DETECTION AND ANTIBIOTIC SUSCEPTIBILITY OF *VIBRIO CHOLERAE* IN *OREOCHROMIS TANGANICAE* (TILAPIA) AND WATER IN LAKE TANGANYIKA, KIGOMA-TANZANIA

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ABSTRACT

Toxigenic *Vibrio cholerae* O1 and O139 are responsible for the production of cholera toxin and subsequently cause cholera. Although cholera has been very rampant around African great lakes, little is known about the status of *V. cholerae* in aquatic reservoirs like fish. This study aimed to determine the occurrence of toxigenic and drug resistant *V. cholerae* in Tanganyika Tilapia (*Oreochromis tanganicae*) and water from Lake Tanganyika in Tanzania. Repeated cross-sectional study design that involved 140 Tilapia fish samples and 60 water samples was carried out in Lake Tanganyika during dry and rain seasons. Bacterial analyses culture and biochemical tests were used for identification of *V. cholera*. Positive isolates were confirmed by PCR of the *ompW* and *ctxA* genes then serotyped with polyvalent O1 antiserum. Antimicrobial susceptibility patterns of *V. cholerae* O1 (n = 65) were performed using disk diffusion method. Results of the study revealed a prevalence of *V. cholerae* at 27.9% (n = 39/140) in Tanganyika tilapia and 33.3% (n = 20/60) in water samples. The proportions of toxigenic *V.cholerae* in Tanganyika Tilapia and water were 11.4% (n = 16/140) and 20% (n = 20/60) respectively. Prevalence of toxigenic *V. cholerae* in fish gills and intestines were 5.7% (n = 16/280) and 0.4% (n = 1/280) respectively. *Vibrio cholerae* were susceptible to Gentamicin (100%), Ciprofloxacin (100%), Cefotaxime (100%), Ceftazidime (73.8%), Chloramphenicol (63.1%), Tetracycline (53.8%) and Naliadixic acid (47.7%). Resistance in *V. cholerae* was observed for Streptomycin (100%), Amoxicillin (95.4%), Ampicillin (81.5%), Sulphamethaxole (80%) and Trimethoprim (72.3%). This study revealed that Tanganyika Tilapia and water in Lake Tanganyika harbor toxigenic, antibiotic resistant *V. cholerae* of the epidemic potentials, with high incidences in wet season. Raising awareness on public health practices (hygiene/sanitation) and rational use of antimicrobial agents to safeguard public health from cholera outbreaks are recommended.

DECLARATION

I, Martin Michael, 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 in any other institution.

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The declaration is hereby confirmed;

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DEDICATION

This work is dedicated to the Almighty God; to my beloved mother Christina Fisoo and beloved father Michael Mtau Kinyonga.

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LIST OF ABBREVIATIONS AND ACRONYMS

APW	Alkaline Peptone Water
ATB	Antibiotics
Bp	Base Pair
CDC	Centre for Disease Control
CLSI	Clinical and Laboratory Standards Institute
CT	Cholera Toxin
ctxA	Cholera enterotoxin sub unit A
DNA	Deoxyribonucleic acid
DRC	Democratic Republic of Congo
EDTA	Ethylindiaminetetraacetic acid
FETA	Fisheries Education and Training Agency
hlyA	Haemolysin Alpha toxin
ICEs	Integrating Conjugative Elements
KIA	Kligler’s Iron Agar
ml	Millilitre
Nacl	Sodium chloride
OmpW	Outer membrane protein
PCR	Polymerase Chain Reaction
PFGE	Pulsed-Field gel Electrophoresis
rDNA	Ribosomal deoxyribonucleic acid
rpm	Revolution per Minute
STSI	Saline Triple Sugar Iron Agar
SUA	Sokoine University of Agriculture
TCBS	Thiosulfate Citrate Bile Sucrose

TCP	Toxin Coregulated Pilus
tcpA	Toxin Coregulated Pilus sub unit A
toxR	Toxin Regulator
TSI	Triple Sugar Iron
TZS	Tanzania Standards
URT	United Republic of Tanzania
UV	Ultra violet
VBNC	Viable But Non-Culturable
WHO	World Health Organization
μl	Microlitre
μm	Micrometre

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background Information

Vibrio cholerae is a facultative anaerobe, Gram negative straight or curved rods; motile with a polar flagella bacteria that causes cholera in human (Lutz *et al*., 2013). The disease is associated with contaminated food, water, poor sanitation practices and unhygienic environmental condition of water sources (WHO, 2016). The fatal effects of cholera are mainly due to the toxin produced by specific serogroups (O1 and O139) of *V. cholerae* (WHO, 2016). The epidemic strains spread across countries and or continents over time, giving rise to cholera pandemics (WHO, 2016).

Repeated cholera pandemic episodes have been recorded in the past 200 years and the disease still remains a significant health issue in many parts of the world (Adabi *et al*., 2009). Since 2000 through 2016, countries that report to WHO have recorded a total of 3.4 million cholera cases and 65,600 deaths. In 2016, major cholera epidemics affected several areas in the world, including Haiti in the Americas, Democratic Republic of Congo (DRC), Somalia, United Republic of Tanzania and Yemen (WHO, 2016). *Vibrio cholerae* represents an enormous public health burden especially in developing countries around the world (Maya *et al*., 2011). Although, cholera is a self-limiting illness, antibiotics are commonly administered as part of the treatment regime (Adabi *et al*., 2009). Several studies have been done to review initial identification and subsequent evolution of antibiotic-resistant strains of *V. cholerae* (Adabi *et al*., 2009). Antibiotic resistance mechanisms including efflux pumps, spontaneous chromosomal mutations, conjugative plasmids, integrative conjugative element (ICE) and integrons of several *V.cholerae* strains have been identified, which may be useful for control of epidemics

(Maya *et al*., 2011). Study conducted by Maya *et al*. (2011) argued that antibiotic use has to be restricted and alternative methods for treating cholera have to be implemented in order to avoid spread of resistance (Maya *et al*., 2011).

Genetic variation by positional mutations or horizontal transfer of foreign genes is one of the adaptation strategies employed by various bacterial species, including *V. cholerae* to survive in different environments (Shukla *et al*., 2008). Continuous detection and monitoring of *V. cholerae* strains in the environment is essential for early prevention of disease and emergency preparedness plans (Mehrabadi *et al*., 2012). Pathogenicity of *V. cholerae* depends on expression of some virulence factors such as Cholerae toxin (CT) that is facilitated by the Toxin-Coregulated Pilus (TCP). These two genes are encoded by the *ctxA* and *tcp* genes, respectively (Ramazanzadeh *et al*., 2015). Another virulence factor is the Outer-Membrane protein *ompW* which is involved in stimulating the immune response via induction of protective immunity. It also plays important role in bacterial pathogenesis by increasing the adaptability of pathogenic strains (Ramazanzadeh *et al*., 2015). Findings of the study conducted by Nyambuli *et al*. (2018) in Lake Tanganyika at Kigoma revealed that sardines, phytoplankton and water harbor drug resistant *V. cholerae*. To date, there are no studies that have determined Tanganyika tilapia as among the host range of toxigenic and drug resistant *V. cholerae* in Lake Tanganyika. This study therefore intended to identify magnitude of *V. cholerae* in Tanganyika Tilapia fish and water at Lake Tanganyika in Kigoma-Tanzania. It also aimed to determine antibiotic susceptibility patters of *V. cholerae* in commonly used antibiotics on cholera treatments.

1.2 Problem statement

Cholera is a major public health problem in developing countries which has been linked to poverty and poor sanitation (WHO, 2016). There are an estimated 3-5 million cholera cases and 100 000 -120 000 deaths due to cholera every year in endemic settings (WHO, 2016). In August 2015 an outbreak was reported to cause 11 563 cases, including 144 deaths in Tanzania (WHO, 2016). Although improved sanitation can reduce the risk of cholera outbreaks, systematic and continuous monitoring of *V. cholerae* in environmental samples is important in prevention of epidemics.

1.2.1 Justification

There has been less information on the occurrence of pathogenic drug resistant *V. cholerae* in aquatic environments. Little is known on contribution of Lake Tanganyika in Kigoma Tanzania on occurrence of toxigenic *V.cholerae*. WHO (2016) suggested that warm water in Lake Tanganyika basin for example is a likely habitat for the El Tor variant of *V. cholerae* O1. In 2016 outbreaks local authorities in Dar es salam, Mwanza and Kigoma Tanzania burned fish markets arguing that fish play a crucial role in cholera transmission (Hounmanou *et al*., 2016). Moreover, study conducted by Nyambuli *et al*. (2018) in Kigoma revealed that, water phytoplankton and sardines act as reservoirs of *V. Cholerae*. Results of this study intended to provide baseline information on virulence, antibiotic susceptibility patterns and potential hosts of *V. cholerae* strains in fish and water. Information from this study will be useful in implementation of preventive interventions against cholera outbreaks.

1.3 Objectives

1.3.1 Overall objective

The overall objective of this study was to detect *V. cholerae* and determining their antibiotic susceptibility patterns in Tanganyika Tilapia and water in Lake Tanganyika, during non-outbreak periods

1.3.2 Specific objectives

I. To establish the prevalence of pathogenic *V. cholerae* O1 in Tanganyika Tilapia and water from Lake Tanganyika.

II. To determine the antibiotic susceptibility patterns of *V. cholerae* isolates.

1.4 Research Questions

I. What is the magnitude of toxigenic *V. cholerae* O1 in Tanganyika Tilapia and water from Lake Tanganyika?

II. What is antibiotic susceptibility pattern of pathogenic *V. cholerae* isolates from Tanganyika Tilapia in Lake Tanganyika?

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Prevalence and Virulence Characteristics of *V. cholerae*

Vibrio cholerae are abundant in aquatic environments, where they are found free-living in water or in association with plankton (Okoh *et al*., 2010). *Vibrio cholerae* favor higher water temperature where by the outbreaks are more frequent during the warmer season (Gurbanov *et al*., 2011). Studies have suggested that fish may act as reservoirs and vectors of *V. cholerae* (Okoh *et al*., 2010). While an estuarine environment represents an ideal setting for the survival and persistence of *V. cholerae* (Schärer *et al*., 2011). Environmental monitoring for the presence of *V. cholerae* and other Vibrio species with pathogenic potential is important as it can identify the potential sources of infections (Gurbanov *et al*., 2011).

The genes for cholera toxin are carried by CTXphi (CTXφ), a temperate bacteriophage inserted into the *V. cholerae* genome. CTXφ can transmit cholera toxin genes from one *V. cholerae* strain to another through horizontal gene transfer (Sjölund-karlsson *et al*., 2011). Genetic diversity of proteins within individual *V. cholerae* explains the versatility of the bacterium in different environments. Chromosome II contains genes necessary for adaptation and growth in unique environments with genes found to be active during human infection (Ukaji *et al*., 2015). Intensive study of toxigenic strains of O1 and O139 yield a set of virulence genes most notably belongs to TCP pathogenicity island, *tcpA*, *tcpI* and *acfB* encoding colonization factor TCP also CTX prophage and *ctxA* encoding CT toxin (Okoh *et al*., 2010).

2.2 Isolation of *V. cholerae* from environmental samples

Recent molecular advances in microbiology have greatly improved the detection of bacterial pathogens in the environment (WHO, 2016). Culture methods have been greatly improved and it allow a researcher to preserve the organism of interest for further studies such as genomic, metabolomic, secretomic and transcriptomic analysis (Ukaji *et al*., 2015). Molecular detection provides data on the presence and type of pathogens which gives a clearer understanding of the ecology and epidemiology of diseases (WHO, 2016). *Vibrio cholerae* serogrups O1, O139, or non-O1/non-O139 are usually determined by a slide agglutination assay while primers in PCR are used to detect species specific gene *ompW* and other virulent genes such as *ToxR, ctxA* (WHO, 2016).

2.3 Vibrio species in Alkaline Peptone Water

Alkaline peptone water (APW) is the media usually recommended as an enrichment broth, used in isolation of *V. cholerae* from clinical samples and non-clinical samples such as suspected food and water samples (Sichewo *et al*., 2013). Enrichment in APW enhances the isolation of *V.cholerae* O1 when few organisms are present (Sichewo *et al*., 2013). Alkaline peptone water (APW) enhances growth of vibrio species while in other way around it suppresses growth of non-Vibrio organism (Sichewo *et al*., 2013). A number of other broth media have been described for enrichment of *V. cholerae*. These include Monsur's enrichment medium which contains Trypticase, potassium tellurite, and sodium taurocholate (bile salts) (Ukaji *et al*., 2015).

2.4 Vibrio species in Thiosulfate Citrate Bile Salts sucrose agar

Thiosulfate Citrate Bile Salts sucrose (TCBS) agar is highly selective for the isolation of *V. cholerae* and *V. parahaemolyticus* as well as other Vibrio species (Ukaji *et al*., 2015).

The TCBS agar contains high concentrations of sodium thiosulfate and sodium citrate to inhibit the growth of *Enterobacteriaceae* (WHO, 2016). This TCBS agar is commercially available and easy to prepare, requires no autoclaving, and is highly differential and selective. It is green when prepared, overnight growth (18 to 24 hours) of *V. cholerae* produces large (2 to 4 mm in diameter), slightly flattened, yellow colonies with opaque centers (Fig. 1) (WHO, 2016).

Figure 1: Overnight colonies of *Vibrio cholerae* on TCBS agar, Source: WHO, 2016

2.5 Kligler's Iron agar (KIA) or Triple Sugar Iron agar (TSI) for *V. cholerae* identification

Kligler's iron agar (KIA) and triple sugar iron agar (TSI) are widely used carbohydrate containing media in diagnostic microbiology (WHO, 2016). The reaction of *V. cholerae* on Kligler's iron agar, which contains glucose and lactose, is similar to those of non-lactose-fermenting Enterobacteriaceae (K/A, no gas, no H2S) (WHO, 2016). Triple sugar iron agar which contains sucrose in addition to glucose and lactose gives reactions of A/A, no gas, and no H_2S. Kligler's iron agar, or Triple sugar iron agar slants are inoculated by stabbing the butt and streaking the surface of the medium (Fig. 2). Slants should be incubated at 35° to 37℃ and examined after 18 to 24 hours. Caps on all tubes of biochemical should be loosened before incubation. If the caps are too tight and

anaerobic conditions exist in the KIA or TSI tube and the reactions of *V. cholerae* may not be exhibited (WHO, 2016).

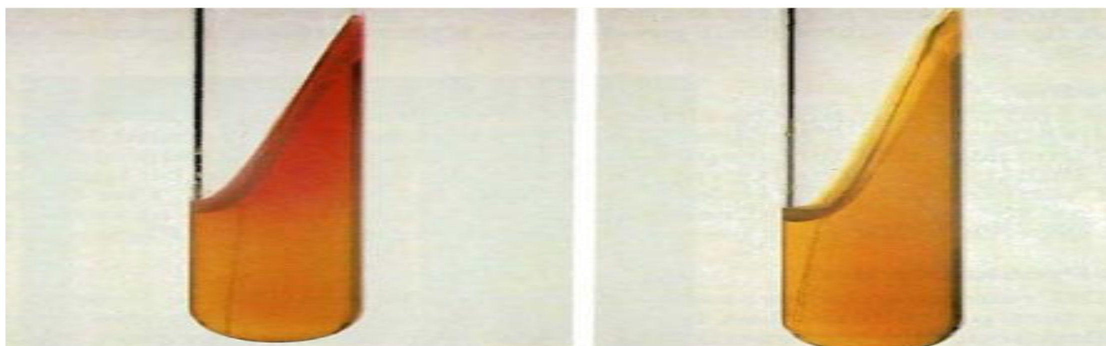

Figure 2: Reactions of *V. cholerae* in Kligler's iron agar and triple sugar iron agar

Source: WHO, 2016

2.6 Oxidase test for *V. cholerae*

The oxidase test is one of the biochemical test used in microbiology to determine if a bacterium produces certain cytochrome c oxidases (Tamplin *et al.*, 1990). It uses disks impregnated with a redox indicator reagent such as *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine (TMPD) (Tamplin *et al.*, 1990). Oxidase test is normally done by placing 2 to 3 drops of oxidase reagent (1% tetramethyl-p-phenylenediamine) on a piece of filter paper (WHO, 2016). In a positive reaction, the bacterial growth becomes dark purple within 10 seconds (WHO, 2013). Organisms of the genera Vibrio, Neisseria, Campylobacter, Aeromonas, Plesiomonas, Pseudomonas and Alcaligenes are all oxidase positive (Tamplin *et al.*, 1990).

2.7 String Test for *V. cholerae*

String test is one of the biochemical test used to differentiate *V. cholerae* from other *Vibrio spp* and other enteric bacteria (Wenpeng *et al.*, 2014). When an isolated colony of

a suspected bacterium is emulsified in sodium deoxycholate (Bile salt), it lyses the cell wall of bacterium releasing the DNA (Wenpeng *et al*., 2014). The suspension loses the turbidity and mixture becomes viscous. A mucoid “string” is formed when an inoculating loop is drawn slowly away from the suspension. The sensitivity of this test is 86% and specificity is only 70% hence other biochemical test should also be used for confirmation of *V. cholerae* (Wenpeng *et al*., 2014). Formation of thread like mucoid string indicate positive test. Most Vibrio are positive, whereas Aeromonas strains are usually negative (WHO, 2016).

2.8 Polymerase Chain Reaction (PCR) assay for detection of O1 and O139 serogroups *V. cholerae*

Deoxyribonucleic acid (DNA) based methods for isolation of *V. cholerae* have been designed for rapid, sensitive analysis of a range of clinical and environmental samples (Singh *et al*., 2002). End point PCR is not quantitative and the presence of the PCR products must be verified using a procedure such as southern hybridization and gel electrophoresis (Singh *et al*., 2002). Three types of targets are used to detect *V. cholerae* in environmental samples by PCR, these are species-specific genes (16S rDNA, ITS, *ompW*), serogroup-specific genes (O1 and O139) and toxin/pathogenic factor genes (*ctx*, *tcpA*, etc.) (Singh *et al*., 2002). Briefly, water, plankton, oyster, and/or sediment samples are collected and concentrated. DNA is extracted from the samples, using a modification of the method of Murray and Thompson (Murray and Thompson 1980) and PCR is performed on the extracted DNA using a multiplex (*ompW* and *ctxA*) primer array. Positive and negative controls are tested in parallel and are included in a eubacteria 16S rDNA PCR reaction on each sample to test template quality (Singh *et al*., 2002).

2.9 Serology for detection of *V. cholerae*

The discovery of monoclonal antibody in the 1980s and subsequently the development of a monoclonal antibody against *V. cholerae* O1, triggered development of direct detection methods for *V. cholerae* (WHO, 2016). Using immunological methods, the mystery concerning the inability to culture *V. cholerae* in environmental samples was reported during inter-epidemic period in Bangladesh. These difficulties arise from several possible factors including low density, inter-specific competition, cell state and health (VBNC, starved).

2.10 Sources and Symptoms of Cholera

The cholera bacterium is usually found in water or food sources that have been contaminated by feces (poop) from a person infected with cholera (WHO, 2008) Cholera is most likely to be found and spread in places with inadequate water treatment, poor sanitation, and inadequate hygiene. Symptoms of cholera can begin as soon as a few hours or as long as five days after infection. Often, symptoms are mild. But sometimes they are very serious (WHO, 2016). About one in 20 people infected have severe watery diarrhea accompanied by vomiting, which can quickly lead to dehydration. Although many infected people may have minimal or no symptoms, they can still contribute to spread of the infection (WHO, 2008).

2.11 Treatment of Cholera

Cholera is treated by replacing lost fluids and electrolytes using a simple rehydration solution, oral rehydration salts (ORS) (WHO, 2013). The ORS solution is available as a powder that can be reconstituted in boiled or bottled water. In conjunction with hydration, treatment with antibiotics is recommended for severely ill patients (WHO, 2013). It is particularly recommended for patients who are severely or moderately

dehydrated and continue to pass a large volume of stool during rehydration treatment. Antibiotic treatment is also recommended for all patients who are hospitalized (WHO, 2008). Antibiotic choices should be informed by local antibiotic susceptibility patterns. In Tanzania, Doxycycline is recommended as first-line treatment for adults, while azithromycin is recommended as first-line treatment for children and pregnant women (WHO, 2008).

2.12 Antibiotic resistance mechanism of *Vibrio cholerae*

Antibiotic resistance is the one of the global health agenda; where by emergence of antimicrobial drug resistance following the introduction of antibiotics is commonly reported (Shukla *et al*., 2008). Resistance to antimicrobial compounds can arise through spontaneous mutations in the bacterial chromosome (Mehrabadi *et al*., 2012). Mutations conferring resistance to the cell wall biosynthesis inhibitor alafosfalin and to the DNA replication inhibitor family of quinolones are well documented in *V. cholerae* (Page *et al*., 2011). A comprehensive study during the 1980 cholera epidemic in the United Republic of Tanzania. Wenpeng *et al*. (2014) revealed that *V. cholerae* genes undergo higher mutation rates than *E. coli* genes, facilitating resistance to antibiotics such as alafosfalin. Horizontal gene transfer via self-transmissible mobile genetic elements, including STX elements is among of the factor for spread of antibiotic resistant *V cholerae* (Burrus *et al.,* 2006). The STX elements belong to the class of integrative conjugative elements (Hochhut *et al*., 2001). The STX elements has ability to harbor genes that provide the bacterium resistance to Sulfamethaxole, Trimethoprim and Streptomycin (Burrus *et al.,* 2006).

Currently many strains of the O1 and O139 serogroups isolated around the globe have acquired SXT elements through natural spread (Hochhut *et al*., 2001). Studies have

revealed that multidrug resistant *V. cholera* is appearing with increasing frequency. Findings of the study by Nyambuli *et al*. (2018) revealed that; *V. cholerae* isolates were resistant to Ampicillin (83.33%), Amoxicillin (100%), Chloramphenicol (50%) and Tetracycline (100%). For this reason, recommendations for antibiotic use for cholera case management should promote their selective use and be based on the antibiotic susceptibility pattern of *V. cholerae* in the area. Antibiotic susceptibility test is usually done by disk diffusion method which is a simple practical and well-standardized technique used to test antibiotic resistance (Okoh *et al*., 2010). The test is performed by applying a bacterial inoculum of approximately $1–2\times10^{8}$CFU/mL to the surface of a large (150 mm diameter) Mueller-Hinton agar plate. The diameter of the zone is related to the susceptibility of the isolate and to the diffusion rate of the drug through the agar (Okoh *et al*., 2010).

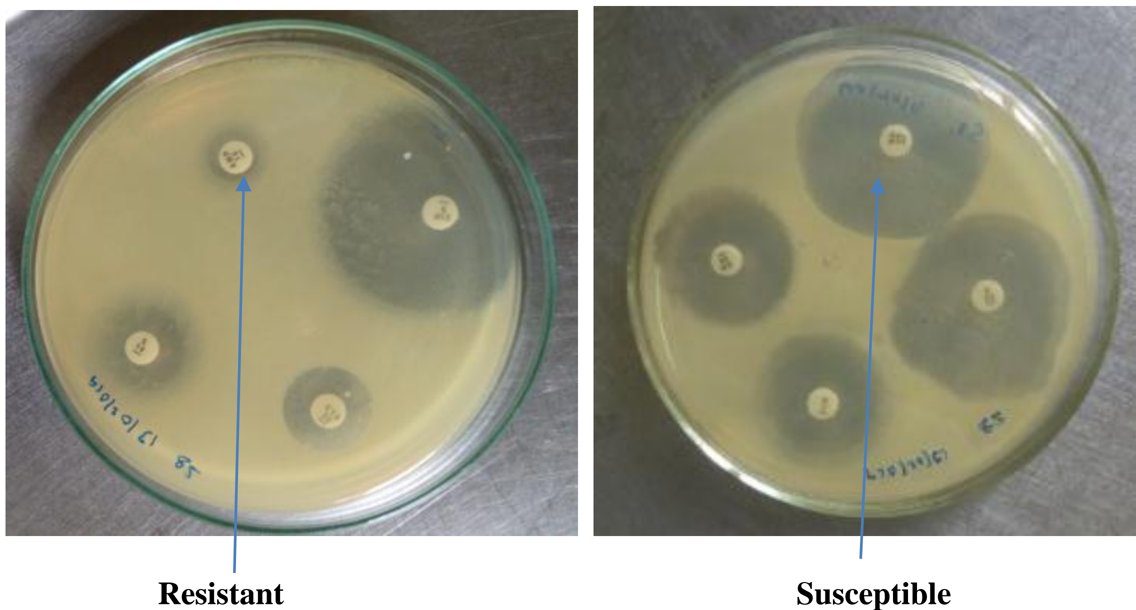

Figure 3: Sample on Mueller-Hinton agar plate indicating the susceptibility patterns of *V. cholerae* on different antibiotics

2.13 McFarland Turbidity

McFarland 0.5 turbidity standards are available from various manufacturers. Alternately, the 0.5 McFarland may be prepared by adding 0.5 ml of a 1.175% (wt/vol) barium chloride dihydrate ($BaCl_2 \cdot 2H_2O$) solution to 99.5 ml of 1% (vol/vol) sulfuric acid. The turbidity standard is then aliquoted into test tubes identical to those used in preparation of the inoculum suspension (WHO, 2016). McFarland standard should be checked by using a spectrophotometer with a 1-cm light path; for the 0.5 McFarland standards.

2.14 Control of Cholera Disease in Tanzania

From 15 August 2015 through 7 January 2018, 33 421 cases including 542 deaths (case fatality rate = 1.62%) have been reported across all 26 regions of the United Republic of Tanzania (Tanzania mainland and Zanzibar) (WHO, 2018). WHO recommends proper and timely case management in Cholera Treatment Centres. Improving access to potable water and sanitation infrastructure, and improved hygiene and food safety practices in affected communities, are the most effective means of controlling cholera. Key public health communication messages should be provided (WHO, 2018). The Ministry of Health (MoH) in Tanzania is collaborating with WHO and other partners (UNICEF and the United States Center of Disease Control and Prevention (CDC) to implement and monitor cholera control activities through the National Task Force (WHO, 2018).

CHAPTER THREE

3.0 MATERIAL AND METHODS

3.1 Study Area

This study was carried out in areas along the Lake shores of Tanganyika at Kigoma Municipality. Kigoma region is located on the shores of Lake Tanganyika at the North-West corner of Tanzania (URT, 1998). The region is situated between Longitudes 29. 5° and 31.5^{o} East and Latitudes 3.5^{o} and 6.5^{o} South of the Equator. The entire region has an area of 45 066 Square kilometers of which 8029 sq.km is water and 37 037 is land area (URT, 1998). 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 (Nyambuli *et al.*, 2018).

The annual rainfall in the region is variable ranging from 600 mm-1500 mm being the heaviest in highlands and intermediate in the lower slopes. Mean daily temperatures range between 25°C in December to January to 28°C in September. About 93.6% of the population in Kigoma region are engaged in agriculture and fishing activities as the main economic activities to sustain their livelihood (URT, 1998). Lake Tanganyika is one of great Lakes in east and central Africa with about 800 fishing sites and 100 000 people estimated to be directly involved in the fisheries (URT, 1998).

3.2 Study Sites

This study was carried out in two sites along Lake Tanganyika in Kigoma municipality, namely Kibirizi (Fig. 3) fish landing site situated in (Mwanga kaskazini) and Katonga (Fig. 6) fish landing site situated in Bangwe ward (Fig. 5). The two landing sites were selected because are they are largest in Kigoma and easily accessed. Bathing, washing,

swimming (Fig. 6) and fetching water were carried out in this sites apart from fishing activities in the selected sampling sites.

Figure 4: Kibirizi fish landing site

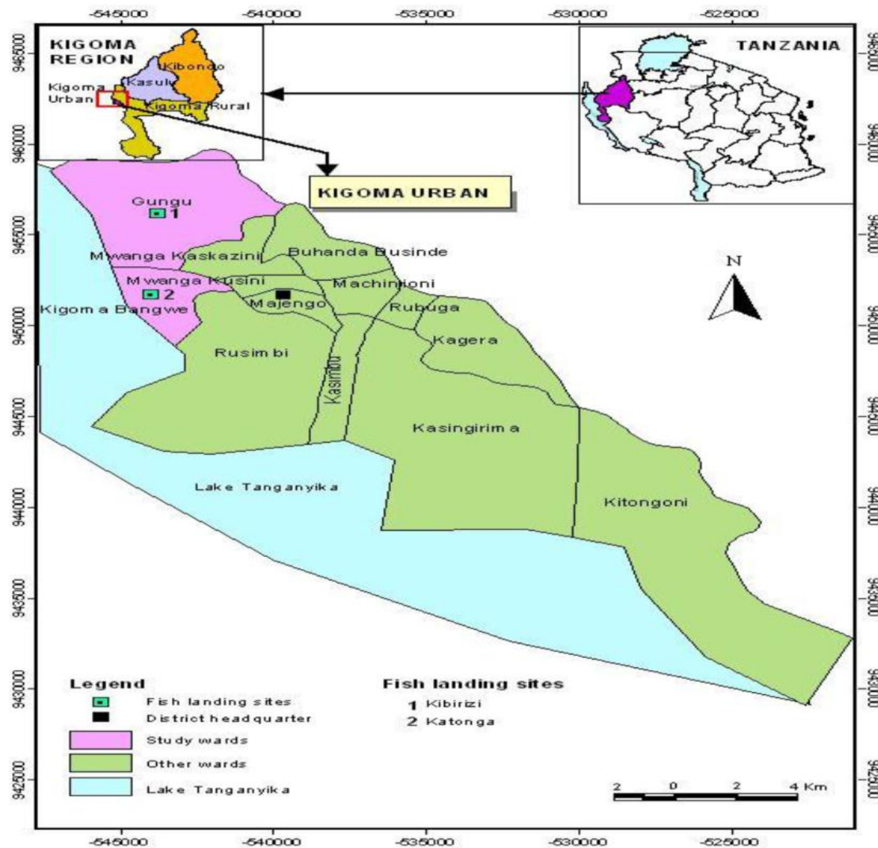

Figure 5: A map of Kigoma Municipality showing study areas

Source: Drawn with QGIS software 2.6.1 using the Tanzania Shape file 2012

Figure 6: Katonga fish landing site

3.3 Study Design

Repeated cross-sectional study design was conducted from September 2018 to March 2019 with sample being collected in two seasons, dry season (September to November 2018) and rain season (February to March 2019), from the same sampling sites (Kibirizi and Katonga fish landing sites) along Lake Tanganyika in Kigoma Municipality.

3.4 Inclusion and Exclusion Criteria

Tanganyika Tilapia from Lake Tanganyika were included in this study for the isolation and identification of *V. cholerae* while those from nearby rivers and ponds were excluded. Fresh water from Lake Tanganyika was included to be part of the study while waste water from rivers, wells and pond were excluded.

3.5 Sample Size Determination

Sample size was estimated using ($n = Z^2PQ / L^2$) as justified by (Chulaluk, 2009). Where; n = required sample size, P = known/ Conservative estimate, Q = (1-P), L = Allowable error for estimation, Z = Point on standard normal distribution curve corresponding to significance level of 5% (its values is 1.96). According to Hounmanou *et al*. (2016)

prevalence of *V. cholerae* in water was 36.7% and 23.3% in fish. Due to limited resource, this study involved sample size of 140 fish samples which is equivalent to 51% of the required sample size and 60 water samples which represent 16.8% of the required samples.

3.6 Sampling Method

Purposive sampling technique was applied to select fish landing sites (Kibirizi and Katonga fish landing sites). Purposive sampling was also used to select water samples and fishermen from which Tanganyika Tilapia (*Oreochromis tanganicae*) were collected.

3.7 Data Collection Method

Study was conducted in two season (dry season in November 2018 and rain season in February 2019). About 70 fish samples and 30 water samples were collected at each sampling season, a total of 140 fish samples and 60 water samples were collected in both seasons with two sites (Kibirizi and katonga) contributing half of each sample respectively. Fish samples were further subdivided into two parts (gills and intestine) whereby gills and intestine were analyzed as separate samples. Samples of fresh fish (gills and intestine) and water in tubes were put in a cool box and transported to Fisheries Training Agency (FETA) in Kigoma to maintain cold chain. Water samples and fish (gills and intestine) were then transported to Kigoma Regional referral hospital laboratory for microbiological analysis. The isolates were then transported to SUA for molecular analysis and antibiotic susceptibility testing.

3.8 Microbiological Isolation of *V. Cholerae*

About 25ml of water samples were added to 225ml of enrichment media (Alkaline Peptone Water) (Oxoid Ltd, Basingstoke, Hampshire, England), while 5.5gm of intestine

and 5.5gm of gills from each fish were added into 50ml of Alkaline Peptone Water (APW) (Oxoid Ltd, Basingstoke, Hampshire, England). Samples in APW were incubated at 37°C for six hours for enrichment before culturing onto Thiosulphate Citrate Bile Sucrose Agar (TCBS). A loop full of enriched culture samples from APW was streaked on TCBS agar plate (Oxoid Ltd, Basingstoke, Hampshire, England). All the plates were incubated at 37ºC for 24 hours. After incubation yellow and shinning colonies on TCBS agar (sucrose fermenting, ≥ 2 mm in diameter) were suspected as *V. cholerae* isolates (Fig 7a). Suspected isolates were then purified on Tryptone Soy Agar (TSA) (Oxoid Ltd, Basingstoke, Hampshire, England) for further confirmation (Fig. 7b).

Purified colonies were then screened for species confirmation on Triple Sugar Iron test (TSI). Uniform yellow colour colonies with no gas (H_2S) formation after overnight incubation at 37 ˚C were regarded as presumptive *V. cholerae* (Fig. 8). Oxidase reaction (BDH Chemical LTD, England) and String test were used for genus confirmation (Fig 9). Positive samples were determined by colour change from yellow to blue or dark purple within 10 seconds for the oxidase test, while formation of thread like mucoid string indicated positive test for string test. Sero-agglutination test was performed using specific *V. cholerae* O1 polyvalent anti-serum, finally DNA was extracted for molecular identification of *V. cholerae ompW* and *ctxA* genes.

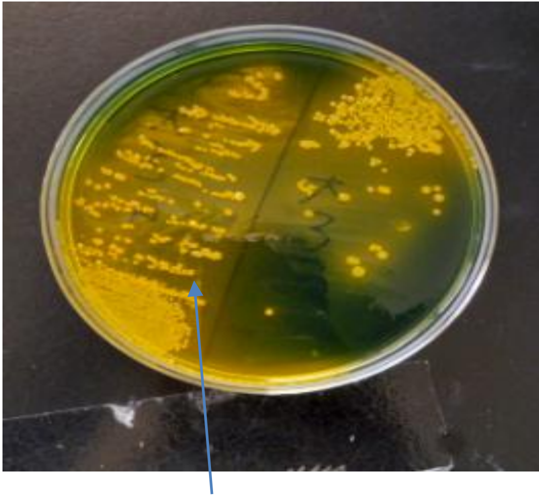

Figure 7a: Yellow colonies of Vibrio species on TCBS

Figure 7b: Purified colonies of Vibrio species on TSA

Figure 8: Triple Sugar Iron indicating *V. cholerae* (clear yellow tubes with no gas) for presumptive biochemical confirmation of *V. cholerae*

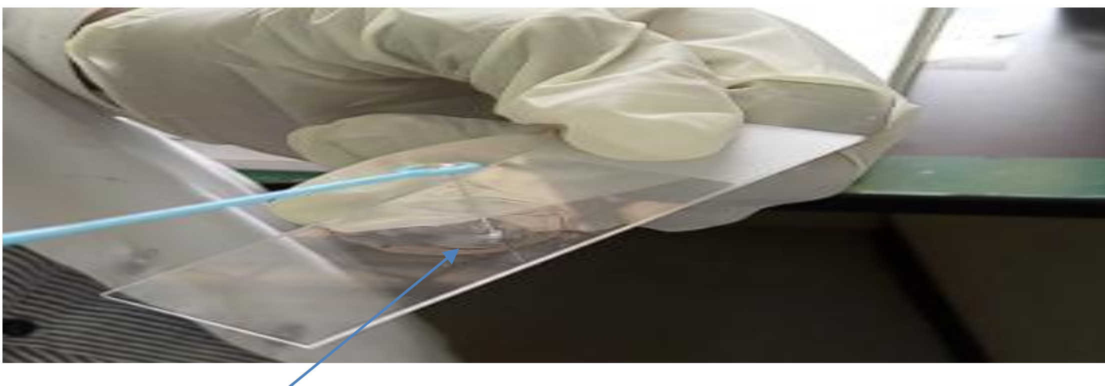

Figure 9: String test for identification of pathogenic *V. cholerae* (arrow)

3.9 DNA Extraction

The DNA extraction was done by using boiling method (Hounmanou *et al*., 2016). Briefly, a loopful of bacteria colonies were picked and mixed with 200 µl of deionized water and boiled for 10 min in water-bath at 100ºC. Cell debris were removed by centrifugation at 12 000xg for 3 minutes. Supernatant (template DNA) DNA was then transferred into Eppendorf tubes and stored at -20ºC for PCR assay (Hounmanou *et al*., 2016).

3.10 Molecular detection of *ompW* and *ctxA* genes by PCR

The assay was conducted in Molecular biology laboratory at SUA by conventional multiplex PCR amplification using GeneAmp PCR System 9700 PCR machine (Applied Biosystems, Foster City, CA, USA) (Nandy *et al*., 2000). Specific genes namely; the outer membrane protein gene *ompW* Size 558bp Sathiyamurth *et al*. (2013) and cholera toxin gene *ctxA* with 301 bp were targeted (Wong *et al*., 2012). The detection of each gene was performed according to Dalusi *et al*. (2015). Double distilled water was used as a negative control while *V.cholerae* (0139NCTC12945 (ATCC51394) strain was used as a positive control for PCR assay (Hounmanou *et al*., 2016).

Table 1: Master Mix solution for *ompW* and *ctxA*

Reagents	Volume (µl)
ompW Forward Primer	0.6
ompW Reverse primer	0.6
ctxA Forward Primer	0.4
ctxA Reverse primer	0.4
Dream Taq Green PCR Master Mix	12
Double distilled water	8
Template DNA	3
Total	25

Sathiyamurthy *et al.* (2013)

Table 2: Cycling profiles of each PCR

Genes	Initial denaturation	Number of cycles	Denaturation	Anneling	Extension	Final extension	Source
ompW	96ºC for 4 min	30	95ºC for 30s	66ºC for 20s	72ºC for 30s	72ºC for 30s	Sathiyamurthy *et al.* (2013)
ctxA	95ºC for 3 min	35	95ºC for 30s	65ºC for 30s	72ºC for 1 min	72ºC for 10 min	Dalusi *et al*. (2015)

Table 3: Primers sequences used for the PCR

Targeted genes	Primer Sequences (5'-3')	Size (bp)	Source
Outer membrane protein gene *ompW*	F-CACCAAGAAGGTGACTTTATTGTG R-GAACTTATAACCACCCGCG	588	Sathiyamurthi *et al*. (2013)
Cholera toxin gene *ctxA*	F-CAGTCAGGTGGTCTTATGCCAAGAGG R-CCCACTAAGTGGGCACTTCTCAAACT	301	Dalusi *et al*. (2015)

Amplified PCR products (10 µl) were resolved by 1% agarose gel electrophoresis with 0.5× Tris-borate- EDTA as the running buffer, in which 0.1 µl/ml of GelRed (Phenix Research) was used as DNA marker. Electrophoretic separation was performed at 120 V for 45min along with 1000 bp PCR ladder as molecular weight marker. The gel was visualized under UV trans-illuminator and recorded using digital camera.

3.11 Determination of antibiotic susceptibility patterns of *V. cholerae*

Antibiotic susceptibility testing was done in 65 samples of isolated *V. cholerae* using disk diffusion method whereby twelve different antibiotics notably Streptomycin (10 µg), Gentamicin (10 µg), Ciprofloxacin (5 µg), Trimethoprim (10 µg), Ceftazidime (10 µg), Tetracycline (30 µg), Chloramphenicol (30 µg), Ampicillin (10 µg), Amoxicillin (10 µg), Cefotaxime (30 µg), Naliadixic acid (10 µg) and Sulphamethoxazole (30 µg), were selected according to selection standards of clinical laboratory standard institute (CLSI, 2014) (Oxoid LTD, England).

Bacteria isolates were inoculated in normal saline and compared to McFarland standard of 0.5 which is equivalent to 10^8 CFU/ml. Normal saline was poured on to 15x150-mm Mueller Hinton (MH) agar and allowed to set for 5min, then antibiotic discs were placed

onto the MH agar and incubated at 37°C overnight (Okoh *et al*., 2010). A measurement of inhibition zone was conducted using a metric transparent ruler and interpreted according to the zone diameter interpretive chart of (CLSI, 2014). Standard strain (O139NCTC12945 (ATCC51394)) which has never been exposed to antibiotics was used as a reference (Hounmanou *et al*., 2016).

3.12 Data Analysis

Microsoft Excel was used to store data. EPI-INFO 7 (version 7.2.0.1) of statistical software was used to calculate proportions of positive *V. cholerae* samples at different sites, then compared by Chi-square test. The confidence intervals (CI) of proportions were set at 95% CI. Interpretation of the antibiotic pattern was carried out according to space zone diameter interpretive chart of (CLSI, 2014). Results are presented in Tables and Figures.

CHAPTER FOUR

4.0 RESULTS

4.1 Prevalence of *V. cholerae* in Tanganyika Tilapia during dry and rain seasons

Study revealed an overall prevalence of *V. cholera* infection in *Oreochromis tanganiceae* of 27.85% (n = 39/140). The prevalence during dry season and rainy seasons were 18.57% (n = 13/70) and 37.14% (n = 26/70) respectively. Proportion of fish samples infected with *V. cholerae* in rainy season was significantly higher (P < 0.05) compared to dry season.

Table 4: Prevalence of *V. cholerae* in Tanganyika Tilapia from dry and rain seasons

	Prevalence during dry season		Prevalence during rainy season				
	N	P (%)	N	P (%)	Total	Chi-square	p-value
Positive	13	18.57	26	37.14	39	6.007	0.014
Negative	57	81.42	44	62.85	101		
Total	**70**	**100**	**70**	**100**	**140**		

Key: N = Number of samples; P (%) = percentage positive

4.2 Proportions of toxigenic *V. cholerae* in Tanganyika Tilapia during dry and wet season

Detection of toxigenic *V.cholerae* was conducted in this study by using PCR targeting cholera toxin gene *ctxA*. Based on this test, the prevalence of toxigenic *V. cholera* infection in Tanganyika Tilapia was 11.42% (n = 16/140). The prevalence during dry season and rainy seasons were 8.6% (n = 6/70) and 14.2% (n =10/ 70) respectively. The difference between proportions of fish samples infected with toxigenic *V. cholerae* during dry season and rainy season was not significant (Table 5).

Table 5: Proportions of toxigenic *V. cholerae* in Tanganyika Tilapia during dry and wet season

	Prevalence during dry season		**Prevalence during rainy season**				
	N	P (%)	N	P (%)	Total	Chi-square	p-value
Positive	6	8.57	10	14.2	16	1.129	0.288
Negative	64	91.42	60	85.8	124		
Total	**70**	**100**	**70**	**100**	**140**		

Key: N = Number of samples; P (%) = percentage positive

4.3 Prevalence of *V. cholerae* in Water Samples during Dry and Rain Seasons

Study revealed an overall prevalence of *V. cholera* infection in water samples of 33.33% (n = 20/ 60). The prevalence during dry season and rainy seasons were 26.66% (n = 8/30) and 40% (n = 12/30) respectively (Table 6). Proportion of water samples infected with *V. cholerae* in rainy season was significantly higher (P < 0.05) compared to dry season.

Table 6: Prevalence of *V. cholerae* in water samples during dry and rain seasons

	Prevalence during dry season		**Prevalence during rainy season**				
	N	P (%)	N	P (%)	Total	Chi-square	p-value
Positive	8	26.66	12	40	20	5.556	0.018
Negative	22	73.33	18	60	40		
Total	**30**	**100**	**30**	**100**	**60**		

Key: N = Number of samples; P (%) = percentage positive

4.4 Proportions of Toxigenic *V. cholerae* in Water Samples during Dry and Wet Season

Findings revealed the prevalence of 20% (n = 12/60) of water samples infected with toxigenic *V. cholerae*. The prevalence during dry season and rainy seasons were 3.33% (n = 1/30) and 36.66% (n = 11/30) respectively (Table 7). Proportion of water samples infected with toxigenic *V. cholerae* in rainy season was significantly higher ($P < 0.05$) compared to dry season.

Table 7: Proportions of toxigenic *V. cholerae* in water during dry and wet season

	Proportion during dry season		**Proportion during dry season**				
	N	P (%)	N	P (%)	Total	Chi-square	p-value
Positive	1	3.33	11	36.66	12	10.417	0.001
Negative	29	96.66	19	63.33	48		
Total	**30**	**100**	**30**	**100**	**60**		

Key: N = Number of samples; P (%) = percentage positive

4.5 Proportions of Toxigenic *V. cholerae* in Fish Gills and Intestine during Dry and Wet Season

To establish difference in magnitude of *V. cholerae* within their host, fish samples were subdivided into two parts (gill and intestine) making total of 280 samples. Findings revealed prevalence of 5.71% (n = 16/280) of *V. cholerae* in fish gills and prevalence of 0.35% (n = 1/280) in fish intestine (Table 8). Proportion of fish gill samples infected with toxigenic *V. cholerae* was significantly higher ($P < 0.05$) compared to fish intestine.

Table 8: Proportions of toxigenic *V. cholerae* in fish gills and intestine during dry and wet season

	Proportions of *V. cholerae* in fish gills (n =280)		**Proportions of *V. cholerae* in fish intestine (n =280)**			
	N	P (%)	N	P (%)	Chi-square	p-value
Positive	16	5.71	1	0.35	10.34	0.001
Negative	264	94	279	99.6		
Total	**280**	**100**	**280**	**100**		

Key: P (%) = percentage positive

4.6 Identification of Toxigenic and non-toxigenic *V. cholerae* by PCR

It was found that 39 isolates from fish samples and 20 from water samples, were *V. cholerae* positive similar to PCR targeting gene *ompW* 588bp (a species-specific genetic marker used for *V. cholerae* detection). However, 16 fish samples and 11 water samples were positive for toxigenic *V.cholerae* similar to PCR targeting gene *ctxA* 301bp (Fig 9 and Fig. 10).

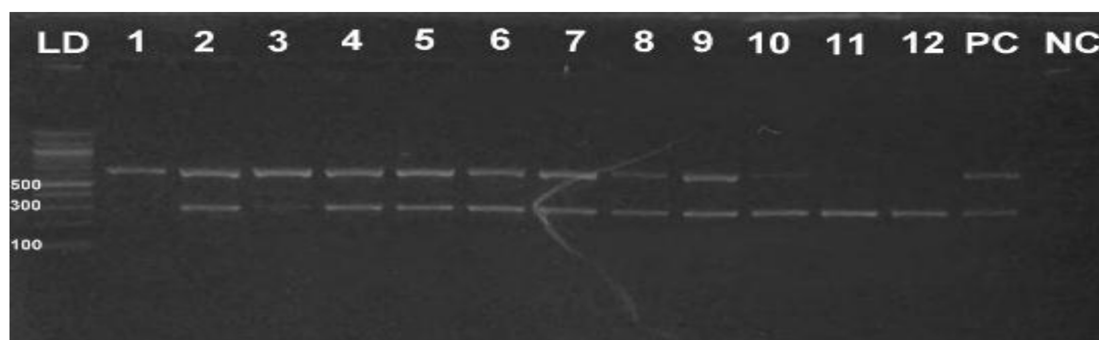

Figure 10: Gel output indicating *Vibrio cholerae* species specific gene *ompW* and cholera toxin gene *ctxA* from fish gills, intestine and water samples

LD: DNA ladder marker, PC: Positive control, NC: Negative control, 1 – 12 are positive samples for *ompW* gene at 588bp while sample number 2 - 12 were positive for cholera toxin gene *ctxA* at 301bp.

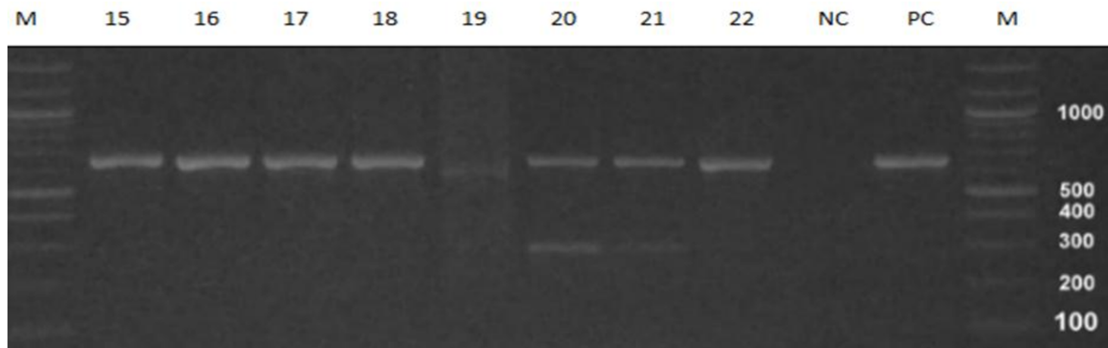

Figure 11: Gel output for *ompW* and *ctxA* genes

M: DNA ladder marker, PC: Positive control, NC: Negative control, 15, 16,17,18,19, 20, 21 and 22 are positive samples for *ompW* gene at 588bp while 20 and 21 are positive for cholera toxin gene *ctxA* at 301bp.

4.7 Serological Identification of *Vibrio cholerae* O1

Sero-agglutination test for confirmation of pathogenic *V.cholerae* O1 indicated that 47.6% (n = 31/65) isolates were positive on polyvalent O1 antiserum. The proportion of O1 positive samples from fish Gills, intestine and water were 27.69% (n = 18/65), 7.6% (n = 5/65) and 12.30% (n = 8/65) respectively (Table 9).

4.8 Antibiotic Susceptibility Patterns of isolated *V. cholerae*

Antibiotic susceptibility patters varies within different antibiotics (Fig. 11). *Vibrio cholerae* in this study were susceptible to Gentamicin 100% (n = 65/65), Ciprofloxacin 100% (n = 65/65), Cefotaxime 100% (n = 65/65), Ceftazidime 73.8% (n = 48/65),

intermediate resistance in Chloramphenicol 63.1% (n = 41/65), Tetracycline 53.8% (n = 35/65) and Naliadixic acid 47.7% (n = 31/65). Resistance was observed in Streptomycin 100% (n = 65/65), Amoxicillin 95.4% (n = 52/65), Ampicillin 81.5% (n = 53/65), Sulphamethaxole 80% (n = 62/65) and Trimethoprim 72.3% (n = 47/65). According to CLSI (2014), every antibiotic has three categories of presentation. This include intermediate, susceptible and resistance. Table 10 summarizes these categories as per each antibiotic.

Table 9: Antibiotic susceptibility pattern of isolated *V. cholerae* isolated in Tanganyika tilapia and water from Lake Tanganyika

Antimicrobial agents tested	**(n = 65)**					
	Susceptible (%)	N	Intermediate (%)	N	Resistance (%)	N
Trimethoprim (W5)	24.6	16	3.1	2	72.3	47
Ceftazidime (CAZ)	73.8	48	0.00	0	26.2	
Tetracycline (TE30)	53.8	35	10.8	7	35.4	23
Chloramphenicol (C30)	63.1	41	1.5	1	35.4	23
Amoxicillin (AMC)	0.00	0	4.	3	95.4	62
Cefotaxime (CTX)	100	65	0.00	0	0.00	0
Sulphamethaxole (RL25)	20	13	0.00		80	52
Ampicillin (AMP10)	3.1	2	15.4		81.5	53
Streptomycin (S10)	0.00	0	0.00	0	100	65
Ciproflaxin (CIP5)	100	65	0.00	0	0.00	0
Gentamycin (CN30)	100	65	0.0	0	0.00	0
Naliadixic acid (NA30)	47.7	31	4.4	3	47.7	31

Cut of points used was based on CLSI, located in appendix 1

CHAPTER FIVE

5.0 DISCUSSION

Vibrio cholerae is an aquatic bacterium that inhabits coastal and estuarine areas, and it is known to have several environmental reservoirs including fish (Kristie *et al*., 2017). Proportions of fish samples infected with *V.cholerae* during dry season and wet season were 18.57% and 37.14% (n = 70) respectively. Proportion of fish samples infected with *V. cholerae* in rain season was high compared to dry season, their difference is significant at $P < 0.05$. Rain is one of the factors which facilitate cholera epidemics due to the fact that it increases chances for bacterial contamination and colonization (WHO, 2016). A study conducted in Mandalay generally exhibited a single annual peak, with an annual average of 312 patients with severe diarrhea dehydration over the past 5 years (2011-2016) and was closely associated with the rainy season (Roobthaisong *et al*., 2017). The prevalence of *V. cholerae* in this study was comparable to previous studies, the study by Hounmanou *et al*. (2016) in Morogoro -Tanzania reported the proportion of contamination of *V. cholerae* in wastewater, vegetables and fish as 36.7%, 21.7% and 23.3% respectively. Findings in this study also revealed that 5.71% (n = 16/280) of subdivided gills samples from fish were infected with *V. cholerae* while 0.35% (n = 1/280) from fish intestine were infected with *V. cholerae*. Difference between proportions of *V. cholerae* in fish gills and intestine was significant at $P < 0.05$.

The prevalence of *V. cholerae* from fish gills and water was high compared to the intestines, this may be due to presence of accessory toxins in fish intestine, that make *V. cholerae* fail to adopt a viable state in fish intestine. Accessory toxins maintain low levels of intestinal colonization of *V. cholerae* in fish intestine (Kristie *et al*., 2017). Furthermore, Mdegela *et al.* (2015) isolated *V. cholerae* from fish and water samples in

Lake Victoria and reported prevalence of 53.7% in surface, gills 17.1%, intestine 4.9%, and 20% in water. Several other studies have isolated the organism from fish and various water sources and associated them with hospitalized patients with diarrhea, peritonitis as well as in immune compromised cases (Sathiyamurthy *et al*., 2013). Serological testing indicated that 31 (47.69%) out of 65 isolates were positive on polyvalent O1 antiserum. This finding underlined the previous study on *V. cholerae* O1 strains isolated from water and fish from Lake Victoria, in Kenya (Onyuka *et al*., 2011). The major risk factors for cholera have been linked to rivers and bathing water contaminated by sanitary effluents (Nyambuli *et al*., 2018). 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 and introduce enteric pathogens into the lake (Mdegela *et al*., 2015).

The antibiotic susceptibility in this study is comparable to the previous findings by Nyambuli *et al*. (2018) and Hounmanou *et al*. (2016) who reported susceptibility patterns of different antibiotics in *V. cholerae*. 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%). Resistant to such antibiotics may be related to their abuse, environmental pollution and overuse in humans and veterinary medicine (Onyuka *et al*., 2011). In another study, Akoachere *et al.* (2013) reported multidrug resistance (92%) in *V. cholerae* isolates (resistant to two or more antibiotics). Antibiotic drug resistance in bacteria isolates may arise through mutation or acquisition of resistance genes on mobile genetic elements like

plasmids, transposons integrons, and integrating conjugative elements (Akoachere *et al.,* 2013).

A review conducted by Yahaya *et al*. (2018), identified 340 publications, of which only 25, reporting from 16 countries in the sub-Saharan African region were eligible, with the majority (20; 80.0%) of the cholera toxigenic *V. cholerae* isolates being serogroup O1 of the El Tor biotype predominantly of Ogawa and Inaba serotypes. According to Yahaya *et al*. (2018) resistance was predominantly documented to trimethoprim-sulphamethoxazole in 50% studies, ampicillin 43.3% studies, chloramphenicol 43.3% of the studies and streptomycin in 30% of the studies (Yahaya *et al*., 2018). 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 (Akoachere *et al.,* 2013).

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

Tanganyika Tilapia (*Oreochromis tanganicae*) and water from Lake Tanganyika are reported by this study, as a reservoir of drug resistant and pathogenic *V. cholerae* of outbreak potential during a non-cholera outbreak periods. Consumption of contaminated water and food including fish containing the microorganism, is one of the factors that facilitate human infection of *V. cholerae* as well as cholera outbreaks.

6.2 Recommendations

According to the findings in this study there is need for continuous surveillance and monitoring of *V. cholerae*, through identification of their host range and drug susceptibility patterns to safeguard public health. Study also identified importance of awareness rising among communities on disease prevention practices as well as rational use of antibiotics. Strategies on cholera control including seasonal surveillance, health education and better health policies should therefore be encouraged and amplified towards potential reservoirs.

With regards to the findings of this study, the following recommendations are suggested.

i. Further scientific studies including whole genome sequencing, to establish genotypic factors influencing pathogenicity of *V. cholerae* and identification of different antibiotic resistance factors at molecular level.
ii. Periodical surveillance of *V. cholerae* with specific use of accurate sample size to get actual picture on epidemiology of the disease.
iii. Health education on effects and transmission cycles of *V. cholerae*.

iv. Adequate education on rational use of drugs specifically antibiotics for the sake of intervening antibiotic resistance among local communities.

v. Good hygiene and handling practices during fish evisceration and processing to avoid cross-contaminations.

vi. Treatment should be guided by susceptibility test results where possible or empirical evidence of antibiotics that are known to be sensitive in specific outbreak

vii. High resisted antibiotics should be withdrawn in prevention and treatment of *V. cholerae.*

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APPENDIX

Appendix 1: Antibiotics zone diameter interpretive chart

S/NO	Drugs	Inhibition zone (mm)		
		Susceptible	**Intermediate**	**Resistance**
1	Trimethoprim (W5)	>= 17	15 - 17	<= 14
2	Ceftazidime (CAZ)	>= 21	18 - 20	<= 17
3	Tetracycline (TE30)	>= 15	13 - 14	<= 12
4	Chloramphenicol (C30)	>= 18	13 - 17	<= 12
5	Amoxicillin (AMC)	>= 17	14 - 16	<=13
6	Cefotaxime (CTX)	>= 21	18 - 20	<= 17
7	Sulphamethaxole (RL25)	>= 21	18 - 20	<= 17
8	Ampicillin (AMP10)	>= 17	14 - 16	<= 13
9	Streptomycin (S10)	>= 17	14 - 16	<= 13
10	Gentamycin (CN30)	>= 15	13 - 14	<= 12
11	Ciproflaxin (CIP5)	>= 21	16 - 20	<= 15
12	Naliadixic acid (NA30)	>= 19	14 - 18	<= 13

Source. CLSI