PREVALENCE, PATHOGENIC MARKERS AND ANTIBIOTIC SUSCEPTIBILITY OF VIBRIO CHOLERAE IN SARDINES, WATER AND PHYTOPLANKTON IN LAKE TANGANYIKA, TANZANIA

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A DISSERTATION SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN PUBLIC HEALTH AND FOOD SAFETY OF THE SOKOINE UNIVERSITY OF AGRICULTURE. MOROGORO, TANZANIA.

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ABSTRACT

Cholera is an epidemic disease of major global and public health significance. A cholera outbreak in Kigoma region of Tanzania is reported to be endemic as it occurs almost every year. The aim of this study was to investigate the prevalence, pathogenic markers and antibiotic susceptibility of *Vibrio cholerae* in sardines, water and phytoplankton in Lake Tanganyika. A total of 120 samples from sardines, water and phytoplankton were collected from October, 2015 to February, 2016. Isolation of *V. cholerae* was carried out as per Tanzania Bureau Standards (2002). All isolates were subjected to PCR for confirmation by detecting the outer membrane protein (ompW). Virulence genes were detected using molecular methods by targeting cholera enterotoxin gene (ctx), the toxin co-regulated pilus gene (tcpA), the toxin regulatory protein (toxR) and the haemolysin gene (hlyA). Then, the isolates were tested for antibiotic susceptibility using antibiotic discs with Ampicillin, Chloramphenicol, Ciprofloxacin, Gentamycin, Amoxicillin and Tetracycline. The findings showed that, 9% (n = 66) of the sardines samples and 3% (n=30) of the water sample harboured *V. cholerae* O1. *Vibrio cholerae* was not detected and isolated in phytoplankton samples (n = 24). One Isolate from water samples harboured both toxR and hlyA, but not ctx and tcpA. *Vibrio cholerae* isolates were resistant to Ampicillin (83.33%), Amoxicillin (100%), Chloramphenicol (50%) and Tetracycline (100%). All of the isolates were susceptible to Gentamicin and Ciprofloxacin. Generally the study concludes that sardines and water are important reservoirs of *V. cholerae*. Regardless the absence of ctx and tcpA, monitoring for *V. cholerae* should be done as they pose threat to human health. Prevention and control regimes should take into considerations the type of antibiotics to be used based on the level of resistance observed in this study.
DECLARATION

I, Nyambuli Sosthenes Muinja, do hereby declare to the Senate of Sokoine University of Agriculture that this dissertation is my own original work and that it has neither been submitted nor being concurrently submitted for a higher in any other institution.

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(MSc. Candidate)

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Prof. Robinson H. Mdegela                   Date

(Supervisor)

_________________________  ________________________
Dr. Abdul A.S. Katakweba                   Date

(Supervisor)
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<tbody>
<tr>
<td>APHA</td>
<td>American Public Health Association</td>
</tr>
<tr>
<td>APW</td>
<td>Alkaline Peptone Water</td>
</tr>
<tr>
<td>ATB</td>
<td>Antibiotics</td>
</tr>
<tr>
<td>Bp</td>
<td>Base Pair</td>
</tr>
<tr>
<td>CDC</td>
<td>Centres for Disease Control</td>
</tr>
<tr>
<td>CLSI</td>
<td>Clinical and Laboratory Standards Institute</td>
</tr>
<tr>
<td>CT</td>
<td>Cholera Toxin</td>
</tr>
<tr>
<td>ctxA</td>
<td>Cholera enterotoxin sub unit A</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>GPSB</td>
<td>Gelatin Phosphate Saline Broth</td>
</tr>
<tr>
<td>GPA</td>
<td>Gelatin Phosphate Agar</td>
</tr>
<tr>
<td>hlyA</td>
<td>Haemolysin</td>
</tr>
<tr>
<td>ICEs</td>
<td>Integrating Conjugative Elements</td>
</tr>
<tr>
<td>ml</td>
<td>Millilitre</td>
</tr>
<tr>
<td>μl</td>
<td>Microlitre</td>
</tr>
<tr>
<td>μm</td>
<td>Micrometre</td>
</tr>
<tr>
<td>Nacl</td>
<td>Sodium chloride</td>
</tr>
<tr>
<td>NIMR</td>
<td>National Institute for Medical Research</td>
</tr>
<tr>
<td>PAHO</td>
<td>Pan American Health Organization</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PFGE</td>
<td>Pulsed-Field gel Electrophoresis</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolution per Minute</td>
</tr>
<tr>
<td>STSI</td>
<td>Saline Triple Sugar Iron Agar</td>
</tr>
<tr>
<td>TAFIRI</td>
<td>Tanzania Fisheries Research Institute</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>TASP II</td>
<td>Trade and Agriculture Support Programme Phase II</td>
</tr>
<tr>
<td>TCBS</td>
<td>Thiosulfate Citrate Bile Sucrose</td>
</tr>
<tr>
<td>TCP</td>
<td>Toxin Coregulated Pilus</td>
</tr>
<tr>
<td>tcpA</td>
<td>Toxin Coregulated Pilus sub unit A</td>
</tr>
<tr>
<td>toxR</td>
<td>Toxin Regulator</td>
</tr>
<tr>
<td>TSI</td>
<td>Triple Sugar Iron</td>
</tr>
<tr>
<td>TZS</td>
<td>Tanzania Standards</td>
</tr>
<tr>
<td>URT</td>
<td>United Republic of Tanzania</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra violet</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organisation</td>
</tr>
</tbody>
</table>
CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

The human population surrounding Lake Tanganyika depends on the fish found in the lake (URT, 1998). The fishery of Lake Tanganyika is of great importance to the surrounding regions since fish are important source of food, provide employment and increase income. Animal derived protein, vitamins, minerals and fat from cattle, goats, and sheep as well as chickens are increasingly becoming scarce, thus fish remains the major source of nutrients in those areas. In the lake, there are two major types of commercial fish; small fish namely Stolothrisa tanganicae (locally known as Dagaa) and the bigger ones Limnothrisa miodon (locally known as Lumbo) that are native fish species of Lake Tanganyika. Both smaller and bigger fish contribute over 60% to the catches of Lake Tanganyika where Tanzania is a major exporter of fish from the lake as compared to the other countries sharing the lake (Kirema-Mukasa, 2012).

Although fish from the lake are good sources of proteins, they are associated with enteric bacterial contamination like Vibrio cholerae, Salmonella spp and E. coli (Sichewo et al., 2013). All bacteria are of public health importance because can cause different diseases to humans (Nourmohamadi and Shokrollahi, 2014). Fish can be contaminated with pathogenic bacteria if washed with contaminated water. Fish can also contaminate water through their gut wastes. Although, the ability of pathogenic vibrios to cause disease depends on the expression of various virulence-factors like toxin- coregulated pilus (Sathiyamurthy et al., 2013), V. cholerae is among the major cause of food-borne infections with a considerable social-economic cost to the affected populations. Every year millions of cholera episodes occur throughout the world especially in developing countries.
In 2015, a total of 42 countries from all continents reported 172,454 cases of cholera to WHO, of which 41% were reported from Africa (WHO, 2016). According to Mayala et al. (2003), from 2001 to 2002, about 75% of the regions in Tanzania reported cholera outbreaks and Kigoma was among the affected regions. Of recent, a total of 20,961 cholera cases, including 329 deaths were reported in Tanzania from October, 2015 - March, 2016 (WHO, 2016).

Cholera outbreaks in Kigoma region of Tanzania are reported to be endemic. The endemicity is contributed by overcrowding of Burundian refugees and poor sanitation (ICT, 2015). In this region, different antibiotics are used to combat the disease especially during the cholera outbreak. It is reported that the V. cholerae strain currently circulating in Kigoma is sensitive to tetracycline, ciprofloxacin, and erythromycin antibiotics (CDC, 2015). V. cholerae 01 strains studied during two epidemic periods in 1997 and 1999 when tetracycline or erythromycin was used for treatment of patients with severe disease. Among the 94 V. cholerae strains isolated in 1997; 98.6%, 93.6% and 81.9% were sensitive to Ciprofloxacin, tetracycline and erythromycin, respectively (Urasa et al., 2000). Moreover, of the 87 strains collected in 1999, 100%, 58.6% and 46% were sensitive to ciprofloxacin, tetracycline and erythromycin, respectively. However, V. cholerae as other bacteria of clinical and public health significance is continuously developing more resistance to newer antibiotics in various places, especially in the developing countries (Ukaji et al., 2015). Despite, the greatest challenge of V. cholerae towards antibiotics therapy is the development of antibiotics drug resistance, there are limited studies so far been conducted in Kigoma to establish the resistance of V. cholerae on the antibiotics used. Therefore, this study was conducted in order to fill the gap in knowledge regarding the prevalence, virulence characteristics and antibiotic susceptibility of V. cholerae in Lake Tanganyika.
1.2 Problem Statement and Justification

1.2.1 Problem statement

High prevalence of cholera in Kigoma is reported at alarming rate since 1978 (Rutagemwa, 2010). In addition, the recurrence of cholera outbreak also reported in the study area (Rutagemwa, 2010). The region has a case fatality rate of 3.06% for a population estimated at 2127930 in 2012 (EPoA, 2015). In May 2015 for instance, 31 people died of cholera and more than 3,000 had acute diarrhoea cases. These were among the refugees in the port of Kigoma town along Lake Tanganyika; nearby Kagunga village (Gruijl and Dobbs, 2015). Cholera outbreak in human is caused by *V. cholerae* that may contaminate water and foodstuffs including fish (Lutz *et al.*, 2013). Moreover, cholera outbreak is associated by poor sanitation practices and unhygienic environmental condition of lake. Fish and water serves as vehicles for the transmission of Vibrio species in human, especially if water used for washing sardines is contaminated with *V. cholerae*. Contaminated fish and water increases the incidence of cholera outbreak and hence more burden to human health (Senderovich *et al.*, 2000). The effect associated with cholera to affected individuals include, severe dehydration and death within hours if left untreated. This leads to great economic losses due to loss of manpower and cost in disease control and prevention.

1.2.2 Justification of the study

The Kigoma Municipal residents depend on the Lake Tanganyika as the main source of fish as food, employment and income. Water from the lake is used for domestic purposes (drinking, bathing and washing of clothes) and food production (URT, 1998). There are limited studies that has been conducted to establish the extent of bacterial contaminants in sardines and water in Lake Tanganyika, thus it necessitate the need to investigate the magnitude of *V. cholerae* from environmental samples in Lake Tanganyika. On the other
hand, a significant increase in the prevalence of *V. cholerae*’s resistance to antibiotic agents is reported (Akoachere *et al.*, 2013). Thus, a test for antibiotic susceptibility was needed to be carried out. The findings from this study therefore will serve as baseline information and an input to policy makers for developing preventive and control measures of *V. cholerae* in Lake Tanganyika and other lakes in the countrywide.

1.3 Objectives

1.3.1 Overall objective

The overall objective of this study was to evaluate the extent of *Vibrio cholerae* contamination and their antibiotic resistance patterns in sardines, water and phytoplankton in Lake Tanganyika, Tanzania.

1.3.2 Specific objectives

i. To determine the prevalence of *V. cholerae* in sardines, water and phytoplankton in Lake Tanganyika, Tanzania

ii. To identify the virulence characteristics of *V. cholerae* isolates in sardines, water and phytoplankton in Lake Tanganyika, Tanzania

iii. To establish the magnitude of antibiotic susceptibility for *V. cholerae* isolates from sardines, water and phytoplankton samples to antibiotics.

1.4 Research Questions

i. What is the prevalence of *Vibrio cholerae* in sardines, water and phytoplankton in Lake Tanganyika?

ii. What are the virulence characteristics from the isolated *V. cholerae*?

iii. What is the magnitude of susceptibility for isolated *V. cholerae* to antibiotics?
CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Identification and Classification of *V. cholerae*

*Vibrio cholerae* is a Gram-negative, non-spore forming, curved rod whose motility depends on a single polar flagellum. The bacterium is part of the Vibronaceae family and it is about 1.4 – 2.6 µm long (Fig. 1), facultative anaerobe, and fermentative metabolism. It is oxidative-positive, reduce nitrate and motile by means of a single, sheathed, polar flagellum (WHO, 2002).

![Figure 1: Showing scanning electron microscope image of Vibrio cholerae](http://remf.dartmouth.edu/Cholera_SEM/)

As Igbnosa and Okoh (2009) uphold, *V. cholerae* can be divided into serogroups (Fig. 2), using specific antisera or monoclonal antibodies against the ‘O’ antigen component of the bacterial lipopolysaccharide. Although there are more than 200 *V. cholerae*, only type O1 and O139 are primarily responsible for cholera outbreaks in the world (WHO, 2002). Also, while the O1 serogroup exists as two biotypes, classical and El Tor the antigenic
factor allows further differentiation into two major serotypes Ogawa and Inaba. The strains of the Ogawa serotype however are said to express A and B antigens and small amount of C antigen, whereas Inaba strains express only A and C antigens. Similarly, a third serotype (Hikojima) though expresses all three antigens, it is rare and unstable (Igbinosa and Okoh, 2009). Growth of *V. cholerae* is stimulated by addition of 1% sodium chloride (NaCl). An important distinction of *V. cholerae* from other *Vibrio* spp is its ability to grow in nutrient broth without NaCl added (WHO, 2002). Biochemical tests, agglutination tests and molecular methods are also used for the identification of *V. cholerae* (Huq *et al.*, 2012).
2.2 Virulence Factors and Pathogenicity of *V. cholerae*

The ability of *V. cholerae* O1 and O139 to cause cholera depend upon the production of cholera toxin (CT) that alters the ionic fluxes across the intestinal mucosa, resulting in substantial loss of water and electrolyte in liquid stools (Bina *et al.*, 2013). The *V. cholerae* O1 strains may produce CT and other virulence factors; non O1 strains rarely
possess these attributes. Regardless the existing knowledge about *V. cholerae*’s mode of infectivity, 70% of human still exhibit diarrhoea when inoculated with a *V. cholerae* strain which lacks CT (DiRita *et al.*, 1991). Strains may produce Haemolysin that are responsible for pathogenesis. Even though different studies on *V. cholerae* O1 and O139 indicate that CT, toxin coregulated pilus (TCP) and toxin regulator (TR) are necessary to cause diarrhoea, studies on non-toxigenic environmental isolates of *V. cholerae* show a secretory response in intestinal tissue that may be caused by other virulence factors (Fraga *et al.*, 2007). Also TCP is the virulence determinant that is required for colonization. The expression of the genes that encode both CT and TCP production are under the protein toxin regulator (toxR). Other virulence factor that contributes to human pathogenesis is hlyA gene (Haemolysin) that confers Vibrio cells capacity to cause blood cell lysis in the infected host (Fooladi *et al.*, 2013). The pathogenesis of *V. cholerae* is well established when it infects human due to ingestion of contaminated water and food. After passage through the acid barrier of the stomach, the organism colonizes the epithelium of the small intestine by means of TCP, hemagglutinin and core-encoded pili which are other colonization factors of *V. cholerae*. Cholera enterotoxin produced by *V. cholerae* is secreted across the bacterial outer membrane into the extracellular environment and disrupts ion transport by intestinal epithelial cells (Igbinosa and Okoh, 2008).

### 2.3 Ecology and Environment of *V. cholerae*

In the aquatic environment, *V. cholerae* is found in association with zooplankton and phytoplankton on the chitinous exoskeletons, crustaceans and in the mucilaginous health of blue-green algae (Igbinosa and Okoh, 2008). The main factors influencing the occurrence and distribution of *V. cholerae* in aquatic environments are water, temperature, salinity, nutrient availability and its association with other marine organisms. Water temperature is considered the most important factor influencing the distribution and
abundance of *V. cholerae* (Igbinosa and Okoh, 2008). The highest concentration of *V. cholerae* occurs when water temperature is between 20°C and 30°C. Vibrios produce a chitinase that bind to chitin. Chitin is a semi-transparent material predominantly mucopolysaccharide that is the principal component of crustacean shells. *Vibrio cholerae* O1 attaches to moulted zooplankton exoskeletons (exuviae) (Fig. 3A) and on *Volvox* spp. (a colonial form of phytoplankton) and the attachment appears as a ‘ring’ pattern (Fig. 3B) (Tamplin *et al*., 1990).  

**Figure 3**: Showing fluorescence photomicrography of *V. cholerae* O1 attached to plankton


Epislein *et al*. (1993) and Sawabe *et al*. (2003) reported that, chitinases and mucinases facilitate the attachment of *V. cholerae* to aquatic organisms, while algae surface films enhances the growth of the pathogen. In the aquatic environment, Vibrios may undergo a series of major physical and metabolic changes. For example Igbinosa and Okoh (2008)
illustrate that *V. cholerae* can be dormancy in response to nutrient deprivation, elevated salinity and / or reduced temperature. In addition, Yam *et al.* (1999) demonstrated strong linear correlations between non-O1, temperature and salinity. *Vibrio cholerae* show an initial rapid decline in total rapids, carbohydrates and a more gradual decline in proteins and DNA due to nutritional deprive.

### 2.4 Antibiotic Susceptibility of *V. cholerae*

The greatest challenge of *V. cholerae* towards antibiotic therapy is the development of antibiotic drug resistance. Strains with transferable, multiple drug resistance were first isolated in 1964 - 1965 in the Philippines (Sack *et al.*, 2001). *Vibrio cholerae* O1 strains isolated in Tanzania in 1999 increased resistance to Tetracycline and Ampicillin compared to strains obtained in 1997 (Urassa *et al.*, 2000). It is restated that the use of antimicrobial in livestock treatment without consulting veterinarians, the use of indigenous herbs and changing the antibiotics (once there is a feeling that the former one was not effective) may result into microbial resistant into human (Katakweba *et al.*, 2012). For instance, Tagoe and Attah (2009) report that antibiotic abuse is common in Ghana due to lack of hospital attendance for proper diagnoses, purchasing antibiotics without doctor’s prescription or pharmacist’s advice and misuse of antibiotics for treating common cold or flu. Antibiotics dispensed for human treatment without prescription may cause the high prevalence of antibiotic resistance (Kagashea *et al.*, 2010). Antibiotic drug resistance in Vibrio species can develop through mutation or through acquisition of resistance genes on mobile genetic elements such as plasmids, transposons, integons and integrating conjugative elements (ICEs) (Sjolund-karlsson *et al.*, 2011). The ICEs commonly carry several antibiotic drug resistance genes and play a major role in the spread of antibiotic drug resistance in *V. Cholerae*. This bacterium carries genes encoding resistance to
ampicillin, chloramphenicol, gentamicin, streptomycin and tetracycline (Amit and Ramamurthy, 2011).

2.5 Isolation and Identification of *V. Cholerae*

The control and prevention of *V. cholerae* infection is largely based on screening of water and food for the presence or absence of bacterium and the genes responsible for their pathogenesis. Traditional microbiological method employs two types of media for enrichment namely; Alkaline Peptone Water (APW) and Gelatin Phosphate Sulphate Broth (GPSB). The Media is followed by Thiosulfate Citrate Bile Salts Sucrose (TCBS) agar and Gelatin Phosphate Agar (GPA). These media use an alkaline pH and a moderate salinity to increase Vibrio growth and decrease growth of non-Vibrio species (TZS 733: 2002). Following an incubation period, presumptive *V. cholerae* can be isolated from these media and confirmed by either biochemical analyses or immediately by PCR. Isolates can then be serogrouped as O1, or non-O1 by a slide agglutination assay using antisera for the O1 antigen or by PCR using primers developed to target O1 coding regions of the genomic DNA. In addition, the distinction between virulent and non-virulent strains can be accomplished using PCR whose first target is species-specific genes followed by the multiple virulence-associated genes (Huq *et al*., 2012). Furthermore, molecular analysis such as ribotyping and pulsed-field gel electrophoresis showed that, *V. cholerae* O1 strains had all of the virulence factors normally found in *V. cholerae* O139. However, in contrast to O1 strains that have a core substituted with an average of 17 repeat units of 4-NH2-4 and 6-dideoxymannose, O139 strains are encapsulated. Furthermore, the bacterial lipopolysaccharide (LPS) of serogroup O139 does not contain any long O-antigen side chains (Faruque *et al*., 2003).
2.6 Prevention and Control of *V. Cholerae* Infections

Measures for the prevention of cholera mostly consist of provision of safe water, proper sanitation, and food safety to populations who do not yet have access to basic services (WHO, 2016). Appropriate media, such as radio, television or newspapers should be involved in disseminating health education messages aiming at communities adopting preventive behaviour for averting contamination. In addition, strengthening surveillance and early warning greatly helps in detecting the first cases and put control measures in place (WHO, 2016).
CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Description of the Study Area

Kigoma region is located on the shores of Lake Tanganyika in the North - West corner of Tanzania. The region is situated between Longitudes 29. 5° and 31.5° East and Latitudes 3.5° and 6.5° South of the Equator. The region shares boundaries with country of Burundi and Kagera region to the North, Shinyanga and Tabora regions to the East, the Democratic Republic of Congo to the West and Rukwa region to the South (URT, 1998). The north-eastern section of Lake Tanganyika belongs to Burundi, the whole of the west coast belongs to the Democratic Republic of Congo, the southern part to country of Zambia and about 480 km of the eastern coast belongs to Tanzania (Kirema-Mukasa, 2012). Lake Tanganyika covers an area of 32 945 km² out of which 13 510 km² lies within the borders of Tanzania. The lake is about 650 km long with an average width of about 80 km and 1,470 m maximum depth (Kiwale, 2003).

3.2 Study Sites

The study was carried out in Kigoma Municipality in areas along the Lake Tanganyika (Tanzania side). The sites under study were Kibirizi (No. 1) fish landing site (3-4 km from Kigoma town) and Katonga (No. 2) fish landing site (a small village in Bangwe Division situated 4-5 km to the south of Kigoma Bay) (Fig. 4). Apart from fishing activities (Fig. 5); bathing, washing, swimming and fetching water were carried out in the selected sampling sites (Fig. 6). These landing sites were selected because they are the largest sites for landing fish from the lake and were easily accessible during sampling which were very important for maintaining the integrity of the sample.
Figure 4: A map of Kigoma Municipality showing study areas
Figure 5: Sun drying sardines on ground racks found in Kibirizi landing site during sample collection

Figure 6: Water from Kibirizi landing site used for domestic purposes
3.3 Study Design and Samples Collected

The study design followed was cross-sectional one. The study was done from October, 2015 to February, 2016. Samples of fresh sardines were purchased from local fishermen immediately after landing at the beach and then stored overnight at Tanzania Fisheries Research Institute (TAFIRI), Kigoma. Fresh sardines were stored in freezer at -18 °C, water and phytoplankton were stored in a fridge at 2 - 8 °C. Samples were transported in cool box with ice cubes at about 2 - 8 °C to National Fish Quality Control Laboratory (NFQCL) Nyegezi, Mwanza for testing.

3.4 Sample Size

Sample size was estimated using the formula derived by Chulaluk (2009).

\[ n = \frac{z^2 \ p \ (1-p)}{\varepsilon^2} \]

\( Z \): 1.96 (assuming 95% confidence interval),
\( \varepsilon \): 5% (maximum error allowed),

\( P \): Prevalence of *Vibrio cholerae* 14.8% in water (Madoroba et al., 2010), 50% in sardines (unknown prevalence) and 2% in phytoplankton (Mansurul, 2011).

Calculated number of samples \( n \) equal to: Water = 193.76, Sardines = 384.16 and Phytoplankton = 30.12. But due to limited resource; only 15% (29.064 = 30) of water samples, 17% (65.307 = 66) of sardines samples and 80% (24) of phytoplankton samples were collected.

3.5 Sampling Procedure

Samples were collected from local fishermen in sterile polythene bags; about 100 g of sardines samples were collected. Dry sardines were stored at ambient temperature 20 – 25 °C and fresh sardines in sterile cool box with ice cubes (2 – 8 °C). About 250 ml of water samples were collected in 250 ml sterile bottles from surface water and stored in sterile
cool box with ice cubes (2 – 8 °C). Approximately 100 ml of phytoplankton samples were collected at depth of about 10 m in order to capture different species of phytoplankton. The Phytoplankton samples were collected according to APHA (1998) whereby 100 Litres of surface water was filtered through a phytoplankton net of 13 µm mesh size. The concentrate that remained at the bottom of the net (about 100 ml) was collected into a sampling bottle ready for laboratory analysis. The distance of sampling site for water and phytoplankton samples collection were 100 m, 200 m and 300 m from the fishing landing site. Containers carrying sardines during fishing at the shore were washed by local fishermen at the landing site when they land. Sardines were washed by fish vendors before sent to the market. Samples were collected from the same area in the Lake in monthly interval during fishing period for five months. A total of 120 samples were collected during the study period (Table 1).

<table>
<thead>
<tr>
<th>Sample type</th>
<th>Kibirizi site</th>
<th>Katonga site</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sardines</td>
<td>33</td>
<td>33</td>
</tr>
<tr>
<td>Water</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>Phytoplankton</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>60</strong></td>
<td><strong>60</strong></td>
</tr>
</tbody>
</table>

### 3.6 Laboratory Analysis

The isolation of *V. cholerae* and antibiotic susceptibility tests were carried out at the National Fish Quality Control Laboratory- Nyegezi, Mwanza. Molecular analysis was carried out at National Institute for Medical Research (NIMR), Mwanza.

#### 3.6.1 Microbiological isolation of *V. cholerae*

The isolation of *V. cholerae* was carried out as per Tanzania Bureau Standards (2002). The protocol is described in details in appendix 1. Briefly, 25 g of sardines (about 20 - 25
whole pieces of sardines) samples were homogenised with 225 ml of APW (Oxoid Ltd, Basingstoke, Hampshire, England) to make the first initial suspension. Another 25 g of sardines samples were homogenised with 225 ml of GPSB (Oxoid Ltd, Basingstoke, Hampshire, England) to make the initial suspension. Both homogenate suspensions were incubated at 37 °C for 24 hours for enrichment.

One hundred ml of water sample were concentrated on 0.45 µm pore diameter membrane filter (Millipore, Bedford, USA) and enriched in APW. Similarly, phytoplankton were concentrated on 0.45 µm pore diameter membrane filter paper (Millipore, Bedford, USA) and transferred into APW. Concentrated water and phytoplankton samples were incubated at 37 °C for 24 hours. A loop full of enriched culture samples from APW was streaked on TCBS agar plate (Oxoid Ltd, Basingstoke, Hampshire, England) and those GPSB enrichment culture were streaked on GPA plate (Oxoid Ltd, Basingstoke, Hampshire, England). All the plates were incubated at 37 °C for 24 hours. After incubation, yellow and shiny colonies on TCBS agar (sucrose fermenting, ≥ 2 mm in diameter) (Fig. 7) and red colonies from GPA plates were suspected as V. cholerae and purified on Saline Triple Sugar Iron Agar (STSI) plate (Oxoid Ltd, Basingstoke, Hampshire, England).

Purified colonies were screened by Gram staining, samples that were Gram negative and commas shaped were tested for Oxidase reaction (BDH Chemical LTD, England) for genus confirmation (all Vibrio species were Oxidase positive). Positive samples (colour changed to blue or dark purple within 10 seconds) were tested by STSI slant for species confirmation. Uniform yellow colour colonies with no gas (H₂S) formation after overnight incubation at 37 °C were regarded as presumptive V. cholerae (Fig. 8). Thereafter, sero-agglutination test was performed using specific V. cholerae O1 anti-serum, and then DNA was extracted for the molecular identification of the organisms.
Figure 7: Yellow colonies of *V. cholerae* on Thiosulfate Citrate Bile Sucrose (TCBS agar)

Figure 8: Yellow colour in Saline Triple Sugar Iron Agar (STSI) test tubes regarded as presumptive *V. cholerae*. 
3.6.2 Slide agglutination test

Agglutination tests for *V. cholerae* somatic O antigens were carried out on a clean glass slide. An inoculating sterile tooth pick was used to remove a portion of the growth colony from the surface of STSI plates. Colonies were emulsified in a small drop of physiological saline and mixed thoroughly by tilting back and forth for about 30 seconds. Suspension was carefully examined to ensure that it was even and did not show clumping due to autoagglutination. Where clumping occurred, the culture was termed “rough” and was not serotyped.

To smoothen a suspension (turbid and free-flowing), a drop of antiserum was added (Antiserum *V. cholerae* O1; Bio-Rad, Marnes-la-conquette, France). Approximately equal volumes (each 10 μl) of antiserum and growth suspension were mixed. The suspension and antiserum were thoroughly mixed then the slide was tilted back and forth to observe agglutination. Occurrence of very strong clumping within 30 seconds to 1 minute was interpreted as positive reaction (PAHO, 1994).

3.6.3 Molecular confirmation and identification of toxigenic *V. cholerae*

3.6.3.1 DNA extraction

The DNA was extracted using a commercial kit - QIAamp DNA blood Mini Kit (QIAGEN GmbH, Hilden, Germany). The kit provides fast and easy methods for purification of DNA for Polymerase Chain Reaction (PCR). The extraction of DNA was done according to the manufacturer’s instructions. Briefly, total DNA was purified from cultured *V. cholerae*; the sample was mixed with 180 μl of lysis buffer and 20 μl proteinase in a 1.5 ml microcentrifuge tube, then mixed by vortex-mix and incubated in the heat block at 56 °C for 1 hour. After incubation, the mixture was briefly centrifuged to remove drops from the inside of the lid of the 1.5 ml microcentrifuge tube, then mixed with 200 μl buffer AL for 15 seconds and incubated at 70 °C for 10 minutes. 200 μl ethanol (96 – 100%) was added to the sample and centrifuged, the spin column was
washed with two buffers (500 μl buffer AW1 and 500 μl AW2) followed by addition of Elution Buffer that extracted DNA taped on the column membrane.

3.6.3.2 Polymerase Chain Reaction (PCR) and Gel Electrophoresis

The assay was conducted by conventional PCR amplification using GeneAmp PCR System 9700 PCR machine (Applied Biosystems, Foster City, CA, USA). Specific genes namely; the outer membrane protein (ompW), cholera toxin (ctx), toxin coregulated pilus (tcpA), toxin regulator (toxR) and haemolysin (hlyA) were targeted as shown in Tables 4 and 5.

The detection of each gene was performed according to Wong et al. (2012), with some slight modifications as uniplex in a final reaction volume of 25 μl as shown on Tables 2 and 3 below.

**Table 2: Master Mix solution for hlyA, ompW and ctx**

<table>
<thead>
<tr>
<th>Reagents</th>
<th>hly A</th>
<th>ompW</th>
<th>ctx</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward primer</td>
<td>0.3</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>0.3</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>10XBuffer</td>
<td>2.5</td>
<td>2.5</td>
<td>5.0</td>
</tr>
<tr>
<td>Dream Taq Green PCR Master Mix</td>
<td>0.2</td>
<td>1.0</td>
<td>0.2</td>
</tr>
<tr>
<td>Double distilled water</td>
<td>13.7</td>
<td>11.5</td>
<td>9.8</td>
</tr>
<tr>
<td>dNTP (10mM)</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Mgcl2 (25mM)</td>
<td>2.0</td>
<td>2.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Template DNA</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>25</strong></td>
<td><strong>25</strong></td>
<td><strong>25</strong></td>
</tr>
</tbody>
</table>
Table 3: Master Mix solution for toxR and tcpA

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Volume (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward primer</td>
<td>1.0</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>1.0</td>
</tr>
<tr>
<td>10XBuffer</td>
<td>2.5</td>
</tr>
<tr>
<td>Dream Taq Green PCR Master Mix</td>
<td>0.2</td>
</tr>
<tr>
<td>Double distilled water</td>
<td>12.3</td>
</tr>
<tr>
<td>dNTP (10mM)</td>
<td>1.0</td>
</tr>
<tr>
<td>Mgcl2 (25mM)</td>
<td>2.0</td>
</tr>
<tr>
<td>Template DNA</td>
<td>5</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>25</strong></td>
</tr>
</tbody>
</table>

The cycling profiles of each PCR are described in the Table 3 and the sequences of the specific primers used are displayed in Table 4 below.

Table 4: Cycling profiles of each PCR

<table>
<thead>
<tr>
<th>Genes</th>
<th>Initial denaturation</th>
<th>Number of cycles</th>
<th>Denaturation</th>
<th>Annealing</th>
<th>Extension</th>
<th>Final extension</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>OmpW</td>
<td>96°C for 4 min</td>
<td>30</td>
<td>95°C for 30s</td>
<td>66°C for 20s</td>
<td>72°C for 30s</td>
<td>72°C for 30s</td>
<td>Sathiyamurthy et al., 2013</td>
</tr>
<tr>
<td>Ctx</td>
<td>95°C for 3 min</td>
<td>35</td>
<td>95°C for 30s</td>
<td>65°C for 30s</td>
<td>72°C for 1 min</td>
<td>72°C for 10 min</td>
<td>Wong et al., 2012</td>
</tr>
<tr>
<td>TcpA</td>
<td>94°C for 10 min</td>
<td>30</td>
<td>94°C for 1.5 min</td>
<td>60°C for 1.5 min</td>
<td>72°C for 1 min</td>
<td>72°C for 10 min</td>
<td>Guhathakurta et al., 1999</td>
</tr>
<tr>
<td>ToxR</td>
<td>94°C for 10 min</td>
<td>25</td>
<td>94°C for 40s</td>
<td>64°C for 40s</td>
<td>72°C for 1 min</td>
<td>72°C for 10 min</td>
<td>Guhathakurta et al., 1999</td>
</tr>
<tr>
<td>HlyA</td>
<td>94 °C for 5 min</td>
<td>35</td>
<td>94 °C for 1 min</td>
<td>58 °C for 1 min</td>
<td>72 °C for 1 min</td>
<td>72 °C for 5 min</td>
<td>Fooladi et al., 2013</td>
</tr>
</tbody>
</table>
Table 5: Primers sequences used for the PCR

<table>
<thead>
<tr>
<th>Targeted genes</th>
<th>Primer Sequences (5'-3')</th>
<th>Size (bp)</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholera toxin gene</td>
<td>F-CAGTCAGGTGGTCTTATGCCAAGAGG</td>
<td>167</td>
<td>Wong et al., 2012</td>
</tr>
<tr>
<td></td>
<td>R-CCCACTAAAGTGGGACCTTCTCAAACT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Toxin regulator</td>
<td>F-CGG GAT CCA TGT TCG GAT TAG GAC AC</td>
<td>900</td>
<td>Kondo et al.,</td>
</tr>
<tr>
<td>(toxR)</td>
<td>R-CGG GAT CCT ACT CAC ACA CTT TGA TGG C</td>
<td></td>
<td>2009</td>
</tr>
<tr>
<td>Outer membrane protein</td>
<td>F-CACCAAGAAGGTGACTTTATTGTG</td>
<td>588</td>
<td>Sathiyamurthy et al.,</td>
</tr>
<tr>
<td>(ompW)</td>
<td>R-GAACATTATAACCCACCGCG</td>
<td></td>
<td>2013</td>
</tr>
<tr>
<td>Toxin coregulated</td>
<td>F-CAC GAT AAG AAA ACC GGT CAA GAG</td>
<td>453</td>
<td>Wenpeng et al.,</td>
</tr>
<tr>
<td>pilus (tcpA)</td>
<td>R-CGA AAG CAC CTT CTT CTA CGT TG</td>
<td></td>
<td>2014</td>
</tr>
<tr>
<td>hlyA</td>
<td>F-GGC AAA CAG CGA AAC AAA TAC C</td>
<td>727</td>
<td>Fooladi et al.,</td>
</tr>
<tr>
<td>(Haemolysin)</td>
<td>R-CTC AGC GGG CTA ATA CGG TTT A</td>
<td></td>
<td>2013</td>
</tr>
</tbody>
</table>

For gel electrophoresis, 10 μl of PCR products was loaded into a horizontal 1.5% agarose gel stained with 0.1 μl/ml of DNA marker GelRed (Phenix Research) dived in 1xTBE (Tris Borate EDTA) buffer. Electrophoretic separation was performed at 100 V for 1 hour along with 1000 (bp) PCR ladder as molecular weight marker. The gel was visualized under UV trans-illuminator and recorded using digital camera. Double distilled DNase free water was used as negative control and DNA from reference strain of *V. cholerae* O139 NCTC 12945 (ATCC 51394) (Culture collections, Public Health England, Porton Down, Salisbury, SP4 OJG, UK) was used as positive control.

### 3.6.4 Determination of antibiotic susceptibility

All confirmed positive isolates were subjected to antibiotic susceptibility testing using the Kirby-Bauer disc diffusion method as described by Nhung et al. (2007). Colonies of each sample were lightly touched with a wire-loop and inoculated in a tube containing sterile normal saline until the suspension became slightly turbid and matched with the 0.5 Mac Farland turbidity standards (Remel, Lemexa, Kamsas). Using a sterile cotton swab, the entire surface of dried Muller Hinton agar plates (Oxoid Ltd, Basingstoke, Hampshire, England) were streaked by the above solution. The inoculated plates were left to dry for about fifteen minutes and six (commonly used in severe Cholera treatments) antibiotic
discs namely Tetracycline (30 µg), Gentamicin (10 µg), Ciprofloxacin (5 µg), Chloramphenicol (30 µg), Ampicillin (10 µg) and Amoxicillin (10 µg) (Oxoid Ltd, Basingstoke, Hampshire, England) were applied and then plates were incubated at 37 °C overnight. After the incubation, the inhibition zone diameters were measured using a transparent plastic ruler and interpreted according to the zone diameter interpretive chart of CLSI (2014) (Appendix 2).

3.7 Data Analysis

Data were entered and stored in Microsoft Excel. Proportions of positive *V. cholerae* samples at different sites were calculated then compared by Chi-square and Fisher exact tests according to the total sizes using EPI-INFO 7 (version 7.2.0.1) statistical software (2016). The confidence intervals (CI) of proportions were set at 95% CI. Results are presented in tables and interpretation of the antibiotic pattern was carried out according to space zone diameter interpretive chart of CLSI (2014).
CHAPTER FOUR

4.0 RESULTS

4.1 Isolation of *V. cholerae*

The findings revealed that sardine samples harboured *V. cholerae* in 9% (n = 66), water sample in 3% (n = 30) which was *V. cholerae* O1 and phytoplankton samples in 0% (n = 24) as shown in the table 6 below.

**Table 6: Antiserum confirmation and ompW confirmed by PCR for *Vibrio cholerae* isolates from water, sardines and phytoplankton samples**

<table>
<thead>
<tr>
<th>Samples</th>
<th>Number of positive V. Cholerae Confirmed by Agglutination Test</th>
<th>Number of positive V. cholerae confirmed in PCR (ompW)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>P (%)</td>
</tr>
<tr>
<td>Water</td>
<td>30</td>
<td>1(3%)</td>
</tr>
<tr>
<td>Sardines</td>
<td>66</td>
<td>0(0%)</td>
</tr>
<tr>
<td>Phytoplankton</td>
<td>24</td>
<td>0(0%)</td>
</tr>
<tr>
<td>Total</td>
<td>120</td>
<td></td>
</tr>
</tbody>
</table>

**Key:** N = number of samples; P = number of positive samples; (%) = percentage positive, ompW = Outer membrane protein

4.2 Identification of *V. cholerae*

It was found that seven (7) isolates were *V. cholerae* positive similar to PCR targeting gene ompW 588bp (a species-specific genetic marker used for *V. cholerae* detection) (Fig. 9). However, out of seven isolates only one sample, No.103 isolate, was confirmed with antisera O1 as *V. cholerae* O1.
Figure 9: ompW gene detection for confirmation of *V. cholerae* by PCR

M: DNA ladder; Lanes 95 to 105 are *V. cholerae* DNA samples; NC: Negative Control (DNA free water); P: Positive control (*V. cholerae* 0139)

4.3 Prevalence of *V. cholerae*

4.3.1 Prevalence of *V. cholerae* in sardines

Although, none of *V. cholerae* was O1 serogroup, out of the sixty six sardines samples, only six (9%) were positive for *V. cholerae*. Samples contaminated with *V. cholerae* from Katonga landing site were high (15%, n = 33) compared to Kibirizi landing site (3%, n = 33) samples. However, the difference was insignificant at P>0.05.

4.3.2 Prevalence of *V. cholerae* in water

Thirty samples were collected from two landing sites. The prevalence of *V. cholerae* in water was 1 (3%) out of 30 samples, however the prevalence was confirmed to be *V. cholerae* O1. The isolate was from Kibirizi landing site (6%, n = 15) samples and none of *V. cholerae* from Katonga landing site (0%, n = 15) samples was isolated. The difference was not significant at 95% level of confidence (p>0.05).
4.3.3 Prevalence of *V. cholerae* in Phytoplankton

Twenty four phytoplankton samples were collected from two different landing sites and none of the *V. cholerae* was isolated from these samples.

4.4 Virulence Factors of *V. cholerae* Isolates

After identification of *V. cholerae* by detecting ompW using primers with the expected 588 bp, all 7 isolates were subjected to PCR for detection of virulence genes.

4.4.1 The Cholera toxR

The toxR was identified in one isolate (*V. cholerae* O1 serogroup) from water sample (no. 103). It harboured the toxR gene identified by PCR using specific primers at 900 bp region of the gene as displayed on the picture in Fig. 10.

![Figure 10: Cholera toxin regulatory protein detected in one isolate (Sample No 103) using PCR](image)

M: DNA ladder; Lanes 95 to 110 are *V. Cholerae* DNA samples; N: Negative Control (DNA free water); P: Positive control (*V. cholerae* 0139)
4.4.2 Haemolysin gene (hlyA)

The hlyA gene was identified by PCR using specific primers at 727 bp, only one isolate *(V. cholerae* O1 serogroup) from water sample was identified as shown in Fig. 11.

![Figure 11: Haemolysin gene (hlyA) detected in one isolate (Sample No 103) using PCR](image)

M: DNA ladder; Lanes 95 to 110 are *V. cholerae* DNA samples; N: Negative Control (DNA free water); P: Positive control (*V. cholerae* 0139)

4.4.3 The Cholera enterotoxin gene (ctx)

All seven *V. cholerae* isolates from sardines and water were further tested for the presence of ctx by PCR using specific primer at 167 bp region of the gene. It was found that neither O1 nor non-O1 *V. cholerae* isolates contained the ctx operon. Only positive control was ctx positive as displayed in Fig. 12.
Figure 12: ctx in positive control using PCR

M: DNA ladder; Lanes 1 to 17 are V. cholerae DNA samples; N: Negative Control (DNA free water); P: Positive control (V. cholerae 0139)

4.4.4 The cholera toxin co-regulated pilus subunit A (tcpA)

The 453 bp region of the gene was amplified on the positive V. cholerae only as displayed on the picture in Fig. 13 and found that none of the seven (7) isolates harboured tcpA.

Figure 13: tcpA in positive control using PCR.
M: DNA ladder; Lanes 95 to 104 are *V. cholerae* DNA samples; N: Negative Control (DNA free water); P: Positive control (*V. cholerae* 139)

**4.5 Antibiotic Susceptibility for *V. cholerae* isolates**

The results in table no. 7 shows that, *V. cholerae* isolates were resistant to Ampicillin and Chloramphenicol (83%), Amoxicillin and Tetracycline (100%), but they were susceptible to Ciprofloxacin and Gentamicin (100%). Further, it indicates that *V. cholerae* O1 isolate was resistant to Ampicillin, Intermediary to Amoxicillin and susceptible to the rest of antibiotics tested (Fig. 14).

Additionally, while 50% of bacteria isolates resisted to more than one antibiotics (Ampicillin, Amoxicillin and Tetracycline), only 33% of isolates resisted to four antibiotics (Ampicillin, Chloramphenicol, Amoxicillin and Tetracycline).

![Figure 14: Antibiotic discs on MH-agar plates with *V. cholerae*](image)
Table 7: Antibiotic susceptibility pattern of isolated *V. cholerae*

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>Resistant in %</th>
<th>Intermediate in %</th>
<th>Susceptible in %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>83.33</td>
<td>16.67</td>
<td>0</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>83.33</td>
<td>16.67</td>
<td>0</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>Amoxicillin</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
CHAPTER FIVE

5.0 DISCUSSION

*Vibrio cholerae* is among of a group of microorganisms whose major habitats are aquatic ecosystems (Igbinosa and Okoh, 2009). In this study, a total of 120 samples (66 sardines, 30 water samples and 24 phytoplankton) from two landing sites were analysed for *V. cholerae*. The findings indicate that both sardines (9%) and water (3%) were positive for *V. cholerae*. Though, the bacterium was isolated from sardines and water, it was not isolated from phytoplankton. This may be due to failure of bacterium to adopt a viable state in phytoplankton when the environmental conditions changed such as low concentrations of nutrients and temperatures. Viable state enables them to carry out metabolic functions and form colonies without being culturable. However, they remain present in the aquatic environment throughout the year, either in free-living or in association with phytoplankton (Huq *et al*., 2012).

Prevalence of *V. cholerae* found in this study is comparable to previous studies. Senderovich *et al.* (2010) reported 71% prevalence of *V. Cholerae* from the intestines of fish species sampled from fresh water habitats in Israel. Traoré *et al.* (2014), on the other hand, found 6% prevalence of *V. cholerae* strains from fish and water samples in Ouagadougou, Burkina Faso. Furthermore, Mdegela *et al.* (2015) isolated *V. cholerae* from fish and water samples in Lake Victoria, Tanzania and reported prevalence of 53.7% in surface, gills 17.1%, intestine 4.9%, and 20% in water.

Although, the *V. cholerae* isolated from sardines in this study were not of the serotypes causing cholera, they may still cause sporadic cases of watery diarrhoea and inflammatory enterocolitis. The type of *V. cholerae* isolated in this study from water is the main cause of
human diarrhoea. Being water borne infection, cholera is transmitted by ingesting food or water contaminated with the bacterium (WHO, 2011).

Several studies have isolated the organism from fish and various water sources and associated them with hospitalised patients with diarrhoea, peritonitis and also in immunocompromised cases (Sathiyamurthy et al., 2013; Faruque et al., 1998; Albert et al., 1993; Dalsgaard et al. 1999). For instance, Faruque et al. (1998) demonstrated that V. cholerae are associated mostly with sporadic cases of diarrhoea and extra intestinal infections in humans. Likewise, in researches conducted by Albert et al. (1993) and Dalsgaard et al. (1999) indicated that V. cholerae was the important cause of diarrhoeal disease in humans.

During this study, serological testing revealed only one isolate of V. cholerae from water sample that agglutinated with antisera O1, the sample were collected at 200 m away from fish landing site. This finding extols well the previous study on V. cholerae O1 strains isolated from water and fish from Lake Victoria, in Kenya (Onyuka et al., 2011). In this study, the isolate was from Kibirizi landing site which is a very active area for bathing, washing and swimming activities, the sample could be contaminated with V. cholerae due to human interaction at the landing site. Apart from human activities in this area, a small river originating from Nyakageni and Msulula springs in Gungu area of the Kigoma –Ujiji Municipality enters the lake at this site. The major risk factors for cholera have been linked to rivers and bathing water contaminated by sanitary effluents (Dalusi et al., 2015). The water from this river may be contaminated with domestic wastes and surface runoff during rain seasons which may contain the bacterium. As it is argued that the major routes of pollutants that can be contaminated by pathogens and accessed to the lake are rivers and or storm water; that drain from residential areas and industrial sources which introduce
enteric pathogens in the lake (Mdegela et al., 2015). So water from this river plays an important role in cholera endemicity in Kigoma town.

*Vibrio cholerae* isolated from sardines and water was further tested for the presence of virulence genes. Result showed that, one isolate of *V. cholerae* O1 from water sample possessed the gene encoding the regulatory protein toxR which controls the coordinated expression of genes associated with pathogenicity in toxigenic *V. cholerae* O1. This finding is similar to what have been reported by Kimani et al. (2014) that in Kenya, the prevalence of toxR gene (24%) in *V. cholerae* O1 from environmental strains were isolated in the coastal and Lake Victoria Basin regions. None of non-O1 isolates contained this gene. Likewise, Hounmanou et al. (2016) reported the absence of toxR in *V. cholerae* isolated from Low quality water, fish and vegetable in Morogoro.

The same isolate *V. cholerae* O1 with toxR, possessed the haemolysin (hlyA) gene. This suggests virulence factor contributing towards cholera pathogenesis (Fooladi et al., 2013; Hounmanou et al., 2016). In this study, all *V. cholerae* isolated including O1 serogroup lacked the established virulent factors (ctx and tcpA). These findings correspond with that of Guhathakurta et al. (1999). They reported the absence of tcpA and ctx in *V. cholerae* O1 and non-O1. Furthermore, Traoré et al. (2014) reported that non-O1 *V. cholerae* in fish and water lacked the ctx gene. Although organisms of the O1 serogroup are frequently isolated from aquatic environments, most of the environmental *V. cholerae* O1 isolated do not produce cholera toxin to which the clinical state of cholera is principally attributed (Igbinosa and Okoh, 2008).

In this study, antibiotic resistance test was carried to the isolated bacteria. *Vibrio cholerae* isolates were resistance to Chloramphenicol (50%), Ampicillin (83.33%), Tetracycline
(100%) and Amoxicillin (100%). Findings of this study accentuate the study conducted in New Bell-Douala, Cameroon that showed Ampicillin resistance (92%), Amoxicillin (88%) and Tetracycline (68%) (Akoachere et al., 2013). Onyuka et al. (2011) reported that; *V. cholerae* O1 isolates from water and fish samples in Lake Victoria Basin of western Kenya were resistant to Tetracycline and Ampicillin (66.7%). On the other hand *V. cholerae* O1 strains isolated in a tertiary-care centre in India were resistance to Ampicillin (64.3%) (Mandal et al., 2012). Traoré et al. (2014) reported that, *V. cholerae* isolates from fish and water were resistant to Ampicillin (50%) in Ouagadougou, Burkina Faso. Although findings of this study show 50% resistant to chloramphenicol, Akoachere et al. (2013) showed 80% susceptible to chloramphenicol. Resistant to the mentioned antibiotics may be related to their abuse and overuse in humans and veterinary medicine (Kagashea et al., 2010; Tagoe and Attah, 2010; Onyuka et al., 2011; Katakweba et al., 2012). However, Tetracycline is the antibiotic mostly used in Kigoma region for cholera treatment (CDC, 2015); this may play a role in persistence and dissemination of pathogenic strains in study area.

In this study, the multi-drug resistance patterns detected among isolates showing 50% isolates resisted to more than one antibiotics and 33% isolates resisted to four antibiotics. Akoachere et al. (2013) reported multidrug resistance (92%) in *V. cholerae* isolates (resistant to two or more antibiotics). Antibiotic drug resistance in bacteria isolates during the study may arise through mutation or acquisition of resistance genes on mobile genetic elements like plasmids, transposons integrons, and integrating conjugative elements (Sjölund-Karlsson et al., 2011).
Due to inappropriate use of antibiotics, different enteric pathogens including *V. cholerae* are becoming increasingly resistant. This underlines the pervasiveness of the pressures that lead to the emergence and spread of antibiotic resistance (Mrityunjoy, 2013). In this study, *V. cholerae* isolates were sensitive to Ciprofloxacin (100%), and Gentamicin (100%). The antibiotic sensitivity in these antibiotics is comparable to the previous findings by Traoré *et al.* (2014), Ukaji *et al.* (2015) and Hounmanou *et al.* (2016) who reported susceptibility of Ciprofloxacin in *V. cholerae*. Ciprofloxacin has been used in Kigoma region for treatments in humans during cholera outbreak (CDC, 2015), the findings thus agree with its use in cholera treatment. WHO also recommends the use of Ciprofloxacin as treatment choice for cholera (Sack *et al.*, 2001).
CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

Human are infected by pathogenic *V. cholerae* due to ingestion of contaminated water and food including fish containing the microorganism. Sardines and water are reported as reservoirs of *Vibrio cholerae* in this study as they tested positive for *V. cholerae* which can acquire toxigenicity at any time they come across vibriophages, which transfers the cholera toxin. At this point we urge that, an awareness raising and public training on basic hygienic practices in the study area should be taken into consideration so as to protect public health from cholera in the near future since *V. cholerae* O1 was detected in water. It should also be noted that in samples where the *V. cholerae* was not detected, environmental conditions could be unfavourable for their growth (viable but non-culturable form-VNC). Under favourable climate conditions VNC *V. cholerae* could revert to transmissible state and become virulent. Cholera control strategies in this area should therefore be encouraged and amplified towards potential reservoirs. Although isolates displayed increased resistance towards Chloramphenicol, Ampicillin, Amoxicillin and Tetracycline, they were susceptible to ciprofloxacin and Gentamicin. *Vibrio cholerae* are the causative agents of cholera epidemics; therefore, identification and detection of *V. cholerae* is very important for providing epidemiologic and public health information.

6.2 Recommendations

- The sources and routes of cholera infection in Kigoma were not clear. The findings of this first study have revealed that water and sardines act as a reservoir of *V. cholerae*. Further studies are required to investigate whether the *V. cholerae* strains in sardines and water from the lake are associated with human diseases such as
cholera outbreak that has been recurring several times in Kigoma. The study should compare the isolates from water, fish and humans.

- Since *V. cholerae* from the environment may be dormant (viable but not culturable) when isolated, phytoplankton samples are recommended to be directly analysed by PCR or fluorescent antibody methods that are culture independent.

- One year calendar monitoring study should be carried out to ascertain ecological and epidemiological factors of *V. Cholerae* bacteria and disease.

- Since this study used minimal sample, another study should be carried out with higher number of samples and more coverage area.

- Lastly, the study recommends that rational and appropriate use of antibiotic agent in veterinary practise and human treatments should be adhered in order to reduce drug resistance.

- Also, high resisted antibiotics should be withdrawn in prevention and treatment of *V. cholerae*.
REFERENCES


Mansurul (2011). Seasonal prevalence of pathogen (*Vibrio cholerae*) in water and plankton samples in the Southern Coastal part of Bangladesh. *India Journal of Science and Technology* 4: 1101-1104.


APPENDICES

Appendix 1: Protocol for isolation of *V. Cholerae* (TZS733:2002: Examination for *Vibrio cholerae* in food stuffs)

25g of Test Portion

- 225mls Alkaline Saline Peptone Water
- 225mls Gelatin phosphate [GP] Saline Broth

Incubate at 37°C for 18 hours

Plating out onto TCBS Agar and incubate at 37°C for 18 hours

Plating out onto GP Agar and incubate at 37°C for 18 hours

**ISOLATION CONFIRMATION**

- Purification onto Saline TSI Plates (Incubate at 37°C for 24hrs)

**PRELIMINARY TESTS**

- Oxidase Test
- Gram Stain Test
- Motility Test

**BIOCHEMICAL TESTS**

- Saline TSI
- Lysine decarboxylase test
- KIA Agar test

Interpretation of Results
### Appendix 2: Antibiotics zone diameter interpretive chart

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>Resistant (mm)</th>
<th>Intermediary (mm)</th>
<th>Sensitive (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>$\leq 13$</td>
<td>14-16</td>
<td>$\geq 17$</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>$\leq 12$</td>
<td>13-17</td>
<td>$\geq 18$</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>$\leq 15$</td>
<td>16-20</td>
<td>$\geq 21$</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>$\leq 12$</td>
<td>13-14</td>
<td>$\geq 15$</td>
</tr>
<tr>
<td>Amoxicillin</td>
<td>$\leq 13$</td>
<td>14-17</td>
<td>$\geq 18$</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>$\leq 14$</td>
<td>15-18</td>
<td>$\geq 19$</td>
</tr>
</tbody>
</table>

**Source:** Clinical and Laboratory Standards Institute (CLSI), 2014