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Antibiotic Susceptibility Patterns and Plasmid Profile of *Vibrio cholerae* from Water Samples in Elele Community, Rivers State, Nigeria

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ABSTRACT: The antibiotic susceptibility patterns and plasmid profile were studied for 18(32.14%) samples of *Vibrio cholerae* isolates recovered from water samples from Elele Community. All isolates showed a multiple resistance patterns to 7 antibiotics namely amoxicillin, cotrimoxazole, nitrofurantoin, gentamicin, tetracycline, ampicillin, chloramphenicol and none of the isolates were susceptible to all antibiotics tested. All 56 isolates were sensitive to ofloxacin and ciprofloxacin. The presence of plasmid was screened and out of the 18 isolates only 1(5.5%) harboured plasmid with 2 bands of 1.19kpb and 1.06kpb as molecular weight. @JASEM

Keywords: Antibiotics, susceptibility patterns, isolates, plasmid

Cholera (frequently called asiatic cholera or epidemic cholera) is an acute diarrhea illness caused by the bacterium *Vibro cholerae* (Bryan *et al*., 1994).It is characterized by painless diarrhea and vomiting. Fewer than 10% of cases develop the severe form of disease, which if not treated can be rapidly life threatening due to dehydration and shock. In severe cases the diarrhea can be very watery and profuse, the loss of fluid can make the disease so serious (Dobson and Carper, 1993).The Vibrios that cause epidemic cholera were subdivided into two biotypes classical and with three serotypes namely ogawa, inaba and hikojima. The association of *Vibro cholerae* with aquatic plants in laboratory microcosm experiment has been demonstrated by Spira,(1981).In this study, *Vibro cholerae* was found to concentrate on the surface of the water by hyacinth, *Eichornia crassipes*, and the most common aquatic plant in Bangladesh. In developing countries, cholera often occur as rapidly progressive, large scale outbreaks these large scale outbreaks cause a high burden of disease and rapidly overwhelm curative health care services, particularly during the complex humanitarian emergencies or in settings where public health systems have been broken down (Swerdlow *et al.*, 1992). *Vibro chlolerae* 0139 Bengal first emerged during 1992 and 1993 and caused large epidemic of cholera in Bangladesh, India and neighbouring countries.

During 1994 to the middle 1995, in most Northern and Central areas of Bangladesh, the 0139 Vibrios were replaced by a new clone of *Vibro cholerae* 01 of the biotype eltor whereas in the Southern coastal regions the 0139 Vibrios continued to exist (Faruque *et al*., 1996).During the late 1995 and 1996, cases of cholera attributable to both *Vibrio cholerae* 01 and 0139 were again detected in various regions of Bangladesh. Since 1996, cholera in Bangladesh has been caused mostly by *Vibrio cholerae* 01 of the biotype eltor and a large outbreak of cholera caused predominantly by *Vibrio cholerae* 0139 occurred in the capital city Dhaka and adjoining areas (Faruque *et al.*,1996). After the seventh pandemic caused by *Vibrio cholerae* 01 biotype eltor reached and initially spread in West African countries in the 1970s, the majority of countries in the Eastern and Southern parts of Africa have experienced major cholera epidemics.

Mozambique was hit in August, 1997 by an epidemic caused by *Vibrio cholerae* 01 biotype eltor with the number of cases totaling more than 10,000 by the end of 1997. The epidemic continued into 1998 and 1999, with *Vibrio cholerae* 01 being introduced into South African (Athan *et al*.,1998).

Cholera is spread by the faeco-oral route, via contaminated water and food. Asymptomatic infections are common especially in case of eltor people excrete bacteria for 10 days. This is sufficient time to ensure continued contamination of the environment (Dobson and Carper,1993).

Following an incubation period of 6 to 48 hours cholera begins with the abrupt onset of watery diarrhea. In diarrhea, give plenty of fluids e.g oral rehydration solution (Prescott *et al*., 2002). Confirmation is best made via a bacteriological culture, culturing should be preferable on a special medium in a bacteriology laboratory e.g Thiosulphate citrate bile-salts sucrose agar (TCBS), polymyxin mannose telloite agar (PMT) or another selective medium.

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Table 1: Antibiotic susceptibility patterns of *Vibrio cholerae* isolates from water samples

Isolate code	OFX	AMX	NIT	GEN	TET	C	PN	CPX	SXT	NB
22B(2)	S	R	R	R	R	R	R	S	R	S
31B	S	R	R	R	R	S	R	S	R	R
8A	S	R	R	R	S	S	R	S	R	R
14A	S	R	R	R	S	S	R	S	R	S
6A	S	R	R	R	R	R	R	S	R	S
7A	S	S	R	S	S	S	R	S	R	S
Pilgrim	S	S	S	S	S	S	S	S	R	S
32B	S	R	R	R	R	R	R	S	R	S
42	S	R	R	R	R	R	R	S	R	S
U4	S	S	S	S	S	S	S	S	S	S
38B(2)	S	S	R	R	R	R	R	R	R	S
76	S	R	R	R	R	R	R	S	R	S
23B	S	R	R	R	R	R	R	S	S	S
30B(2)	S	R	R	R	R	S	R	S	R	R
15A	S	R	R	R	R	R	R	S	S	S
16A	S	S	R	S	S	S	R	S	R	S
3A	S	R	R	R	S	S	R	S	R	S
U2	S	R	R	S	R	R	R	S	R	R
EKE-NGUR (B)	S	R	S	S	S	R	S	S	R	S
OKWU 4	S	S	S	S	S	R	S	S	S	S
MH_2(B)	S	S	S	S	S	S	S	S	R	S
OKWU 3 (B)	S	R	R	R	R	R	R	S	S	R
MADONNA H1	S	R	R	R	R	R	R	S	R	S
Ozaraegbe	S	R	R	R	R	S	R	R	R	S
32(B)(2)	S	R	R	R	R	R	R	S	R	S
11A	R	R	R	R	S	R	R	S	R	S
MADONNA H2	S	R	R	R	S	R	R	S	R	R
1A	S	R	R	S	R	R	R	S	S	S
9A	S	R	R	S	R	R	R	S	R	S
5	S	R	R	R	R	R	R	S	S	S
2	S	R	R	R	R	R	R	S	R	S
37(B) S	S	R	R	R	R	R	R	S	R	S
B	S	R	R	S	S	S	R	S	R	S
45	S	R	R	S	R	R	R	S	R	S
10A	S	S	S	S	S	S	R	S	R	S
23(B)2	S	R	R	R	R	S	R	S	S	S
2A	S	R	R	R	S	S	R	S	R	S
17A	S	R	R	S	S	S	R	S	R	S
4A	S	R	R	S	S	S	R	S	S	S
13A	S	S	R	S	S	S	R	S	R	S
79	S	R	R	R	R	R	R	S	R	R
5A	S	R	R	R	S	R	R	S	R	S
Eke-Nguru	S	S	S	S	S	S	S	S	R	S
Elele	S	R	R	S	S	R	R	S	R	S
20B(2)S	S	R	R	R	R	R	R	S	S	S
MH1B	S	S	R	S	S	S	R	S	R	S
OKWU 3	S	R	R	S	R	S	R	S	R	S
U3	S	R	R	S	S	S	R	S	R	S
36B	S	R	R	S	R	R	R	S	R	S
3	S	S	R	S	R	R	S	S	R	S
36(B)2	S	R	R	S	R	S	S	S	R	R
OKWU 5 S	S	S	R	S	R	S	R	S	R	R
MH2	S	R	R	S	R	S	R	S	S	S
Owerri 2	S	R	R	S	R	S	R	S	R	S
20B	S	R	R	S	R	R	R	S	R	S
31B(2)	S	S	R	S	R	R	R	S	R	S

KEYS: AMX- Amoxycillin; NIT - Nitrofuranatoin; GEN- Gentamicin; PN- Ampicillin; TET- Tetracycline; C- Chloramphenicol; NB- Norfloxacin; OFX- Ofloxacin; SXT- Cotrimoxazole; CPX - Ciprofloxacin; R- Resistance; S- Sensitive

Antibiotics resistance is of great importance in developing countries due to the increasing use of antibiotics in humans, animals and agriculture (Cheesbrough, 2000). In developing countries the

situation is particularly serious for the following reasons: Antibiotics can be obtained outside of recognized treatment centres, and taken without medical authorization or supervision. This leads to inappropriate use of antibiotics and their being taken at sub-optimal dosages.

Developing countries are often unable to afford costly second line antibiotics to treat infections due to resistant organisms. This results in prolonged illness with longer periods of infectivity and further spread of resistant strains. In recent years resistance of first line antibiotics such as ampicillin, tetracyclines, chloramphenicol and sulphonamides has been increasing. (Chessbrough, 2000).

Vibro cholerae 01 and *Vibro cholerae* non-01 demonstrates a lower frequency of resistance to ampicillin. However, from 1995 to 1999, strains of *Vibro cholerae* 01 were susceptible to chloramphenicol and trimethroprim-sulphamathoxazole but 2000 to 2001 4% and 10% of strain later developed resistance to those drugs (Tjaniadi *et al*., 2003). Plasmids are small extra double stranded DNA molecules usually circular and can exist independently of host chromosome and are present in many bacteria and some yeast and fungi. They have their own replication origin and are autonomously replicable and stably inherited (Nester *et al*., 2002 and Prescott *et al.*, 2002).

Plasmid coded and chromosome coded antibiotic resistance in bacteria seem to function by different mechanism. Resistance to streptomycin is chromosomally mediated resistance based on a change in the 30s subunit of the ribosome, whereas, the plasmid mediated resistance involves an enzyme attached to the antibiotic molecule (Levis, 1993).

MATERIALS AND METHODS

The study period was from August to November 2006 after receiving permission from the institutional sample collection ethical committee. The study was conducted in Elele Community. Samples were collected in universal bottles containing alkaline peptone water from suspected streams, rivers, wells and boreholes in Elele Community. The samples were collected at an interval of 2 hours and transferred into the universal bottles to keep the Vibrios alive because the alkaline peptone water inhibits the growth of the normal gastrointestinal tract organisms and at a pH level of 8.6 – 9.0

Sampling processing (culturing): The samples were cultured on TCBS (Thiosulphate citrate bile-salts sucrose agar) which is a selective media that inhibit the growth of other organisms to allow the growth of vibrio species to be more clearly demonstrated and contains substances e.g like salts, or other chemicals, dyes. The samples were cultured using pour plate method by pipetting 1ml of sample from the universal bottle containing peptone water (alkaline) into the petri dish and then poured the prepared TCBS agar and swirl anticlockwise, clockwise, up and down then allowed to solidify before incubating for 18 – 24 hours at 37^0C.

Identification of isolates:-Gram staining: A thin smear of the pure isolate was made on a clean grease free slide, fixed by passing over the bursen flame three times. The fixed dried smear was covered with crystal violet stain for 30-60 seconds. Rapidly washed off with clean water, and covered smear with Lugol's iodine which acts as a mordant which helps increase the affinity between the cell and dye then washed with clean water. It was decolourized (few seconds) with acetone-alcohol and washed immediately with clean water. The smear was then counter stained with neutral red for 1 minute, washed off with clean water and placed in a draining rack for the smear to air dry. It was then examined microscopically with the oil immersion objective to report the bacteria and cells.

String test: The string test uses fresh growth from non-selective agar and is useful for ruling out non *Vibro* species particularly *Aeromonas* species. The string test may be performed on a glass microscope slide or plastic petri dishes by suspending 18-24 hours growth from heart in fusion agar (HIA) in a drop of 0.5% aqueous solution of sodium deoxychlorate. If the result is positive, the bacterial cells will be used by the sodium deoxychlorate, the suspension will loose turbidity and DNA will be released from the used cells causing the mixture to become viscous. A mucoid "string" is formed when an inoculating loop is drawn slowly away from the suspension. *Vibrio cholerae* strains are positive, whereas *Aeromonas* strains are usually negative.

Antibiotic susceptibility testing: As antibiotic resistance increases in many parts of the world monitoring the antibiotic susceptibility of *Vibrio cholerae* 01 and 0139 has become increasingly important. The disc diffusion method presented is a modification of the Kirby Bauer technique (1997) that has been carefully standardized by National Committee for Clinical Laboratory Standards (1997). The antibiotics used for the susceptibility include

ciprofloxacin (10mg), chloramphenicol (10mg), gentamicin (10mg), ofloxacin (10mg), ampicillin (25mg), tetracycline (25mg), cotrimoxazole (20mg), and norfloxacin (10mg). An aliquot of 0.1ml of alkaline peptone water containing the inoculated isolate with the turbidity corresponding to that of the Mc Farland's turbidity standard after proper adjustment was used to flood the nutrient agar plate.

Table 2: Percentage of Susceptibility pattern of *Vibrio cholerae* isolates

Isolate	Number of isolates	OFX	AMX	NIT	GEN	TET	C	PN	CPX	SXT	NB
Vibrio Cholerae	56	55 (98.2)	14 (25.0)	7 (12.5)	28 (50.0)	21 (37.5)	26 (46.4)	8 (14.3)	54 (96.4)	12 (21.4)	46 (82.1)

KEYS: AMX- Amoxycillin; NIT - Nitrofuranatoin; GEN- Gentamicin; PN Ampicillin; TET- Tetracycline; C - Chloramphenicol; NB - Norfloxacin; OFX - Ofloxacin; SXT - Cotrimoxazole; CPX- Ciprofloxacin

The suspension in excess was discarded and the plate allowed to stand on the bench for 3-5 minutes. The multi discs containing various antibiotics were carefully and aseptically placed on the surface of the medium. The plates were incubated at 37^0C for 24 hours and then observed zones of inhibition measuring 18mm and above were regarded as indicative of sensitivity. The zones of inhibition between 13 – 17mm were regarded as intermediate and the ones less than 12mm resistant (Bauer *et al.,* 1997).

Table 3: Antibiotic resistance and plasmid profile of isolates obtained from *Vibrio cholerae.*

Isolates Codes (Kpb)	Antibiotics Patterns	No. with Plasmids	Plasmids Size
36B	AMX, NIT, TET, C, PN, SXT	0	
76	AMX, NITM GEN, TET, C, PN, SXT	0	
45	AMX, NIT, TET, C, PN, SXT	0	
Owerri 2	AMX, NIT, TET, PN, SXT	0	
79	AMX, NIT, GEN, TET, C, PN, CPX, XT, NB	0	
791	AMX, NIT, GEN, TET, C, PN, CPX, SXT	0	
MADONNA H1	AMX, NIT, GEN, TET, C, PN, SXT	0	
451	AMX, NIT, TET, C, PN, SXT	0	
MADONNA, H2	AMX, NIT, GEN, C, PN, SXT, NB	2	1.19, 1.06
11A	OFX, AMX, NIT, GEN, C, PN, SXT	0	
32B	AMX, NIT, GEN, TET, C, PN, SXT	0	
31B	AMX, NIT, GEN, TET, PN, SXT, NB	0	
20B	AMX, NIT, TET, SXT, C, PN	0	
6A	AMX, NIT, GEN, TET, C, PN, SXT	0	
30B(2)	AMX, NIT, GEN, TET, PN,SXT, NB	0	
Owerri 2 (1)	AMX, NIT, TET, PN, SXT	0	
22 B (2)	AMX, NIT, GEN, TET, C, PN, SXT	0	
23 B (2)	AMX, NIT, GEN, TET, PN	0	

KEYS: AMX- Amoxycillin; NIT - Nitrofuranatoin; GEN- Gentamicin; PN- Ampicillin; TET- Tetracycline; C Chloramphenicol; NB - Norfloxacin; OFX- Ofloxacin; SXT- Cotrimoxazole; CPX- Ciprofloxacin

Isolation of plasmids DNA: 44 grams of TCBS Agar was dissolved in 500ml of distilled water and autoclaved at 121^0C for 15 minutes before allowed to cool at 470^0C. The isolates from the slants were then inoculated and incubated for 18 – 24 hours to check if the organism were still viable and pure cultures. The culture was again sub cultured into tubes containing Lauren Broth and incubated for 18 – 24 hours. The tubes were centrifuged for 3500xg left with only the pellet. A micro pipette was used to pipette 200µl of buffer 1A (400m MTris, 20m MNa EDTA, Acetic acid to pH 8.0) into the eppendorf tube of the tube containing the cell pallet and placed on the vortex mixer. The vortex mixer is used to mix the cell pallet with buffer 1A,after vortexing 400 µl of lysing solution (4% sodium dodecyl sulphate, 100m MTris) was added and mixed gently by inverting the tube rapidly 20 times at room temperature, after inverting the tubes 300µl ice cold buffer 2B (3m Sodium Acetate Acetic acid to pH 5.5) which is an anti-lysing buffer was added, vortex and stored tube in ice then centrifuge at 3000xg for 15 minutes before transferring the supernatant containing the cell DNA to a fresh eppendorf tube. 700µl of chloroform was added, vortex, centrifuge at 3000xg for 10 minutes. The supernatant was transferred to a fresh tube, this should be done twice. The DNA was precipitated by adding 1ml absolute ethanol, vortex and store tube in

ice for 2 hours. Centrifuge at 3000xg for 30minutes in a microfuge. The supernatant was removed by gentle aspiration and wash pallet with 7% ethanol centrifuge at 3000xg for 5minutes, this should be done twice, decent and air dry for 5minutes. The nucleic acid was redissolved by adding 60µl of buffer 3C (10m Mtris, 2mM Na2, EDTA, Acetic acid to pH 8.0), vortex and stored at 20^0C.

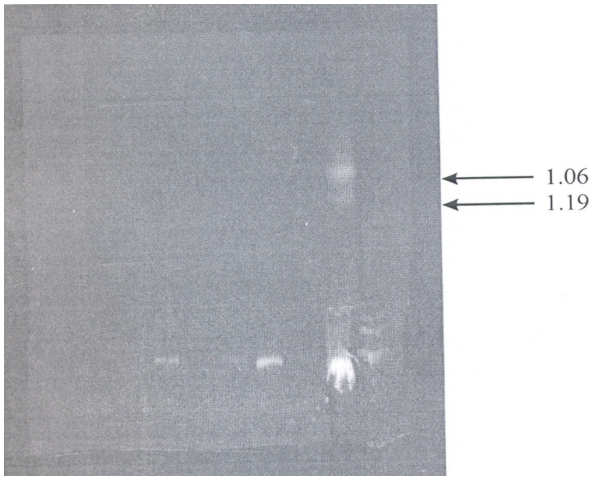

Fig 1: Separation of DNA Molecular weight on agarose gel stained with ethdium bromide (1). Line A = standard bacteriophage lambda DNA fragments, Line B, C, D, E, F, G, H. I, J are DNA fragments of test isolates.

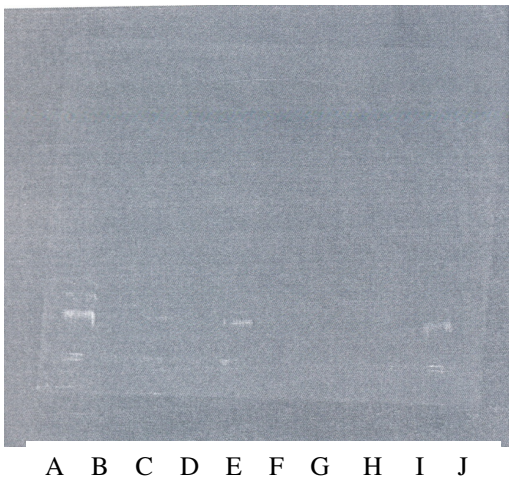

Fig 2: Separation of DNA Molecular weight on agarose gel stained with ethdium bromide (2). Line A = standard bacteriophage lambda DNA fragments, Line B, C, D, E, F, G, H. I, J are DNA fragments of test isolates.

Characterization of plasmid DNA by Agarose gel electrophoresis:-

Preparation of gel and gel shots: 0.8% agarose gel was prepared in TBE (Tri Boric acid ethylene diamine tetraacetic acid) buffer and allowed to boil intermittently until solution becomes clear. It was cooled to 45^0C and 7µl of Ethidium bromide was poured into the conical flask containing the agarose gel. The gel was poured into the gel plate with comb in place and allowed to solidify. The gel tray and comb were removed and the gel was put into the bank containing the gel buffer. 2µl of the tracing dye (Bromophenol Blue) with 1µl of the marker were mixed and loaded into the first well. 2µl of the bromophenol blue with 20µl of the sample were mixed and loaded into the remaining wells.

Electrophoresis: The power pack was connected to the tank and electrophoresis allowed to proceed at a voltage (60-64mv) running from the negative to the positive direction for 24 hours. The gel was viewed on the ultraviolet transilluminator and photographs were taken using protective goggles. The relative distance moved by the test plasmid DNA was compared with that of the marker running side by side.

RESULTS AND DISCUSSION

Cholera is a serious problem in developing countries when the outbreak caused substantial death, disease and economic loss (Hart and Kariuki, 1998).

According to this survey of antibiotics susceptibility, table 1 showed that ciprofloxacin and ofloxacin were mostly sensible compared to other antibiotics which are not in accordance with the work of Hart and Kariuki (1998) who reported that the extensive use of the ciprofloxacin for treating diarrheal infection might have promoted incidence of ciprofloxacin resistance to *Vibrio cholerae*. All the *Vibrio cholerae* strains were resistant to cotrimoxazole and strains resistance to tetracycline were in accordance with the work of Hart and Kariuki (1998) who reported almost all the *Vibrio cholerae* strains were resistance to cotrimoxazole versus none of *Vibrio cholerae* strains isolated during 1998-1999. In this current study, we observed that Vibrio isolates exhibited expanding resistance to nitrofurantoin and cotrimoxazole. According to the survey for major epidemics of cholera in Latin America,Tanzania, Bangladesh and Zaria. norfloxacin as shown in table 2 (82.1%) is widely used as an alternative to tetracycline (37.5%) for the treatment which corresponds to the present survey gotten from Elele Community, Rivers State, Nigeria. The plasmid isolation screened as shown in

table 3 and figure 1 was in accordance with the work of Dalsgard *et al*., (1997) who reported that the plasmid isolation screened has plasmid with two bands. It is amply clear that long term surveillance programs are essential to identify changes in the spectrum of microbial pathogens causing serious infection and to monitor for treads in antibiotic resistance patterns. The information gleamed from the surveillance efforts is integral to the designing approaches to the therapy of serious infections and appropriate control measures for antibiotic resistance pathogen. The current study highlights the necessity of simple water supply and hand hygiene practices, including point-of-use chlorination and safe water vessels and to ensure the long term sustainability of interventions aimed at the introduction of safe water system at high risk of pathogens transmitted through the faecal-oral route and to regulate the case of antibiotics.

Further studies should be carried out on plasmid curing and DNA polymerase chain reaction isolated from cholera samples.

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