VIRULENCE CHARACTERISTICS AND ANTIBIOTIC SUSCEPTIBILITY OF *VIBRIO CHOLERAE* IN LOW QUALITY WATER, FISH AND VEGETABLES IN MOROGORO, TANZANIA

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ABSTRACT

Cholera, one of the world's deadliest infectious diseases, remains rampant and frequent in Tanzaniaand thus thwartexisting control measures. The current study wasundertaken to evaluate the occurrence of toxigenic *Vibrio cholerae* in low quality water, fish and vegetables during the non-outbreak period in Morogoro, Tanzania. From October 2014 to February 2015, 60 wastewater samples, 60 fish samples from sewage stabilization ponds and 60 vegetable samples were collected. Samples were subjected to bacteriological analyses for identification of *V. cholerae*and confirmed by detection of the outer membrane protein (OmpW) by Polymerase Chain Reaction (PCR). The isolates were then tested for antibiotic susceptibility and forvirulence genes including, cholera enterotoxin gene (*ctx*), the toxin co-regulated pilus gene (*tcpA*), toxin regulatory protein (ToxR) and the haemolysin gene (*hlyA*). The proportion of contamination of*V. cholerae*in wastewater, vegetables and fish was 36.7%, 21.7% and 23.3% respectively.Two isolates from fish gills were*V. cholerae* O1 and tested positive for c*tx* and *tcpA*.One of them contained in addition the *hlyA* gene while 5isolates from fish intestines tested positive for *tcpA*.The *V. cholerae* isolates displayed a strong resistance to Ampicillin and Amoxicillin followed by a moderate resistance to Tetracycline. However, they were susceptible to Gentamicin, Chloramphenicol and Ciprofloxacin.It is concluded that pathogenic, toxigenic and antibiotic resistant *V. cholerae* species are present and persist in aquatic environments and can be isolated even during the non-outbreak periods. This is of serious public health importance and one of the great challengestoCholera control programmes.

DECLARATION

I, HOUNMANOU, YAOVI MAHUTON GILDAS, do hereby declare to the Senate of Sokoine University of Agriculture that this dissertation is my own original work, done within the period of registration and that it has neither been submitted nor being concurrently submitted for a higher degree award in any other institution.

_____   _____
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The declaration is hereby confirmed:

_____   _____
Prof. Robinson H. Mdegela   Date
Supervisor

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ACKNOWLEDGEMENTS

DEDICATION

This work is dedicated to the Almighty God;to my beloved Mum Affi Josephine Noutchet, my brothers Claver and Hermann and my late father Comlan Roger Hounmanou.

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LIST OF ABBREVIATIONS AND ACRONYMS

APW	Alkaline Peptone Water
ATB	Antibiotics
Bp	base pair
CLSI	Clinical and Laboratory Standards Institute
ESBL	Extended Spectrum beta lactam
GDP	Gross Domestic Products
IFCRC	International Federation of Red Cross and Red Crescent societies
LQW	Low Quality Water
M	Mole
MORUWASA	Morogoro Urban Water Supply and Sanitation Authority
PAHO	Pan American Health Organization
PCR	Polymerase Chain Reaction
rpm	Revolution Per Minute
TCBS	Thiosulfate Citrate Bile Sucrose
TSI	Triple Sugar Iron
WHO	World Health Organisation

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background Information

Low quality wateris an important resource for water-scarce regions of the world. It is increasingly used for various domestic purposes and income generation activities such as gardening and fishing in areas having low rainfall and water shortage (Mok *et al.*, 2014).Humans in these areas, who use low quality water for agricultural activities and thus for food production,are predisposed to a number of waterborne hazards comprising chemical, physical or biological ones. Among biological hazards are bacteria such as *Vibrio* species which are of a great interest because they are oneof the most common organisms in surface waters of the world (Adeleye *et al.,* 2010). Although there are about 12 *Vibrio* species which are harmful to human, the most important ones are *Vibrio cholerae*, *V. parahaemolyticus* and *V. vulnificus* (Nhung *et al.,* 2007; Raissy *et al.,* 2012). However, *Vibrio cholerae* (O1 and O139), thecausative agent of Cholera is a known and dangerous source of high morbidity and mortality worldwide and mostly in sub-Saharan African countries including Tanzania (WHO, 2008); Benin (IFCRC, 2013) and others. Moreover, these bacteria, autochthonous of aquatic environments are transmitted to human via faecal-oral route (Daniel and Shafaie, 2000). Humans get infectedthrough ingestion of raw or undercooked contaminated foodstuff such as legumes and fish or cross-contaminated ready-to-eat food but also through poor personal hygiene and sanitation like lack of hand washing (Daniel and Shafaie 2000). These cholera risk factors are highly found in Morogoro whereby the river water and water from sewage stabilization ponds are used for vegetable irrigation, fishingas well as for domestic activities. Therefore,Morogoro population wouldhighly be vulnerable tocholerainfections if the causing bacteriawill bepresent in these waters which safety are questionable and uncertain.

The present study aimedto assess the safety of Morogoro river water and water from Morogoro sewage stabilization ponds, as well as,fish and vegetablesproduced out of such watersby studying the presence ofpathogenic *V.cholerae.*

1.2 Problem Statement

In endemic areas, several studies have reported a seasonal pattern of occurrence of *V. cholerae* and cholera (Mishra *et al.,* 2012; Fooladi *et al.,* 2013). However, since the seventh cholera pandemic reached the country in 1974, the disease has been reported almost every year in Tanzania regardless seasons (WHO, 2008 and 2013). There are therefore existences of neglected reservoirs of the causative agent, *V. cholerae*. The inadequate information on all possible sources of toxigenic strains of *V. cholerae*complicatescholera prediction, prevention and control and contributes to frequent and unexpected outbreaks of cholera in Tanzania. Therefore, there is a need to assess whether toxigenic strains of *V. cholerae* could occur in environmental isolates during the non-outbreak period. Additionally, the massive use of antibiotics in prophylaxis during previous cholera outbreaks in the world resulted in the emergency of multidrug resistant strains of *V. cholerae* (Mandal *et al.,* 2012).Current surveillance of antibiotic susceptibility pattern of the organism not only from clinical isolates but also from environmental isolates in endemic regions is necessary as the environment could serve as a reservoir for resistant strains.

1.3 Study Justification

Cholera, a worldwide deadly infectious disease remains highly frequent in Tanzania. The reasons are multiple and multiform and need to be addressed. However, the occurrence of toxigenic *V. cholerae* has always been associated exclusively with outbreaks (Mishra *et*

al., 2012; Fooladi *et al.,* 2013). Thus, there has been an underestimation of other reservoirs of cholera causing organisms especially during the non-outbreak period.

Furthermore, in cholera outbreak,antibiotics are used to reduce the shedding of the bacteria (thereby reducing spread of the disease), treating severe illness (by reducing volume of diarrhoea), and also to reduce duration of disease and hospitalisation (Mandal *et al.,* 2012).The massive use of antibiotics as prophylactic measures during cholera outbreaks, resulted in selection of multidrug resistant strains of*V. cholerae* (Mandal *et al.,* 2012). Current data on the antibiotic susceptibility pattern of *V. cholerae* is therefore needed for efficient control of cholera outbreaks.

Moreover,cholera occurrence risks factors such as poor sanitation and poor water supply keep growingin Tanzania. For instance, the increased use of low quality water for vegetableirrigation, fishing and domestic purposes in populations experiencing water scarcity like Morogoro seems threatening as far as cholera is concerned.This study was therefore carried out in order to illuminateon whethertoxigenic *V. cholerae* could be isolated from low quality water and its related human activities during the non-outbreak period. Findings from this study would raise awareness among different stakeholders including users, regulatory agencies and agencies involved in management and treatment of LQW.

1.4 Objectives

1.4.1 Main objective

The main objective of the study was to evaluate the occurrence of toxigenicand antibiotics resistant *V.cholerae* inlow quality water, fish and vegetables during thenon-outbreak period in Morogoro, Tanzania.

1.4.2 Specific objectives

i. To determine the magnitudeof*V.cholerae*in water, fish and vegetables in Morogoro, Tanzania.

ii. To establish the virulence characteristics of the isolated *V.cholera*e.

iii. To elucidate the antibiotic susceptibility pattern of isolated *Vibriocholerae.*

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Description of *Vibrio cholerae*

Vibrio cholerae is a Gram-negative, facultative anaerobic, curved rod shaped bacterium that belongs to the family of Vibrionaceae. With about 1.4–2.6mm long, the bacterium is oxidase-positive, reduces nitrate, and is motile by means of a single, sheathed, polar flagellum (Fig. 1).Currently, the organism is classified into more than 206 serogroups based on the "O"somatic antigens. However, only the O1 serogroup and the recently discovered O139 are associated with epidemic and pandemic cholera. Isolates of the O1 serogroup of *V. cholerae* have been further divided into two biotypes namely Classical and El Tor and into three serotypes, Inaba, Ogawa, and Hikojima. The non-O1 and non-O139 strains are occasionally isolated from cases of diarrhoea and from a variety of extra-intestinal infections, from wounds, and from the ear, sputum, urine, and cerebrospinal fluid.Though these are not of epidemiological importance, they are of great food safety concern and any ready-to-eat foodstuff is supposed to be free from the non-O1 and non-O139 *V. cholerae* (WHO, 2002).
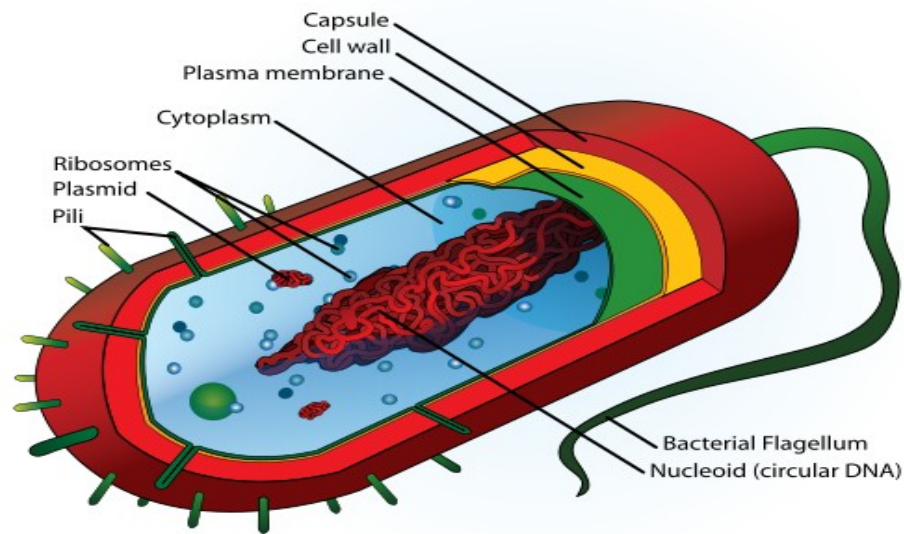
Figure 1:*Vibrio cholerae* cell

Source:[www.biologyduide.net/biol1/3b_exchange.htm]

2.2 History of Cholera Outbreaks in Tanzania

Since the seventh Cholerapandemic reached the country in 1974, the disease has been reported almost every year in Tanzania. With a case fatality rate averaging 10.5% between 1977 and 1992, the country witnessed its first major outbreak in 1992 recording 18 526cases with 2 173 deaths(WHO, 2008). In 1997, an epidemic of cholera which started at the end of January in Dar es Salaam accounted for 40,249 cases and 2,231 deaths. However, from 2002 to 2006, most Tanzanian regions reported cholera cases and nine of them including Morogoro reported morethan 2 000 cases during this five years period (WHO, 2008). Between 1st January and 31st December 2006, a total of 14,297 cases including 254 deaths were reported from 16 regions (out of 21) including Morogoro (WHO, 2008).

In 2013, WHO reported about 25, 762 cases of cholera with 490 deaths from 18 countries including Tanzania (WHO, 2013). In early 2015, cholera outbreak was reported in Kigoma (from January to March 2015) and killed five people with 170 hospitalized patients,

showing the rampant status of Cholera in Tanzania (Pesatimes, 2015). The most recent one is the ongoing large-scale cholera outbreak that has left thirteen people dead and affected 815 people in Dar es Salaam, Morogoro, Pwani and Iringa regions in Tanzania as per the situation from 15th August to 3rd September 2015 (WHO, 2015). These outbreaks are attributed to poor water sanitation and hygiene, poor food safety and high population dynamic (WHO, 2015). However, it is important to highlight that these outbreaks occur in the middle of a dry season in these regions.

2.3 Molecular Mechanism of Virulence in *Vibrio cholera*e

Vibriocholerae enterotoxin (the cholera toxin) is a product of *ctx* genes. c*tx*-A encodes the A subunit of the toxin, and *ctx*-B encodes its B subunit (Sanchez and Jan*,* 2011). The genes are part of the same operon. The *ctx*operon and the *tcp* operon (toxin co-regulator pilus concerned with fimbriae synthesis) are part of a regulon, the expression of which is controlled by environmental signals, including temperature, pH, osmolarity, and certain amino acids (Faruque *et al.,* 1998).Proteins involved in control of this regulon expression were identified asTox-R, Tox-S and Tox-T. Tox-R is a transmembranous protein with about two-third of its amino terminal part exposed to the cytoplasm (Todar, 2008). Furthermore, Tox-R dimers bind to the operator region of *ctx*-AB operon and activate its transcription(Todar, 2008). Tox-R and Tox-S appear to form a standard two-component regulatory systems with Tox-S functioning as a sensor protein that phosphorylates and thus converts Tox-R to its active DNA binding form (Sanchez and Jan, 2011).Tox-T for its case,is a cytoplasmic protein that is a transcriptional activator of the tcp operon(Sanchez and Jan, 2011). Additionally, expression of Tox-T is activated by Tox-R, while Tox-T, in turn, activates transcription of tcp genes for synthesis of pili. Faruque *et al.* (1998)stated that Tox-R interacts with Tox-S in order to sense some change in the environment and transmit a molecular signal to the chromosome which induces the transcription of genes for attachment (pili formation) and toxin production, hence the usefulness of targeting the

Tox-R during environmental investigations. Furthermore, it is reasonable to expect that the environmental conditions that exist in the humangastrointestinal tract (temperature, low pH, high osmolarity, etc.), as opposed to conditions in the extra-intestinal (aquatic) environment of the vibrios, are those that are necessary to induce formation of the virulence factors necessary to infect (Akoachere *et al.,* 2013).This can explain why environmental isolates foundduring the non-outbreak period are commonly non-toxigenic.

2.4 Pathogenesis and Ecology

Poor sanitation practices in highly populated areas harbouring endemic toxigenic strains are the source of occasional outbreaks due to contamination of drinking water and/or improper food preparations. Contaminated water with free-living *V. cholerae* cells are the main origin of epidemics, followed by contaminated food, especially water products like fish and vegetables produced with contaminated water.Infection due to *V. cholerae* begins with the ingestion of contaminated water or food (Nair and Bartram*,* 2000). After passage through the acid barrier of the stomach, the organism colonizes the epithelium of the small intestine by means of the toxin-coregulated pili and other colonization factors such as the different haemagglutinins, accessory colonization factor, and core-encoded pilus (Nair and Bartram*,* 2000)(Figure 2). Thereafter, cholera enterotoxin produced by the adherent vibrios is secreted across the bacterial outer membrane into the extracellular environment and disrupts ion transport by intestinal epithelial cells. The subsequent loss of water and electrolytes by the cells leads to the severe diarrhoea characteristic of cholera (Reidl and Klose, 2002).

Moreover, *V. cholerae* exists as natural inhabitants of aquatic ecosystems mostly under warm climate (water temperature).Non-O1 and non-O139 strains are more frequently isolatedfrom rivers and estuarine areas than O1 and O139strains, and interestingly most environmental O1 strainsare non-toxigenic (Reidl and Klose,2002).It was postulated that under stressful conditions the vibrios are converted into a viable but non-culturable

(VNC)form that cannot be recovered by standard culture techniques and such VNC forms are able to cause infection and thusrevert to the culturable form (Faruque *et al.,* 1998).

Further studies have illustratedthe ability of *V. cholerae* O1 to associate with a variety ofzooplankton, phytoplankton, blue-green algae and various other aquatic organisms. These associations (symbiosis)prolong survival, and presumably the vibrios gain nutrientsfrom the host(Faruque *et al.,* 1998).

Furthermore, in endemic areas, cholera epidemics occur in a regular seasonal pattern. However, Mukhopadhyay*et al.*(1998) reported that differences in genetic properties are often noticed among *V. cholerae* O1 and O139 strains isolated during different epidemics, showing thus a clonal diversity among epidemic strains. These events have raised questions about whether seasonal epidemics are caused by periodic appearances of the same strains of *V. cholerae* or are due to a continual emergence of new toxigenic clones from non-toxigenic progenitors.
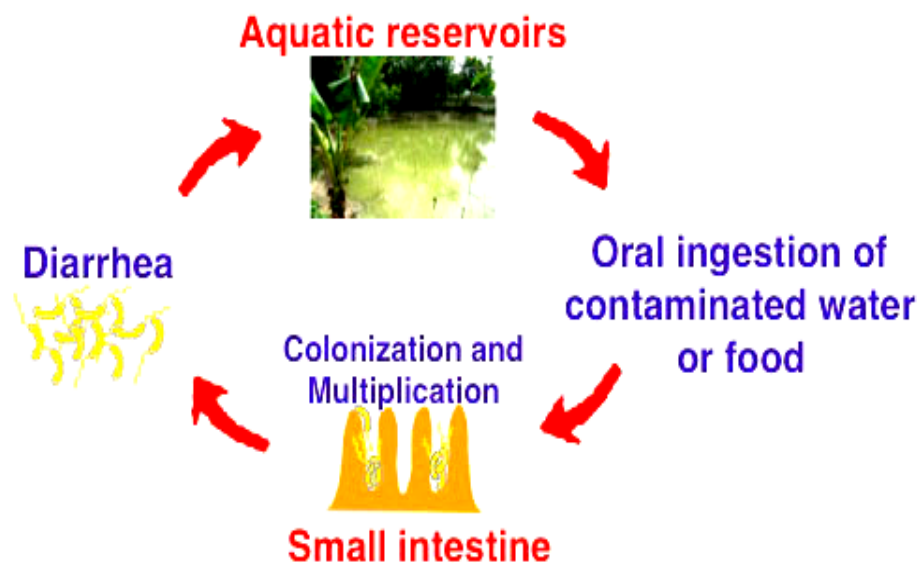
Figure 2: Cholera cycle
Source: [www.research.pomona.edu/jan-liu-liu-lab-research/]

2.5 Role of *Vibrio cholerae* bacteriophage CTXΦ in the Survival and Persistence of Toxigenic Strains of *Vibrio cholerae*

A study conducted in Bangladesh has reported that under appropriate conditions, toxigenic *V. cholerae* strains can be induced to produce extracellular CTXΦ phage particles (Faruque *et al.,* 1998). Therefore, the naturally occurring strains of toxigenic *V. cholerae* O1 and O139 are inducible lysogens of CTXΦ. Furthermore, it seems possible that in the natural ecological settings, unidentified environmental factors induce lysogenic CTXΦ in toxigenic *V. cholerae*, resulting in the release of extracellular CTXΦ particles into the aquatic environment(Faruque *et al.,* 2002). The cell-free phage particles participate in the emergence of novel toxigenic strains of *V. cholerae* through interactions with non-toxigenic strains which exist in the environment and in the human population that consumes the environmental waters. The phage (CTXΦ) uses TCP as its receptor, and hence the phage can infect only *V. cholerae* cells expressing TCP(Faruque and Mekalanos, 2012). This further supports the assumption that in natural settings, CTXΦ probably plays an important role in the origination of new toxigenic strains of *V. cholerae.* Moreover, it has been demonstrated that CTXΦ infects recipient *V. cholerae* strains more efficiently in the intestinal environment, where virulence factors such as TCP are adequately expressed(Faruque *et al., 2002*). While the conversion of non-toxigenic *V. cholerae* is favoured within the gastrointestinal tract of the mammalian host, the natural selection and persistence of the novel toxigenic strains may involve both intestinal and environment factors, the immune status of the host population, and antigenic properties of the new pathogenic strain. The induction of CTXΦ lysogens is probably controlled by precise environmental signals such as optimum temperature, sunlight, and osmotic conditions(Faruque and Mekalanos, 2012).

2.6 Low Quality Water Uses in Urban Agriculture and Domestic Purposes

As in many other developing countries, Tanzania is also witnessing a dramatic demographic growth. According to the Tanzanian National Bureau of Statistic, the population hadincreased to 44.9 million in 2012with the urban population increasing faster than the rural one (NBS, 2013). In order to cope with urban economic difficulties, most of immigrant urban dwellers are enterprising income-generation activities in the informal sector (Foeken *et al.,* 2004). One of these activitieswhich are widespread in many developing countries including Tanzania cities is the urban agriculture (notably vegetable gardening) whereby urban dwellers produce food for their livelihoods and earn extra income (Dougnon *et al.,* 2012). Nevertheless, the main sources of water used for the irrigation in such production system are piped water, rivers, channels from natural springs, and wells (Foeken *et al.* 2004). For populations experiencing water scarcity, the main sources of water remain the reclaimed wastewater and sewage water.Wastewater reuse could therefore, have some adverse effect on human, animal and environmental health since the use of untreated sewage can have serious health implications to farmers and consumers, and can irreversibly degrade the environment (Flynn *et al.,* 1999).

Wastewater users are therefore exposed to a number of chemical, physical and biological hazards which can cause severe diseases and permanent adverse effects. For instance, Duc *et al.* (2012) explained that direct contact with polluted water from the Nhue River (Switzerland) used for food production was associated with risk of diarrhoeal diseases. Likewise, Jiwa *et al.* (1991) reported that Morogoro River, one of the main irrigation water sources in this Municipality was faecal contaminated (presence of faecal coliforms:*Escherichia coli, faecal streptococci and Clostridium perfringens*). Similar observations were made by Okoh *et al.* (2010) who isolated pathogenic *Vibrio* species from wastewater used for vegetable production in South Africa.

Furthermore, reports from the Morogoro Urban Water supply and Sewage Authoritypointed out that thepopulation living in the upstream areas of the Morogoro River affects water quality and quantity through human related activities done by communities living at the catchment areas(MORUWASA, 2010).Specifically, the contamination was due to the use of improper sanitation facilities and poor farming practices (improper use of fertilisers) and thus increase water related diseases and infections.

The focus in this study was on toxigenic *V. cholerae* known as water related pathogen and one of the major diarrhoeal disease causing agents (Cholera).As displayed in Fig. 3 and 4,several activities are carried out in Morogoro using LQW.

Figure 3: Use of LQW in sewage stabilisation ponds for fishing and irrigation of vegetables irrigation at MZUMBE

Figure 4: Use of LQW in Morogoro River for domestic and recreational purposes

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Area

This study was carried out in Morogoro Urbanand its peri-urban areas (mainly Mzumbe). The Municipality is located about 195 km from the west of Dar es Salaam and situated on the lower slopes of Uluguru Mountains. It lies at the crossings of longitude 37.0° east of Greenwich Meridian and latitude 4.49° south of Equator. With a total land area of 531 km^2 representing 0.4 % of the total regional area (Fig. 5). Morogoro municipality has only one division with 29 administrative wards and 272 hamlets (MMC, 2015) and a total population of 315,866 individuals (NBS, 2013).

The climate of Morogoro municipality is characterized by an average daily temperature of 30 ^{o}C with a daily range of about ±5 ^{o}C. The highest temperature occurs in November and December, during which the average maximum temperature is about 33°C. The minimum temperature is generally recorded in June and August when the temperatures falls down to about 16°C. The average relative humidity is about 66 % and drops down to as low as 37%. The total average annual rainfall ranges between 821 mm to 1,505 mm. Heavy rains

occur between March and May and short rains occur between October and December each year (MMC, 2015).
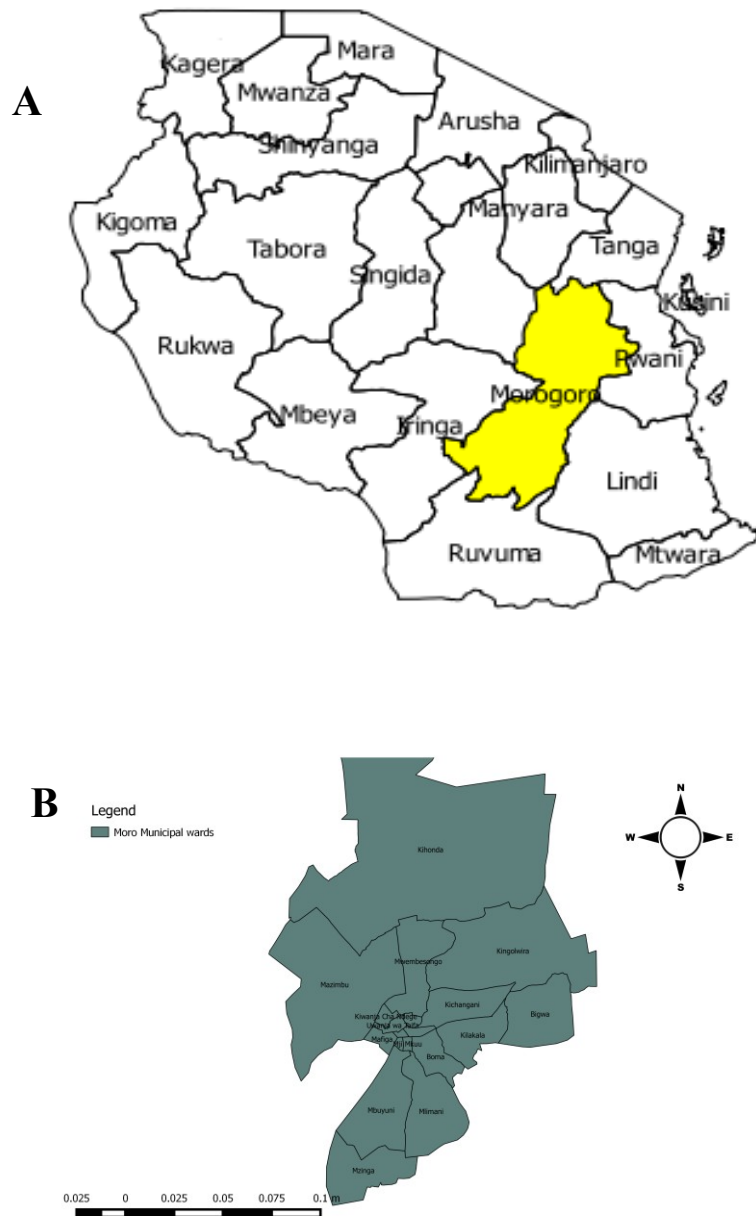
Figure 5: Map of the study area

Legend: A = Tanzania Map highlighting Morogoro Region in Yellow;

B = Morogoro Municipality withdrawn from Morogoro Region's Map

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

3.2 Study Design

The study was across-sectional study conducted on water, fish and vegetables collected from the study area.

3.3 Study Materials

During this study, the main materials were water samples, fresh fishTilapia,and African sharp tooth catfish and Chinese cabbage. In the laboratory, subsamples were taken from fish gills and intestines. This is because fish filet (the main edible part) is regarded as sterile and bacteria that occur in itare due to cross-contaminants from the environment (water), the gills, the intestines or the handlers (Hammad *et al.,* 2012; Cho *et al.,* 2014). Gills and intestines are thus representative parts of fish to give inferences on possible microorganisms present in fish.

3.4 Sample Size Determination

The formula used to estimate the samples size was:

$$n = \frac{z^2 * p * (1-p)}{d^2}$$ ...(1) (Chulaluk, 2009), where z = student t value for an expected

confidence level $z_{0.05/2} = z_{0.025} = 1.96$ at 95% confidence interval (two sided); p: prevalence of *Vibrio cholerae*14.8% in water (Madoroba *et al.,* 2010); 11.1% in fish (Sathiyamurthy *et al.,* 2013) and 50% in vegetables (for unknown prevalence). This gives: n water =193.76; n fish =151.63; n vegetables = 384.16.

Nevertheless, as the aim of this study is not to establish the actual prevalence of *V. cholerae* but rather, to assess the occurrence of toxigenic *V. cholerae*isolates in environmental bodies during a non-outbreak period, there is no obligation to reach the calculated sample size. Therefore, 60 watersamples, 60 fish samplesand 60 vegetable samples (a total of 184 samples) were collected and analysed.

3.5 Sampling Procedure

Water samples were collected from Morogoro River, Mafisa and Mzumbe sewage stabilisation ponds. Vegetables, the Chinese cabbage samples were collected from Mzumbe and Funga-Funga production sites, while fish samples (Tilapia and African sharp tooth catfish) were exclusively obtained from Mzumbe sewage stabilisation ponds. Briefly, water sample werecollected in 100ml sterile bottle following the water sampling methods for microbiological analysis described in the guidelines for drinking water quality (WHO, 1997).Samples were thus put in sterile cooler box and transported to the laboratory.

Water sampleswere collected from the upstream to the downstream of the River at approximately every 4 Km interval, mainly at the level of Uluguru Mountain (origin), under the bridge of Morogoro town, at Funga-Funga vegetable production site and finally at Mafisa where the river water meets the effluent water of the sewage stabilisation ponds. Other water samples were collected from the two inlets of Mafisa ponds and at the outlet. Further water samples were collected from the inlet and the outlet of Mzumbe sewage treatment ponds and at Mzumbe vegetable production site.

A total of 12 water samples, 20 fish samples (10 per site) and 20vegetable samples (10 per site; 3 leaves=1sample) werecollected per sampling time. Water was collected five times while fish and vegetableswere sampled thrice.Sample collection was repeated in order to track the organisms throughout the study period because the life span of *V. cholerae*cell in aquatic environment is for two weeks to one month maximum (Nair and Bartram, 2000). Furthermore the repetition is to facilitate effective and efficient laboratory manipulationby minimizing the work load. Collected samples were transported to the laboratory in cooler

box and for fish samples, subsamples were taken from their intestines and gills. All samples wereprocessed for isolation and identification of pathogenic *V. cholerae*.

3.6 Ethical Considerations

Prior samples collection, authorisations were obtained in the form of written Research Permit from Morogoro Municipal Council (Appendix 3), Mzumbe University (Appendix 4) and MORUWASA (Appendix 5).

3.7 Laboratory Analysis

To carry out this study, bacteriological isolation followed by molecular identification and antibiotic susceptibility test were performed.

3.7.1 Isolation of *Vibrio cholerae*

A bacteriological procedure for isolation of *Vibrio* specieswas carried out as per US-FDA Bacteriological Analysis Manual (Elliot *et al.,* 2001). A clear diagram of this protocol is displayed in Appendix 1. Briefly, after 18h enrichment in Alkaline Peptone Water (SIGMA, CHEMICAL. Steinheim, Germany), a loopful of sample was streaked on Thiosulfate Citrate Bile Sucrose agar, TCBS agar (ACCUMIX, Tulip Diagnostics (P) LTD, Verna, India). Yellow colonies on TCBS agar (sucrose fermenting, $\geq$ 2 mm) suspected as *Vibrio cholerae* were purified on Trypticase Soy Agar, TSA (MERCK KGaA, Darmstadt, Germany). Purified colonies were screened by Gram staining. Samples that were Gram negative and comma shaped were tested for Oxidase reaction (Oxidase, Becton, Dickinson and Company, Mexico) for genus confirmation. Positive samples were then tested by Triple Sugar Iron, TSI (OXOID LTD, Basingstoke, Hampshire, England) for species confirmation; uniform yellow colour with no gasformation (hydrogen sulphide: H_2S) after overnight incubation at 37˚C were regarded as presumptive*Vibrio*

cholerae(Elliot *et al.,* 2001). Thereafter, sero-agglutination test was performed using specific *Vibrio cholerae* O1 anti-serum, thenDNA was extracted for the molecular identification (Appendix 1). Fig. 6 and 7 relate some steps of the practical works.

Figure 6: Water sampling and colonies purification on TSA agar

Figure 7: Fish handling at the laboratory

3.7.2 Slide agglutination test

Agglutination tests for *V. cholerae* somatic O antigens was carried out on a clean glass slide. An inoculating sterile tooth pick was used to remove a portion of the growth from the surface of TSA (MERCK KGaA, Darmstadt, Germany). 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 auto agglutination. Where clumping occurred, the culture was termed "rough" and was not serotyped.

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

3.7.3 Molecular confirmation of*Vibrio cholerae* and identification of virulence genes
3.7.3.1 DNA extraction

Twenty-four hours colonies from TSAwere picked up and mixed with 200 μl of sterile distilled water and boiled for 10 min in water-bath at 100 ℃. Cell debris were removed by centrifugation at 10,464 xg for 3 minutes, and the supernatant containing the template DNA was taken into a fresh Eppendorf tube and stored at -20℃ till PCR assay (Park *et al.,* 2013).

3.7.3.2 Polymerase Chain Reaction(PCR)and Gel Electrophoresis

The assay was conducted by conventional Polymerase Chain Reaction amplificationusing TECHNE TC-4000 (Bibby Scientific Ltd) PCR machine. Specific genes and proteins targeted were,OmpWprotein(outer membrane protein) for confirmation of *Vibrio cholerae,* the *ctx* gene (cholera toxin gene),the *tcp*A gene (toxin coregulated pilus) and the *hly*A gene (Haemolysin).

TheOmpW protein is scientifically known as species specific for *Vibrio cholerae* (Garrido *et al.,* 2014; Wei *et al.,* 2014). The c*tx* is the main virulencegene present in both O1 and O139 serotypes) (Wong *et al.,* 2012). The *tcp*A gene intervenes in fimbriae synthesis allowing the organism to be fixed in the intestinal epithelium (Wenpeng *et al.,* 2014) and the*hlyA*gene confers to the Vibrio cells the ability to cause blood cell lysis in the infected host (Fooladi *et al.,* 2013).The ToxRproteincodes for the transcription of the *ctx* and *tcp* genes (Kondo *et al.* (2009).

The detection of each gene wasperformed as uniplex in a final reaction volume of 25 µlcontaining 0.8 µM concentration of each primer, 2 x Dream Taq Green PCR Master Mix (Thermo Scientific, Nairobi, Kenya) and5 µl of DNA as shown on Table 1 below.

Table 1: Master Mix solution

Reagents	Volume (µl)
Forward primer	0.2
Reverse primer	0.2
Dream Taq Green PCR Master Mix	10
Double distilled water	9.6
Template DNA	5
Total	**25**

The cycling profiles of each PCR are described in the table 2 and the sequences of the specific primers used are displayed in Table 3.

For the gel electrophoresis, 10μl of PCR products was loaded into a horizontal 1.5% agarose gel stained with 0.1 μl/ml of DNA marker GelRed (Phenix Research) and run in 1xTBE (Tris Borate EDTA) buffer. Electrophoretic separation was performed at 100 V for 1hour along with 1000 (bp) PCR ladder as molecular weight marker. The gel was visualized under UV trans-illuminator and recorded as JPEG file using a SAMSUNG digital camera.

During analyses, double distilled DNase free water was used as negative control and DNA from reference strain of *Vibrio cholerae* O139 NCTC 12945 (ATCC 51394) (Culture collections, Public Health England, Portion Down, Salisbury, SP4 OJG, UK) served as positive control.

Table 2: Cycling profiles of each PCR

Genes/proteins	Initial denaturation	Number of cycles	Denaturation	Annealing	Extension	Final extension	Reference
OmpW	96ºC for 4 min	30	95ºC for 30s	66ºC for 20s	72ºC for 30s	72ºC, 10min	Sathiyamurthy *et al.*, 2013
ctx	95ºC for 3 min	35	95ºC for 30s	65ºC for 30s	72ºC for 1 min	72ºC, 10min	Wong *et al.*, 2012
*tcp*A	94°C for 2 min	30	94°C for 1 min	60°C for 1 min	72°C for 1 min	72ºC, 10min	Wenpeng *et al.*, 2014
*hly*A	94 °C for 5 min	35	94 °C for 1 min	58 °C for 1 min	72 °C for 1 min	72 °C, 5min	Fooladi *et al.*, 2013
ToxR	94°C for 4 min	30	94°C for 1 min	60°C for 1.5 min	72°C for 1.5 min	72°C, 10 min	Kondo *et al.*, 2009

Table 3: Primers sequences used for the PCR

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

3.7.4 Antibiotics susceptibility testing

All confirmed positive isolateswere subjected to antimicrobial 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 matches the 0.5 Mac Farland turbidity standards (Remel, Lemexa, Kamsas). Using a sterile cotton swab, an entire surface of dried Muller Hinton agar plate (OXOID LTD, Basingstoke, Hampshire, England) was streaked by the above solution. The inoculated plate was left to dry for about five minutes and six (commonly used antibiotics in severe Cholera treatments) antibiotic discs notably Tetracycline, Gentamicin, Ciprofloxacin, Chloramphenicol, Ampicillin and Amoxicillin (OXOID LTD, Basingstoke, Hampshire, England) were then applied using a dispenser and 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 interpretivechart ofCLSI (2007) (Appendices 2).

3.8 Data Analysis

Data were stored using Microsoft Excel computer program. Proportions of positive *Vibrio cholerae* samples at different sites and different sample types were thereby calculated then compared by Chi-square and Fisher exact tests based on the total sizes using EPI-INFO 7 statistical software. The confidence intervals (CI) of proportions wereset at 95% CI. Proportions of isolates resistant and susceptible to each of the six tested antibiotics were analysed using the same process. Results were presented in graphs and tables. Interpretation of the antibiotic pattern was carried out as displayed in table 4 obtained from the zone diameter interpretive chart ofCLSI (2007).

Table 4: Antibiotics zone diameter interpretive chart

Antibiotics	Resistant (mm)	Intermediary (mm)	Sensitive (mm)
Ampicillin	≤13	14-16	≥17
Chloramphenicol	≤12	13-17	≥18
Ciprofloxacin	≤15	16-20	≥21
Gentamicin	≤12	13-14	≥15
Amoxicillin	≤13	14-17	≥18
Tetracycline	≤14	15-18	≥19

CHAPTER FOUR

4.0 RESULTS

4.1 Identification of *Vibrio cholerae*Using PCR

After biochemical tests, DNA was extracted from all the presumptive *Vibrio cholerae* colonies (Fig. 8) and submitted to PCR analysis targeting a species specific gene of the outer membrane protein (OmpW) using a specific set of primers. As shown inFig.9, the 588 bp region of the OmpW gene was amplified in most of the presumptiveisolates submitted to the analyses.
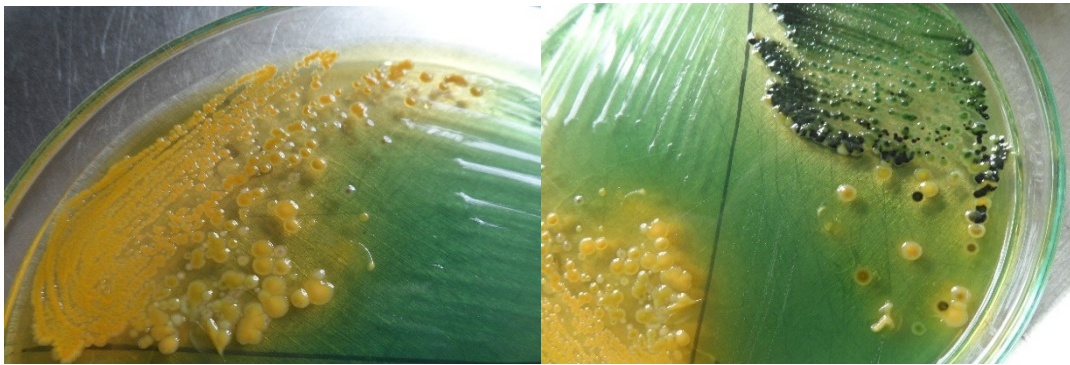
Figure 8: Yellow colonies on TCBS agar considered as presumptive *V. cholera*
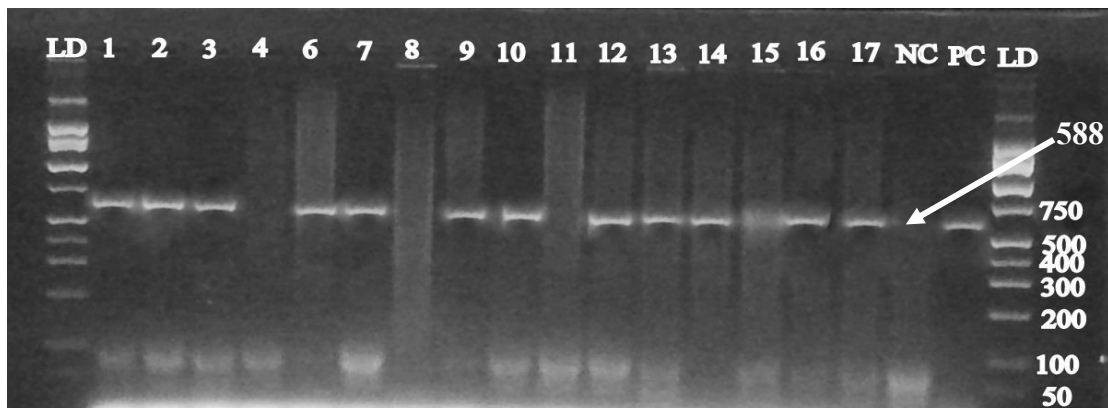
Figure 9: OmpW detection for confirmation of *Vibrio cholerae* by PCR

LD: DNA ladder; lines 1 to 17 are samples; NC: Negative Control; PC: Positive control

4.2 Occurrence of *Vibrio cholerae*

4.2.1   Detection of *Vibrio cholerae*in LQW

Sixty water samples were collected from three different sites and analysed as per protocol

during this study. Out of the 60 samples, 22 (36.7%) were positive for*V. cholerae* but none

of them was *V. cholerae* O1. Additionally, out of the three sites, water samples collected

from Mafisa sewage stabilisation ponds had the highest proportion of contamination

with*V. cholerae*(46.66%, n=15), followed by Morogoro River (35%, n=20) and Mzumbe

sewage stabilisation ponds (32%, n=25). Although the proportionat Mafisa was

grosslygreater than those of Mzumbe and Morogoro River, the difference was not

statistically significant (p>0.05).

4.2.2   Distribution of*V. cholerae*throughout thesewage stabilisation ponds

From the influents to the effluents of the sewage stabilisation ponds of Mafisa and

Mzumbe, the frequency of isolated *V. cholerae* varied. For Mafisa, they ranged between

30% (n=10) and 80% (n=5) from the influent to the effluent water samples respectively

and 20% (n=10) to 40% (15) for Mzumbe(Fig. 10).The increasein contamination level

between the inlet and the outlet samplesboth from Mafisa and Mzumbewas statistically

insignificant(p>0.05).

Likewise, even though the frequency of isolation of *V. cholerae* from water at the outlets

of Mafisa ponds (80%) was greater than the one of Mzumbe ponds (40%), the difference

was not significant (p>0.05).
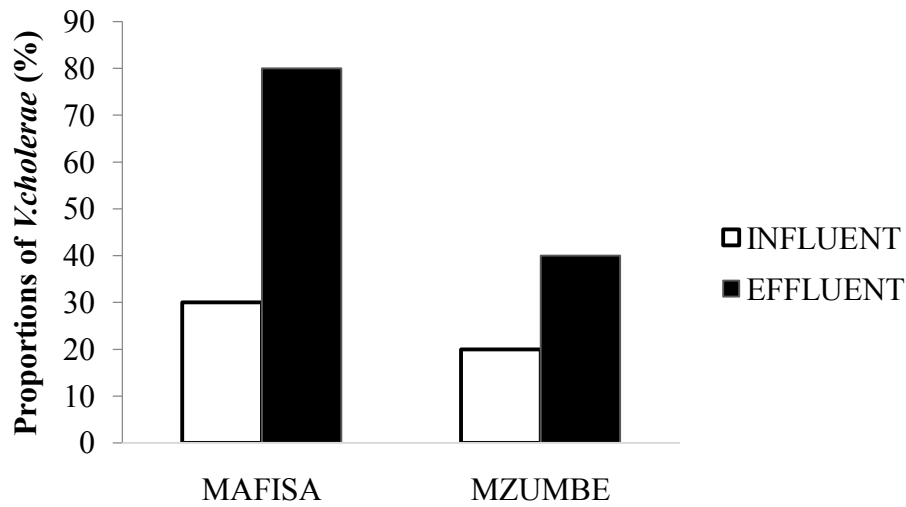
Figure 10: Comparison of proportion (%) of*V. cholerae* between inlets and outlets waters

from Sewage stabilization ponds

4.2.3 Detection of *V. cholerae* in Vegetables

As per study protocol, 60 vegetable samples specifically theChinese cabbageleaves were collected from two production sites: Mzumbe vegetables production site (irrigated with outlet LQW from Mzumbe sewage stabilisation ponds) and Funga-Funga vegetables production site (irrigated with LQW from Morogoro River). Out of the 60 samples, 13 (21.7%) were positive. However, none of theseisolates was*V. cholerae*O1. Funga-Funga production site appeared to harbour more contaminated vegetables (36.66%, n=30) than Mzumbe production site does (6.66%, n=30) (Fig. 11). The difference was significant (p<0.05).
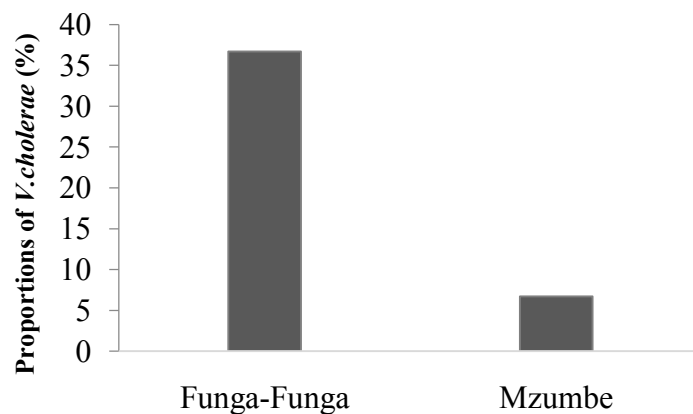
Figure 11: Comparison of proportions (%) of*V. cholerae* in vegetables from Funga-Funga and Mzumbe production sites

4.2.4 Detection of *Vibrio cholerae* in fish

Two different fish species were collected during this study. Tilapiaand African sharp tooth catfish.Sixty fish samples including 30 Tilapia and 30 Catfish were collected from Mzumbe sewage stabilisation ponds and subjected to bacteriological analyses. In the laboratory two subsamples were withdrawn from each of the 60 samples giving a total of 120 samples made of 60 intestine samples and 60 gills samples. With 28 positives, the overall prevalence was 23.33% (n=120).Moreover, out of the 28 isolates, 2 (7.14%) were confirmed *V. cholerae* O1. However, none of the catfish samples harboured the bacteria. Furthermore, this contamination rate differs at subsamples level. Out of the 60 intestine samples, 15 harboured *V. cholerae*(25%) and 13 tested*V. cholerae* positive from the 60 gills samples (21.66%)(Fig. 12). The difference was however insignificant (p>0.05).
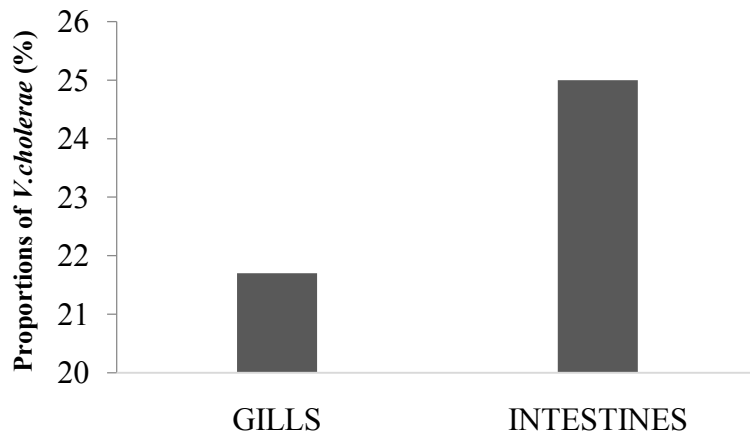
Figure 12: Comparison of proportions (%) of isolated*V. cholerae* in fish intestines and

gills

4.2.5 Occurrence of *V. cholerae* O1 in Fish samples

Two of the 28 positive samples obtained from all intestines and gills tested positive*V.*
cholerae O1 (7.14%). These 2 pathogenic *V. cholerae* O1 isolates were from gills samples

and represented 15.38% of the 13 positive gills' samples. However, the overall prevalence

of *V. cholerae* O1 in all 60 fish gills samples was3.33%.

4.3 Virulence Characteristics of Isolated *Vibrio cholerae*

4.3.1 Cholera enterotoxin gene (*ctx*)

The main virulence factor of *Vibrio cholerae* is the possession of the cholera toxin gene

(*ctx*) which encodes the production of cholera toxin, the main cause of the disease. From

our study, the cholera toxin gene (*ctx*) was identified in two isolates. These two were

previously confirmed as *V. cholerae* O1 isolates. They harboured the *ctx* gene identified

by PCR using specific primer at 167 bp region of the gene as displayed in Figure 13.

Furthermore, none of the non O1 isolates contained the *ctx* gene.
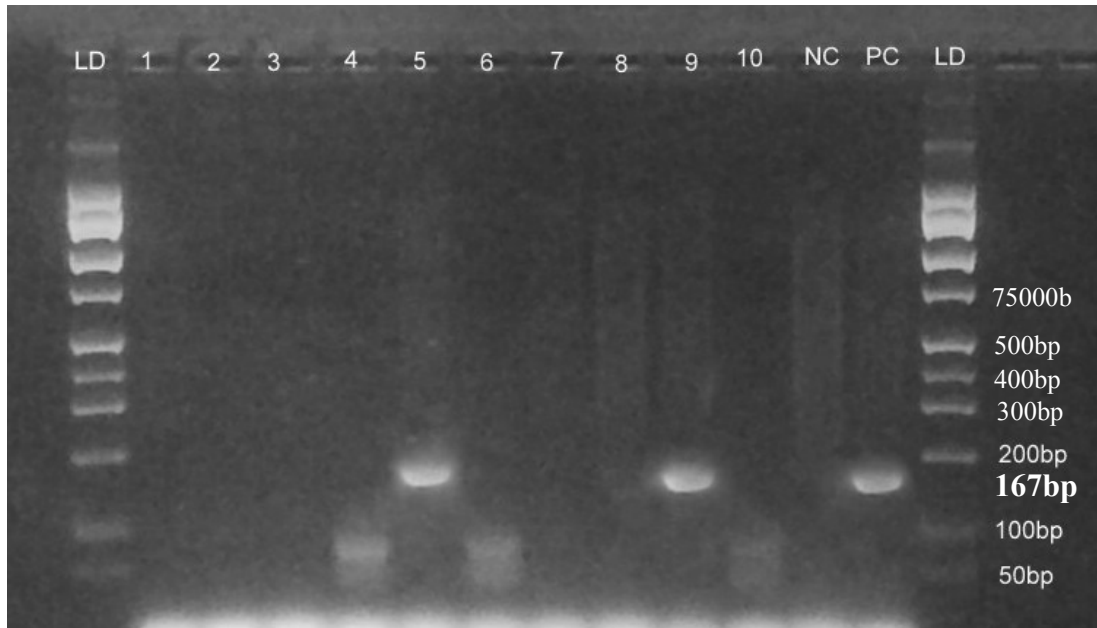
Figure 13: Cholera toxin gene (ctx) detected in fish isolates using PCR

LD: DNA ladder; lines 5 and 9 are positive samples; NC: Negative Control; PC: Positive control

4.3.2 Cholera toxin co-regulated pilus subunit A (*tcp-A*)

This gene intervenes in fimbriae synthesis, allowing the organism to be fixed in the host's intestinal epithelium. It plays thus a key role in the pathogenesis of *Vibrio cholerae* after the *ctx* gene. Out of the 63 tested isolates, the 453 bp region of the *tcp*-A gene was amplified in 7 samples (including those harbouring the *ctx* gene) as described inFigure 14. All the *tcp-A* positive isolates were from fish samples.
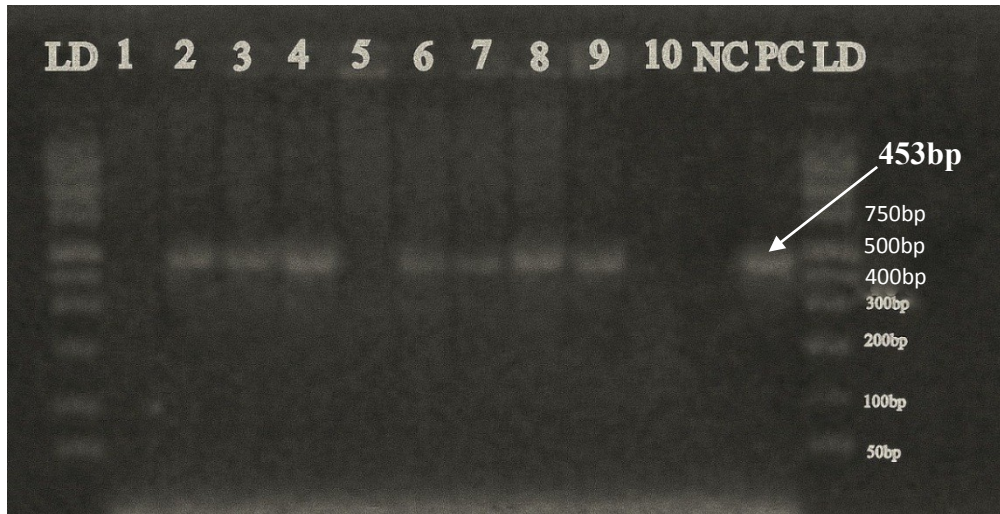
Figure 14: Cholera toxin co-regulated pilus gene (*tcpA*) in fish isolates using PCR

LD: DNA ladder; 1 to 16 are samples; NC: Negative Control; PC: Positive control

4.3.3 Haemolysin gene(*hly*A)

The *hlyA* gene confers to the *Vibrio cholerae* cells the ability to cause blood cell lysis in the infected host and thus is an important virulence factor of the bacterium. Of the 63 tested isolates, only one contained the *hlyA* gene. As displayed inFigure 15 below, the 727 bp region of the gene was amplified in one isolate which was one of the O1 isolates from fish gills.
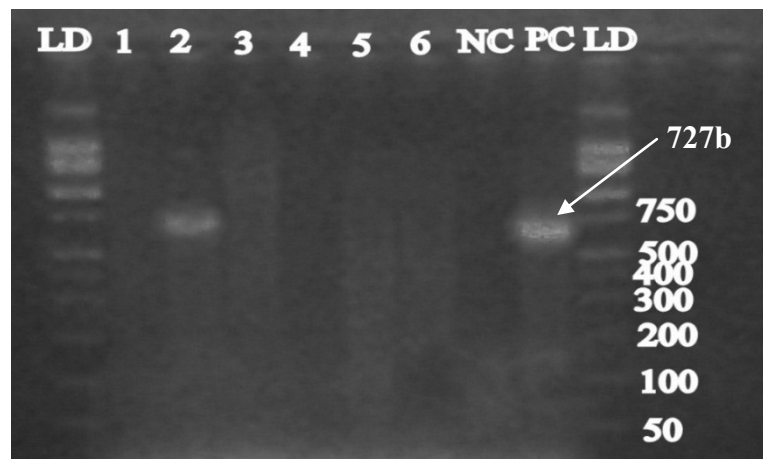
Figure 15: Cholera haemolysin gene (*hly*A) in fish isolate using PCR

LD: DNA ladder; 1 to 16 are samples; NC: Negative Control; PC: Positive control

4.3.4 Cholera toxin regulatory protein (ToxR)

All 63 *V. cholerae* isolates (from water, fish and vegetables) were tested for the presence of cholera toxin regulatory protein (ToxR) which regulates the production of the cholera toxin gene using PCR. Expected to produce a band around 900 bp region according to the primer used, none of the isolates including the positive controls was ToxR positive.

4.4 Antibiotics Susceptibility Pattern of Isolated *Vibrio cholerae*

All *V. cholerae*isolates were subjected to antibiotic testing as per study protocol for establishment of their antibiogram based on six commonly used antibiotics in severe cholera cases. As displayed in the Table 5, the tested antibiotics were Tetracycline (TE), Gentamicin (CN10), Ciprofloxacin (CIP5), Chloramphenicol (C30), Ampicillin (AMP10) and Amoxicillin (AML 10). The isolates demonstrated a very strong resistanceto Ampicillin (93.65%) and Amoxicillin (87.20%) (Fig.16). Furthermore, they displayed a moderate resistance against Tetracycline whereby about 26.98% of them were confirmed, even though 60.31% were susceptible to the same antibiotic. Nevertheless, the isolated *V. cholerae*, were susceptible to a number of antibiotics mainly Gentamicin (93.65%), Chloramphenicol (92.06%) and Ciprofloxacin (90.47%).

Moreover, the antibiotic susceptibility scheme of the toxigenic *Vibrio cholarae* O1 was not different from that of the non-toxigenic isolates ones. The two toxigenic O1 *V. cholerae* isolates were resistant to Ampicillin and Amoxicillin while sensitive to Gentamicin, Chloramphenicol, and Ciprofloxacin. Moderate resistance was displayed to Tetracycline.

Furthermore, the susceptibility scheme of the isolates did not vary with respect to the sample type based on origin. Therefore, Ampicillin and Amoxicillin remained the two

main antibiotics towards which all isolates from water, vegetables, fish intestines and fish gills developed strong resistance. Same observation was recorded with Gentamicin, Chloramphenicol, Ciprofloxacin and Tetracycline towards which the isolates were sensitive.

Figure 16: Antibiotics discs on MH-agar plates after 24h incubation

Table 5: Antibiotic susceptibility pattern of *V. cholerae* isolates(n=63).

Antibiotics	Resistant (%)	Intermediary (%)	Susceptible (%)
Ampicillin	93.65	1.58	4.76
Chloramphenicol	1.58	6.34	92.06
Ciprofloxacin	6.35	3.17	90.47
Gentamicin	3.17	3.17	93.65
Amoxicillin	87.30	4.76	7.93
Tetracycline	26.98	12.69	60.31

CHAPTER FIVE

5.0 DISCUSSION

Vibrio cholerae are natural inhabitants of aquatic environments,hence water plays a central role in thetransmission of cholera. In order to circumvent potentialcholera threats, it is paramount to determine the prevalence of *V. cholerae* in aquatic environments**.** From the findings of this study, the high level of non-O1 *V. cholerae* in the sewage system may be ecologically normal but remains threatening since people use such water for other human related activities. As a matter of fact, vegetables irrigated with the water revealed high contamination by *V. cholerae* whereby about 21.66% (n=60) of the tested Chinese cabbage harboured the organism. Upadhyay *et al*. (2013) also isolated these bacteria from water bodies in India. Sathiyamurthy *et al.* (2013) demonstrated theclinical and epidemiological relevance of non O1 *V. cholerae* isolated from water have.They cause non choleric diarrhoea and gastroenteritis in humans. More interestingly, Rahman *et al.*(2008) concluded that the pathogenic strains of *Vibrio cholerae* that cause cholera in humans are derived from environmentalnon-pathogenic strains. Thisinformation is thus important for raising awareness on theextent to which irrigation with wastewater sources in Morogoro are contaminatedwith *V. cholerae.* Therefore,appropriate measures should be taken to curb thepublic health threats of diarrhoea and prevent eventual occurrence of real cholera outbreaks.

Additionally, human exposure to *V. cholerae* through the use of contaminated wastewater remains serious in Morogoro due to inability of the sewage ponds to efficiently remove these pathogens. The prevalence of *V. cholerae* in the effluents water of both studied sewage ponds were higher than those of the inlets. This situation suggests that there is either further contamination of the sewage water between the inlets and the outlets by

humans and animals including birds or further growth and proliferation of the bacteria throughout the system. According to Alonso *et al*. (2006), water of waste stabilization ponds' outlet is supposed to be cleaner and safer than the one of the inlet with respect to bacterial content. This discrepancy could be explained by the fact that *V. cholerae* are autochthonous of aquatic environments (Faruque *et al.,* 1998) and could therefore be recovered at any point of the sewage plants independently on those isolated at the inlet. Also, as the chemical content of water at the inlet is too high and contain other competitive organisms, *V. cholerae* could not survive better than from the facultative to the maturation ponds where water is cleaner. Besides, studies have illustrated the ability of *V. cholerae* to associate with a variety of zooplankton, phytoplankton, blue-green algae (cyanobacteria) that prolong its survival (Faruque *et al.,*2002; Faruque and Mekalanos, 2012). Such organisms might naturally be in high concentration in the maturation ponds and favour the survival of *V. cholerae*.

The high prevalence of *V. cholerae* in wastewater could be the reason of its high prevalence observed in vegetables irrigated using these waters. Dougnon *et al.* (2012) demonstrated a strong correlation between the microbial load of irrigation waters and the irrigated vegetables. According to these authors, the higher the bacterial load of the used irrigation water, the higher the bacterial contamination in the irrigated vegetables will be. Such situation is a serious public health threat when considering the size of vegetable markets in Tanzania. Our findings are in accordance with that of Mrityunjoy *et al.* (2013) who established a high bacterial load of *V. cholerae* in vegetables samples in Bangladesh and concluded how it was a serious public health problem. Besides, vegetables produced with such water are consumed not only locally in the surrounding villages but are also exported to other city centres of the country like Dar es Salaam and to other neighbouring countries in the region (Foesken *et al.,* 2004). Therefore, a large number of people are

exposed to *V. cholerae.* Moreover, there aresome non-pathogenic viable but not culturable forms of *V. choleare* in foodstuffs that can survive heat and other stresses and get into human gut with appropriate conditions for their growth (Ottaviani *et al*., 2009). Such scenario could induce the occurrence of pathogenic forms leading to the disease despite thoroughcooking. Additionally, with respect to the nutritional advice which suggests that vegetables should not be too much cooked, the risk remains rampant.

Therefore, the situation should be considered seriously by policy makers. Vegetables from the largest production site of Morogoro (Funga-funga), which are transported to Dar es Salaam and other places in the country are highly contaminated with *V. cholerae* (36.66%, n=30). Although such isolates of *V. cholerae* are non O1, thus non-pathogenic, several studies demonstrated their role in severe non choleric diarrhoea (Sathiyamurthy *et al.,* 2013) and possible cholera conditions in cases where they get into the human gut and gets stimulated (Faruque *et al*., 2002; Rahman *et al*., 2008). For instance, non-O1 and non-O139 strains of *V. cholerae* have been associated with occasional outbreaks of cholera (Singh *et al.,* 2002).

Pathogenic *V. cholerae* from fish samples confirmed O1 by sero-agglutination and ctx positive by PCR was isolated from two out of 60 samples. These choleragenic isolates were all obtained from Tilapia samples while all the Catfish samples tested negative. The absence of *V. cholerae* in catfish collected from the sewage system could be because Tilapia and catfish do not co-exist in the same ponds. In fact, catfish samples were found in the anaerobic ponds and some in facultative ponds while Tilapia were essentially obtained from maturation ponds. Besides, these results showed that water samples from the maturation ponds were more contaminated by *V. cholerae* than the first ponds. This is due to the fact that the anaerobic ponds contain a number of chemicals which thwart the

growth of microorganisms (Mdegela *et al.,* 2010). Therefore, it is normal that catfish, mostly found in anaerobic ponds may be free of *V. cholerae* whereas Tilapia remain contaminated by the bacteria due to their coexistence in the same ponds. Additionally, catfish are described as having the ability to harbour their surrounding chemicals, and are therefore commonly used to assess water pollution based on their biomarker responses (Mdegela *et al.,* 2010). Such chemical content might thus not allow the growth of microorganisms like *V. cholerae*. The isolation of *ctx* positive *V. cholerae* O1 in a non-cholera outbreak from fish samples shows a serious cholera threat and justifies the unexpected occurrence of cholera outbreaks in Tanzania. The presence of the toxin co-regulated pilus (*tcp*-A) in the isolates is a serious threat to public health because as described by Sanchez and Jan (2011), this gene encodes for the fimbriae synthesis which allows the bacteria to be fixed to the host's intestinal epithelium. Once fixed, the organism can multiply in the gut and regulate cholera toxin production because of the local intestinal conditions leading to serious cholera occurrence (Wenpeng *et al.,* 2014). Such situation was demonstrated by Rahman *et al*. (2008) and Faruque and Mekalanos (2012).

Moreover, the detection of the haemolysin gene is another serious threat. This gene as well as the *tcpA* were obtained from seafood samples by Ottaviani *et al*. (2009) who concluded that their presence in environmental samples testifies that not only clinical samples are toxigenic. The haemolysin gene allows the organism to cause blood cell lysis in the infected individual and leading to anaemia in the infected individuals (Fooladi*et al.,* 2013). The presence of toxigenic species of *V. cholerae* from aquatic environment in non-outbreak period could explain the frequent occurrence of cholera outbreaks (Akoachere *et al.*, 2013). Therefore, the diversity of reservoirs of toxigenic cholera causing agent has a direct negative impact on cholera prevention, resulting in frequent cholera outbreaks. This

conclusion could explain the fact that cholera outbreaks have become very frequent in Tanzania where they occur every year in many regions of the country.

Although fish gills and intestines are not always consumed, they can cross-contaminate the filet during evisceration and processing and thus reach consumers. They currently constitute infectious materials to fish handlers and processors who have to respect good hygienic and processing practices during fish processing to avoid cross-contamination of the filet. Unlike intestines, fish gills are rarely removed during preparation and are consequently consumed sometimes. Therefore, apart from the risk of cross contaminating the cooked meal with toxigenic *V. cholerae* encountered during processing, the consumption of an undercooked fish containing the gills is a serious cholera occurrence risk factor in human. Similar observation was previously made by other scientists such as Senderovich *et al.* (2010), Sathiyamurthy *et al.* (2013) and Mrityunjoy *et al.* (2013). Senderovich *et al.* (2010), for instance confirmed ten different fish species including Tilapia (*Sarotherodon galilaeus*) from various aquatic environments comprising ponds and rivers as containing *V. cholerae* in their intestine. Though, these studies established the presence of *V. cholerae* in fish, the current one stands as the first to prove the presence of *Vibrio cholerae* O1 containing the cholera toxin, the toxin regulated pilus and the haemolysin gene in fish from freshwater in non-outbreak period in Tanzania.

Moreover, fish playing a crucial role in cholera transmission was also suspected by Kigoma region Authorities in Tanzania during the most recent outbreak whereby fish markets were banned from access in the region as control measure (Pesatimes, 2015). As *V. cholerae* is part of water microbiota, fish would have thereby been contaminated. However, toxigenic *V. cholerae* O1 were not found in water samples. This supports the suggestion of Sanchez and Jan. (2011) who stated that the non-pathogenic organisms

could be the progenitors of pathogenic ones. Furthermore, Onyuka *et al.* (2011) isolated *V. cholerae* O1 from fish samples in Kenya during the non-outbreak period and concluded that in cholera endemic areas these microorganisms exist in biofilm-like aggregates in which the cells are in conditional viable state. Such conditions are further explained by Akoachere *et al.* (2013) who reported that the presence of toxigenic *V. cholerae* O1 in water during the non-outbreak period in Cameroun is positively correlated with the physico-chemical characteristics such as temperature (around 32.7°C), pH (alkaline) and salinity (1.62 – 11.03 ppt) of the water. In this case, one could conclude that the physico-chemical conditions mainly pH and salinity of fish could be the factors maintaining the persistence of toxigenic organisms in fish. Moreover, Evans *et al.* (2005) reported that in osmoregulation and iron balance in fish, high concentrated chloride solution is secreted by fish gills whereby the mechanisms of NaCl secretion by the gill epithelium are clearer than the mechanisms of NaCl uptake from the environment. This suggests that the persistence of pathogenic *V. cholerae O1* isolates in gills could be attributed to the salt content of this tissue. The high salt content of gills (Evans *et al.,* 2005) could be the reason of the persistence of the bacteria in gills unlike water and vegetables. Additionally, high intestinal bicarbonate secretion (HCO^{3-}) by fish intestinal epithelium was demonstrated to lower the intestinal pH (Wilson *et al.,* 2003; Grosell, 2006). This offers an appropriate alkaline pH necessary for the growth and the persistence of toxigenic *V. cholaerae* leading to their isolation in fish intestines.

On the other hand, Faruque *et al.* (1998), pointed out that pathogenic strains of *V. cholerae* are commonly isolated from environmental samples only during outbreak period. Nevertheless, there were no cholera outbreaks in Morogoro during this study, nor has been one during the study period of Akoachere *et al.* (2013) in Cameroun although toxigenic *V. cholerae* O1 were isolated in these studies. The discrepancy could be explain by the fact

that pathogenic isolates of *V. cholerae* in fish could originate from non-pathogenic progenitors as described by Rahman *et al*. (2008); Sanchez and Jan (2011) and maintained in fish by their physico-chemical characteristics (Akoachere *et al.,* 2013). Moreover, Faruque *et al.* (2002) reported that it is possible that in the natural ecological settings, unidentified environmental factors induce lysogenic phage CTXΦ in toxigenic *V. cholerae*, resulting in the release of extracellular CTXΦ particles into the aquatic environment. Therefore, the cell-free phage particles participate in the emergence of novel toxigenic strains of *V. cholerae* through interactions with non-toxigenic strains.

Results of this study has revealed that although the *V. cholerae* O1 isolates contain the *ctx* and *tcp* genes, they were ToxR negative and so did the positive control. This situation is abnormal because according to Sanchez and Jan (2011), Rahman *et al.* (2008) and many other authors, ToxR is the central regulator of *ctx* and *tcp* genes. The absence of ToxR in these isolates could be probably due to the quality of the purchased primers or other PCR reagents conservation failure. However, further clarification is needed on this case which can be ascertained by sequencing the genome of the isolates in order to locate the source of the problem.

Although rehydration plays a pivotal role in reducing mortality during cholera epidemics, antibiotics have been used to reduce the shedding of the organism (thereby reducing spread of the disease), treating severe illness (by reducing volume of diarrhoea), and also to reduce duration of disease and hospitalisation (Mandal *et al.,* 2012). However, the resistance of microorganisms including *V. cholerae* to antibiotics has become a serious health challenge worldwide. Almost all of the isolates including the toxigenic ones were multidrug resistant and strongly resistant toAmpicillin and Amoxicillin with moderate resistant to Tetracycline. Similar situation was described by Akoachere *et al.* (2013) who

found that *V. cholerae* isolates developed resistance in the range of 92 to 64% towards these three antibiotics. Results of this study are constant with many other studies like the one of Chikwendu*et al.* (2014). In case severe cholera case is reported, health care workers need to be aware of such relevant information in order to take a wise decision regarding the best drug to be prescribed to patients. Fortunately, isolated *V. cholerae* are demonstrated to be highly sensitive to a number of antibiotics including Gentamicin, Chloramphenicol and Ciprofloxacin (Akoachere *et al.*, 2013; Mrityunjoy *et al.*, 2013).

However, some dissimilarities are reported between these studies regarding the situation of chloramphenicol and tetracycline resistances. However, Faruque *et al.* (1998) reported that the resistance pattern of *V. cholerae* depends on the geographical location and the time of isolation. Moreover, the cause of the resistance to drugs by the isolates could be explained by various reasons including the presence of antibiotics released in the sewage system by the connected hospitals and humans by several pathways (Chikwendu*et al.*, 2014). Therefore antibiotic residues that persist in the sewage due to their misuse and release in the sewage could be the basis of occurrence of antibiotics resistant organisms; a serious public health threat.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

Vibrio choleae aetiological agent of cholera, a deadly diarrheal disease was isolated from low quality water and its related human activities in Morogoro Tanzania from October 2014 to February 2015 during a non-outbreak period. These isolates were obtained from water, fish and vegetable samples. Two of them were confirmed *V. cholerae* O1.Three main virulence genes were detected in these isolates notably cholera toxin gene, the leading cause of the disease and others virulent genes mainly the toxin co-regulated pilus and the haemolysin gene which play important roles in the pathogenesis of the bacteria. The isolates demonstrated a strong resistance to Ampicillin and Amoxicillin followed by a moderate resistance against Tetracycline.

It comes out of this study that multi-toxigenic *V. cholerae* species are present and persist in environmental samples and can be isolated even during the non-outbreak periods. The environment could therefore serve as reservoir for virulence strains of *V. cholerae*.This is of serious public health importance because it could lead to cholera control programmes which do not usually take environmental parameters in account.

6.2 Recommendations

With respect to the findings of this study, the following recommendations are formulated.

  i.   Periodical surveillance on bacteriological quality of LQW to inform policy makers on appropriate wastewater reuse challenges.

 ii.   Adequate wastewater treatment before utilisation for irrigation.

iii.   Thorough cooking of vegetables and fish.

iv.   Fish gills removal during fish processing.

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

vi.   Use of active antibiotics (Gentamicin, Chloramphenicol and Ciprofloxacin) in hospital centres to reduce the shedding of the organism (thereby reducing spread of the disease), to treat severe illnesses (by reducing volume of diarrhoea), and also to reduce duration of disease and hospitalisation.

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

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APPENDICES

Appendix1: Isolation and confirmation of *Vibrio Cholerae*(USFDA BAM 2001)

Sample preparation	Homogenise 25g or ml of sample in 225 ml of APW
Enrichment	Incubate at 37°C for 18hrs
Isolation	Streak enrichments to TCBS agar at 37°C; incubate overnight
Purification	Typical Yellow colonies (≥ 2 mm) are subcultured on TSA + 2% NaCl at 37°C and incubated overnight
Identification	**Gram staining** (G-, comma shaped with falagelum)
Biochemical identification	Single purified colony is tested for **oxidase** reaction: positive result= colour change to blue or dark purple within 10 seconds
	Positive samples are transferred to **TSI** tubes for species confirmation: uniform yellow colour with no gas formation after overnight incubation at 37°C are confirmed *Vibrio cholerae*
DNA extraction	DNA Extraction from pure cultures of positive samples from TSI is done by heating method described by Parck *et al.,* 2013
Confirmation	PCR for confirmation of *Vibrio cholerae* targeting a species specific gene, the *ompW* followed by sero-agglutination test using anti-serum *Vibrio cholerae* O1
Virulence detection	PCR was performed for identification of virulence genes of *Vibrio Cholerae*: the *Ctx, ToxR, TcpA and HlyA* using specific primers.

Appendix2: ATB testing per Kirby-Bauer method

Inoculation

A loopful of purified colonies is added to sterile normal saline in order to match the 0.5 Mac-Farland turbidity

Transfer

Streak a swab of the above to MH agar and leave for 5 min

Culture and ATB testing

Add ATB testing discs and incubate at 37°C overnight

After the incubation, the inhibition zone diameters is measured using a transparent plastic ruler and interpreted according to zone size by using the Kirby Bauer chart

Appendix 3: Morogoro Municipal's Research Permit

HALMASHAURI YA MANISPAA MOROGORO

Simu/Nukushi Na: 023 – 2614727

Barua Pepe: info@morogoromc.go.tz
Tovuti: www.morogoro.go.tz.
Unapojibu taja:

Ofisi ya Mkurugenzi wa Manispaa,
S.L.P. 166,
MOROGORO
TANZANIA

Kumb. Na. R.10/MMC-24/.....   **Tarehe:** ..12../...09. , 2014

Mkuu wa Chuo,
...........SUA..............,
S.L.P.....3000........,
........MOROGORO......,

Yah: **MAOMBI YA KUFANYA UTAFITI**

Tafadhali husika na kichwa cha habari hapo juu.
Napenda kukujulisha kuwa kibali kimetolewa kwa Mwanachuo
..Ya.M...Gildas..Hounmanou ambaye ni mwanafunzi katika Chuo
chako kufanya utafiti katika Halmashauri ya Manispaa ya Morogoro
kuanzia tarehe..18./09.../2014 kwa gharama zake mwenyewe. hadi July 2015

Tunamtakia mafunzo mema.

Mayala P.M K.NY. MKURUGENZI WA MANISPAA
MOROGORO
Kny:MKURUGENZI WA MANISPAA
MOROGORO

Nakala: MOROGORO MANICIPAL...
          P.O. 166,
          MOROGORO.

     "   Y.M.GILDAS. HOUNMANOU
          S.L.P. 3000......,
          MOROGORO

Appendix 4: Mzumbe University's Research Permit

MZUMBE UNIVERSITY
(CHUO KIKUU MZUMBE)

E-mail::mu@Mzumbe.ac.tz	P.O.BOX 101
Tel: +255 023 2604380/1/3/4	**MZUMBE**
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Cell: +255 0754 694029	
Website: www.mzumbe.ac.tz	

Ref. No. MU/OF/R.2/1/43   Date: 17th September, 2014

TO WHOM IT MAY CONCERN

RE: PERMISSION CLEARANCE TO STUDENT AND RESEACHER

Mr. Gildas Hounmanou is a Masters student at Sokoine University of Agriculture – SUA.

He wants to facilitate a research function to our Oxidation ponds on the following activities:-
1. Taking water samples
2. Fishing
3. Taking vegetable samples around the oxidation ponds

This permission is granted from September, 2014 up to July, 2015

Thank you in advance for your cooperation.

Regards,
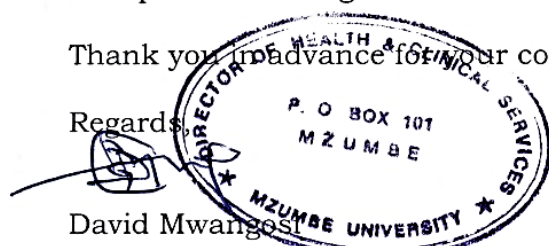
David Mwangosi
For: DIRECTOR HEALTH AND CLINICAL SERVICES
MZUMBE UNIVERSITY HEALTH CENTRE

Cc: Deputy Vice Chancellor – Administration and Finance
         Security Officer – Mzumbe University

Appendix 5: MORUWASA's Research Permit

MAMLAKA YA MAJISAFI NA USAFI WA MAZINGIRA MOROGORO

MOROGORO MANISPAA
Telegrams: "MTO"
Simu: +255 23 2614 182
          +255 23 2614 214
Faksi: +255 23 2614 145
Barua pepe: uwsamg@raha.com
Tovuti: www.moruwasa.co.tz

Mkurugenzi Mtendaji,
Mamlaka ya Majisafi na Usafi wa Mazingira,
S.L.P 5476,
MOROGORO

Unapojibu tafadhali taja:

Ref.No.UWSA /T/1/1/VOL XII/1   19TH September, 2014

Principal,
Vice Chancellor,
Sokoine University Of Agriculture,
P.O.BOX, 3000
MOROGORO

Re: UNIVERSITY STAFF, STUDENTS AND RESEARCHERS CLEARANCE

We Morogoro Urban Water Supply and Sanitation Authority (MORUWASA), accepted your students MR. Y.M GILDAS HOUNMANOU (MSc Public Health and Food safety) to undertook research in PREVALENCE AND ANTIBIOTICS SUSCEPTIBILITY OF PATHOGENIC VIBRIO SPECIES IN LOW QUALITY WATER, FISH AND VEGETABLES IN MOROGORO, TANZANIA starting from September 2014 to July 2015.

When he arrives at MORUWASA he will contact Eng.S.Ishengoma who is our Sewerage Engineer for more information.

We wish him all the best.

Rahma S.Sabuni,
For.MANAGING DIRECTOR

Copy. Eng. S.Ishengoma – For information and assistance to the students.