ISOLATION AND MOLECULAR CHARACTERIZATION OF *Vibriocholerae*FROM WATER SOURCES IN ZARIA, KADUNA STATE, NIGERIA

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BY

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DEPARTMENT OF MICROBIOLOGY,

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JANUARY, 2015

DECLARATION

I declare that the work in this thesis entitled "ISOLATION AND MOLECULAR CHARACTERIZATION OF *Vibriocholerae*FROM WATER SOURCES IN ZARIA, KADUNA STATE, NIGERIA" has been carried out by me in the Department of Microbiology. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this thesis was previously presented for another degree or diploma at this or any other institution.

Gyamndah Hope BULUS _______________ ____________

Signature Date

CERTIFICATION

This thesis entitled " ISOLATION AND MOLECULAR CHARACTERIZATION OF *Vibriocholerae*FROM WATER SAMPLES IN ZARIA, KADUNA STATE, NIGERIA" by GYAMNDAH HOPE BULUS meets the regulations governing the award of the degree of Master of Microbiology of the Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

This thesis is dedicated to God Almightyand tomy late grandparents Rev. (Dr.) and Mrs. Mari Habu.

ABSTRACT

Water is life and has a profound effect on human health. *Vibrio cholerae* is autochthonous in aquatic environment and is an important human waterborne pathogen causing cholera.This study was designed to determine the presence of *Vibrio cholerae* in some water sources in Zaria, Nigeria. A total of 207 water samples from wells, boreholes, tap, street vendors, and rivers were collected from Samaru, Sabon Gari and Zaria city and filtered using the membrane filtration technique. The filtrate on the filter paper was plated on thiosulphate citrate bilesalt sucose agar(selective medium)with prior enrichment on alkaline peptone water. Presumptive isolates were identified and characterized using both conventional biochemical method and Microgen (Microgen^{TM} GnA+B–ID System) identification kit. Isolates were serotyped using *Vibrio cholerae* antisera kit (Deben Diagnostics, Ltd.) and confirmed using PCR to detect presence of *rfb*O1, *rfb*O139 and *omp*W (species specific) genes. Data were analyzed to determine percentage distribution and prevalence of the pathogen and Krsukal-Wallis test was employed to acertain the significance ($P \leq 0.05$). Out of nine (9) *Vibrio* species isolated, the conventional method identified two (2) isolates as *V. cholerae* while the PCR confirmed five (5) isolates. *Vibro cholerae* of the non O1/ non O139 serogroup were isolated from well, river and street vended water with a prevalence of 2.4% in Zaria. Overall, *V. cholerae* was predominant in river (33.3%) followed by well (4.9%) and least predominant in street vended water (2.7%). None was isolated from borehole and pipeborne water. Water samples from Sabon Gari were most contaminated with an occurrence of 3.2% followed by Samaru (2.6%) while the least contaminated water samples were from Zaria city (1.5%). Results of this study suggest that the use of PCR targeting *ompW* species specific gene of *V.cholerae* in

combination with the conventional method would be an important tool in proper identification of *V. cholerae*. Isolation of *V. cholerae* from some of the water sources indicates that water plays a significant role in transmission of the organism, and drinking water from such contaminated sources could pose a threat to public health. Hence, government should ensure that potable water is made available and accessible to the populace.

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CHAPTER ONE

1.0 INTRODUCTION

Water is essential to life and health; however, more than one billion people worldwide do not have access to safe drinking water (WHO, 2000). According to the National Water and Sanitation Policy-Final Draft (2004), contaminated water and insanitary conditions are the cause of prevalent water and sanitation-related preventable diseases such as cholera, typhoid, diarrhoea, dracunculiasis and schistosomiasis.

Cholera is an acute diarrhoeal infection caused by ingestion of food or water contaminated with the bacterium *Vibrio cholerae*. It has a short incubation period, from less than 1 to 5 days (Todar, 2008), and produces copious, painless, watery diarrhoea that can quickly lead to severe dehydration (WHO, 2011a). Cholera is an extremely virulent disease. It affects both children and adults and can kill within hours. Cholera spreads easily in areas with poor sanitation and hygiene, and lack of safe drinking water supplies. The disease is now considered to be endemic in many countries and the pathogen causing cholera cannot currently be eliminated from the environment (WHO, 2008).

Vibrio cholerae, the causative agent for cholera is a Gram-negative bacterium of the family Vibrionaceae (Willey*et al*., 2008). It is a free-living organism found in many waters and estuaries (Willey*et al*., 2008). The main reservoirs of *V. cholerae* are people and aquatic sources such as brackish water and estuaries, often associated with algal blooms. Recent studies indicate that global warming creates a favorable environment for the bacterium (WHO, 2011a). *V. cholerae* produces a potent enterotoxin called choleragen that is responsible for the symptoms of cholera (Sack *et al*., 2004). It is serologically classified as belonging to the O antigenic group (Chatterjee and Chaudhuri, 2004).Of more than 200 known serogroups of *V. cholerae*, the two well-known serogroups, O1 and O139 have been associated with epidemic cholera (Gaffga *et al*., 2007). Serogroup O1 has two biotypes: Classical and El Tor, each of which has three serotypes: Inaba, Ogawa, and Hikojima (Fykse *et al*., 2007). The El Tor biotype of the O1 serogroup first emerged in Indonesia at the beginning of the seventh pandemic in 1961, became prevalent in the Ganges delta of the Indian subcontinent, and then spread into other countries (Wang *et al*., 2010). The first non-O1 serogroup epidemic, which emerged in southern India and then spread into many Asian countries, was caused by an O139 strain of *V. cholerae* (Albert *et al*., 1993; Bhattacharya *et al*., 1993).The primary connection between humans and pathogenic strains is through water, particularly in economically reduced areas that do not have good water purification systems (Faruque *et al*., 1998). From 1970, *V. cholerae* O1 El Tor has gradually spread to most of Africa with case-fatality rates between 4% and 12% (WHO, 1997) and has been responsible for a number of epidemics in Africa and Nigeria (Williams et al., 2010).

1.1 Statement of Research Problem

Cholera has been a substantial burden in the developing world for decades and it is endemic in Africa, Asia, South and Central America (Zuckerman *et al*., 2007). Cholera remains a global threat to public health and a key indicator of lack of social development. While the disease no longer poses a threat to countries with minimum standards of hygiene, it remains a challenge to countries where access to safe drinking water and adequate sanitation cannot be guaranteed. Recently, the re-emergence of cholera has been noted in parallel with the ever increasing rise of vulnerable populations living in unsanitary conditions (WHO, 2011a). Over the past two centuries, seven pandemics of cholera have been recorded and an eighth is emerging (Richards and Watson, 2006). The seventh pandemic, caused by *V. cholerae* serogroup O1, originated in 1961 in Indonesia and spread to Asia, Europe, and Africa. By 1991, it had reached South America and, within about a year, an estimated 533,000 cases and 4,700 deaths occurred in 19 countries (Taneja *et al*., 2003). This pandemic continues today. An eighth pandemic involving a new strain of *Vibrio cholerae* (serogroup O139) originated in Bengal, India in 1992 and quickly spread to Bangladesh and Thailand (Tauxe *et al*., 1998). The first African country to be affected by the seventh pandemic was Guinea. Between September 1970 and February 1971 the epidemic spread from Guinea through Sierra Leone, Liberia, Côte d'Ivoire, Ghana, Togo, Benin, Nigeria, and Southern Cameroon. From 1991 to 1996, the number of cases remained high and ranged between 70,000 and 160,000 (WHO, 1997).

Every year there are an estimated 3-5 million cholera cases and 100,000 – 120,000 deaths due to cholera (WHO, 2011a). In 2005, the reported incidence of cholera in Africa was 95 times that in Asia and 16,600 times that in Latin America. In 2007, the reported rate of death from cholera in Africa was seven times that in Asia; no cholera-related deaths have been reported in Latin America since 2001 (Gaffga *et al*., 2007). In a review by Aminu and Medugu (2011), it was observed that cholera is endemic in Nigeria since the occurrence of the first outbreak in 1970 occurring almost every year since the first outbreak. In July 16, 2008, it was reported that cholera claimed six lives leaving 30 others hospitalized in Zaria LGA of Kaduna State (Isere and Osemwenkhae, 2010).

1.2 Justification

The growing number and frequency of major cholera outbreaks are causing concern (WHO, 2010). According to Crowcroft (1994), cholera is one of only three diseases notifiable worldwide. Notifications to WHO do not specify the serogroup or biotype and are not sufficient to follow the progress of pandemics. Epidemics of cholera caused by toxigenic *V. cholerae* O1 and O139 (Bengal strain) represent a major problem in most developing countries. Even non-O1 and non-O139 serogroups, though reported less frequently are increasingly being shown as the causative agents of cholera (Das and Gupta, 2005). Since *Vibrio cholera*e is autochthonous to the aquatic environment, monitoring this bacterium in water sources is important for the control of cholera (Choopun *et al*., 2002).

Conventional culture methods currently used for diagnosis of *V. cholerae* remains the gold standard (Alam *et al*., 2010), but the process however, is laborious and time consuming (Maheshwari *et al*., 2011). Furthermore, close relatedness among *V. cholerae* and certain other members of *Vibrio* species such as *V. mimicus* or *Aeromonas species* with respect to their biochemical properties has often made identification of the organism quite difficult (Maheshwari *et al*., 2011). Therefore there is the need to characterize the organism using PCR which represents a rapid procedure with high sensitivity and specificity.

1.3 Aim and Objectives

1.3.1 Aim

The aim of this study was to isolate and characterize*Vibrio cholerae* from water samples in Zaria, Nigeria.

1.3.2 Objectives

The specific objectives of this research were to:

i. Isolate *Vibrio cholerae* from river, well, borehole, street-vended and pipeborne water sources.

ii. Identify the isolates using biochemical and serological characteristics.

iii. Carry out the molecular characterization of the isolates using polymerase chain reaction.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Water

Water is a chemical compound with the formula H_2O (containing an oxygen and two hydrogen atoms). It is a colourless, tasteless and odourless liquid that has a boiling point at 100^oC and freezes at 0^oC (under the pressure of 760 mm Hg) (Kołwzan *et al*., 2006). Water is a liquid at standard ambient temperature and pressure but it often exists on earth with its solid (ice) and gaseous state (water vapor or steam). It occurs in nature and contains dissolved salts and gases. Water covers 70% of the earth's surface, and thus, it is the most essential habitat of life(Kołwzan *et al*., 2006).

2.1.1 Sources of Water

Rain, snow, hail and sleet are precipitated upon the surface of the earth as and may be considered as the original or natural source of all the water collected from roof run-off and ground surfaces (CDC, 2009). Water can be obtained from two sources: surface and ground water.

i. Surface water is water that collects on the ground or in a stream, river, lake, reservoir or ocean. It is constantly replenished through precipitation and lost through evaporation and seepage into ground water supplies(CDC, 2009).

ii. Ground water is water located below the ground surface in pores and spaces in the rock which can be obtained by digging or drilling wells (CDC, 2009).

2.1.2 Uses of Water

Of all the resources required for sustaining ecosystems and the services they provide for human health and wellbeing, water is arguably the most important. In contrast to all other resources, no living organism can survive in the complete absence of water, making it an essential ingredient necessary for all life (UNEP, 2009). Water is considered to be the most important resource for sustaining ecosystems, which provide life-supporting services for people, animals and plants. It is used daily for different purposes and the amount of water used is vastly larger than most people realize (UNICEF, 2012). Water can be used directly or indirectly forhousehold or domestic, agriculture, recreational activities, industrialand thermoelectric power use(WHO, 2011b).

2.1.3 Water and Health

Water is one of the most vital natural resources for all life on earth. The availability and quality of water always have played an important part in determining not only where people can live, but also their quality of life (WHO, 2011b). Even though there always has been plenty of fresh water on earth, water has not always been available when and where it is needed, nor is it always of suitable quality for all uses. Scarcity and misuse of fresh water pose a serious and growing threat to sustainable development and protection of the environment. Human health and welfare, food security, industrial development and the ecosystems on which they depend, are all at risk, unless water and land resources are managed more effectively in the present decade and beyond than they have been in the past (UN, 1992).

When water is unsafe and sanitation non-existent, contaminated water can kill (UNICEF, 2012). The quality of drinking water is closely associated with human health and providing safe drinking water is a major public health priority (Semenza *et al*., 1998). Many water sources in developing countries are unhealthy because they contain harmful physical, chemical and microbiological agents. To maintain good health however, not only must a water supply be safe to drink, it must also be;

i. available in sufficient quantity for cooking, hand washing, personal bathing, cleaning, and laundering clothes,

ii. easily and safely accessible by all the community without the need to carry heavy containers of water over long distances or having to visit sites where insect vectors of disease breed,

iii. available all of the time or when it is needed,

iv. available at affordable cost (Cheesbrough, 2006).

Typically, microbiological hazards are of great concern (Mara and Horan, 2003). Water can be significant means of transmission of infectious agents or pathogens and the greatest microbial risks are associated with the ingestion of water contaminated with faeces (from humans or animals) containing pathogenic microorganisms (Mara and Horan, 2003; WHO, 2011b; Fawell and Nieuwenhuijsen, 2003).

Many illnesses, contaminants, and injuries can be water, sanitation, or hygiene-related. Waterborne diseases are caused by organisms that are directly spread through water. Water-related illnesses can be acquired due to a lack of water for good hygiene, lack of sanitation, or increasing insect populations that breed in water and then spread disease (CDC, 2012). Waterborne and other water-related diseases consist mainly of infectious diarrhoea, typhoid, cholera, salmonellosis, shigellosis, amoebiasis, and other protozoan and viral intestinal infections (WHO, 2011b).

2.2 *Vibrio cholerae*

2.2.1 Taxonomy

Kingdom: Bacteria

Phylum: Proteobacteria

Class: Gammaproteobacteria

Order: Vibrionales

Family: Vibrionaceae

Genus: Vibrio

Species: *V. cholerae*

Binomial name:*Vibrio cholerae*

2.2.2 Bacteriology

Vibrio cholerae is a non-invasive, gram negative, non-spore forming, and comma-shaped curved rod 2 to 4 μm long that is oxidase-positive and motile by means of a polar flagellum (Brooks *et al*., 2010; PHAC, 2010). Itis highly motile with a distinctive rapid to and fro movement which has been likened to swarming gnats (Rugel and Klose, 2012).

The flagellum facilitates chemotactic-directed movement toward the preferred colonization site within the intestine (Camilli and Mekalanos 1995; Butler and Camilli 2004), and also contributes to biofilm formation within the environment (Watnick and Kolter 1999). *Vibrio* motility is best seen using dark-field microscopy, but the vibrios can also be seen using transmitted light microscopy (Cheesbrough, 2006). *V. cholerae* grows well on thiosulfate-citrate-bilesalt-sucrose (TCBS) agar, on which it produces yellow convex, smooth, round colonies that are readily visible against the dark-green background of the agar (Brooks *et al*., 2010). *V. cholerae* is an aerobe and facultative anaerobe. It can grow over a wide temperature range of 16–40 ºC with an optimum of 37ºC (Cheesbrough, 2006). The bacterium can survive freezing and can grow within a pH range of 5 to 9.6 (PHAC, 2010) andis non-halophilic (Cheesbruogh, 2006).

Vibrio cholerae can assume a variety of survival forms, including a shift to what has been termed a viable but non-culturable form, which is often associated with biofilms (Morris, 2011). Viable but non-culturable cells can leave their dormant stage and multiply again, resulting in an explosion of their concentration in the environment (Cabral, 2010). Strains can also assume a rugose phenotype (identifiable on culture by a characteristic rough or wrinkled appearance), in which the microorganism produces large quantities of an amorphous exopolysaccharide, leading to formation of a biofilm that is resistant to chlorine, ultra-violet (UV) light, and other standard disinfectants (Camilli and Mekalanos, 1995; Butler and Camilli, 2004; Morris, 2011). The bacterium has been described to switch between the smooth and rugose colony morphotypes contributing to its environmental survival (Valeru *et al*., 2012). The exopolysaccharide (EPS) materials of rugose colony-forming *V. cholerae* strains were recognized as a heavy, fibrous, electron-dense, ferretinstained layer surrounding the cells, but smooth colony *V. cholerae* do not appear to have this EPS layer surrounding it. The cell surface EPS materials confer a rugose colony morphology and resistance to osmotic and oxidative stresses. The regulation of EPS synthesis in bacteria is complex and involves multiple systems utilizing both positive and negative regulation (Valeru *etal*., 2012).

Vibrio cholerae as a species includes both pathogenic and nonpathogenic strains that vary in their virulence gene content. This bacterium contains a wide variety of strains and biotypes, receiving and transferring genes for toxins, colonization factors, antibiotic resistance, capsular polysaccharides that provide resistance to chlorine and new surface antigens such as the O139 lipopolysaccharide and O antigen capsule (Heidelberg *et al*., 2000).

2.2.3 Ecology

Primarily, *V.* cholerae as a speciesis an ubiquitous inhabitant of the aquatic environment around the world (Singh *et al*., 2001; Tamrakar *et al*., 2009). Its distribution depends on water temperature (optimal growth at water temperatures >20°C) and salinity. In areas with seasonal variations in water temperatures, the microorganism shows clear seasonality: environmental counts increase during warmer periods and decline or become non-detectable during cold weather (Morris, 2011). This *Vibrio* species not only survives in riverine, estuarine, and coastal waters around the world but also lives in association with crustacean copepods, such as *Acartia tonsa* and *Gerris spinolae*, and with aquatic plants, either in the viable and culturable state or in the viable but non-culturable state (Roy *et al*., 2008). It has also been found in association with chironomid egg masses and water hyacinth and can be carried by gulls, other birds, and mammals (Morris, 2011).

The persistence of *V. cholerae* in aquatic environments has been linked to association with chitin, an ubiquitous component of the cell walls of fungi and the cell walls of arthropods (Pruzzo *et al*., 2008; Mitchell and Gu, 2012). Chitin serves as a carbon and nitrogen source for vibrios and other aquatic bacteria (Heidelberg *et al*., 2000). *V. cholerae* attachment to chitinaceous surfaces leads to biofilm formation, which in turn can serve a protective role, enhancing the survival of the bacteria. *V. cholerae* cells growing on chitin are stimulated to take up free DNA present in the environment (Meibom *et al*., 2005; Mitchell and Gu, 2012).

2.2.4 Genome of *V. cholerae*

The genome of *V. cholerae* is remarkedly diverse, exhibiting a high degree of clonal diversity (Thompson *et al*., 2004). It consists of two circular chromosomes (1 and 2) together totaling about 4 million base pairs of DNA sequence and 3885 predicted open reading frames (ORF's). Chromosome 1 contains 2,961,146 base pairs while chromosome 2 contains 1,072,314 base pairs with an average G + C content of 47.7% and 46.9% respectively (Heidelberg*et al*., 2000; Willey*et al*., 2008). Crucial genes for toxicity, regulation of toxicity, virulence and important cellular functions, such as DNA replication, transcription and protein synthesis are located in the larger chromosome (Willey*et al*., 2008).The second chromosome contains housekeeping and other essential genes in the genome, including essential genes for metabolism, heat-shock proteins and 16S rRNA genes, which are ribosomal sub-unit genes used to track evolutionary relationships between bacteria (Heidelberg *et al*., 2000). Genes responsible for identification of *V. cholerae* are also located in the chromosome some of which include *omp*W and *rfb* genes (Reidl and Klose, 2002).

OmpW gene is a species specific gene unique to *V. cholerae* and not found in other members of *Vibrio* species (Madroba and Momba, 2010, Nandi *et al*., 2000). It is highly conserved among *V. cholerae* strains and serves as a good species specific marker for different serotypes and serogroups (Tamrakar *et al*., 2009; Masheshwari *et al*., 2011). The outer membrane protein (OMP) *ompW* of *V. cholerae* has been described as a 22kDa molecule (Nandi *et al*., 2005). Nucleotide sequence of this gene was first reported by Jaiajakumari and Manning in 1990 which was demonstrated to be present in chromosome II (small chromosome) of the bacterium (Heidelberg *et al*., 2000; Nandi *et al*., 2000). The nucleotide sequence of the *ompW* gene in *V. cholerae* has not changed and the gene appears to be conserved among different *V. cholerae* srains (Sheik *et al*., 2012) and this led to the development of primers targeting the gene for rapid identification of the microorganism by Nandi *et al*. (2005). Several functions were proposed for the *ompW*-related proteins in these bacteria, including their pore forming (Jeanteur *et al*., 1992) and colicin receptor properties (Pilsl *et al*., 1999). It may also play a role in the adherence process, which is likely to facilitate the survival of the organism within the host or environment or both (Nandi *et al*., 2000).

The rfb gene is a cluster of genes responsible for O – antigen biosynthesis in *V. cholerae* (Nandi *et al*., 2000) for the generation of serogroup specific determinants, possessing both environmental and virulence properties (Stroeher *et al*., 1997; Nandi *et al*., 2000; Reidl and Klose, 2002).

The genome of *V. cholerae*has also been found to contain a distinctive class of integrons, which are gene expression elements that acquire open reading frames (ORFs) and convert them to functional genes (Mazel *et al*., 1998). This permits the bacteria to entrap genes from other microorganisms and thus constitutes a mechanism for the clustering and spread of pathogenic genes as well as genes for other biochemical functions.The activity of integrons within *Vibrio* and other bacteria appears to play a major role in genetic evolution of bacteria; including the emergence of newly pathogenic and even pandemic forms of *V. cholerae* (Faruque *et al*., 1998). *Vibrio* integrons, in particular, are capable of harboring a large number of integrated genes (Rowe-Magnus *et al*., 2003). These super-integrons allow bacteria to take in and "try out" a large array of "new genes that may confer on the bacteria a selective advantage to survive and thrive under changing environmental conditions or express new virulence traits during the course of disease evolution(Mitchell and Gu, 2012).

2.2.5 Cell structure and metabolism

Since *Vibrio cholerae* is a gram negative bacteria, the cell wall consist of lipopolysaccharides (LPS) (Nesper *et al*., 2000). The lipopolysaccharides (LPSs) of gram-negative organisms are the most abundant molecules on the cell surface and provide a protective barrier to hydrophobic agents and detergents (Stroeher *et al*., 1992). LPS and capsule structures represent important virulence factors and typing determinants of pathogenic *V. cholerae* strains (Reidl and Klose, 2002). LPS consists of three distinct regions or domains:

i. lipid A, a hydrophobic domain that associates with the outer membrane (forms part of the lipid bilayer),

ii. the core oligosaccharide, and

iii. a terminal, immunogenic polysaccharide domain termed the O antigen which is serotype specific (Kierek and Watnick, 2003).

This outermost region, the O antigen, provides the major antigenic variability of the cell surface and on the basis of this heat-stable polysaccharide the species *Vibrio cholerae* is serogrouped (Stroeher *et al*., 1992; Kierek and Watnick, 2003).The lipopolysaccharide (LPS) of *V. cholerae* O139 differs from that of O1 in that the LPS can be described as semirough, i.e., it does not appear to possess long O antigen chains but instead has a heavily substituted core (Stroeher *et al*., 1997). The monosaccharide compositions of the *V. cholerae* O1 and O139 O antigens differ considerably. The O-antigen of *V. cholerae* O1 consists of a homopolymer of approximately18 perosamine (4-NH_2-4,6-dideoxymannose) residues consistingof dideoxy-phosphomannose substituted with tetronate (Reidl and Klose, 2002) whereas the O139 polysaccharide is distinguished by a D-galactose 4,6-cyclophosphate moiety (Knirel*et al*., 1995; Kierek and Watnick, 2003)

2.3 *V. cholerae* Strains

2.3.1 *V. cholerae* O1

V. cholerae serogroup O1 is the etiologic agent of cholera (Murray *et al*., 1999). The organism was first discovered by Filippo Pacini in 1854 however, the relationship of cholera to contaminated water was elucidated in 1855 by John Snow in a study of sources of a cholera epidemic in London (Blake, 1994). The bacterial etiology of cholera wasconfirmed by Robert Koch in 1883 when he isolated the cholera bacillus from pond water during a cholera outbreak in Egypt (Koch, 1884; Murray *et al*., 1999).

Vibrio cholerae O1 has two biotypes—El Tor and classical (Kanungo and Sur, 2012), classified based on phenotypic characteristics (Table 1) (Kitaoka *et al*., 2011) and defined genotypic differences (Safa *et al*., 2010). Both biotypes have further been subdivided based on antigenic factors (Table 2.2) into three serotypes designated Inaba, Ogawa and Hikojima (Islam *et al*., 2004) which is rare as well as unstable (Mandal *et al*., 2011).

2.3.2 *V. cholerae* O139

V. cholerae O139 was first recognized in South Asia in 1992 as a cause of epidemic cholera (CDC, 2012; WHO, 2011). This organism was reported to have originated from *V. cholerae*O1 El Tor by lateral transfer of a genomic island substituting the O139 for the O1 antigen, but is otherwise almost identical to *V. cholerae*O1 El Tor (Harris *et al*., 2012). However, this organism does not produce the O1 LPS and lacks at least some of the genetic material necessary for production of the O1 antigen. Furthermore, like many strains of non-O1 *V. cholerae* and unlike *V. cholerae* O1, this organism produces a polysaccharide capsule (Kaper *et al*., 1995; Brooks *et al*., 2010).

Of more than 200 known serogroups of *V. cholerae*, the two well-known serogroups O1 and O139 have been associated and reported as the etiological agents of cholera (Fykse *et al*., 2007; CDC, 2010). Cholera is an acute, diarrheal illness caused by infection of the intestine with the bacterium *Vibrio cholerae* and is transmitted by contaminated food or water.

Table 2.1: Phenotypic differences between *V. cholerae* classical and El Tor O1 strains

Test	**Classical**	**El Tor**
Haemolysis of sheep erythrocytes	-	+
Agglutination of chicken erythrocytes	-	+
Voges–Proskauer reaction	-	+
Polymyxin B resistance	s	R
Phage IV	s	R
Phage 5	r	S

Key: + positive, - negative, s sensitive and r resistant.

Table 2.2: Antigenic determinants of *V. cholerae* (Todar, 2008).

Serotype	**O Antigens**
Ogawa	A, B
Inaba	A, C
Hikojima	A, B, C

Key:

A, B and C = O antigens referred to as O factors

The infection is often mild or without symptoms, but sometimes it can be severe. Approximately 5-10% of persons will have severe cholera which in the early stages includes diarrhoea, sometimes described as “rice-water stools,” is profuse, painless and watery, with flecks of mucus in the stool (Pearson, 2011).Vomiting is often present, occurring a few hours after the onset of diarrhoea (Kaper *et al*., 1995) the vomitus is generally a clear, watery, alkaline fluid (Sack *et al*., 2004), rapid heart rate, loss of skin elasticity, dry mucous membranes, low blood pressure, thirst, muscle cramps and restlessness or irritability. Persons with severe cholera can develop acute renal failure, severe electrolyte imbalances and coma. If untreated, severe dehydration can rapidly lead to shock and death (Pearson, 2011).

Profuse diarrhoea produced by cholera patients contains large amounts of infectious *Vibrio cholerae* bacteria that can infect others if ingested, and when these bacteria contaminate water or food will lead to additional cases.

2.3.3*V. cholerae* non O1/non O139

Non-O1 and non-O139 *Vibrio cholerae* are the third most commonly reported group of *Vibrio* bacteria. On the average, 44 cases of non-O1 and non-O139 *Vibrio cholerae* were reported to the CDC each year since 2000 (CDC, 2013). In recent years, until the emergence of the O139 serogroup, all isolates that were identified as *V. cholerae* on the basis of biochemical tests but that were negative for the O1 serogroup were referred to as ‘‘non-O1 *V. cholerae*’’ (Kaper *et al*., 1995). In earlier years, the non-O1 *V. cholerae* was referred to as non-cholera vibrios or nonagglutinable vibrios. These non-O1/non-O139 strains have been divided into serogroups O2 through O138 on the basis of the lipopolysaccharide (LPS) somatic antigen. The great majorities of these strains do not produce cholera toxin and are not associated with epidemic diarrhoea (Morris, 1990). They are highly heterogeneous with considerable serological diversity and vary in virulence property (Luo *et al*., 2013). These other serogroups are usually isolated from patients with mild diarrhoea or from the environment (Fykse *et al*., 2007). Infections are seasonal with a peak in the late summer and early fall, coinciding with the warmest water temperatures (CDC, 2013).Symptoms include the following:

i. Gastroenteritis: people with gastroenteritis caused by non-O1, non-O139 *Vibrio cholerae* can have symptoms of differing severity ranging from mild diarrhea to severe watery diarrhea. Fever and bloody diarrhea are not typically seen in gastroenteritis due to non-O1, non-O139 *Vibrio cholerae.*

ii. Sepsis due to non-O1 and non-O139 *Vibrio cholerae* is seen in immunocompromised patients and in people with liver disease. Its symptoms include fevers, chills, and decreased blood pressure (shock).

iii. Non-O1 and non-0139 *Vibrio cholerae* has rarely been reported to cause wounds, infections with redness and swelling at the site of infection.

2.3.4 Host Defences

Gastric acidity, mucus secretion, and intestinal motility are nonspecific defenses that play important roles in preventing *V. cholerae* infection. Also, genetic factors may be important since the toxin must bind to specific cellular receptors in order to function. It has been suggested that intestinal secretory immunoglobulin A (sIgA) protects against colonization of the mucosa preventing attachment of the Vibrios to the mucosal surface and neutralize or prevent binding of the cholera enterotoxin (Nelson *et al*., 2009).

2.3.5 Epidemiology

In the past 200 years, there have been 8 main cholera pandemics, with the disease being most common in tropical and subtropical areas. Most of the cases are in the Indian subcontinent and Africa (in 2002 the WHO estimated that 97% of the cholera cases were in Africa). There are several million cases of cholera each year and in endemic areas, these tend to be most common in children aged 2-9 and in women who are of child-bearing age. Epidemics in endemic areas tend to occur during the hot season(PHAC, 2010).

Infection with *V. cholera* appears to exhibit three major epidemiologic patterns: heavily endemic, neoepidemic (newly invaded, cholera-receptive areas), and, in developed countries with good sanitation, occasional limited outbreaks. These patterns probably depend largely on environmental factors (including sanitary and cultural aspects), the prior immune status or antigenic experience of the population at risk, and the inherent properties of the vibrios themselves, such as their resistance to gastric acidity, ability to colonize, and toxigenicity (Brooks *et al*., 2010; PHAC, 2010).

In heavily endemic regions,*V. cholerae* infection exhibits some periodicity; this may vary from year to year and seasonally, depending partly on the amount of rain and degree of flooding. Because humans are the only reservoirs, survival of the *V. cholerae* during interepidemic periods probably depends on a relatively constant availability of low-level undiagnosed cases and transiently infected, asymptomatic individuals. During epidemic periods, the incidence of infection in communities with poor sanitation is high enough to frustrate the most vigorous epidemiologic control efforts. Although transmission occurs primarily through water contaminated with human feces, infection also may be spread within households and by contaminated foods. In neoepidemic cholera-receptive areas, spread of cholera usually depends on traffic of infected human beings, although spread between adjacent communities can occur through bodies of water contaminated by human faeces (Brooks *et al*., 2010)**.**

2.3.6 Diagnosis

The diagnosis is suggested by severe, watery diarrhoea. For rapid diagnosis, a wet mount of liquid stool is examined microscopically. The characteristic motility of *Vibrios* is stopped by specific anti-somatic antibody. Other methods are culture of stool or rectal swab samples on TCBS agar and other selective and nonselective media; the slide agglutination test of colonies with specific antiserum; fermentation tests (oxidase positive); and enrichment in peptone broth followed by fluorescent antibody tests, culture, or retrospective serologic diagnosis. More recently the polymerase chain reaction (PCR) and additional genetically-based rapid techniques have been recommended for use in specialized laboratories (Nelson *et al*., 2009).

2.3.7 Control

Control measures of *V. cholerae* infection can be sanitary, immunologic and or chemotherapeutic.

Sanitary: Application of sanitary principles that involve protecting drinking water and food from contamination with human faeces are important in controlling outbreaks. However this is not feasible in underdeveloped areas (PHAC, 2010).

Immunologic: Traditional parenteral inactivated vaccine strains are available though not recommended for widespread use as they only provide protection for 3-6 months. Oral vaccines that provide protection for several years (up to 3) are available (Hill *et al*., 2006; PHAC, 2010).

Chemotherapeutic: Fluid and electrolyte replacement followed by the WHO's oral rehydration solution is the recommended treatment for dehydration. Administering antibiotic like ciproflaxin, doxycycline or co-trimoxazole reduce the duration of the illness (PHAC, 2010;Kanungo and Sur, 2012).

2.3.8 Other *Vibrio* Infections

These include infections with other *Vibrio* species apart from *V. cholerae* some of which are *V. parahaemolyticus*, *V. fluvialis*, *V. alginolyticus*, *V. vulnificus*, *V. mimicus*, *V. damsel* amongst many others (Cunha, 1994; Brooks *et al*., 2010). *Vibrio* species can produce multiple extracellular cytotoxins and enzymes that are associated with extensive tissue damage and that may play a major role in the development of sepsis. Infected individuals often present with gastroenteritis, wound infection and septicaemia (Cunha, 1994; Brooks *et al*., 2010).

Infections are mostly acquired through consumption of contaminated raw or undercooked shellfish such as oysters, clams, mussels, or crabs. Exposure of wounds to contaminated seawater, injury caused by contaminated seashells, and shark and alligator bites are potential alternative sources of infection. Because *Vibrio* species are natural inhabitants of sea water, infections are commonly reported in states or countries bordered by large bodies of sea water. Persons with underlying medicalconditions, such as alcoholism, cirrhosis, or malignancy, and recipients of organ transplants are at increased risk of *Vibrio* infections and serious complications (Cunha, 1994; Willey *et al*., 2008; Brooks *et al*., 2010).

Persons of any age who consume or are exposed to contaminated food or water are at risk of developing *Vibrio* infection, especially if they have underlying medical conditions such as advanced liver disease (Cunha, 1994).

2.4Life Cycle

The life cycle of *Vibrio cholerae* has two stages; an in-the-body stage and an in-the-environment stage. In the body, the bacteria enter through oral ingestion, pass through the stomach and intestine, colonize within the intestines and acquire virulence and cause diarrhoea.In the environment, the bacteriamultiply as any other bacteria via binary fission while living in aquatic environments and from there, the bacteria can be picked up by any organism unawares, most particularly humans when they are drinking water (Jude*et al*., 2009).

2.5Transmission

Two routes of transmission of *V. cholerae* have been recognized, the first one occurs from aquatic reservoirs in the environment (primary transmission), and the second one occurs from previously infected individuals (secondary transmission); once the primary transmission has initiated an outbreak, secondary transmission causes epidemics in the endemic areas (Ruiz-Moreno*et al*., 2010). In endemic areas, water is usually the main vehicle of transmission, although this may occur via food and thus infection due to *V. cholerae* begins with the ingestion of contaminated water or food. Transmission in non-endemic areas is more commonly associated with consumption of foods, such as raw or undercooked seafood, imported from endemic regions (Mandal *et al*., 2011). *Vibrio cholerae* is spread mainly via the faecal–oral route. Some of the best-known sources of infection are as follows:

i. Drinking water that has been contaminated at its source, during storage or usage;

ii. Contaminated foods, vegetables that have been fertilized with human excreta (night soil) or 'freshened' with contaminated water;

iii. Soiled hands can also contaminate clean drinking water and food;

iv. Fish, particularly shellfish taken from contaminated water and eaten raw or insufficiently cooked (Pearson, 2011).

2.6Pathogenesis of *V. cholerae*

After entering the host via contaminated food or water, *V. cholerae* penetrates the intestinal mucosa and attaches to microvilli of the brush border of gut epithelial cells. It then produces a heat labile enterotoxin which consists of two subunits: subunit A which is split into two peptides, A_1 and A_2 linked by a disulfide bond and subunit B with five identical peptides. The subunit B rapidly binds the toxin to cell membrane gangliosides hence promoting entry of subunit A into the cell membrane resulting in large increase of adenylate cyclase activity and in the concentration of intracellular cyclic adenine monophosphate (cAMP) (Todar, 2008; Brooks *etal*., 2010) shown in Figure 2.1 .

The net effect of the toxin is to cause cAMP to be produced at an abnormally high rate which stimulates mucosal cells to pump large amounts of chloride into the intestinal contents. Water, sodium and other electrolytes follow due to the osmotic and electrical gradients caused by the loss of chloride ion (Cl^-). The lost water and electrolytes in mucosal cells are replaced from the blood. Thus, the toxin-damaged cells become pumps for water and electrolytes causing the diarrhea, loss of electrolytes, and dehydration.

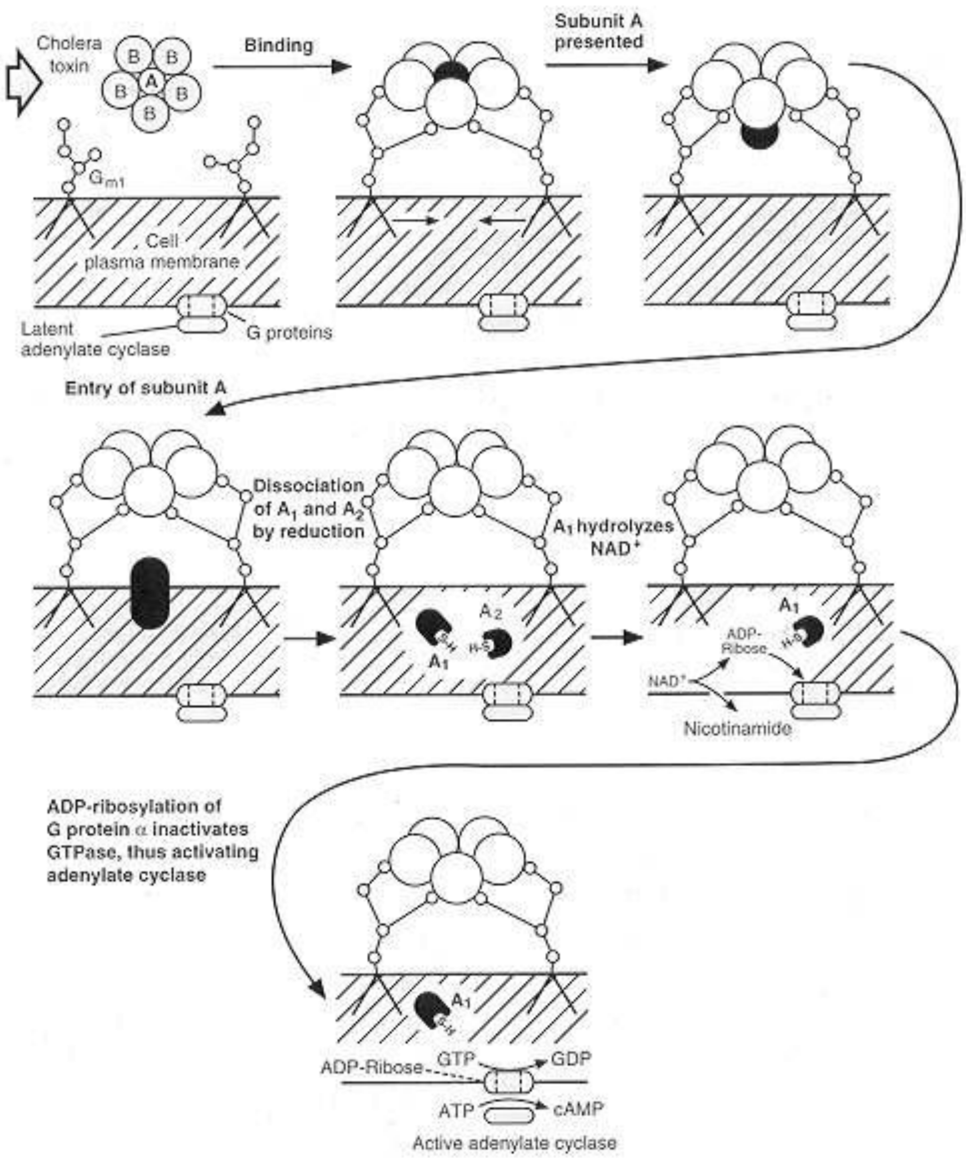

Figure 2.1: Mechanism of action of enterotoxin produced by *V. cholerae* (Todar, 2008).

2.7 Identification of *V. cholerae*

The detection of *Vibrio* species is based on traditional phenotypic techniques as well as more recent molecular tools (Tamplin, 2001; Vandenberghe *et al*., 2003). Generally, conventional culture-based methods involve selective pre-enrichment of samples, plating onto selective solid media followed by morphological, biochemical and serological characterization (Igbinosa and Okoh, 2008). Standard operating procedures optimized for the detection and identification of V. cholerae (Colwell and Huq, 1994; Tantillo *et al*., 2004) include inoculation of the test portion into the selective enrichment medium alkaline peptone water (APW) and incubation at optimum temperatures, and streaking onto the selective medium; thiosulfate citrate bilesalt sucrose agar (TCBS).

Presumptive colonies subcultured on non-selective media such as nutrient agar (NA) or trypticase soya agar (TSA) are subjected to microscopic and biochemical analysis, such as Gram staining, motility, oxidase, arginine dihydrolase, lysine decarboxilase, ortho-nitrophenylgalactopyranoside (ONPG), carbohydrate fermentation and halophilic characteristics according to Bergey's Manual of Systematic Bacteriology (Farmer and Hickman, 1992). Serological methods have been used successfully for identification of *Vibrio* species in food and the aquatic environment. Vibrio species may be serotyped according to both somatic 'O' and capsular polysaccharide 'K' antigens.

Traditional standard microbiological techniques for detection of *Vibrio* species are, however, slow, laborious and often require several days to be performed. Moreover, conventional phenotypic assays characterized by low sensitivity may fail to detect strains of bacteria present in the samples at low levels and with unusual phenotypic profiles (Farmer and Hickman, 1992). Molecular methods for identification of *Vibrio* species have increased lately, especially the use of polymerase chain reaction (PCR)-based techniques to amplify specific DNA sequences, as well as digestion of these fragments with restriction enzymes (Igbinosa and Okoh, 2008).

2.7.1 Polymerase Chain Reaction (PCR)

Polymerase chain reaction (PCR) is a technique in molecular biology to amplify a single or a few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence (BioMedicine, 2012; Encyclopedia Britannica, 2013). The principle involves amplification of specific regions of a DNA strand (target DNA); this can be a single gene, part of a gene or a non-coding sequence. Most PCR methods typically amplify DNA fragments of up to 10 kilo base (kb) pairs although some techniques allow for amplification of fragments up to 40kb in size.

2.7.2 Stages and Components of PCR

PCR usually consists of a series of 20-35 cycles. It is however most commonly carried out in three steps, often preceded by one temperature hold at the start and another at the end(Walker and Rapley, 2000). There are three basic steps in PCR and these include:

i. Denaturation: The double stranded DNA is denatured by heating the reaction mixture to above 90°C. The region within the complex DNA which is to be specifically amplified (target DNA) is made accessible.

ii. Annealing: Reaction mixture is cooled to 40 - 60°C. The precise temperature is critical and each PCR system has to be defined and optimized. This step allows hybridization of the two oligonucleotide primers which are present in excess to bind to their complementary that flank the target DNA. The annealed oligonucleotides act as primers for DNA synthesis (since they provide a free 3' hydroxyl group for DNA polymerase).

iii. Extension or elongation: This is also known or termed as the DNA synthesis step and is carried out by a thermostable DNA polymerase most commonly, *Taq* DNA polymerase. DNA synthesis proceeds from both 3' ends of the primers until the new strands have been extended along and beyond the target DNA.

A final elongation step is frequently used after the last cycle to ensure that any remaining single stranded DNA is completely copied. This differs from the elongation step only that it is longer typically 10-15 minutes. A final hold of 4-15°C for an indefinite time is often employed to allow short term storage of the reaction especially if the reaction are run over night and cannot be removed immediately after the cycling (Walker and Rapley, 2000).

PCR is an invaluable procedure that has innumerable uses (Glick and Pasternak, 2003). It is the best-studied and most widely used target amplification technique and has developed into a mainstay technique in many molecular biology laboratories (Persing *et. al*., 1993). PCR was developed around 1983 by Kary B. Mullis, who won a Nobel Prize in Chemistry for the invention (Mullis, 1990). Since then, PCR has been widely used as a diagnostic and research tool. Its applications are continually growing and are widespread over many scientific disciplines, including molecular biology, microbiology, genetics, clinical diagnostics, forensic science, environmental science, hereditary studies and paternity testing.

PCR has now become a frequently used detection method, and several PCR protocols have been developed for *V. cholerae* (Singh *et al*., 2001; Gubala, 2006; Fykse *et al*., 2007). This technique includes species-specific PCR targeting the outer membrane protein, which is very sensitive, specific and efficient for *V. cholerae* identification (Nandi *et al*., 2000). Even so, molecular-based techniques have some inherent limitations that include amplification bias; hence, a polyphasic approach that consists of both classical and molecular based techniques is preferable for identification of *V.cholerae* (Vandamme *et al*., 1996).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study area

Zaria is a major city in Kaduna State in Northern Nigeria as well as being a Local Government Area with a population ranging between 500,000 and 1 million. It is located at about 652.6m above sea level, some 950km from the coast along latitude 11°07'5" N and longitude 7°43'43" E. It possesses a tropical continental climate with a pronounced dry season lasting up to 7 months (October – May). It experiences a brief period of hot but dry weather in March and April. The rainy season lasts from May to September with long-term annual rainfall of 1040 mm. Zaria's economy is primarily based on agriculture. Staples are guinea corn and millet and cash crops include cotton, groundnut and tobacco. Because Zaria is north of the rail junction at Kaduna, it has equal rail access to the seaports at Lagosand Port Harcourt. The concentration of the institutions of higher learning has made Zaria famous regionally, nationally, and internationally (http://www.collinsmaps.com/maps/Nigeria/Kaduna/Zaria/P220126.00).

3.2 Sample size

Sample size was determined using the equation as described by Naing *et al.*, (2006)

$$n = \frac{z^2 p q}{d^2}$$

Where n = sample size of water

z = standard normal distribution at 95% confidence interval = 1.96

p = prevalence of previous study 11.8% = 0.118

q = (1 – p) = 1- 0.118 = 0.882

d = precision (allowable error), taken as 5% = 0.05

$$\text{Thus, } n = \frac{1.96^2 \times 0.118 \times (1 - 0.118)}{0.05^2}$$

n = 159.9

Therefore, approximately 207 water samples were collected.

3.3 Sample collection and transportation

Water samples were collected from Samaru, Sabongari and Zaria city from the following sources: well (covered and uncovered) (Appendices I, II and III), pipeborne (Appendices IV and V), borehole (hand pumped and motorized) (Appendices VI, VII, VIII and IX), street vendors (also known as 'mai ruwa'), and river (Appendix X) in Zaria. Samples were collected from the various sample points as described by Cheesbrough (2006) as follows:

i. Collection from tap: The tap was turned on full and allowed to run for 1 minute to flush the nozzle and to allow stagnant water in the pipe to be discharged. The sterile 1L sample bottle was then filled from a gentle flow of the water after which the bottle was capped, covered and labelled.

ii. Collection from river: The cap and cover of a sterile 1L sample container was aseptically removed. Facing the mouth of the bottle upstream (i.e. towards the flow of water), the neck was plunged downwards about 30cm below the water surface and then tilted to fill completely. The bottle was then carefully capped, covered and labelled appropriately.

iii. Collection from borehole: The hand pump was operated continuously for 5 minutes to pump out water after which water was collected into a sterile sample 1L bottle by allowing the water from the pump to flow directly into the bottle. The cap and cover were then carefully replaced and the bottle labelled appropriately.

iv. Collection from well: Using a clean rope a small heavy piece of metal was tied to a sterile 1L sample bottle after which the cap was aseptically removed. The bottle was then lowered into the well and allowed to fill up. The bottle was then carefully raised out of the well, capped, covered and appropriately labelled.

v. Street-vended water was purchased from street vendors hawking on streets of Samaru, Sabon Gari and Zaria city.

One liter of water sample from each source was collected in separate sterile containers, labeled appropriately and transported at $4^{o}C$ in an insulated cold box for further analysis within 2 hours of collection (Bacteriological Analytical Manual, 2004) immediately to the Post graduate laboratory of the Department of Microbiology, A.B.U, Zaria.

3.4 Enrichment and isolation of *Vibrio cholerae*

Using the membrane filtration technique (CDC, 1994), 1liter of each water sample was filtered through a 0.45µm membrane (Millipore) filter with a suction pump (Appendix XI). Using a sterile forceps the membrane filter was placed aseptically in 100ml of alkaline peptone water (APW) and enriched for 6 - 8 hours at 35 – 37^{o}C. After the enrichment, the membrane filter was placed on already prepared sterile thiosulphate citrate bile-salt sucrose (TCBS) agar (Oxoid Ltd., Basingstoke, England) plate with a sterile forceps. The inoculated TCBS plates were then incubated at 35 – 37^{o}C for 18 – 24 hours (CDC, 1994).

After incubation, large (2 – 3mm) yellow, smooth and slightly flattened colonies with opaque centers and translucent peripheries were presumably identified as *Vibrio specie*(Appendix XII) and further subcultured on already prepared sterile nutrient agar (NA) (Oxoid Ltd., Basingstoke, England) slants for further analysis (Bacteriological Analytical Manual, 2004 and CDC, 1994).

3.4.1 Gram staining

Presumptive isolates were gram stained as follows: a smear of an 18-24 hour colony of the presumptive isolate was made on a grease-free glass slide and heat-fixed over the bunsen flame. It was then covered with crystal violet for about 1 minute and washed with water. The smear was thereafter covered with Grams iodine for about 1 minute and then washed with water. Decolorization of the smear was carried out by addition of acetone with gentle agitation for 10-30 seconds after which it was washed with water then neutralized with safranin for 10-30 seconds before finally washing with water and allowed to dry. The gram stained slides were then viewed under a microscope using the oil immersion objective (x100).

3.5 Biochemical Characterization of Isolates

Conventional biochemical tests were performed on the presumptive isolates of the suspected *Vibrio specie* colonies and further identified to species level using Microgen ™ GnA+B-ID System, UK kit. Conventional tests performed included the following:

Oxidase test: 2 to 3 drops of oxidase reagent (1% tetramethyl-*p*-phenylenediamine) was placed on a piece of filter paper in a clean petri dish. A colony of the isolate was immediately smeared across the wet paper using a sterile wooden applicator stick. A dark purple color developing within 10 seconds indicated a positive test (Appendix XIII). Color development after 10 seconds was disregarded and result interpreted as oxidase negative.

Motility:Sulphur, indole and motility (SIM) medium (Oxoid Ltd., Basingstoke, England) were inoculated with the isolate by making a fine stab with an inoculating needle to a depth of l-2cm short of the bottom of the tube and incubated at 37°C for 24 to 48hours. A positive motility was recorded when there was no defined growth along the line of inoculation with the medium being cloudy. If the organism is not motile, a sharply defined growth restricted to the line of inoculation with the rest of the medium remaining clear was recorded as a negative result. The production of black colour indicates the production of hydrogen sulphide (H_2S).

String test: a large colony (18 – 24 hour growth) of the isolate was emulsified in a drop of 0.5% aqueous solution of sodium deoxycholate (bile salt) on a clean glass slide. Positve reaction was recorded for an isolate when mucoid "string" was formed on drawing the inoculating loop slowly away from the suspension due to lysis of the bacterial cells by the sodium deoxycholate (bile salt) causing the release of DNA from the lysed cells thereby resulting in a viscous mixture giving a positive result (Appendix XIV). On the other hand, results were read negative when no mucoid string was formed.

The Microgen GN-ID system consisted of 24 (GN A+B) standardized biochemical substrates in microwells to identify the family *Enterobacteriaceae* and other non-fastidious gram negative bacilli (oxidase negative and positive).

Principle:The Microgen GN-ID system comprises two separate microwell strips GN A and GN B. Each microwell test strip contains 12 standardized biochemical substrates GN A: lysine, ornithine, H_2S, glucose, mannitol, xylose, O.N.P.G, indole, V.P, citrate and T.D.A. GN B: gelatin, malonate, inositol, sorbitol, rhamnose, sucrose, lactose, arabinose, adonitol, raffinose, salicin and arginine. The dehydrated substrates in each well are reconstituted with a saline suspension of the organism to be identified. If the individual substrates are metabolized by the organism, a colour change occurs during incubation or after addition of specific reagents as shown on the substrate reference table in the appendix. The results are interpreted using the Microgen Identification System Software (MID-60) to identify the test organism.

Procedure: A colony of 18-24 hour culture if the isolate was emulsified in 5ml sterile 0.85% saline and mixed thoroughly to give a suspension. The adhesive tape sealing the microwell test strip(s) was carefully peeled back; using a sterile pasteur pipette, 3-4 drops of the bacterial suspension was added to each well of the microwell test strip (s). After inoculation, wells 1, 2, 3 and 9 (GN A microwell test strip) and well 24 (GN B) were overlayed with 3-4 drops of mineral oil after which all the wells were sealed with the adhesive tape earlier removed and incubated at 35-37°C for 48 hours. After incubation, the adhesive tape was removed and all positive reactions were recorded using the substrate reference table as a guide (Appendix XV) on the forms provided however, specific wells that required additional reagents were read as follows:

i. Two drops of Kovac's reagent were added to well 8 and results was recoded after 60 seconds.

ii. One drop each of VPI and VPII reagent to well 10 and result read within 15-30 minutes.

iii. One drop of TDA reagent was added to well 12, results were read after 60 seconds.

iv. Nitrate reduction test was performed on well 7 after reading the ONPG result by adding 1 drop each of Nitrate A and B reagent after which the result was read after 60 seconds.

On the Microgen GN-ID A+B report form on which the substrates have been organized into triplets (sets of 3 reactions) with each substrate assigned a numerical value (1, 2 or 4), the sum of the positive reactions for each triplet formed a single digit and a total of 9 digits of the Octal code was generated. This was then entered into the Microgen Identification System Software (MID-60) that finally identified the presumptive isolates to specie level.

3.6 Serological Identification of Isolates

The serological identification of the isolates were performed using *V. cholerae* O1 antisera (polyvalaent and monovalent Inaba and Ogawa antisera) and *V. cholerae* O139 antiserum (Denka Seiken Co., Ltd, Tokyo, Japan) according to manufacturer's instruction as follows: A clean glass slide was partitioned into two sections using wax pencil. A drop each of physiological saline (30μl) and antiserum were placed onto one section to serve as control. To another section, a portion of growth of 18-24 hours colony of confirmed *V. cholerae* isolate from NA plate was suspended in 0.5ml of physiological saline using a sterile inoculating loop and emulsified. A drop of antiserum was then added to the antigenic suspension and mixed thoroughly by tilting the slide back and forth for one minute after which the agglutination pattern was observed. Presence of agglutination was recorded as positive while absence of agglutination was recorded as negative.

3.7 Molecular Characterization of Isolates

Molecular characterization of the isolates was carried out using polymerase chain reaction (PCR) to detect the presence of *ompW*(species specific) and *rfb*O1 and *rfb*O139 genes specific for the O1 and O139 serogroups respectively of *Vibrio cholerae.* This was done using the primers shown in Table 3.1.

3.7.1 DNA Extraction

Isolates were inoculated in 10ml of LB (Luria Betani) medium and incubated at 35-37°C for 24 hour following which the cells were harvested by centrifuging at 8000rpm for one minute. The cells were then suspended in 200μl of distilled water. 500 to 700μl of lysis buffer (Rlt) was added and incubated at room temperature for 10-15 minutes. The lysed cells were transferred into clean spin columns and centrifuged at 8000 rpm for 2 minutes. 250μl of wash buffer (RPE) was added and centrifuged at 8000rpm for 2 minutes. 250μl of Rwl was added and centrifuged again at 8000rpm for 2 minutes, the spin columns were centrifuged again for 1 minute. The DNA was then eluted with 100μl of elution buffer into sterile labeled ependorff tube and stored at 4°C until ready for use.

Table 3.1: Primers used for PCR

Targetgene	**Primer sequence**	**Amplicon size (bp)**	**Reference**
ompW(F)	5'- GGTAGCCTTGGTGATATTGGTG-3'	208	Goel *et al*., 2010
ompW(R)	5'- TAGCAGCAAGTCCCCATGAGT-3'		
O1rfb(F)	5'- GTTTCACGAACAGATGGG- 3'	192	Hoshino *et al*., 1998
O1rfb(R)	5'- GGTCATCTGTAAGTACAAC-3'		
O139rfb(F)	5'- AGCCTCTTTATTACGGGTGG-3'	449	Hoshino *et al*., 1998
O139rfb(R)	5'- GTCAAACCCGATCGTAAAGG-3'		

3.7.2 Polymerase chain reaction (PCR)

Amplification of the target DNA was performed in a thermal cycler (GeneAmp – Applied Biosystems 9700) (Appendix XVII) using PCR tubes containing a reaction mixture volume of 50µl. Each of the reaction mixtures contained 7µl of template DNA, 1µl each of forward and reverse primer, 25µl of PCR Master Mix (Fermentas, Life Scsiences, UK) containing 0.05µl Taq DNA polymerase, reaction buffer, 4mM of $MgCl_2$, and 0.04mM of each dNTP (dATP, dCTP, dGTP and dTTP) and made up to 50µl with 16µl of nuclease free water (Fermentas, Life Scsiences, UK).

The reaction mixture was subjected to an initial denaturation at 95°C for 5 minutes and an amplification of 30 cycles, each of which consisted of three steps in the following order denaturation at 95°C for 30 seconds, annealing of template DNA at 55°C for 30 seconds and extension of the primers at 72°C for 1minute. The final extension was performed at 72°C for 7 minutes and the PCR product was kept at 4°C prior to use.

3.7.3 Agarose gel electrophoresis

The PCR products were electrophoresed through 1% agarose gel to resolve the amplified products as follows: A mixture of powdered agarose and Tris-Acetate EDTA (TAE) buffer was boiled and allowed to cool to about 56°C after which 5µl of ethidium bromide was added. The solution was poured into a gel mold with the combs in place and kept at room temperature for the gel to solidify. The agarose gel was placed in an electrophoresis apparatus and the TAE buffer was added to cover the gel. A total of 20µl of the DNA together with a molecular weight marker (100bp), a gel loading dyewas loaded into the wells using a micropipette. After the wells were filled, a voltage of 100V for 30 minutes was used to run the electrophoresis. The amplified products were then visualized under ultra violet (UV) light and the gel was documented using Molecular Imager® Gel Doc^{TM} XR+ Imaging System (BIO-RAD).

3.8 Data Analysis

Data generated from the research were analyzed using Statistical Package for Social Sciences version 19 (SPSS Inc., Chicago, USA). Kruskal-Wallis test was used to determine the level of significance of the occurrence of *V. cholerae* in the borehole, well, pipeborne, street vended and river water sources analyzed of which P values less than or equal to 0.05 were taken to be significant.

CHAPTER FOUR

4.0 RESULTS

A total of 207 water samples comprising borehole, well, pipeborne, street vendor, and river sources from Samaru, Sabon gari and Zaria city were analyzed. Nine (9) samples yielded smooth yellow, circular, and slightly flattened colonies on TCBS agar which were Gram negative short curved rods. Biochemical studies using a combination of conventional methods and standard kit confirmed 2 of the isolates as *V. cholerae* (Table 4.1 and 4.2).

The serology results in Table 4.3 shows that none of the *V. cholerae* isolates agglutinated with the polyvalent and monovalent O1 *V. cholerae* antisera. Also, no agglutination was observed with the O139 *V. cholerae* antiserum. This indicates that the *V. cholerae* isolated from the water samples belonged to the non-O1/non-O139 serogroup.

The DNA of the *V. cholerae* and other *Vibrio* species isolated subjected to PCR and results of the amplified PCR products are shown in Plate 4.1. Amplifications were observed with the *omp*W gene for 5 of the isolates confirming the biochemical characterization of the isolates. However, no amplification was observed with *rfb*O1 and *rfb*O139 genes indicating that the genes were absent and this result confirmed the serological test that none of the isolates were of the O1 or O139 *V. cholerae* serogroups.

Comparing the conventional and molecular results in the Table 4.4,out of 207 water sources sampled, 9 presumptive *Vibrio* species were isolated of whichbiochemical test using the Microgene kit confirmed 2 *V. cholerae* isolates (0.97%) while the PCR detected 5 (2.42%).

Table 4.1:Morphological and biochemical identification of presumptive *Vibrio* spp

Sample no	**Morphology on TCBS agar**	**Gram reaction**	**Oxidase test**	**String test**	**Motility**	**Identification (Presumptive)**
104	Smooth, yellow, circular and slightly flattened	Pink short curved rods	+	+	+	*Vibrio* spp
105	Smooth, yellow, circular and slightly flattened	Pink short curved rods	+	+	+	*Vibrio* spp
110	Smooth, yellow, circular and slightly flattened	Pink short curved rods	+	+	+	*Vibrio* spp
116	Smooth, yellow, circular and slightly flattened	Pink short curved rods	+	+	+	*Vibrio* spp
124	Smooth, yellow, circular and slightly flattened	Pink short curved rods	+	+	+	*Vibrio* spp
129	Smooth, yellow, circular and slightly flattened	Pink short curved rods	+	+	+	*Vibrio* spp
138	Smooth, yellow, circular and slightly flattened	Pink short curved rods	+	+	+	*Vibrio* spp
173	Smooth, yellow, circular and slightly flattened	Pink short curved rods	+	+	+	*Vibrio* spp
206	Smooth, yellow, circular and slightly flattened	Pink short curved rods	+	+	+	*Vibrio* spp

Key:

+ = positive

Table 4.2: Biochemical identification of *V. cholerae* using Microgen GnA+B – ID System

Sample number	**OXI**	**MOT**	**NIT**	**LYS**	**ORN**	**H_2S**	**GLU**	**MAN**	**XYL**	**ONP**	**IND**	**UR**	**VP**	**CIT**	**TDA**	**GEL**	**MAL**	**INO**	**SOR**	**RHA**	**SUC**	**LAC**	**ARA**	**ADO**	**RAF**	**SAL**	**ARG**	**OCTAL CODE**	**ORGANISM**
104	+	+	+	+	–	–	+	+	–	+	+	–	+	+	–	–	–	–	–	–	+	–	–	–	–	–	–	746660100	*V. cholerae*
105	+	+	+	–	–	–	+	+	–	+	+	–	–	+	–	+	–	–	–	–	+	–	+	–	–	+	+	706624123	*V. fluvialis*
129	+	+	+	+	–	–	–	–	–	+	+	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	740600100	*V. alginolyticus*
138	+	+	+	–	–	–	+	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	706400000	*V. parahaemolyticus*
206	+	–	+	+	+	–	+	+	–	–	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	566240000	*V. carchariae*

Key:

OXI– Oxidase

MOT– Motility

NIT– Nitrate reduction

LYS – Lysine decarboxylase

ORN – Ornithine decarboxylase

H_2S – Hydrogen sulphide

GLU – Glucose

MAN – Mannitol

XYL – Xylose

ONP – Ortho-Nitrohenyl β-galactoside

IND – Indole

UR – Urease

VP – Voges-Proskauer

CIT – Citrate

TDA – Tryptophan deaminase

GEL – Gelatin liquefaction

MAL – Malonate

INO – Inositol

SOR – Sorbitol

RHA – Rhamnose

SUC – Sucrose

LAC – Lactose

ARA – Arabinose

ADO – Adonitol

RAF – Raffinose

SAL – Salicin

ARG – Arginine

Table 4.3: Serological identification of the *V. cholerae* isolates

Sample no.	***V. cholerae* O1 Antiserum** Polyvalent	Ogawa	Inaba	***V. cholerae* O139 Antiserum**	**Identification**
104	–	–	–	–	Non-O1/Non-O139 *V. cholera*
173	–	–	–	–	Non-O1/Non-O139 *V. cholera*

Key:
- = Negative

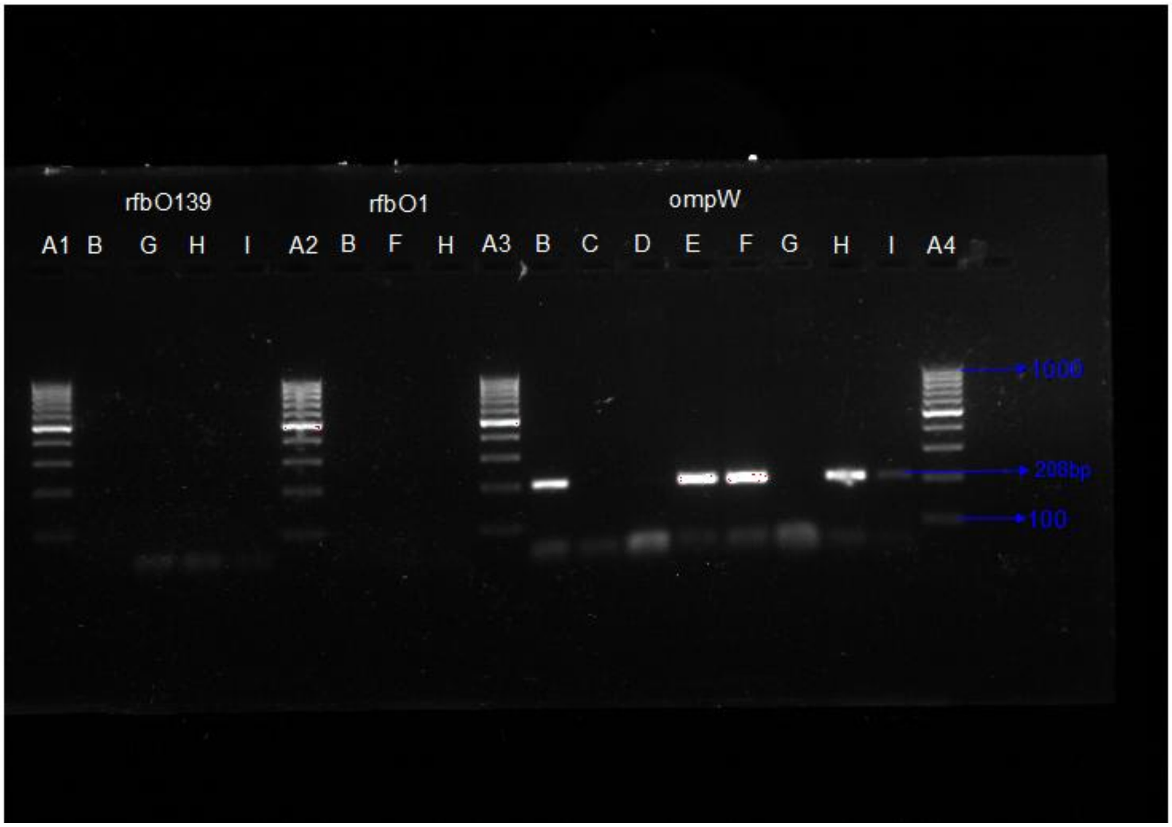

Plate 4.1: Agarose gel electromatogram of PCR products showing *ompW*, *rfb*O1 and *rfb*O139 genes of *V. cholerae*.

Key:
Lanes A1, A2, A3 and A4 represent 100bp molecular ladder.
Lanes A1 to A2 represents isolates tested for *rfb*O139 gene.
Lanes A2 to A3 represents isolates tested for *rfb*O1 gene.
Lanes A3 to A4 represents isolates tested for ompW gene of which B, E, F, H, and I lanes represent isolates exhibiting positive *V. cholerae* with amplicon size of 208bp *omp*W gene but negative for *rfb*O1 and *rfb*O139 genes.

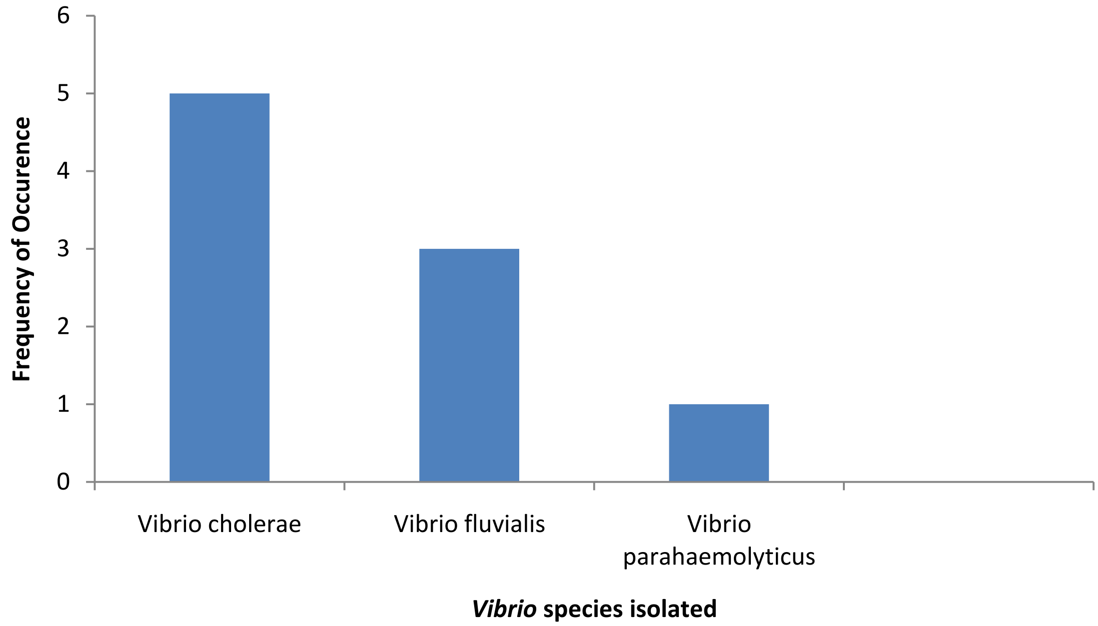

Fig 4.1: Frequency of occurrence of *Vibrio* species isolated from various water sources in Zaria.

Table 4.4: Comparison of conventional method and PCR assay in detection of *V. cholerae*

Source		**Conventional method**			**PCR analysis**	
	N	**Vs**	**Vc**	**(%)**	**Vc**	**(%)**
Borehole	62	2	0		0	
Well	61	5	1		3	
Pipe borne	44	0	0		0	
Street vendor	37	1	1		1	
River	3	1	0		1	
Total	207	9	2	(0.97)	5	(2.42)

Key:
N = number of samples
Vc = *Vibrio cholerae*
Vs = *Vibrio* species
% = percentage positive

The prevalence of *V. cholerae* in water samples (borehole, well, pipeborne, street-vended and river) from different sites in Zaria is shown in Table 4.5. The result shows that prevalence of *V. cholerae* in Zaria was 2.4%. *V. cholerae* was isolated from well, river and street vended water with water sample from river having the highest occurrence (33.33%) followed by samples from well water (4.9%) while the least occurrence was obtained in street vended water (2.7%). *V. cholerae* was however not isolated from water samples from boreholes and taps in all the three sampling sites.

With regard to the various sites of sampling (i.e. Samaru, Sabon Gari and Zaria city) as shown in Table 4.5, water samples from Sabon Gari were the most contaminated with an occurrence of 3.2%. *V. cholerae* was isolated only from well water (10.5%) in that location. *V. cholerae* isolated from water samples in Samaru had an occurrenceof 2.6% with the organism being predominant in street-vended water (6.3%) followed by well water(4.2%). Water samples from river, boreholes and wells in this site had an occurrence of 0.0% as the organism was not isolated from them. Water samples from Zaria city were the least contaminated with *V. cholerae* with an occurrence of 1.5% of which the organism was isolated only in water sample from the river source.

From the Kruskal Wallis test as shown in Table 4.6, there was a significant difference and association between the sources of water and occurrence of *V. cholerae* in Zaria with $P = 0.03$ ($P \leq 0.05$). For Samaru and Sabon Gari, $P \geq 0.05$ (0.729 and 0.331 respectively). This indicates that there was no significant difference and association between the source of water and occurrence of the organism in the two sample sites. However in Zaria city, there was a significant difference and association between the source of water and occurrence of the organism with $P = 0.00$ ($P \leq 0.05$). There was an inverse relationship between the mean ranks of water source and occurrence of the organism; the lower the mean rank the greater the occurrence while the higher the mean rank, the lesser the occurrence.

Table 4.5: Distribution of *V. cholerae* from different water sources in Zaria

	***Vibrio cholerae* isolated from various water sources**							
	Samaru		**Sabon Gari**		**Zaria city**		**Total**	
Source	**N**	**p (%)**	**N**	**p (%)**	**N**	**p (%)**	**N**	**p (%)**
Borehole	22	0 (0.0)	17	0 (0.0)	23	0 (0.0)	62	0 (0.0)
Well	24	1 (4.2)	19	2 (10.5)	18	0 (0.0)	61	3 (4.9)
Pipeborne	14	0 (0.0)	16	0 (0.0)	14	0 (0.0)	44	0 (0.0)
Street vendor	16	1 (6.3)	9	0 (0.0)	12	0 (0.0)	37	1 (2.7)
River	1	0 (0.0)	1	0 (0.0)	1	1 (100)	3 (33.3)	1
Total	77	2 (2.6)	62	2 (3.2)	68	1 (1.5)	207	5 (2.4)

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

Table 4.6: Comparative occurrence of *V. cholerae* in water sources in Zaria using Kruskal-Wallis test

Sources	Samaru		Sabon Gari		Zaria city		Total	
	N	**mr**	**N**	**mr**	**N**	**mr**	**N**	**mr**
Borehole	22	40.00	17	32.50	23	35.00	62	106.50
Well	24	38.40	19	29.42	18	35.00	61	101.41
Pipeborne	14	40.00	16	32.50	14	35.00	44	106.50
Street vendor	16	37.59	9	32.50	12	35.00	37	103.70
River	1	40.00	1	32.50	1	1.00	3	72.00
χ^2	772.037		62	4.602	68	67.000	207	16.344
P		0.729		0.331		0.000*		0.003*

Key:
N = number of samples
mr = mean rank
χ^2 = chi square
P = P - value
* = Statistically significant P-values (where P $\leq$ 0.05).

CHAPTER FIVE

5.0 DISCUSSION

*Vibrio cholerae*is an autochthonous inhabitant of the aquatic environment and this constitutes a reservoir for survival which aids in transmission of the bacterium as earlier discussed in the review of literature. In this work, *V. cholerae*was isolated from some of the water sources (well, street vendor and river) and were of the non-O1/non-O139 serogroup; none belonged to the O1 or O139 serogroup (Table 4.3; Plate 4.1). Non-O1/non-O139 *V. cholerae* serogroup in the past has been considered of negligible importance (Singh *et al*., 2001). Presently, this strain can no longer be ignored (Kumar*et al*., 2008) as several studies have revealed isolation of the organism from various water sources and its association with hospitalized patients presenting with diarrhoea, peritonitis and also in immunocompromised cases in several parts of the world (Singh *et al*., 2001; Wiwatworapan and Insiripong, 2008; Rozemeijer *et al*., 2009; Smith *et al*., 2009; Elhadi *et al*., 2012; Luo *et al*., 2013). In a research conducted by Marin *et al*. (2013), *V. cholerae* strains from sequential outbreaks in Nigeria were characterized and it was observed that a lineage of *V. cholerae* non-O1/non-O139 serogroup was circulating with atypical El Tor strains isolated from patients with cholera-like cases. Their findings indicated that non-O1/non-O139 serogroups were the important causes of diarrhoeal diseases in Nigeria. Studies have shown that the presence of this serogroup in water is of public health importance as their presence suggests that environmental conditions are suitable for survival of epidemic *V. cholerae* O1 in the water source (Smith *etal*., 2009). Also, the strain may act as reservoirs of virulence genes in the environment which could lead to the emergence of new epidemic or virulent variants by lateral transfer of virulent genes which can bring about diarrhoeal infections (Elhadi *et al*., 2012; Luo *et al*., 2013).

In this study PCR amplification of the *omp*W gene confirmed the presence of 5 *V. cholerae* isolates as against 2 confirmed by the biochemical test performed (Table 4.4; Plate 4.1). In some cases biochemical tests may not be able to differentiate *V. cholerae* from other *Vibrio* speciesas a result of sharing serological markers and phenotypic characters among *Vibrio* species (Yadava *et al*., 2013). PCR on the other hand is able to identify and characterize organisms based on genotypic traits hence, more sensitive. A similar study was conducted by Baron *et al.* (2007) and result of their work showed that PCR can be used for checking doubtful biochemical identification of *V. cholerae* using the *ompW* specie-specific gene.Outer membrane protein (*ompW)* gene sequence is one that is highly conserved among *V. cholerae* strains and serves as a good species specific marker for different biotypes or serotypes of *V. cholerae* (Tamrakar *et al*., 2009).

From the result of this work, the overall prevalence of *V. cholerae* isolated from water samples in Zaria was 2.4% (Table 4.5). Although the prevalence was low, there was a significant difference and association between the source of water and occurrence of *V. cholerae* in the water samples in the environment ($P = 0.003 \leq 0.05$) (Table 4.6). This is of great concern as most of the populace use water from these sources for drinking and other household needs which can aid transmission of the pathogen. In Nigeria, majority of the rural populace do not have access to potable water and therefore, depend on well, stream and river water for domestic use (Shittu *et al*., 2008). Zaria is known to be one of the oldest towns in Northern Nigeria which is very large with a growing population from different parts of the world. However, the town has been known to have unending water problems despite being blessed with an abundant water resource (Lukman *et al*., 2009). This was confirmed in the course of this study during sampling as it was observed that most of the populace depended on water from wells and boreholes for domestic use. Also street vendors were seen at all times hawking water in large gallons pushed in trolleys.

Out of the three water sources form which *V. cholerae* was isolated (river, street-vended and well), the microorganism was found to be most predominant in water from river source with a percentage occurrence of 33.3% followed by well water (4.9%) and the least predominant in street-vended water (2.7%) (Table 4.5).It was observed that a lot of human and agricultural activities such as open defeacation by individuals and irrigation farming were performed by farmers close to the river banks. These farmers mostly use manure comprising of animal dungs (or droppings) as fertilizers to grow their crops. Also, nomads (herdsmen) were seen with their cattle grazing on harvested farm lands close to the bank of the river as the herds also drink from the water. The river body may have possibly been contaminated as a result of run-off water from human faecal matter and manure (containing faecal droppings of the animals). In a study conducted in Zaria, *V. cholerae* was isolated from households engaged in livestock farming who use the animal wastes as manure for their vegetable gardens, suggesting animals to be a source of contamination of *V. cholerae* (Abakpa *et al*., 2013). An important source of contamination of surface and ground waters is run-off water from agricultural and pasture lands, and urban areas (Cabral, 2010). Faecal bacteria enter surface water by direct deposit of feces and by overland run-offs. The movement of human and animal wastes into surface waters can be a major factor contributing to the pollution of available water in many regions (Cabral, 2010).

According to Aulet *et al*. (2007) agricultural activities modify the aquatic environments, thus generating conditions that allow survival of *V. cholerae* non-O1, non-O139 in water. Also, researchers have reported non O1/non O139 *V. cholerae* as ubiquitous and belonging to autochthonous flora of streams, rivers, brackish, estuarine and coastal waters (Rozemeijer *et al*., 2009; Kumar, *et al*., 2010) and this may also suggest why the organism was isolated from the river. Although Zaria city had the lowest occurrence of *V. cholerae* of 1.5% of all the sample sites, the observations made during sampling and evidence from research and literature discussed suggest why there was a high level of occurrence of the organism in the river source and a statistically significant result in Zaria city with $P = 0.00 \leq 0.05$ (Table 4.6).

From our results, Sabon gari had the highest occurrence of 3.2% as compared to other sites with the organism being predominant only in well water (10.5%) even though it was least predominant in the same source in Samaru (4.2%) (Table 4.5). Although statistical analysis showed that there was no significant difference and association between the occurrence of the organism and source of water in these locations, traditionally wells have been and still are an important source of water supply. In the course of this study, it was observed during sampling that most of the wells were hand dug and poorly covered or not covered at all. Also, poor drainage systems were located near some wells with stagnant water observed around some of them. Some wells were seen to be located less than 100m to septic tanks. Containers used to draw water from the well (commonly known as 'guga') were often seen left at the side of the well, in direct contact with soil and other sources of environmental contamination. These probably suggest some of the possible reasons for contamination of the wells with the organism. According to Tamrakar *et al*.(2009), major source of ground water contamination could be as a result of leakage of contaminated surface water along wells in cholera endemic areas. Other possible source of contamination could be seepage of surface water through soil layers when natural filtration capacity of the soil fails, improper construction, shallowness, proximity to toilet facilities, sewage, refuse dump sites and various human activities around the well (Shittu *et al*., 2008; Bitton 1994). Results of this research are similar to that of Umoh *et al*. (1983) who isolated non O1 *V. cholerae* from well water during an outbreak of gastroenteritis in Katsina. The outbreak was linked to faecal contamination of well water facilitated by vendors who sold the water to various households in the area. Similarly in 2010 outbreak of cholera was speculated to be directly related to sanitation and water supply. The hand dug wells and contaminated ponds being relied on by most of the Northern states as sources of drinking water was a major transmission route during the outbreak (Adagbada *et al*., 2012). Ground water is one of the nation's most important natural resource and is an important source of drinking water for mostly rural populations that supply their own domestic water from local wells (WHO, 2011c; USGS, 2013). Microbial contamination of groundwater used for drinking purposes is a dominant concern in many countries (Mara and Horan, 2003). Ground water sources such as wells and springs are often believed to be of good quality with regard to bacterial pathogens transmitted by faecal-oral route (Shittu *et al*., 2008; WHO, 2012). However, such sources are readily contaminated by faecal material especially where there are potential sources of contamination nearby or where contaminants may be carried by surface water (WHO, 2012).

In this research, *V. cholerae* was predominant in street vended water in Samaru with a prevalence of 6.3% higher than that of well water (4.2%) in the same location(Table

4.5). Although the result was not statistically significant, during sampling it was observed that some most of the street vendors purchased water from private owners of motorized boreholes that pump the water directly into large storage tanks with hose attached for transfer into containers for the street vendors while a few obtained water from public wells. Some of these containers or gallons were observed to have cracks which are not properly sealed while some use dirty polythene bags to seal the leaking edges. Interaction with some street vendors revealed that the containers are not washed frequently. While the source of contamination of the street-vended water may have varied and could not be determined, evidence suggests that the contamination may have occurred from any of the factors observed during sampling. There have been number of health concerns associated with water supplied to consumers by water vendors (WHO, 2011c).In 1996 there was an outbreak of severe diarrhoea in Kano. In an attempt to find the possible risk factors, Hutin *et al*. (2003) conducted a research and their results showed that street vended water was associated with the illness. Similarly, consumption of street-vended water has also been reported to be a significant risk factor associated with cholera epidemic in Sierra Leone (Nguyen *et al*., 2014) and Latin America (Ries *et al*, 1992; Weber *et al.*,1994; Tauxe *et al.*,1995). Vended water is common in many parts of the world where scarcity or lack of infrastructure limits access to suitable quantities of drinking water (WHO, 2011c). Although water vending is more common in developing countries, it also occurs in developed countries. Nigeria is also not an exception because municipal water supply is not available for all households, water vendors can be observed at all times in Zaria. Water vendors use a range of mode of transport to carry water for sale directly to the consumer including tanker trucks, wheel barrows or trolleys. These include access to adequate volumes and concern regarding inadequate treatment or transport in inappropriate containers, which can result in contamination (WHO, 2011c).

The non-detection of the pathogen in water samples from taps and boreholes analyzed in this study (Table 4.5) may be a reflection of the water treatment process and depth of the boreholes respectively. Another possible reason could be due to the ability of the pathogen to survive in a viable but non-culturable state. During environmental stress in the natural ecosystem *Vibrios* may undergo a series of physical and metabolic changes resulting to a viable but non-culturable (VBNC) state rather than dying when exposed to reduced nutrient levels (Igbinosa and Okoh, 2008). In the viable but non-culturable state, bacteria are metabolically active but do not form colonies when plated onto conventional culture media and have been shown to retain their virulence factors. Under favorable conditions, often season-dependent, they can revert to the vegetative state for their growth and multiplication (Oliver, 1995).Water from a relatively protected source cannot be guaranteed to be free from contaminating bacteria when consumed. In a typical urban setting in a developing country, where the water supply is intermittent, drinking-water is collected and stored, in various ways, for one or more days in the household (WHO, 2011c). If the water is not handled correctly, the processes of collection and storage provide ample opportunity for contamination. It has been recently reported that *V. cholerae* O1 and non-O1 can shift to a rugose form associated with the production of an exopolysaccharide which promotes cell aggregation (Morris, 1990). This rugose form resists both chlorine (even at levels exceeding 2 mg/l) and other disinfectant in potable water and is likely to contribute to the waterborne transmission of cholera (Rice *et al*., 1992; Morris, 1990). Contamination of drinking-water can also occur as result of wastewater influx in old or damaged network systems.

CHAPTER SIX

6.0 SUMMARY, CONCLUSION AND RECOMMENDATIONS

6.1 Summary

This study serves as a baseline, confirming the presence of *V. cholerae*of the non-O1/non-O139 serogroup in some well, street-vended and the river water sources analyzed. The prevalence of *V. cholerae* in Zaria was 2.4% of which Sabon Gari had the highest percentage distribution of 3.2% followed by Samaru (2.6%) and the least being 1.5% in Zaria city. This study has also established the fact that a combination of both conventional and molecular techniques targeting species specific gene (*ompW*) is essential for effective identification of *V. cholerae*.

6.2 Conclusion

In this study, we report the presence of *V. cholerae* of the non-O1/non-O139 serogroup isolated from some well, river and street-vended water samples analyzed Zaria with a prevalence of 2.4%.The microorganism was not isolated from borehole and pipeborne water sources. *V. cholerae* was found to be predominant in river (33.3%), followed by well water (4.9%) and lastly, street-vended water (2.7%). Water samples from from Sabon Gari were the most contaminated with an occurrence of 3.2% followed by that from Samaru while the least contaminated water samples were from Zaria city with an occurrence of 1.5%.

The study also revealed that the use of PCR targeting species specific gene (*omp*W) in combination with conventional microbiological techniques is essential for proper identification of *V. cholerae*.

From the results of this research, we can conclude that water plays an important role as a transmission vehicle for *V. cholerae* considering the presence of the pathogen in the water samples (well, street-vended and river) thus indicating that the environment may also be conducive for the survival of other serogroups such as O1 and O139 which are etiologic agents of cholera. This infers that consumption of water from such sources may constitute a risk to public health.

6.3 Recommendations

i. Health-based targets and water quality improvement programmes in general should be implemented by government in the context of a broader public health policy, including initiatives to improve sanitation, waste disposal, personal hygiene and public education on mechanisms for reducing both personal exposure to hazards and the impact of personal activity on water quality.

ii. In combination with conventional methods which serve as gold standard for detection of *V. cholerae*, the use of PCR targeting *ompW* gene which is species specific should be encouraged for appropriate identification of the microorganism.

iii. Surveillance studies should be conducted by health organizations including both State and Federal Ministry of Health, to prevent occurrence of outbreaks of *Vibrio cholerae* which could possibly result in an epidemic.

iv. Government should provide a means of ensuring proper distribution and management of municipal water supply to enhance accessibility of potable water to consumers.

v. Local authorities should implement surveillance programmes for water providedby vendors and, where necessary, develop education programmes to improve thecollection, treatment and distribution of water to prevent contamination.

vi. Further research should be conducted to study toxigenicity, genome sequence and relationship between environmental and clinical strains of *Vibrio cholerae*which can help in development of vaccines.

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APPENDICES

Appendix I: Sampled household well beside a septic tank

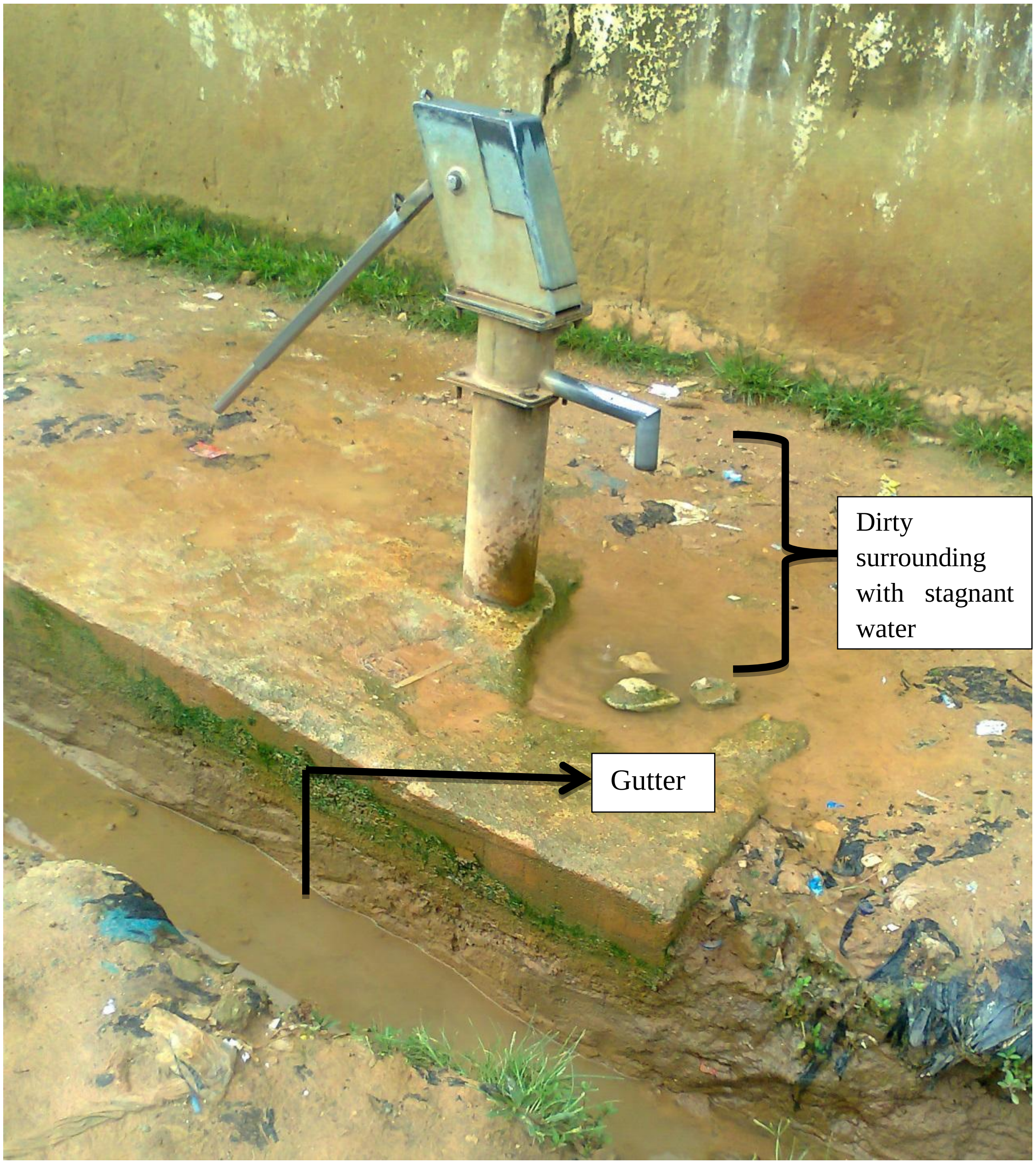
Appendix II:Sampled public well close to a refuse dump site.

Appendix III:Sampled public well

Appendix IV:Sampled pipeborne water source

Appendix V:Sampled pipeborne water sourcelocated near a gutter

Appendix VI:Sampled borehole (hand pump).



Appendix VII:Sampled borehole (hand pump).

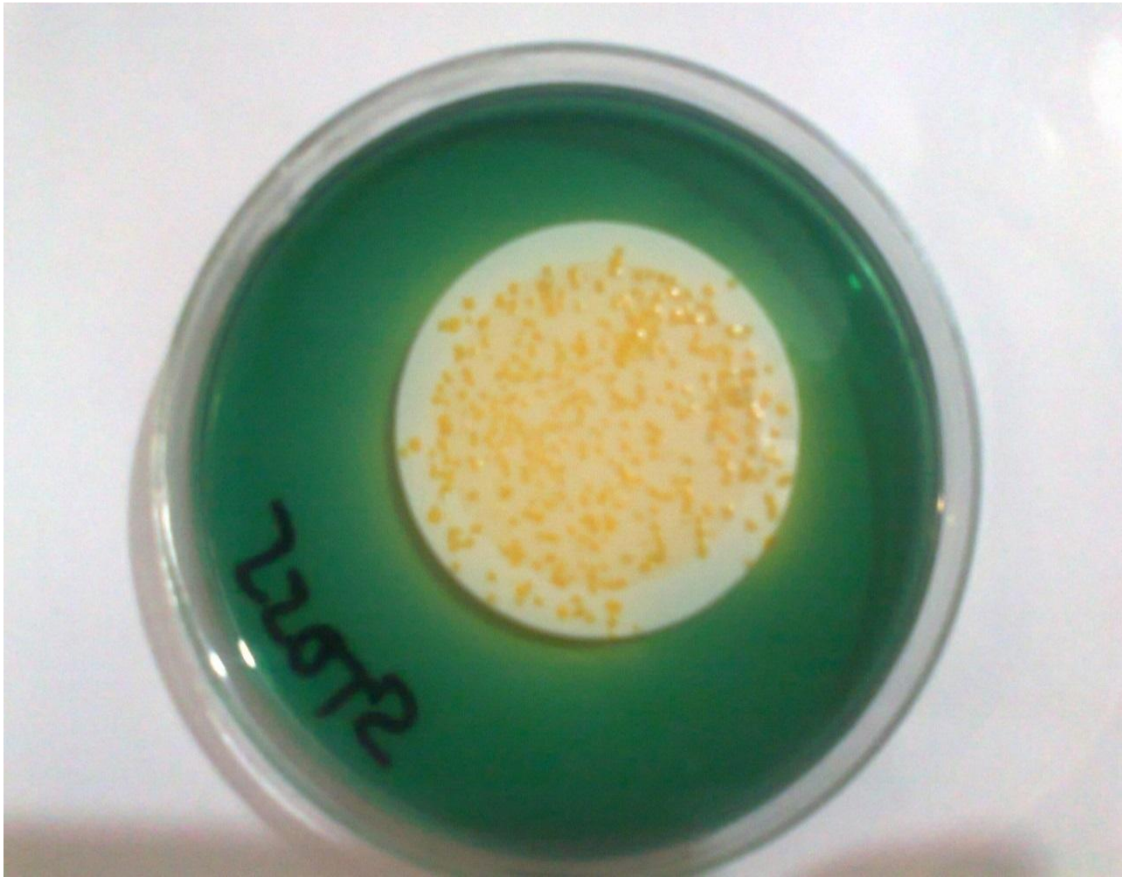
Appendix VIII:Sampled borehole (motorized) with water stored in tanks.

Appendix IX:Sampled borehole (motorized) with tap outlet.

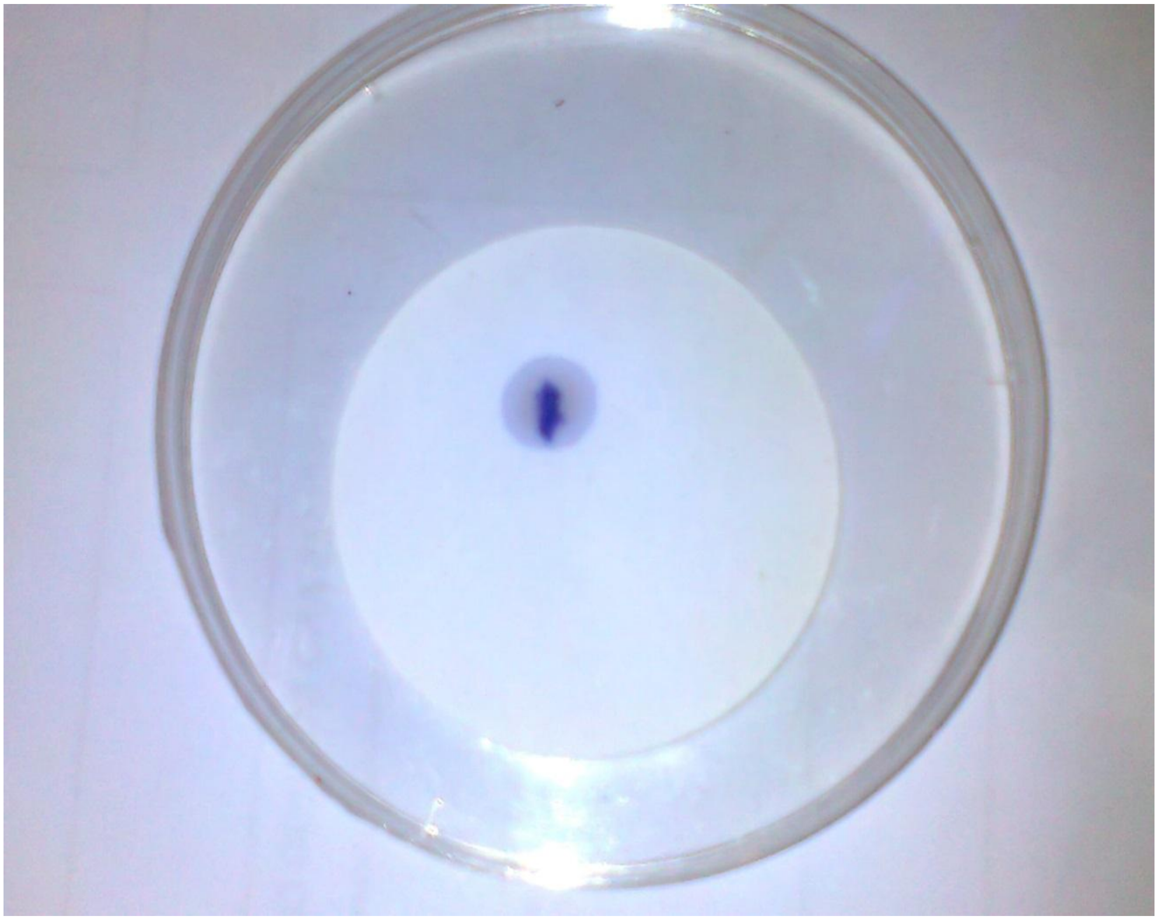
Appendix X: Sampled River source at Sabon Gari.

Appendix XI: Set up of filtration apparatus used to filter the sampled water

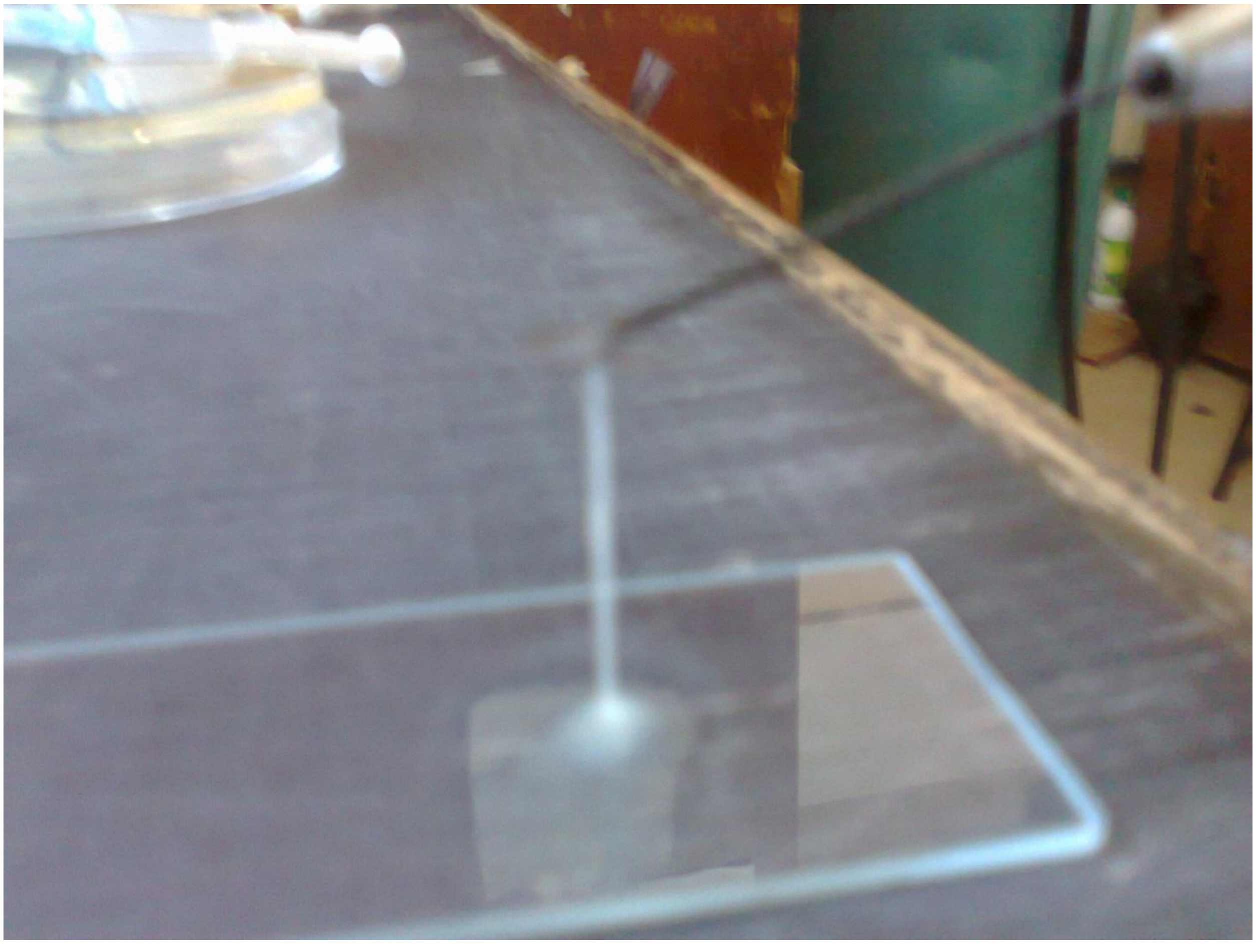
Appendix XII:Colonial morphology of presumptive *Vibrio* specie on TCBS agar



Appendix XIII:Positive oxidase reaction



Appendix XIV:Positive string test



Appendix XV:Microgen GnA + B Substrate reference table

Well	Reaction	Description	Positive	Negative
1	Lysine	Lysine decarboxylase-Bromothymol blue changes to green/blue indicating the production of the amine cadaverine	Green / Blue	Yellow
2	Ornithine	Ornithine decarboxylase-Bromothymol blue changes to blue indicating the production of the amine putrescine	Blue	Yellow / Green
3	H_2S	H_2S production- Thiosulphate is reduced to H_2S that reacts with ferric salts producing a black precipitate.	Brown/ Black	Straw
4 5 6	Glucose Mannitol Xylose	Fermentation- Bromothymol blue changes from blue to yellow as a result of acid produced from the carbohydrate fermentation	Yellow	Blue / Green
7	ONPG	Hydrolysis - ONPG hydrolysis by Β - galactosidase results in the production of yellow ortho-nitrophenol	Yellow	Colourless
7a	Nitrate (for oxidase positive organisms)	Reduction of nitrate to nitrite is indicated by formation of a red colour on addition of nitrate A and B reagents	Red	Colourless/ yellow
8	Indole	Indole is produced from tryptophan and gives a pink/red complex when Kovac's reagent is added.	Pink / Red	Colourless
9	Urease	Hydrolysis of urea results in the formation of ammonia leading to an increase in pH which turns phenol red from yellow to pink/red	V. deep pink	Straw-pale salmon colour
10	VP	Acetoin production from glucose is detected by the formation of pink/red complex after addition of alpha naphthol and creatine in the presence of KOH	Deep pink/ Red	Colourless to pale pink
11	Citrate	Utilisation of citrate leading to a pH increase giving a colour change in bromothymol blue from green to blue.	Blue	Yellow / Green
12	TDA	Indolepyruvic acid is produced from tryptohan by tryptophan-deaminase giving a cherry red colour when ferric ions are added.	Cherry red	Straw colour
13	Gelatin	Proteolytic enzymes liquefy gelatin resulting in black particles being dispersed throughout the well	Black	Colourless
14	Malonate	Inhibition of the conversion of succinic acid to fumaric acid occurs when sodium malonate is the only source of carbon. An isolate incapable of using this substrate result in the accumulation of succinic acid and the organism does not grow. A positive reaction is the result of the use of sodium malonate at the	Blue	Yellow

		same time that ammonium sulphate is used as the nitrogen source giving sodium hydroxide which increases the alkalinity giving a blue colour.		
15	Inositol	Fermentation- Bromothymol blue changes from blue to yellow as a result of acid produced from the carbohydrate fermentation	Yellow	Blue
16	Sorbitol			
17	Rhamnose			
18	Sucrose			
19	Lactose			
20	Arabinose			
21	Adonitol			
22	Raffinose			
23	Salicin			
24	Arginine	Arginine is converted to ornithine, ammonia and CO_2 by arginine dihydrolase resulting in an increase in pH and a change in colour of the bromothymol blue from green to blue. At 48 hours green reactions are negative.	Green / Blue Blue	Yellow/ Yellow Green

Appendix XVI:Biochemical reactions using Microgen Gn A+B kit

Appendix XVII:PCR using GeneAmp PCR system 9700 thermocycler