PRODUCTION OF SOME VIRULENCE FACTORS UNDER DIFFERENT GROWTH CONDITIONS AND ANTIBIOTIC SUSCEPTIBILITY PATTERN OF *Aeromonas hydrophila*

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ABSTRACT

The production of some virulence factors under different growth conditions and antibiotic susceptibility pattern of Aeromonas hydrophila were investigated in this study. The virulence factors tested on the isolates included haemolytic activity, exopolysaccharide (capsule) and toxin production. Other cell property evaluated was antibiotic resistance. Of the several chemotherapeutants tested, streptomycin had Minimal Inhibitory Concentration (MIC) and Minimal Cidal Concentration (MCC) at 25 µg/ml; flumequine, MIC 15 µg/ml and MCC 20 µg/ml; nitrofurantoin, MIC 20 µg/ml and MCC at 20 µg/ml; chloramphenicol, MIC 15 µg/ml and MCC 30 µg/ml and nalidixic acid, MIC 25 µg/ml, MCC 30 µg/ml, respectively. Virulence characteristics were apparent from the study; the properties exhibited such as ß-haemolytic activity (F= 1.32551; p < 0.001) and toxin production (F = 0.141; p > 0.05) were evidence of the pathogenic potential of A. hydrophila.

Keywords: Virulence, Haemolytic activity, Susceptibility, Antibiotics, *Aeromonas hydrophila*

INTRODUCTION

Virulence is an important property of microorganisms in relation to their pathogenicity and is defined as the capacity of the organism to invade tissues, multiply and produce toxic effects. It is estimated by the minimal lethal dose (MLD), which is the smallest dose of the organism (number or weight of cells from a culture) that will kill a particular species of animal. Virulence depends on two factors that may be largely independent of one another; namely, the invasiveness or aggressiveness, and the toxigenicity or toxin - producing property of the organism. Thus, the tetanus bacillus for instance is highly toxigenic but only weakly aggressive; in contrast, the Pneumococcus is markedly aggressive but its toxicity is minimal (Cruickshank *et al.*, 1980).

Aeromonas hydrophila has been isolated from both polluted and unpolluted water worldwide (Schubert, 1976), and its ability to produce virulence factors like enterotoxin, haemolysin, endotoxins, cytotoxins (Barney *et al.,* 1972; Berheimer and Avigad, 1974; Chopra and Houston 1999), as well as its antibiotic resistance potential (Chopra and Houston, 1999; Albert, 2000; Okpokwasili and Okpokwasili, 1994) has been documented. Chopra *et al.*, (2000), reported a cytotoxic enterotoxin production; in a study of nine isolates of the organism, 69 % were found to produce cytotoxin and haemolysin. Fatal and non-fatal infections caused by the organism have been reported. *A. hydrophila* has also been observed as a life threatening pathogen, associated with a variety of clinical manifestations, including septicemia (Seatha *et al.*, 2004; Mathewson and Dupont, 1992), meningitis (Seatha *et al.*, 2000); endocarditis (Davis *et al.,* 1978), corneal ulcers (Feaster *et al.,* 1978), wound infections (Shackelford *et al.*, 1973; Mani *et al.*, 1995), peritonitis (Salton and Schick, 1973) and acute diarrheal diseases (Albert, 2000). *A. hydrophila's resistance* to chemotherapeutants such as; ampicillin, colistin sulphate, sulphonamide, tetracycline and cotrimoxazole, has been reported (Okpokwasili and Okpokwasili, 1994). Earlier reports indicated that the environmental strains of *A. hydrophila* were capable of producing toxins and that isolates recovered from healthy and moribund fish were cytotoxic and most strains were enterotoxic in the Rabbit Ileal Loop (RIL) and suckling mouse tests. The organism was concluded to produce an *Escherichia coli* ST-like (heat stable) and heat labile toxins (Kaper *et al.,* 1980).

This study investigated the expression of some virulence characteristics and antibiotic resistance of *A. hydrophila* isolated from fish with a view of establishing its pathogenicity.

MATERIALS AND METHODS

Isolation, identification, enumeration and maintenance of *Aeromonas hydrophila:* The bacterium*, Aeromonas hydrophila* was isolated from the fish *Epiplatys bifasciatus*, of the family, Cyprodontidae, from Taylor Creek, a fresh water creek in Yenegoa, Bayelsa State of Nigeria. Reference strain, ATCC 7966 (*A. hydrophila*), made available by G. S. C Okpokwasili, served as the control.

Culturable aerobic heterotrophic bacterial counts were obtained after appropriate serial dilutions and plating in Rimler Shotts agar and Tryptone Soy agar (TSA) plates (Shotts and Rimler, 1973). The *Aeromonas hydrophila* strain was tested for production of various virulence factors, namely,

capsule or slime production, α or β haemolysis, antibiotics susceptibility as well as toxin production following cultivation in the hydrocarbons: gasoline, toluene, kerosene and diesel oil as sole carbon sources and glucose as control.

Substrate Utilization by *A. hydrophila*: Production of some virulence factors under different growth conditions was determined using the vapour phase transfer method (Mills *et al.,* 1978) as modified by Okpokwasili and Amanchukwu (1988). The components of the medium were: 0.42 g $MgSO_4.7H_20$; 0.297 g KCl; 0.85 g $KH_2 P0_4$; 0.42 g Na $N0_3$; 1.27 g $K_2H P0_4$; 20.12 g NaCl, and 20 g TSA agar powder. The methods of Harrigan and McCance (1976) were adopted for the characterization and identification of the isolates. Purity of the samples was maintained by inoculation on TSA slants at 4 °C, and sub-culturing fortnightly onto freshly prepared TSA slants and stored in a refrigerator.

Haemolysis: Alpha or Beta haemolytic activity of culture filtrates of *A. hydrophila* on Ox red blood cells was used to determine the ability of the organism to produce haemolysin (pathogenicity factor) in different hydrocarbon substrates. One hundred (100) ml of Mineral Salts Broth (MSB) distributed in 5 ml volumes into 20 test tubes was supplemented with 0.1, 0.25, 0.5 and 0.1 ml of each hydrocarbon: gasoline, toluene, kerosene, diesel and glucose, the control. These were inoculated with the test organism and incubated at 37 ^oC for 24 h, after which the culture was centrifuged at 3000 rpm for 15 min to clarify and then filtered through a Whatman No. l filter paper. Equal (1-mL) volumes of the filtrate and 1 % Ox RBC (washed thrice in saline, resuspended in same solution and diluted appropriately to obtain the working concentration) were mixed in clean test tubes and incubated in a water bath at 37 ^oC for 1 h. The mixture was then centrifuged at 3000 rpm for 5 min to remove unlysed RBC and debris. The Optical density (OD) of the supernatant at 420 nm was read in a Spectronic-20 to determine the degree of haemolysis (α or β), as a measure of haemolysin produced in the culture filtrate. Similar determination was carried out with culture filtrate of glucose-supplemented MSB as control.

Capsule (Exopolysaccharide) Formation: The method described by Cruickshank *et al.,* (1980), was adopted for the capsule formation test. Approximately 10 ml volume of *A. hydrophila* was grown in mineral salts medium (MSM), mended with 1% (w/v) glucose and maintained at pH 7.2 in a mechanical shaker (swirling flask) at 13 ^oC at 100 rpm for 5 days. Swirling flask procedures described by Pazur and Forberg (1980), were adopted for the exopolysaccharide capsule production in liquid broth as most procedures currently in use do not achieve selective release of exopolymers from the cells. This procedure was found to enhance clumping of cells as a result of the selective pressure exerted by the swirling process. Increase in the number and size of observable clumps in the suspension and the presence of collar of cells on the walls of the swirling flasks at the liquid-air interface correlated with subsequent increase in the culture biomass detected visually. Capsular material was not easily stained and remained relatively uncolored with a comparatively weak dye such as Methylene blue. Consequently, the India ink wet mount procedures for exopolysaccharide capsule examination was adopted in this study and was found most appropriate, as the black dye sharply outlined the edge of the capsule. The capsular and slime layers were observed in a wet preparation in which India ink was added for contrast (Duguid, 1959). The thickness of the capsule observed virtually was qualitatively scored as high (+++), moderate (++), or low (+).

Antibiosis: Antibiotic susceptibility testing was done using the minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) procedure (Cruickshank *et al.,* 1980). The following antibiotics in their various concentrations (µg/ml), were used: kamamycin monosulphate (50 µg/ml), streptomycin sulphate (25 µg/ml), furazolidone (60 µg/ml), ampicillin (100 µg/ml), amikacin (65 µg/ml), flumequine (20 µg/ml), nalidixic acid (30 µg/ml), tetracycline (100 µg/ml), erythromycin (60 µg/ml), cephalothin (65 µg/ml), chloramphenicol (30 µg/ml), gentamycin (70 µg/ml), oxolinic acid (25 µg/ml), nitrofurantoin (20 µg/ml).

A two-fold serial dilution of each antibiotic was prepared in Tryptone soy broth (TSB), and 0.1 ml volume of a 12 h TSB culture of the test organism was added to each dilution. Two controls were included, a blank containing TSB + antibiotic but no bacterial inoculation and TSB with bacterial inoculation but no test antibiotics. Following incubation at 37 ^oC for 24-48 h, each culture was examined for bacterial growth. All dilutions with no apparent growth (indicated by lack of turbidity) was subcultured in TSA, and the highest dilution showing no growth in the TSA subculture were taken as containing the minimal bactericidal concentration.

Toxin Production: The potential for toxin production of the pre-grown isolates was tested by growth in Tryptone Soy broth (TSB) amended with 0.6% yeast extract and incubated at 37°C for 48h. The toxin was harvested from the spent broth by centrifuging at 4000 rpm for 15 mins. The supernatant liquid containing the toxin was partially purified by re-dissolving in 10 ml of distilled water and filtered through Whatman No. 1 filter paper, after which 55 ml of the extracted toxin was used for the virulence assay.

Virulence Studies: Ten 3-months old each of three fish species, *Clarias gariepinus, Clarias gariepinus* X *heterobranchus bidorsalis* hybrid, and *Oreochromis mossambicus* obtained from African Regional Aquaculture Centre (ARAC), Aluu, about 500 m northwest of the University of Port Harcourt, Choba Campus, Port Harcourt Rivers State were introduced into 3 litres of pre-sterilized pond water in clean aquaria. Each aquarium was covered with wire

gauze. To different aquaria was added 0.1, 0.25, 0.5 and 1.0 ml of supernatant of TSB culture fluid of the *A. hydrophila* supplemented with 0.6 % yeast extract and clarified by centrifugation at 4000 rpm for 15 min. After 96h of exposure the number of fish that died were counted and recorded. Following the preliminary tests, confirmatory experiment was carried out with higher doses or concentrations of the culture filtrate. Consequently, 5 ml, 10 ml, 15 and 20 ml of the filtrate were administered to the test fish by the bathe method (immersion of filtrate into the aquaria). All experiments were carried out in triplicates.

Fish Behavioural Studies and Bioassay: Significant behavioural responses of fish to the administered toxin were studied following 96 h of exposure, and the percentage death or survival recorded. The lethal dose of the toxin that could kill fifty percent of the fish samples (LD_{50}) was then determined using the graphic procedure (Litchfield and Wilcoxon, 1949) for estimating the median effective dose and the dose percent effect curve. The interpolated value at 50 % mortality ratio gave the LD_{50} of each toxin at the corresponding concentrations.

Data Analysis: The data for the total heterotrophic bacterial count and substrate utilization test were tested by 2-way analysis of variance with substrate (five levels) and volume of substrate (four levels) as fixed factors.

Haemolytic activity was measured as optical density and/or absorbance (at 420 nm) of liberated haemoglobin using the Spectronic-20. Analysis of variance was then used to test the difference between means as well as their level of significance.

Data from the bioassay and mortality ratios were analysed by 2 -way or 3-way analysis of variance as appropriate. Where data were not normally distributed, appropriate transformation was applied to the values before the analysis. The data obtained from these were used to calculate the fifty-percent lethal dose (LD_{50}) using the graphic method (Litchfield and Wilcoxon, 1949).

RESULTS

Substrate Utilization by *A. hydrophila:* The ability of *Aeromonas hydrophila* to utilize the various substrates as sole sources of carbon and energy has been outlined in Table 1. There was also a remarkable difference in the pattern of growth, and hence the optical density of each substrate at the various concentrations. A significant main effect of concentrations of substrate ($F = 5.3319$; $p < 0.01$) on bacterial counts was observed. Gasoline, toluene and kerosene were utilizable, only at low concentrations. Utilization of the substrate by *A. hydrophila* decreased with increase in hydrocarbon concentration. A significant difference was observed in the growth of *A. hydrophila* while utilizing diesel oil ($F = 12.5693$; $p < 0.001$). A gradual but appreciable increase in turbidity occurred, signifying the ease in which *A. hydrophila* utilized the substrate (Table 1).

Haemolytic Activity: β-haemolysis was observed from haemolytic activity of Ox Red Cells by *A. hydrophila* (Table 2). From analysis of variance, gasoline 0.0983, and diesel oil, 0.5178, had a significant mean difference of 0.4195. There was no significant statistical effect of concentration of substrate ($F = 0.2292$; $p > 0.05$) on the isolate. However, a significant main effect of substrate ($F= 1.32551$; $p <0.001$) was evident.

The substrate differential haemolysis of Ox red blood by *A. hydrophila* was shown in Figure 1. The peak depicted by diesel oil is an indication of the less toxic and/or sublethal or benign constituents of the diesel oil compared to those of gasoline, toluene or kerosene. A similar pattern of sublethal effect was shown by glucose, the control. The higher toxic level of gasoline was shown by the zero slope of its differential haemolysis (Figure 1).

Capsule Formation: Result of the capsule production test by *A. hydrophila* was presented in Table 3. Growth on glucose was observed to enhance minimal capsule production, while high capsule production generally occurred among the hydrocarbon grown isolates

Antibiotic Sensitivity: The result of the antibiotic sensitivity, aimed at determining the most effective chemotherapeutants against *Aeromonas hydrophila* through their Minimal Inhibitory Concentration (MIC) and the Minimal Cidal Concentration (MCC) are presented on Table 4. Chemotherapeutants with good antimicrobial activity included: streptomycin (MIC and MCC at 25 μg/m), flumequine, (MIC 15 μg/ml and MCC 20 μg/ml), Nitrofurantoin, (MIC 20 μg/ml and MCC 20 μg/ml); chloramphenicol, (MIC 15 μg/ml and MCC 20 μg/ml). Nalidixic acid, (MIC 25 μg/ml and MCC 30 μg/ml). The order of efficacy was as follows: flumequine > Nitrofurantoin > Oxolinic acid > Chloramphenicol > Streptomycin > Nalidixic acid.

Fish Behaviour: Table 5 presents the different behaviors observed in the various fish samples following exposure to the culture fluid aimed at determining the toxicity of the medium. Time taken to achieve death of each species correlated with the concentration of the culture fluid used. High concentration produced a concomitant increase in the death rate at reduced time interval. *O. mossambicus* showed least resistance to the culture fluid. Significant behavioral changes like erratic movement were observed at about 6 h after treatment. *O. mossambicus* was observed to be susceptible to high doses of the treatment, while *C. gariepinus* species took a longer time to manifest observable effects at about 9 h. The hybrid of *H. bidorsalis* X *C. gariepinus* was the most resistant even at a higher dose, manifesting observation changes at the 11 h following treatment.

Table 1: Utilization of Hydrocarbon Substrates by *Aeromonas hydrophila*

Substrate	Concentration (ml^{-})	OD $_{420\ nm}$ Over Time (in Days)							
		0	1	2	3	4	5	6	7
Gasoline	0.1	0.002	0.013	0.017	0.022	0.026	0.030	0.036	0.041
	0.25	0.001	0.015	0.022	0.028	0.033	0.039	0.044	0.048
	0.5	0.003	0.010	0.008	0.007	0.005	0.005	0.004	0.002
	1.0	0.002	0.006	0.004	0.004	0.003	0.002	0.002	0.001
Toluene	0.1	0.001	0.019	0.023	0.027	0.032	0.035	0.038	0.042
	0.25	0.003	0.022	0.028	0.031	0.037	0.041	0.046	0.049
	0.5	0.004	0.012	0.010	0.010	0.009	0.008	0.008	0.006
	1.0	0.002	0.010	0.008	0.007	0.006	0.006	0.004	0.003
Kerosene	0.1	0.003	0.021	0.028	0.033	0.039	0.045	0.049	0.055
	0.25	0.002	0.027	0.032	0.038	0.046	0.047	0.045	0.050
	0.5	0.004	0.018	0.015	0.013	0.011	0.011	0.010	0.008
	1.0	0.002	0.016	0.013	0.009	0.007	0.007	0.006	0.005
Diesel Oil	0.1	0.002	0.029	0.037	0.044	0.052	0.058	0.065	0.069
	0.25	0.001	0.033	0.041	0.048	0.057	0.064	0.072	0.078
	0.5	0.004	0.038	0.049	0.056	0.062	0.069	0.074	0.082
	1.0	0.002	0.043	0.055	0.064	0.071	0.074	0.082	0.089
Glucose	0.1	0.004	0.027	0.032	0.037	0.045	0.050	0.059	0.064
	0.25	0.002	0.031	0.035	0.043	0.049	0.057	0.065	0.069
	0.5	0.002	0.036	0.038	0.049	0.053	0.061	0.072	0.077
	1.0	0.003	0.041	0.043	0.055	0.059	0.069	0.078	0.088

Table 2: Haemolytic Activity of *A. hydrophila*

Concentration of Substrate	OD $_{420\ nm}$	Gasoline	Toluene	Kerosene	Diesel Oil	Glucose
0.1	0.165	0.201	0.206	0.345	0.329	0.2492
0.25	0.096	0.183	0.195	0.412	0.344	0.246
0.5	0.070	0.093	0.116	0.601	0.407	0.2574
1.0	0.062	0.80	0.109	0.713	0.502	0.2932
$\bar{x}$	0.0983^{b}	0.1393^{b}	0.1565^{b}	0.5178^{a}	0.3955^{a}	

Means with different superscripts are significant

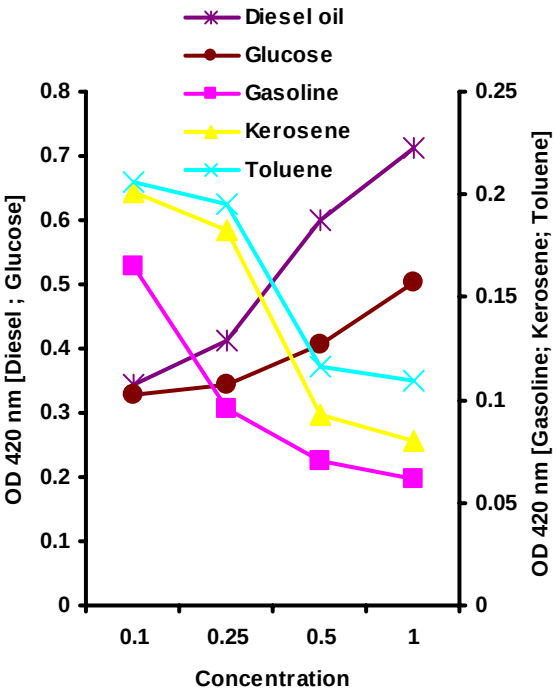

Figure 1: Substrate differential haemolysis of Ox red blood by *A. hydrophila*

Very slow death was observed after several days of the treatment: 24 h – 96 h for *O. mossambicus* and 36 – 96h for *C. gariepinus* species and *the* hybrid of *H. bidorsalis* X *C. gariepinus* respectively.

Bioassay: Response of fish to treatment with the culture fluid is presented in Table 6. The effect was more pronounced at a higher concentration of the toxin as more death occurred at 15 than at 20 ml concentrations. However, there was no statistical difference in the effect of various concentrations used for the bioassay ($F = 0.141$; $p > 0.05$). The effectiveness of the treatment expressed in the mortality rate of fish was shown in Figure 2. *O. mossambicus* showed more instantaneous response to treatment, followed by *C. gariepinus* and lastly by the hybrid of *H. bidorsalis* X *C. gariepinus* which were observed to be most resistant. The percentage survivors of samples were observed to decrease with increased concentration of toxin, with complete death recorded in the 20 ml volume. However, the hybrid of *H. bidorsalis* X *C. gariepinus* were more resistant, and so more survivors were observed even at the 20 ml lethal dose of the toxicant than the other samples. There was nevertheless, no significant statistical effect of mortality rate of fish ($F = 0.393$; $p > 0.05$). The LD_{50} is represented in (Figure 3).

DISCUSSION

This study had analysed the virulence potential of *Aeromonas hydrophila*, as well as its susceptibility pattern to a wide range of antibiotics with a view of determining its pathogenicity. Virulence was observed

Table 3: Capsule Production by *A. hydrophila*

Substrate	Concentration (ml)			
	0.1	0.25	0.5	1.0
Glucose	-	+	+	+
Gasoline	+++	++	+	-
Toluene	+++	+++	++	+
Kerosene	+++	+++	+++	++
Diesel Oil	+++	+++	+++	++

+++ High production; ++ Moderate production; + Low production, - No capsule production

Table 4: Minimal Inhibitory Concentration (MIC) and Minimal CIDAL Concentration (MCC) of Chemotherapeutants and Susceptibility Pattern of *A. hydrophila*

Chemotherapeutants	MIC (µg/ml)	MCC (µg/ml)
Kamamycin	45	50
Streptomycin	25	25
Furazolidone	40	60
Ampicillin	100	100
Amikacin	45	65
Flumequine	15	20
Nitrofurantoin	20	20
Tetracycline	80	100
Erythromycin	40	60
Cephalothin	50	65
Chloramphenicol	15	30
Gentamycin	50	70
Oxolinic acid	20	25
Nalidixic acid	25	30

Table 5: Behavioural Response of Fish Following Intoxication

Behavioural Response	Time of Response (h) after Treatment		
	O. mossambicus	***C. gariepinus***	***H. bidorsalis* X *C. gariepinus***
Fish appear active at surface, gulping air	6 h	9 h	11 h
Fish lying listlessly near water surface	6 h	9 h	11 h
Erratic swimming movements	7 h	10 h	12 h
Fish twisting onto side, exposing abdomen	8 h	11 h	14 h
Slower and irregular movements	10 h	14 h	16 h
Fish on bottom of aquaria, fins folded	12 – 24 h	18 h	20 h
Floating on the surface lifeless, with operculum, mouth open, bulging eyes exposed	24 – 96 h	24 – 96 h	24 – 96 h

TABLE 6:Effect of Toxin on Mortality of Fish Samples

Fish	Mortality Concentration of Toxin (ml)							
	0.1	0.25	0.5	1.0	5	10	15	20
O. mossambicus	0	0	0	0 .2	0 .2	0 .4	0 .7	0.9
C. gariepinus	0	0	0	0	0 .1	0.1	.3	0.8
H. bidorsalis* X *C. gariepinus	0	0	0	0	0	0.1	0.3	0.4

to be an important property of *A. hydrophila* in relation to its pathogenicity; and depended on two factors that may be largely independent of one another; namely, the invasiveness or aggressiveness, and the toxigenic or toxin - producing property of the organism. The ability of *Aeromonas hydrophila* to utilize the various substrates as sole sources of carbon and energy is shown in this study.

There was a significant main effect of concentration of substrate ($F = 5.3319$; $p < 0.01$) at lower concentrations more than higher concentrations. Comparatively, gasoline supported less growth than diesel oil. This may be due to its properties: short-chain carbon length (C_5 – C_9); specific gravity, 0.68 – 0.77; boiling point 30 – 200; flash point – 40 as well as the presence of additives such as anti-knock, mercaptans, anti-oxidants and corrosion inhibitors, which are toxic to microorganisms. Diesel oil, on the other hand, has carbon chain C > (14); boiling point, 180 – 360 and flash point 77. Diesel oil was remarkably utilized probably due to its rich mineral content such as sulphur and some heavy metals (cations) some of which are essential in the synthesis of amino acids in microorganisms (Atlas, 1995). From analysis of variance, significant main effect of 0.1925 (8.130 – 7.9375) was evident. Similarly, toluene and diesel oil had significant main effect of 0.145 (8.130 – 7.985). Toluene has carbon atoms C_{10} - $C_{14;}$ specific gravity 0.78; boiling point 160 – 285, flash point, 77 and complex inhibitory chemicals like lead anti-knock additives (Bertoni *et al.,* 1996).

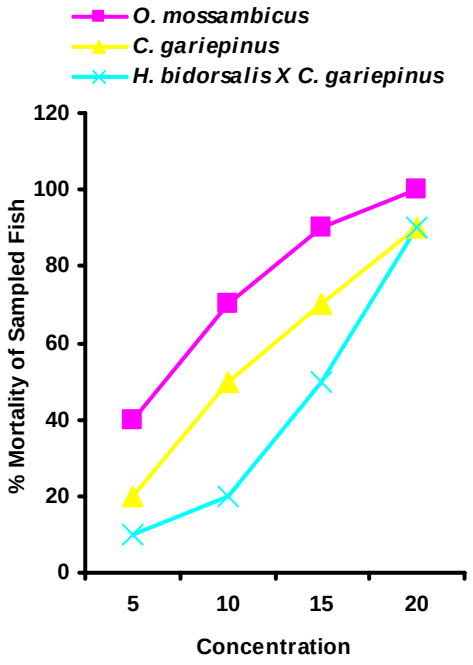

Figure 2: Percentage response of fish to *A. hydrophila*

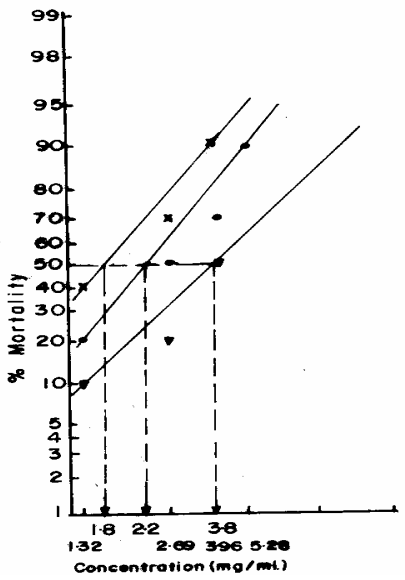

Figure 3: LC_{50} of samples in response to *A. hydrophila*
***x---x Tilapia; o---o Clarias; >---< Clarias* hybrid**

Gasoline, toluene and kerosene were utilized only at low concentrations as they are thought to be poor substrates because of their toxic effect on microorganisms. Data from analysis of variance showed no significant main effect of concentration of substrates of gasoline and toluene, 7.985 – 7.9375 (0.0475); kerosene and toluene, 8.0175 – 7.985 (0.0325). This is attributable to their close carbon range (C_5 - C14), aliphatic components as well as inhibitory additives. The toxicity is probably due to disorganization of cytoplasmic membrane resulting mainly from non-specific effect on membrane proteins including those associated with transport and oxidation (Dutta and Harayama, 2001). Utilization of substrate at sub lethal concentrations might also be genetically regulated, in consonance with the findings of Kozlovsky *et al*., (1993) who reported that genetic information for synthesizing enzymes responsible for degrading these hydrocarbons is enclosed in the extra-chromosomal genetic elements (plasmid) and that the enzymes of a degradative pathway are plasmid – specific. Glucose was maximally utilized as a carbon source, and may represent growth conditions of *A. hydrophila* in its natural environment (Rom-ling *et al.,* 1994) at uncontaminated sites.

Haemolysins are substances that bring about the haemolysis or dissolution of red blood cells. Haemolysin was produced by all the *Aeromonas hydrophila* isolates. This was indicated by the β - haemolysin (ability to lyse red cells completely) exhibited by the organism, in consonance with the reports of Barney *et al*., (1972); Beiheimer and Avigad, (1974), Chopra and Houston (1999). This phenomenon suggests the production of two types of haemolysin responsible for such phenomenon: a heat stable glycolipid, and a heat-labile phospholipase C (PLC), which can both lyse and agglutinate human red cells and platelets respectively (Shortridge *et al.,* 1990*)*. Capsule production was also demonstrated in the study, conforming to the reports of Kenne and Lindeberg, (1983); Sutherland, (1983) who showed that microorganisms produce a large number of structurally diverse extracellular polysaccharides (EPS) with resultant unique rheological properties. The phenomenon of capsule formation is of prime importance among pathogenic bacteria as it enhances the virulence of the organisms as it acts as a defense for the organism against bactericidal factors in body fluids. In capsulate bacteria, the slime is generally similar in chemical composition and antigenic character to the capsular substances. Microbial exopolymers were observed to occur as slime fibers loosely associated with or dissociated from the cells.

Generally, bacterial sensitivity to antibiotics increases with increase in antibiotic concentration. It is noteworthy that the efficacy of antibiotics varies. While some are able to exhibit high bacteriostatic and bactericidal effect on a wide range of organisms at low concentrations, some can only do so at a very high dosage. Of the several chemotherapeutants (in μg/ml) used during this study, streptomycin, flumequine, nitrofurantoin, chloramphenicol and nalidixic acid (in order of potency) were drugs of choice, as resistance of the bacterium was very low, and the drugs had both bacteriostatic and bactericidal effect on the organism. In general, *Aeromonas* strains show a drug sensitivity (susceptibility) to the quinolones (flumequine and oxolinic acid) and the nitrofurance; nitrofurantoin according to the reports of Okpokwasili and Okpokwasili, (1994). However, high resistance to such drugs as: ampicillin, cephalothin, erythromycin, tetracycline, furazolidine and amikamicin observed in the study correlates with the findings of Okpokwasili and Okpokwasili (1994). Drug resistance may be natural or acquired characteristic of microorganisms. It is inferred from

the study that the resistance of *A. hydrophila* to used drugs could have resulted from impaired cell wall or an envelope penetration, enzyme inactivation or altered binding sites. Similarly, acquired drug resistance could have been as a result of mutation, adaptation or gene transfer; and spontaneous mutation, which could have occurred at low frequency. However, Aoki *et al.*, (1981), Toranzo *et al.*, (1985) reported that genetic resistance may be chromosomally or plasmid – mediated, and that plasmid - mediated resistance is typical of the Gram-negative enteric pathogens (such as *A. hydrophila)*. By the process of conjugation, resistant plasmids might have been transferred both between the bacterial strains. Such resistance factors could have coded for multiple antibiotic resistance due to possession of resistant factors (R – plasmids) thereby rendering the drugs impenetrable to the bacterial cell as well as causing conversion of an active drug to an inactive product by enzymes produced by the organism (inactivating enzymes), which correlates with the findings of Röling *et al,* (2002). Nevertheless, it is possible that the observed high resistance pattern of *A. hydrophila* to antibiotics used in the study could have resulted from drug abuse, especially since the drugs in question are cheaper, and more readily available, a scenario about which Toranzo *et al.*, (1985), had warned against in a bid to check drug misuse and the prevalence of bacterial resistance and the associated risk of transfer of resistance to pathogens especially of the aquatic organisms which may induce gastroenteritis. It is further inferred that antibiotic resistance to *A. hydrophila* might also have displayed an intrinsic resistance to the inhibitory or lethal effects of the drugs. Such resistance might depend, for example, on the absence or inaccessibility of those structural and/or functional features against which the antibiotic is effective.

Toxin production evident in the study conforms to the findings of Xu *et al.,* (1998) who reported a heat-labile enterotoxin produced by *A. hydrophila* that produced fluid accumulation in Rabbit Ileal Loop (RIP), and an enterotoxic response in Y – adrenal cells. In a study of 9 isolates of *A. hydrophila*, 69 % were found to produce cytotoxin, and haemolysin Chopra *et al.*, (2000). Kaper *et al.*, (1980) and Sanyal *et al.,* (1978) reported that all strains of *A. hydrophila* isolated from diarrheic and healthy individuals, animals and drinking water, river water and sewage were enterotoxigenic.

From results of previous studies, Barney *et al.*, (1972); Beiheimer and Avigad, (1974); Chopra and Houston (1999), Aeromonas, (*A. hydrophila)* was shown to produce endotoxins, cytotoxin and haemolysin. Xu *et al.*, (1998) and Albert, (2000) on the other hand reported a heat-labile enterotoxin produced by *A. hydrophila* similar to that described in this study, which produced fluid accumulation in RIL and an enterotoxic response in Y-1 adrenal cells. Similarly, Chopra *et al.*, (2000), reported that *A. hydrophila* can produce an enterotoxin, but it is different from that of *Vibrio cholerae* and enterotoxigenic *E. coli*

Exposure of fish to the *A. hydrophila* toxins extracted during the study resulted in some behavioural responses in the tested fish. However, the toxic effect was more pronounced at high, rather than sublethal or low doses. However, *H. bidorsalis* X *C. gariepinus* proved more resistant to the toxic than the other species. This might be attributed to the improved vigor and immuno-competence exhibited by the hybrid. The toxic effect was more pronounced at a higher concentration of the toxicant as more death occurred at 15 than at 20 ml concentrations. The effectiveness of the toxins is therefore expressed in the mortality rate of test samples. The LD_{50} of fish samples shown in Fig., indicated that *O. mossambicus* had LD_{50} at 1.32; *C. gariepinus* at 2.69 and *H. bidorsalis* X *C. gariepinus* at 3.96. The vulnerability of O. *mossambicus* is thus shown by its LD_{50}, 1.32, attributed to such factors as trauma or stress that might have been encountered by the fish while the ability of the other two species to withstand the toxic effect at least a longer period than the *O. mossambicus* is shown by their respective LD_{50}. However, the LD_{50} of the various fish samples was achieved at higher concentrations of the toxins.

In conclusion, results of this study indicated that exopolysaccharide capsule formation, antibiotic resistance, haemolysin production, endo and exo-toxin production are pathogenic potentials of *A. hydrophila.* These features are evidence that the organism is an outstanding life threatening pathogen worthy of further investigation.

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