ISOLATION AND ANTIBIOTIC SUSCEPTIBILITY OF *Vibrio cholerae* FROM VARIOUS WATER SOURCES IN MAKURDI, NIGERIA.

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ABSTRACT

Cholera remains a global threat to public health especially in developing countries. Africa has been the continent with the highest number of officially reported cholera cases since 1996. It is impossible to prevent cholera from being introduced into an area but the spread of the disease within an area can be prevented through early detection, confirmation of cases, and appropriate preventive measures. Cholera remains a major public health problem in many developing countries. Cholera, an enteric diarrheal disease caused by the gram-negative bacterium, Vibrio cholerae (either type O1 or O139) continues to be a worldwide health concern. Robert Koch then successfully isolated the agent of cholera and created an awareness of cholera toxins in 1888. The research work is therefore important in several ways both to the health personnel and individuals within the society. This study will provide knowledge on Vibrio cholerae as a waterborne infectious agent and also suggest ways of preventing cholera outbreaks in our society. It will also be of importance to the health personnel and our local community health workers by providing awareness for various preventive measures to be put in place to avoid cholera outbreaks in our society. Antibiotics are biochemicals produced by microorganisms that inhibit the growth of or kill other microorganisms. Bacterial resistance is the capacity of bacteria to withstand the effects of antibiotics that are intended to kill or control them. The study will therefore also help us ascertain which antibiotic is most effective against Vibrio cholerae.

Keywords: *Vibrio, cholerae, Antibiotics, cholerae, water.*

1. INTRODUCTION

Vibrio cholerae is a Gram-negative, rod-shaped bacterium. The bacterium's natural habitat is aquatic. It is a facultative anaerobe and has a flagellum at one cell pole as well as pili. *Vibrio cholerae* is usually transmitted by ingestion of contaminated water, as sewage-contaminated water remains the primary vehicle for cholera outbreaks. *Vibrio cholera* was first accurately described as the cause of cholera by Italian anatomist, Filippo Pacini in 1854 but his discovery was not widely known until Robert Koch and his collaborators, 30 years later (1883), isolated the organism and publicized the knowledge and means of fighting the disease. Cholera, an enteric diarrheal disease caused by the gram-negative bacterium, *Vibrio cholerae* (either type O1 or O139) continues to be a worldwide

health concern (WHO, 2004). Robert Koch then successfully isolated the agent of cholera and created an awareness of cholera toxins in 1884 (Finkelstein, 2002).

Cholera remains a global threat to public health especially in developing countries (WHO, 2008). Africa has been the continent with the highest number of officially reported cholera cases since 1996 (Kindhauser, 2003). It is impossible to prevent cholera from being introduced into an area but the spread of the disease within an area can be prevented through early detection, confirmation of cases, and appropriate preventive measures (Chomvarin, 2007). *Vibrio cholerae* enterotoxin is the major virulence factor that causes diarrhea which symptoms include vomiting, watery diarrhea, dehydration, and sometimes leg cramps which happen as a result of electrolyte imbalance (Choo pun *et al*., 2002).

Cholera incidence is influenced by a changing micro-ecology of *Vibrio cholerae*, the vulnerability of people through exposure to health risks, resistance to infection through immunity and/or nutritional status, and environmental, socio-economic, and behavioural changes (Collins, 2003). *Vibrio cholerae* may be established as an endemic or recurrent pathogen in a given place. *Vibrio cholerae* when entering and colonizing the human host must endure changing environmental factors such as temperature, acidity osmolarity, intestinal growth inhibitory substances, and immune system factors (CFSPH, 2006).

Toxin-coregulated pilus (TCP) is then necessary for colonization of the small intestine. Children and adults can be infected with this disease. About 20% of those who are infected develop acute, watery diarrhea, and about 10–20% of these individuals develop severe watery diarrhea with vomiting. If these patients are not promptly and adequately treated, the loss of such large amounts of fluid and salts can lead to severe dehydration and death within hours. The case-fatality rate in untreated cases may reach 30–50% (WHO, 2004). Treatment is straightforward (basically rehydration) and, if applied appropriately, should keep the case-fatality rate below 1%. Cholera is usually transmitted through faecally contaminated water or food and remains an ever-present risk in many countries. Cholera is most likely to be found and spread in places with inadequate water treatment, poor sanitation, and poor hygiene (CFSPH, 2006). New outbreaks can occur sporadically in any part of the world where water supply, sanitation, food safety, and hygiene are inadequate.

The greatest risk occurs in over-populated communities and refugee settings such as IDP camps characterized by poor sanitation, unsafe drinking water, and increased person-to-person transmission. The incubation period is very short (2 hours to 5 days), therefore the number of cases can rise extremely quickly (Weink *et al*, 2008).

In Benue State, a lot of people have been displaced from their ancestral homes due to crises and are now crowded in internally displaced camps. Under such a crowded arrangement, periodic assessment of the hygienic condition of both the environment and means of livelihood is of paramount concern and water cannot be left out. The problem of water shortage in such camps puts the people at risk they resort to any source of water for cooking, drinking, and other domestic uses. Microbiological analysis of water to ascertain the portability at the Internally displaced persons' camps is therefore necessary in order to educate the people and bring the attention of concerned authorities for necessary intervention in order to improve the living conditions of the refugees.

2. METHODS AND MATERIALS

2.1 Experimentation Site

This study was performed at the Internally Displaced Persons (IDP) Camp, Benue State, NIGERIA.

2.2 Materials needed for isolation and identification of *Vibrio cholerae*

1. Alkaline peptone water (APW)
2. Thiosulfate citrate bile salts sucrose agar (TCBS)
3. Incubator
4. MacConkey agar
5. Gloves
6. Syringe
7. Sterile water bottle (container)
8. Petri dish

9. Test tubes

2.3 Experimental method

The water specimens (samples) were collected in sterile containers at the Internally Displaced Persons (IDP) Camp and transported to the laboratory for bacteriological analysis. Selection of the isolation method depended on the type of water sample to be cultured, also the salinity of the water source was also a determining factor (Morris *et al*., 2004). For instance, it was considered that samples from marine and estuarine environments may contain numerous other Vibrio species that grow as well as *Vibrio cholerae* in alkaline peptone water (APW) and on Thiosulfate citrate bile salts sucrose agar (TCBS). Bacteriological analysis of the water samples was carried out by dilution in 10-fold increments (serial dilution) about 10-3 in order to reduce the number of competing microorganisms. Incubation of the alkaline peptone water will inhibit the growth of some competitors, particularly other *Vibrio* species because they do not grow at this elevated temperature (about 37^0C). Specimens from freshwater may not require dilution before culturing or incubating because at 37^0C they contain relatively fewer Vibrios and Vibrio-like organisms (American Water Works Association, 2009)

2.3.1 Preparation and Quality Control of Media and Reagents

Follow the manufacturer's instructions to prepare media. After autoclaving, cool the medium. Agar should be poured into Petri dishes. Freshly prepared plates may be used same day or stored in a refrigerator (Turnidge *et al.*, 2003).

2.3.2 Tests for determining biotypes of *Vibrio cholerae* 01

The differentiation of *Vibrio cholerae* 01 into classical and El Tor biotypes is of public health and epidemiologic importance in helping identify the source of the infection, particularly when cholera is first isolated in a country or geographical area. Biotyping is not appropriate for *Vibrio cholerae* non-01 (Barrett *et* al., 2004). The El Tor biotype is currently predominant throughout the world. The classical biotype is seen only rarely in most places, with the exception of Bangladesh (WHO, 2002). The tests shown below are used in determining the biotype of *Vibrio cholerae* 01.

i. Voges-Proskauer test

The Voges-Proskauer test has been used to differentiate between the El Tor and classical biotypes of *Vibrio cholerae* 01. Classical biotypes usually give negative results; El Tor isolates are usually positive.

ii. Oxidase test

The oxidase test is a biochemical reaction that assays for the presence of cytochrome oxidase. In the presence of an organism that contains cytochrome oxidase enzyme, the reduced colourless reagent becomes an oxidized coloured product.

There are many method variations to the oxidase test but the filter paper test method will be used for the sake of this project's research work;

1. Soak a small piece of filter paper in 1% Kovacs oxidase reagent and let dry

2. Use a loop and pick a well-isolated colony from a fresh (18-24 hours culture) bacterial plate and rub it onto treated filter paper.

3. Observe for colour changes

4. The microorganisms are oxidase positive when the colour changes to dark purple within 5-10 seconds. Microorganisms are delayed oxidase positive when the colour changes to purple within 60-90 seconds. Microorganisms are oxidase negative if the colour does not change or it takes longer than 2 minutes

iii. Urease test

The urease test identifies those organisms that are capable of hydrolyzing urea to produce ammonia and carbon dioxide. It is primarily used to distinguish urease-positive bacteria from other Enterobacteriaceae.

2.3.3 Antimicrobial Susceptibility Testing (Agar Disk Diffusion Method)

Antimicrobial therapy is helpful, although not essential in treating cholera patients. Antimicrobial agents reduce the duration of illness, the volume of stool, and the duration of shedding of *Vibrio cholerae* in the faeces (Jorgensen *et al., 2003*). Antimicrobial agents recommended for treating cholera patients include tetracycline, doxycycline, furazolidone, erythromycin, or chloramphenicol (WHO, 2004). Ciprofloxacin and norfloxacin are also effective.

Antimicrobial agents suggested for use in susceptibility testing of *Vibrio cholerae* include:

1. Ciprofloxacin

2. Chloramphenicol

3. Tarivid

4. Septrin

5. Streptomycin

6. Gentamycin

7. Augmentin

8. Pefloxacin

2.3.4 Procedure for Agar Disk Diffusion

i. Mueller-Hinton susceptibility test agar

The Mueller-Hinton agar should always be used for disk diffusion susceptibility testing.

ii. McFarland turbidity standard

A McFarland 0.5 standard should be prepared prior to beginning susceptibility testing. The McFarland standard is used to adjust the turbidity of the inoculum for the susceptibility test.

iii. Preparation of inoculum

Each culture to be tested should be streaked onto a non-inhibitory agar medium (e.g. blood agar) to obtain isolated colonies. After incubation at 37^{o}C, select 4 or 5 well-isolated colonies with an inoculating loop, and transfer the growth to a tube of sterile saline or nonselective broth (Mueller-Hinton broth). Incubate the broth at 35^{o}C until turbid, and then adjust the turbidity to proper density.

iv. Inoculation procedure

Within 15 minutes after adjusting the turbidity of the inoculum suspension, dip a sterile cotton swab into the suspension. Pressing firmly against the inside wall of the tube just above the fluid level, rotate the swab to remove excess liquid. Streak the swab over the entire surface of the medium three times, rotating the plate approximately 60 degrees after each application to ensure an even distribution of the inoculum. Finally, swab all around the edge of the agar surface.

v. Antimicrobial disks

The working supply of antimicrobial disks should be stored in the refrigerator (4^0C). Upon removal of the disks from the refrigerator, the package containing the cartridges should be left unopened at room temperature for approximately 1 hour to allow the temperature to equilibrate. This reduces the amount of condensation on the disk. If a disk-dispensing apparatus is used, it should have a tight-fitting cover, be stored in the refrigerator, and be allowed to warm to room temperature before use.

Apply antimicrobial disks to the plates as soon as possible, but no longer than 15 minutes after inoculation. Place the disks individually with sterile forceps, and then gently press down onto the agar. In general, place no more than 12 disks on a 150-mm plate and no more than 4 disks on a 100-mm plate. This prevents overlapping of the zones of inhibition and possible error in measurement. Diffusion of the drug in the disk begins immediately; therefore, once a disk contacts the agar surface, the disk should not be moved.

2.3.5 Pathogenesis of *Vibrio cholerae*

Cholera toxin is an AB entero-toxin. After entering the body through contaminated food and water *Vibrio cholerae* colonizes the small intestine by adhesion, motility, and chemotaxis. Then it secretes the cholera entero-toxin. Cholera toxin has 5 B sub-units and an active A sub-unit (AB5). B subunits bind to oligosaccharides of G**M1** ganglioside. Conformational change occurs allowing the presentation of the A subunit to the cell surface. The A subunit enters the cell and it transfers the ADP-ribosyl of NAD to adenylate cyclase which activates it with the help of GTP and regulatory protein) to form cAMP. This produced cAMP pumps out large amounts of chlorine and water and prevents the entry of Na+ ions and water resulting in dehydration in cholera patients.

3. Results and Discussions

3.1. Results

Figure 1; Microbial load (*Vibrio cholerae*) of water samples on Thiosulphate-Citrate-Bisalt Sucrose Agar

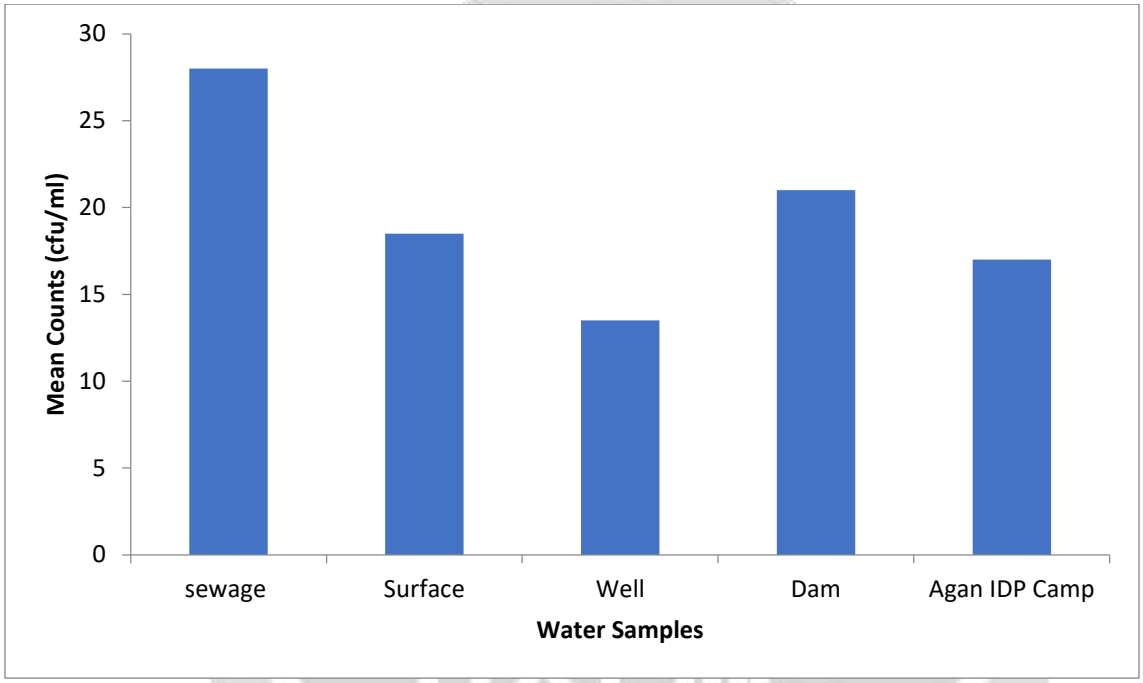

Table 1: Cultural and morphological characteristics of *Vibrio cholerae* isolates from the various water samples

S/N	Isolates Code	Colony colour	Colony Shape	Gram staining	Morphology	Identified organisms
1	Sewage water	Yellow	Curved	-	Rod	*Vibrio cholerae*
2	Well water	Yellow	Curved	-	Rod	*Vibrio cholerae*
3	Surface water	Yellow	Curved	-	Rod	*Vibrio cholerae*
4	Dam water	Yellow	Curved	-	Rod	*Vibrio cholerae*
5	Agan IDP Camp	Yellow	Curved	-	Rod	*Vibrio cholerae*

Keywords:
- = Negative
+ = Positive

Table 2: Biochemical characteristics of *Vibrio cholerae* isolates from the various water Samples

Isolate code	Catalase	Oxidase	Urease	Indole	VP	Identified organism
SW	+	+	-	+	-	*Vibrio cholerae*
WW	+	+	-	+	-	*Vibrio cholerae*
SSW	+	+	-	+	-	*Vibrio cholerae*
DW	+	+	-	+	-	*Vibrio cholerae*
AgW	+	+	-	+	-	*Vibrio cholerae*

Keywords:
- = Negative
+ = Positive
VP = Voges proskauer
SW = Sewage water
WW = Well water
SSW = Surface water
DW = Dam water.(AgW = Agan IDP Camp water)

Table 3; Results of the antibiotics tested against the *Vibrio cholerae* isolates

Pefloxacin	30mg	14.5 ± 2.12	16.5 ± 0.71	14.0 ± 0.00	18.0 ± 1.41	13.5 ± 0.71
Gentamycin	10mg	13.5 ± 0.71	13.0 ± 0.00	13.0 ± 0.00	18.0 ± 1.41	13.5 ± 0.71
Augmentin	30mg	10.5 ± 0.71	15.0 ± 1.41	15.0 ± 0.00	18.5 ± 0.71	12.0 ± 1.41
Ciprofloxacin	10mg	18.5 ± 2.12	14.0 ± 0.00	13.0 ± 0.00	17.5 ± 0.71	16.0 ± 1.41
Streptomycin	10mg	16.5 ± 0.71	17.0 ± 0.00	15.5 ± 0.71	16.0 ± 1.41	10.5 ± 0.71
Septrin	30mg	14.5 ± 0.71	15.0 ± 0.00	15.0 ± 0.00	21.0 ± 1.41	14.0 ± 1.41
Tarivid	30mg	17.5 ± 3.54	14.5 ± 0.71	12.5 ± 0.71	18.5 ± 0.71	13.5 ± 2.12
Chloramphenicol	30mg	11.0 ± 0.00	14.5 ± 0.71	12.5 ± 0.71	18.5 ± 0.71	13.5 ± 2.12

3.2. DISCUSSION

The study on isolation and antibiotic susceptibility test of *Vibrio cholerae* from different water sources was undertaken. The result of the study which was spared from sewage water, well, dam, surface, and Agan IDP Camp water source showed that all the water samples used for analysis were contaminated with *Vibrio cholerae.* The water sources such as the dam and surface water were used for various unhealthy purposes such as defecation and dumping sites. This could have accounted for the presence of the organism in the water sources. The Sewage water samples had the highest frequency of *Vibrio cholerae* while the well water had the lowest. Contaminated wastewater has been associated with the spread of cholera. This is further validated by the high incidence of *Vibrio cholerae* in water samples from various studies, indicating that they are natural inhabitants of aquatic environment. For instance, many waste water treatment plants in South Africa still release final effluent containing significant amount of enteric pathogens such as *Vibrio cholerae*.

A research work conducted in the bacteriological section of National Public Heaath Laboratory, Teku from march to September 2005 in which stool samples were studied and processed. Among the processed sample, 53 *Vibrio cholera*e cases were found. All isolated *Vibrio cholerae* were sensitive to Ampicillin, Ciprofloxacin, Erythromycin and Tetracycline whereas all were resistant to Septrin, Cotrimoxazole and Tarivid. Ampicillin, Ciprofloacin, Erythromycin and Tetracycline were found to be more potent antibiotics against *Vibrio cholerae* isolated during the study.

Another study undertaken during cholera epidemics in North West Ethiopia from August 2006 to September 2008 in which diarrheic stools were processed per the standard microbiology procedures at Bahir Dar Regional Health Laboratory. Antimicrobial susceptibility tests were performed using disc diffusion technique per Kirby-Bauer method. All *Vibrio cholerae* were resistant to chloramphenicol while erythromycin, tetracycline and ciprofloxacin had the least resistance which suggests that Ciprofloxacin could be used in treatment of adult cholera cases whereas erythromycin is alternative for young children

In previous studies carried out by the WHO and other agencies, a single dose of ciprofloxacin, tetracycline, doxycycline, and furazolidine has been shown effective in reducing the duration and volume of diarrhea.

This study was carried out at the University of Agriculture, Benue state. The result showed that Ciprofloxacin has more zones of inhibition as against the other antibiotics on all the water samples. Augmentin proved to be the least effective antibiotic. The results imply that ciprofloxacin is more effective against the other antibiotics. This therefore shows the efficacy of ciprofloxacin in the treatment of cholera.

Antibiotics represent a major class of antimicrobial agents. By definition, antibiotics are biochemicals produced by microorganisms that inhibit the growth of or kill other microorganisms. The discovery and use of antibiotics have revolutionized medical practice in the twentieth century. The determination of antibiotic susceptibility of a pathogen is therefore important in selecting the most appropriate one for treating a disease. There are several procedures used by clinical microbiologists to determine the sensitivity of microorganisms to antibiotics. One of them is the Kirby-Bauer Disc Method which is used to determine which antibiotic is most effective against the *Vibrio cholerae*. The procedure followed is simply that a filter disc impregnated with various antibiotics is applied to the surface of the agar plate (Mueller-Hinton agar) containing the microorganisms (*Vibrio cholerae*) to be tested and the plates are incubated at 37^{o}C for 24 hours. As the substance diffuses from the filter paper into the agar, the concentration decreases. At some particular distance from each disc, the antibiotic is diluted to the point that it no longer inhibits microbial growth. The effectiveness of a particular antibiotic is shown by the presence of growth-inhibition zones. These zones of inhibition (ZOIs) appear as clear areas surrounding the disc from which the substances with antimicrobial activity diffused. The diameter of the zone of inhibition is measured with a meter rule.

4. Conclusion

Based on the results obtained from this study and other relevant studies to investigate *Vibrio cholerae* (Elhadi et al., 2004; Noorlis et al., 2011), It is confirmed that *Vibrio cholerae* are important pathogens associated with sewage water, surface water such as the River Benue and dam water. Consequently, there is the risk of an outbreak in areas without proper drinking water sources. Inadequate cooking and contamination of raw food obtained from the river are the main contributing factors to these cases. Many of the contaminated foods from the river might be consumed in insufficiently cooked mode and it might increase the possibility of infections. It is therefore necessary to consume well-cooked food from the river and use clean and good water sources to decrease the risk of getting such infections.

Mueller-Hinton agar was used for the antibiotic susceptibility testing. After incubation, the plates were examined and the diameter of the zones of inhibition was measured to the nearest whole millimeter using a meter rule. The meter rule is held on the back of the petri dish, which is illuminated with reflected light. The endpoint is complete inhibition of growth as determined visually (ignoring faint growth or tiny colonies which can be detected by very close scrutiny) or large colonies growing within the clear zone of inhibition (which represent resistant colonies). The result showed that Ciprofloxacin has more zones of inhibition as against the other antibiotics on all the water samples. Augmentin proved to be the least effective antibiotic. The results imply that ciprofloxacin is more effective against the other antibiotics and is found to be the more potent antibiotic against *Vibrio cholerae* isolated during the study.

5. Recommendation

1. Wastewater treatment facilities should be installed to ensure the treatment of waste before disposal.

2. An adequate supply of safe drinking water and good food hygiene should be ensured

3. Preventive measures such as sanitary education and water decontamination should be carried out.

4. Oral Rehydration Therapy (ORT) is the most important medical advanced treatment and should be ensured in cases of emergence of cholera

5. Antibiotics should be given only in severe cases. Ciprofloxacin is one of the few antibiotics that has been shown to be most effective and is therefore recommended in the treatment of cholera

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