INVESTIGATING THE VIRULENT GENES AND ANTIBIOTIC SUSCEPTIBILITY PATTERNS OF *V. CHOLERAE* O1 IN ENVIRONMENTAL AND CLINICAL ISOLATES IN ACCRA

BY

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THIS THESIS IS SUBMITTED TO THE UNIVERSITY OF GHANA, LEGON IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF A MASTER OF PHILOSOPHY DEGREE IN MOLECULAR CELL BIOLOGY OF INFECTIOUS DISEASES

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DECLARATION

I, DAVID ABANA, do hereby declare that with the exception of references to other people's work, which have been duly acknowledged, this thesis is the outcome of my own research conducted at the Department of Cell, Molecular Biology and Biochemistry, University of Ghana, College of Basic and Applied Science under the supervision of Dr. Lydia Mosi and Dr. David Opare. Neither all nor parts of this project have been presented for another degree elsewhere.

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ABSTRACT

Background: Cholera has been endemic in Ghana, since it was first detected in 1970. Outbreaks have often started in slums of the city especially Accra. In spite of the overwhelming nature of cholera outbreak that has resulted in high mortality and morbidity, there is little information on the reservoir of the causative agent. It has however, been shown that long-term survival of the bacteria may be attained in watery environments or in humans with no signs of cholera. Consequently, cholera outbreaks may be triggered predominantly in large densely populated urban areas. The perennial environmental reservoir of toxigenic *V. cholerae* O1 has not yet been identified in West Africa due to inadequate research. With environmental cholera sources being suspected in Ghana, enhanced monitoring of aquatic reservoirs and drinking water is therefore important particularly within urban agglomerations. This project was conducted to investigated clinical and environmental isolates of *V. cholera*e O1 in Accra, determine their virulent genes, their antibiotic susceptibility pattern and the environmental factors maintaining their persistence in the environment.

Method: Water samples from various sources (taps, dug wells, streams and storage tanks) were analyzed for contamination with *V. cholerae* O1 by culturing on TCBS and TSA plates using standard methods. Forty clinical isolates from a previous cholerae outbreak were included in the study. Antibiotic susceptibility patterns of the bacteria were determined by the disc diffusion method. Virulent genes were identified by analyzing for genes for ctx, tcpA ($tcpA_{El}$ $tcpA_{Cl}$), zot, ompW, rbfO1 and attRS by PCR. Physicochemical characteristics of water (pH, temperature and salinity, total dissolved solids and conductivity) were investigated using standard methods. One-

way ANOVA and student t- test were used to analyze the relationship between physicochemical factors and the occurrence of *V. cholerae* O1. Differences were considered significant at $P \leq 0.05$.

Results: Eleven *V. cholerae* O1 strains isolated from streams, storage tanks and wells during the study period were added to 40 clinical isolates for analysis. All isolates were resistant to one or more of the eight antibiotics used. Multidrug resistant was observed in over 97% of the isolates. All isolates had genes for at least one virulence factor. V*ibrio cholera* toxin gene was detected in 42 (82.4%) isolates. Approximately 81.8% of the isolates were positive for $tcpA_{ET}$ gene, but also harbored the $tcpA_{cl}$ gene. Isolates were grouped into thirteen genotypes based on the genes analyzed. pH salinity, TDS and conductivity significantly correlated positively with isolation of *V. cholerae* O1.

Conclusion: *V. cholerae* serotype Ogawa biotype El tor is the main biotype circulating in Ghana with the emergence of a hybrid strain. Multidrug resistant *Vibrio cholerae* O1 with different genotypes and pathogenicity as well as the non 01/0139 are present in water sources in the study area. Temperature, salinity, TDS and conductivity are among the factors maintaining the persistence of the *V. cholerae*. These findings indicate an urgent need for the appropriate use of antibiotics, provision of potable water supply and regular disinfection of water from contaminated sources to prevent outbreak of cholera.

DEDICATION

This research work is first and foremost dedicated to the Almighty God for His mercies and kindness that has been showered upon me throughout my academic life. My profound appreciation goes to my parents for their unflinching love, care, support and encouragement over the years.

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LIST OF ABBREVIATIONS

µL	Micrometer
TCBS	Thiosulfate citrate bile salt sucrose
TSA	Ttyptose soya agar
CDC	Center for Disease Control
CT	Cholera Toxin
TCP	Toxin co-regulated pilus
VPI	*Vibrio. cholerae* Pathogenicity Island
ZOT	Zonular occludens toxin
WHO	World Health Organization
g	Grams
WHA	World Health Assembly
CFR	Case fatality rate
L	Liter
GHS	Ghana Health Service
NOVC	Non O1/O139 *Vibrio cholerae*
FAO	Food and Agriculture Organization
mL	milliliter
CFTR	cystic fibriosis transmembrane conductance regulator
RDT	Rapid detection test
^{o}C	Degree Celcius

APW	Alkaline peptone water
PCR	Polymerase Chain Reaction
PFGE	Pulse-field gel electrophoresis
ORS	Oral rehydration solution
IV	Intravenous
rpm	Revolutions per minute
ICE	Integrating conjugative elements
DNA	Dioxyribose nucleic acid
NPHRL	National Public health reference laboratory
OMP	Outer membrane protein
TDS	Total dissolved solids
UV	Ultra violet
NCCLS	National Committee for Clinical Laboratory Standards
CFU	colony forming unit
ATCC	American Type Culture Collection
PBS	Phosphate buffered saline
ZR	Zymo research
VBNC	Viable but non-culturable
MIC	Minimum inhibition concentration
MDR	Multi drug resistance

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

Cholera is acute secretory diarrhoea caused by the Gram-negative bacterium *Vibrio cholerae* (Nelson *et al.,* 2009). It is one of the earliest and well–known human pathogens. However, there is still room for more research into the transmission and progression of the disease. The transmission between humans and the environment still remains unknown particularly in the area of the evolutionary response of the causative agent to environmental and host-driven selective pressure. (Chun *et al*., 2009). The gram-negative *Vibrio cholerae* O1 and O139 are responsible for epidemic cholera; a harsh and acute diarrhoea disease (Nelson, *et al* 2009). The disease has caused harm to civilizations throughout history, and to date, there have been as many as seven pandemic records (Mizikar, 2012). The current pandemic still affects millions of people and causes more than 100,000 deaths annually. The bacterium has become common in places that have not reported the disease for years (Mizikar, 2012). In Haiti, more than 700,000 people contracted the disease, resulting in more than 8,500 deaths since the beginning of the cholerae outbreak in Haiti after the 2010 earthquake (CDC, 2015).

The aquatic surroundings such as rivers, estuaries and oceans are the accustomed territory for *V. cholerae,* where it can be found as free-living cells or attached to biotic or abiotic surfaces (Lutz *et al.,* 2013). There are more than 200 documented serogroups, but only O1 and O139 serogroups have been linked to epidemics (Goel *et al.,* 2008). Other serogroups commonly identified as non-O1/O139 (Safa *et al.,* 2010) coexist in the environments with the O1/O139 strains (Chatterjee *et al.,* 2009).

For *V. cholerae* O1 or O139 to be able to cause epidemics disease, it must have the ability to express virulent factors such as, cholera toxin (CT) and toxin co-regulated pilus (TCP). These factors are located together within two regions in the *V. cholerae* chromosome. The ctx genetic element which has been reported to cover the genome of a filamentous phage (ctxΦ) (Fujinaga, 2003) and the *V. cholerae pathogenicity* island (VPI) that codes for the TCP, a type IV pilus that functions in colonization and acts as a receptor for ctxΦ. ctxΦ is a transportable phage and encodes the cholera toxin (Feng *et al.,* 2004). Thus, expression of ctx gene should be preceded by ctxΦ. The environmental strains have been reported not to produce the cholera toxin gene, hence, lack the ability to cause epidemic cholera until they are infected by phage (Rivera *et al.,* 20O1). Other agents of enteropathogenicity are haemolysin (hlyA), heat stable enterotoxin (stn/sto), hem-agglutinins, neuraminidase, outer membrane protein, shiga-like toxin (stx), a ToxR regulatory protein and the zonulaoccludens toxin (zot) (Carvalho *et al.,* 2006). Genes that code for cholera toxin (ctx) and toxin co-regulated pilus (tcp) have been associated with toxigenic *V. cholerae* which they acquire from their marine environment (Dixit *et al.,* 2008). The aquatic environment therefore, acts as a major part in the ecology, transmission, and epidemiology of *V. cholerae*. The discovery of *V. cholerae in* the aquatic environment cannot therefore, be underestimated in the management and prevention of the disease.

1.2 Problem Statement

It is reported that about 3.5 million people are affected every year with between 100,000 to 130,000 deaths per year, globally (WHO, 2013). There has been a radical upsurge in the number of reported cases and nearly all developing countries are facing the danger of either cholera outbreak or the threat of an epidemic in recent years (WHO, 2014). The World Health Assembly in May, 2011,

re-affirmed the emergence of cholera as a key global public health treat and adopted resolution WHA 64.15, encouraging member states to embrace the execution of an integrated and comprehensive move towards the control of cholera. There was an increase in the number of cholera related deaths from 4948 in 2009 to 7543 in 2010, which represents a 52% increase with a case fatality rate (CFR) of 2.3%. These numbers were recorded in 32 countries of which 20 countries were in Africa. The African countries accounted for 3397 deaths and 45% of the global total. Haiti reported 3990 deaths, accounting for 53% of the global total. All these cases were reported in a spade of 70 days (WHO 2011).

Ghana witnessed its first cholera outbreaks in 1970. Between 1970 and 2012, Ghana has recorded a total of 5,498 cholera deaths, according to data compiled by the World Health Organization. (2014). A total of 1,546 deaths were recorded between 1970 and 1980 while 2,258 deaths were recorded between 1981 and 1990. From 1991 to 1999, cholera claimed 1,067 lives, and between 2000 and 2012, 627 deaths were recorded (CDC, 2015). In 2011, 10,628 cholera cases with 105 deaths were reported, while a total of 9,542 cholera cases with 100 deaths were reported in Ghana in 2012. However, in 2013, Ghana was spared the agony of cholera deaths, despite some reported cases of the disease in the country. Ghana recorded its highest cholera outbreak in 2014, with a total reported case of 26,286 with 211 deaths (Case Fatality Rate of 0.8%) (GHS, 2014).

According to the Ghana Health Services, from June, 2014 to November, 2014, 26,286 with 211 deaths reported cases were recorded in Ghana. This represents a CFR of 0.8%. These cases were reported from 123 out of the 216 districts in the country. Greater Accra Region accounted for 72% of all the cases reported (GHS, 2014)

1.3 Justification

In spite of the overwhelming nature of cholera outbreak that has resulted in high mortality and morbidity, there is little information on the reservoir of the causative agent. It has however, been shown that long-term survival of a bacteria may be attained in watery environments or in humans with no signs of cholera (Nelson, *et al* 2009). Consequently, cholera outbreaks may be triggered predominantly in large densely populated urban areas (Akoachere, *et al.*, 2014). The perennial environmental reservoir of toxigenic *V. cholerae* O1 have not yet been identified in West Africa due to inadequate research (Rebaudet, *et al.*, 2013).

Water is assumed to be the main reservoir for the bacteria.in Ghana. therefore,enhanced monitoring of aquatic reservoirs and drinking water is important, particularly, within urban agglomerations. According to Mourino-perez *et al.,* (2003), sea water and plankton samples from Peru were positive for *V. cholerae* O1 and found to contain ctx toxin (Mourino- Perez, 2003). Also, Chomvarin *et al.*(2007), reported that toxigenic *V. cholerae* is found in marine environment and is transmitted through drinking water and still remains one of the most important cause of morbidity and mortality (Chomvarin *et al.*, 2007). However, no perennial environmental reservoir of toxigenic *V. cholerae* has been identified in Ghana, which could be as a result of lack of appropriate studies *(*Rebaudet, *et al.*, 2013*).* Studies have shown a seasonal pattern of occurrence of the causative agent of cholera (WHO, 2004). This phenomenon have been attributed to the fluctuation in seasons and changes to environmental factors such as temperature, pH, salinity and other factors such as nutrient concentration that have a direct bearing on the occurrence of *V. cholerae (*Ahmed, *et al.*, 2011). Since there is a link between the epidemiology of the disease and the ecology of *V. cholerae* in the environment, an increase in understanding of the factors that supports its survival and multiplication is essential for public health protection.

Antibiotics have been use to complement rehydration in moderate to severe forms of cholera in order to cut down on consumption of inadequate hospital consumables such as oral and intravenous fluids to sustain hydration (Kuma *et al.,* 2014). Since the introduction of antibiotics in cholera management, there have been reported cases of the emergence of antimicrobial drug resistance. In the global survey that was conducted in 1976, it was revealed that *V. cholerae* was susceptible to most of the antibiotics that were prescribed. About 3% of the isolates were resistance to some selected antibiotics.

This trend has however, changed for the past decade with several reported cases from countries that are endemic for cholera reporting increased resistant strains of *V. cholera*. The most common antibiotics that were reported to be losing its potency are tetracycline, ampicillin, kanamycin, streptomycin, sulphonamides, trimethoprim and gentamicin. This trend is as a result of the indiscriminate use of the drugs (Gelbíčová, *et al.*, 2016).). To halt this problem, there is the need to be selective in the use of antibiotics and this selection should be based on the antibiotics susceptibility patterns of *V. cholerae* in the local setting. Work done by Kuma *et al.,* (2014) revealed multiple drug resistant epidemic strains of *V. cholerae* O1 and recommended the constant surveillance of frequently used antimicrobial agents.

Ghana currently has limited data showing the relationship between clinical isolates and that of environmental isolates. Research in this area has therefore, become expedient and data could be made available to serve as a platform for controlling and prevention of annual disease outbreak.

1.4 Hypothesis

Environmental isolates of *V. cholerae* have the same virulent genes and antibiotic susceptibility patterns as clinical isolates in a cholera endemic country such as Ghana.

1.5 Aim

To investigate the genomic virulence and antibiotic susceptibility patterns of *V. cholerae* in environmental and clinical isolates in Accra.

1.6 Specific Objectives

1. To isolate and characterize *V. cholerae* with pathogenic potential from the environment.
2. To evaluate the physicochemical factors that maintains its persistence in the environment.
3. To determine the antibiotic susceptibility patterns of clinical and environmental isolates.
4. To compare the virulent genes between clinical and environmental isolates.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 History of Cholera

Cholera is second to malaria as the leading cause of death among children below 5years world-wide (Mizikar, 2012). *Vibrio cholerae* is the etiological agent of epidemic cholera (Alam *et al.*, 2015). Cholera is characterized by an acute onset of rice-watery diarrhoea that leads to dehydration and loss of electrolytes (Nelson *et al.*, 2009). Back in the 15th century BC, a cholera-like disease was found in Sanskrit. Later, the disease was confirmed to be cholera and has spread over continents. Between 1817 and 1923, cholera disease has caused six worldwide pandemics (Sack *et al.*, 2004). During the 1848 London epidemic, an English physician and a leader in the adoption of anesthesia and medical hygiene, John Snow established the concept that diseases are caused by living organisms and backed the theory of waterborne transmission of cholera (Vandenbroucke, 1997).

Robert Heinrich Herman Koch, a celebrated German physician successfully isolated the organism responsible for cholera in 1883, during the fifth pandemic. He also postulated the existence of the cholera toxin (CT) in 1884 (Finkelstein, 2000). The crude cholera toxin was observed to be responsible for the stimulation of the fluid secretion from the small intestine by a group of bacteriologists in Calcutta in 1953 (De and Chatterjee, 1953). In 1854, an Italian scientist Filippo Pacini, independently found a comma-sharped organism in a stool sample under a microscope which was later revealed as *V. cholera*.

Since the beginning of cholera, there have been seven episodes of cholera pandemic in history. Although, the cause of the earliest pandemic is not known, it is widely speculated that the classical *V. cholera* O1 was responsible. The fifth and sixth pandemic was however, caused by the classical

V. cholera O1 (Hinmam, 2016). The seventh pandemic was caused by a new biotype called EL tor (Quilici *et al.,* 2010). In December 1992, a new biotype called O139 which was christened the Bengal to indicate where it was first isolated caused a massive epidemic in Banagladesh (Taneja *et al.*, 2009). This was believed to be the beginning of the eighth pandemic.

2.2 The Global Picture

Cholera, which was in a decline has over the past recent years re-emerged as a serious infectious disease with a global upsurge (WHO, 1995). However, in 1994, 94 countries in the world reported cases of cholera to the World Health Organization. This was the highest number of countries to report the disease to the world health organization (WHO, 1995). This prompted the World Health Organization to include cholera as one of the disease that needs to be reported to the organization. Currently, cholera is among the diseases required to be notified under the International Health Regulation. Cholera, which was previously thought to be endemic in the Indian sub-continent, has reached Africa and now endemic in many African countries.

The disease persists due to poor sanitation, improper waste disposal and lack of potable drinking water. Since 1996, Africa has consistently reported the highest incidence of cholera to the World Health Organization (WHO, 2015). For instance, in 2006, African countries accounted for 99% of globally reported cases of cholera. According to the World Health Organization, cholera still remains a major global treat to public health and wellbeing; particularly in developing countries with inadequate sanitary conditions (WHO, 2007). A total of 101,383 cases were reported in 2004 as against 131,943 cases in 2005; an increase of 30% of globally reported cases. This figure further increased by 79% between 2005 and 2006 whereas the number of reporting countries still remained

the same. The global fatality rate also increased from 1.72% in 2005 to 2.66% in 2006. (WHO, 2007)

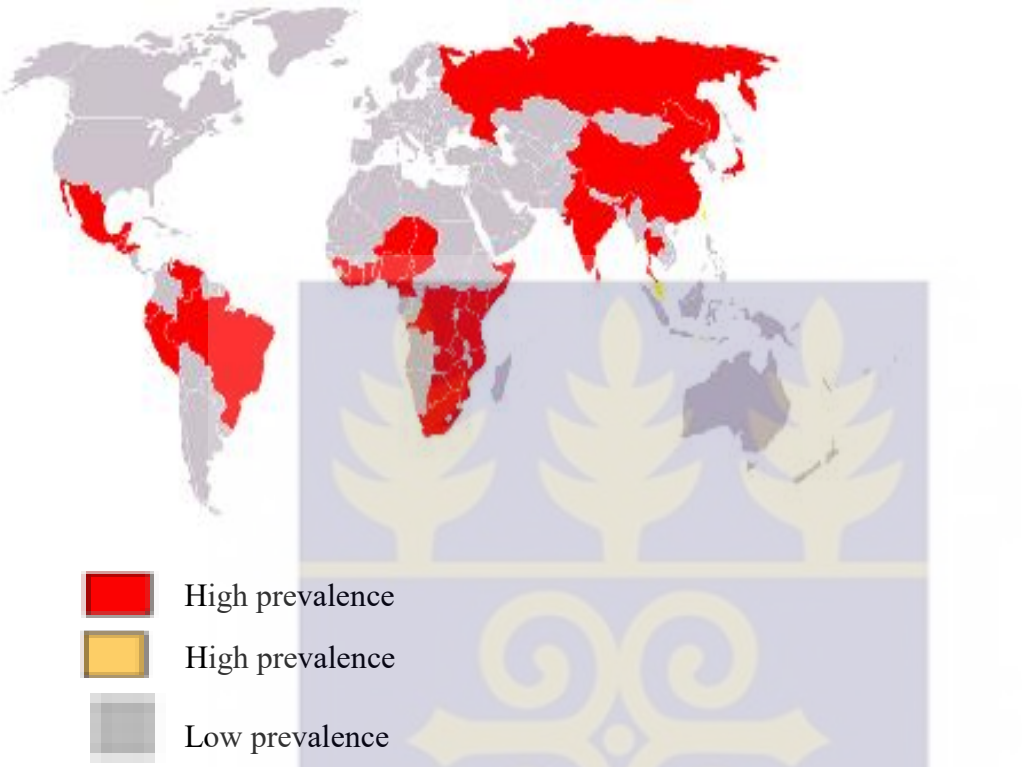

Fig: 2.1. Global trend of *V. cholerae* (CDC, 2015)

The global trend of *V. cholerae* shows that many countries in Africa as well as south America and Europe had reported cases of cholera.

2.3 Causative Agent

A member of the Vibrionaceae, *V. cholerae* is responsible for cholera outbreak. It is found mostly in coastal and marine environment (WHO, 2016). The bacterial thrives best in saline environment, although water with low salinity can support its growth if such water bodies are warm and have adequate organic nutrients (Rebaudet and Piarroux, 2015). *V. cholera* is able to utilize chitin as a source of carbon and nitrogen. It is usually associated with shellfish, zooplankton, blue crabs, copepods and other aquatic animals (Kirn *et al.*, 2005). It has been suggested that, there is a lateral

transfer of gene in water leading to the speculation that chitin may be inducing competence in *V. cholerae* especially during zooplankton bloom (Meibom, 2005). *V. cholerae* is able to enter a state of viable but non-culturable state in water, which is also referred to as conditionally viable environmental cells (Faruque *et al.*, 2005).

The species of *V. cholera* are composed of over 200 serogroups, based the lipopolysacharides O surface antigen (Mizikar, 2012). Out of the over 200 serogroups, only O1 and O139 are associated with epidemic cholera. The O1 serogroup is further divided into two biotypes; the classical and El tor. The antigenic factors allow for a further differentiation into their serotypes; these are the Ogawa which expresses the A and B antigen, the Inaba which expresses A and C and Hikojima that expresses all three antigens A, B and C (Keddy *et al.*, 2013). The Hikojima however, are rare to find and unstable in the environment. There are other serotypes of *V. cholera* known as the non-O1, non-O139 *V. cholera* (NOVC). The NOVC are able to cause sporadic cases of small outbreaks but not epidemics and pandemics (Arvanitoyannis, 2010). The serogroups O139 was first identified in 1992 during a cholera epidemic in South Asia (Ramamurthy *et al.*, 1993). It is almost identical to the O1 El tor biotype and it is said to have been derived from the O1 El tor.

Bacteraemia is usually not associated with choleragenic *vibrios*, this is because of the ability of the toxin to suppress the induction of inflammation during infection (Engel *et al.*, 2016).

2.4 Epidemiology

Thousands of people continue to suffer the repercussions of cholera globally every year with a case fatality rate of about 2 to 4% (WHO, 2013). There have been both pandemic and epidemic patterns to cholera occurrence. It has persisted for many years in Asia and Africa. In Asia, cholera is more pronounced after the monsoon rains and appears to be seasonal in it occurrences (Sack *et*

al., 2004). The most disease burdened countries are in the African continent with more cases recorded from Africa during the present seventh cholera pandemic (Sudre and Bompangue, 2009). The incidence rate is common among children under five years and at times affects neonates. Cholera epidemics occur in a long cycle of prevailing endemic disease. There is a close relation between the declining levels of immunity among a population from a previous outbreak and a cycle of climate variability (Koelle *et al.,* 2005). Countries such as Ethiopia, Angola, Zimbabwe, Pakistan, Somalia, Nigeria, Sudan, Ghana, among others have witnessed devastating epidemics of cholera in the past decades (Mizikar, 2012). Populations that have no record of cholera outbreak have no or low levels of immunity against cholera (Jutla *et al.,* 2011). This results in all age groups being at risk when there is an epidemic. This was the case in Haiti where there was no incidence of cholera before 2010 (Wrębiak, 2010) High case-fatality rates are also increased due to poor sanitation, poor health infrastructure, logistical problems and mismanagement in epidemic situations. Environmental factors that result in changes in surface water such as temperature, nutrient and pH results in the proliferations of phytoplankton and zooplankton which have direct bearing on the epidemiology of cholera (Jutla *et al.,* 2011). Floods and other natural disasters such as earthquakes, cyclones that disrupt public health and sanitation also increase the chance of cholera outbreaks (Morris, 2011). The infectious dose of cholera can be as low as 10^3 in a healthy individual, although, it is estimated at 10^5 to 10^8 (Nelson *et al.,* 2011). Cholera has an incubation period between 12 hours and 5 days depending on the immune status of the individual (Weil *et al.,* 2009).

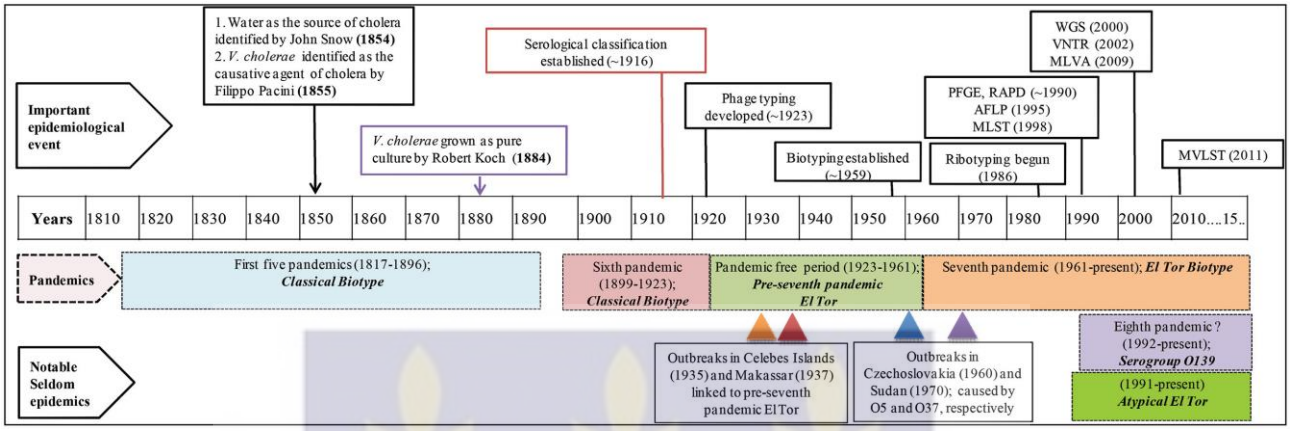

Fig: 2. 2. Timeline of cholera epidemiology since 1817 (Rahaman *et al.*, 2015)

Timeline of cholera epidemiology since 1817. The upper panel shows important scientific advances that have changed the landscape of cholera epidemiology. Pandemics and their putative causative strains are shown in shaded boxes with dashed boarders. The lowermost panels show infrequent outbreaks and their causative strains, with triangles indicating time of outbreaks.

2.5 Pathogenesis

The most important features of cholera and its pathogenesis are well documented. *Vibrio cholera* infection begins with the ingestion of the organism during eating or drinking contaminated food or water. *V. cholera* is susceptible to acidic conditions and hence, most of the bacteria are killed by the gastric acid in the alimentary canal (Yildiz, 2007). The small numbers of bacteria that escape the acidic conditions are able to colonize the epithelium of the small intestines where they release their toxin, which is the primary virulent factor by means of the toxin-coregulated pilus. Other factors that may take part in colonization are the accessory colonization factor, core-encoded pilus and the different haemagglutinins (Sack *et al.,* 2004).

The toxin is a protein exotoxin that belongs to the AB family (Chao *et al.,* 2010). It has one enzymatically active A subunit which is responsible for stirring up toxicity and a cell binding pentamer B domain for binding and cell entry. There are dissimilar forms of the AB toxin, those

that consist of one polypeptide chain and can be cleaved into A and B components. There are also others that are made up of the individual A and B subunits which accumulate during the process of intoxication (Fujinaga *et al.,* 2003). The existence of the AB_5 family of toxins has also been discovered. The AB_5 is made up of a single A-subunit and a homopetameric B-subunit. The assembly of the subunit prior to secretion from the organism forms a holotoxin. Cholera toxin is a typical example of the AB_5 toxin family (Fujinaga *et al.,* 2003). The A subunit is a 27kDa which is composed of an A_1 chain and an enzymatically active A_2 subunit. Both A_1 and A_2 are attached to the B subunit through a non-covalent bond via the A_2 chain. Cleavage between A_1 and A_2 subunit into two polypeptides is made possible due to the presence of a serine-protease cleavage site between residues 192 and 195. Before the A_1 chain can infiltrate the cytosol of the host cell the disulphide and peptide bonds must be broken. The B subunit is made up of 11.5 kDa peptides which is assembled non-covalently into a stable homopentamer and allows for binding to the ganglioside GM1 on the pm (Ewers *et al.,* 2010). After colonization, the B subunits binds to the surface receptors of the GM1 ganglioside and the A subunit then traffics its way intracellularly where it's enzymatically activates the adenylate cyclase thereby increasing intracellular cyclic AMP. The high level of cAMP leads to the activation of cystic fibrosis transmembrane conductance regulator (CFTR), which causes a sudden efflux of chlorides and water via the apical chloride channel and secretory diarrhoea.

A filamentous bacteriophage, ctxφ encodes the genes for cholera toxin (Jesen *et al*., 2006). There are different versions of the bacteriophage that encodes the toxin. Both El tor and classical biotypes have different bacteriophage. The toxin-coregulated pilus located at the cell surface serves as a point of attachment for the ctxφ (Nelson *et al*., 2009) which is found in the genomic island known as Vibrio Pathogenicity Island (VPI-1) (Chao *et al*., 2010). For *V. cholerae* to be virulent it must

acquire VPI-1 followed by ctxφ. The VPI-1 was found to be present in all O1 El tor strains responsible for the seven pandemics. However, the seventh pandemic strains has a second *Vibrio* pathogenicity island VPI -@ (Chao *et al*., 2010).

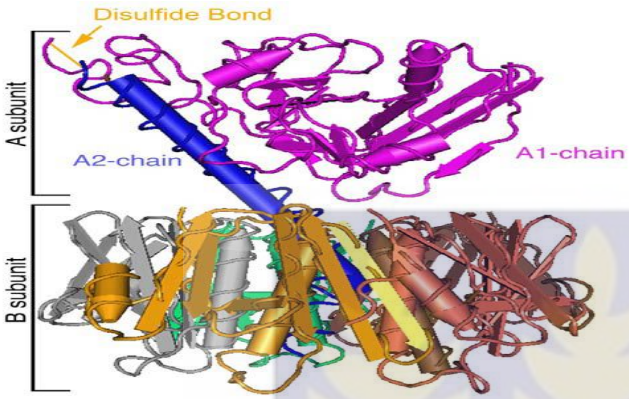

Fig: 2.3. Structure of *V. cholerae* toxin (Wernick *et al*., 2010).
The structure shows both α subunit and β subunit of the toxin. Both sub units are linked together by A2 – chain.

2.6 Transmission

Unlike symptomatic patients infected with *V. cholerae* O1 and O139 strains that can shed the organism for between two days to two weeks, asymptomatic patients can only shed it for few days (Nelson *et al.*, 2009; Weil, 2009). There is ample evidence of household transmission (Weil, 2009). *V. cholerae* can exist in stool as individual planktonic cells as well as in biofilm-like aggregates (Nelson, 2007). Once they find themselves in the environment, they can transform into conditional viable environmental cells within 24hours (Nelson, 2008). The conditional viable cell can become pathogenic on reintroduction into a human being. However, the infectious dose in this new state is not yet established.

Examination of stool samples from patients has also revealed the presence of bacteriophage that are lytic for *V. cholerae* O1 and O139 as well as environmental water (Jensen 2006). *Vibrio cholerae* O1 gains a hyperinfectivity phenotype once it leaves an infected person (Mudrak &

Tamayo, 2012). This new state reduces its infectious dose to between 10 and 100. Hyperinfective organisms can persist in the environment for 5 to 24 hours once they are shed; indicating that, person- to- person transmission is more active than those that have acclimatized in the environment. The concentration of *V. cholerae O1* and O139 in stool samples of patients, the differences in infectivity between planktonic cells and stool aggregates, the rate of spread amongst people, the number of lytic bacteriophage in stool and water samples and the concentration of conditionally viable environmental cells for transmission between environment – to – person transmission are other key components of cholera transmission models (Tuite, 2011). Cholera epidemics usually follow an increased presence of pathogenic strains in the environment.

2.6.1 Host Susceptibility

There are several host factors that determines a person susceptibility to cholera infections. Immunity to *V. cholera* infections is affected when an individual is infected with enteropathogenic bacteria, parasites or fungi. These infections exert an immunomodulatory effect on the immune system; thereby, impairing its function (Chowdhury, *et al.*, 2010). Another host factor is the difference in gastric acidity levels in the stomach. A low acidic level in the abdomen increases the susceptibility to cholera infections (Van, *et al.*, 1990). This implies that, people who produce less stomach acids are more prone to cholera infections. This includes children, the aged, and people on drugs that have the potential to reduce stomach acidity such as proton pump inhibitor (such as omeprazole) and histamine-2 blockers (such as ranitidine) (Sack *et al.*, 2004). Blood group has also been associated with susceptibility to cholera infections. People with blood group O have been linked to an increased chance of cholera infection in different geographical area (Mizikar, 2012).

2.7 Diagnosis

There are still many challenges that impede early detection of cholera outbreaks. Although all watery diarrhoea cases are given the same treatment regardless of which organism is responsible, according to the World Health Organization, management of cholera should be distinct. This is because *V. cholera* is very virulent and its epidemic potential cannot be under-estimated. Growing of the bacteria on a culture plates still remains the gold standard for the isolation of *V. cholerae* O1 and O139. Rapid diagnostic tools are mostly used in African settings during outbreaks in areas where stool culture is not possible.

The rapid methods using rapid diagnostic test kits (RDT's) based on antigen antibody reaction are however, not a substitute for culture methods due to its low sensitivity (Alam *et al.,* 2010). Notwithstanding its limitation, in new settlement, outbreaks among rural areas, displaced persons or a breakdown in the transport chain to a laboratory, the rapid test method comes in handy. The rapid test kits are used in point-of-care facilities by trained personnel in field conditions to aid in early detection of outbreak. The rapid test kits are friendly to use and not expensive. They have longer shelf life (mostly more than one year), can be stored without a refrigerator and also do not require highly trained personnel (Dick *et al.,* 2012). The kits are developed to detect the lipopolysacharides of the outer membrane of *V. cholerae* O1 and O139 by monoclonal antibodies. The various rapid detection kits come with different sensitivities and specificities depending on the reference standard used (Alam *et al.,* 2010).

The usefulness of the rapid diagnostics test kits is limited due to its low sensitivity and specificity which usually ranges between 93-98% and 67-96% respectfully (Alam *et al.,* 2010). Where there is both epidemiological and clinical evidence in an outbreak settings with more than 10 persons presenting same illness, the pre-test prevalence of cholera within the population will either lean

towards 100% or 0%. The specificity and sensitivity of the test kits improves as the prevalence in the test population approaches each end. If the cause of the outbreak is *V. cholerae,* the rapid kits will have over 80% positive results. However, if it is due to other causes, it is likely to have over 60% negative. The results should thus, be able to provide a clear indication as to whether the cause of the outbreak is due to *V. cholerae* or not (CDC, 2015). The kits are not particularly helpful with environmental samples as its sensitivity is below 50% (Bhuiyan *et al.,* 2003).

2.7.1 Phenotypic Characterization

Owing to the fact that the rapid diagnostics detection kits comes with many challenges, and the need for other information on the isolated organism such as strain characterization, its antimicrobial susceptibility patterns, and other epidemiological information, the traditional culture methods remains critical in cholera management. Errors with respect to phenotypic identification of *V. cholerae* are usually not reported, but can lead to inappropriate use of laboratory resources and personnel (Smith *et al.,* 2011). *V. cholerae* can grow on most basic media, but selective media is recommended for initial isolation. The selective media of choice is Thiosulfate - Citrate Bile-salt Sucrose (TCBS). The culture method must be preceded by enrichment in Alkaline Peptone Water (APW). The enrichment allows *V. cholerae* outgrowing other organism. The use of both APW and TCBS is advice for when isolating *V. cholerae* from patients suspected of cholera, asymptomatic patients and environmental samples. *V. cholerae* are sucrose fermenters and appears yellow measuring between 2-4 mm on TCBS. After traditional culture method, serological confirmation is a must in phenotypic identification of *V. cholerae*. Polyvalent antisera against O1, monovalent Ogawa, Inaba and Hikojima are more effective for serological test. The antisera are more stable and have a long shelf-life when stored appropriately. The test must however be done

using 24 hours' culture from a non-selective media since growth on TCBS often results in false negative reactions. This is because of the increasing patterns of antimicrobial resistance, that calls for continues surveillance of both environmental and clinical strains (CDC, 2015). This further enhances the key role played by the traditional culture method in *V. cholerae* case management.

2.7.2 Molecular Characterization

Recent advances in diagnostics have led to characterization of bacteria at the molecular level using their amino acid sequences. Only few laboratories in Africa however, have the capacity to perform molecular characterization. Characterization of *V. cholerae* O1 and O139 has become essential in supporting phenotypic and serological identification of pathogens. It also assists in the epidemiological studies of disease in local and international settings (Reimer *et al.*, 2011). Data from molecular methods are used to support phenotypic test results; the cost of molecular techniques has been reducing and hence should be considered as part of a mechanism for rapid and accurate diagnosis (Kirigia *et al.*, 2009).

The best molecular method is the one that can target the nucleic acid sequence that encodes the two virulence genes (CT and TCP). The outbreak potential of *V. cholerae* from the environment can be determined using molecular methods. Again, molecular diagnoses including the detection of DNA sequences specific O1 and O139 antigen biosynthesis helps in the confirmation of toxigenic *V. cholerae.* Automated real-time polymerase chain reactions (PCR) using fluorescence is used to detect PCR product. The target gene has always been the ctxA gene encoding the A subunit (Singh *et al.*, 2002). Multiplex PCR can also be used to detect more than one target gene. This new technique has an added benefit in that, it is able to differentiate between the biotype classical and El tor of the O1 serogroups (CDC, 2013). *V. cholerae* genotyping gives a measure of

how closely related a strain is to the other, this is important for accurate epidemiological investigations (Lee *et al.,* 2006). For comparison, laboratories standardized methods should be followed. Currently, the subtyping method that possibly meets the internationally accepted criteria for *V. cholerae* is the pulse-field gel electrophoresis (PFGE). This method was standardized by pulseNet international.

2.8 Sign and Symptoms

Cholera has a striking clinical presentation. It is accompanied by watery diarrhoea for which one loses as much as 1 L of water within few hours. Fatality can occur due to hypotensive shock as a result of loss of water, a situation referred to as cholera gravis (Chowdhury *et al*., 2010). Symptoms usually manifest after one to two days of incubation but can have an early onset within hours or delayed for about five days. It begins with steady watery diarrhoea usually painless, but this can become voluminous within a short period of time with vomiting (Sack *et al.,* 2004).

In an endemic region, people can have the infection without any symptoms. These asymptomatic patients can still shed the organism to spread the infection. Stools from patients with cholera may contain faecal matter in the early onset but if purging continues for a while, a characteristic rice-water stool is observed. The rice water stool is opaque white and looks like water that has been used to wash rice (Chowdhury *et al.,* 2010). Severe cholera can cause the affected to have about 250 mL/kg of stool per normal body weight in a day (Mizikar, 2012). Due to distension of fluid in the bowel few people may have stomach cramps or discomfort.

In rare occasions, diarrhoea due to cholera is accompanied by fever (Wrębiak, 2010). Physicians are therefore advice to suspect secondary infections when diarrhoea due to cholera, is accompanied by fever. Complication of cholera sets in when there is massive dehydration and loss of

electrolytes. This may lead to patients becoming lethargic; they may have decreased skin turgor, wrinkled hands and feet or have sinking eyes with cold clammy skin and dry mouth (Arvanitoyannis, 2010). Hypoglycemia sets in after dehydration which is one of the most common causes of lethality especially amongst children (Sajeev *et al.*, 2014). Hypoglycemia is triggered by less glucose intake during the acute stage of illness and defective neoglucogenesis which is next to less storage. When there is an exchange of extracellular potassium with intracellular hydrogen as a result of loss of potassium and correction of acidosis hypoglycemia sets in. This is more pronounced in children with a history of malnutrition or people with an impaired immune system with reduced body stores for potassium (Jackson *et al.*, 2013, CDC, 2013).

Another complication due to cholera is the loss of bicarbonate and lactic acidosis as a result of poor perfusion. This leads to blood pressure drop because peripheral pulse becomes rapid and thread and so becomes had to palpate. There is decreased urine output (Tariq *et al.*, 2009). Cholera sicca is a rare complication where fluid accumulates in dilated intestinal loops leading to circulatory collapse even sometimes death before the passage of first stool (Guerrant *et al.*, 2003). Presentation of cholera symptoms differs between endemic and epidemic settings. In an endemic setting, asymptomatic patients range from 40% to 80% (Nelson *et al.*, 2009). Just as other enteropathogens with mild diarrhoea, cholera can assume similar forms in an endemic region making it difficult to diagnose. It is however, concentrated among people with reduced immunity, migrants, children and inhabitants who have not been previously exposed. In an epidemic setting, however, all age groups are at risk with a high case fatality rate. This is no differences in the symptoms of cholera caused by O1 or O139 strain (Das *et al.*, 2011).

2.9 Management

The cornerstone of cholera management is replacing loss water and electrolytes. Much was not achieved in earlier attempts at using oral rehydration. This was as a result of lack of understanding on the physiological sodium-glucose co-transport. Later in the 1960's, rehydration gained grounds as the most successful approach in the management of cholera by the addition of equal concentration of sodium and glucose to optimize sodium uptake in the small intestine whiles replacing fluid loss (Guerrant *et al.*, 2003). Currently, cholera management has assumed a different dimension with several approaches.

Case fatality rate of cholera has reduced significantly to less than 0.2% with the present standard of care even in areas where resources are hard to come by. Nevertheless, there still exist a lot of obstacles especially during epidemics. This includes the best method for rehydration and this can lead to an increase in mortality rate of more than 10% (Walton *et al.*, 2011). The World Health Organization has therefore, established a guideline for the management of cholera. These guidelines are simple and can easily be understood and applied in clinical settings (PAHO, 2013).

2.9.1 Cholera Cots

One of the common mistakes in the management and care for cholera patients is the underestimations of the volume of fluid loss and the amount required to replenish. An average of about 200 mL/kg of isotonic fluid is required for patients with severe cholera within the first 24 hours of onset (WHO, 2010). This has made the use of cholera cots one of the basic tools in cholera management in endemic settings. The cots are inexpensive and are used to estimate the volume of stool loss. It is equipment covered with a plastic lid with a hole created in the middle to enable stool to be collected. It does not require highly trained health workers to manage the cots and

calculate the volume of fluid loss and estimate the replacement needs. The volume of stool collected in the receptacle is collected and the volume is estimated. This gives an indication of the amount of fluid replacement required. Fluid losses through urine in the initial stages of therapy are insignificant hence the amount of fluid in the receptacle is a reflection of stool losses. However, urine should be collected in a different receptacle during rehydration to avoid the vicious circle of increasing urine output and also avoid over- hydration (CDC, 2013 and PAHO, 2013).

2.9.2 Rehydration

The primary objective of managing patients with cholera is the replacement of lost fluid. The World Health Organization (WHO) has therefore recommended ways for replacing lost fluid for severe dehydration, some dehydration and no dehydration (PAHO, 2013). Severe dehydration: immediate replacement of lost fluid through intravenous (IV) administration within 3-4 hours is essential. Severe dehydration can lead to hypovolemic shock and requires immediate attention until normal blood circulation is restored. The use of lactated ringer solution is recommended, however, an isotonic sodium chloride solution can be used as a substitute. Oral rehydration solution (ORS) are rich in potassium, bicarbonates and glucose than standard intravenous fluids, hence oral rehydration should begin as soon as patients are capable of drinking. Approximately 100 mL/kg IV in 6 hours to 30 mL/kg in the first hour followed by 70 mL/kg in the preceding 5 hours is recommended for patients less than one year (WHO, 2010). For cholera patients, older than one year the following are recommended, 100 mL/kg IV within 3 hours to 30 mL/kg as quickly as possible then 70 mL/kg in the next two hours. This should be done under strict monitoring and observation (PAHO, 2013 and WHO, 2013). Homemade rehydration fluids are also encouraged for patients with no sign of dehydration (Ramakrishna *et al.*, 2000).

2.9.3 Antibiotics

The primary treatment for cholera is rehydration with oral or intravenous fluids (WHO, 2004). For moderate to severe cases, antimicrobial agents may reduce the volume and duration of diarrhea (Gelbíčová, *et al*., 2016). Different antibiotics such tetracyclines (e.g., doxycycline), fluoroquinolones (e.g., ciprofloxacin, norfloxacin), macrolides (e.g., erythromycin and azitromycin), and trimethoprim/sulfamethoxazole have mostly been used to treat cholera (WHO, 2004). However, antimicrobial drug resistance can undermine the success rate of antimicrobial therapy. There have been several reported cases of resistance to tetracycline and fluoroquinolone by *V. cholerae*, and multidrug resistance is said to be increasing (Kitaoka *et al.*, 2011).

Drug resistance in *Vibrio* spp. can develop through several means such as mutation or through acquisition of resistance genes on mobile genetic elements, such as plasmids, transposons, integrons, and integrating conjugative elements (ICEs). ICEs integrate and replicate with the host chromosome and can excise themselves and transfer between bacteria by conjugation (Wozniak *et al.*, 2009). ICEs have been implicated to commonly carry several antimicrobial drug resistance genes and play a significant role in the spread of antimicrobial drug resistance in *V. cholerae*. In 1992, the first ICE was described in O139 Bengal isolates in Madras, India and was christened SXT after the resistance phenotype it conferred (trimethoprim/sulfamethoxazole). Since 1992, there are many reported cases of O139 and O1 isolates haven acquired the SXT or a closely related ICE (Kitaoka *et al.*, 2011). Drug-resistant *V. cholerae* has become a global health concern because resulting infections can be more severe and difficult to treat. Infections with drug-resistant *V. cholerae* result in higher case-fatality rates, prolonged hospitalizations, more secondary infections, and this causes an increase in health care costs (Iwanaga *et al*., 2004).

2.9.4 Nutritional interventions

Malnutrition can be avoided in patients with cholera after replacing the lost fluid by resumption of high energy diet. This helps to reduce a possible complication, hypokalemia and hypoglycemia. In infants, breast feeding along with oral rehydration is encouraged. Zinc supplement have also been shown to reduce the duration of diarrhoea and volume of fluid loss in children (Roy *et al.*, 2008). WHO recommends 10 mg/day, 20 mg/day for 10 days for children below 5 years and 6 months respectively (WHO, 2005). In underdeveloped regions, children with diarrhoea have also benefited from vitamin A additions (Mayo-Wilson, 2011).

2.9.5 Public health and other forms of management

In an event of an outbreak of cholera, public health officials are usually inundated with many cases to manage. It is therefore, crucial to adopt other forms to manage and minimize transmission rate. The creation of treatment centers, public announcement, training of volunteers in case management, provision of potable water and good sanitation. Where possible, radio advertisement and mobile handsets messaging should be adapted in public education. This helps to ensure patients seek early medical attention especially in the area of homemade oral rehydration therapy and sanitation. Other public health components include, proper disposal of waste and bodies of people who have died during the outbreak, local, regional and national coordination is also important (Mizikar, 2012).

2.10 Prevention

2.10.1 Public education

The current response to cholera outbreak is more reactive than proactive. This approach, though, is able to prevent high fatality rate; it has failed to prevent cases of cholera. This therefore, calls for integrated strategies to link all health systems in other to prevent its occurrence. Health education for instance is a key to the prevention of cholera amongst the high-risk groups (children, pregnant women and immune-compromised patients) (Martin *et al.*, 2012; Barzilay *et al.*, 2013). Health education should be centered on the provision of and use of safe and adequate drinking water, the practice of good personal hygiene, proper disposal of waste, prompt reported of cases to avoid wide spread in the event of an outbreak (WHO, 2013).

2.10.2 Surveillance

Seasonality of cholera has been reported in many endemic countries occurring annually usually during raining seasons (Mishra *et al.*, 2012). Surveillance systems are therefore, essential in providing early warning systems. This will lead to a well-coordinated approach and assist in the preparation of preparedness plans. Surveillance systems have aided many countries to utilize geographical and mathematical data systems to assess spatial distribution of the disease causing organism at local levels. This helps to zone case clustering areas and disease prone areas (Tuite, *et al.*, 2010). The prediction of an outbreak and mounting appropriate strategies can further be enhanced by modeling techniques using climate data, remote sensing and geographical information systems (Colwell *et al.*, 1996). In many poor countries where provision of adequate water and sanitation is skewed, data from surveillance systems will help local authorities in feature planning in other to give priority to disease prone areas.

2.10.3 Vaccination

Due to the difficulties in implementing public health and personal preventive measures; it has occasioned the century-long search for cholera vaccines. WHO has therefore, approved for use some cholera vaccines. Although, the vaccines are safe and effective against *V. cholerae*, cholera vaccines are yet to be integrated into worldwide cholera control programs (Martin *et al.*, 2012). Two vaccines prequalified for use by WHO are the oral killed vaccines. One of such oral killed vaccines is Dukoral (Wc-rBs, crucell, Sweden). It is made up of several biotypes and serotypes of *V. cholerae* O1 and is supplemented with 1mg per dose of recombinant B subunit of the cholera toxin. Results from clinical trials on this vaccine showed that, it confers about 85% to 90% protection in the first six months regardless of age group and sex after administration of two doses within two weeks. After six months' protection tends to drop significantly among children but was still active in adults and 2years older children (Martin *et al.*, 2012).

Another vaccine shanchol (Shantha Biotechnics-sanofi Pasteur, India) is composed of several biotypes and serotypes of *V. cholerae O1* and O139. It however, lacks the B subunit of the *V. cholerae* toxin (WHO 2010). ORCVAx was formulated in Vietnam in 1997 and was modified in 2004. This bivalent vaccine has been administered to both adults and children in many endemic areas of Vietnam (WHO, 2010).

The Swiss serum and vaccine institute in Bern have developed CVD 103-Ngr cholera vaccine. It is available in many European countries, Latin America and Canada. Although, not approved by WHO, the vaccine is made of an attenuated *V. cholerae* O1 prepared by recombinant DNA. It has been tested in many countries including developed and developing nations that are endemic little or no cholera outbreaks (Chen *et al*., 2013). A review on cholera vaccines currently available in

2011 showed that, cholera vaccines provides 50-60% efficacy in preventing episodes of cholera in the initial 2 years after primary administration. After 3 years, a booster dose is required (Sinclair, *et al.*, 2011).

CHAPTER THREE

3.0 METHODOLOGY

3.1 Study Site

This study was conducted in four communities in the greater Accra region (Teshie, James Town, Chorkor and Nima). The selected communities are densely populated and lies along the coastal belt of Accra with one community (Nima) further away from the coastal belt. The communities have two equatorial climates with two seasons; the dry season which usually starts from November and ends in April and the raining season which starts from May to September; there are always intermittent rains during the dry season. Records from the Ghana Health Service, National Public Health Reference Laboratory (NPHRL) indicates that the selected communities were the most affected during the 2014 cholera outbreak that hit the country (GHS, 2015). Access to potable water has been a major problem in these communities with the inhabitants relying on various forms of storage tanks to store water for drinking and other domestic activities. Streams in these communities are used for irrigation and other recreational activities.

3.2 Study Design

A total of 244 water samples were collected from taps, streams and storage tanks in the selected communities from October to January and analyzed for *Vibrio cholera* O1 and O139. Forty clinical isolates from the selected communities at the National Reference Laboratory were also included in the study. The antibiotic susceptibility patterns of the isolates were determined. The genes responsible for virulence were also determined by amplifying their specific regions using PCR. The genes of interest were the cholera toxin (ctx), toxin co-regulated pilus (tcpA $_{\text{El Tor}}$ and tcpA

$_{classical}$) Zonular Occludence Toxin (zot), the attachment site (attRS) and the outer membrane protein (ompW). The relationship between some environmental factors such as temperature, pH, salinity and the occurrence of *V. cholerae O1* and O139 were also studied.

3.3 Sample Collection

From October, 2015 to January, 2016, a total of (244) water samples were collected aseptically from randomly selected streams and household sources including shallow wells, storage containers and taps into a 500ml sterile bottles. A total of (11) streams, (45) shallow wells, (98) storage tanks and (90) tap water were sampled within the time period. To collect tap water, the openings of the taps were cleaned with 70% alcohol soaked in cotton wool. The tap was then open and allowed to flow for few seconds to flush out any contaminants. For wells and streams, a sterile rope was tied to the neck of the sample container and immersed into the water until the container was full. Stream samples were collected at a depth of about 30 to 40 cm 2 to 5 m away from the banks.

Fig: 3.1. Sources of water from which samples were taking for this research A- Shallow well, B-Tap, C- Storage tank, D- stream

3.4 Determination of Environmental Parameters

Water temperature, salinity, conductivity and total dissolve solids (TDS) were determined on site immediately after samples were collected using Extech instrument (Exstik II conductivity/TDS salinity EC400). The pH of each sample was recorded with cybescan (Eutech instruments).

3.5 Isolation and Characterization

Ten milliliters (10 mL) of each sample was transferred into triple strength alkaline peptone water (APW) (Oxoid, Basingstoke, United Kingdom) pH 8.5 and incubated at 37 ºC for 6-8 hours for enrichment. A loop full of the enrichment broth was picked just beneath the surface of the broth and streaked onto thiosulfate citrate bile salt sucrose (TCBS) agar. The plates were incubated at 37 ºC for 24 hours. Presumptive colonies (yellow, measuring 2.4 mm) were sub cultured onto Tryptose Soya Agar (TSA) (Oxoid, Basingstoke, United Kingdom) for 24 hours to obtain pure cultures. Biochemical analysis was done on the pure culture. Gram negative, and oxidase positive isolates were subjected to serotyping.

3.6 Gram Staining of Presumptive *V. cholerae*

With a sterile cooled loop, a drop of saline solution was placed on a clean glass slide. The loop was heated and cooled again. A very small sample of a bacterial colony was picked and gently and stirred into the drop of saline on the slide to create an emulsion. The smear was allowed to air dry. After the smear has air-dried, the slide was held at one end and passed through a flame of Bunsen burner for two to three times with the smear-side up to heat fix. The smear was flooded with crystal violet and allowed to stand for 1 minute. The slide was gently tilted and rinsed with distilled water using a wash bottle. The smear was again flood with gram's iodine and allowed to stand for 1 minute. The slide was tilted slightly and rinsed with distilled water using a wash bottle. The smear was decolorized using 95% ethyl alcohol by tilting the slide slightly and applying the alcohol drop by drop for 5 to 10 seconds until the alcohol runs almost clear. The smear was immediately rinsed with distilled water. Safranin was used to flood the smear as a counter-stain for 45 seconds and the

slide tilted and rinsed with distilled water. The slide was blot dried with a bibulous paper and observed using light-microscope under oil-immersion.

3.7 Serotyping of *V. cholerae* positive isolates

The slide agglutination technique as described by Akoachere *et al.* (2013) was used. *V. cholera* O1 and O139 polyvalent antiserum (Mast Group and Denka Seiken, Japan) was used for the analysis. Two drops of sterile 0.85% normal saline solution was drop on a clean microscopic slide. With an inoculation loop, an isolated colony from an overnight culture on TSA was emulsified into each of the saline solution. A drop of *V. cholerae* antiserum was dropped onto one of the emulsified isolates and the other a drop of saline as a control. The reagent was mixed by tilting the slide back and forth for 60 seconds' whiles viewing under indirect light against a dark background. Distinct clumping or agglutination within the time period, without clumping in the saline control was regarded as a positive result. Specimens that showed agglutination only with Ogawa-type serum was reported as *V. cholerae* O1 serovar Ogawa. Isolates that were negative for both O1 and O139 were described as non O1, O139 *V. cholerae*.

3.8 Antibiotics Susceptibility Testing

The susceptibility pattern of the isolates to antimicrobial agents was determined using the disc diffusion (Kirby-Bauer) methods as described by the National Committee for Clinical Laboratory Standards (now Clinical and Laboratory Standards Institute) (NCCLS Doc. M2-A6; 2003). A loop full of each isolate was emulsified in 3 mL sterile normal saline in a test tube and the density measured with McFarland densitometer (Grant-bio Den-1 no. 05O102-1109-0368. England) with a total aerobic plate count of 10^6 CFU/mL. A sterile cotton wool was dipped into the standardized

suspension of the bacterial culture and used to spread the surface of Mueller-Hinton plates evenly (Oxoid, Basingtoke, United Kingdom). The plates were allowed to dry for a few minutes. Antibiotic discs (Oxoid, Basingstoke,United Kingdom) with the following concentrations, tetracycline (30 µg), doxycycline (30 µg), trimethroprim/sulfamethoxazole (cotrimoxazole) (25 µg), ciprofloxacin (5 µg), chloramphenicol (30 µg), erythromycin (15 µg), azithromycin (30 µg) and nalidixic acid (30 µg) were place on the plates. Distance between discs was about 15 mm to prevent overlapping of zones of inhibition. The plates were then incubated at 37 ºC for 24 hours, and the zones of inhibition measured with protocol 3 symbiosis Cambridge UK. Each zone of isolates was compared with the recorded diameters of the control organism *E. coli* ATCC 25922 to determined susceptibility or resistance. For the purpose of analysis, all isolates with intermediate zones of inhibition were classified as resistant.

3.9 DNA Extraction

DNA was extracted using Fungal/Bacterial DNA extraction Kits (Zymoresearch Corporation, California, U.S.A) following the manufacturer's protocol. About 50–100 mg *V. cholerae* (wet weight) cells were suspended in 200 µL of phosphate buffered saline (PBS) in a Zymoresearch (ZR) bashingbead lysis tube and 750 µL lysis Solution was added to it. The tubes were secured in a bead beater fitted with a 2 mL tube holder assembly and processed at maximum speed (Eppendorf Centrifuge, Hamburg, Germany) for 5 minutes. The ZR bashingbead lysis tube was centrifuged in a microcentrifuge at 10,000 x g for 1 minute.

A total of 400 µL supernatant was transferred to a zymo-Spin IV Spin Filter in a collection tube and centrifuge at 7,000 x g for 1 minute. A volume of 1,200 µL of bacterial DNA binding buffer was added to the filtrate in the collection tube and 800 µL of the mixture transferred to a zymo-

Spin column in a collection tube and centrifuged at 10,000 x g for 1 minute. The flow through was discard from the collection tube and the process was repeated. DNA pre-wash buffer of volume (200 µL) was added to the zymo-Spin column in a new collection tube and centrifuged at 10,000 x g for 1 minute. A volume of 500 µL of Bacterial DNA wash buffer was added to the zymo-Spin column and centrifuged at 10,000 x g for 1 minute. The zymo-Spin column was transferred to a clean 1.5 mL micro centrifuge tube and 100 µL DNA elution buffer added directly to the column matrix. The resulting solution was centrifuged at 10,000 x g for 30 seconds to elute the DNA. The supernatant containing the DNA was kept in a refrigerator and used later for PCR.

3.10 Detection of Virulent Genes

The *V. cholerae* isolates was examined for the presence of cholera toxin regulatory genes, cholera toxin (ctxA), toxin-coregulated pilus (tcpA $_{\text{El tor}}$), (tcpA $_{\text{classical}}$), zonular occludence toxin (zot), *V. cholerae* outer membrane protein (ompW), O1 somatic antigen (rbfO1) and attRs by PCR assay. Oligonucleotide Primers were synthesized by Inqaba Biotechnical Industries (pty) ltd., South Africa.

Table 3.1. List of primers used for PCR amplifications

Primer	Forward (F) and reverse (R) sequences	Expected band size	Reference
ctx	F-5′−CTCAGACGGGATTTGTTAGGCACG−3′ R-5′−TCTATCTCTGTAGCCCCTATTACG−3′	302bp	Akoachere *et al.*, 2013
tcpA El Tor	F5′−GAAGAAGTTTGTAAAAGAAGAACAC−3′ R-5′−GAAAGGACCTTCTTTCACGTTG−3′	472bp	Akoachere *et al.*, 2013
tcpA classical	F-5′− CACGATAAGAAAACCGGTCCAAGAG-3′ R-5′−ACCAAATGCAACGCCGAATGGAGC3′	618bp	Akoachere *et al.* (2013)
zot	F-5′−TCGCTTAACGATGGCGCGTTTT−3′ R-5′−AACCCCGTTTCACTTCTACCCA−3′	947bp	Akoachere *et al.* (2013)
ompW	F-5′− CACCAAGAAGGTGACTTTATTGTG −3′ R-5′− GAACTTATAACCACCCGCG −3′	588bp	Srisuk *et al.*, 2010
rbfO1	F-5′− TCTATGTGCTGCGATTGGTG −3′ R-5′− CCCCGAAAACCTAATGTGAG −3′	638bp	Srisuk *et al.*, 2010
attRS	F-5′− CCTTAGTGCGTATTATGT −3′ R-5′− ACATAATACGCACTAAGG −3′.	630bp	Srisuk *et al.*, 2010

Amplification of the target gene was carried out by a PCR reaction. A total volume of 25 µL containing 15.87 µL of distilled water, 0.13 µL of *Taq* polymerase, 5µL of PCR buffer, 0.5 µL of dNTPs, 0.5 µL of forward and reverse primer each and 2.5 µL of template DNA was used for each amplification process. Two negative controls were included in each PCR reaction. The cycling profile was initial denaturation of template strand at 94 ºC for 5 minutes, followed by 39 cycles of amplicon denaturation at 94 ºC for 1 minute, primer annealing at 54 ºC for 1 minute and elongation at 72 ºC for 1 minute with final extension at 72 ºC for 10 minutes. Amplification was performed

in a thermocycler (Biometra, Gottingen, Germany). PCR products were separated on 1% Agarose gel electrophoresis containing 2 µL of ethidium bromide. The amplified PCR products were visualized under UV.

CHAPTER FOUR

4.0 RESULTS

Fifty-one (51) *V. cholerae* isolates were used for this study out of which 78.4% (n=40) were archived samples from Public Health Reference Laboratory (Korle Bu) isolated from cholera patients between 2014 and 2015. The environmental samples 21.6% (n=11) were isolated from different water sources. Among the clinical isolates, 50% (n=20) were reported isolated cases in 2014 whiles the other 50% (n=20) were isolated in 2015 from selected communities in Accra. For the environmental isolates, 45.5% (n=5), 4.4% (n=2) and 4.1% (n=4) were isolated from stream, shallow wells and storage tanks respectively as shown in table 4.1.

Table: 4.1. Demographical Data on Prevalence of *V. Cholerae* O1 in the Different Water Samples

Water Source	No. of Samples	*V. Cholerae* Positive.	O1 Positive	O139 Positive	None. O1,O139
Tap	90	5(5.6)	0(0)	0(0)	5(5.7)
Stream	11	8(72.7)	5(45.5)	0(0)	3(27.3)
Shallow Wells	45	6(13.3)	2(4.4)	(0)	4(8.9)
Storage. Tanks	98	14(14.3)	4(4.1)	0(0)	10(10.2)
TOTAL	**244**	**28(11.5)**	**11(4.5)**	**0(0)**	**22(9.0)**

4.1 Culturing of clinical and water samples to detect presence of *V. cholerae*

The different water samples were cultured on TCBS plates to detect the presence of *V. cholerae*. Presumptive positive and clinical isolates were also revived and cultured on TSA plates to obtain pure isolates for further analysis. Out of the 244 water samples cultured, only 28 (11.5%) had the growth of *V. cholerae*.

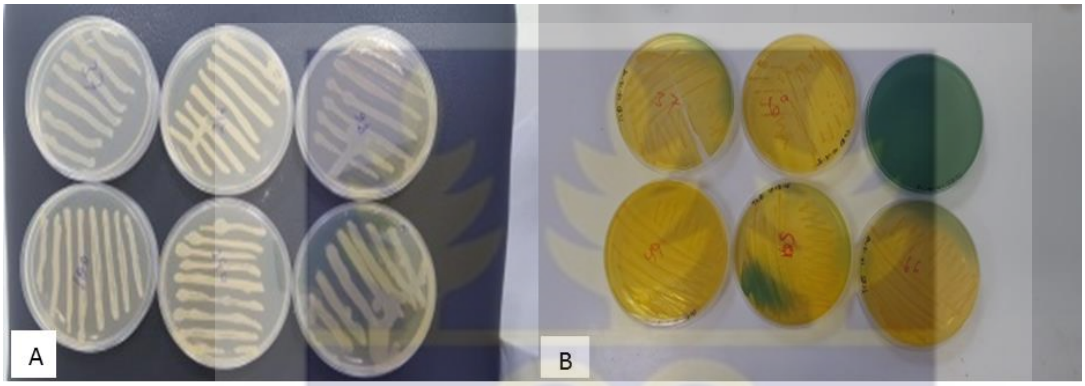

Fig. 4.1. Presumed *V. cholerae* isolates on TSA (A) and TCBS (B) plates after 24 hours incubation at 37ºC. Isolates on TSA are cream mucoid whiles isolates on TCBS appears yellow and creamy.

4.2 Identification of *V. cholerae* Isolates Using Microscopic Method

Identification of *V. cholerae* after gram staining revealed all the bacteria as gram negative, comma shaped bacteria. No gram-positive, isolates were observed after gram staining indicating that the isolates were pure.

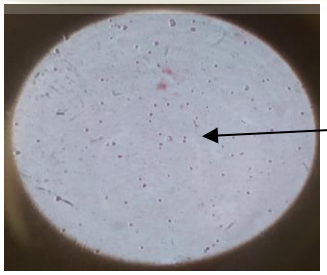

Fig: 4.2. Gram stain of presumed *V. cholerae* isolates under a microscope. *V. Cholerae* appears comma shaped under a microscope

4.2 Serotyping of the *V. cholerae* isolates

Out of the 28 environmental and 40 clinical *V. cholerae* isolates (n=68), 51 were found to be O1 serogroup with serotype Ogawa (Table 4.2). None was Inaba or Hikojima. As represented in table 4.2. Thus, only 51 *V. cholerae* were used for further analysis.

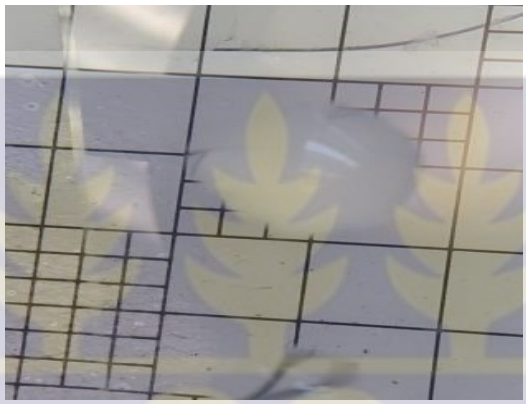

Fig: 4.3 Serological analysis of isolate using the O1 polyvalent antiserum. Agglutination observed represents a positive *V. cholerae* strain

Table: 4. 2. Serotyping and Biotyping of *V. cholerae*

Typing method	Test performed	Serotypes	Number of positive strains (%)	
			Clinical	Environmental
Serotyping	Agglutination (mast group and Denka Seika Kit, Japan)	**Ogawa**	**40(100)**	**11(100)**
		Inaba	0(0)	0(0)
		Hikojima	0(0)	0(0)

4.3 Antibiotic Resistant Patterns

All isolates were screened against eight antibiotics (Table 4.3). Among the clinical isolates, erythromycin and nalidixic acid recorded the highest resistant with 92.5% (n=37) and 72.5% (n=29) respectively. However, 90.0% (n=36) and 85.0% (n=34) of the *V. cholerae* were susceptible to ciprofloxacin and doxycycline respectively. None of the isolates were sensitive to all antibiotics whiles one isolate was resistant to all the eight antibiotics used. None of the environmental isolates was resistant to ciprofloxacin and doxycycline with both recording 100% (n=11) sensitivity. Erythromycin and nalidixic acid again were the least effective against the environmental isolates recording 18.2% (n=2) and 27.3% (n=3) sensitivity respectively as shown in table 4.3.

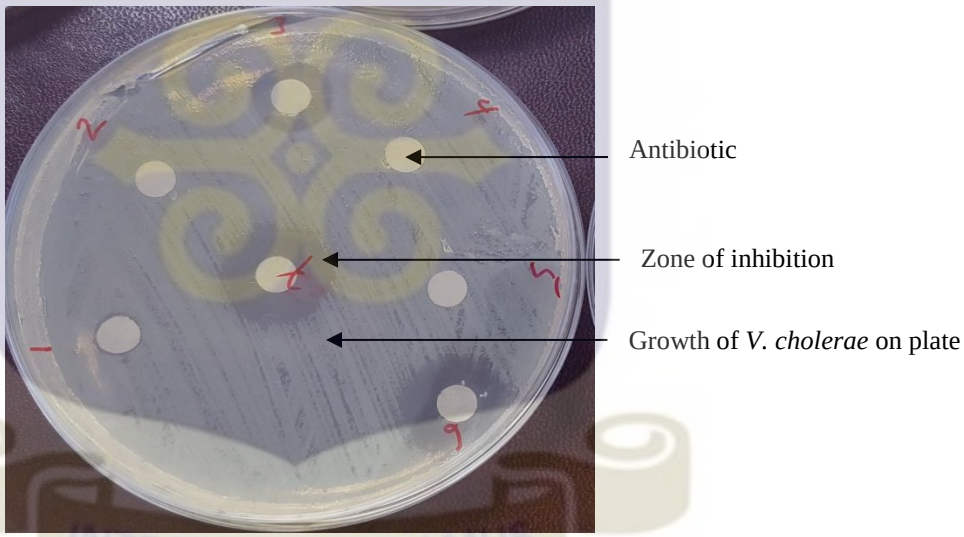

Fig: 4.4. Zones of inhibition of *V. cholerae* on Mueller-Hinton Agar using antibiotic disc with different concentrations as per the manufacturer. Tetracycline (30 µg), Doxycycline (30 µg), Trimethroprim/sulfamethoxazole (cotrimoxazole) (25 µg), Ciprofloxacin (5 µg), Chloramphenicol (30 µg), Erythromycin (15 µg), Azithromycin (30 µg) and Nalidixic acid (30 µg).

Table: 4. 3. General antibiotic susceptibility pattern of *V. cholerae*

ANTIBIOTICS USED	CLINICAL ISOLATE (40)		ENVIRONMENTAL ISOLATES (11)		TOTAL ISOLATES (51)	
	NO. RESISTANT (%)	NO. SENSITIVE (%)	NO. RESISTANT (%)	NO. SENSITIVE (%)	NO. RESISTANT (%)	NO. SENSITIVE (%)
Azithromycin	17 (42.5)	23 (57.5)	6 (54.5)	5 (45.5)	23 (45.1)	28 (54.9)
Ciprofloxacin	4 (10)	36 (90)	0 (0)	11 (100)	4 (7.8)	47 (92.2)
Chloramphenicol	21 (52.5)	19 (47.5)	7 (63.6)	4 (36.4)	28 (54.9)	23 (45.1)
Erythromycin	37 (92.5)	3 (7.5)	9 (81.8)	2 (18.2)	46 (90.2)	5 (9.8)
Trimethoprim-sulfamethoxazol	26 (65.0)	14 (35.0)	4 (36.4)	7 (63.6)	30 (58.8)	21 (41.2)
Tetracycline	14 (35.0)	26 (65.0)	1 (9.1)	10 (90.9)	15 (29.4)	36 (70.6)
Nalidixic acid	29 (72.5)	11 (27.5)	8 (72.7)	3 (27.3)	37 (72.5)	14 (27.5)
Doxycycline	6 (15.0)	34 (85.0)	0 (0.0)	11 (100)	6 (11.8)	45 (88.2)

4.4 Physicochemical Parameters

The highest temperature was recorded in storage tanks with a temperature of 33.2^{o}C followed by tap water with a temperature of 33.0^{o}C. The lowest temperature was however, recorded in streams with a temperature of 25.6^{o}C. There was generally no significant difference in the temperatures for the various water sources (p=>0.05) with the exception of stream and storage tank (p= 0.02), stream and tap water (p=0.005) (F= 4.966) as shown in figure 4.5.

The lowest pH values were recorded in well water (pH=4.78) with the highest been recorded in tap water (pH=8.75). The only significant difference between the various water sources with regards to pH was observed in storage tanks and well (p=0.03) and storage tank and tap water (p=0.005) with no significant difference among the rest (p=>0.05). The average Salinity, total dissolved solids and conductivity values were between 25.6, 4.97 and 9.83 to 705, 1126 and 1412 respectively. There was no significant difference among the various water sources with respect to conductivity (p >0.05) with the exception of storage tanks and well (p=0.0003).

Total dissolved solids recorded a significant difference between storage tanks and well (p=0.00O1), tap water and well (p=0.002) with no significant difference between the rests (p> 0.05). With regards to salinity there was a significant difference between the entire water source (p<0.0O1) except storage tank and well (p=0.2) as represented in figure 4.5.

Table: 4. 4. General physicochemical parameters (pH, temperature, TDS and conductivity) of water sources.

WATER SOURCE		SAL	TDS	COND	TEMP (°C)	pH
STREAM	HIGH	693	1116	1398	33	7.95
	LOW	4.97	7.88	9.83	25.6	7.42
TAP	HIGH	291	467	583	33.0	8.75
	LOW	37.1	59.6	74.1	27	6.29
S. WELLS	HIGH	705	1126	1412	31.6	8.63
	LOW	41.1	65.8	82.3	27.8	4.78
S. TANKS	HIGH	328.0	524.0	656.0	33.2	8.09
	LOW	35.2	56.4	70.5	28.1	5.71

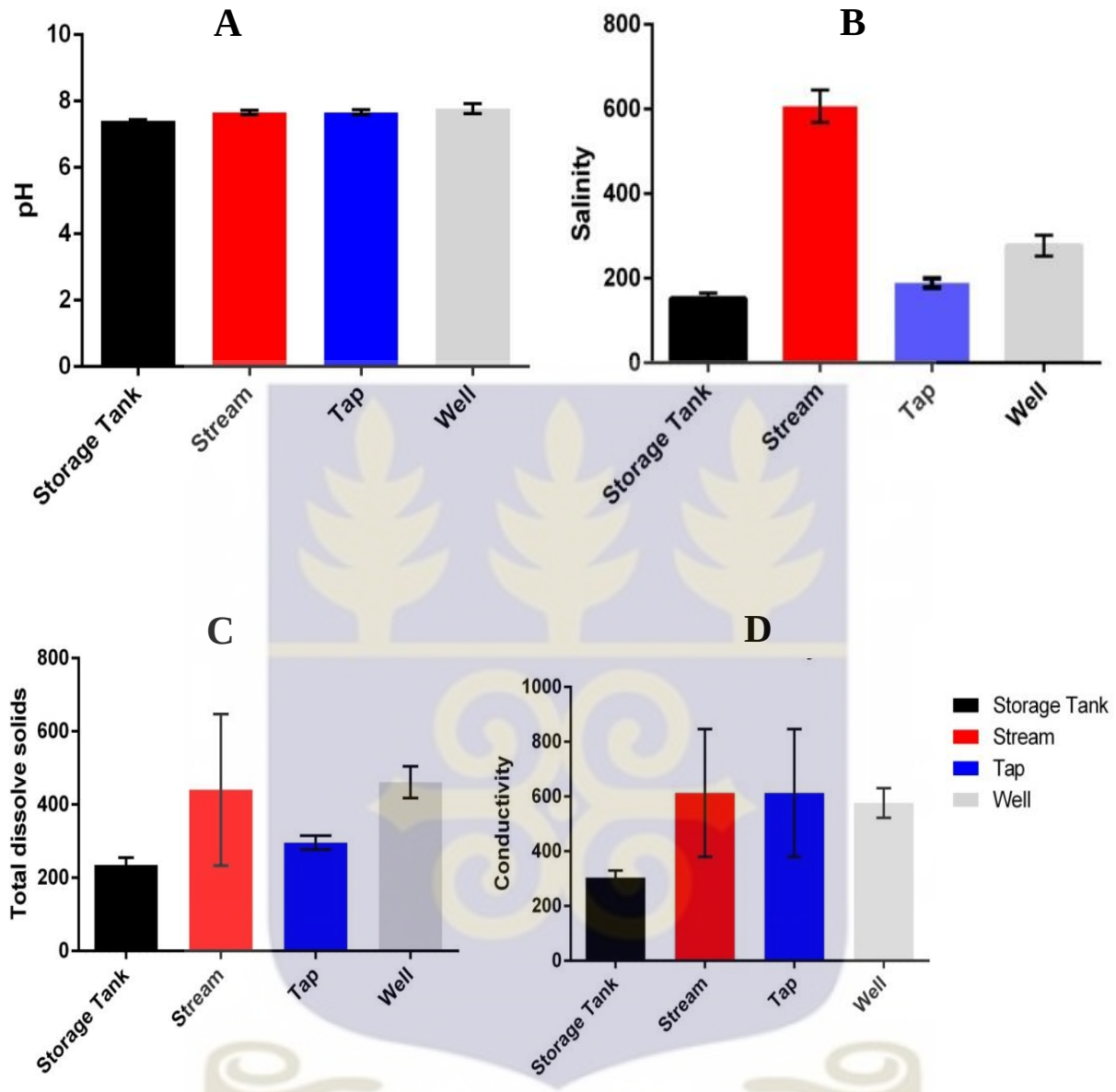

Fig: 4.5. Differences in the physicochemical parameters (A-pH, B- Salinity, C-Total Dissolved Solids D-Conductivity.) of water with respect to the source of collection.

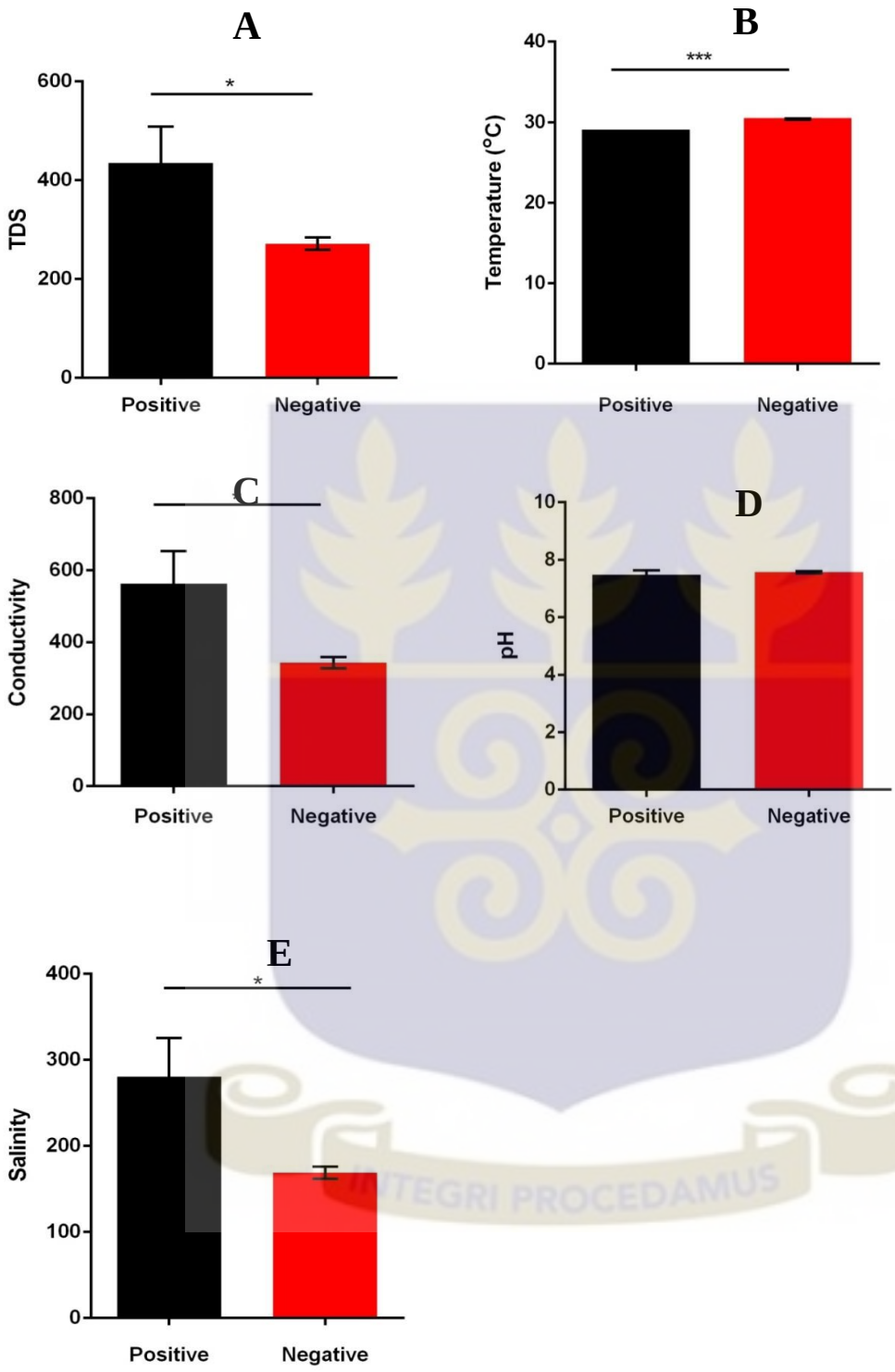

Fig: 4.6. Effect of Physicochemical Parameters (A-Total dissolved solids, B-Temperature, C-Conductivity D-pH, E- Salinity) on occurrence of *V. Cholerae O1* in the sample area.
*** = p<0.05, *** = p<0.0001.**

4.5. Genotyping and Detection of Virulent Genes

PCR results revealed the presence of at least one of the virulent genes in both clinical and environmental isolates as shown in table 4.5. Ctx gene was detected in 85% (n=34) and 72.7% (n=8) of the clinical and environmental isolates respectively. All isolates (100%) were positive for the gene coding for tcpA $_{\text{El Tor.}}$ A total of 77.5% (n=31) and 81.8% (n=9) of clinical and environmental isolates had zot and tcpA $_{\text{classical}}$ genes respectively. The ctx attachment site attRS was found to be present in 82.5% (n=32) and 63.6% (n=7) for both clinical and environmental isolates respectively. RbfO1 gene coding for the serogroups O1 was detected in 90% (n=36) and 81.8% (n=9) in clinical and environmental isolates respectively. Two isolates representing 5% and 18% of the clinical and environmental isolates were found to lack all the genes except the gene coding for tcpA $_{\text{El Tor}}$. A total of 55% (n=22) and 36.4% (n=4) were positive for all the genes while 77.5% (n=31) and 81.8% (n=9) were found to contain both tcpA $_{\text{El Tor}}$ and tcpA $_{\text{classical}}$ genes.

4.6. PCR Product of *V. cholera*, Biotype El tor

PCR revealed the presence El tor biotype gene (475bp) in 100% of both clinical and environmental isolates as shown in the electrophoretogram below. This indicates the prevalence of the El tor biotype in the research area. As much 77.5% and 81.8% of clinical and environmental isolates harbored with the El tor isolates harbored classical genes. None of the isolates were found to contain only the classical gene.

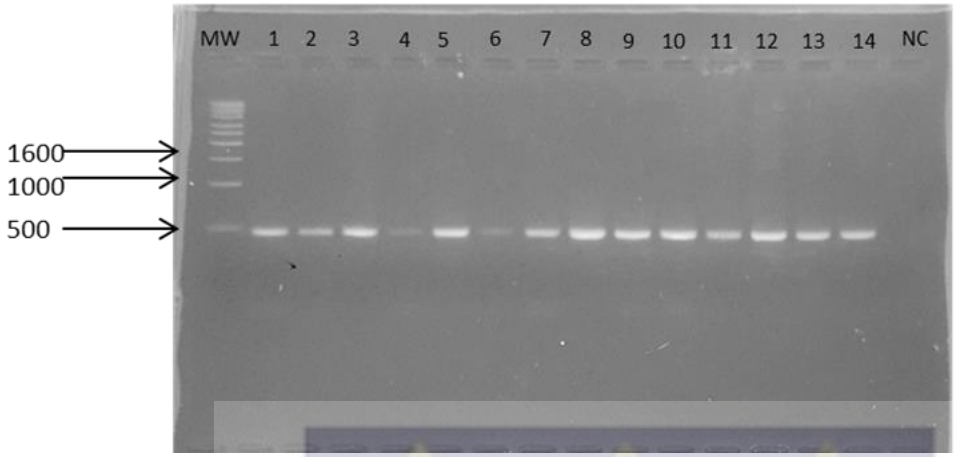

Fig: 4.7. An electrophoretogram of PCR product of *V. cholerae* El tor gene isolates. Lane 1: 1 kb plus DNA ladder; NC: negative control; lanes 2 to 14: a PCR product (475bp) of *V. cholerae* isolates 1-14 for El tor.

4.7 PCR Product of *V. cholera* toxin gene (ctx)

Analysis of the 51 *V. cholera*e for the presence of the cholera toxin gene (ctx), detected the ctx gene with 302 bp in 42 (82.4%) of the isolates. This shows that a high percentage of the cholera toxin gene present among the isolates.

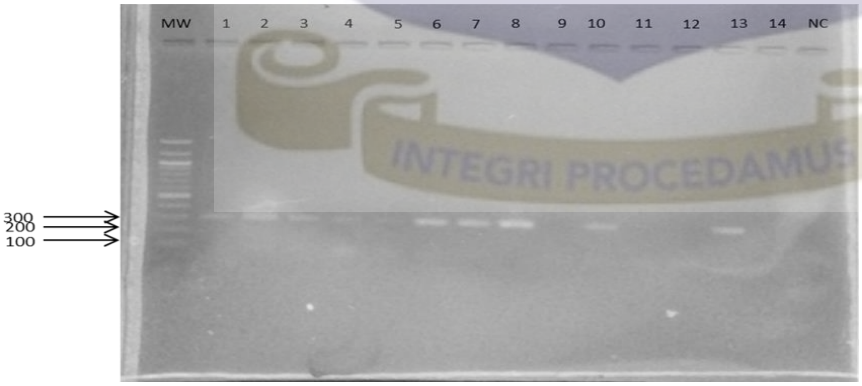

Fig: 4.8. An electrophoretogram of PCR product of the cholera toxin gene (ctx). Lane 1: 100 bp DNA ladder; NC: negative control; lanes 2 to 14: PCR product (302 bp) of *V. cholerae* isolates for ctx gene

Table: 4.5. Virulent genes in *V. Cholerae* O1 isolated from clinical and environmental samples

Gene	Total Number Positive (%)	
	Clinical	**Environmental**
ctx	36 (90)	7 (63.6)
zot	31 (77.5)	9 (81.8)
attRS	38 (95)	8 (72.7)
ompW	35 (87.5)	6 (54.5)
tcpA $_{\text{El Tor}}$	40 (100)	40 (100)
ctcpA $_{\text{classical}}$	31 (77.5)	9 (81.8)
rbfO1	36 (90)	9 (81.8)

Based on the genes analyzed, 11 and 5 genotypes of *V. cholerae* O1 were identified for clinical and environmental isolates respectively. Genotyping of the genes revealed genotype$tcpA_{El}^{+}ompW^{+}ctx^{+}attRS^{+}rbfO1^{+}zot^{+}tcpA_{cl}^{+}$as the most predominant genotype with 55% (n=22) and 36.6% (n=4) in clinical and environmental isolates respectively. Genotype El tor^{+}ompW^{-}ctx^{-}attr^{+}rbfO1^{+}zot^{+}classical^{+}was the least detected with 2.5% (n=1) and 9.1% (n=1) in both clinical and environmental isolates respectively as shown in table 4.6.

Table: 4.6. Genotypes of *V. Cholerae* O1 isolates

Genotype	Number of Isolates (%)		
	Clinical	**Environmental**	**Total**
El tor^{+}ompW^{+}ctx^{+}attrRS^{+}rbfo1^{+}zot^{+}classical^{+}	22 (55)	4 (36)	22(51)
El tor^{+}ompW^{-}ctx^{+}attrRS^{+}rbfo1^{+}zot^{+}classical^{+}	0(0)	2(18)	2(3.9)
El tor^{+}ompW^{+}ctx^{-}attrRS^{+}rbfo1^{+}zot^{+}classical^{+}	1(2.5)	0(0)	1(2.0)
El tor^{+}ompW^{-}ctx^{-}attrRS^{+}rbfo1^{+}zot^{+}classical^{+}	1(2.5)	1(9.1)	2(3.9)
El tor^{+}ompW^{+}ctx^{+}attrRS^{-}rbfo1^{+}zot^{+}classical^{+}	2(5.0)	2(18.0)	4(7.8)
El tor^{+}ompW^{+}ctx^{+}attrRS^{+}rbfo1^{+}zot^{-}classical^{+}	0(0)	4(36.0)	4(7.8)
El tor^{+}ompW^{+}ctx^{+}attrRS^{-}rbfo1^{+}zot^{-}classical^{-}	1(2.5)	0(0)	1(2.0)
El tor^{+}ompW^{-}ctx^{-}attrRS^{-}rbfo1^{-}zot^{-}classical^{-}	2(2.5)	0(0)	4(7.8)
El tor^{+}ompW^{-}ctx^{+}attrRS^{-}rbfo1^{+}zot^{+}classical^{-}	3(7.5)	0(0)	1(2.0)
El tor^{+}ompW^{+}ctx^{+}attrRS^{+}rbfo1^{+}zot^{+}classical^{-}	1(2.5)	0(0)	3(5.9)
El tor^{+}ompW^{-}ctx^{-}attrRS^{-}rbfo1^{-}zot^{+}classical^{-}	1(2.5)	0(0)	1(2.0)
El tor^{+}ompW^{-}ctx^{+}attrRS^{-}rbfo1^{-}zot^{-}classical^{-}	2(5.0)	0(0)	1(2.0)
El tor^{+}ompW^{-}ctx^{+}attrRS^{-}rbfo1^{-}zot^{-}classical^{-}	1(2.5)	0(0)	1(2.0)

CHAPTER FIVE

5.0 DISCUSSION

Cholera continues to pose a serious treat and public health significance especially in developing countries. This has been compounded by the lack of infrastructure and economic development leading to inadequate potable water supply and poor sanitation. Between June to November 2014, Ghana recorded its highest cholera outbreak in the country's history (GHS, 2014) with most of the cases occurring in Accra, the capital city of Ghana. Since cholera is associated with poor sanitation and water supply, high prevalence rate reflects lack of access to basic health needs (CDC, 2013). This therefore, calls for proper provision of basic health needs and adequate data on cholera prevention and control strategies to curb the menace.

The results from this study, shows low prevalence of *V. cholerae* O1 strains in environmental samples. Out of the 244 water samples analyzed, only 11 (4.5 %) were found to belong to the O1 serogroup, whiles 22 (9.0 %) isolates belong to non-O1, O139 serogroup as shown in Table 4.1. Contrary to this study, previous studies have reported the absence of *V. cholerae* O1 in environmental samples (Akoachere *et al.*, 2013, Gaudart *et al.*, 2013, Baron *et al*, 2013). However, similar patterns of low prevalence of *V. cholerae O1* in environmental samples comparable to what was found in this study have been reported in Haiti (Alam *et al.,* 2014) and other endemic areas such as Bangladesh (Hug *et al.,* 2005). Alam *et al.,* 2014, have also reported the presence of *V. cholerae* O1 Ogawa biotype El-tor strains in the environment in frequencies comparable to what was observed in this study (Alam *et al.,* 2014). It has been reported that majority of *V. cholerae* in environmental isolates belongs to the non O1/ O139 serogroups (Louis *et al.,* 2003), as was evident in this study. The non O1/O139 serogroups have also been isolated from cases of cholera-like diarrhoea and other intestinal infections in clinical settings (Issa *et al.,* 2009).

It has been noted that, the pathogen thrives best in saline environment, but can also survive in low saline environment provided the environment is warm and has adequate organic nutrient (Alam *et al*., 2015). Therefore, the low presence of the pathogen in tap water could be attributed to its low salinity and lack of organic matter. It could also be due to the fact that the source is regularly disinfected. *V. cholerae* are noted for existing in a viable but non-culturable state (VBNC) in an unfavorable condition (Wu *et al.,* 2016). This could also be a contributing factor for the low levels of isolates from the various sources. Owing to the fact that VBNC forms could revert to transmissible state when conditions becomes favorable, it is advisable to implement cholera control strategies in endemic areas even in the absence of *V. cholerae* detection in the environment.

The prevalence of *V. cholerae* in the study area could be higher than what is reported by this study since we did not include VBNC *V. cholerae*. The current study has also revealed the co-existence of O1 and non O1 strains in the environment. Studies by Faruque *et al*. have also reported similar co-existence and possible gene transfer during co-existence of O1 and non O1/O139 serogroups resulting in the emergence of novel pathogenic strains. This study has also emphasized the absence of O139 serogroup in the African continent as reported by similar studies across Africa (Faruque *et al.*, 2003). Contrary to observation by Akoachere *et al*. (2013), who recorded zero isolates of *V. cholera* O1 from water samples, this current study isolated *V. cholerae* O1 from streams (5), storage tanks (4) and shallow wells (2). This could be due to a spillover from the 2014 major outbreak in Ghana resulting in domestic contamination or the open nature of some of the storage tanks and wells as was evident during sampling. It however, agrees with report in Zimbabwe where *V. cholerae* was isolated in domestic water samples and streams after the 2009 major cholera outbreak (Chambers, 2009). This therefore, confirms water sources as one of the reservoirs of the organism.

The highest number of isolates from streams was not surprising, as streams are used as dump site for human, animal and domestic waste. It is reported that about 70% of city dwellers in Ghana lack access to public places of convenience and hence, resort to the use of streams and gutters (GHS, 2013). Most of the wells in the sampling sites were not covered which allowed easy contamination of the water in the wells. Some were also located close to gutters and communities which allowed for easy contamination. Similar works in Orissa, revealed that wells (shallow underground water source) are easily contaminated with *V. cholerae* and people close to wells are more affected than those in distant areas (Mukherjee *et al.*, 2011). Absence of the pathogen in tap water shows the important role that safe drinking water plays in the fight against cholera and other enteric diseases. This is because tap water is well treated before distribution and contaminations can only results through broken pipes along distribution lines.

Serotyping of the *V. cholerae* isolates revealed the presence of 100% (n=40) and (n=11) for both clinical and environmental isolates respectively. No Inaba or Hikojima was recorded in this study. Serotype Ogawa has been found to predominate in Africa (Thapa Shrestha *et al.*, 2015). A recent study by Eibach *et al* (2016), revealed the presence of 95.7% Ogawa and only 1.1% Inaba serotype among the *V. cholerae* isolates.

It has been shown that effective use of antibiotic reduces the duration of diarrhoea, volume of stool losses by up to 50% and also reduces the duration of shedding of viable organisms in stool by patients from several days to 1-2 days (Folster *et al.*, 2014). Eight antibiotics recommended by the WHO and Ghana Health Services (Tetracycline, doxycycline, ciprofluxacin, erythromycin, Chloramphenicol, azitromycin, trimethoprim/sulfamethoxazole, nalidixic acid) were used in this study (GHS, 2011). This study demonstrated a loss of sensitivity to a lot of antibiotics such as erythromycin and nalidixic acid used for first line treatment. Similar incidence has also been

reported in Democratic Republic of Congo by Miwanda *et al.* (2015). Erythromycin and nalidixic acid recorded the lowest level of sensitivity 92.5% (n=37) and 72.5% (n=29) respectively. Thepa Shrestha *et al*. (2015) reported 100% resistance of both clinical and environmental isolates to nalidixic acid in Kathmandu city in Nepal (Thepa Shrestha *et al*., 2015). This study is in line with a work in Ghana by Kuma *et al.* (2014) who reported 94.4% resistance to erythromycin. This revealed resistance trends in macrolides as observed in this study. The report however, recorded only 44.6% resistance to nalidixic acid as against the 72.5% resistance reported in this current study. Des *et al.* (2013) also reported 100% resistance to nalidixic acid. Similar trends have also been seen in South America (Goel *et al.,* 2010, Choudhry *et al*., 2011,). A survey from 2004 to 2005 in Cameroon showed that all *V. cholerae* strains were susceptible to tetracycline and resistant to nalidixic acid which is in agreement with what was observed in this study (Kansakar *et al.,* 2011). A total of 58.8% (n=30) isolates were resistant to trimethoprim/sulfamethoxazol. Similar resistant trend has also been reported in Haiti during the 2010 cholera outbreak in the country (Folster *et al.,* 2014).

The steady increasing resistance by *V. cholerae* to nalidixic acid between 2010 and 2012 in Ghana was also similar to that observed in the 2010 Haitian cholera outbreak (Nelson *et al.,* 2010). Resistance was also found in sulfamethoxazole/trimethoprim, Chloramphenicol and tetracycline. Similar resistance patterns have been reported from various countries worldwide including Ghana (Quilici *et al.,* 2010, Marin *et al.,* 2013). Since 1980, there has been a stepwise increase in the minimum inhibitory concentrations (MIC) to quinolones (Folster *et al.,* 2014). The 45.1% resistance to azitromycin was in contrast with report by Kuma *et al* in 2014, who reported 0% resistance among 11 clinical isolates. This difference could be due to the differences in sample size and passage of time. Eibach *et al.* (2016) have reported an increasing trend of *V. cholerae* O1

to most antibiotics between 2011 and 2014 in Ghana. Resistance to these antibiotics could be due to large scale abuse or extensive use of these antibiotics for the treatment of other infectious diseases in Ghana other than for the treatment of cholera. This may have resulted in the selection of resistant strains.

Although virulence factors do not include drug resistance, it may play a vital role in the selection, persistence and dissemination of pathogenic strains making it difficult to eradicate. Ciprofloxacin and doxycycline recorded the highest percentages of sensitivity towards *V. cholerae* O1 with 92.2% and 88% respectively. This was however, not surprising as similar reports have also been made (Kuma *et al.*, 2014; Shretha *et al* 2015, Eibach *et al.*, 2016). The effect of ciprofloxacin and doxycycline also compares favorably with reports in India that reported high levels of sensitivity to ciprofloxacin and doxycycline (Goel *et al.*, 2010). The resistance pattern in the environmental isolates in this current study was not different from what was observed in the clinical isolates. It was however, noted that none of the environmental isolates was resistant to both ciprofloxacin and doxycycline. Similar trends have also been reported by Thapa Shrestha *et al* in Nepal (Thapa Shrestha *et al.*, 2015).

One limitation of this study is that only eleven environmental isolates were used and hence cannot be said to be conclusive on the general antibiotics resistance pattern observed among the environmental isolates. All clinical isolates were resistant to one or more antibiotics indicating the emergence of multidrug resistance (MDR) strains. This observation is similar to other MDR's strains which have been observed in all parts of the world (Thompson *et al.*, 2011). A studies in Ghana on clinical isolates in 2006 reported 31% *V. cholerae* isolated to be MDR (Thompson *et al.*, 2011). These MDR's reported in 2006 may have been disseminated into the environment resulting in the high number of MDR's recorded in this study. It could also be as a result of

spontaneous mutation or the horizontal transfer of resistance genes by co-existing with gut coliforms microflora and *Vibrio* spp (Wang *et al*., 2016). It could also be due to changes in the chromosomal DNA (Baranwal *et al.*, 2002).

Chromosomal mutations have been found to be responsible for enconding DNA gyrase and topoisomerase IV subunits resulting in its affinity for antibiotics. This mutation has been implicated to confer resistance to quinolones on *V. cholerae*. The steady increase in resistance to quinolones could also be as a result of mutations in gyrA and parC genes due to environmental and drug pressure as was reported in other study (Kim *et al.*, 2010). Studies has shown that, *V. cholerae* strains may contain a mutation in only the gyrA gene and additional mutation can be gained in parC by these strains over time, increasing their quinolones resistance. This phenomenon could also account for the differences in drugs resistance between this study and earlier studies in Ghana. It is therefore, possible that the resistance to any of the above named antimicrobial compounds is due to spontaneous chromosomal mutations.

The effectiveness of ciprofloxacin and doxycycline is re-assuring as WHO recommends the use of doxycycline and ciprofloxacin as treatment of choice for cholera (Gelbíčová, *et al.*, 2016).

Cholera dynamics through a change in pathogen, host, reservoir, species abundance or population interaction have been reported to be influenced by environmental factors such as pH, salinity, total dissolved solids and temperature (Del Refugio *et al.*, 2005). This study therefore, investigated the physicochemical factors in relation to the occurrence of *V. cholera* O1. Temperature ranged from 25.6^{o}C to 33.20C for the various water sources. Generally, there was no significant difference in the temperature of the various waters sources ($P>0.05$, $F=4.966$). However, there was a significant

difference in temperatures between storage tanks and stream (p=0.02), stream and tap water (p=0.005).

This report found a correlation between temperature and the occurrence of *V. cholerae* O1 in the various water sources. This observation contradicts with reports by Akoachere *et al.*, (2014) who reported no correlation between temperature and occurrence of *V. cholerae* O1 in water source. Although, other studies have also reported no correlation in isolation pattern of *V. cholerae* and maximum temperature recorded, others have reported temperature correlation with the occurrence of *V. cholerae* (Del Refugio *et al*., 2005 and Blackwell *et al*., 2008) This study is only suggestive but a longer duration of sampling in our study area will permit valid conclusions about the influence of temperature on the occurrence of *V. cholerae.* The pH values ranged from slightly acid (4.78) to slightly basic (8.75). There was a significant difference in the pH for storage tanks, tap water and well ($p<0.05$, $F= 4.87$). However, there was no significant difference between tap water, well and stream ($p=>0.05$). The optimal pH for *V. cholerae* isolation has been reported to be 7.0 to 8.5. The organism gets inactivated below a pH of 4.5 (WHO, 2007). The pH values recorded in this study are similar to the optimal pH of 8.5 as reported by Akoachere *et al* (Akoachere *et al*., 2014). There was no correlation between *V. cholerae* occurrence and pH ($p>0.05$) as seen in figure 4.6. This finding is similar to report by Blackwell and Oliver (Blackwell & Oliver., 2008). It is however, in variance with report by Akoachere *et al.* (2014), who also reported a significant correlation between pH and occurrence of *V. cholerae*. Values for salinity, conductivity and total dissolved solids ranged from as low as 4.97, 7.88 and 9.83 to 705, 1126 and 1412 respectively. Significant difference was observed in salinity for all the various water sources except storage tanks and well ($p=0.05$, $f=25.74$). Most people in the study area get water from tap water and store in their storage tanks and this could have accounted for the salinity indifference

between storage tanks and tap water. Significant difference was also seen in tap water and well and storage tank and well with respect to total dissolved solids. This difference observed between tap water and well can be attributed to the open nature of most of the wells in the study area enabling contamination of the wells. This could also be accounting for the difference in conductivity observed between storage tank and well (p=0.05) with no significant difference noticed in the rest of the various water sources with regards to salinity, conductivity and total dissolved solids. There was a strong correlation between the occurrence of the pathogen and the three physicochemical parameters. This report is in line with reports by Louis *et al*. (2003) and Akoachere *et al*. (2014), who also reported similar findings. Salinity has also been demonstrated to have a strong influence on the growth of *V. cholerae* especially at higher temperatures (Louis *et al.,* 2003).

V. cholerae genotyping shows the level of relatedness of the strain and its importance in epidemiological study or the virulent nature of the infection. All the *V. cholerae* isolates; both clinical and environmental, were of the tcpA $_{\text{El tor}}$ biotype. However, tcpA $_{\text{classical}}$ gene was also identified in 77.5% and 81.8% of the clinical and environmental isolates respectively. Even though, it is being reported that *V. cholerae* was either one of the two biotypes, recent study conducted, reported the presence of *V. cholerae* strains that harbors both the tcpA $_{\text{El tor}}$ and tcpA $_{\text{classical}}$ gene (Das *et al*., 2016). It is reported that *V. cholerae* O1 El Tor strains has a better adaptability in the environment and are able to colonize more effectively in the intestinal lumen than the classical biotype. However, the classical biotype possess cholera toxin that are able to cause more fluid accumulation that the El Tor (Ghosh *et al*., 2010) this implies that, the tcpA $_{\text{El tor}}$ carrying the tcpA $_{\text{classical}}$ gene popularly known as the 'El Tor hybrid' is more virulent than strains

with a single biotype (Ghosh-Banejee *et al*., 2010; Eibach *et al*., 2016). This may account for the high pandemic cholera outbreak in 2014 in Ghana.

One of the genetic elements associated with virulence in pathogenic O1 and O139 *V. cholerae* are a lysogenic filamentous bacteriophage which encodes the cholera toxin (ctx). The dissemination of this bacteriophage may be associated with the derivation of toxigenic *V. cholerae* strains from non-toxigenic progenitors. Therefore, the higher the prevalence of the ctx gene, the more virulent or toxigenic the cholera outbreak will likely be. In this study, we identified 85% and 72.7% of the ctx gene in both the clinical and environmental isolates. This could also explain the fast and large spread of the 2014 cholera outbreak in Ghana. Zonular occludens toxin (zot) has been found to increase the permeability of the small intestinal mucosa by affecting the structure of the intercellular tight junctions. This gene was found in 77.5% of the clinical and 81.8% of the environmental isolates. Zot gene has been postulated to be present in toxigenic *V. cholerae* strains and likewise the ctx gene in zot positive strains (Ghosh *et al*., 1997; Jiang., 20O1). However, contrary to these studies, we identified 15% (n=6) of the clinical isolates possessing the ctx gene without the zot gene as well as 10% (n=4) of the zot gene without the ctx gene in the clinical *V. cholerae* isolates. Similar to this is a study conducted by Akoachere *et al*. (2013) who reported the presence of zot gene without ctx gene in a non-toxigenic *V. cholerae*. These findings may suggest that the zot gene can occur independently of the ctx gene and as such can be used to explain the ability of some *V. cholerae* strains to cause illness in the absence of the cholera toxin. The absence of the ctx gene could be due to ctxφ prophage genome missing or disrupted by mutations. This could mean that these isolates without the ctx genes are defective a phenomenon which is very common among all bacteria genera.

The ompW gene has been found to be specie-specific for *V. cholerae* and its presence confirms the presence of the bacteria. The presence of both ompW and rbrO1 genes is an indication that the isolates were *V. Cholerae* and belong to the O1 serogroup 1 antigen of the "O" side chain of the LPS (serogroup). The absence of both rbfO1 and ompW genes in two clinical and four environmental isolates could be due to the fact that these isolates belong to different serogroups of *V. Cholerae*. In all, the predominant genotype among the *V. cholerae* isolates is El tor^{+}, ompW^{+},ctx^{+},attRS^{+},rbfO1^{+},zot^{+},classical^{+}. These type of the *V. cholerae* have the el tor biotype genes harboring the classical genes as well as all the virulent genes used in this study. It is thus, not surprising that the severity of the 2014 cholera outbreak was almost uncontrollable.

5.2 CONCLUSION

This study showed an increasing trend in multidrug resistant *Vibrio cholerae* O1 with pathogenic potential in water sources. The pathogenic strains co-exist with non O1/O139 which are non-pathogenic in study area. However, ciprofloxacin and doxycycline still remain effective in the treatment of *V. cholerae* in both clinical and environmental isolates. *V. Cholerae* serotype Ogawa biotype El Tor is the main biotype circulating in Ghana with the emergence of hybrid strains. The virulent genes associated with cholerae outbreak can be identified in both the clinical and environmental isolates of *V. cholerae*. Temperature, salinity, total dissolved solids and conductivity are among the factors maintaining the persistence of the organism in different water sources. Findings indicate an urgent need for the appropriate use of antibiotics and provision of potable water supply in study area and in addition, regular disinfection of water from contaminated sources to prevent outbreak of cholera.

5.3 RECOMMENDATION

Based on our findings, we recommend:

- The expansion of potable water distribution and provision of appropriate sanitary infrastructures in the study area.
- Continuous monitoring of both clinical and environmental isolates for their antibiotic profile.
- Routine "well" inspection and disinfection as measures for disease prevention in study area.
- Appropriate use of antibiotics to reduce drug resistance.
- Detection of conditional viable cells in water sources to establish the true prevalence of *V. cholerae* in the study area.
- Further studies to determine the virulent genes among the *V. cholerae* non-O1/139 strains

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Appendix A

ANTIBIOTICS SUSCEPTIBILITY OF THE ISOLATES

CLINICAL ISOLATE	AZI	CIPRO	CHLO	ERYT	TRIM-SULF	TETRA	NAL-ACID	DOXY
CI1	R	R	R	R	R	R	R	R
CI2	R	S	R	R	R	S	S	S
CI3	R	S	R	R	S	S	R	S
CI4	S	S	R	R	S	S	R	S
CI5	S	S	R	R	R	S	R	S
CI6	S	S	S	R	R	R	R	S
CI7	R	S	S	R	R	R	S	S
CI8	R	S	S	R	R	S	S	S
CI9	S	S	S	R	R	S	R	R
CI10	S	S	S	R	S	R	R	S
CI11	R	R	R	R	S	R	S	S
CI12	S	S	S	R	R	R	R	S
CI13	R	S	S	R	R	S	S	S
CI14	R	S	S	R	R	S	R	S
CI15	S	S	R	R	S	S	R	S
CI16	S	S	R	R	S	R	R	S
CI17	S	S	R	S	R	S	R	S
CI18	R	S	R	R	R	S	S	R
CI19	R	S	R	R	S	S	S	S
CI20	S	S	S	R	R	S	R	S
CI21	S	S	R	R	R	S	R	S
CI22	S	S	S	R	S	S	R	S
CI23	S	S	S	R	R	S	R	R
CI24	R	S	R	R	R	R	R	R
CI25	R	R	R	R	R	R	S	S
CI26	S	S	S	R	S	R	R	S
CI27	S	S	S	R	R	S	R	S
CI28	S	S	R	R	S	S	R	S
CI29	R	R	S	R	R	S	R	S
CI30	R	S	S	R	R	R	R	S
CI31	R	S	R	R	R	S	R	S
CI32	S	S	R	R	S	S	R	R
CI33	S	S	S	S	R	R	S	S
CI34	S	S	S	R	S	R	S	S
CI35	S	S	R	R	R	S	R	S
CI36	S	S	R	R	R	S	R	S
CI37	R	S	R	R	R	R	R	S

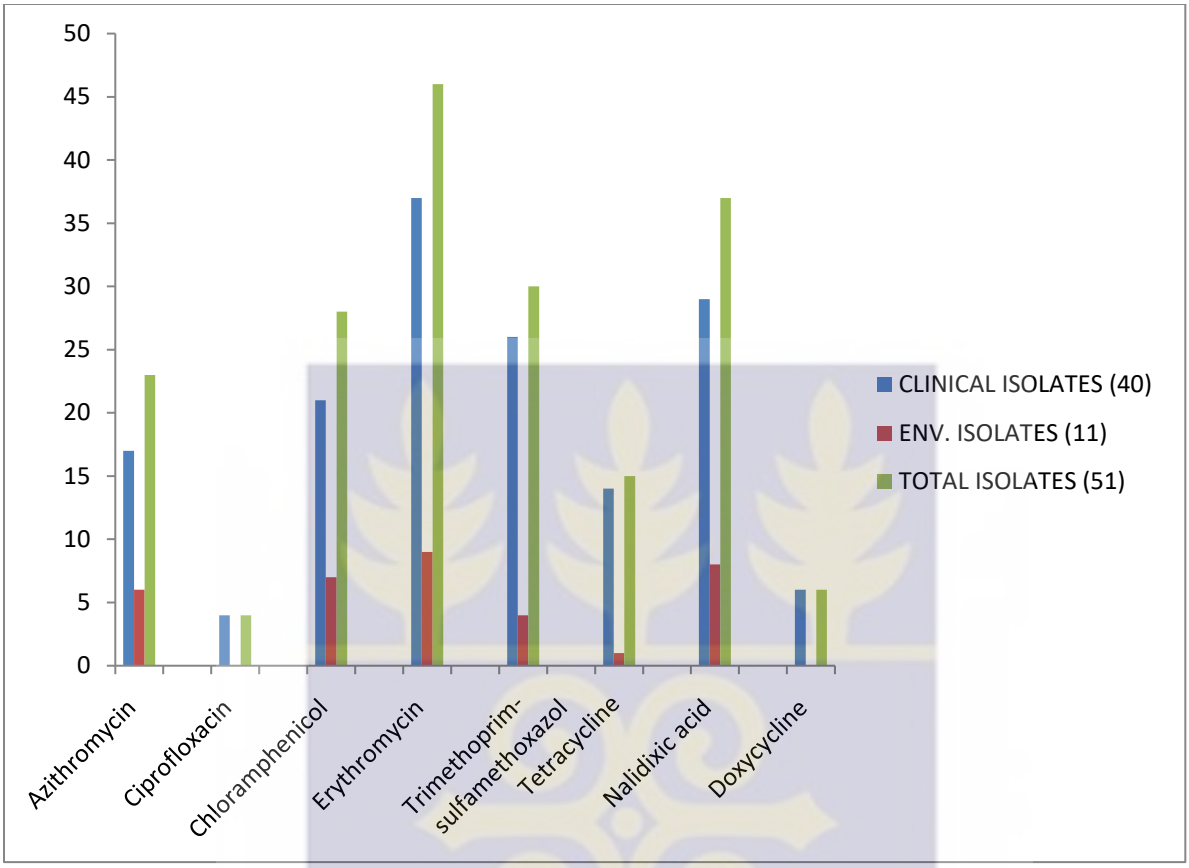
CI38	R	S	S	R	S	S	R	S
CI39	S	S	R	R	S	S	S	S
CI40	S	S	S	R	R	S	R	S
				ENVIRONMENTAL ISOLATES				
EI1	R	S	R	R	S	S	S	S
EI2	S	S	R	R	R	S	R	S
EI3	R	S	S	R	S	S	R	S
EI4	S	S	R	R	S	S	R	S
EI5	R	S	S	S	S	S	S	S
EI6	R	S	R	R	R	S	R	S
EI7	R	S	R	R	S	S	R	S
EI8	S	S	R	R	R	R	R	S
EI9	S	S	S	R	R	S	S	S
EI10	S	S	S	S	S	S	R	S
EI11	R	S	R	R	S	S	R	S

REAL VALUES OF ANTIBIOTICS SUSCEPTIBILITY OF ISOLATES

	CLINICAL ISOLATES							
	AZI	CIPRO	CHLO	ERY	TRIM-SULFA.	TETRA	NALID. ACID	DOXY
CI1	6.8	8.2	0	0	0	0	8	9.1
CI2	8	26.3	0	0	0	16.4	23.1	23.8
CI3	7.3	26.4	8.1	7	18.7	18.7	12	30
CI4	24.1	28.1	9.8	11	19.6	23.1	11.5	19.7
CI5	30.3	19.5	7	9.6	0	22	9	16.9
CI6	23.6	16.6	19.8	0	0	7.4	6	18.4
CI7	11.2	17	16.9	0	0	4	16.9	20
CI8	9.7	21.6	20.4	0	0	16.3	17.5	18.6
CI9	24.9	27.3	18.8	0	0	18.4	0	11
CI10	31.1	17.6	16.7	0	17.4	0	0	28.6
CI11	9.4	9.5	8	0	15.5	11.7	18	31
CI12	24	21.8	23.1	0	0	6.2	7	28.5
CI13	12.3	20.7	18.9	0	0	17.4	19.6	28.4
CI14	10.6	16.4	17.6	0	0	18.8	0	29.1
CI15	20.4	17.3	5.7	0	17.2	26.1	0	26.4
CI16	21.6	18.8	4	0	18.4	0	0	17.4
CI17	19.1	15.7	9	16.2	0	18.7	0	19.5
CI18	11.2	16.4	6	4	9.5	17.7	16.5	12
CI19	8.2	20.1	4	8	15.7	17.4	15.9	17.5
CI20	16.3	22.2	0	5.6	6.9	22.9	5.9	20.7
CI21	17.1	17.7	0	10.1	7.8	26.3	11	26.1
CI22	21	15.7	17.9	0	17.7	17.3	9	27.4
CI23	20.2	16.8	17.4	6	0	20	4	9.5
CI24	10	18.1	6	9	0	0	6	7.4
CI25	11.7	9.6	8	5	0	0	16.5	16.4
CI26	18.4	18.3	18.8	0	16.3	7.4	0	19.5
CI27	17.6	17.9	27.4	14.3	0	21.1	5.6	19.4
CI28	16.6	17.4	11.8	0	18.5	19.4	7	20.7
CI29	12.4	7.9	16.6	0	6	18.6	0	26.5
CI30	9.3	19.4	18.5	8.4	9	11	0	28.3
CI31	10.1	16.8	12	7.1	5.9	18.7	0	19.7
CI32	17.3	31.8	8	7.6	15.7	28	0	13.6
CI33	16.6	22.9	17.4	15.5	0	5	17.3	19.6
CI34	205	25.3	19.9	0	15.1	6.1	18.3	17.6
CI35	19.4	18.9	9.6	13.9	0	19.7	0	24.5
CI36	17.4	16.7	4.9	0	0	18	0	28.5
CI37	9.9	26.8	8.8	0	7.4	7	0	28.5
CI38	8.3	17.7	15.9	0	18.6	18.4	0	29.6
CI39	9.2	17.9	10	0	19.6	23.6	17.4	19.6

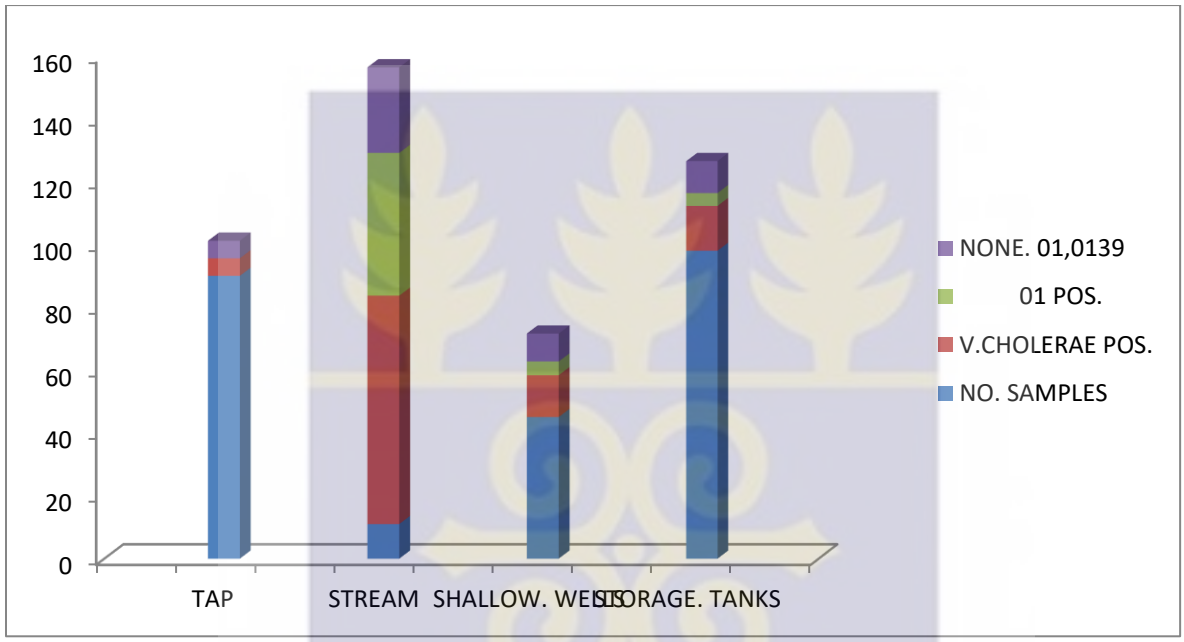
CI40	15.6	21.1	16.6	8	7.5	29.1	8	28.6
				ENVIRONMENTAL ISOLATES				
EI1	4.6	26.1	12	0	17.1	16.1	16.8	17.4
EI2	15.6	30.2	11.5	0	4	17.4	0	20.4
EI3	7.5	24.6	24.2	0	16.4	18.3	0	19
EI4	17.3	16.7	12.1	0	15.7	19.5	0	18.2
EI5	9.2	16.1	16.8	16.5	18.2	22.6	16.3	19.6
EI6	8.5	16.3	7.4	0	0	31.1	0	24.4
EI7	10.2	19.9	9.4	0	19.6	22.4	0	27.8
EI8	22.5	20.4	10.5	4.2	4	5.9	0	26.4
EI9	19.3	21.3	17.7	0	23.8	19.6	19.6	31.5
EI10	16.8	17.5	19.5	17.4	22	17	6	22.9
EI11	11.1	15.7	7.5	6.4	15.8	16.5	0	15.8

Graph showing the antibiotics resistance pattern of the isolates



Appendix B

A graph showing the Demographical Data on Prevalence of *V. Cholerae* O1 in the Different Water Samples



Appendix C

SOLUTION PREPARATION

C 1.1 1X TAE buffer

To prepare the working solution of 1X TAE buffer, 20 ml of the 50X TAE buffer (Thermo Scientific) was measured and diluted with distilled water to a total volume of 1 L. The pH of this solution was 8.0

C 1.2 AgaroseGel (1.5%)

To prepare 1.5% agarose solution, 1.5 g of agarose was weighed and dissolved in 100 ml 1X TAE buffer.

C 1.3 Ethidium bromide solution (10 mg/ ml)

To make 10 mg/ mlethidium bromide solution, 0.1 g ethidium bromide tablet was weighed and dissolved in 10 ml 1X TAE buffer

C 1.4 Concentrations of Antibiotic Disk prepared

Antibiotic	**Disk Concentration (µg/µl)**
Azithromycin	30
Erythromycin	15
Doxycycline	30
Ciprofloxacin	5
Trimethroprim-sulfamethoxazole (cotrimoxazole)	25
Nalidixic acid	30
Chloramphenicol	30
tetracycline 30µg	30

C 1.4 PCR MIX FOR BACTERIA, *V. CHOLERAE O1*

Reagent	**1X**
PCR buffer	5
10µM dNTPs	0.5
2.5µM Forward primers	0.5
2.5µM Reverse primers	0.5
Nuclease free water	15.87
5U/µl Polymerase	0.13
DNA Template	2.5
TOTAL	**25µl**

C 1.4 **An electrophoretogram of PCR product of *V. cholerae* isolates**