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Molecular detection of hemolysin and aerolysin gene of *Aeromonas* spp. isolated from African tigrine frog (*Hoplobatrachus occipitalis*)

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ABSTRACT

Objective: To determine the hemolysin (*hlyA*) and aerolysin (*aerA*) genes of *Aeromonas* sp. isolate collected from frog *Hoplobatrachus occipitalis* in Côte d'Ivoire.
Methods: A total of 210 fresh healthy frogs and 384 smoked frogs were collected in three local markets. Biochemical identification, hemolysin test and detection of virulence gene by PCR were performed.
Results: The results revealed that 148 strains of *Aeromonas* sp. were isolated only from 48 (22.9%) fresh frogs. Strains identified were *Aeromonas hydrophila* and *Aeromonas sobria*. While all 384 smoked frogs were not contaminated by *Aeromonas* sp. Of the 148 isolates analyzed, 105 (70.1%) had the β-hemolytic activity on the blood agar. PCR reaction showed that virulence genes were widely distributed among the *Aeromonas* sp. isolates. The *hlyA* gene (84.4%) was the most frequent virulence factor present in the isolates. Three genotypes were recorded including $hlyA^+aerA^+$ (58.8%), $hlyA^+aerA^-$ (25.0%) and $hlyA^-aerA^+$ (15.5%).
Conclusions: The presence of the two genes of virulence indicates that there is a risk for consumers and this requires the measure of assurance for the sanitary safety of products.

1. Introduction

The edible frog *Hoplobatrachus occipitalis* (*H. occipitalis*) is one of the important species of Anura family that is considered a delicacy as local people catch them for food because of their taste and fleshy legs[1]. It serves as an important source of animal protein and is highly appreciated by the consumers[2]. But, because its insalubrious living areas, various diseases occur frequently in frog. So far, some pathogenic microorganisms such as *Aeromonas* sp., *Bacillus cereus*, *Salmonella* and *Trypanosoma* isolated from frog could contaminate the consumers and represent a public health risk[3-5]. Among pathogen bacteria that can be incriminated, *Aeromonas* sp. plays an important role in susceptibility of disease in frog. The genus *Aeromonas* sp. is a ubiquitous Gram-negative, non-spore-forming bacilli or coccobacilli that is generally motile, halophilic, usually oxidase- and catalase-positive and belongs to the family Aeromonadaceae. It is widely distributed in the soil, foodstuffs and aquatic environment[6]. Since times immemorial, they are important zoonotic pathogens of poikilotherms, but they are now emerging as important human pathogens. The three main pathogenic species of the genus are *Aeromonas hydrophila* (*A. hydrophila*), *Aeromonas caviae*, and *Aeromonas sobria* (*A. sobria*)[7]. *Aeromonas* sp. is one of the pathogenic agents of red leg disease of frog and it poses a serious threat to aquaculture industry as well as to human health[8,9]. Aeromonads have implicated in food and water borne disease outbreaks in different parts of the world. They have been associated with human gastroenteritis and various extraintestinal diseases, such as skin and soft tissue infections, traumatic wound infections and lower respiratory tract/urinary tract infections[6]. The mechanism of pathogenesis of *Aeromonas* spp. is complex, unclear and virulence is considered to be multifactorial[10]. The main virulence factors of *Aeromonas* spp. pertaining to the pathogenicity of the disease are the expression of extra cellular toxins and enzymes. *A. hydrophila* produces several extracellular virulence products such as proteases, hemolysin (*hlyA*),

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aerolysin (*aerA*) and cytolytic enterotoxins that are related to its pathogenicity[11,12]. Moreover, the presence of fimbria, flagella and capsule helps in the attachment of the bacteria to the host surface[6].

The edible frog *H. occipitalis* is one of the important species of frog mostly consumed by 55.2% of people in Côte d'Ivoire[13]. At the moment, there is no raniculture in Côte d'Ivoire. The production of commercialization is informal and frogs are harvested in ponds, rivers and swamps by collectors who delivered them to wholesale and retail market. Various activities are undertaken in Côte d'Ivoire to promote the breeding of frog. Frog meat, principally *H. occipitalis,* is increasingly consumed in many households to cover the deficit in animal protein. However, there are no data taking account the health risk associated with consumption of frog meat. This fact has motivated this work whose goal was to determine the virulence genes of *Aeromonas* sp. isolated from frog *H. occipitalis*. Specifically, it came to seek the presence of *hlyA* and *aerA* genes in *Aeromonas* sp. of *H. occipitalis*.

2. Materials and methods

2.1. Sample collection

A total of 210 fresh healthy frogs and 384 smoked frogs *H. occipitalis* purchased from three local markets (Issia, Sinfra and Daloa) were included in this study. Each frog was placed in sterile bag and put in a glass. Samples were constituted by 210 g skin, 210 g intestines and 210 g muscles of fresh frogs and 384 muscles of smoked frogs.

2.2. Biochemical identification of Aeromonas strains

About 5 g muscle and skin (each) and 1 g of intestine were weighed, aseptically removed and homogenized using a pestle and mortar for 2 min. The homogenate was enriched in 45 mL and 9 mL peptone buffer water (BioRad, Marnes La Coquette, France), respectively, and incubated at 37 °C for 24 h according to Sarkar *et al.* with slight modification[14].

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 *Aeromonas* sp. colonies which were dark green opaque with dark center were selected for further identification. Biochemical tests used for identification included oxidase, catalase, urease, indole production, gas and H_2S from glucose, lactose and lysine decarboxylase tests. The capacity of *Aeromonas* sp. to grow at different concentrations of NaCl (0%, 2%, 4% and 6%) was also performed in brain heart infusion broth. All strains were confirmed using Galery API 20 NE (BioMérieux, Marcy l'Etoile, France).

Hemolysin production was determined by spreading *Aeromonas* sp. isolates on blood agar (Bio-rad, Paris, France) containing 5% of sheep blood and incubated at 37 °C for 24 h according to Majeed method[15].

2.3. Detection of virulence gene of Aeromonas sp.

Genomic DNA from *Aeromonas* sp. strains was extracted using the heat-shock method according to Balsalobre *et al.*[16]. The 16S rRNA gene of all the strains of *A. hydrophila* was amplified by using the universal primer as described by Sahu *et al.*[12]. The detection of virulence gene was performed using a pair of primers H1 and H2 to amplify 597 bp *hlyA* gene and 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.

Primers	Genes	Sequences (5'–3')	Size (bp)
16S rRNA1	16S rRNA	AAGAGTTTGATCCTGGCTCAG	1 500
16S rRNA2		GGTTACCTTGTTACGACTT	
H1	*hlyA*	GGCCGGTGGCCCGAAGATGCAGG	597
H2		GGCGGCGCCGGACGAGACGGG	
A1	*aerA*	GCCTGAGCGAGAAGGT	416
A2		CAGTCCCACCCACTTC	

2.4. Data analysis

All data collected were double entered and analyzed using software SPSS. The differences were considered to be significant at $P < 0.05$ using the *Chi*-square and Kruskal Wallis tests.

3. Results

Result from the present study showed that all 384 smoked frogs were not contaminated by *Aeromonas* sp. However, *Aeromonas* sp. was isolated from intestine, skin and muscles of 48 (22.9%) frogs among 210 fresh healthy frogs of specie *H. occipitailis* analyzed. Per locality that was observed on Issia site with 89 frogs examined showed a contamination rate of 29.8% as compared with Daloa (24.3%) and Sinfra frogs (11.7%). The difference was significant between contamination rates and the sites of sampling (*Chi*-square = 6.60; degree of freedom = 2; $P = 0.037$).

A total of 148 bacterial strains of *Aeromonas* sp. (Table 2) were isolated from fresh healthy frogs. The isolation rates of these 148 strains upon anatomical parts of frog were as follows: 42.4% intestine, 19.5% skin and 8.6% muscle. Out of 148 isolates, 105 (70.1%) isolates were β-hemolytic positive and 43 (29.0%) isolates were non-hemolytic. The hemolytic activity of *A. hydrophila* (72.6%) was higher than *A. sobria* β-hemolytic activity (65.7%).

Table 2
Aeromonas species isolated from different sites.

Species	Issia	Daloa	Sinfra	Total
Aeromonas sp. isolated	81	43	24	148
A. hydrophila	69	29	15	113
A. sobria	12	14	9	35

All 148 strains bacteriologically identified like *Aeromonas* sp. were positive by PCR with amplification of 1 500 bp fragments of 16S rRNA gene specific for *Aeromonas* sp. (Figure 1).

PCR assay, using primer H1 and H2, showed that 125 (84.4%)

of *Aeromonas* sp. isolated were positive for *hlyA* genes (Table 3). PCR products of *aerA* genes with A1 and A2 primers that amplified 416 bp sequence (Figure 2) were found in 110 strains of *Aeromonas* sp. (74.3%). Kruskall Wallis test showed that there was no significant difference in the prevalence of *hlyA* and *aerA* genes production found per *Aeromonas* sp. related to sampling sites ($P > 0.05$). Three genotypes in *Aeromonas* sp. isolates were found. The $hlyA^+/aerA^+$ genotype was the most common genotype among *Aeromonas* sp. isolates with a prevalence of 58.8% (87) of isolates. Table 3 shows that the prevalence of genotypes $hlyA^+/aerA^+$ ranged between 55.2%–73.3% for *A. hydrophila* and 25.0%–60.0% for *A. sobria*.

Table 3
Summary of the presence of *hlyA* and *aerA* genes in 148 *Aeromonas* spp. by PCR according to sampling sites [*n* (%)].

Species	Sites	$hlyA^+$	$aerA^+$	$hlyA^+/aerA^+$	$hlyA^+/aerA^-$	$hlyA^-/aerA^+$
A. hydrophila	Issia	60 (87.0)	52 (75.4)	43 (62.3)	17 (24.6)	9 (13.0)
	Daloa	23 (79.3)	22 (75.9)	16 (55.2)	7 (24.1)	6 (20.7)
	Sinfra	12 (80.0)	14 (93.3)	11 (73.3)	1 (6.7)	3 (20.0)
A. sobria	Issia	10 (83.3)	5 (41.7)	3 (25.0)	7 (58.3)	2 (16.7)
	Daloa	13 (92.9)	10 (71.4)	9 (60.0)	4 (28.6)	1 (7.1)
	Sinfra	7 (77.8)	7 (77.8)	5 (55.6)	2 (22.2)	2 (22.2)
Total		125 (84.4)	110 (74.3)	87 (58.8)	38 (25.0)	23 (15.5)

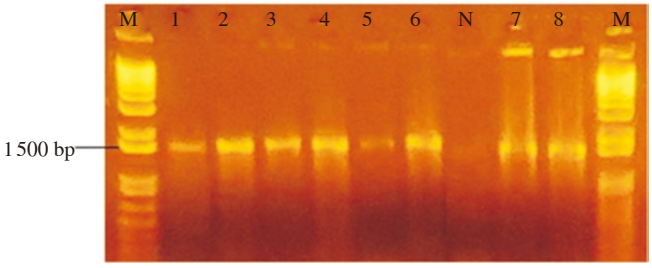

Figure 1. Electrophoretic profile of amplification product of 16S rRNA gene of *Aeromonas* spp.
M: DNA size marker (1 kb +); Lane 1: Positive control for *A. hydrophila*; Lane 2–5: *A. hydrophila* strains; Lane 6–8: *A. sobria* strains: N: Negative control.

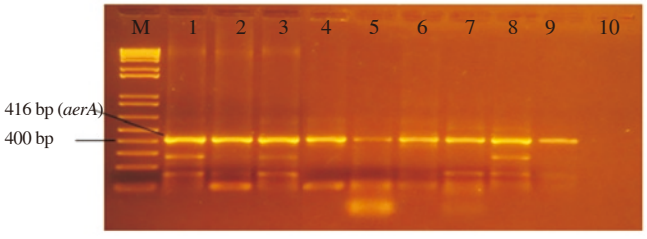

Figure 2. Electrophoretic profile of amplification product of *aerA* gene of *Aeromonas* spp.
M: DNA size marker (1 kb +); Lane 1–3: Positive strains of *A. sobria*; 4: Positive control for *A. hydrophila*; Lane 5–9: Positive strains of *A. hydrophila*; Lane 10: Negative control.

4. Discussion

On the whole, 22.9% (48/210) of frogs in this study were contaminated by *Aeromonas* sp. The value was obtained which denoted that *H. occipitalis* could play an eventual role in the transmission of this microorganism. Our findings also confirm that frogs sold in ours markets are harvested in the insalubrious areas. This may explain the contamination rates (22.9%) of *Aeromonas* sp. recorded from healthy *H. occipitalis*. However, future microbiology studies of water and diet of frog may help to clarify this finding. On the contrary, our result was lower than that of Tiberti who recorded 37.5% of contamination rate of *Rana temporaria* collected in area Brescia (Northern Italy)[8]. This report was in disagreement with the work of Rodrigues *et al.* who indicated the absence of *Aeromonas* sp. in frozen frog leg[18]. The differences between the findings of various authors and those of this study might be due to several factors such as the number of analyzed samples, season, geographical location and hygiene conditions.

This report mentioned that all of 384 smoked frogs surveyed were not contaminated by *Aeromonas* sp. The result could be probably related by the possible elimination of microorganisms during process of smoking by the action of temperature as reported by Adebayo-Tayo *et al.*[19].

A total of 105 *Aeromonas* sp. strains including 82 (72.6%) *A. hydrophila* and 23 (65.7%) *A. sobria* have β-haemolytic activity. The results of haemolytic activity in this study were confirmed by several researchers. About 100% of *A. hydrophila* having haemolytic activity on 5% sheep blood agar were reported by Abdel-Tawab *et al.*[20] and 83.3% were reported by Majeed[15].

In the current study, two virulence genes (*hlyA* and *aerA*) were identified in *A. hydrophila* and *A. sobria* isolates from the three localities. All of 148 isolates analyzed had at least one of the genes of the virulence studied. These *hlyA* genes were found in 95 strains of *A. hydrophila* (84.1%) and 30 strains (85.7%) of *A. sobria* carried this gene and it was the factor most commonly obtained in this study. The presence of *hlyA* had been shown by Ge *et al.* who was found that all (100%) of *A. hydrophila* isolated from *Rana temporaria* produced *hlyA* gene[21]. The main clinical symptoms infected by *A. hydrophila* in frogs are hemorrhage in the legs, especially in the hind limb. Ge *et al.* explained that *hlyA* gene should be main reason of leading to this septicemia[21]. Additionally, of the 148 *Aeromonas* sp. isolates investigated, 110 (74.3%) carried the *aerA* gene. The role of *aerA* gene in the pathogenicity of *Aeromonas* sp. genus was previously demonstrated. This presence indicated that it is virulent strains because *aerA* gene is one of the most important virulence factors for *A. hydrophila* bacteria[22].

Three genotypes among *Aeromonas* sp. isolates were found in this study $hlyA^+aerA^+$, $hlyA^+aerA^-$ and $hlyA^-aerA^+$. The $hlyA^+aerA^+$ (62.0%) for *A. hydrophila* and 48.6% for *A. sobria* were most frequent virulence in isolates from heath frogs *H. occipitalis*. This result is in accordance with Oleiwi *et al.* who also found high prevalence (52.38%) of $hlyA^+aerA^+$ from raw and drinking water[23]. The presence of both genes *hlyA* and *aerA* in our strain indicated that *Aeromonas* sp. isolated in these frogs was highly virulent, which suggests their high capability to cause disease in frog. This also represents a risk for human health mainly for frog meat consumers because genotypes $hlyA^+aerA^+$ had been implicated in human diarrheal disease and there is a significant correlation between this genotype and diarrhea[17].

In several countries, the cases of infections caused by *Aeromonas* sp. have been reported[24-26]. Therefore, no data were reported on aeromoniosis diseases in Côte d'Ivoire. These results should be a warning that sanitary control measures have to be taken.

This results revealed that 22.9% of edible fresh frog *H. occipitalis*

sold from the market of middle west of Côte d'Ivoire harbored potential pathogenic *Aeromonas* species. Two virulence genes, *hlyA* and *aerA* have been detected and this indicates the pathogenicity of this bacteria. However, no *Aeromonas* sp. strains was found in smoked frog. This finding highlights the necessity to consume smoked form of frog meat or eat fresh form after good cooking in order to avoid or reduce the risk of food borne disease. In view of pathogenic natural of *Aeromonas* sp., the breeding of frog will be requisite to continuous monitoring of the quality of water which is essential to minimize the health risk due to *Aeromonas*.

Conflict of interest statement

We declare that we have no conflict of interest.

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