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Virulence of *Aeromonas hydrophila* Isolated from Fresh Water Catfish

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Abstract

Background: A large proportion of Nigerians consume fish as the source of protein in their meals. This may be attributed to health factors, preference and affordability for low income earners. The incidence of *Aeromonas hydrophila* in fresh catfish may constitute a significant health risk to the consumer if there is a horizontal transfer to man as it has been reported to be pathogenic. This study examined the possibility of fresh water catfish being a reservoir of pathogenic *Aeromonas hydrophila*. **Method:** *Aeromonas hydrophila* was isolated from the different organs of fresh water catfish (*Clarias gariepinus* and *Ictalurus punctatus*) obtained from Kporoko river in Lokoja. *Aeromonas hydrophila* was identified using both phenotypic and genotypic methods. The pathogenic traits of the *Aeromonas* species such as biofilm formation, production of haemolysin, enterotoxin and enzymes were determined. **Results:** *Aeromonas hydrophila* occurred in all the examined fish organs (fish, liver, kidney, skin and gut) of *Clarias gariepinus* but occurred only in the skin, intestine, kidney and gut of *Ictalurus punctatus* examined, but the incidence of *Aeromonas hydrophila* was prevalent in the gut of all the fishes analyzed. All the *Aeromonas* isolates analysed in this study produced biofilm, haemolysins and lipase enzymes. They also produced enterotoxins with values ranging between 0.069 - 1.138. **Conclusion**: The occurrence of *Aeromonas* in fresh catfish possessing these pathogenic traits is of great public health significance to man as it indicates the likelihood of man being predisposed to toxigenicity when the toxin concentration reaches a lethal value. It is therefore recommended that the internal organs of fresh catfish be thoroughly cleaned and cooked before consumption.

Keywords

Aeromonas, Biofilms, Fresh Water Catfish, Enterotoxins

1. Introduction

Aeromonas species are ubiquitous, Gram-negative, non-spore forming, motile, aerobic or facultatively anaerobic bacteria with bacillary or coccobacillary morphology. They have been found in brackish, fresh, estuarine, marine, chlorinated and unchlorinated water supplies worldwide, with highest numbers obtained in warmer seasons of the year [1]. *Aeromonas* occur in a wide variety of retail fresh foods of animal origin such as sea foods, raw milk, meats and poultry [2]. *Aeromonas* species have been isolated from blood, cerebrospinal fluid, exudates from otitis media, urine, peritoneal fluid, necrotic muscle, infected heart valves and bones [3]. Presently, as a putatively emerging enteric pathogen, *Aeromonas* species have the inherent capability to grow in water distribution systems, especially in biofilms, where they may be resistant to chlorination [4]. *Aeromonas* strains from a variety of retail foods such as fish and poultry products can act as possible vehicles for the dissemination of food borne *Aeromonas* gastroenteritis in humans [5]. The infectious nature of *Aeromonas* species was first demonstrated in United States of America by [6]. Since then, interest in the organism has increased, because of its effect on human health, poultry and aquatic animals [6]. Interest in the role of *Aeromonas* infection in humans, animals and food borne disease has increased in recent years [7]. Although the "food poisoning" potential of the organism has not been completely established, the association with human gastroenteritis strongly suggests that *A. hydrophila* produces a heat labile enterotoxin and a heat stable cytotoxic enterotoxin which is known to cause watery or bloody and mucoid diarrhea in infants as confirmed by [8]. *A. hydrophila* has been recognized as causative agents of human meningitis, endocarditis, osteomyelitis, wound infections and gastrointestinal or diarrheal disease [9].

The pathogenicity of *A. hydrophila* is a concern to public health authorities and probably accounts for significantly more cases of gastroenteritis than is apparent at present. Indeed, a large proportion of cases of gastroenteritis are still been classified with unknown or unidentified etiology [10]. It also causes diarrhea in susceptible host and in animals [11]. It is characterized by intestinal symptoms including bacteremia, fever, chills, abdominal pain, nausea, vomiting and diarrhea [12]; extra-intestinal symptoms which include human meningitis, endocarditis, osteomyelitis and wound infections [9]. Other symptoms in humans include cellulitis, septicemia, urinary tract infections, hepatobilliary infection, ear infections, and suppurative arthritis in patients with leukaemia [13].

Aeromonas infection of fish could originate from water that is contaminated with the infectious agents or feeding fish with poultry manure contaminated with *Aeromonas* species. Some studies in Nigeria have incriminated *Aeromonas* as a fish pathogen. Man, also stands the risk of infection when handling or consuming improperly cooked fish [14].

In Nigeria, there is increasing awareness of the organism with its economic and public health significance. [15] conducted studies on incidence and bio typ-

ing of *Aeromonas* species on snails and water samples in Port Harcourt, Rivers state and found out that these samples were likely reservoirs and therefore constitute high sources of human infection. Report on human cases of aeromoniasis in Nigeria is gaining ground. *A. hydrophila* has been isolated from children with diarrhea in Zaria, Port Harcourt and Lagos [16].

Virulence factors of *Aeromonas are* associated with the structural components of the bacterial cell and with exotoxins that are secreted during bacteria metabolism [17]. The nature of virulence in *Aeromonas* species is complex and apparently varies between strains. Some of the virulent factors produced by *Aeromonas* species include biofilm formation, lipase production, haemolysin, cytotoxicity, enterotoxin production and antimicrobial resistance. The aim of this study is to determine the virulence characteristics of *Aeromonas hydrophila* isolated from fresh water catfish.

2. Materials and Methods

2.1. Sample Collection

A total of twelve (20) fresh catfish belonging to two different species (*Clarias gariepinus* and *Ictalurus punctatus*) were collected from fish vendors by convenience sampling which was based on the availability of different species of catfish at Kporoko village river side in Ajaokuta local government, Kogi State. They were carried in sterile plastic buckets containing water from the river where they were caught and transported alive to the Microbiology Laboratory of Salem University for examination. This method is most appropriate as it reduces the rate of cross infection [18].

2.2. Isolation of *Aeromonas hydrophila* from Fresh Catfish

The fishes were identified to the species level based on published methods [19].

The fish samples were transported to the laboratory in sterile plastic buckets and were analyzed immediately. Processing of samples was done based on established methods [20]. Each fish was sacrificed by pitting before examination. The stomach contents were removed by searing an area of the stomach wall with heated knife, longitudinal and transverse incision was made to expose the GIT contents. The kidney, intestine, skin, liver and gut were removed using a sterile knife. Each of the organs was macerated using a sterile mortar and pestle. Serial dilution was carried out by weighing 1 g of each macerated organ into 9 ml of sterile water. 0.1 ml of (10^{-4} and 10^{-5}) dilution was each inoculated onto tryptic soya agar and incubated at 28˚C for 24 h.

The method of [21] and [22] was employed in isolation of *Aeromonas* sp. This involves enrichment, selective plating, and detection of colonies, preliminary identification and complete biochemical identification.

The colonies were picked from the tryptic soya agar with the aid of a sterile platinum loop and inoculated onto prepared MacConkey's agar and was incubated at 28˚C overnight. From MacConkey agar plates, circular colonies (about

2 - 3 mm in diameter) with characteristic pale, translucent, and colonies with white or cream colour was picked and sub-cultured on sterile nutrient agar slants. They were stored in the refrigerator for further biochemical tests.

2.3. Identification of *Aeromonas* Species through Biochemical Tests

Biochemical identification was based on standard techniques [23] [24]. All the media and reagents for biochemical tests were prepared according to the instruction of the manufacturers. The isolates on MacConkey agar was subjected to Gram staining, Catalase test, Oxidase test, Urease test, Indole test, Motility test, Citrate utilization, Carbohydrate fermentation test, Methyl red test, Starch hydrolysis, Voges-proskauer test, Gelatin hydrolysis and growth in 6.5% NaCl.

2.4. Molecular Characterisation of Test Isolates

The method of [25] was employed in the genetic identification of the isolates.

2.4.1. Bacterial DNA Extraction

Bacterial culture (10 mL) was centrifuged at 10,000 ×g for 5 min and re-suspended in 400 µl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Then the cells were lysed by the addition of 200 µl of 10 mg ml^{-1} lysozyme and incubated at 37˚C for 60 min. The preparation was incubated for 10 min with 40 µl of 10 mg ml^{-1} proteinase K at room temperature followed by the addition of SDS to a final concentration of 1% and incubated at room temperature until the preparation was clear. Eighty microliters of 0.5 M EDTA was added and mixed gently and the solution was de-proteinated by sequential phenol: chloroform: isoamyl alcohol (24:23:1 v/v) extraction. Isopropanol was added to precipitate DNA and after centrifugation, washed with 70% alcohol and dried using Maxi Vacuum dryer. TE buffer (pH 8.0) 500 µl was added until further use. The DNA concentration was estimated by visual comparison with the standard DNA size markers after electrophoresis on 0.8% agarose TAE (tris-acetate EDTA) gels stained with 0.5 mg ml^{-1} ethidium bromide (Sigma Chemicals Co, USA).

2.4.2. PCR Amplification

The amplification was performed using high fidelity PCR system (GeneAmp PCR System 2400). PCR products were visualized by ethidium bromide staining on 1.5% agarose gel electrophoresis. PCR reactions has been carried out by following the series of standardizing experimental protocol as annealing temperature, concentration of $MgCl_2$, template DNA, Taq DNA polymerase, dNTP's and primers. The PCR reaction components consists of 200 mm dNTP, 20 pmoles of primer, 2 units of Taq DNA polymerase enzyme, assay buffer with working concentration of 1.5 mM $MgCl_2$, 20 - 30 ng template DNA in an assay volume of 25 mL. The 16S rRNA gene of the PCR products were sequenced using these universal primers: forward primer 27F; 5'-AGA GTT TGA TCC TGG CTC AG-3' and reverse primer 1492R; 5'-GGT TAC CTT GTT ACG ACT T-3' (Chromous Biotech Ltd., Bengaluru, India) [26].

2.4.3. Analysis of 16S rDNA PCR Product

Thermal cycling was performed with GeneAmp PCR System 2400. Amplification reactions were performed in a 25 μL volume, containing: 20 mmol/L Tris-HCl (pH = 8.4), 50 mmol/L KCl, 2.0 mmol/L $MgCl_2$, 200 umol/L of dNTPs, 1 umol/L of each primer, 30 ng of genomic DNA and 1.5 U of Taq DNA polymerase. The temperature profile was as follows: initial denaturation at 95˚C x× 3 min, 35 cycles of denaturation at 94˚C × 1 min, annealing at 55˚C × 1 min, and extension at 72˚C × 2 min, and final extension at 72˚C × 3 min. The PCR products were analyzed by electrophoresis on 1.5% agarose gels and stained with ethidium bromide. 100 bp ladder was used for evaluating the size of amplicons. The DNA bands were visualized and documented using a Gel documentation system (Biorad gel documentation system 2000).

2.5. Determination of Virulence Characteristics of *Aeromonas hydrophila*

2.5.1. Determination of Enterotoxin Production by Test Isolates

Enterotoxin activity was determined by the suckling mouse test according to the method employed by [27] and [28]. The test organisms were cultured in tryptone soy broth supplemented with 0.6% (w/v) yeast extract and brain heart infusion broth and incubated for 24 h at 37˚C in a shaker incubator at 300 rpm. Cell-free supernatant was obtained by centrifuging the culture at 3000 rpm for 30 min followed by filtration of the supernatant using a 0.45 μm Millipore filter. Cell-free supernatant (100 μL) mixed with 0.001% Evans blue dye (Merck, Darmstadt, Germany) was inoculated into a 2-4-day old mice intragastrically using a gastric tube. After 3 h, the mice were sacrificed by inhalation. The bowels were removed and weighed. Test isolates were considered positive if the ratio of intestinal weight to the remaining body weight of the mice inoculated with the strain was greater than 0.080.

$$\text{Enterotoxin production} = \frac{\text{intestinal weight of inoculated mice (kg)}}{\text{remaining body weight without bowels (kg)}}$$

2.5.2. Determination of Hemolysin Production by Test Isolates

Hemolysis test was carried out as described by [29]. Blood agar was prepared by adding 5% to 10% of sterile sheep blood to sterile tryptic soya agar. The test isolate was inoculated on the blood agar and incubated at 28˚C for 24 - 48 h. The plates were then observed for hemolysis. Complete clearing of the blood red coloration in agar immediately surrounding growth indicated a positive result (β-hemolysis), while no change in the agar immediately surrounding growth indicated a negative result.

2.5.3. Lipase Production

Lipase detection was performed on Tween 20 agar [30]. Isolates of *Aeromonas hydrophila* was cultured on tween 20 agar, composed of peptone, 10 g/L; NaCl, 5 g/L; $CaCl_2 \cdot 2H_2O$, 0.1 g/L; agar-agar, 20 g/L; tween 20, 10 mL. the plate was incu-

bated for at 28˚C for 24 to 48 h. Clear zones of precipitation on the tween 20 agar plate confirmed lipase production.

2.5.4. Biofilm Production by Test Isolates

Biofilm or slime production by the test organism was determined using the method of [31] and [32]. The medium (1L) was prepared using a composition of brain heart infusion broth; 37 g/L, sucrose; 50 g/L, agar No 1 (Oxoid); 10 g/L and Congo red dye or stain (BDH Ltd.); 0 8 g/L and distilled water; 1 L. Congo red stain was prepared separately and alone as a concentrated aqueous solution and autoclaved at 121˚C for 15 min. The sterile congo red stain was then added to the prepared, sterile agar with a temperature of 55˚C. Plates of the medium were inoculated and incubated aerobically for 24 h at 37˚C. A positive result was indicated by black colonies with a dry crystalline consistency. Non-slime producers usually remained pink, though occasional darkening at the centre of the colonies was observed. An indeterminate result was indicated by a darkening of the colonies but with the absence of a dry crystalline colonial morphology.

3. Results

Fifteen (15) out of the fifty (50) organisms isolated from fresh water catfish were Gram negative, motile, non-spore formers, tested positive to oxidase, catalase, indole, methyl red, gelatin hydrolysis and urease tests and were considered to belong to the genus *Aeromonas*. The PCR product with band size of 1.2 kb molecular gene size was confirmed as *Aeromonas hydrophila*.

Aeromonas hydrophila was more prevalent in the gut of fishes as compared to the other organs of the fish with the highest percentage occurrence of 26.7% as shown in Table 1.

Most of the isolates produced haemolysin, amylase, lipase, biofilm, and enterotoxin as shown in Table 2.

Figure 1 shows the occurrence of *Aeromonas hydrophila* in the different parts and organs of *C. gariepinus* and *I. punctatus.* The gut of *C. gariepinus* had the highest microbial count of *Aeromonas hydrophila* while *A. hydrophila* occurred most in the gut and skin of *I. punctatus*.

4. Discussion

Previous reports on the investigation of aeromoniasis among fishes, poultry and

Table 1. Frequency of occurrence of *Aeromonas hydrophila* in various fish organs.

Fish Organ	Number of *Aeromonas hydrophila* Isolated	% Occurrence
Intestine	3	18.8
Gut	5	31.3
Liver	2	12.5
Skin	3	18.8
Kidney	3	18.8

Table 2. Virulence characteristics of *Aeromonas hydrophila* isolated from fresh catfish.

Isolates	Haemolysin production	Lipase production	Enterotoxin production	Biofilm production
CG1/I	+	+	+(0.107)	+
CG1/L	+	+	+(0.150)	+
IP2/G	+	+	−(0.069)	+
CG2/I	+	+	+(0.138)	+
CG2/G	+	+	−(0.070)	+
IP1/G	+	+	+(0.099)	+
CG2/L	+	+	+(0.125)	+
CG1/K	+	+	+(0.107)	+
CG1/G	+	+	+(0.110)	+
IP2/S	+	+	+(0.099)	+
IP1/K	+	+	−(0.070)	+
CG2/S	+	+	+(0.105)	+
IP1/I	+	+	+(0.135)	+
CG2/K	+	+	+(0.076)	+
IP1/S	+	+	+(0.136)	+
CG1/G	+	+	+(0.096)	+

Key: CG1/I, CG1/L, CG2/I, KeCG2/G, CG2/L, CG1/K, CG1/G, CG2/S, CG2/K, CG1/G= *Aeromonas hydrophila* isolated from different parts (I—Intestine, L—Liver, G—Gut, K—Kidney, S—Skin) of *Clarias gariepinus.* IP2/G, IP1/G, IP2/S, IP1/I, IP1/S = *Aeromonas hydrophila* isolated from different parts (I—Intestine, G—Gut, S—Skin) of *Ictalurus punctatus.*

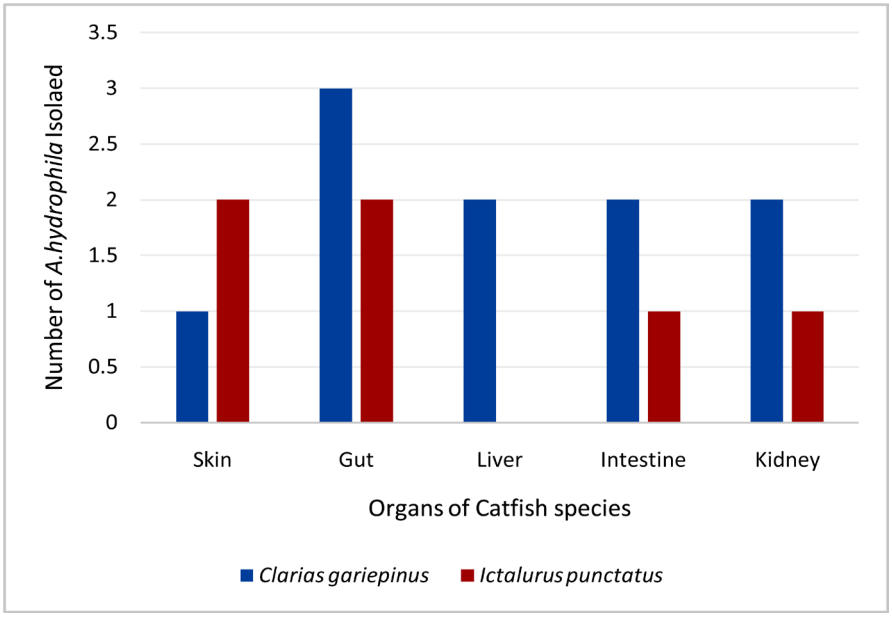

Figure 1. Occurrence of *Aeromonas hydrophila* isolated from *C. gariepinus* and *I. punctatus.*

human population were based on clinical signs and much effort has not been directed at isolating and characterizing *Aeromonas* sp. from fishes or man in Nigeria [2] [33].

Aeromonas hydrophila had the lowest and highest occurrence in the liver and gut of the pond-raised catfishes respectively. The predominance of *A. hydrophila* in the gut of fishes may be attributed to the presence of *A. hydrophila* in contaminated water in which the fish lives and feeds which then infects the gastrointestinal organs of the fish.

The high occurrence of *Aeromonas hydrophila* reported in this study warns on public food safety problem in Nigeria. In a similar study by [34] in Zaria, Nigeria, *Aeromonas* species were isolated from the GIT, gills and skins of various fishes, from both fresh, sewage, chlorinated and non-chlorinated water. The occurrence of *Aeromonas hydrophila* in the intestine, kidney, liver, skin and gut of fishes is in agreement with studies carried out by [35] [36] [37]. In most parts of Nigeria, people handle fish with bare hands and sometimes consume improperly cooked fish which may promote the horizontal transfer of the organism from the fish to man.

This study shows that majority of the *Aeromonas* isolated produced hemolysins and enterotoxins. These toxins are responsible for lethality, hemolysis and enterotoxigenicity. Their production by organisms found in food signals public health concern. The secretion of these extracellular proteins including enterotoxin, haemolysin and aerolysin are associated with bacterial virulence. Previous studies have shown that hemolytic toxins, haemolysin A and aerolysin A contribute to the virulence of *A. hydrophila* in fish and human host [38].

The result of this study implies that majority of the *Aeromonas* tested produced lipase. Lipase production may provide nutrients and may also constitute virulence factors by interacting with human leukocytes or by affecting several immune system functions through free fatty acids generated by lipolytic activity [39].

Biofilm production is a very important virulent characteristic exhibited by *Aeromonas* and the isolates used in this study all produced biofilms. Biofilms enhances adherence of the organism to specific host tissues and thereby produce invasive microbial colonies and diverse illness [40]. Biofilms enhance stability and protect *Aeromonas* against external factors such as antibiotics [41]. Biofilms provide cell nutrients in higher concentrations than the surrounding environment via the nutrient-rich solute retained in the interstitial region of the extracellular polymeric matrix [42] [43]. Biofilms act as reservoirs in which some aeromonads are able to persist for several years and emerge later in favorable conditions [44].

5. Conclusion

This study showed that *Aeromonas hydrophila* occurred in various organs of the fish samples examined and also exhibited several virulent characteristics. Fresh

catfish contaminated with *Aeromonas* may cause toxicity and other gastrointestinal infection to man which may be lethal especially if the toxins produced are in high concentrations. It is therefore recommended that fresh catfish be cooked thoroughly before consumption. Fish handlers should ensure their hands are properly washed after catching the fishes from the river as their hands could serve as a vector introducing the organism to their gastrointestinal tract especially when used in consuming ready to eat foods.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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