l)	Mean	5.3	4.09	3.81	3.47	3.48
	Std Dev	1.54	0.831	0.73	0.26	0.43
Water pH	Mean	7.9	7.91	8.26	7.78	8.09
	Std Dev	0.75	0.32	0.40	0.38	0.42
Air Temperature (0°C)	Mean	23.88	20.09	23.48	23.2	28.9
	Std Dev	2.44	0.97	1.03	1.99	1.4
Water temperature (0°C)	Mean	23.47	19.28	22.46	21.22	28.97
	Std Dev	1.31	0.37	0.69	0.75	2.7

Significant difference was observed in the above physicochemical parameters. This was the case in the depth of water in R. Migori (0.18± 0.16m) and R. Sondu Miriu (0.2±0.16 m). Dissolved oxygen showed very strong significance at 5.3 ±1.54 mg/l and 4.09 ±0.8 mg/l in R. Migori and R. Sondu Miriu respectively. Water temperature showed strongly significance at 28.9± 2.6 °C in Bondo swamp. There was significant

change in salinity (158.9µm/cm), water temperature (0.20°C), dissolved oxygen (3.1mg/l) and pH (4.43).

Analysis of the data using ANOVA showed that there was significant difference between the depth, salinity, oxygen concentration, pH, water, temperature and the time of sample collection within the rivers and water pan. This was an indication that these parameters depend on seasons and other geomorphological parameters. These are shown in the figures 2 to 6.

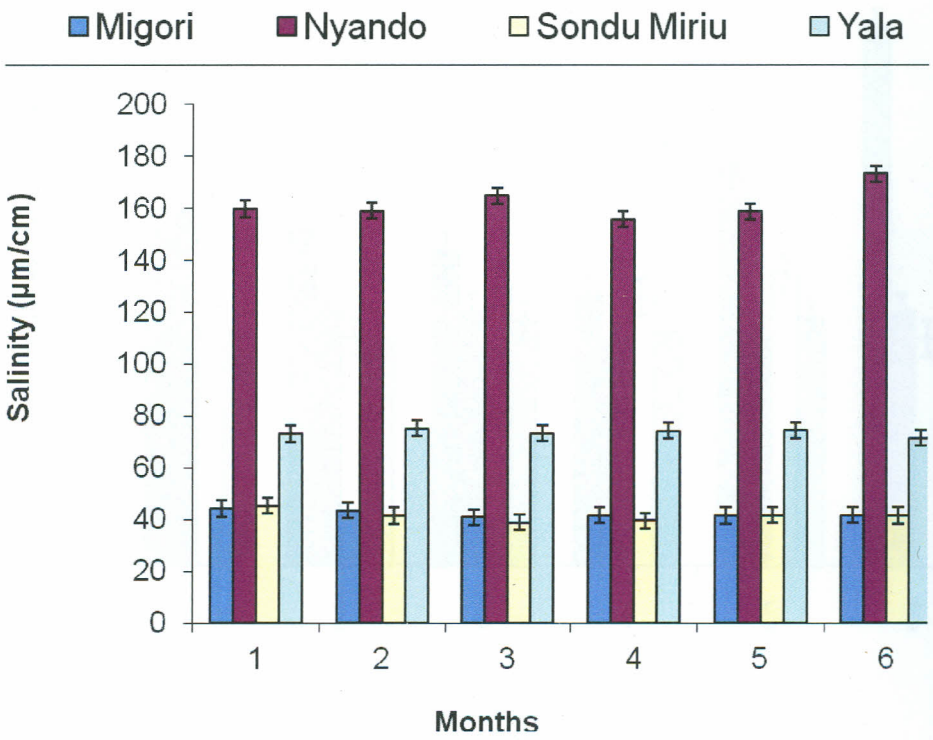


Legend: 1- July 2013; 2&3- August 2013; 4 & 5 - September 2013; 6- November 2013

Fig. 2: Average water depth (centimeters) of the rivers during study period (months)

There was statistical significant difference in the depth of the river and the season at p-value 0.001 ($\alpha = 0.05$). The depth of water was highest in August in R. Migori (24.6cm) and R. Yala (21.8cm). It was highest in July in R. Nyando (23.4cm) and R. Sondu Miriu (19.8cm). However, the depth of water was lowest in November in R. Migori (21.4cm). It was lowest in late August in R. Nyando (21.9cm). It was also

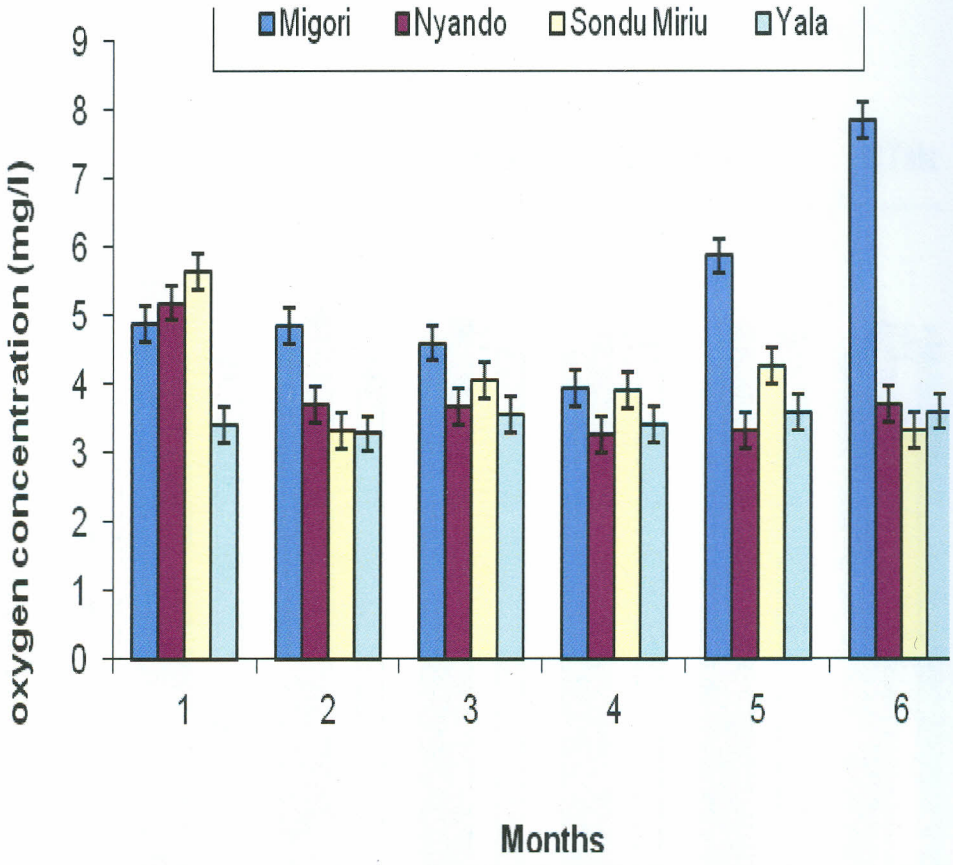
lowest in late September in R. Sondu Miriu (18.1cm) and finally lowest in July in R. Yala (20.6cm).



Legend: 1- July 2013; 2&3- August 2013; 4 & 5 - September 2013; 6- November 2013

Fig.3: Average salinity (µm/cm) of the rivers during study period (months)

There was statistical significance difference in the salinity of the river and the season at p-value 0.001 ($\alpha = 0.05$). Salinity was highest in November in R. Migori (44.4µm/cm) and R. Nyando (173.3µm/cm), in R. Sondu-Miriu it was highest in July (41.7µm/cm) while in R. Yala it was highest in August (75.2 µm/cm). However, salinity concentration was lowest in late August in R. Migori (40.9 µm/cm) and R. Sondu-Miriu (38.8µm/cm). It was lowest in September in R. Nyando (155.8µm/cm). Finally, it was lowest in November in R. Yala (71.5µm/cm).

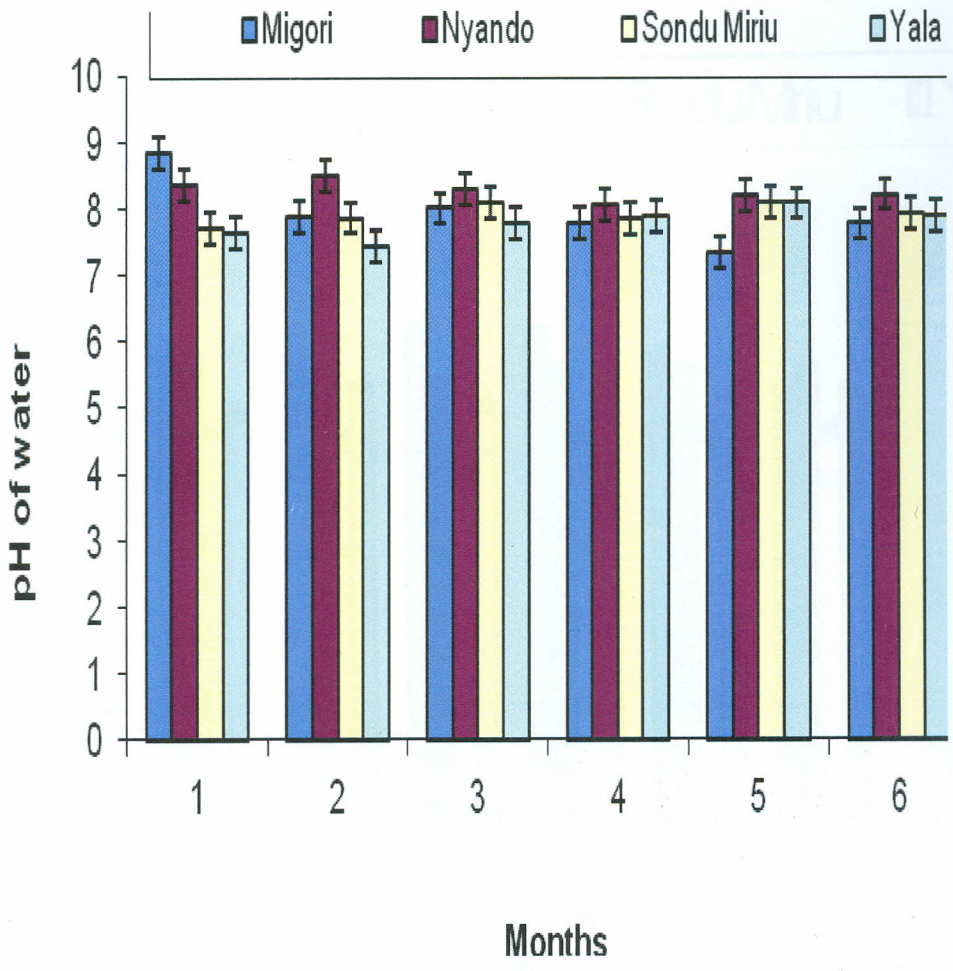


Legend: 1- July 2013; 2&3- August 2013; 4 & 5 - September 2013; 6- November 2013

Fig. 4: Average oxygen concentration (mg/l) of the rivers during study period (months)

There was significance difference in the oxygen concentration in water as well as the season at p-value 0.001 (α= 0.05) using ANOVA. Oxygen concentration was highest in November in R. Migori (7.9mg/l) and R. Yala (3.6 mg/l) while it was highest in July in R. Nyando (5.2 mg/l) and R. Sondu Miriu (5.6 mg/l). However, the oxygen concentration was lowest in September in R. Migori (3.9 mg/l) and R. Nyando (3.3

mg/l). It was lowest in November in R. Sondu Miriu (3.3 mg/l) and finally lowest in July in R. Yala (3.6 mg/l).

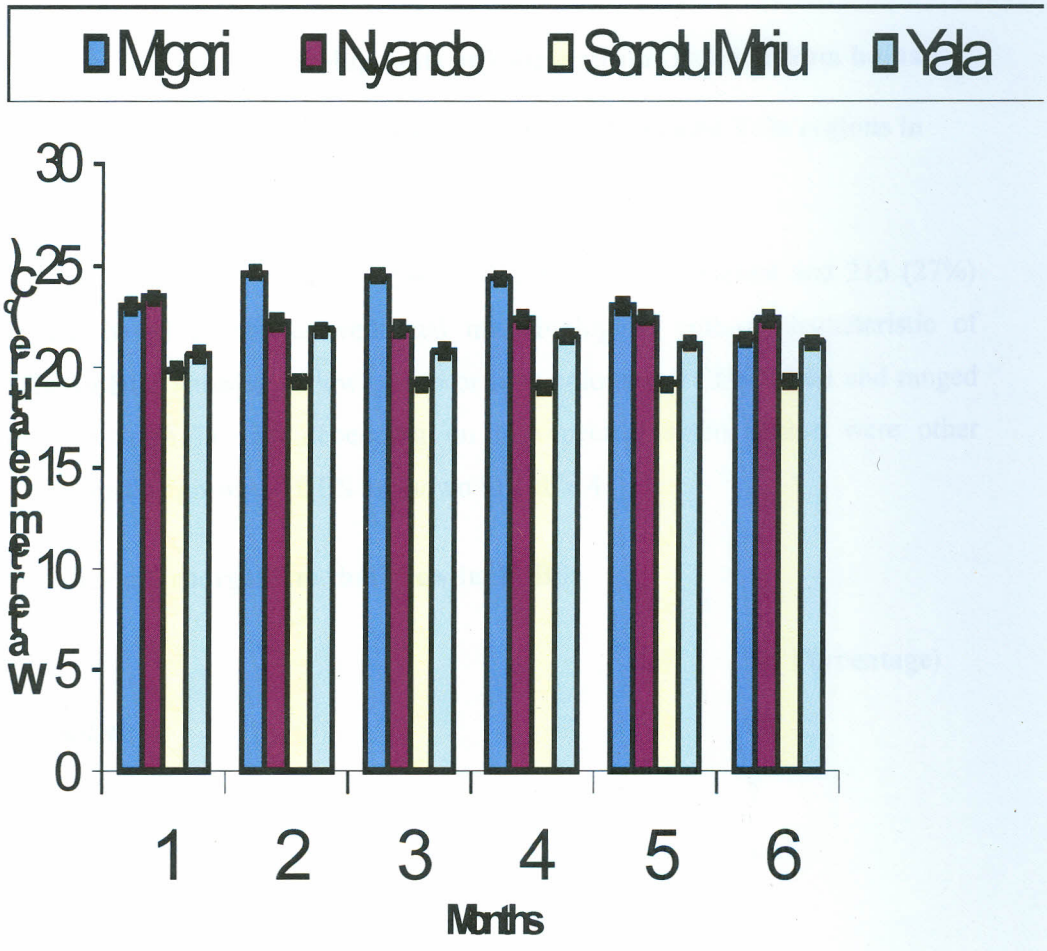


Legend: 1- July 2013; 2&3- August 2013; 4 & 5 - September 2013; 6- November 2013

Fig. 5: Average pH of the rivers during study period (months)

There was significance difference in the pH of water and the season at p-value 0.001 (α= 0.05) using ANOVA. The pH was highest in July in R. Migori (8.8) and R. Nyando (8.4) while it was highest in September in R. Sondu Miriu (8.08) and R. Yala

(8.08). However, pH was lowest in Septemberin R. Migori (7.3) and Sondu Miriu (7.7). It was finally lowest in August in R. Yala (7.4).



Legend: 1- July 2013; 2&3- August 2013; 4 & 5 - September 2013; 6- November 2013

Fig. 6: Average temperature (°C) of the rivers during study period (months)

There was statistical significant difference in the water temperature and the season at p-value 0.001 (α= 0.05). Water temperature was highest in August in R. Migori (24.6°C) and R. Yala (21.9 °C). It was also highest in July in R. Nyando (23.4 °C) and

R. Sondu Miriu (19.8 °C). The temperature was lowest in November in R. Migori (21.4 °C) and in late August in R. Nyando (21.8 °C). However, the temperature was lowest in in September and in July in R. Sondu Miriu (18.9 °C) and R. Yala (20.6 °C) respectively.

The analysis shows that salinity and oxygen are negatively correlated while the others are positively correlated.

4.2 Phenotype and colony forming units of Vibrio strains isolated from human stool and water from Migori, Nyando, Sondu, Bondo and Yala regions in Western Kenya

Out of all the 811samples collected, 596 (73%) were environmental and 215 (27%) were clinical based on the conventional microbiological culture characteristic of *Vibrio*. The isolates showed yellow, green or blue colour in TCBS media and ranged between 2 to 3mm in size depending on the specific strain. There were other microorganisms that grew in TCBS as shown in Table 4.

Table 4: Other microorganism that grew in TCBS

Species	**%(Percentage)**
Vibrio alginolyticus	14
E. coli	5
Salmonella species	5
Aeromonas spp and Enterococus spp	26
Proteus spp	37
Other bacteria	11

Out of the environmental samples, 11 (1.8%) were found to be of *Vibrio* species (Table 5). In addition, of the clinical samples, 4 (1.9%) were found to be of *Vibrio* species (see table 6) based on their growth characteristics in various media. Out of the 11 environmental samples, eight were identified as *V. cholerae* while three were identified as *V. Vulnificus.* The four *Vibrio* strains isolated from the clinical samples were *V.*

cholerae. The *V. cholerae* 01 serotype identified was of biotype EI Tor and serotype Inaba and Ogawa. Both Inaba and Ogawa serotypes were isolated from the environmental samples while isolates from clinical samples were all ogawa serotype. All the positive samples were collected in June, July, September, October and November. These concided with the short rainfall period (October to December) and long rainfall period (April to June). The *V. cholerae* from the water and stool samples were the same.

The CFU from *Vibrio* isolates from both water (Table 5) and stool (Table 6) samples were within amount of 10^6 to 10^7 capable of causing cholera to a healthy person.

Table 5: Colony Forming Units (CFU) of *Vibrio* isolates from water in respective sites

No.	Samples	CFU per ml	*Vibrio* strains
1	MW 11	$6X10^6$	*V. vulnificus*
2	MW 33	$8X10^6$	*V. vulnificus*
3	MW 28	$1.3X10^7$	*V. vulnificus*
4	MW 27	$1.2X10^7$	*V. cholerae*
5	SM 6	$5X10^6$	*V. cholerae*
6	NW 67	$7X10^6$	*V. cholerae*
7	BW 52	$1.6X10^7$	*V. cholerae*
8	BW 58	$1.3X10^7$	*V. cholerae*
9	BW 69	$6X10^6$	*V. cholerae*
10	BW 79	$2.0X10^7$	*V. cholerae*
11	Y2	$4X10^6$	*V. cholerae*

Legend: MW – River Migori water sample; SM – River Sondu-Miriu water sample; NW – R. Nyando Water sample; BW – Bondo swamps water sample; Y – River Yala water sample

Table 6: Colony Forming Units (CFU) of *Vibrio* isolates from stool samples in respective sites

No.	Samples with *Vibrio* species	CFU per ml	***Vibrio* strains**
1	NS 64	$6X10^6$	*V. cholerae*
2	NS 67	$6X\ 10^6$	*V. cholerae*
3	NS 44	$4X10^6$	*V. cholerae*
4	MS 51	$8X10^6$	*V. cholerae*

Legend: NS –Stool sample from Nyando District hospital; MS - Stool sample from Migori District hospital

4.3 Genotype of Vibrio strains obtained from clinical and water from Migori, Nyando, Sondu, Bondo and Yala regions in Western Kenya

The genotypic characteristics of the fifteen *Vibrio* isolates was determined (see 3.9) by PCR using 16S rRNA Vib 1, Vib 2 and PI primers and the amplicons are shown in Plates 1-4. The study showed that the isolates were *Vibrio* strains (Plate 1). Polymorhism was also observed in the study which was presented by more than one band in the PCR gel band (Plate 2). This showed the presence of acquired gene.

The isolates were confirmed to contain rrn genes (Plate 2). The isolates were also confirmed to be Type B *V. vulnificus* by the presence of 273bp and 825bp (Plate 3). The presence of *V. cholerae* was confirmed by *ctx* gene (564bp) (Plate 4) an indication that the isolates were pathogenic.

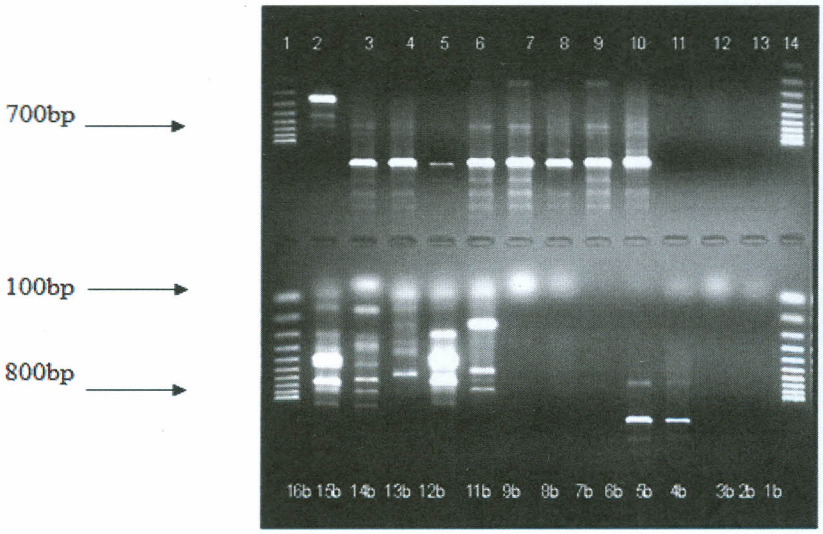


Plate 1: PCR gel band showing the presence of *rrn* gene that transcribes Bacterial 16S rRNA used for identification of *Vibrio* species. Lanes 1 and 14= L=Molecular DNA marker 100bp, lanes 2-9 isolated *Vibrio* strains, lane 10 = positive control of fresh *Vibrio*, lane 12-13=3b and 2b are negative controls (PCR water)

Primer pair:

16S rRNA-1 (length 20)-5′- AGRGTTYGATYMTGGCTCAG -3′

16S rRNA – 2 (length 19) -5′-GGYTACCTTGTTACGACTT - 3′

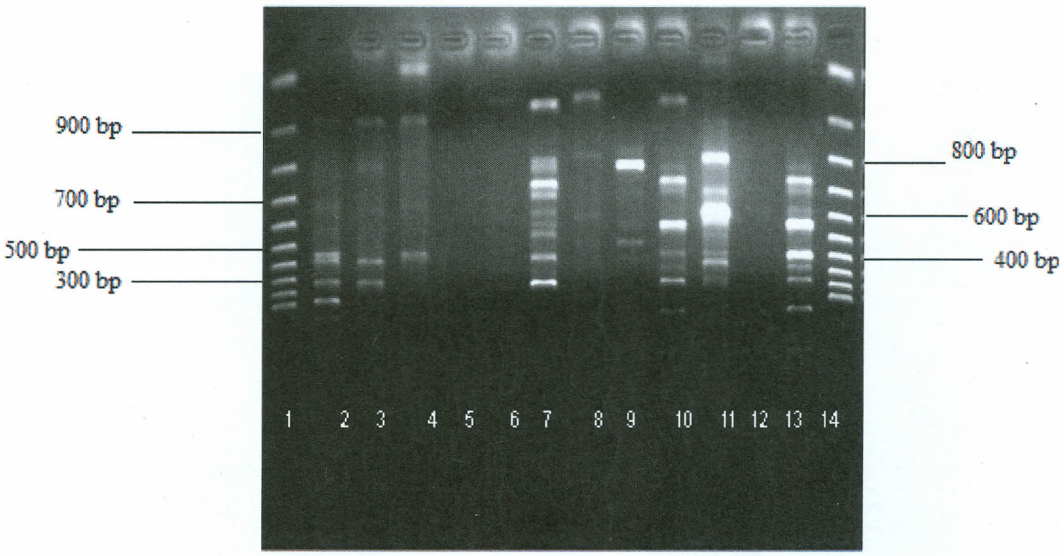


Plate 2: PCR gel band showing *rrn* gene (Vv 16S rRNA) used for identification of *Vibrio vulnificus*. Lane 1 and 14= L=Molecular DNA maker 100bp, lane 2 = positive controls of fresh *Vibrio*, lane 5= negative control (PCR water), lane 3, 4, 6-13 isolated *Vibrio* strains

Primer pair:

Vib 1 (length 20)-5′- GTGGTAGTGTTAATAGCACT -3′

Vib 3 (length 21) -5′- GCTCACTTTCGCAAGTTGGCC - 3′

The obtained results after performing ordinary quantitative PCR showed that isolates 3, 7,10,11,13 showed polymorphisms of genes an indication that there was more than one gene acquired from other *Vibrio* strains in the isolated *Vibrio* species.

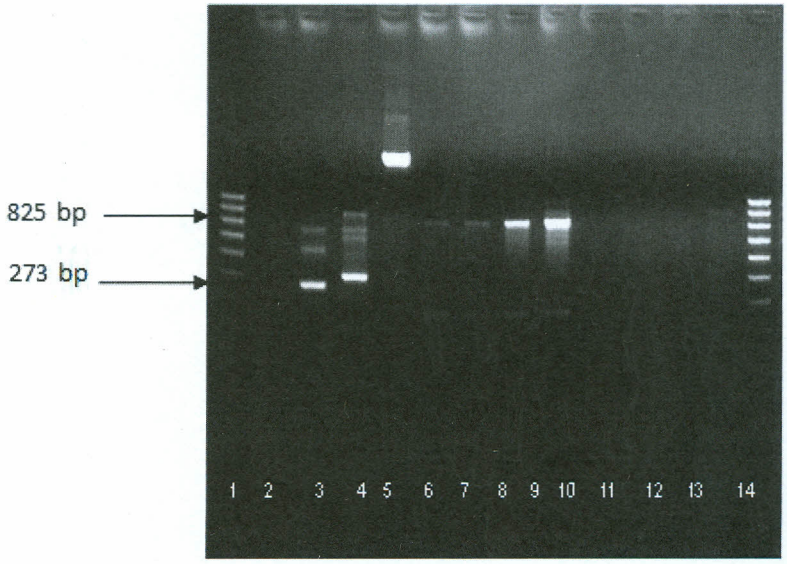


Plate 3: PCR gel of the *Vibrio* isolates showing presence of 273bp and 825bp

This confirmed the presence of Type B *V. vulnificus.* Lane 1 and 14= L=Molecular DNA maker 100bp, lane 13 negative controls (PCR water). Lanes 2-9 isolated *Vibrio* isolates. The primer pairs used were:

Vib 2 (length 18)-5′- TCTAGCGGAGACGCTGGA -3′

Vib 3 (length 21) -5′- GCTCACTTTCGCAAGTTGGCC - 3′

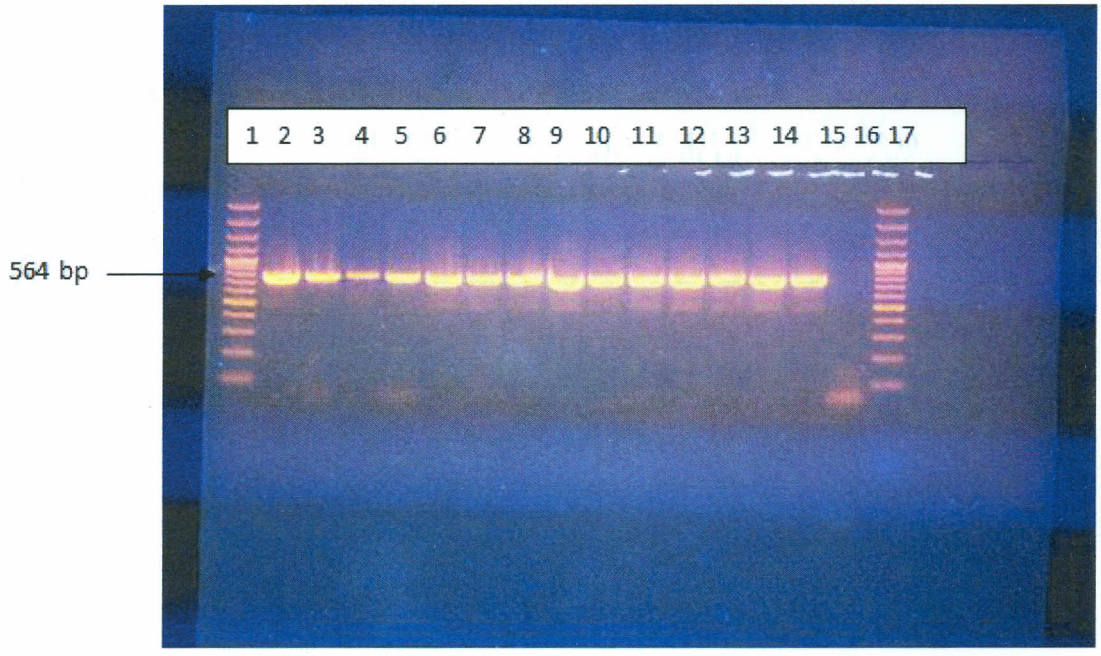


Plate 4: PCR gel band showing *ctx* gene (564bp) found in the *Vibrio* strain.

This confirms the presence *Vibrio cholerae* in the isolates. Lane 1 and 17= Molecular DNA marker 100bp, lanes 2 -14= isolated *Vibrio cholerae*, lane 15 = positive control, lane 16 = negative control (PCR water). Primer pair used was:

P1

TGAAATAAAGCAGTCAGGTG

P2

GGTATTCTGCACACAAATCAG

The obtained results indicate that isolates 2-14 were Vibrio cholerae and had a gel ladder head resolved at 564 base pair (100 bp gene marker ruler was used). This acted as an indicator for the presence of ctx gene within the isolates for Vibrio identification (Plate 4).

CHAPTER FIVE

DISCUSSION

5.1 Relationship between physicochemical parameters and Vibrio strains in R. Migori, R. Nyando, R. Sondu, Bondo Swamp and R. Yala regions in Western Kenya

Vibrio cholerae infection is dynamic and this is due to climate change (Colwell, 1996). As much as improvement of hygiene is a factor that has sometimes brought cholera to check, physicochemical parameters have continued to contribute to unpredictable outbreak of cholera. Cholera is therefore correctly referred to as emerging and re-emerging disease based on its epidemiological characteristics (WHO, 2010). This is attributed to the fact that of late there has been sporadic outbreak of cholera in the country (Kenya) with Homa Bay and Migori counties bearing the major brunt where fourteen lives were lost (Angela and Maurice, 2015) and Siaya county where five lives were lost (Nelson and Derrick, 2016). This has so far spread to various counties in the country e.g. Mombasa, Kwale (Ujamaa area in Likoni), Nakuru (Rhonda and Bondeni) and Nairobi (Mathare, Kibera and Kawangware) where several lives have been lost (Calvin, 2015). Based on this study, in terms of parametric figures, considering that the positive samples from Migori were 1.1% (0.7% from water and 0.4% from human stool samples) and those from Siaya were 0.3% (03% from water and 0% from human stool samples), one could easily disregard this as of no significance but due to the above outbreak of cholera no findings however minimal should be treated with seriousness to avoid taking any chances of outbreak since such percentages lead to loss of lives.

Increase in surface temperature and variation in tropical atmospheric thermodynamic state are expected to increase the upper limit of the distribution of tropical winds (Oouchi *et al.*, 2006; Bender *et al.*, 2010). In this regard, when we consider the environments in which this *Vibrio* strains were isolated, it was found that the water where cholera strain was isolated had a pH range of between 7.7 - 8.2 ($P \leq 0.01$),

temperature of 22 - 28°C ($P \leq 0.01$), water sanity of 17-161.2 µm/cm (0.2 to 2.3% ($P \leq$ 0.01). These physicochemical conditions correlated well with the epidemiological distribution of cholera and conditions required for *V. cholerae* and *V. vulnificus* to thrive (Kim *et al.,* 2001). It is documented by Kelly (1982) and Tamplin *et al.,* (1982) that *V. vulnificus* is frequently isolated at temperatures greater than 20°C with water salinity between 1.6 and 2.3%. Thus the physicochemical conditions reported in this study favoured the existence and isolation of *V. vulnificus* (20%) of the total collected *Vibrio* strains (both from clinical and the environment) while *V. cholerae* was 80% of the total collected *Vibrio* strains. The high *V. cholerae* population in the environment is expected to be self-regulated by sero group specific lytic phage (01 and 0139) leading to rapid outbreak like that experienced in 2015 in Nyanza starting from Migori where there were 637 cases and 14 deaths (IFRC, 2015). This is in support to the cholera episode in Western Kenya that affected 790 cases and 53 deaths being reported from 10 districts (Mohamed *et al.,* 2012).

In addition, according to Colwel *et al.,* 1994, seasonal factors such as rainfall and hours of sunlight, contribute directly to the physical and chemical characteristics of water, that in turn affect plankton populations whose blooms provide habitat to *V. cholerae* flora (Huq *et al.,* 1995, Huq *et al.,* 2001). Temperatures of ≥25°C, pH of ≥7.0 and salinity range of 0.01 to 3.3% are documented to enhance *V. cholerae* counts (Huq *et al.*, 1984; Lobitz *et al.*, 2000; Loi *et al.*, 2003). It was observed that water temperature directly correlates with cases of cholera in this study. In this regard, the average temperature of between 22-28°C was attained in this study that fostered the colonization of *Vibrio* in the respective environment. This was coupled with salinity between 0.2 - 2.3% that was not within the range of salt concentration requirement for *Vibrio* to thrive. However, as documented by Thompson *et al.,* (2004) Vibrio can thrive on 0% salinity although it has an absolute growth requirement for sodium (Thompson *et al.,* 2004). According to Randa and Polz (2004) the surface water salinity is bound to change. However, the reported salinity in this study was too low for both *V. parahaemolyticus, V. vulnificus* and *V. cholerae* to thrive hence the would be reduction of their population density (Borroto, 1997; Thompson *et al.*, 2004). Despite this, *V. cholerae* has the ability to thrive in salinity of 0, and this explains why

it was isolated in this study with a CFU of between $4X10^6$/ml to $2X10^7$ ml in a pH of between 7.7 to 8.2 a fact that is supported by studies by Thompson *et al* .(2004).

Based on these findings, it is reported that when conditions become favourable *Vibrio* transform from non-typeable, non- pathogenic to pathogenic strain and cause rampant abrupt infections like those recently reported in Migori and Homa Bay counties where a total of 14 lives were lost and 637 and 143 confirmed cases respectively (IFRC,2015). The new infections were attributed to drinking contaminated water that would have correlated with the period when bacteria were transforming from non-pathogenic to pathogenic state immediately after the start of the rainy season in the region and thriving conditions became favourable as explained therein.

In addition, factors like water salinity have been found to greatly affect proliferation of *Vibrio* strains. In this regard, response to fluctuating water salinity in the region between (0.2 to 2.3%) would have contributed immensely in the presence, emergence and re-emergence of the respective *Vibrio* isolates observed in this study. It is documented that *Vibrio* thrive in water salinity of 0.5-17% which based on this study was attained (0.2 to 2.3%) and thus enhanced their survival (Randa and Polz, 2004; Thompson *et al*., 2006). The salinity plays a great role in the growth of *Vibrio* strains since they need the salt to form biofilm and use it for other physiological activities.

5.2 Phenotype and colony forming units of Vibrio strains isolated from human stool and water from Migori, Nyando, Sondu, Bondo and Yala regions in Western Kenya

Serologically, the type of *V. cholerae* identified in the study region was inaba and ogawa. This was an important finding since earlier studies according to Dickens *et al.*, 2013 reported the presence of the former (inaba) and not the later type of *V. cholorae*. The presence of ogawa type was also recently confirmed in the recent outbreak of cholera in Migori and Homabay counties by Kenya Medical Research Institute – CDC in Kisumu, Western Kenya (MoH, 2015). This knowledge on the type of *V. cholerae* species in these areas is important in controlling and prevention of outbreak of cholera. This strain identification confirms the periodic outbreaks experienced in these regions

where non-01/non-0139 serogroups have been linked with cholera- like illness sporadically (Dalsgaard *et al.*, 1995; Dalsgaard *et al,.* 2001; Onifade *et al.,* 2011). It is documented that the non-01/non-0139 *V. cholerae* strains are frequently isolated from environment, particularly from sea food and aquatic sources (Chatterjee *et al.,* 2009) and are highly heterogeneous with considerable serological diversity and vary in virulence properties as discussed herein. In this regard, levels of serogroups 01 and 0139 in the environment begin to increase rapidly during an outbreak where levels of their respective lytic vibriophages rise in response. Once lytic Vibriophage concentrations are high enough, bacteria levels begin to drop rapidly and levels of lytic vibriophages eventually follow (Faruque *et al.*; 2004; Nelson *et al.*, 2009). This explains the low isolation levels of *V. cholerae* in this region. The environmental strains seem to constitute reservoir of potential pathogenic strains to human diarrhoeal infections (Rivera *et al.,* 2001; Singh *et al.,* 2001; Faruque *et al.,* 2003; Faruque *et al.,* 2005) where they carry key virulence genes such as cholera toxin (CT) and toxin co-regulated pili (TCP) which are usually carried by epidemic strains (Faruque *et al.,* 2004).

Some *Vibrio* (e.g. Vibrio E1ToR) produce soluble hemolysins. Others (*V. cholerae*) digest red blood cells without liberating a soluble hemolysin. They remove myxovirus receptors from the red blood cell surface by means of a receptor-destroying enzyme (RDE) neuramidase. This gives *V.cholerae* the opportunity to share the ecology with *V. vulnificus* of biotype 3 that contain the gene for cytotoxin making it critical for pathogenicity (Colodnen *et al.*, 2004). The existence of this strain (*V. vulnificus*) also leads to hemolysins resulting to necrosis of the tissue surrounding the wounds. However, Vibrio cholerae strains have been found to be able to possess a range of bacteriocins (Mitra *et al.,* 1980) that lowers the existence of other bacteria species in the environment. Therefore, the low population of *V. cholera* could be a result of upsurge of *V. vulnificus* in the environment. This is reported in patients with elevated serum iron levels as a predisposing risks factor to septicemia. Therefore, it is reported in this study that there is a confirmation of genetic overlap of the two *Vibrio* strains that is also observed in PCR amplification (Plate 2) in this study.

Aeronomonas spp is also found in the blood in persons with seriously impaired host defense or endocondition. It can cause fresh water wound infections. Thus its isolation in this study is an indication of its hemolysin activity which is shared by *V. vulnificus*, *V. cholerae*, *E. coli* and *Salmonella* spp.

Increased global temperature and sea level rise are expected to alter the geographic range of *Vibrio* spp and extend their growing season (Baker-Austin *et al.,* 2010). Regardless of the role of salinity, temperature probably has the most important effect on the prevalence and levels of the pathogenic *Vibrios*. In this study, *Vibrio cholerae* count ranged from $4x10^6$ /ml to 2.0×10^7 /ml in the water samples with the highest counts being from Bondo swamp. The range was from 4×10^6 to 8×10^6 in stool samples with the highest counts being from Migori district hospital. This was within the infectious dose (10^6 to 10^7 colony forming unit) that is required to cause cholera to a healthy person (Childers and Klose, 2007). These findings also support those of Franco *et al.,* 1997 in which increases in the number of *V. cholerae* in the environment mainly triggered by temperature, are followed by outbreak of cholera. It should also be noted that CFU counts are a rough approximation, and should be treated with caution. In instances where all biofilm cells transform to VBNC state or a technique fails to detect and quantify VBNC, it may be wrongly interpreted that an antibiotic has effectively eliminated all biofilm cell contaminants (Li *et al.*, 2014) this may result into detrimental health effects. Since CFU count technique cannot detect and quantify VBNC, it is therefore unsuitable for quantification of most biofilm-forming bacteria. In addition, the CFU method does not count inactive or damaged biofilm cells (Davey, 2011). To avoid underestimating cell counts by counting one colony per biofilm cluster rather than one colony per biofilm cell (Uppuluri *et al.,* 2006), effective disruption of biofilms from surfaces and disintegration into individual cells is crucial (Welch *et al.*, 2012). However Mouriño-Peréz and coworkers (2003) among others have found that CFU plate counts give a good correlation to fluorescent microscopy counts ($R2 = 0.72$, $p < 0.0001$) in treatments with Dissolved Organic Carbon (DOC) addition. Therefore estimation of Vibrio spp. abundances from total bacterial abundances is not possible, and molecular-based or culture-based identification of Vibrio spp. is required.

5.3 Genotype of *Vibrio* strains obtained from clinical and water from Migori, Nyando, Sondu, Bondo and Yala regions in Western Kenya

16S rRNA was used to identify the *V. vulnificus* which was further confirmed by species specific primers for *vv* responsible for expression of haemolysin. This gene is expressed to phenotypic identification of *Vibrio* strains and its amplification in these isolates acted as a confirmation of the presence of *V. vulnificus* in the samples (Nadja *et al.,* 2013). However, it is worth noting that the *V. vulnificus* genetic make- up had genes from other *Vibrio* strains in the region as depicted in Plate 1 an aspect of heterogeneity of the strains that could be as result of recombination of genes which is normally observed in high frequency recombination (Hfr) in specialized transduction. In this regard, some phages (temperaturate phages) as those seen on Vibrio species are able to establish a state known as lysogeny in which expression of phage genes and replication of the phage is inhibited. In many cases the prophage is inserted into the bacterial DNA and replicates as part of the chromosome. When lysogeny breaks down and the phage enters the lytic cycle, it is excised from the chromosome by recombination between sequences at each end of the integrated prophage. If this recombination event happens in the wrong place, an adjacent region of bacterial DNA is incorporated into the phage DNA. All the progeny of this phage will then contain this bacterial gene which will therefore be transduced at a very high frequency once the transducing phase has been isolated. This explains the possibility of activities of *Rec* genes used by *Vibrio* to acquire additional characteristics to enable them survive during harsh conditions apart from attaining phage lytic cycle in addition to expressing genes responsible for adverse conditions e.g., outer membrane protein (*ompW*) a phenomenon only endowed with *V. cholerae* strains making it a highly suitable genetic maker for the organism. This identification of the *Vibrio cholerae* strain from the possible *V. mimicus*, *Aeromonas* Spp associated with their biochemical characteristics makes identification process difficult. This has been necessitated by lack of virulence markers such as cholera toxin, toxin-coregulated pilus (TCP) that are known to be associated with the pathogenic strain of *V. cholerae* 01 or 0139 (Morris *et al.,* 1990; Kaper *et al.*, 1995). These characteristics are made evident with the fact that *toxR* gene found in both *V. cholera* and *V. parahaemolyticus* fails to display species-specific identification properties between *V. parahaemolyticus* and *V. cholerae*. The gene probe

fails to detect *V. cholerae* despite 52% identifying their *toxR* gene sequence with *V. parahaemolyticus* (Kim *et al.,* 1999). In so doing the isolates become ubiquitous and may have spatial distribution leading to sporadic infection like that reported in Migori (Angela and Maurice, 2015) and Siaya (Nelcon and Derick, 2016). This is typical of *V. cholerae* that belongs to non-01/non-0139 serogroup which can be isolated in abundance from aquatic or estuarine sources causing sporadic cases or limited outbreaks of diarrhea in human (Moris *et al.,* 1990). This aspect is depicted in Plates 1 and 2 where polymorphism of the isolates is displayed, an indication of failure to display species–specific identification properties between *V. parahaemolyticus* and *V. cholerae*. In addition, although the precise function of the *ompW* protein in *V. cholerae* is not well document, it may play a role in the adherence process, which is likely to facilitate the survival of the organism within the host or in the environment or both leading to sporadic attacks as envisaged in this study. It is reported that while the *ompW* gene is present in the smaller chromosome (there are two chromosomes in *V. cholerae*), the *toxR* gene is located in larger chromosome of the organism (Trucksis *et al.,* 1998). This serves as a survival mechanism for this organism. Based on these findings, it is evident that the use of 16SrRNA sequence as the only identification method is not very adequate due to polymorphism observed herein in the respective isolates. This is guided by lack of appreciable differences between the sequences occurring in *V. cholerae* and other members of *Vibrionaceae* family as reported in other studies (Kita-Tsukamoto *et al.,* 1993). For further and precise identification, genotypic methods were used where *V. cholerae* toxins genes were sought for by polymerase chain reaction.

Genotypically, the analysis of species specific *ctx*A genes responsible for cholera toxin that is a preserve of 01/0139 was observed (Fields *et al.,* 1992). Of the analyzed isolates, twelve had bands of 564bp. This acted as an indication that there were *V. cholerae* isolates in the water and human stool samples from the study area which was enhanced by change in temperature between 22-28°C. However, this temperature was much lower than the optimal growth temperature (30- 35°C) required by *V. parahaemolyticus* for its growth (Jay *et al.,* 2005). Thus lack of its isolation in the study samples.

The existence of some aspects of genome polymorphism within the isolates observed in this study was an indication of the presence of other genes that may have been acquired from the environment through phage mediated integration mechanism. *Vibrio cholerae* is the causative agent of the diarrheal disease cholera which is usually acquired by oral ingestion of bacterium with concentrated water or food (Dirita, 1992). In response to specific environmental conditions such as temperature, pH or Osmolarity (Gardel and Mekalanus, 1994). *V. cholerae* expresses several virulence determinants including the cholera toxin (CTX), a toxin coagulating pilus (TCP), the accessory colonization factor and a major outer membrane protein (OmpU) (Peterson and Mekelenos, 1988).

Environmental signals optimal for CTX production also stimulates the expression of TCPA (Iredell and Manning, 1994) and OmpU, a porin that may also function as an adhesin (Sperandio et *al.*, 1996). It coordinates expression of *ctxAB*, *tcP A* to *F* and some *acf* genes which is due to the action of several regulating proteins that function in a branched regulatory cascade in which this activator protein ToxR and TcpP, are required for activation of toxT transcription (Hase and Mekalanos, 1998) and ToxT, a member of the AraC family with transcriptional activators (Higgins *et al.,* 1992) that activates expression of other virulence genes, including *ctxAB* and *tcpA* to *F* (Dirita *et al.*, 1991). The *OmpU* gene is in a ToxT-independent branch of this cascade and is activated directly by *ToxR* (Champion *et al.*, 1997; Crawford *et al.,* 1998). In this regard, the amplification of *ctx* gene (564bp; Plate 4) is an indication that the gene was being expressed and is its regulation by *ToxR* and *TcpP* which are required for the activation of *toxT* transcription and *toxT* that activates the expression of other virulence genes including *ctxAB* and *tcpA*. The CTX genetic elements are more stable in the classical strains than E01 Tor biotype or 0139 strains, presumably because the *ctx* genetic elements in classical strains besides being widely separated are never flanked on both sides by RSI elements (Mekalanos, 1983). In contrast to either EI Tor biotype or 0139 strains, one or both copies of *CTX* genetic elements are flanked by repeat sequence 1 (RSI) (Mekalanous, 1983).

The most frequent genotype in clinical and environmental *V. choerae* 01 strains is *hylA*, *hap*, *CtxA*, *zot*, *toxR*, *tcp1*, *tcpA*, *rtxA*, *VasA*, *vark* and v*asa*, *vark* and *vas H* of which *ctxA* that is associated to *toxR*, *tcpF*, *tcp* was amplified in this study.

All *V. cholerae* 0139 isolates are expected to be positive for *tcp I*, *hop* and *T6ss*, *hlyA*, *ctxA*, *zot*, *toxR* and *rtxA* of which this study amplified *ctxA* or cassette of *tcpS* and *toxR*. This also corroborates with the findings by Singh *et al.*, 2001 that showed that cholera-toxin producing strains have the ability to produce secretogenic toxins.

CHAPTER SIX

CONCLUSION AND RECOMMEDATIONS

6.1 CONCLUSION

i. Physicochemical parameters increased the growth of *Vibrio* strains in R. Migori, R. Nyando, R. Sondu, Bondo Swamp and R. Yala. In this study, there was a positive correlation of colony forming unit and temperature, salinity and depth of water. The colony forming unit ranged from 4×10^6 /ml to 2.0×10^7 /ml in the water and from 4×10^6 to 8×10^6 in the stool. This was within the range that has been reported to cause cholera hence the explanation for cholera outbreak in some parts of the study region and could be used as reference point in determining possibilities of outbreaks.

ii. There is relationship between phenotypic identification and colony forming unit of *Vibrio* strains as a method of identification of isolates from human stool and water from Migori, Nyando, Sondu, Bondo and Yala regions in Western Kenya. This study established the presence of both *V. cholerae* and *V.vulnificus* in Migori, Sondu-Miriu, Nyando, Bondo and Yala and their favourable conditions. The serogroup of the *V. cholerae* found in this region was inaba and ogawa 01/0139 serogroup. The *V. vulnificus* found in this study was type B. This type B *V. vulnificus* was from water samples collected from R. Migori. The environmental conditions therein in the study area could not support the survival of *V. parahemolyticus* hence its absence in human stool and water samples from the study area as discussed therein.

iii. The genotype of *Vibrio* strains obtained from human stool and water from Migori, Nyando, Sondu, Bondo and Yala regions in Western Kenya in this study was *ctxA* that is associated to *toxR*, *tcpF*, *tcp*. The genome polymorphism observed in this study was an indication of the presence of other genes that may have been acquired from the neighboring strains through other gene transfer methods e.g. transformation, conjugation and transduction that needs to be studied further.

6.2 RECOMMENDATION FROM THIS STUDY

1. Physicochemical parameters which increase the growth of *Vibrio* strains in the study area should be on check to act as indicator to possible outbreak.

2. This study showed the presence of serotype of *V. cholerae,* inaba and ogawa. This information can be used to control and manage the outbreak caused by these serotypes. Strategies for control of *Vibrio vulnificus* in this region should also be put- in place and periodic surveillance should be encouraged for both *V. cholerae* and *V. vulnificus*.

3. The virulent genes of *V. cholerae* and *V. vulnificus* in the study region can inform development of vaccine against *V. cholerae* in the region. Polymorphisms shown in this study is a clear indication of gene transfer between the *Vibrio* species an indication that more than one antibiotic may be used to treat cholera at a given time.

6.3 RECOMADATIONS FOR FUTURE STUDIES

1. There is need to study the mechanistic applications of physicochemical factors that enhance survival of the *V. cholerae* in the environment in this region and other regions in the country.

2. The type of *Vibrio* strains in the other parts of Kenya namely Coastal, Nairobi and Nakuru regions that are prone to cholera outbreak need to be established for epidemiological purposes taking into account the ogawa strain as opposed to inaba alone.

3. There should be investigation on the antimicrobial that the *V. cholerae* in this region is resistance to based on the genetic heterogeneity displayed by the isolates in this study.

4. There should be a study on the cause of gene polymorphism observed in this study.

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