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Detection of *act* and *alt* Enterotoxin Genes in *Aeromonas* Strains Isolated from *Hoplobatrachus occipitalis* Frogs Intended for Human Consumption in Côte d'Ivoire

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Abstract *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. In Côte d'Ivoire, the consumption of frogs appears as a solution to solve animal proteins problems in West regions. This research was investigated in order to evaluate the presence of enterotoxins genes in *Aeromonas* strains found on edible frog. *Aeromonas* research was carried out on 210 edible *Hoplobatrachus occipitalis* frogs purchased in different markets in central western Côte d'Ivoire. After isolation of *Aeromonas* strains by culture methods, the polymerase chain reaction (PCR) technique was used for the detection of *alt* and *act* enteroxins in isolated strains. The results showed contamination of the frogs by *Aeromonas sobria* (9.1%) and *Aeromonas hydrophila* (13.8%). The *alt* and *act* enteroxin genes were detected in 34.5% and 17.0% of the isolated *Aeromonas* strains, respectively. This presence of virulence gene requires the implementation of health surveillance measures to avoid cases of *Aeromonas* contamination among consumers.

Keywords: *Aeromonas, Enterotoxins genes, Frog, Côte d'Ivoire*

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1. Introduction

Frogs are amphibians of the order Anurans. They are distributed in nature on all continents. Today these animals are gradually disappearing and the causes mentioned by several studies are, among others, cases of disease, climate change and overexploitation by humans either for food or for scientific research [1,2,3]. Regarding food, the craze around frog meat around the world is due to its organoleptic and nutritional quality.

In Côte d'Ivoire, this consumption is mainly observed in the west of the country by several ethnic groups [4]. These animals are captured in unsanitary aquatic areas conducive to the development of microorganisms. This could represent a health risk for consumers because the captured frogs escape adequate health inspection. Indeed, it is reported that frogs are capable of transmitting to consumers, several pathogenic bacteria such as *Aeromonas* sp, *Salmonella* sp and *Mycobacterium* [5,6,7].

Aeromonas has long been considered an opportunistic pathogenic bacterium for humans but is currently recognized as an emerging pathogen [8]. Indeed, *Aeromonas* is involved in several infections including gastroenteritis, sepsis, wound infections, meningitis and endocarditis [9]. This microorganism is responsible for 85% of cases of gastrointestinal infections encountered in humans [10]. Those at risk are children, the elderly and the immunocompromised [8]. In frogs, the species *A. hydrophila* is responsible for "red-legged diseases" and is implicated in the mass mortality of frogs in captivity and in the wild [11].

In Côte d'Ivoire, research on the pathologies of frogs is fragmentary. These captured animals are sold on different markets and in view of the consumption rate reported by [4] which is 52.7%, it is necessary to assess the quality of the frogs sold on these markets in order to prevent possible contamination. Thus the aim of this work was to evaluate the presence of enterotoxins genes in *Aeromonas* strains found on edible frog.

2. Material and Methods

2.1. Frog Sample Collecting

During the period from December 2016 to February 2017, a total of 210 apparently healthy fresh edible frogs

(*Hoplobatrachus occipitalis*) were purchased from retailers in various supply markets located in central western Côte d'Ivoire. Frog samples were collected at the markets of Daloa, Issia and Sinfra. The collectors capture generally these frogs during the night in rivers and shallows then transported very early to the markets to be sold. The purchased frogs were individually placed in sterile Stomacher bag which were labeled and stored in a cooler containing ice and then transported to the laboratory for analysis.

2.2. Isolation and Identification of *Aeromonas*

After individual dissection under aseptic conditions from each frog in the laboratory, organs such as intestine, skin and muscle were removed and stored in tubes for testing for *Aeromonas*.

The method described by [12] was used to achieve the isolation of *Aeromonas* sp. A total of one gramme of each organs skin, intestine and muscle taken individually was crushed and homogenized in 9 mL of buffered peptone water (Bio-Rad, France) using a Stomacher. Each suspension obtained was incubated at 37°C for 24 h for enrichment. After incubation an aliquot (0.1 mL) of each of the enrichment suspensions was inoculated in Aeromonas agar (Sigma Aldrich, India) supplemented with 30 mg / L ampicillin and the Petri dishes were incubated at 37°C for 24 h.

The presumptive colonies of Aeromonas sp. which are characterized by a green color with a black center on Aeromonas agar supplemented with 30 mg/L of ampicillin were subcultured on an alkaline nutrient agar (Bio-Rad, France) and incubated at 37°C for 24 h. From the pure colonies, a biochemical identification was carried out.

Identification of presumptive *Aeromonas* isolates was performed by subculture of five presumptive *Aeromonas* sp colonies on alkaline nutrient agar (Bio-Rad, France). Incubation was carried out at 37°C for 24 h. From the colonies of the pure culture, an identification was carried out by the determination of morphological, biochemical and cultural characters in nutrient broth at different concentrations of NaCl (0 % to 6%). These strains obtained were confirmed using an API 20 NE gallery (BioMérieux, France).

2.3. DNA Extraction

The DNA extract was obtained by the heat shock method as described by [13] from strains of *Aeromonas* sp. revived by culture and incubated in brain heart broth (Bio-Rad, France), 24 h at 37°C.

2.4. Detection of *act* and *alt* Genes by PCR

The cytoxic enterotoxin (*act*) and heat-labile enterotoxin (*alt*) genes were detected by monoplex PCR according to the method of [14] using a pair of primers (Table 1).

The PCR was performed in a final volume of 25 µL containing 4 µL of dNTPs mix (0.2 mM), 4 µL of $MgCl_2$ (25 mM) (Promega Madison wi USA); 0.2 µM of each primer (IDT, USA); 1.5 µL of Taq polymerase (5U / µL) (Promega, France), 5 µL of 10X PCR buffer, 5 µL of DNA and milliQ water.

PCR conditions were done in a Thermocycler (TECHNE) with the following program : an initial denaturation at 95°C for 5min following by 35 cycles consisted of denaturation: 95°C for 15s, annealing: 57°C for 30s (*act* gene) *and* 69 °C for 30s (*alt* gene), extension: 72°C during 30s and a extension at 72°C for 30 following final extension during 10 min at 72°C. PCR products 232bp and 361bp were revealed using a UV transilluminator (Cleaver Scientific LTD) after an electrophoresis in a 1.5% agarose gel in Tris-borate-EDTA (0.5X) (Sigma Aldrich, USA) during 1h at 100 Volt.

Table 1. Primers used for amplification of *act* and *alt* genes

Primers	Genes	Sequences (5'- 3')	Size (bp)	Reference
AHC-F	*act*	AGAAGGTGACCACCAAGAACA	232	[14]
AHC-R		AACTGACATCGGCCTTGAACTC		
AHL-F	*alt*	TGCTGGGCCTGCGTCTGGCGGT	361	
AHL-R		AGGAACTCGTTGACGAAGCAGG		

3. Results and Discussion

The study of frogs sold in the markets revealed the presence of two species of *Aeromonas*. These were *Aeromonas hydrophila* and *Aeromonas sobria*, which respectively contaminated 13.8% (29/210) and 9.1% (19/210) of the frogs analyzed. Our results corroborate with those of the work of [11] who also found a rate of contamination (8.8%) on *Rana catesbeina* and *Rana clamitan*. These results confirm that *Aeromonas* is a ubiquitous bacterium in water and is able to contaminate any food from water. The presence of these strains can be justified by the hygienic quality of the catching areas. Indeed, these frogs come from highly polluted aquatic environments generally conducive to the development of pathogenic microorganisms including *Aeromonas* [15]. This presence of *Aeromonas* in apparently healthy frogs indicates the importance of asymptomatic carriage of *Aeromonas* sp in amphibians and the possible role it may play in the transmission of this microorganism.

The results of this study show the presence of virulence genes in *Aeromonas* strains. The PCR, molecular detection technique demonstrated heat-labile cytotonic enterotoxins (*act*) and heat-labile cytotoxic enterotoxins (*alt*) in *H. occipitalis*.

The *act* enterotoxin gene (232 bp) was found in 51 strains of *Aeromonas* sp. with a prevalence of 34.5% (Figure 1). While the *alt* gene with a size of 361 bp was the least encountered in the samples. A total of 25 strains of *Aeromonas* sp. or 17.0% harbored this gene (Figure 2). Chi-square test showed that production of *act* and *alt* genes by *Aeromonas* strains was not significantly related to harvest sites ($P > 0.05$).

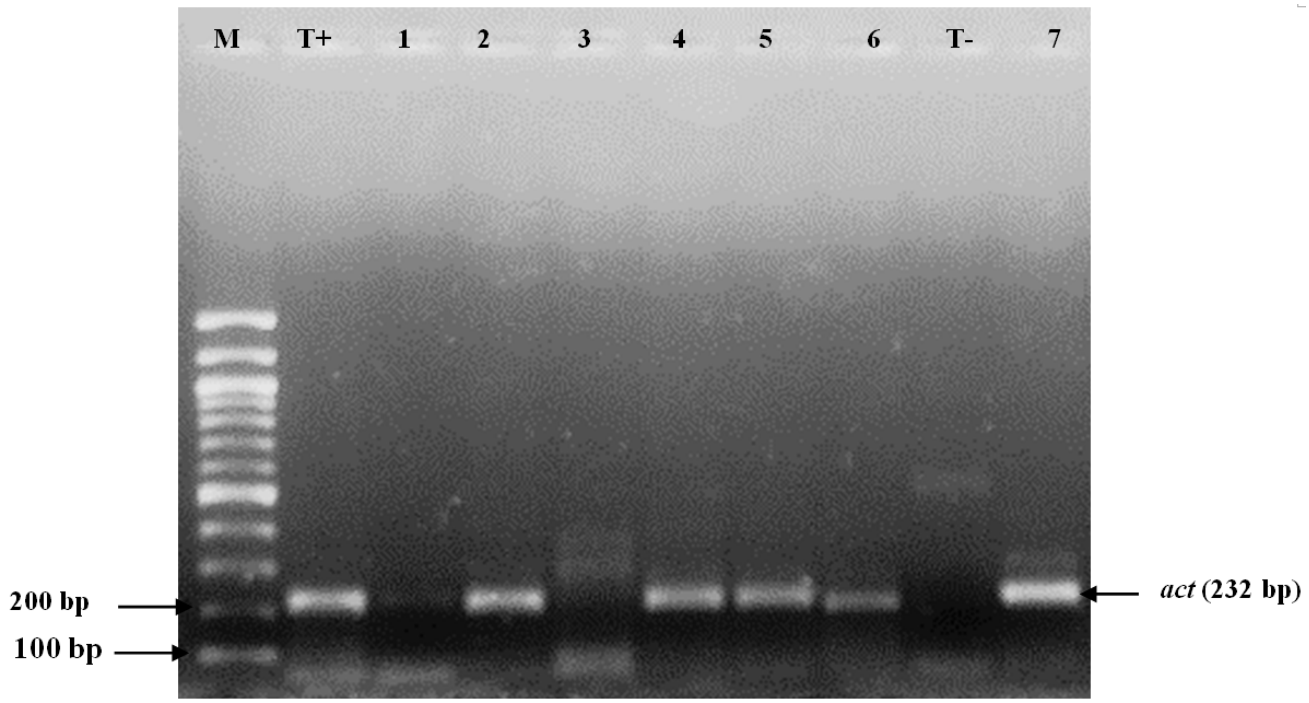

Figure 1. Electrophoretic profile of *act* gene in *Aeromonas* species on 1.5% agarose gel (M : Molecular marker 100 bp (Sigma Aldrich, Saint Louis, USA); T+ : positive control for *A. hydrophila*; Lane 2, 4, 5, 6, and 7: strains tested positive by the presence of the *act* gene (232 bp); T–, negative control)

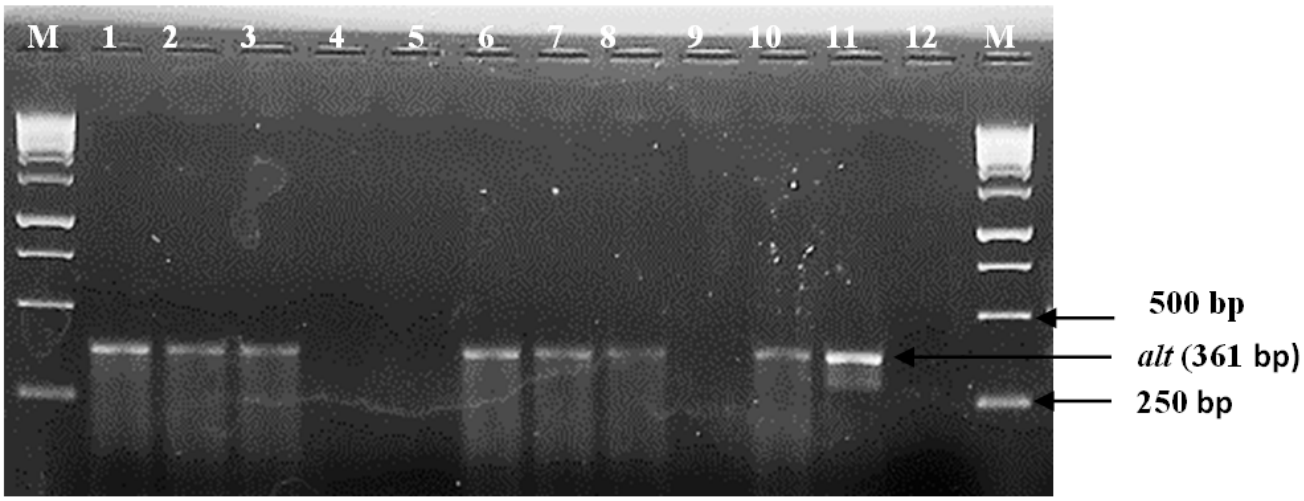

Figure 2. Electrophoretic profile of *alt* gene in *Aeromonas* species on 1.5% agarose gel (M : Molecular marker 1kb (Sigma Aldrich, Saint Louis, USA); lane 1 : positive control for *A. hydrophila*; Lane 2, 3, 6, 7, 8, 10 and 11 : strains tested positive by the presence of the *alt* gene (361 bp); 12 : negative control)

Our results are in agreement with those of [16] and [17] who showed that the strains of *Aeromonas* sp. can produce a variety of virulence factors including heat labile cytotonic enterotoxins (*act*), heat labile cytotoxic enterotoxins (*alt*). Among these identified genes, the *act* gene is capable of increasing the level of TNF (Tumor Necrosis Factor) and IL-1 (Interleukin 1) in the macrophage cell line and other inflammatory cytokines resulting from tissue lysis. According to [18], these cytotoxic toxins released by *Aeromonas* sp. are the cause of enterocyte death and the very severe symptoms of dysentery in humans. The presence of these genes in our strains indicates that *Aeromonas* sp. isolated in these frogs was virulent to animal. This therefore suggests their ability to cause disease in frogs. These bacteria cause internal bleeding in the frog, characteristic of red leg disease.

Note that this species of frog is the most popular and most consumed in Côte d'Ivoire [4]. This presence of the *act* and *alt* genes would constitute a real risk for human health, mainly in consumers of frogs if the meat consumed is undercooked.

4. Conclusion

This study is the first case of detection of *alt* and *act* enterotoxins genes on *Aeromonas* strains isolated from amphibians in Côte d'Ivoire. In light, this shows the involvement of *H. occipitalis* frogs in the transmission

of potentially pathogenic *Aeromonas* in the aquatic environment. These animals, now largely consumed by the populations of western Côte d'Ivoire, should be monitored to avoid contamination.

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Conflict of Interest

The authors declare that there is no conflict of interest regarding the manuscript.

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