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MOLECULAR CHARACTERIZATION AND ISOLATION OF VIBRIO SPECIES FROM MOUNTAIN TOP UNIVERSITY RIVER AND THE UNIVERSITY OF LAGOS LAGOON. GHAJIGA PHEEBEMI RACHEAL 18010104006 A RESEARCH PROJECT SUBMITTED TO THE DEPARTMENT OF BIOLOGICAL SCIENCES, COLLEGE OF BASIC AND APPLIED SCIENCES, MOUNTAIN TOP UNIVERSITY, MAKOGI, IBAFO, OGUN STATE, NIGERIA. IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF BACHELOR OF SCIENCE (B.Sc.) IN BIOTECHNOLOGY AUGUST, 2021. DECLARATION I hereby declare that this project report was written under the supervision of PROF.

STELLA SMITH is a product of my own research work. Information derived from various sources has been duly acknowledged in the text and a list of references provided. This research project report has not been previously presented anywhere for the award of any degree or certificate. GHAJIGA PHEEBEMI R. Date CERTIFICATION This is to certify that this research project titled "MOLECULAR CHARACTERIZATION AND ISOLATION OF VIBRIO SPECIE FROM MOUNTAIN TOP UNIVERSITY RIVER AND UNIVERSITY OF LAGOS LAGOON'' was carried out by GHAJIGA, PHEEBEMI RACHEAL, with matriculation number 18010104006. This project meets the requirements governing the award of Bachelor of Science (B.Sc.)

Degree in Biotechnology department of biological sciences of Mountain Top University, Ogun State, Nigeria and is approved for its contribution to knowledge and literary presentation. GHAJIGA PHEEBEMI R. Date PROF. STELLA SMITH Date (Project Supervisor) DR. C. AYOLABI Date (Head of Department) DEDICATION I dedicate this project to God Almighty for giving me the grace, empowerment, good health and all I needed to make this work a success and secondly to my dear parents, Mr. & Mrs.

Ghajiga and to my sister Pheekanmilla Sarah Ghajiga for their guidance, support, understanding, sacrifice and kind support. I also dedicate this work to my course mates and friends for their support in the course of my four- year study of Biotechnology in Mountain Top University. May the Almighty God bless you all! Amen.

MOLECULAR CHARACTERIZATION AND ISOLATION OF *VIBRIO SPECIES* FROM MOUNTAIN TOP UNIVERSITY RIVER AND THE UNIVERSITY OF LAGOS LAGOON.

GHAJIGA PHEEBEMI RACHEAL

18010104006

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IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF BACHELOR OF SCIENCE (B.Sc.) IN BIOTECHNOLOGY

AUGUST, 2021.

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3-10-22

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(Project Supervisor)

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May God in His infinite mercies continue to bless you all, Amen!

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ABSTRACT

Isolation and identification of the causative agent is the initial step towards understanding the nature of a disease in any environment. The aim of this study was to investigate the isolation, identification and molecular characterization of *Vibrio* species from the water samples in Mountain Top University, Ogun state and University Lagos lagoon, Lagos Nigeria.

Vibrio species is the most common species of bacterium associated with water bodies and its aquaculture bacteria are also responsible for acute diarrhea illness in human beings. Consumption of raw sea fish or contaminated seafood is responsible for severe gastroenteritis.

Isolates were evaluated for characterization and identification using biochemical tests. The result obtained from the biochemical test confirms that the bacterial isolates belong to the species *Vibrio.*

Vibrio species isolated from water samples were characterized by PCR amplification identified by 16S ribosomal RNA gene sequence analysis (Molecular-based detection technique).

Out of the 20 isolates obtained from the isolation of water samples from Mountain Top University River and the University of Lagos lagoon on TCBS agar, four isolates were positively characterized as *Vibrio* spp.

The results showed that *Vibrio* species were present but significantly low in Mountain Top University River and the University of Lagos lagoon. This indicates the presence of a potential reservoir in MTU and UNILAG water bodies, and consumption of fishes or contaminated water might cause water borne infection.

Key Words: *Vibrio* species, PCR, Isolation, characterization.

CHAPTER ONE

1.0 INTRODUCTION

The transmission of cholera is very high and is characterized by watery diarrhea. Symptoms can be noticed within 13 hours to 5 days after consuming contaminated food or water. Adults and children both suffer from the effects of cholera, and if left untreated, can result in death within hours. (World Health Organization, 2017).

Individuals are exposed to avoidable health risks due to a lack of, insufficient, or ineffective management of water and sanitation services. Waterborne infections affect children more than adults. Improved access to water sources leads to improved health and, as a result, increased school attendance, which has a positive long-term impact on their lives. The World Health Organization (WHO) published a report in 2017 that stated that the contamination with an illness like cholera has been linked to contaminated water and poor sanitation. *Vibrio cholerae* in food and water, is responsible for the illness well-known as cholera. (WHO, 2017). *Vibrio* species are abundant in the aquatic environment, well-organized, attached to plankton, and found in various tissues and organs of marine animals. Common sources include urban water supplies and ice made from urban water. (Nair. 2004).

Vibrio is a Gram-negative bacterium, curved-rod (comma) shaped, or asporogenous rods that are straight and are motile having one polar flagellum when grown in liquid medium (Kaysner*et al*., 2004). Amongst others, they rank the topmost common toxin-producing microorganisms found in seawater, marine, coastal, and estuarine waters around the world. (Craig Baker- Austin et al. 2107). Several species of *Vibrio* are pathogens. A variety of marine species, such as prawns and crabs, carry them when humans are exposed to them; they have been known to cause fatal infections. More than 70 species of *vibrios* belonging to the genus *Vibrio*, are classified as halophilic or non-halophilic based on their need for sodium chloride to grow. Human infectious illnesses can be caused by over 12 out of a 100 forms of *Vibrio* species, among which are three particular disease causing species namely *Vibrio cholerae, Vibrio parahaemolyticus and Vibrio vulnificus.* A very fast means of contacting infections caused by *Vibrio* is by eating, ingesting or handling raw shellfish (oysters) or coming in contact through skin contact with contaminated sea water. (Potasman, Paz, and Odeh et al 2002; Daniels *et al*. 2002 infested water. (Daniels *et al*., 2002). *Vibrio* infections are the most common types of infections. Since the majority of *Vibrio*

infections are linked to the ingestion of infected food, they are often classified as foodborne illnesses.

Foodborne illness is a disease caused by consuming contaminated food as defined by the World Health Organization (WHO) (Velusamy *et al*., 2010). In Nigeria, *Vibrio cholerae* infections appear to have increased in recent years. Climate change effect in driving the risks connected with *Vibrio* has piqued people's interest (Craig Baker-Austin et al. 2010). As a result, *Vibrio* infections have long been associated with tropical and subtropical environments (Iwarnoto et al., 2010).

Based on the structure of the *O* antigen, strains of *Vibrio cholerae* are divided into more than 200 different serogroups. Historically, the serogroup O1 has been the primary cause of epidemic cholera, but in 1992, a new epidemic serogroup, the O139, emerged (Faruque, 2003). Two key virulence factors are encoded by pathogenic (toxigenic) *Vibrio cholerae* strains: cholera toxin (CT) and the toxin-coregulated pilus (TCP), the manifestation of which causes the acute watery diarrhea linked with cholera. (Herrington, 1998). A lysogenic bacteriophage (CTX) and a reservoir of pathogenicity, respectively, encode CT and TCP (Faruque et al., 2003). The rest of the *Vibrio cholerae* strains are known as "non-O1, non-O139" strains and are not responsible for cholera outbreaks. Because they rarely have the CT and TCP genes, these strains are often described as "non-toxigenic". Nonetheless, many of these non-O1/non-O139 species encode alternative virulence genes and have been linked to a variety of diseases in people, including gastroenteritis and extraintestinal infections. (Dalsgaard et al., 2014). The RTX toxin (Chow et al. 2001), outer membrane proteins (including OmpU) (Mathur et al., 2007), and hemolysins are examples of auxiliary virulence factors (Rahman et al., 2008).In *Vibrio cholerae*, a cluster of rtx genes produces a multifunctional, auto-processing rtx (repeats in toxin) toxin that facilitates immune evasion. *hly*A and other hemolysins are toxins that form pores that break open eukaryotic cells. (Heuser 2001). The existence of these virulence factors in non-O1/non-O139 strains is thought to contribute to their ability to survive in the wild. (Sakib, 2018).

The need for DNA extraction is to obtain a relatively pure form of it for further investigations. Several DNA extraction methods are available including but not limited to boiling, alkaline, salting out, phenol-chloroform, DNA extraction from silica columns etc. For the purpose of this study we would be using the boiling method of extraction.

In an aquaculture scenario, accurate identification of the causal agent is essential for devising suitable preventive treatments. Bacterial identification methods that have been used in the past have significant flaws. As a result, a variety of molecular approaches are becoming more common for identifying various aquaculture-related bacterial diseases.

Recent molecular improvements in biotechnology have greatly improved the detection of bacterial pathogens in the environment. These advancements and a downward trend in the cost of molecular detection methods have contributed to increased frequency of detection of pathogenic microorganisms where traditional culture-based detection methods have failed. Culture methods also have been greatly improved, and the confluence of the two suites of methods provides a powerful tool for detection, isolation, and characterization of pathogens. While molecular detection provides data on the presence and type of pathogens, culturing methods allow a researcher to preserve the organism of interest for "-omics" studies, such as genomic, metabolomic, secretomic, and transcriptomic analysis, which are rapidly becoming more affordable. This has yielded a clearer understanding of the ecology and epidemiology of microorganisms that cause disease. (Wiley et al., 2012).

Polymerase chain reaction (PCR) has generated great benefits and allowed scientific advancements. PCR is an excellent technique for the rapid detection of pathogens, including those difficult to culture.

Conventional PCR is qualitative, and has been in use in detecting, monitoring and identifying bacteria from a set of environmental or aquacultural samples and is the center of molecular diagnostics.

Kary Mullis created the polymerase chain reaction (PCR) in 1985 which made synthesizing large copies of DNA possible without cloning. The method depends on the ability of a DNA polymerase to extend a primer with the template strands. (Mullis *et al.,* 1985). Since its description, this technology has caused a veritable revolution in biological research, establishing the agreement of basic biological processes in areas involving diagnoses and genetic improvements for plants and animals (Spolidorio et al., 2005).

Polymerase chain reaction (PCR) procedures are rapid and highly specific for detecting many pathogens (Anzar and Alarcon 2008).

Identification and detection of *Vibrio* spp.through conventional culture and biochemical test methods is a laborious and time - consuming process. Currently, deoxyribonucleic acid (DNA) based typing techniques are frequently used to generate strain-specific fingerprinting and have proven to be useful tools in detecting a single copy of a target DNA sequence of cells that are present in very limited amounts (Chakraborty *et al*., 2008; Velusamy *et al*., 2010).

The most important information and fresh insights into the identification of closely related marine bacteria have come from techniques PCR-based molecular techniques, such as Random Amplified Polymorphism DNA (RAPD), 16S rRNA sequence identification, colony hybridization with species-specific probes, Fluorescent In Situ Hybridization (FISH), Ribotyping, Restriction Fragment Length Polymorphism (RFLP), and Amplified Fragment Length Polymorphism (AFLP) etc., has produced the most useful information and new insights into the identification of closely related marine bacteria. Analysis of 16S rDNA and other housekeeping gene sequences are the most popular and precise DNA sequence-based identification approaches currently utilized to identify closely related *Vibrio* species. (Chartterjee et al., 2012). The PCR technique is useful in molecular characterization and exact identification of *Vibrio cholerae* using 16S rDNA sequences. (Leslie *et* al., 2013).

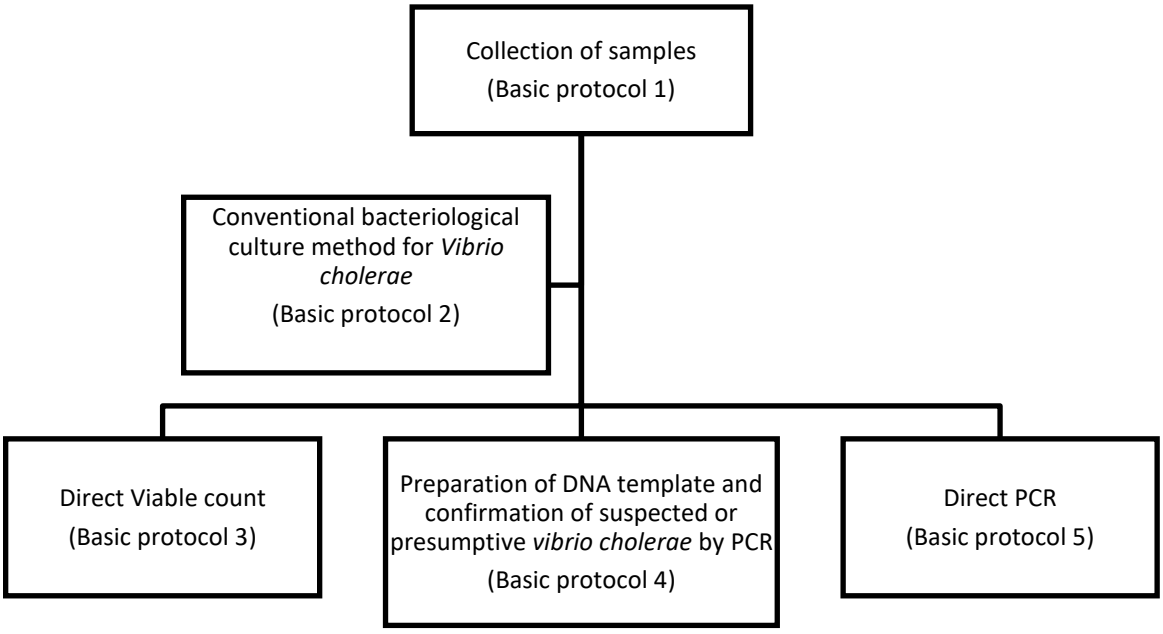

Figure 1.1 Flowchart of methods (protocols) used to detect, isolate and characterize V. *cholerae* from MTU River and University of Lagos Lagoon.

1.4 Aims and objectives.

The goal of this research was to isolate *Vibrio* species from Mountain Top University River and University of Lagos lagoon and molecularly characterize them in order to show the significance of *Vibrio* species present in both sites.

- To isolate and characterize *Vibrio* species from MTU river and University of Lagos lagoon.
- To confirm the *Vibrio* spp using 16S rRNA gene PCR.

Statement of problem.

Most of the water borne diseases are caused by *Vibrio* species, the significant amount (either high or low) found in a particular site could serve as a potential health challenge to man and in the development of aquaculture with great economic losses around the world because of their high morbidity and mortality rates (mortality ≥ 50%). (Al- Taeel et al., 2017).

Significance of study.

Identifying and understanding the prevalence, mode of action and mode of transmission of these organism *Vibrio* species, provides insights and adequate knowledge in handling and in treating illnesses associated with *Vibrio* spp. Identification of *Vibrio* species is based mainly on their morphological, physiological, and biochemical characteristics (Alsina et al., 1994), hence the reason for culturing, isolating and molecular characterization of *Vibrio* species. In doing these, grounds can be established for further research to be carried out which can advance or improve already established grounds.

CHAPTER TWO

LITERATURE REVIEW

2.0 *Vibrio* Species

Vibrio cholerae is an example of a non-invasive organism that solely affects the small intestine by the release of enterotoxin and is the etiological agent of cholera, whereas *Vibrio parahaemolyticus* and *Vibrio vulnificus* are considered intrusive germs that primarily impact the colon (Famer. 1992).

V. fluvialis and *V. vlnificus* are emerging human and foodborne pathogens associated with outbreaks and occasional occurrences of severe diarrhea (Bhattacharjee, et al., 2010). Blistering gastroenteritis, skin wounds, or a medical condition known as primary septicemia are all indications of *V. vulnificus*, and the infection is extremely harmful for persons who have had a chronic liver disease for a long time. (Liang, 2013).

In fish and other aquatic animals, halophilic *Vibrio* species have been documented to produce severe morbidity, mortality, or infections (Cano-Gómez, 2009). In general, the breakout of *Vibrio* species in aquaculture has a direct influence on a country's economy and poses a public health danger.

Vibrios are gram-negative organisms that have straight or curved rod-like forms and range in length from 1.4 to 2.6 meters. They can be motile or non-motile; motile species use three flagella at one end to move around. Many horizontal unsheathed flagella produced by most *Vibrio* species are chemoorganotrophic, non-endospore-forming bacteria that grow in the absence of molecular oxygen. They vary from pseudomonads in that they have both fermentative and respiratory metabolism and are usually oxidase positive (Dryselius, 2007). Oxygen is the final electron acceptor. They are unable to fix nitrogen; ammonium salts are the most common source of nitrogen. With the exception of *V.metschnikovii*, nearly all *Vibrio* pathogens test positive for oxidase. (Lai, 2009)

Most *Vibrio* species have been reported to be affected by *Vibrio*-static agent 0/129, which is used as a diagnostic test (Salter. 2009).

They have the ability to stop and absorb a wide range of carbon, phosphorus, and nitrogen substrates, as well as produce the external enzymes chitinase and laminarase, which make plentiful nutrients available to the indigenous bacteria (Zubkov, et al, 2009). Furthermore, they have evolved an adaptive mechanism to cope with ever-changing environmental conditions, which includes shrinking to an ultra-microbial morphology (0.4m diameter) (Denner, 2002). *Vibrio* species are halophilic, meaning they need 2 to 3 percent sodium chloride (NaCl) to thrive (Sridhar, 2013) Apart from *V. mimicus* and *V. cholerae, Vibrio* species are referred to as halophilic organisms because they do not thrive on medium without sodium chloride (Tortora, 2013). The importance of salinity in the growth of *Vibrio* species reveals the dynamics of abundance in the aquatic ecosystem (Tantillo, et al., 1999). Pathogenic *Vibrio* species of human origin are divided into two groups based on the diseases they cause: one group causes extra intestinal disorders, and the other group causes gastrointestinal ailments. Biochemical approaches, which are described further below, have long been used to develop *Vibrio* species-specific diagnostic assays. Squids and other aquatic organisms have symbiotic interactions with some *Vibrio* species that are known to emit light (Ruby, 1996). Some organisms, including fish, coral, and frogs, have been known to be morbid to other *Vibrio* species (Akram, et al., 2013).

2.1 Serologic Identification of *Vibrio* species.

Antisera are one of the most rapid and most used specific methods of identifying *Vibrio* species. The minimum identification of *Vibrio* species requires only serologic confirmation of the presence of serotype antigens with suspect isolates. However, a more complete characterization of the organism may be necessary and may include various biochemical tests as the determination of other characteristics (Jorgensen, 1999).

2.1.1 Serogroups of *Vibrio species.*

Currently, there are more than 130 serogroups of *Vibrio* species, based on the presence of somatic O antigens. However, only the O1 serogroup is associated with epidemic and pandemic cholerae. Other serogroups may be associated with severe diarrhea, but do not possess the epidemic potential of the O1 isolates and do not agglutinate in O1 antisera (Turindge, 1999).

2.1.2 Serotypes of *V. cholerae* O1

Isolates of the O1 serogroup of *Vibrio cholerae* have been further divided into three serotypes, Inaba, Ogawa, and Hikojima (very rare). Serotype identification is based on agglutination in antisera to type-specific O antigens. Identifying these antigens is valid only with strains that are negative polyvalent O1 antisera. Isolates that agglutinate weakly or slowly with serogroup O1 antisera but do not agglutinate with either Inaba, or Ogawa antisera are not considered to be serogroup O1 (Washington, et al., 1999).

2.1.3 Slide agglutination

An agglutination test for *Vibrio cholerae* somatic O antigens was carried out on a glass slide. An inoculating loop was used to scoop out portions from the brain heart infusion broth (BHI) used to activate the cells from the glycerol used in preserving them. Emulsification was done for the inocula/growths in a drop of physiological saline and mixed thoroughly by tilting back and forth for about 30 seconds. The suspensions were examined carefully ensuring an even suspension and avoidance of clumping due to agglutination was ensured. These were carried out on both UNILAG and MTU samples each.

The suspension became smooth (turbid and free-flowing), then a drop of 10µl antiserum was added to the suspensions. The suspensions and antiserums were mixed thoroughly, and then tilted back and forth to observe for agglutination. The reactions were positive with a very strong clumping appearing within 30 seconds to 1 minutes of tilting back and forth.

2.2 Biochemical Identification of *Vibrio* species.

1. Oxidase test

Fresh growth of 24 hrs. from brain and heart infusion broth (BHI) was used in conducting this test. Three drops of oxidase reagent (one % tetramethyl-*p*-phenylenediamine) were added on a piece of filter paper in a petri dish. With a sterile wooden spatula, the culture was smeared across the wet paper. The growth became dark purple within 10 seconds, indicating a positive reaction for *Vibrio*

2. Carbohydrates

Glucose broth was inoculated from fresh growth from both samples, then incubated at 37 ^{0}C for 72 hours. Positive for fermentation, broth fermented by *Vibrio species* after 72 hours.

2. Salt broths

0% and 1% salt broth were inoculated very lightly from fresh growth from both samples. The inocula were made light as lightly as possible to prevent visible turbidity before incubation of the broths. The broths were incubated at 37^{0}C for 24 hours. After 24 hours growths were observed, Positive, indicating a presence of *Vibrio*.

3. Gram staining

On a clean, grease free slide, a smear of suspension was created with a loopful of the isolate. It was air-dried and heat-fixed. Drops of crystal violet were poured and kept for about 30 seconds to one minute and rinsed with water. It was then flooded with gram's iodine for 1 minute and rinsed with water. 70% alcohol was added for about 10-20 seconds and rinsed with water. Safranin was added for about 1 minute and rinsed with water. It was then air dried and Observed under Microscope. Positive gram-negative bacteria, as it retained the primary stain.

5. Catalase Test

Using an inoculating loop, a small amount of the isolate was transferred to the surface of a clean, dry glass slide; a drop of 3% H_2O_2 was added and observed for the evolution of oxygen bubbles, catalase positive.

6. Decarboxylase test

A drop of 18-24 hour brain heart infusion broth culture was added to each of the three decarboxylase broths (arginine, lysine, and ornithine). Then a 4 mm layer of sterile mineral oil was added to each of the tubes. The tubes were then incubated for 4 days at 35-37°C at room temperature. The tubes were then observed for color change at 24 hours. Yellow color broth was observed as a negative reaction in *Vibrio* spp for Lysine decarboxylation. For ornithine decarboxylation, purple broth coloration was observed i.e. positive for *Vibrio* spp**.**

Table .2.1 Biochemical tests for V*ibrio species*.

S/N	TESTS	1	2	3	4
1.	Lysine	-	+	-	+
2.	Ornithine	+	+	-	+
3.	Moller's decarboxylase	-	+	-	-
4.	Motility	+	+	+	+
5.	Oxidase	+	+	-	+
6.	Growth with 0% NaCl	+	+	+	-
7.	Growth with 6% NaCl	+	+	+	+
8.	Gas from glucose	-	+	-	-
9.	Catalase	+	+	+	+
10.	Gram staining	+	+	+	+

1= *V.cholerae*, 2 = *V.parahaemolyticus*, 3 = *V.mimicus*, 4 = *V.vulnificus*,

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Sample collection

Water samples were collected from the rivers from two randomly selected areas in Mountain Top University, with the use of sterile500 mL Duran Schott bottles. The same was also repeated for University of Lagos Lagoon, from which water samples were collected from the Lagoon from 2 randomly selected areas. The lid from the sample containers was removed, and without touching the inside of the bottle or lid, the container was filled with 500 mL of water for both sample sites. The lids were then tightly closed to prevent leakage. The samples were properly labeled and transported on ice to the laboratory for analysis.

3.2 Sample Processing and Isolation of *Vibrio* Species

Water samples were analyzed immediately upon arrival inthe laboratory. For each water sample, 25 mL was incubated in 25 mL of alkaline peptone water containing sodium hydroxide with a pH of 8.8 at 37^{o}C for 24 hours. After 24 hours, 100 mL was dispensed on thiosulfate citrate-bile-salt-sucrose (TCBS) agar. The plates were incubated aerobically at 37^{o}C for 24 hours. After incubation, distinct yellow colonies (presumptive *Vibrio cholerae*) were subcultured by streaking over the surface of TCBS media with a loop in a zigzag manner, and plates were incubated aerobically at 37^{o}C for 24 hours. Pure isolates were collected using a loop to scoop and dispensed into Eppendorf tubes containing 20% glycerol, and stored at -80^{o}C for further analysis.

3.3 Subculturing

The inoculating loop was used to pick yellow colonies (presumptive *Vibrio cholerae*) from the primary culture/selective media. This was done by using a sterile inoculating loop to touch the top-center of the colony, making sure not to touch the surface of the agar or any other surrounding bacteria (even if they too were presumptive *Vibrio cholerae)* and streaked onto TCBS agar plates, them which was followed by a 24-hour incubation period at 37$_{0}$ C.

3.4. EXTRACTION OF GENOMIC DNA FROM *VIBRIO* SPECIES

3.4.1 PROCEDURE

Genomic DNA extraction from the bacteria cells was carried out by boiling method. The 1.5 mL microcentrifuge tube was first labeled. Then one mL of already vortexed bacterial broth culture was dispensed into one of the labeled microcentrifuge tubes, and then placed in the centrifuge at 10,000 rpm for 5 minutes before the cells were collected. The supernatant was discarded leaving the pellets (cells). One 1 mL of sterile water was dispensed into the tubes with pellets and a cell suspension was made by vortexing, and then cells were washed by centrifuging at 10,000 rpm for 5 minutes, this was repeated. Then 200 µL of sterile water was dispensed into the tubes with pellets and a cell suspension was made by vortexing. Tubes were then placed in a heating block at 100^0 C for 10 minutes to boil the cells. Afterwards, tubes with cells were placed in ice for cell lyses. After 10 minutes, the lysed cells were vortexed slightly and centrifuged at 10,000 rpm for 5 minutes. The supernatant (containing DNA) was gently aspirated into another micro centrifuge tube and stored at-20^0C

3.5. PCR PROCEDURE

The nutrient broth was firstly sterilized in an autoclave at 121^0 C for 15 minutes; the reagent was mixed in a 0.2 µL PCR tube. The reagent was then kept on ice. The tubes were vortexed, so that all the composition mixed properly. The primers used for the amplificationwas the 16S rRNA (V.16S-700F-5- CGG TGA AAT GCG TAG AGA T -3 and V. 16S-1325R- 3 -TTA CTA GCG ATT CCG AGT TC -5) for the detection of *Vibrio* spp. And was performed in a 10µL reaction mixture comprising 5x master mix, 1mM $MgCl_2$, Taq polymerase, PCR buffer deoxynucleoside triphosphate mix, forward and backward primer and DNA template. Reaction mixtures were heated at 95^0 C for 5 minutes the initial denaturation step, then it was followed by 35 cycles of denaturation at 95^0 C for 2 minutes. Primer annealing at 42^0 C for 30 seconds, and primer extension at 72^0 C for 4 minutes. Then the final extension was at 72^0C for 10 minutes. The samples were held at time was 4ºC at ∞. All tubes were placed inside the thermocycler and programmed. Gel electrophoresis was carried out for the amplicon using the UV transilluminator. A 1400 bp ladder was used as the molecular size marker.

The DNA was prepared for loading by the addition of 2µL of DNA sample, 1/10 volume loading buffer and 2µL of PCR product, into wells created by combs placed in the gel. This was done one sample per well. Analysis by agarose gel electrophoresis was carried out and with the molecular weight marked included in at least one well on the same gel. The amplification products were visualized after electrophoresis at 50V for 45 min on a 1.8% gel. Thirteen *Vibrio* DNA extracts in 2µL from both samples' sites were thoroughly mixed with 4µL loading dye placed in wells in another tank filled with gel and TBE buffer in order to access the DNA strength and bands integrity of the *Vibrio* spp.

Table 3.2 PCR PROTOCOL

Reactions	Temperature	Time
Initial denaturation	95^0 C	5 minutes
Denaturation	95^0 C	2 minutes
Annealing	42^0 C	30 seconds
Elongation/Extension	72^0 C	4 minutes
Final Extension	72^0 C	10 minutes
Hold	4^0 C	∞

Table 3.3 PCR Reaction Components (16S rRNA amplification)

Reagent	Initial	Final	Volume
Master mix	5χ	1χ	2µL
f 16s	20	0.25 µL	0.125 µL
r 16s	20	0.25 µL	0.125 µL
H_2O		5.75 µL	5.75 µL
DNA		2µL	2 µL
Total			**10 µL**

3.6 Bacterial Genomic DNA by Agarose Gel Electrophoresis (1.8%)

The electrophoresis tank was assembled by fitting the rubber dams and positioning the combs. A conical flask was filled with 200mL of 1x Tris Borate EDTA buffer (TBE) buffer, with 3g of agarose powder into the buffer and mixed by swirling. Then mixture was brought to a boil for about 1 to 2 minutes in a microwave until a clear mixture was observed. Mixture was allowed to cool at room temperature ensuring the gel did not solidify while cooling. Then 2 drops of ethidium bromide were added to the gel. After cooling, gel was poured into the already assembled gel cast and allowed to solidify for about 40 minutes. After the gel had solidified, combs were carefully removed from the cast and then the gel was submerged in a running buffer (1x TBE) in the gel tank. The well was placed toward the cathode. Then the samples were loaded into the gel carefully using micropipettes. The samples ran up through half the area of the gel. The gel was removed from the electrophoresis tank and observed on a UV transilluminator.

3.7 METHODS

3.8 Preparation of Alkaline peptone water – (Enrichment Broth)

Casein in peptone, 10.0g of and 5.0g of sodium chloride were weighed respectively, using a weighing balance from the Biotechnology laboratory. The Casein in peptone and sodium chloride were dissolved in 1000ml distilled water with Sodium Hydroxide to adjust the pH using a magnetic stirrer. The magnetic stirrer enhanced the effective dissolution. The medium was poured into 15mL of (25) Duran Schott bottles, each bottle containing about 2/2 volume with the medium. The bottles were loosely capped and then autoclaved under 121^0C for 15 minutes. The sterilized media were collected and allowed to cool.

3.9 Preparation of Sodium Hydroxide

Sodium hydroxide, 10g of was weighed, and then dissolved in 1000ml distilled water. Then, trickles were released into the enrichment broth to adjust the reaction of the medium (8.8) using a pH meter.

3.10 Preparation of Thiosulfate citrate-Bile-Salt-Sucrose (TCBS) agar.

Thiosulfate citrate-bile-salt-sucrose (TCBS) powder, 10g of was weighed using the electronic balance. 50mL of the distilled water was measured using the graduated beaker.

The broth powder was suspended in the 50mL of the distilled water. The mixture was brought to a boil at 100^0 C on an electric heater to dissolve completely. Then it was allowed to cool.

3.11. Plating of Thiosulfate citrate-bile-salt-sucrose (TCBS) agar.

10mL of the medium was poured into each of the labeled Petri dishes swirled three times clockwise and three times anticlockwise to obtain a uniform distribution of the agar medium before setting. They were allowed to solidify and packed into white polythene bags inverted and stored.

3.12. Preparation of Nutrient Broth (50 ml)

Nutrient broth powder of 6.5 g was weighed using the electronic balance. 50 ml of the distilled water was measured in a graduated measuring cylinder. The broth powder was suspended in 50ml of the distilled water and placed on an electric stirrer for proper dissolution of mixture. After which 1 ml each was dispensed into eppendorf tubes, these tubes with the media were then sterilized at 121^0 C for 15 minutes.

3.13. Materials for Alkaline peptone broth

Casein in peptone 10.0g, 5.0g NaCl, 1000ml distilled water, 10g NaOH, 91g Thiosulfate citrate-bile-salt-sucrose (TCBS) agar, Nutrient Broth, agar plates, 1.5 L of 75% ethanol, pipette tips, bunsen burner, inoculating loops, bacterial cell spreader, nutrient agar, 20 ml centrifuge tubes, 1x Tris Borate EDTA (TBE) buffer, agarose powder, DNA ladder, ethidium bromide (EtBr), autoclaved tips, All other reagents were of general purpose or were obtained from the stores of Microbiology and Biotechnology.

3.14. MATERIALS

1.5mL microcentrifuge tubes, autoclaved pipette tips (100-1000µL, 10-100µL), gloves, microcentrifuge tube racks, overnight broth culture, 70% ethanol wipes, and distilled water.

3.15. MATERIALS FOR PCR

Crude DNA template, PCR Master Mix (7.5 mM $mgCl_2$,1Mm dNTPs, 0.4M Tris-HCl, 0.1 M $(NH_4)_2$ SO_4, 0.1%), Nuclease free water, PCR primers, Taq DNA polymerase, molecular weight ladder, TAE buffer, 3µl ethidium bromide staining solution, thermal cycler (BioRad), heating blocks, 1.5 mL microcentrifuge tubes, PCR tube, agarose gel machine, gel tray and comb, UV transilluminator. Pure isolates stored in glycerol (cryopreservation) were resuscitated in 6.5g of Nutrient broth. This was done by aliquoting 1 mL of isolate and 1 mL of nutrient broth into fresh tubes.

3.16. Methods of sterilization

Twenty-four Petri dishes were obtained from the Microbiology and Biotechnology department. These Petri dishes were wrapped in transparent polythene bags, and also alkaline peptone water in Duran Schott bottles were placed in the autoclave together with the secondary enrichment broth Nutrient agar wrapped with aluminum foil. The inoculation loops and needles were flamed until red hot with bunsen burner flame and allowed to cool before and after use. All glass wares were sterilized by wrapping in aluminum foil and heated in an oven at 180^0.

3.17. PRECAUTIONS

- Aseptic techniques were observed at every stage of work.
- Personal protective equipment was worn, some of which included a covered shoe, nose mask, lab coat, hand gloves etc.
- Ensured that the inoculating loop cooled before picking the organism when subculturing in order not to kill organisms of interest.
- Ensured that the petri-dish was inverted during incubation.
- Ensured proper timing most especially during autoclaving.

CHAPTER FOUR

4.0 RESULTS AND DISCUSSION

4.1 RESULTS

Vibrio species are gram-negative, facultative anaerobic, motile, catalase positive, oxidase-positive bacteria that attack fermentative sugars. Thio- sulfate citrate bile salts sucrose agar (TCBS agar) was used for isolation and enumeration of *Vibrio* spp. TCBS agar medium was able to grow *Vibrio species* as it was a selective media and inhibited the growth of other *Pseudomonas* species.

Biochemical and physiological characteristics of *Vibrio* species were done. All isolates were gram-negative after viewing under light microscope after gram staining and they all were positive to glucose fermentation. All isolates showed results for oxidase, catalase and motility tests.

Potentially pathogenic *vibrio* were detected in four isolates (10%) (n=4) In particular, *vibrio* spp. was identified from 4 samples from the Mountain Top University river and none from the University of Lagos Lagoon from the PCR analysis.

This present study was aimed at isolating and molecular characterization, of *Vibrio* spp from the water bodies at,

i) Mountain Top University River, Ogun State
ii) The University of Lagos Lagoon, Lagos state.

Large pointed yellow and green colonies were seen growing on 20 TCBS agar spread Petri plates after incubation for 48 hours.

After isolation, some samples appeared to be green, while others yellow raised circular and smooth colonies on TCBS agar which indicates the presence of possible *Vibrio* species within both samples. Enrichment, culturing, sub-culturing, biochemical tests and molecular based methods have been applied for the isolation and identification of *Vibrio* species. The morphological characteristics of the isolates are shown in Table 4.1.

Table 4.1: Morphological characterization of *vibrio* isolates on Thio-sulfate citrate Bile Salt (TCBS) Agar.

Isolate ID	**Color**	**Shape**	**Size**	**Opacity**	**Elevation**	**Surface**
SMV	Yellow & Metallic Green Sheen	Circular	Small	Opaque	Raised	Smooth
LAG	Yellow/ Metallic Green Sheen	Circular	Small	Opaque	Raised	Smooth

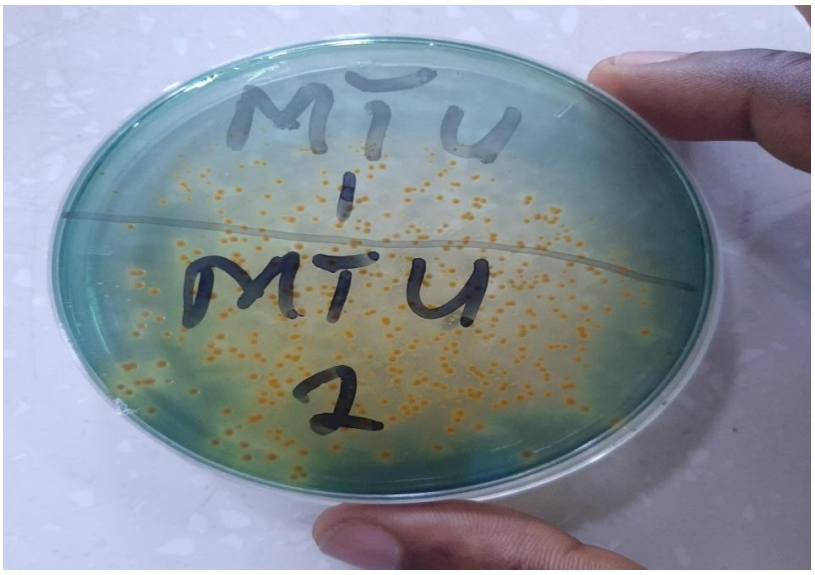

Figure . 4.1. *Vibrio* spp growth or colonies on TCBS agar from Mountain Top University river.

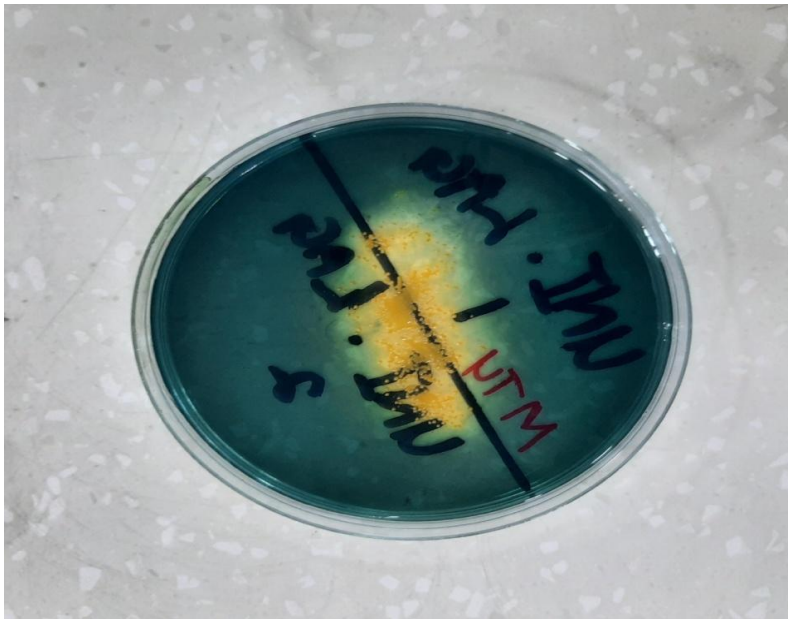

Figure .4.2. *Vibrio* species growth colonies on TCBS agar from the University of Lagos Lagoon.

Table 4.1. Total viable count of bacterial isolates for MTU and UNILAG water samples on Thio - Sulphate citrate Bile Sucrose (Agar TCBS).

Sample	Labeling Code	Total Viable count
MTU Water sample	SMV1	19
MTU Water sample	SMV2	TMC
MTU Water sample	SMV3	81+
MTU Water sample	SMV4	45
MTU Water sample	SMV5	9+
MTU Water sample	SMV6	22
MTU Water sample	SMV7	17
MTU Water sample	SMV8	94+
MTU Water sample	SMV9	29
MTU Water sample	SMV10	41
UNILAG Water sample	LAG 1	200+
UNILAG Water sample	LAG 2	100+
UNILAG Water sample	LAG 3	37+
UNILAG Water sample	LAG 4	TMC
UNILAG Water sample	LAG 5	13
UNILAG Water sample	LAG 6	12
UNILAG Water sample	LAG 7	60+
UNILAG Water sample	LAG 8	30
UNILAG Water sample	LAG 9	17+
UNILAG Water sample	LAG 10	TMC

4.2 Molecular analysis

PCR amplification from extracted DNA of the isolates were carried out based on 16S rRNA sequence analysis. The 1400 bp 16S rRNA PCR revealed a low percentage (10 %) of *Vibrio* spp from the water samples. Therefore, the isolates were confirmed as *Vibrio* spp based on the amplification size (1300 bp), as shown inFigure3.1 and their morphological, cultural, physiological, biochemical and molecular characterization.

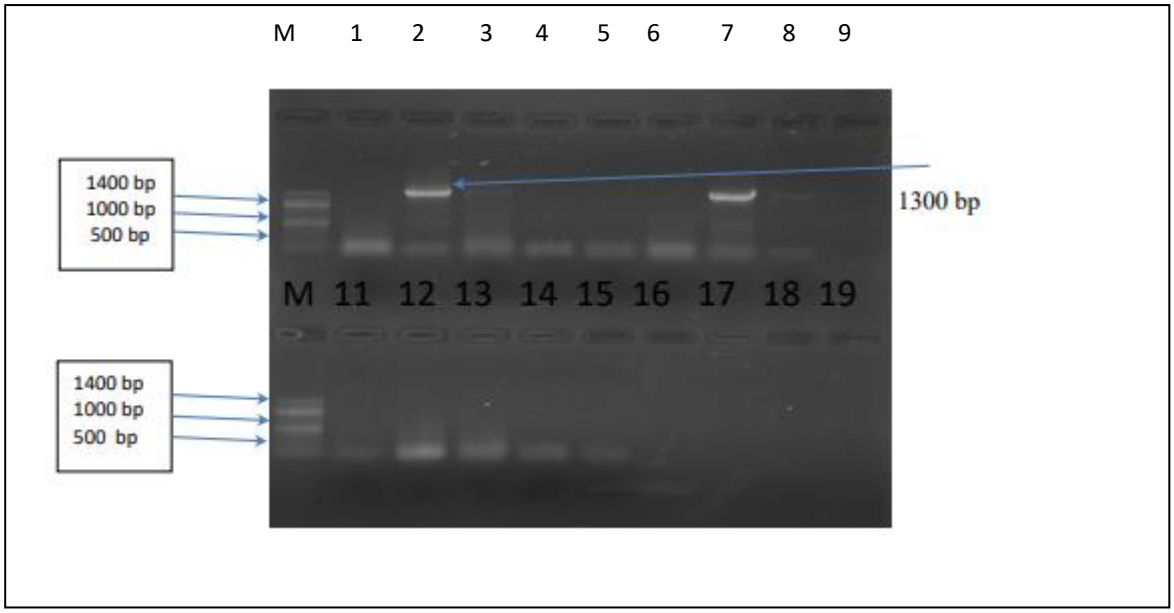

Figure 3.3 Agarose gel electrophoresis showing amplicons of the 16S rRNA gene of *Vibrio* spp. Lane M = DNA marker, lane 2 – 3 = fragments of positive isolates of *Vibrio* spp from MTU isolates, Lane 12 – 13 fragments of positive isolates of *Vibrio* spp from, UNILAG lagoon's isolate.

Figure3.4 Agarose gel electrophoresis showing the DNA strength and integrity of *Vibrio* spp, from MTU river (Lane 1 to 10) and UNILAG lagoon water (Lane 11 to 20).

4.3 DISCUSSION

Understanding the natural ecological environment of infectious pathogens outside the human body, brings about essential understanding of the epidemiology of the diseases involved, and above all to prevent diseases caused by exposure to these disease-causing organisms.

Four (20%) isolates were identified as *Vibrio* spp through amplification of the 16S rRNA analysis. There was more copious growth of *Vibrio* spp from the Mountain Top University River samples (SMV) compared to that of Unilag Lagos lagoon.

The total viable count was higher in the Unilag sample with a count of over 200, than the total viable count of SMV. Given that all PCR components were closely monitored and that comparable observations have been reported in earlier research as pertains to this study, according to (Anwar *et al.*, 2012) other isolates that did not amplify, could be an indication of difficulties caused by dead DNA cells or a loss of cell membrane integrity.

In this study, most of the *Vibrio* spp were detected in MTU's river samples from the PCR analysis carried out. However, there were possible potential *Vibrio* species from the viable counts and biochemical tests conducted consisting of several colonies from both subcultured water sources.

These water sources can serve as a potential route for the transmission of *Vibrio* spp to humans when ingested or through the consumption of raw or undercooked contaminated foods. Thus, the need to take precautions in handling or consuming water or sea food from these sites.

CHAPTER FIVE

5.0 CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

The water samples were found to be contaminated with pathogenic bacteria *Vibrios spp.* Results reveal that both sample sites UNILAG Lagoon and MTU water were contaminated. This remains a public health concern, therefore, posing potential risk to humans from the consumption of untreated water or the consumption of raw seafood from these sources which can lead to illnesses such as gastroenteritis, cholera, diarrhea etc.

5.2Recommendations

The 16S rRNA gene is considered the standard marker for *Vibrio* phylogeny though since the gene evolves slowly, the differences between species are limited and therefore often unable to resolve closely related bacterial strains (Sawabe *et al.*, 2007). Suggestions are that molecular-based detection techniques including PCR can be useful for a fast and accurate determination of the pathogens.

It is recommended that further molecular analysis .i.e. specie specific identification should be employed using multiplex PCR, conventional PCR or even real time PCR for analysis of various strains and virulence genes determination in a study.

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