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Isolation and Identification of *Aeromonashydrophila*, Pathogen of Farmed Tilapia (*Oreochromisniloticus*) in Region Agneby-Tiassa in côte d'Ivoire

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Abstract: ***The pathogen responsible of mortality of farmed tilapia fish in region Agneby-Tiassa in Côte d'Ivoire was investigated. A total of 76 dead fish or showing clinical signs of disease were collected from a private farm in said region. The analyses of samples consisted to isolation of Aeromonas hydrophila, biochemical characterization and molecular detection of virulence gene by PCR. Eleven biotypic profils of A. hydrophila were differentiated in the use or not of lysine decarboxylase, mannitol, rhamnose, gelatin and by fermentation or not of lactose. Biotypes B1 to B4 recorded in the skin, were characterized by positive lysine decarboxylase, positive gelatin, positive mannitol and negative rhamnose. Biotype B10 was showed a negative lysine decarboxylase in the liver of tilapia. Biotypes B5 to B7 of the kidney were differentiated in positive mannitol and positive lysine decarboxylase. The PCR reaction revealed the presence of aerA and hlyA virulence genes in isolated A. hydrophila strains. The biochemical and molecular characterizations allowed to know and to highlight the high virulent of the isolated germ. Hence the interest of the treatment of tilapia production basins against this pathogen.***

Keywords: *Oreochromisniloticus*, *Aeromonas hydrophila*, virulence, Côte d'Ivoire

1. Introduction

Bacterial diseases in cultured fish were considered as the main problem of aquaculture system in Côte d'Ivoire. Fish farms have been facing great problems due to bacterial fish diseases that cause severe damage and mortality in Côte d'Ivoire [1]. *Aeromonas* species are opportunistic pathogens in fish and humans [2]. These bacteria were long considered as opportunistic pathogens for human but they are currently recognized as emerging pathogens [3]. Indeed, *Aeromonas* species are responsible for wide range of human diseases that vary in severity from a self-limiting gastroenteritis to potentially fatal septicemia and traveler's diarrhea, in addition to extra intestinal symptoms such as meningitis, endocarditis and osteomyelitis with a high mortality rate in immune compromised person [4], [5]. *Aeromonashydrophila* is responsible for the « *Aeromonas*septicemia » disease and was involved in massive mortality of farmed fish and in the freshwater [6]. The bacterium is widely distributed in aquaculture and it has been shown to inflict diseases in warm water fish including tilapia [7]. Motile*Aeromonas* septicemia (MAS) is a more dramatic bacterial disease affecting various species of fish and shellfish, feral as well as farmed in both fresh and seawater and cause a serious problem for the fish farming industry in Côte d'Ivoire as well as in other countries [8], [9].*A. hydrophila* has ability to grow at refrigerated temperature so it considered as food borne pathogen of emerging importance [10]. There are many extracellular proteins produced by *A. hydrophila* which were associated with pathogenicity and environmental adaptability. The main virulence factors that have effect on pathogenicity of *Aeromonas* species are enterotoxins, aerolysin and hemolysin in addition to other factors such as adhesin and mucinase production[11]. Aerolysin and hemolysin genes are responsible for haemolytic toxins (aerolysin and hemolysin) production of *A. hydrophila*[12]. Aerolysin gene is recorded to be the putative virulence gene produced by some strains of *A. hydrophila,* which produced an extracellular, soluble, hydrophilic protein exhibiting both hemolytic and cytolytic properties [13]. Aerolysin is a cytolytic pore-forming toxin (PFT) which creates unregulated pores in the membrane of targeted cells. The mature toxin binds to eukaryotic cells and aggregates to form holes (Approximately 3 nm in diameter) leading to the destruction of the membrane permeability barrier and osmotic lysis [14]. Our study aimed to isolate and identify *A. hydrophila* of diseased farmed tilapiain region Agneby-Tiassa, in addition to target its most virulence genes. In this region, fish mortality in aquaculture systems is a common and timely phenomenon. The importance of virulence genes study is to detect the potential pathogenicity of the organism for prevention of infection.

2. Materials and Methods

Field investigation and collection of fish

The study was conducted in a private farm in region Agneby-Tiassa in Côte d'Ivoire.A total of 76 tilapia (39,9±0,2 g ; 13,7±4,1 cm) showing clinical signs of disease were collected. Fish sample were placed in sterile bag and put in the glass. The farm contained twenty eight stocking ponds each with an average area and depth of 1500 m^2 and

70 cm respectively with a good water exchange system. Tilapia was stocked at a high density of about 2000 fishes per 1000 square meter. The infected fish were brought to the Fish Disease Laboratory of the Department of Aquaculture, Nangui Abrogoua University for bacterial analysis. Water samples were also taken from the ponds for the determination of water quality parameters (temperature, pH, dissolved oxygen).

Bacteriological analysis

Nile tilapia was carefully examined by direct observation before and after dissection for possible clinical signs or symptoms of disease.

About 5 g skin and gill (each) and1g of liver or kidney were weighed, aseptically removed and homogenized using a pestle and motar for 2 min. The homogenate was enriched in 45 mL and 9 mL soy trypticase broth (BioRad, France), respectively, and incubated at 37 °C for 24 h according to Sakar *et al.*[15].

After incubation, an aliquot (0,1 mL) of the enrichment was inoculated in *Aeromonas* agar (Sigma Aldrich, India) containing ampicillin (30 mg/L) and incubated at 37 °C for 24 h. Five presumptive *A. hydrophila* colonies per box which were dark green opaque with dark center were selected for further identification. Biochemical tests used for identification included Gram's staining, motility, oxydase, lactose, oxydation/fermentation, esculin hydrolysis and O/129 sensitivity. The capacity of *A. hydrophila* to grow at different concentrations of NaCl (0%, 2% and 6%) was also performed in brain heart infusion broth. All strains were confirmed using Galery API 20 E (BioMérieux, France).

Molecular detection of virulence genes of *A. hydrophila*

Genomic DNA from *A. hydrophila* strains was extracted using the method according to Gevers *et al.*[16] with slight modification. The detection of virulence genes was performed using a pair of primers H1 and H2 to amplify 597 bp *hlyA* geneand A1 and A2 to amplify 416 bp *aerA* gene based on Aslani and Hamzeh method [17]. The sequences of all primers used in this study were manufactured by Integrated DNA Technologies (Table 1).

Table 1: Sequences of primers using in this study

Genes	Primers	Sequences (3'-5')	Size (pb)
hlyA	H1	GGCCGGTGGCCCGAAGATGCAGG	597
	H2	GGCGGCGCCGGACGAGACGGG	
aerA	A1	GCCTGAGCGAGAAGGT	416
	A2	CAGTCCCACCCACTTC	

3. Results

Water quality of ponds and clinical signs of diseased fish

The temperature, pH and dissolved oxygen (DO) content of water collected from ponds were 24-28 °C, 6.9-7.9, and 3-6 mg/L, respectively. The disease signs of the Nile tilapiaobserved were hemorrhagic ulceration at the base of the fins and skin,tail rot, loss of scales. After dissection of the freshly dead fish, kidney, and liver were hemorrhagic, swollen and the presence of congested blood vessels were also noted (Figure 1).

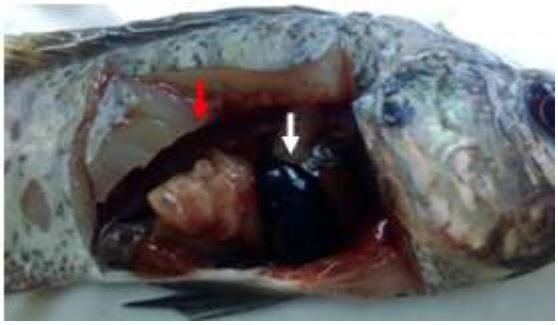

Figure 1: Clinical signs of diseased farmed tilapia (*O. niloticus*). Swollen and blackish liver (white arrow), and Kidney (white arrow)

Biotypes of strains of *A. hydrophila* isolated

Morphologically the isolated colonies showed yellowish opaque, round, convex, smooth edged, and semi-translucent colonies on TSA plates. They were gram-negative, rod-shaped bacteria. The isolated colonies were recorded in kidney, liver and skin of every infected tilapia sample.In the analysis of biochemical characteristics, eleven biochemical profils (B1 to B11) were obtained for *A. hydrophila* (Table 2). Biotypic profils of *A. hydrophila*were differentiatedin the use or not of lysine decarboxylase (LDC), mannitol (MAN), rhamnose (RHA), gelatin (GEL) and by fermentation or not of lactose.*A. hydrophila* strains were also showed good growth on the media containing 0% NaCl but no growth had found with 6% NaCl.Nevertheless, all strains were Gram-negative, motile, oxidase-positive, fermentative, and resistant to O/129 sensitivity test and they hydrolyzed esculin. Biotypes B1 to B4 recorded in the skin, were characterized by positive lysine decarboxylase, positive gelatin, positive mannitol and negative rhamnose. Biotype B10 was showed a negative lysine decarboxylase in the liver of tilapia. Biotypes B5 to B7 of the kidney were differentiatedin positive mannitol and positive lysine decarboxylase.

Table 2: Identified Biotypes of*A. hydrophila*isolated from tilapia

Biotypes characteristics	Identified biotypes of *A. hydrophila*										
	Skin				Kidney			Liver			
	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	B11
Gram stain	BG^-	BG^-	BG^-	BG^-	BG^-	BG^-	BG^-	BG^-	BG^-	BG^-	BG^-
Motility	+	+	+	+	+	+	+	+	+	+	+
Oxydase	+	+	+	+	+	+	+	+	+	+	+
Lactose	-	+	+	-	-	+	+	+	-	+	+
O/129 sensitivity	R	R	R	R	R	R	R	R	R	R	R
O/F	F	F	F	F	F	F	F	F	F	F	F
Esculin hydrolysis	+	+	+	+	+	+	+	+	+	+	+
NaCl 0%	+	+	+	+	+	+	+	+	+	+	+
NaCl 2%	-	+	+	-	+	-	+	+	+	+	-
NaCl 6%	-	-	-	-	-	-	-	-	-	-	-
LDC	+	+	+	+	+	+	+	+	+	-	+
CIT	-	+	+	-	-	-	+	-	-	+	-
VP	+	-	-	+	+	+	-	+	+	-	+
GEL	+	+	+	+	-	+	+	-	+	+	+
MAN	+	+	+	+	+	+	+	+	-	+	+
INO	-	-	-	-	-	-	-	-	-	-	-
SOR	-	-	-	-	-	-	-	-	-	-	-
RHA	-	-	-	-	-	+	-	+	-	-	-
ARA	+	-	-	+	+	+	-	+	+	-	+

B : biotype ; B1-B4 :skin biotypes ; B5-B7 : kidney biotypes ; B8-B11 : liver biotypes ; (+) : positive reaction ; (-) : negative reaction ; R : resistance ; F : fermentation ; O/F : oxydation/fermentation ; CIT : Citrate utilization ; LDC : lysin decarboxylase; VP : acetoin production ; GEL : Gelatine ; MAN : mannitol ; INO : inositol ; SOR : sorbitol ; RHA : rhamnose ; ARA : arabinose

Virulence genesdetected in strains of A. hydrophila

A total of two *A. hydrophila* strains (B2 and B10) out of the tested 11 strains carried *aerA* gene (Figure 2). The DNA bands of two others *A. hydrophila* strains (B3 and B7)of PCR amplified indicated that these strains harbor the *hlyA* gene (Figure 3). None of these virulence genes were observed in the seven others strains of *A. hydrophila*.

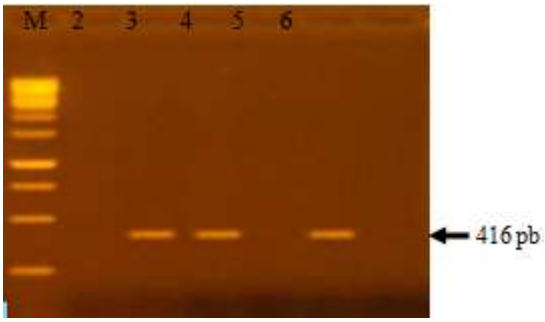

Figure 2: Electrophorese of *aerA* gene of *Aeromonas hydrophila*

M : DNA size marker (1kb +) ; Lane 3 : Positive control for *A. hydrophila*; Lane 4, 6 : Positive strains (B2 and B10) of *A. hydrophila*

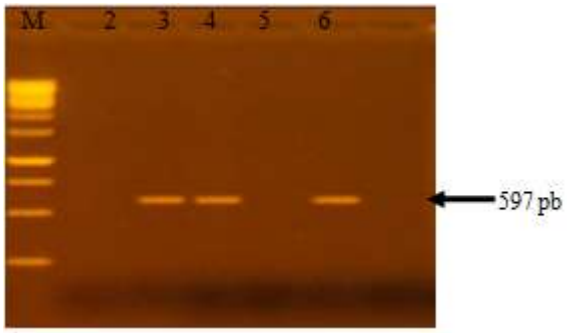

Figure 3: Electrophorese of *hlyA* gene of *Aeromonas hydrophila*

M : DNA size marker (1kb +) ; Lane 3 : Positive control for *A. hydrophila*; Lane 4, 6 : Positive strains (B3 and B7) of *A. hydrophila*

4. Discussion

Clinical signs associated with the Aeromonad infection observed in the present study were hemorrhagic ulceration at the base of the fins and skin,tail rot, loss of scales. Internally, the kidney and liver were hemorrhagic and swollen. Consistent with the current findings, hemorrhagic septicemia was also reported in fish *Chanoschanos* due to *A. hydrophila* infection especially when fish are under stress [18,19]. [20] and [21]have stated that the opportunistic pathogen (such as *A. hydrophila*) causes infection only when the host resistance has been lowered by environmental stress factors, such as high organic load, overcrowding, and sub-lethal oxygen levels. Therefore, an extremely higher stocking density of the fish (about 2000 fishes per 1000 square meter) observed in the current investigation may be a major risk factor of the disease sensibility. In addition, the low water depth (70 cm in average) in the ponds with sudden increase of temperature (from 24°C to 28°C) could be other contributing factors for the infection. Palumbo *et al*[22] observed a broad temperature band (20 °C to 35 °C) in which all strains of *A. hydrophila* grew optimally.

The morphological and biochemical investigations revealed the presence of *A. hydrophila* isolated from tilapia samples. Strains of*A. hydrophila* was Gram-negative rod, motile, oxidative, fermentative, O/129 resistance and comparable to that recorded by Woo and Bruno [23], Sabur[24], Cantas *et al*.[25]. All strains of *A. hydrophila*were positive esculin hydrolysis. The results were agreement with those of Erdem *et al.*[26] who also found a positive result from esculin hydrolysis test for *A. hydrophila* strains. *A. hydrophila* strains were also showed good growth on the media containing 0% NaCl but no growth had found with 6% NaCl.Popoff [27] indicated that motile *Aeromonas* do not grow in nutrient broth containing 5% NaCl. Ten strains *A. hydrophila* obtained in this study were identical to those reported by Obi *et al.*[28] who identified strains with lysine decarboxylase (LDC) positive. Several strains *A. hydrophila* with negative LDC were also reported [29, 30].

A total of two *A. hydrophila* strains out of the tested 11 strains carried *aerA* gene indicating the virulence of the bacterium Wong *et al.*[31] and Zhu*et al.*[32] reported that strains of *A. hydrophila* involving aerolysin genes were very virulent. The primary toxin haemolysins produced by some *Aeromonas* species is termed 'aerolysin', a heat-labile-haemolysin and it possesses both hemolytic and enterotoxic activity which expressed by many strains of *A. hydrophila* [33]. Ullmann *et al.*[34] used PCR method for diagnostic purposes of cytotoxin-encoding genes of aerolysin (*aer*A) and haemolysin (*hly*A).Buckley [35] explained the mechanism of action of aerolysin gene, he revealed that *A. hydrophila* exported autolysins as a protein which was activated by protolysis the activated autolysin blinded to the eukaryotic cell receptor glycophorin and oligomerizes, forming holes in the erythrocytes membrane so called hole forming toxin aerolysin.In the current study, the *hlyA* gene was found in two others strains of *A. hydrophila* carried this gene. Sarkar *et al.*[36] revealed that the presence of hemolysin gene in strains of *A. hydrophila* showed that it is the virulent strains and as a result infection in human by contacts during handling of fish. Secondary contamination also may be due to catching, handling and etc. and may also contribute for its distribution.

5. Conclusions

To best of our knowledge, we showed the initial evidence of the presence of *A. hydrophila* in farmed tilapia fish in region Agneby-Tiassa in Côte d'Ivoire.*A. hydrophila* could be one of the major pathogens of tilapia farming in Côte d'Ivoire. Our current findings contribute to the fight this pathogen in tilapia production basins. However, further studies are needed to demonstrate serological characterization of the organism.

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