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Antibiotics Resistance and Plasmid Profile of *Aeromonas hydrophila* Isolates from Cultured Tilapia and Catfish

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Abstract: We investigated the antibiotics resistance and plasmid profile of *Aeromonas hydrophila* isolates from cultured tilapia and catfish. A total of 28 strains of *A. hydrophila were* isolated from thirty tilapia and catfish samples from a fish farm in Abakaliki, Ebonyi State and antibiotic resistance and plasmid profile of *A. hydrophila* isolates studied. Biochemical tests were carried out using standard methods while antibiotics resistance tests were by disk diffusion technique. Isolates were screened for the presence of plasmid DNA using the method of Doly and Birnboim and agarose gel electrophoresis run and viewed under ultraviolet light after staining with ethidium bromide. Results showed that the 28 isolates had the same morphological characteristics (Yellow colonies) on *Aeromonas* agar base selective media, microscopy showed that they were rod shaped and motile. All the 28 strains were resistant to ampicillin but susceptible to augmentin, gentamycin, pefloxacin, tarivid, streptolysin, septrin, chloranphenicol, sparfloxacin, ciprofloxacin and amoxicillin antibiotics. Out of the 28 isolates, only four strains harboured plasmids. Biochemical characterization showed that all the strains were positive to oxidase, catalase, urease and hydrogen sulphide; could utilize citrate, ferment glucose but couldn't utilize lysine, arginine and ornithine.

Key words: Plasmid DNA · Antibiotic Resistance · Tilapia · Catfish · *Aeromonas hydrophila*

INTRODUCTION

Aeromonas hydrophila is a ubiquitous and gram-negative rod-shaped bacterium which is pathogenic to terrestrial and aquatic animals including humans [1]. *Aeromonas*spp. is said to be one of the most important bacteria among the agents of bacteria fish diseases [2]. They are associated with Hemorrhagic septicaemia, tail and fin rot, ulcer and red-sore diseases [3]. *A. hydrophila* dwells in fresh water ponds and in gastrointestinal tracts of healthy fish. These bacteria can grow and produce toxins even under refrigerated conditions which is an indication that refrigeration cannot be effective enough to control the pathogen [4, 5].

Animals reared in aquaculture facilities are susceptible to numerous bacterial diseases [6]. A. hydrophila infections occur most often during environmental changes, stresses, changes in temperature, in contaminated environments and when an organism is already infected with a virus or another bacterium. It can also be ingested through food products contaminated with the bacterium, such as seafood, meats and even certain vegetables such as sprouts. It can also be transmitted by leeches [7]. A. hydrophila is not as pathogenic to humans as it is to fishes and amphibians. One of the diseases it can cause in humans is gastroenteritis which occurs mostly in young children and people who have compromised immune systems or growth problems. This bacterium is linked to two types of gastroenteritis. The first type is a disease similar to cholera, which causes rice-water diarrhea. The other type is dysenteric gastroenteritis, which causes loose stools filled with blood and mucus. Dysenteric gastroenteritis is the most severe out of the two types and can last for several weeks. A. hydrophila is also associated with cellulitis. It also causes diseases such as myonecrosis and

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eczema in people with compromised or suppressed (By medication) immune systems. In very rare cases, A. hydrophila can cause necrotizing fasciitis.An outbreak of A. hydrophila can cause serious diseases. Although, large scale outbreaks in humans have not yet been reported, outbreaks among vertebrates have occurred.

Although infections due to *Aeromonas* may be self limiting, treatment with antibiotics is generally necessary to limit the progression and persistence of the disease, particularly in vulnerable groups, such as the young, elderly and immunocompromised individuals [8, 9]. Due to careless use of antibiotics, various pathogenic microbes are gaining resistance to different antibiotics. The growing antibiotic resistance of pathogenic bacteria worldwide is compounding factor that limits the effective management of bacterial infections. Antibiotics resistance is a major problem when dealing with *A. hydrophila* infections. An increase in antibiotic resistance of the genus *Aeromonas* particularly has been reported [10, 11]. Waters receiving human and animal waste water discharges, which are expected to contain antimicrobial agents likely to exert a selective pressure and commensal resistant bacteria are capable of transferring their resistances to autochthonous bacteria. Consequently, the freshwater indigenous flora may become a reservoir for antimicrobial genes and the reuse of these waters for humans and animals may contribute to the limitation of antimicrobial's efficiency [12]. The molecular epidemiology of tetracycline- resistant *Aeromonads*, especially *Aeromonassalmonicida*has been well documented, indicating that tetracycline resistance is plamid-encoded [13]. George*et al*. [14] in the study of antimicrobial resistance of mesophilic*Aeromonas*species isolated from two European rivers found as many as 49% tetracycline-resistant *Aeromonas* species. Neither sulphamethoxazole nor trimethoprim alone is very active against *Aeromonas*species but cotrimoxazole is generally efficient due to the strong synergy between the drugs [15]. Most of the isolate in the study performed by Grave *et al*. [16] were resistant to first generation quinolones (Pipemidicacid and oxolinic acid) but clinically susceptible to fluoroquinolones (From pefloxacin (54 %) to ciprofloxacin (98 %)). Antibiotic resistance frequencies and profiles varied according to the source of the strains [17]. Indeed, for several decades, tetracycline has been widely used in clinical medicine, veterinary and agriculture contributing to higher levels of microbial resistance especially among the genus *Aeromonas* [18].

This study was aimed at determining the incidence of drug resistance among strains of *A. hydrophila* isolated from cultured Tilapia and Catfish. The successful transfer of a resistance plasmid was also reported which shows the potential for the spread of drug resistance in fish culture systems.

MATERIALS AND METHODS

Sample (Fish) Collection and Processing: A total of thirty (30) catfish samples (15 males and 15 females) were purchased from the Chi-boy farms in Abakalik, Ebonyi state and were taken in a sterile container to the Microbiology laboratory of Ebonyi state University, Abakaliki. Each of the fish samples were dissected using a surgical blade and samplecollected from the intestinal tracts. About 2g each were weighed, ground in sterile mortar and distributed into 5 test tubes. Thereafter, 10ml of alkaline peptone water was added into each of the test tubes and incubated at 37?8C for 24h to get the stock culture.

Serial Dilution and Isolation of the *Aeromonas hydrophila*: Exactly10^3 Serial dilution was used during the plating on the *Aeronomas* agar base media (From Zigma Andrich USA). Distilled water (0.9ml) was transferred into 3 sterile test tubes and 0.1ml of stock culture added to the first test tube. After this, 0.1ml was transferred from the first test tube to the second test tube and down to the third test tube. Finally, 0.1ml aliquot was aseptically drawn from the third test tube ($10^{\wedge 3}$) of each sample and innoculated on clean sterile petri dishes containing *Aeromonas* agar base media using spread plate method. The plates were incubated at 37°c for 24hours and culture observed for yellow colonies.

Purification of Mixed Culture: The yellow colonies were sub-cultured on nutrient agar using streak plate method and later sub-cultured back on *Aeromonas* agar base selective media. Blood agar haemolysis test was also carried out on the pure isolate to assay for the presences of haemolysin gene.

Biochemical Tests: The assumed *Aeromonas* species were defined to species by carrying out a Gram stain and also biochemical tests for oxidation; motility, catalase enzyme, urease enzyme, utilization of citrate as well as hydrogen sulphide; lysine, arginine, ornithine and fermentation of glucose following standard methods Hayashi *et al*. [19].

Antibiotic Susceptibility Testing by Disk Diffusion: This was carried out following the modified method of The *A. hydrophila* test microorganism were furtheranalysed for their sensitivity to some conventional antibiotics: Augmentin (25µg), Gentamycin (10 µg), Pefloxacin (10 µg), tarivid (30 µg), Streptolysin (30 µg), Septrin (30 µg), ampicillin (10 µg), chloramphenicol (30 µg) Sparfloxacin (10 µg), Ciprofloxacin (10 µg) and Amoxycillin (30 µg) using modified disk diffusion method of Bauer *et al*. [8]. The *A. hydrophila* strains were inoculated in 10 ml of sterile nutrient broth and incubated at 37°C for 16-18 hours. The Aeromonas Agar Base (AAB) plates were inoculated by the use of sterile cotton swab. The discs were placed on the nutrient agar plates inoculated with the *A. hydrophila* strains in aseptic conditions and adequately separated from one another to avoid overlapping and characterization of strains as susceptible or resistant was based on the size of the inhibition zones around each disc according to the manufacturer's recommendations.

Plasmid Isolation: The twenty eight strains of *A. hydrophila* were screened for plasmid DNA by the modified procedure of Kaper *et al*. [20] and were analysed using electrophoresis for 2 hours at 35 mA on a 2 % agarose gel in TBE buffer. The gels were stained with ethidium bromide and viewed under u.v. light. The plasmids molecular masses were evaluated by comparing them with standards.

RESULTS

Isolates, Microscopic Analysis and Blood Agar Heamolysis

Microorganism: Twenty-eight strains of pathogenic *A. hydrophila* both from male and female African catfish and tilapia were isolated in this study. The pathogenic strains were AH01, AH02, AH03, AH04, AH05, AH06, AH07, AH08, AH09, AH10, AH11, AH12, AH13, AH14, AH15, AH16, AH17, AH18, AH19, AH20 AH21 AH22, AH23 AH24, AH25, AH26, AH27 and AH28.

The 28 pure isolates showed the morphological characteristics (Yellow colonies) of *A. hydrophila* on *aeromonas* agar selective media containing ampicillin. Microscopic analysis showed that they were rod shaped and motile. All the isolates from the fish samples were haemolysin negative.

Plasmid DNA Analysis of *A. hydrophila* from Male Catfish and Tilapia in Comparism to Female Catfish and Tilapia: Plasmid DNA were detected in *Aeromonas hydrophila* strains isolated from the fish samples. Lane m is the ladder (The standard), lane –ve is the control, lane is band for the plasmid DNA isolated from male catfish sample which is 1500 base pairs (bp) and lane t is band for the plasmid DNA isolated from male tilapia fish sample which is 1500 bp (Plate 1).

Table 1: Biochemical Characteristics of the Pure Isolates

Tests	Strains form male tilapia n=15		Strains form female tilapia. n=15	
	Atypical reaction		Atypical reaction	
Gram staining	-	15	-	15
Citrate utilization	+	15	+	15
Motility	+	15	+	15
Hydrogen Sulphide	-	15	-	15
Catalase	+	15	+	15
Oxidase	+	15	+	15
Urase	-	15	-	15
Glucose fermentation	I	15	I	15
Ornithine	-	15	-	15
Lysine	-	15	-	15
Arginine	-	15	-	15
lactose	+	15	+	15
Sucrose	+	15	+	15
mannitol	+	15	+	15
Maltose	+	15	+	15
Inositol	-	15	-	15
Voges-proskauer	+	15	+	15

Table 2: Antimicrobial Patterns of the 28 strains of*A. Hydrophila* Isolates

A. hydrophila strains	Antibiotics	Antimicrobial resistance	Antimicrobial susceptibility
AH28,AH27,AH15	Au(25µg)	ApSp	AmGe
AH20,AH19	Ge(10µg)	ApCh	AmGePe
AH3,AH5,AH10	Pe(10µg)	ApChSp	AmPeSt
AH1,AH12,AH16	Ta(30µg)	ApTaSp	AmStCi
AH14,AH11	St(30µg)	ApSp	AmCi
AH8,AH9,AH13,AH17	Se(30µg)	ApChSp	AmStCi
AH4,AH6	Ch(30µg)	ApAuTa	AmGeSt
AH2	Sp(10µg)	ApSp	AmStCi
AH22,AH24,AH26	Ci(10µg)	ApCh	AmCiPe
AH25,AH23,AH21,AH1	Am(30µg)	ApSpTaAu	AmStGePe

Test for ampicillin(Ap), augmentin(Au), gentamycin(Ge), pefloxacin(Pe), tarivid(Ta), streptolysin(St), septrin(Se), chloranphenicol(Ch), sparfloxacin(Sp), ciprofloxacin(Ci) and amoxycillin(Am).

Less than (<) 12 mm means resistance to the antibiotics and greater than(>)12 mm means susceptibility to the antiobiotics

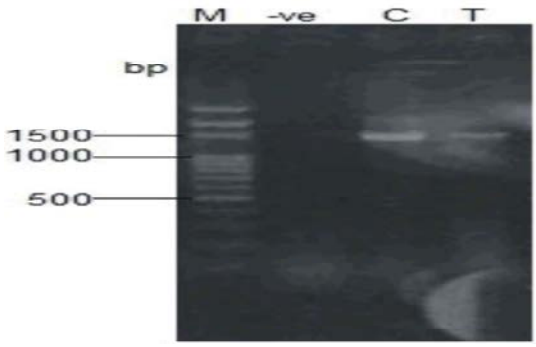

Plate 1: Gel picture ofplasmid DNA from *A. hydrophila* strains isolates from male catfish and tilapia samples

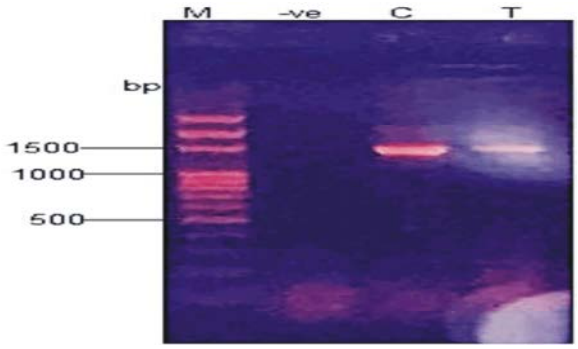

Plate 2: Gel picture ofplasmid DNA from *A. hydrophila* strains isolates from female catfish and tilapia fish samples

Plasmid DNA was also detected in *A. hydrophila* strains isolated from female catfish and tilapia samples. Lane m is the ladder (the standard), lane –ve is the control, lane c is band for the plasmid DNA isolated from the *A. hydrophila* strains which is 1500 base pairs (bp) and lane t is band for the plasmid DNA from the bacteria strains which is 1500 bp (Plate 2).

Plasmid Analysis A: Plasmid analysis showed that two strains of *A. hydrophila* from both male catfish and tilapia samples each had 1500bp plasmid (Plate 1).

Plasmid Analysis B: Plasmid analysis showed that two strains *A. hydrophila* from both female catfish and tilapia samples each had 1500bp plasmids (Plate 2).

DISCUSSION

In this study, the results of the biochemical tests indicated that the isolates were gram negative rods, motile and oxidase, catalase, hydrogen sulphide and urease positive, can utilize citrateand negative to lysine, arginine, ornithine and can ferment glucose, this observation is as a result of the unique morphological and biochemical characteristics of *A. hydrophila* as However, identification and characterisation of *A. hydrophila* could also be done by molecular method Meyers *et al*. [24] and Nawaz *et al*. [25].

The results of antimicrobial resistance showed that all the twenty eight strains of *A. hydrophila* isolates from farm raised catfish and tilapia were resistant to ampicillin while strain AH28, AH27andAH15 showed resistance to both ampicillin and sparfloxacin. Strain AH20 and AH19 were resistance to ampicillin and chloramphenicol. This observation may be as result of difference in antimicrobial profile of *A. hydrophila* as reported by Popoff [26] Who reported that characterization of *A. hydrophila* isolated from tilapia showed that 48 % of the isolates were resistant to tetracycline, 57 % to streptomycin and 43 % isolates were resistant to tetracycline antibiotics. More so, strain AH3, AH5 and AH10 showed resistance to ampicillin, sparfloxacin and chloramphenicol meanwhile, strain AH1, AH12 and AH16 showed resistance to ampicillin, chloramphenicol and tarivid. StrainAH14, AH11 showed resistance to ampicillin and sparfloxacin. This observation contradicted the report of Boonyarat-palin 1989 who reported that *A. hydrophila* isolates were majorly ampicillin-resistant. However, Ogata and Egusa [27] stated that many aeromonads from aquaculture are resistant to antibiotics used in the ecosystem over time which is in line with our observations. Strain AH8, AH9, AH13 and AH17 were resistance to ampicillin, chloramphenicol and sparfloxacinwhile strainAH4 and AH6 showed resistance to ampicillin, augmentin and tarivid. Also, Strain AH2 showed resistance to ampicillin and sparfloxacin. Furthermore, strain AH22, AH24, AH26 and AH25, AH23, AH21, AH18 showed resistance to ampicillin, chloramphenicol and ampicillin, sparfloxacin, tarivid and augmentin respectively. These observations may be as a result of the continuous use of these antibiotics in fish farms. However, results gotten from this study also showed that all the twenty eight strains of *A. hydrophila* isolates from farm raised catfish and tilapia were susceptible to amoxicillin antibiotics with AH28, AH27, AH15 strains showing susceptibility to amoxycillin and gentamycin, AH20 and AH19 strains were susceptible to amoxicillin, gentamycin and pefloxacin. This observation may be as a result of the non-frequent use these antibiotics in the farm. [7] reported that streptomycin and erythromycin antibiotic were not allowed to be used by the aquaculture industry in U.S.and the isolates of *A. hydrophila* in his study were susceptible to theses antibiotics. StrainAH3, AH5 and AH10 were susceptible to amoxicillin, pefloxacin and streptolysin, strain AH1, AH12 and AH16were susceptible to amoxicillin, streptolysin and ciprofloxacin. Strain AH14 and AH11were susceptible to amoxicillin and ciprofloxacin.StrainAH8, AH9, AH13 and AH17 were susceptible to amoxicillin, streptolysin and ciprofloxacin, strain AH4 and AH6were susceptible to amoxicillin, gentamycin and ciprofloxacin.StrainAH2 showed susceptibility to amoxicillin, streptolysin and ciprofloxacin. This observation may be as a result of the non-frequent use these antibiotics in the farm as reported by Oni [28]. Strain AH22, AH24 and AH26 showed susceptibility to amoxicillin, ciprofloxacin and pefloxacin while strain AH25, AH23, AH21 and AH18 were susceptible to amoxicillin, streptolysin, gentamycin and pefloxacinantibiotics. This observation may be as result of difference in antimicrobial profile of *A. hydrophila.* [8] reported that characterization of *A. hydrophila* isolated from tilapia showed that 48 % of the isolates were resistant to tetracycline, 57 % to streptomycin and 43 % isolates were resistant to tetracycline antibiotics and Similarly, Boonyarat-palin 1989 reported that strains of *A. hydrophila* isolates were susceptible to most of these antibiotics.In comparison, earlier analysis suggested that *A. hydrophila* isolates from fish were susceptible to chloramphenicol as well as erythromycin [29]. This kind of disparities in the frequency of resistance will be associated with the frequency and variety of antimicrobial antibiotics used in dealing with *Aeromonas* infections in various geographical areas [12].

Plasmids facilitate resistance transfer in aquaculture, earlier reports suggested that plasmids encoding antibiotic resistance of *A. hydrophila* differ in size from 85•6 to 150 kb [29]. Additionally, the paucity of accounts were physical proof of the transmissible plasmids possessed by fish bacteria, is actually worthy of note that untreated effluents from hatcheries harbour huge amounts of bacteria that are pathogenic to fish and other homoeothermic animals [5, 6, 11]. In this study, four strains out of the twenty eight strains of *A. Hydrophila* isolates harboured plasmids of 1.5 kb (Table1), this observation is similar to the report of De Paola *et al*. [12], Hayashi [18] and Hayashi *et al.* [19] who reported that different fish bacteria harbour plasmids of different sizes that facilitate resistance transfer in aquaculture system.

CONCLUSION

Antibiotics resistance and plasmid profile of pathogenic *A. hydrophila* bacteria from cultured tilapia and catfish gives important epidemiological data hence fish farmers' should be enlightened on the human health and environment implications of these data.

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