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A TRIAL TO PREVENT *VIBRIO CHOLERAE* IN ALBINO MICE USING AUTOGENOUS BACTERIN

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ABSTRACT

This study was carried out to evaluate the protective effects of autogenous bacterin against *Vibrio cholerae* ETOR type isolated from Ubahudara and Aruolah streams in Uli community, Ihiala L.G.A, Anambra State. A total of 16 water samples were drawn from each stream and screened for the presence of *Vibrio cholerae* using pour plate method. The colonies generated from the primary isolation wassub cultured, characterized and identified using their colony description, morphological and biochemical characteristics. The pathogenic potentials of the organism on mice were investigated by challenging the mice orally using 0.5 ml of the inoculum (10^8cells/ml). The infected mice were kept under observation for two weeks for clinical signs, mortalities, gross lesions, histopathological changes and re-isolation from the internal organs after sacrificing the mice. The protective effect of bacterin was investigated using *in vivo* method. The result revealed that *Vibrio cholerae ETOR* type was significantly ($P< 0.05$) seen more in Ubahudara stream (67.44%) than Aruolah stream (32.56%). There were gross clinical abnormalities; kidney and liver congestion, perihepatits and fluid accumulation in the intestines. The histopathological examination revealed marked mono nuclear cell infilteration, disintegration of cartilage surrounding the bronchiole, necrosis of the tubules of kidney and red pulp of the spleen. The mean counts of the organism were significantly ($P< 0.05$) most in the lungs, followed by the spleen, kidney and the liver was the least. The results of *in vivo* activity showed that this autogenous bacterin was effective in reducing pathological changes. Its effect was significant ($P< 0.05$) when compared with the infected non-treated mice. Thus, this study has proven the efficacy of bacterin in preventing *Vibrio cholerae* ETOR type in albino mice for short term.

Key words: *Vibrio cholerae,* autogenous, bacterin, histopathological

INTRODUCTION

The name *Vibrio* was derived from Filippo Pacini, who isolated microorganism that he called “vibrions” from cholera patient in 1885 because of their motility. Vibrios are Gram-negative, curved, rod-shaped bacteria that are natural inhabitants of the marine, fresh water, and estuarine environments (McLaughlin *et al.*,1995). The Centre for Disease Control (CDC) estimated that 8,028 *Vibrio* infections and 57 deaths occur annually in the United States. Transmission of *Vibrio* infections is primarily through the consumption of raw or undercooked shellfish or exposure of wounds to warm seawater (Levine *et al. 1993).Vibrio parahaemolyticus, Vibrio vulnificus* and non-01/ non-0139 *Vibrio cholerae* have been isolated from humans suffering non-cholera vibrio infections (vibriosis), usually associated with the consumption of raw or under cooked seafoods and intake of contaminated natural waters (Yam *et al.*2000).

The primary environmental variables influencing this occurrence of pathogenic *Vibrios* in streams are temperature and salinity (Lipp *et al.* 2002). The optimum temperature for growth of this organism is 37°C, with possibilities for growth ranging from 16 to 42°C (Borroto *et al.* 2000). Floods and droughts may affect not only the concentration of the bacterium in this environment, but also its survival, through the effect exerted by these environmental changes on salinity, sunlight, pH, and nutrient concentrations (Bouma, 2001). *Vibrio cholerae* has optimum pH of 8.5, salinity of 15%, inhibited at temperature below 15^{o}C and killed at temperature of 65^{o}C and above (Borroto *et al.* 2000). The mainstay of the case management of cholera is treatment of dehydration using Oral Rehydration Therapy (ORS) or IV fluids (Ringer lactate) and electrolytes (Sack *et al.* 2006). In cholera

management, antibiotic prophylaxis is usually not part of intervention but essential for disease treatment in severe cases.

However, *Vibrio cholerae* strains from endemic and outbreaks situation within the last decade revealed interesting patterns of antibiotic resistance to commonly used antimicrobial agents. (Coppo *et al.*1995). Eighty six strains of *Vibrio cholerae* O1 (79 Ogawa serotype and 7 Inaba serotype) from 1992 outbreak in Nigeria were less sensitive to ampicillin, penicillin, cloxacillin, cotrimoxazole, streptomycin, and tetracycline (Olukoya *et al.*1995). The 1995 study also described *Vibrio cholerae* strains with 4.5 kilobase to 150 kilobase plasmids specifying resistance to ampicillin, tetracycline, and trimethoprim (Olukoya *et al.*1995). In Nigeria, existing prevention and control strategies are multi-sectoral. Epidemic Preparedness and Response (EPR) approaches including registration of cases, case management and public health measures targeting personal hygiene and water treatment as well as emergency responses from both governmental and non-government agencies have contributed to the reduction in case fatality rates over the years and should be sustained. Nevertheless, the need to explore more viable approaches cannot be overplayed if the infection has to be wholly curtailed. Due to its endemicity in Nigeria, surveillance systems can provide early alerts to outbreaks, therefore leading to coordinated response.

More importantly, it is necessary to introduce intervention measures that address the root problems of poor sanitation and unsafe water supplies in order to prevent future cholera epidemics. In this regards, perhaps, prevention of the disease is the best way to counter subsequent outbreaks. Simple measures as boiling the water for drinking, washing and cooking purposes, treatment of infected facilities, sewages and drainage systems, proper disposal of infected materials such as waste products, clothing, and beddings, treatment of infected faecal waste water produced by cholera victims and sterilisation of utensils either by boiling or by using chlorine bleach. Studies have also indicated that use of soap and hand washing promotion can achieve a 26 to 62% decrease in the incidence of diarrhoea in developing countries (Bouma, 2001).

Early detection and initiation of treatment of these infections are very important, particularly for cholera and invasive *Vibrio* infections, because these may rapidly progress to death (*Vollberg* and Herrara, 1997). Prevention of *Vibrio* infections requires a heightened awareness of these infections by clinicians, laboratory technicians, and epidemiologists. There are at least pathogenic *Vibrio* species recognized to cause human illness (Janda *et al.* 1998). The *Vibrio* species of most medical significance include: *Vibrio cholerae*, *Vibrio vulnificus*, and *Vibrio parahaemolyticus.*

Many researchers have studied different ways of controlling *Vibrio cholera*e infections using vaccine. Many researchers have stated that cholera vaccine areabout 85% effective during the first six monthsand 50–60% effective during the first year. The effectiveness decreases to less than50% after two years. When a significant portionof the population is immunized benefits fromherd immunity may occur among those not immunized.(Besser *et al.* 2005) also reported that no cholera vaccine is currently licensed and available for use by overseas travellers. The previously licensed vaccine in the United States was only about 50% effective and provided only 3 to 6 months of protection. Newer recombinant DNA vaccines like whole cell/recombinant B-subunit (WC/rBS) have demonstrated protection for more than 1 year. At the moment, neither the CDC nor the WHO recommends routine use of the cholera vaccine for travellers, since it may create a false sense of security and does not affect cholera severity. From the studies, there were no effective vaccine control regimens that have been designed in order to control this menace infection. Therefore this work has been designed to try other measures of controlling *Vibrio cholerae.*

MATERIALS AND METHODS

Area of study: The study was conducted in Aruolah and Ubahudara streams at Uli, Ihiala L.G.A., Anambra State. Within the location of the streams, the major anthropological activities are domestic works.

Sample Collection: The containers used for sample collection were washed with detergent and water, and was thoroughly rinsed with water and sterilized with 70% ethanol. Thecontainers were inverted on a swabbed bench, allowing the tiny droplets in the containers to dry up, and were aseptically closed. Water sample were collected by lowering the plastic container inside the water body, 30 cm deep, allowed to overflow before withdrawing the container. The sampling points were approximately 100 mm away from one another. The samplings were done in triplicate. After collection, the sample were covered and placed in a cooler containing ice block to maintain the temperature during transportation for laboratory analysis.

Isolation and identification of the *Vibrio cholerae:* This was carried out by aseptically inoculating 1.0 ml of the sample on Thiosulphate citrate bile salt sucrose (Biotech) agar, using pour plate method and incubated at 37°C for 48 h. After 48 h incubation the grown colonies were sub-cultured, characterized and identified using their colony descriptions, morphological and biochemical characteristics (Arora and Arora, 2008).

Experimental Mice: A total of twenty (24) mice of mixed sex obtained from animal keeping house at Nnobi, Anambra State were used for this study. The mice were kept in separate, thoroughly cleaned and disinfected cages and provided with feeds and water ad libitum.

Inoculation of the mice: This was carried out using the method of Iheukwumere *et al*. (2017). Broth culture of the isolate was centrifuged at 3000 r.p.m for 10 minutes. The sediment was diluted with sterile phosphate buffer saline (PBS) and adjusted to the 10^8CFu/ml using McFarland matching Standard. Then the mice (six in number) were inoculated orally with 0.5 ml of the inoculums while the control group (six in numbers) were only given distilled water. The infected mice were carefully observed for the clinical manifestation of the organism for a period of 4 weeks. The number of deaths was also observed. After 4 weeks, some of the infected mice sacrificed and gross examination of internal organs morphologies was carried out.

Re-isolation of the organism from the infected organs: The internal organs of the infected mice were harvested and portions were aseptically macerated in peptone water and serial diluted using ten-fold serial dilution. Samples were inoculated into peptone water, incubated at 37^{o}C for 24 h (Iheukwumere *et al*., 2017).

Histopathological Study: This was carried out using the modified method of Iheukwumere*et al*.(2017). This study was done in Animal PathologyDepartment, University of Nigeria, Nsukka. After 4 weeks, the mice were autopsied. The internal organs were removed, portion of these organs were washed with PBS and stored in formalin solution for histopathological examination.

Protection of infected mice: This was carried out using the modified methods of Iheukwumere *et al*. (2017).

Preparation of autogenous bacterin: This was carried out by the modified method of Iheukwumere *et al.* (2017). The isolate was grown on nutrient agar at 37^{oC} for 24 h. Growth was harvested in normal saline and inactivated with 1% formol saline at room temperature for 24 h. Using 0.5 Macfarland matching tube, washed concentrate of inactivated bacterium was suspended in normal saline to contain 10^8Cfu/ml. The sterile autogenous bacterin was obtained by adding equal volume of incomplete Freund's adjuvant to adjusted washed concentrate of inactivated bacterium and kept at refrigerator until when used. The autogenous bacterin was giving to the experimental mice at first day in dose of 0.2ml/mouse and boostered at a second dose at 7days in dose of 0.4ml/mouse. The autogenous bacterin in the two shots was giving subcutaneously through the abdominal cavity.

Quality control tests on the prepared autogenous bacterin: The prepared autogenous bacterin was tested for purity, complete inactivation, sterility and safety. ThePurity test was done before inactivation of the isolate. It was done to confirm that the broth culture of the isolate did nit contaminated by other bacteria before inactivation. This was done by subculturing the broth culture into TCBS agar and incubated at 37^{o}c for 24 h. The colony was Gram stained, examined and finally confirmed using unique biochemical reactions.Complete inactivation test was carried out to ensure that the isolate was completely inactivated. The TCBS agar was inoculated with the autogenous bacterin, incubated at 37^{o}C for 48 h. No visible growth of the isolate was seen. In the Sterility test, the prepared autogenous bacterin was confirmed to be free from any fungal contaminants by inoculating it into Sabouraud Dextrose Agar (SDA) plate and incubated at room temperature for 7 days. In the Safety test, two mice were aseptically inoculated subcutaneously with a large dose of the prepared autogenous bacterin (ten-fold of the normal dose). The mice were observed daily for five (5) successive days for any signs of local reactions, clinical signs or deaths.

Experimental design: The mice were grouped into three (3) groups which include group A and B. Each group contained total of six mice. The treatments to the group were as follows:In the first group(A), Blank Control (only distilled water) was given. The second group (B) contain autogenous bacterin, 0.2 ml/mouse for the first dose and boostered on the 7th day with 0.5ml/mouse. The third group were mice Infected without treatment; the experimental mice were then exposed to the isolate via oral route after 14 days. The mice were carefully monitored for a period of 2 weeks (Iheukwumere *et al*., 2017).

Detection of the humoral immune response: Just before the first dose of the autogenous bacterin (zero hour), the mice were randomly selected and their blood were collected. Also just before the second booster dose,

another blood sample was also collected on 14th day. The blood samples were allowed to separate. The separated sera were used against the isolate for agglutination reaction and the antibody titer against the isolate was determined and recorded (Wafaa *et al*. 2012).

Examination of protected mice: The protected mice were carefully observed for the clinical manifestation of the inoculated organism for period of 2 weeks, the protection rates of the inhibitory substances were determined, and the mice were sacrificed and gross examination of the morphologies of internal organs and intestine were carried out. Also the internal organs were harvested and some portions of these organs were cultured on TCBS agar, and incubated at 37^{o}C for 48 h. The counts were taken and the colonies were identified morphologically and biochemically (Wafaa *et al*.2012). The remaining portions of the organs were subjected to histopathological examination (Iheukwumere *et al*., 2017).

Statistical Analysis: The data generated from this study were represented as mean ±Standard deviation and then charts. The test for significance at 95% confidence interval was carried out using ANOVA (Iheukwumere *et al*., 2017)

RESULTS

Vibrio cholera was characterized and identified using its morphology, colony description and biochemical reactions(Table1). The isolate agglutinates fowl RBCS, haemolyse sheep RBCS and tested positive to catalase test, oxidase test, nitrosoindole test, string test, Vp test, glucose test, Sucrose test, arabinose test, saccharose test, citrate test, and motility test. The prevalence of *Vibrio cholera* in water samples collected from two major streams used in Uli community is shown in Table 2. The result of the present study revealed that *Vibrio cholerae* was significantly ($P > 0.05$) seen more in water samples collected from Ubahudara stream as compared to water samples from Aruolah stream. The gross morphologies of the internal organs and intestines of the infected mice are shown in Table 3. *Vibrio cholerae* caused fluid accumulation in the intestine, hypertrophy of the liver and kidney, liver and kidney congestion, perihepatitis and death of some mice. The mean growth counts of *Vibrio cholerae* from the internal organs of the infected mice is shown in Table 4. The mean growth of *Vibrio cholerae* is significantly ($P > 0.05$) seen most in the lungs, followed by the spleens and liver shows the least count. No *Vibrio cholerae* was isolated from the internal organs of the control mice.Table 5 shows the micro-agglutination antibody titers in the sera of mice after vaccination with locally prepared autogenous bacterin against *Vibrio cholerae*. There was no agglutination observed at day 0, before first autogenous bacterin dose. A maximum of 80 titer value was observed in the sera of two mice before booster autogenous bacterin dose, while a maximum of 160 titer value was observed in the sera of three mice before infection. No agglutination was recorded among the control mice before first autogenous bacterin dose, before booster autogenous bacterin dose and before infection. The gross morphological examination of the internal organs of protected mice against *Vibrio cholerae* is shown in Table 6.There was hypertrophy of the liver and Liver congestion in the mice protected with autogenous bacterin. The result of re-isolation of the *Vibrio cholerae* from the internal organs of protected mice is shown in Table 7. Seventy-seven colonies of the organism were re-isolated from positive control liver, one hundred and twenty-four from the lungs, forty-two and one hundred and five from the spleen. Nineteen and twenty-six were re-isolated from the kidney and spleen of those protected with autogenous bacterin respectively. Percent-Organ body weight ratio of the protected mice is shown in Table 8.There is a high Percent-Organ body weight ratio of the organs of control mice compared to the organs of mice protected with autogenous bacterin.

Table 1: Characteristic and identity of *Vibrio cholerae*

Parameter	*Vibrio cholerae*
Appearance on TCBS Agar	Yellow colonies
Elevation	Convex
Size (mm)	Entire
Gram Reaction	–
Shape	Comma shaped (Vibrio)
Catalase	+
Oxidase	+
Nitrosoindole test	+
String test	+
Agglutination with Fowl RBCS	+
VP test	+
Glucose	+
Sucrose	+
Arabinose	–
Saccharose	+
Citrate	+
Motility	+

Table 2: Prevalence of *Vibrio cholerae* in water samples collected from two major streams used in Uli

Streams	Total Isolate	Percentage%
A	**29**	**67.44**
B	**14**	**32.56**
Total	**43**	**100**

A= Ubahudara stream

B= Aruolah stream.

Table 3: Gross morphologies of the internal organs and intestines of the infected mice

Parameter	Observation
Haemorrhage of the liver	Absent
Haemorrhage of the lungs	Absent
Haemorrhage of the kidney	Absent
Haemorrhage of the heart	Absent
Fluid accumulation in the intestine	Present
Hypertrophy of the liver	Present
Hypertrophy of the kidney	Present
Liver congestion	Present
Kidney congestion	Present
Pericarditis	Absent
Perihepatitis	Present
Death	Present

Table 4: Mean growth of *Vibrio cholerae*from the infected organs

Organs	*Vibrio cholera* counts (CFu/g)	
	Test	Control
Liver	77	—
Lungs	124	—
Kidney	42	—
Spleen	105	—

Table 5: Microagglutination antibody titers in the sera of mice after vaccination with locally prepared autogenous bacterin against *V.cholerae*

Isolate	Day	Interval	Total	Antibody titerof the mice serum at different dilutions dilutions						
				0	20	40	80	160	320	640
Vibrio cholerae	0	BFBD	6	6	0	0	0	0	0	0
	7	BBBD	6	0	0	4	2	0	0	0
	7	BI	6	0	0	0	0	2	1	3
Control	0	BFBD	6	6	0	0	0	0	0	0
	7	BBBD	6	6	0	0	0	0	0	0
	7	BI	6	6	0	0	0	0	0	0

BFBD-Before First Autogenous bacterin Dose, BBBD-Before Booster Autogenous bacterin Dose, BI-Before Infection

Table 6: Gross morphological examination of the internal organs of protected mice against *Vibrio cholerae*

Parameter	Observation		
	F_2	A	F_1
Haemorrhage of the liver	—	—	—
Haemorrhage of the lungs	—	—	—
Haemorrhage of the kidney	—	—	—
Haemorrhage of the heart	—	—	—
Fluid accumulation in the intestine	—	—	+++
Hypertrophy of the liver	—	+	+++
Hypertrophy of the kidney	—	—	—
Kidney congestion	—	—	+
Liver congestion	—	+	+++
Pericarditis	—	—	—
Perihepatitis	—	—	+++
Death	—	—	++

A=Autogenous bacterin, +++ = High , ++ = Moderate, + = Mild, F_1 = Positive control (Infected mice without protection), F_2 = Negative control (Healthy mice)

Table 7:Re-isolation of the *Vibrio cholerae* from the internal organ of protected mice

Organ	**Total mean count(Cfu/g)×10^2**		
	A	**F_1**	**F_2**
Liver	**-**	**77**	**-**
Lungs	**-**	**124**	**-**
Kidney	**19**	**42**	**-**
Spleen	**26**	**105**	**-**

A= Autogenous bacterin, F_1=Positive control, F_2=Negative control

Table 8: Percent-Organ body weight ratio of the protected mice

Organ	Percent-Organ body weight Ratio (%)		
	A	**F_1**	**F_2**
Liver	0.048	0.081	0.042
Lungs	0.026	0.043	0.018
Kidney	0.010	0.019	0.007
Spleen	0.003	0.007	0.001

A = Autogenous bacterin, F_1 = Positive control, F_2 = Negative control

DISCUSSION

Biochemical reactions, morphological examination and colony description of *Vibrio cholerae* isolated in the study collaborates with the report of Basu, (2000), who worked on biochemical tests for *Vibrio cholerae*. Similar conclusion was drawn by (Besser *et al.,2005*).The variation in *Vibrio cholerae* loads among the two streams could be attributed to the fact that Ubahudara stream is more close to human settlement and more anthropological activities takes place in this stream than Aruolah stream. Similar conclusions were drawn by Faruque *et al*.(2000). Wenjing (2000) also reported that most drinking water contamination can be attributed to human activities.The accumulation of fluid in the intestines collaborates with the report of Muanprasat *et al.*(2012). The gross morphological examination of internal organ of infected mice against *Vibrio cholerae* revealed congestion in liver and kidney. This agrees with the report of Basu (2000). Oliver *et al.*, (2007) also reported the death of mice associated with systemic spread of infection caused by choleragen toxin released by *Vibrio cholerae.* The significant growth of *Vibrio cholerae* observed from the internal organs of infected mice showed that the organism was able to invade and multiply in these organs. Similar result was reported by Basu, (2000). In the present study, significant antibiodies produced by vaccinated mice before booster autogenous bacterin dose (BBBD) and before infection (BI) agree with the report of Hlady and Klontz (2003). The antibody was able to offer little protection to the mice.The result of the gross morphological examination of the internal organs of protected mice against *Vibrio cholerae* revealed the hypertrophy of the liver and liver congestion. This could be due to the production of hemolysin-cytolysin toxin as a result of choleragen toxin activation. These agree with the report of Oliver (2007), who worked on systemic spread of infection caused by choleragen toxin released by *Vibrio cholerae.* The low count of Vibrio *cholerae* associated with the internal organs of protected mice after infection shows that autogenous bacterin was able to protect the organs against the invading organism (Colwell *et al*. 1996). Autogenous bacterin offered a promising protection on the mice against *Vibrio cholerae* and this could be due to production of antibody against *Vibrio cholerae*. The slight increase in percent-organ body weight ratio of the protected mice when compared with that of negative control could be attributed to the side effect caused by the autogenous bacterin.(Oliver, 2007).

CONCLUSION

The study revealed the effect of autogenous bacterin on *Vibrio cholerae*. It can also be stated that this autogenous bacterin can be used in the treatment of Vibrio infection for short term since it offered a promising protection on the mice against its causative agent, Vibrio *cholerae*.

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