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## Original Research Article

# Pathogenicity of *Aeromonas hydrophila* isolated from diseased sharp teeth catfish, *Clarias gariepinus*, with special reference to the lethality of its extracellular products

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**ABSTRACT**

A study was conducted to investigate the pathogenicity and the median lethal dose ($LD_{50}$) of *Aeromonas hydrophila* isolated from clinically diseased catfish against apparently healthy homologous fish to evaluate the lethality of extra-cellular products (ECPs) of the isolated strain in vivo. For pathogenicity experiment, five different concentrations of *Aeromonas hydrophila* strain BNS 01614 including $3\times 10^8$, $1.5 \times 10^8$, $1.5 \times 10^7$, $1.5 \times 10^6$ and $1.5 \times 10^5$ CFU/fish used via intra peritoneal. The results revealed that pathogenicity of BNS 01614 was confirmed by the mortality of 30 % to 100 % of all tested fish within 4 to 12 days with $LD_{50}$ $1.5 \times 10^7$ CFU/fish. The Concentrated extracellular products (ECPs) of the selected bacterium were prepared and confirmed to be toxic in *Clarias garipineus* with $LC_{50}$ of 20µg.

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## 1. Introduction

Aquaculture has been the fastest growing food-producing industry worldwide in the past few decades and is expected to continue to be the hugest industry in the future. As it expands rapidly, the aquaculture industry faces many challenges, particularly infectious diseases (Yeh et al., 2005). Sharp teeth catfish, *Clarias gariepineus* are considered as important food fish and offered an important protein source in Egypt and many other developing countries. Egypt production of farmed catfish is exceeded 7,547 tons, in 2011 (Macfadyen et al., 2011). However, the fish has suffered from a disease condition in intensive culture system by the bacteria *Aeromonas hydrophila*. *A. hydrophila* was frequently observed in various species of diseased farmed and wild freshwater fishes in

different locations of Egypt (El-Refaey, 2013). The extracellular products (ECPs) secreted by *A. hydrophila* are considered as essential virulent factors as they contain various proteins that possess cytotoxic, cytolytic, haemolytic, and enterotoxic properties, such as haemolysin (Allan and Stevenson, 1981) and protease (Yu et al., 2006). the objectives of this study are to: (1) perform biochemical and molecular identification for *A. hydrophila* BNS 01614 strain isolated from local outbreak in commercial fish farm at Beni-seuf Governorate; (2) detect the virulence of the pathogen and its $LD_{50}$ (median lethal dose) in the catfish; (3) determine the lethality of extracellular products (ECPs) of the selected *A. hydrophila* strain in vivo.

## 2. Materials and methods

### 2.1. Ethical statement

All the in vivo procedures were performed in strict accordance with the recommendations and ethical guidelines for the care of animals used for experimental and other scientific purposes. All the experimental protocols were approved by the Institutional Animal Care and Use Committee of Beni-Suef University, Beni-Suef, Egypt (www. bsu.edu.eg/IACUC.aspx).

### 2.2. Experimental fish

A total number of 140 apparently healthy *C. gariepinus* fish were collected from El-fashn fish farm. They were transferred alive to the wet lab of Fish Department, Faculty of Veterinary Medicine, Beni-suef University, Beni-Suef Governorate. Randomly 20 individuals were subjected to microbiological investigation to ensure that fish bunch is free from pathogenic microorganisms. The rest fish bunch was divided into groups according to their size and weight, and then acclimated for one week in fiberglass tanks supplied with chlorine free tap water and continuous aeration. During acclimatization period, fish were fed on commercial fish feed (25%, protein, Zoo Control, Egypt) at a rate of 2% of their body weight.

### 2.3. Bacterial isolation and identification

*A. hydrophila*, BNS 01614 isolated from outbreak of clinically diseased catfish showed characteristic signs of motile Aeromonas septicemia. The isolated bacterium was sub-cultured and morphologically, biochemically and molecularly identified and verified as described by Balsalobre et al., (2009); Alsaphar et al., (2012); Pinto et al., (2012). The identified bacterium was then kept in BHI glycerol 20% (V/V) at -80 °C

### 2.4. Pathogenicity experiment

The selected *A. hydrophila* strain was inoculated into 5 ml of BHIB (Oxoid®) tubes ($n$=4) and incubated for 20 hrs at 26° C. After elapsing of incubation time, the bacterial cell pellets were obtained by centrifugation using high speed cooling centrifuge (Centurion scientific, core life sciences®, Inc. US) at 10000 rpm, 10°C, 10 min. The revealed pellets were washed 3 times with sterile fish physiological saline (SFPS) (0.65% NaCl). They were re-suspended in SFPS and justified to match Macferland tubes No. 1 and 0.5 equivalents to $3x10^8$ and $1.5x10^8$ CFU/ml, respectively. The bacterial suspension was tenfold serial diluted to obtain the desired final concentrations (table 1).

The pathogenicity experiment represented 6 fish groups including control and 5 experimental ones each for single concentration (table 1). Sixty fish individuals were distributed in sex glass aquaria, 10 in each. All fish groups were IP injected at a dose of 0.1 ml/fish with exception. The exception is that the control one was injected with SFPS instead of bacterial suspensions. All groups were monitored for 14 days post injection for any abnormalities and /or mortalities. Clinical signs and cumulative mortalities were daily recorded till the end of the experiment. Randomly, re-isolation together with PCR detection was performed to assist Khoch's postulate.

### 2.5. Preparation, concentration and total protein determination of ECPs

#### 2.5.1. Preparation of ECPs of selected *A. hydrophila* BNS 01614 strain

The desired ECPs from isolated *A. hydrophila* strain were prepared according to the methodology described by Zhang et al., (2014) with a little modification. Briefly, isolated *A. hydrophila* strain was inoculated into trypticase soya broth (TSB, Oxoid®) tube (5 ml) and incubated for 3 hrs at 26° C. Under aseptic condition the content of the cultured tube was inoculated into a flask containing 250 ml TSB and incubated on a rotatory shaker (SK – L330-Pro, SCI – Logex, Rhsin Land, USA) for 18 hrs at 26° C. After elapsing of incubation time, the ECPs were obtained by centrifugation using high speed cooling centrifuge at 5000 rpm at 10º C for 20 min. Then, 40 ml of the collected ECPs was filtered using syringe filter 22µ (Avdec®, Japan) to get rid of any bacterial cells. Additionally, a loopful from filtered ECPs was platted on TSA (Oxoid®) to ensure complete sterility of ECPs. Concurrently, the obtained 40 ml ECPs were concentrated 8 times using cellulose tube (Thermo Scientific® SnakeSkin® 10K MWCO Dialysis Tubing, Cat. # PI88243) and polyethylene glycol (MW 6000-7500, El Nasr Pharmaceutical Chemical, Egypt). The yield concentrated ECPs (5 ml) were dialyzed against Hank's balanced salt solution (HBSS, calcium chloride 0.14 g/L, potassium chloride 0.4 g/L, potassium mono basic phosphate 0.06 g/L and magnesium hydrochloride 0.1 g/L) buffer with several buffer change for 24 hrs at 4º C to remove any traces of TSB. Then, the obtained concentrated ECPs were kept at – 20° C for further work.

**2.5.2. Determination of total protein contents of crude concentrated ECPs**

The total protein content of the aforementioned crude ECPs was assayed according to Burtis et al., (1999) following the manufacturer's instructions of a commercial kit for protein analysis (Diamond co., Egypt).

**2.6. Determination of Toxicity of crude ECPs of *A. hydrophila* in catfish**

Sixty fish individuals were distributed in sex glass aquaria, 10 in each. The toxicity experiment represented 6 fish groups including control and 5 experimental ones. The experimental groups ($n$=5) were subjected to designed injection regime illustrated in (table 2). Briefly, all experimental fish groups were IP injected with crude ECPs at doses of 10, 20, 30, 40, and 50 µg/fish. Control fish were injected with saline instead of ECPs. All groups were monitored for 14 days post injection for any abnormalities and /or mortalities. Clinical signs and cumulative mortalities were daily recorded till the end of the experiment.

## 3. Results

**3.1. Characteristics of selected *A. hydrophila* BNS 01614 strain**

Results of morphological, biochemical and molecular characters of selected *A. hydrophila* BNS 01614 are shown in table 3 & fig. 1

**3.2. Pathogenicity determination of selected *A. hydrophila* BNS 01614 strain**

Results of designated experimental infection with *A. hydrophila* BNS 01614 in catfish are summarized in (table 4 & fig 2). Signs of septicemia (external & internal, as scattred petechial haemorhges all over the skin, at base of fines and anal opening with enlarged hemorrhagic liver, distended gall bladder and spleen) stared to appear 24hrs post infection accompanied with abnormal behavioral changes including abnormal swimming, listless and the fish became lethargic. Cumulative mortalities were stared 48hrs post infection and extended till the end of the experimental period (14 days). The cumulative mortalities percentages were differed according to the doses of selected *A. hydrophila* strain used. Ultimately, the cumulative mortalities were 100, 60, 50, 40, and 30% within 5, 7, 9, 10 and 12 days post infection, respectively. No mortalities were recorded in the control fish.

**3.3. Determination of total protein contents of crude ECPs obtained from isolated *A. hydrophila* BNS 01614 strain**

The protein contents of the concentrated crude ECPs of *A. hydrophila* were determined as mentioned in materials and methods section and adjusted to a concentration of 0.5µg/µL.

**3.4. Determination of toxicity of crude ECPs of isolated *A. hydrophila* BNS 01614 strain**

Results of designated toxicity experiment with ECPs of *A. hydrophila* BNS 01614 in catfish are summarized in (table 5). The toxic effects of concentrated crude ECPs of the selected *A. hydrophila* BNS 01614 developed rapidly 12 hrs later post injection accompanied with vigorous pictures of septicemia. Cumulative mortalities were 100, 90, 70, 50 and 30% represented after injection of 50, 40, 30, 20 and 10µg/fish within 12-24, 24, 72, 96 and 120hrs, respectively, and the $LC_{50}$ was found to be 20µg/fish. Notably, the clinical signs were observed after 4 days post injection (fig. 3) with the low doses of ECPs used.

**Table 1. Designed regime for pathogenicity test**

| Fish group | No. of fish in each group | CFU/ml | Route of injection | Dose/fish/ml |
|---|---|---|---|---|
| Group 1 | 10 | $3\times10^8$ | IP | 0.1 |
| Group 2 | 10 | $1.5\times10^8$ | IP | 0.1 |
| Group 3 | 10 | $1.5\times10^7$ | IP | 0.1 |
| Group 4 | 10 | $1.5\times10^6$ | IP | 0.1 |
| Group 5 | 10 | $1.5\times10^5$ | IP | 0.1 |
| Control | 10 | SFPS | IP | 0.1 |

**Table 2. Designed regime for determination ECPs toxicity of selected *A. hydrophila* BNS01614 strain**

| Fish group | No. of fish in each group | Dose/fish (µg) | Route of injection |
|---|---|---|---|
| Group 1 | 10 | 10 | IP |
| Group 2 | 10 | 20 | IP |
| Group 3 | 10 | 30 | IP |
| Group 4 | 10 | 40 | IP |
| Group 5 | 10 | 50 | IP |
| Control | 10 | SFPS | IP |

**Table 3. Morphological, biochemical and other characteristics of selected *A. hydrophila* BNS 01614 strain**

| Test | Results |
|---|---|
| Colonial morphology | White smooth, rounded colonies |
| Gram stain | Gram negative, rods |
| Motility | + |
| Oxidase | + |
| Catalase | + |
| Indol production | + |
| Citrate utilization | + |
| H2S production | + |
| Glucose | + |
| Mannitol | – |
| Sucrose | + |
| Arabinose | + |
| Sorbitol | – |
| Insitol | – |
| Rhamnose | – |
| Maltose | + |
| Growth at 5 ºC | – |
| Growth at 15 ºC | – |
| Growth at 20 ºC | + |
| Growth at 26ºC | + |

**Table 4. Cumulative mortalities among fish groups challenged with different concentrations of *A. hydrophila* BNS 01614strain**

| Concentration CFU/ml | Number of fish dead | Mortality % | Post infection days of mortality |
|---|---|---|---|
| $3\times10^{8}$ | 4 | 100% | 2days |
| | 6 | | 3days |
| $1.5\times10^{8}$ | 3 | 60% | 4days |
| | 3 | | 7days |
| $1.5\times10^{7*}$ | 2 | 50% | 6days |
| | 3 | | 9days |
| $1.5\times10^{6}$ | 2 | 40% | 8days |
| | 2 | | 10days |
| $1.5\times10^{5}$ | 1 | 30% | 9days |
| | 2 | | 12days |
| control | - | - | - |

*The median lethal dose ($LD_{50}$)

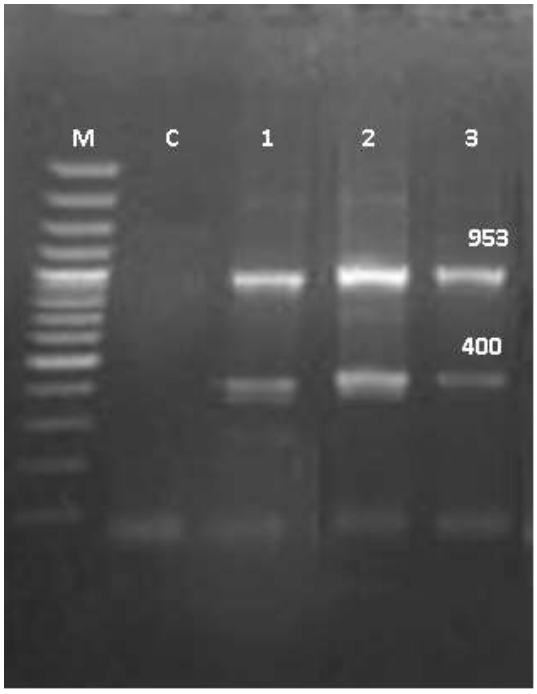


**Fig. 1** Electrophoresis analysis of duplex PCR assay for isolated *A. hydrophila* BNS 01614 strains. M, 100-3000 bp DNA ladder. Lane C, control negative. Lane 1, control positive *A. hydrophila* (BNS N#3). Lanes 2&3, investigated isolate represent bp953 for 16SrRNA and 400 bp for complex genes.

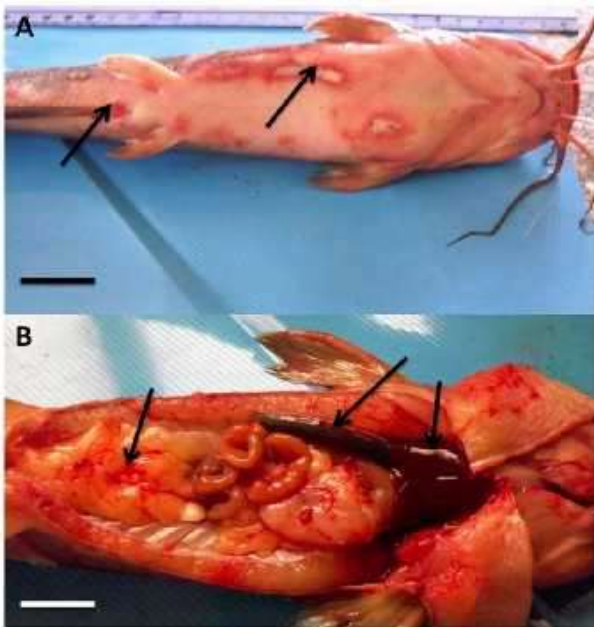


**Fig. 2** *C. gariepinus* showed the typical picture of hemorrhagic septcimia caused by experimental infection with the selected A. hydrophila BNS 01614 strain. (A), scattered hemorrhagic patches together with ulceration of external body surface (*arrows*); (B), internal lesions including diffuse hemorrhagic lesions within the body cavity together with congested, friable and enlarged liver with distention of the gall bladder (*arrows*). Scale bar = 4 cm.

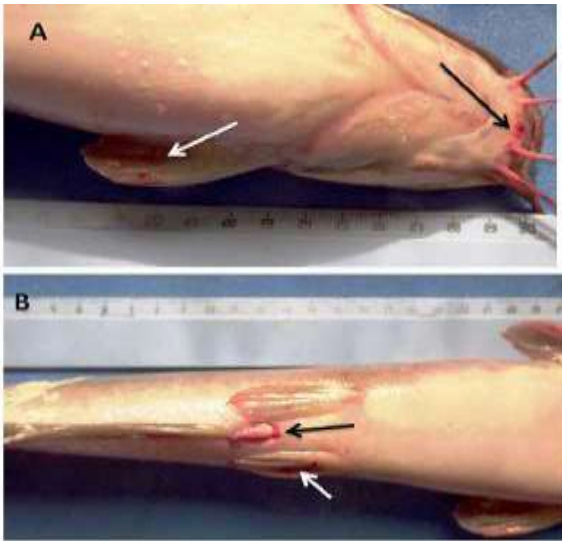


**Fig. 3** *C. gariepinus* showed signs of acute toxicity 24 hrs after injection of crude ECPs of selected *A. hydrophila* BNS 01416 strain. (A), haemorhgic patches at the base of lower jaw, barbells and base of pectoral fin (*arrows*); (B), haemorhges of genital papilae and at the base of anal fin (*arrows*)

## 4. Discussion

Motile aeromonas septicemia , caused by pathogenic members of the genus *Aeromonas*, considered as serious fish disease that severely impact freshwater aquaculture worldwide (Jayavignesh et al., 2011; Kavitha et al., 2014).The seriousness of *A. hydrophila* infection is its expanding fish host range (Austin and Adams, 1996; Zhang et al., 2014). Serious epizootics have been reported in catfish (Noor El-Din et al., 2014) and Nile tilapia (El-Barbary et al., 2010). Despite the fact that, *A. hydrophila* is a bacterium of fish, it infects humans posing a public health threats as it has zoonotic importance causing diarrhea and gastro-enteritis in man (Vally et al., 2004).

Results generated from basic morphological, physiological and biochemical conventional phenotypic characterization, revealed that the isolated selected strain BNS 01614 belonged to genus *Aeromonas*. Although these characteristics didn't exactly match this of Bergey's Manual of Determinative Bacteriology 9$^{th}$Edition (Holt et al., 1994) at the species level, they matched some those previously reported (Reyet al., 2009; Mohammed et al., 2013; Natiq et al., 2014; Ali et al., 2014). Moreover, Beaz-hidalgo et al., (2013) noted that the variable biochemical characteristics among *A. hydrophila* stains probably may be due to the presence and/or absence of plasmids that responsible for metabolic activities of *A. hydrophila*. Alternatively, bacterium-specific gene can be used as targets for PCR amplification to permit more specific detection as well as subspecies and strain differentiation. Previous conventional PCR studies demonstrated that the primer sets designated for amplification of 16SrRNA and *act/hlyA/aer* complex and Aerolysin genes were sensitive enough for identification and virulence determination of *A. hydrophila* by amplification of the specific 953 and 400 bands, which are specific fragments of 16SrRNA and *act/hlyA/aer* genes, respectively, (fig. 1) (Balsalobre et al., 2009; Pintoet al., 2012; Akhtar et al., 2013). Concerning the mortality patterns associated with experimental infection with selected *A. hydrophila* BNS 01614, the generated results revealed that serious mortalities extended in unique cumulative sub-acute patterns with mortality rates ranged between 30-100 %. These patterns were become obvious with both low and high doses of selected *A. hydrophila* BNS 01614 used in the experimentally infected fish groups (Table 4& fig. 2). These mortalities probably may be due to spreading growth of the bacterial patches from the site of infection, which typically secrete proteases, haemolysine, aerolysin, cytolytic, cytotoxic and entero-toxins (Favre *et al.*, 1993; Chopra and Houston, 1999) causing severe destruction and necrosis of the host tissues and enter the blood stream causing systemic septicemia (Sirirat et al., 1999; Sha et al., 2002; Balsalobreet al., 2009; Sarkar et al., 2013).

Regarding the $LD_{50}$ of selected *A. hydrophila* BNS01614, the results showed that the concentration of $1.5 \times 10^7$ CFU/mL could cause 50% cumulative mortalities in the tested *C. garipineus* within 9 successive days (Table 4). Intriguingly, those results were differed than the those reported by Pridgeon et al., (2013); Sahu et al., (2011); Behera et al.,(2013) and Alsapher et al.,(2012) who recorded that the $LD_{50}$ of their selected *A. hydrophila* strains in channel catfish, rohu spp., *Labeo rohita* and *C. carpio* were $1.3\times10^5$, $1.7\times10^4$, $1\times10^7$ and $0.3\times10^8$ CFU/mL, respectively. The difference in the $LD_{50}$ may be attributed to the nature of isolates, differences in fish species and experiment conditions as well.

Ultimately, the results within the represented study showed that the $LC_{50}$ of ECPs of the selected *A. hydrophila* BNS 01614 was 20µg/fish, which caused 50% mortalities in the tested *C. gariepinus* within 4 successive days (Table 5). The obtained results were different from those reported by Pridgeon et al., (2013) who recorded that the $LC_{50}$ of ECPs of *A. hydrophila* in channel catfish was 16µg/fish. In addition, Kalil and Mansour (1997) and Sahu **et**

al., (2011) recorded the lethal toxicity of crude ECPs of *A. hydrophila* at a dose of 0.1 mL/fish in Nile tilapia and rohu spp. were 100% by IP injection and the death was occurred within a day after injection. Nevertheless otherwise specified, the differences in the lethal toxicity and/or $LC_{50}$ of ECPs of *A. hydrophila* may be in close relationship with the virulence of the *A. hydrophila* strain, ECPs contents, culturing conditions, fish species and experiment conditions.

## 5. Conclusion

The present work generated some information on the pathogenicity of *A. hydrophila* and lethality of its ECPs *in vivo*. This study proved that *A. hydrophila*, though opportunistic,is a serious pathogen for catfish could cause severe septicemic picturein catfish. As a ubiquitous species, *A. hydrophila* are available in water, fish body, and other aquatic animals. So, proper preventive measures should be taken in consider for prevention of the disease.

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