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RESEARCH ARTICLE

IN VIVO PATHOGENICITY TEST OF *AEROMONAS HYROPHILA* ON FROG *HOPLOBATRACHUS OCCIPITALIS* UNDER EXPERIMENTAL CONDITIONS

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Abstract

In vivo pathogenicity test of *Aeromonas hydrophila* were done in this experimental study. The objective of this investigation was to evaluate the virulence properties of *Aeromonas hydrophila* isolated from frogs collected in swamps. To carry out the experiments 24 frog *Hoplobatrachus occipitalis* with an average weight of 49.25± 4.21 g were collected and infected with different concentation of *Aeromonas hydrophila* cells (10^7, 10^6 and 10^5 CFU/ml) and physiological saline (0.85% NaCl) by intramuscularly injection in Laboratory. The observations of different batches of frogs during the 96 hours of experimentation showed that the clinical signs of the disease were the weakening of the animal, the cessation of swimming and the appearance of red spots on the hind legs. Death animals were recorded after 24 hours of injection with a concentration of 10^7 CFU/ml. The highest mortality rate (33.3%) was obtained 48 hours after injection of the bacteria. In this study, the lethal dose (LD_{50}) of *A. hydrophila* obtained from the cumulative mortalities was $5.8x10^6$ CFU/ml.

Introduction:-

The edible frog *Hoplobatrachus occipitalisis* one of the important species of frog mostly consumed by 55.2% of people in Côte d'Ivoire (Ble et al., 2016). Various activities are undertaken in Côte d'Ivoire to promote the breeding of frog. To solve the problem of the high demand for frog meat, work to set up farms had been initiated in several localities. Thus, the National Agency for Support to Rural Development (ANADER), established from 1997, twelve frog breeding farms of the species *Rana esculenta* in the region of Man (Avit et al., 1999). This breeding project aimed to satisfy protein needs, produce large frogs in all seasons and ensure the quality of the meat produced.

The breeding of the frog species *Xenopus muelleri* had also been initiated in 1998 in the North of the country but ended in failure due to the lack of control of the feeding and reproduction strategy of the frogs (Tohé, 2009). Since ANADER abandoned the experimental farms for reasons related to the politico-military crisis of 2002, the entire production-to-consumption sector has become exclusively artisanal and informal.

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The frogs are collected from unsanitary swamps and ponds of dubious sanitary quality. These areas are known to be the sites of development of pathogenic microorganisms. The development of raniculture is thus confronted with several diseases that are spread in the living environment of these animals. Indeed, diseases have now become a primary constraint to the culture of many aquatic species, impending both economic and social developments and a significant constraint on aquaculture production and trade (Smith, 2006).

At the parasitic level, frogs harbor several zoonotic pathogenic parasites causing lesions, malformations and death in the amphibian and sometimes human population following consumption of undercooked frog meat. These include Trypanosomas sp. Folleyellid sp. of Alaria sp. (Portier, 2012; Aisien et al., 2015), Rickettsiae and several genera of helminths, protozoa and Microfilariae (Jurku and Modry, 2004; Sailasuta et al., 2011). Amphibians decline globally due to infection with virulent bacteria, despite their vital roles in the ecosystem. It is recognized that bacteria are one of the important causative agents of frog diseases. Among pathogen bacteria that can be incriminated, *Aeromonas hydrophila* plays an important role in susceptibility of disease in frog. Historically, *Aeromonas hydrophila* 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 (Tiberti, 2011 ; Dahdouh et al., 2016). It is also implicated in the mass mortality of frogs in captivity and in the wild. *Aeromonas hydrophila* causes disease by expressing virulence factors such as aerolysin, a hemolytic toxin that destroys redblood cells and a cytolytic pore-forming toxin, which in combination are sufficient to cause 64% mortality in experimentally infected northern leopard frogs, *Lithobates pipiens* (Zachary, 2016).

Thus, the good control of *Aeromonas hydrophila* seems to be essential for the establishment of ranaculture. The aim of this experiment was to study the virulence properties of *Aeromonas hydrophila* isolate from frogs collected in swamps.

Material And Methods:-

Constitution of the experimental batches of frogs

During this experiment, 24 apparently healthy frogs with an average weight of 49.25± 4.21 g captured in the shallows were divided into four batches (N°1 to N°4) of 6 frogs each in aquariums (A1 to A4). These frogs were placed under observation in the aerated aquariums for one week before the injection of the bacterial suspension and sterile NaCl (0.85%), in order to adapt to the experimental conditions. The aquariums were first washed with 70% alcohol then rinsed with water to avoid any formation of bacterial biofilm (Lamontagne, 1991). During the experimental period, the water temperature was maintained at 27.6±0.4°C.

Preparation of different concentrations of *A. hydrophila*

The strain of *Aeromonas hydrophila* used in this study was isolated from frogs in the work of Blé et al. (2017). This strain harbored the *hlyA* and *aerA* virulence genes that are most implicated in frog red-leg disease and human infections (Ge et al., 2011; Stratev et al., 2012). The preparation of the different bacterial concentrations was carried out according to the protocol of Lamontagne (1991). The strain was inoculated onto nutrient agar and incubated at 37°C for 24 h. At the end of the incubation time, two colonies were picked and inoculated into 5 ml of brain heart broth (BCC), which was incubated for a period of 8 hours. After 0.1 ml of the inoculum was suspended in 10 ml of BCC. This preparation was then incubated at 37° C. for 18 hours and then centrifuged at 6000 rpm for 20 min. The pellet was resuspended in 10 ml of saline solution (0.85% NaCl). The bacterial load of this suspension equivalent to the 0.5 McFarland standard (~10^8 CFU/ml) was determined using a densimât. This suspension was considered as the stock suspension of dilution 10^0. From this stock suspension, the successive dilutions of 10^{-1}, 10^{-2} and 10^{-3} were carried out with a sterile saline solution (0.85% NaCl). These dilutions made it possible to obtain the respective bacterial concentrations of 10^7; 10^6 and 10^5 CFU/ml.

Inoculation of bacteria to frogs

The frogs of batch N°1 (control batch) or aquarium A1 were inoculated intramuscularly with 0.1 ml of a physiological saline (0.85% NaCl, pH 7.2) using a sterile syringe. While the frogs of batches N°2, N°3 and N°4 were administered individually by the intramuscular route with dose of *A. hydrophila* 10^7 , 10^6 and 10^5 CFU/ml cells respectively per 0.2 ml (Table 1). After inoculation of bacteria, injection site was again swabbed with 70% alcohol and the frogs released into the aquarium water.

Table 1:- Different Concentration of *A. hydrophila* innoculated.

Batches formed	N°1	N°2	N°3	N°4
Substance administered	NaCl	*A. hydrophila*	*A. hydrophila*	*A. hydrophila*
Concentration	0.85 %	10^7 CFU/ml	10^6CFU/ml	10^5CFU/ml
Number of frog per group	6	6	6	6

Monotoring of experimental batches

Once bacterial injected, each batch of frogs was observed every two hours on the first day and twice a day for others days. The frog were observed carefully for visible external symptoms and behavioral changes. These observations were aimed at looking for the appearance of clinical signs of *Aeromonas hydrophila* and cases of mortalities. The signs sought were: the general condition of the frog, the presence of redness in the legs and lower abdomen, the flow of blood from the mouth or from the extremities and the ad-mortem time.

Determination of lethal dose (LD_{50})

The lethal dose (LD_{50}) is the concentration of bacteria causing 50% mortality of animals in a batch. It was calculated according to the method of Reed and Muench (1938) using following formula:

$$LD_{50} = \text{Antilog (Dilution above 50\% } - \text{ Proportionate Distance)}$$

$$\text{Proportionate Distance} = \frac{\text{Mortality above 50\%} - 50}{\text{Mortality above 50\%} - \text{Mortality below 50\%}}$$

Results and Discussion:-

Evolution of mortality according to the duration of injection.

Determination of in vivo pathogenicity in healthy frogs showed that the strain of *A. hydrophila* used in this study was virulent. However, it takes a much higher concentration of bacteria for this strain to induce weakening and mortality in frogs. In this study, the pathogenicity of *A. hydrophila* was evaluated after injection of different bacterial concentrations into frogs. No case of mortality was recorded in the control group. The monitoring of the frogs during the 96 hours of experimentation showed that the clinical signs of the disease were the weakening of the animal, the cessation of swimming and the appearance of red spots on the hind legs. In additionaly frogs injected fishes with *A. Hydrophila* showed reddening and swelling at the site of infection.

It appears from these analyzes that the strains used could cause the death of the frogs. The first case of frog mortality without clinical signs 24 hours after injection was obtained with a concentration of 10^7 CFU/ml. The highest mortality rate (33.3%) was obtained 48 hours after injection of the bacteria. Beyond 48 h, the number of deaths decreased and remained constant (Figure 1).

The high mortality rate at 48 h of injection would be linked to the physiological stress during the handling of the animal which must have weakened the defense system which was not able to fight the bacterial infection before 48 h. Beyond 48 h, the reduction in mortality could be explained by the fact that the animal was able to counter the infection after 48 h. This observation was also made by Lamontagne (1991).

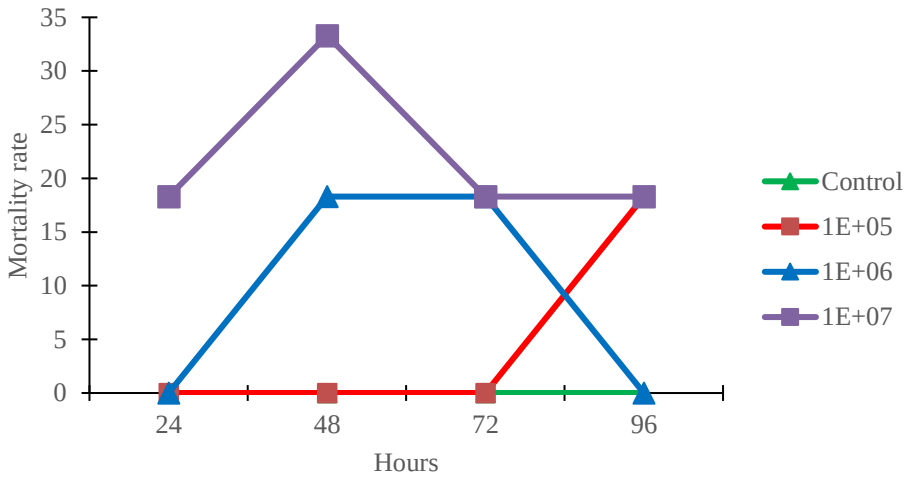

Figure 1:- Evolution of mortality after administration of *A. hydrophila* in frog *H. occipitalis.*

Evolution of mortality according to the bacterial concentration

Analysis of Table 2 shows that 44.4% of the 18 frogs that were injected with the concentrations of *A. hydrophila* died at the end of the 96 hours of experimentation. Concentrations of 10^7; 10^6 and 10^5 spawned respectively 5; 2 and 1 died during the study. In this study, the lethal dose (LD_{50}) of *A. hydrophila* obtained from the cumulative mortalities was $5.8x10^6$ CFU/ml. This bacterial concentration is considered to be the median concentration which causes 50% mortality in the population of a batch of frogs or the concentration which kills half of the animals.

This LD_{50} value for *A. hydrophila* is lower than that reported by Lamontagne (1991) who obtained $6x10^7$ CFU/ml with the same bacterium in an experiment carried out on frogs. Similar in vivo virulence tests of *A. hydrophila* have been carried out on other animal species such as fish in several studies with different LD_{50} obtained. The results was reported by various workers as $1.72x10^7$ - $1.14x10^8$ CFU/ml (Samal et al., 2014) ; $2.1x10^4$ CFU/ml in tilapia at 5 days challenge with viable cells (Khalil and Mansour, 1997), 2.4 $x10^6$ CFU/ml in fish Labeorohita (Haniffa et al., 2015). Our results clearly indicate that this opportunistic bacterium *A. hydrophila* can contribute to massive mortality and economic loss in aquaculture and particularly in frog farms.

Table 2:- Determination of LD_{50} for virulent *A. hydrophila* in *H. Occipitalis.*

A. hydrophila (CFU/ml)	Initial number	Died	Survivied	Death Ratio	Survival Ratio	Mortality	Cumulative Mortality
10^7	6	5	1	5/6	1	5/6	83.3
10^6	6	2	4	2/6	5	7/12	68.75
10^5	6	1	5	1/6	10	8/18	44.44

$LD_{50=}$ $5.8x10^6$ CFU/ml

Conclusion:-

The results show that strains of *A. hydrophila* are highly pathogenic for frogs under the condition of this study. This toxicityis at the LD_{50} of $5.8x10^6$ CFU/ml. Dead frogs with clinical signs have been observed following injection of a high concentration of *A. hydrophila*. These bacteria are usually found in the aquatic environment. Therefore, for the establishment of raniculture, hygiene measures related to water quality must be applied. Further studies need to be conducted to assess the impact of *A. hydrophila* on other amphibian population.

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