

Pathogenicity of *Aeromonashydrophila* and lethality of its extra-cellular products in sharp teeth catfish, *Clariasgariepinus*, (Burchell)

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Abstract

A study was conducted to investigate the pathogenicity and the median lethal dose (LD₅₀) of *Aeromonashydrophila* 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 *Aeromonashydrophila* strain BNS 01614 including 3×10^8 , 1.5×10^8 , 1.5×10^7 , 1.5×10^6 and 1.5×10^5 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 LD₅₀ 1.5×10^7 CFU/fish. The Concentrated extracellular products (ECPs) of the selected bacterium were prepared and confirmed to be toxic in *Clariasgariepinus* with LC₅₀ of 20 µg.

Key Words: *Aeromonashydrophila*, catfish, LD₅₀, Pathogenicity

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, *Clariasgariepinus* 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 *Aeromonashydrophila*. *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 LD₅₀ (median lethal dose) of *A.hydrophila* in the catfish; (3) determine the lethality ofextracellular products (ECPs) of the selectedstrainin 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. Bacterialisolation 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, biochemicallyandmolecularlyidentifiedandverifiedas described by Balsalobre et al.,

(2009); Alsapharet 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 3×10^8 and 1.5×10^8 CFU/ml, respectively. The bacterial suspension was tenfold serial diluted was performed 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 saline 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 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 (Oxoid®) and incubated on a rotatory shaker (SK – L330- Pro, SCI – Logex, Rhsin Land, USA) for 18hrs at 26°C. After elapsing of incubation time, the ECPs were obtained by centrifugation using high speed cooling centrifuge (Centurion scientific, core life sciences®, Inc. US) at 5000 rpm at 10°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 plated 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 (HPSS, 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 4° C to remove any traces of TSB (Oxoid®). 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 six 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 & photo 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 & photo 2). Signs of septicemia (external & internal, asscattred petechial haemorrhges allover 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. Determiration 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. hydrophilawere* determined as mentioned in materials and methods section and adjusted to a concentration of 0.5µg/µL.

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

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 LC₅₀ was found to be 20µg/fish. Notably, the clinical signs were observed after 4 days post injection (photo, 3) with the low doses of ECPs used.

4. Discussion

Motile aeromonas septicemia (MAS), 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. hydrophilain*infection is its expanding fish host range (Austin and Adams, 1996; Zhang et al., 2014).Serious epizootic syndrome has 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 threat as it has zoonotic importance causing diarrhea and gastroenteritis in man (Vally et al., 2004).

Results generated from basic morphological, physiological and biochemical characterization, using conventional phenotypic characteristics, 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 9th Edition (Holt et al., 1994) at the species level, they matched some those previously reported (Reyet et 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* strains 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 primers A16S1 and *act/hlyA/aer* complex primers were sensitive enough to identification and virulence determination of *A. hydrophila* by amplification of the specific 953-bp band and 400-bp specific fragments of 16srRNA and *act/hlyA/aer* genes (Balsalobre et al., 2009; Pinto et al., 2012; Hussain 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 & photo 2). These mortalities probably may be due to spreading growth of the bacterial patches from the site of infection, which typically secrete proteases, haemolysins, 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 bloodstream causing systemic septicemia (Sirirat et al., 1999; Sha et al., 2002; Balsalobre et al., 2009; Sarkar et al., 2013).

Regarding the LD₅₀ of selected *A. hydrophila* BNS 01614, the results showed that the concentration of 1.5×10^7 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 LD₅₀ of their selected *A. hydrophila* strains in channel catfish, rohuspp, *Labeorohita* and *C. carpio* were 1.3×10^5 , 1.7×10^4 , 1×10^7 and 0.3×10^8 CFU/mL, respectively. The difference in the LD₅₀ 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 LC₅₀ of ECPs of the selected *A. hydrophila* BNS 01614 was 20µg/fish, which caused 50% mortalities in the tested *C. garipineus* within 4 successive days (Table5). The obtained results were different from those ones reported by **Pridgeon et al., (2013)** who recorded that the LC₅₀ of ECPs of *A. hydrophilain* 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 rohuspp. 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 LC₅₀ 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 picture in 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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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	3×10^8	IP	0.1
Group 2	10	1.5×10^8	IP	0.1
Group 3	10	1.5×10^7	IP	0.1
Group 4	10	1.5×10^6	IP	0.1
Group 5	10	1.5×10^5	IP	0.1
Control	10	PBS	IP	0.1

Table 2. Designed regime for determination EC₅₀s 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	PBS	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	+
H ₂ S production	+
Glucose	+
Mannitol	-
Sucrose	+
Arabinose	+
Sorbitol	-
Inositol	-
Rhamnose	-
Maltose	+
Growth at 5 °C	-
Growth at 15 °C	-

Growth at 20 °C	+
Growth at 26°C	+

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

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

*The median lethal dose (LD₅₀)

Table 5. Toxicity of ECPs of selected *A. hydrophila* BNS 01614 in catfish

Dose (μg)	Number of fish dead	Mortality %	Post infection days of mortality
10	1	30%	3days
	3		5days
20*	3	50%	3days
	2		4days
30	4	70%	2days
	3		3days
40	5	90%	1day
	4		
50	6	100%	12hours
	4		24hours
control	-	-	-

*LC₅₀ of ECPs

Photo 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 isolates represent bp953 for 16SrRNA and 400 bp for complex genes

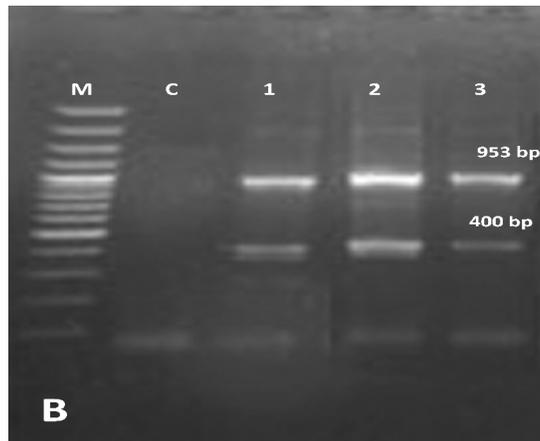


Photo 2. *C. garipinus* showed the typical picture of haemorrhagic septicemia caused by experimental infection with selected *A. hydrophila* BNS 01614 strain. (A), scattered haemorrhagic patches together with ulceration of external body surface (arrows); (B), PM lesions after including diffuse hemorrhagic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

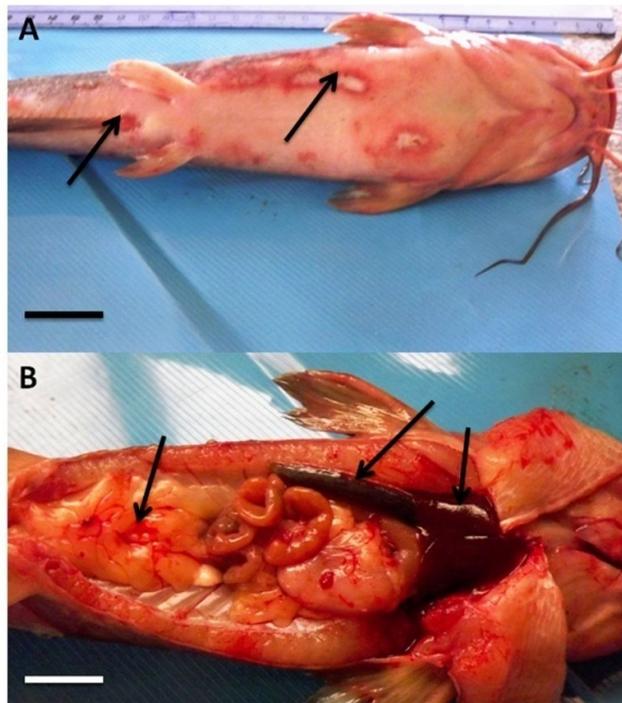


Photo 3. *C. garipinus* showed signs of acute toxicity 24 hrs after injection of crude ECPs of selected *A. hydrophila* BNS 01416 strain. (A), haemorrhagic patches at the base of lower jaw, barbels and base of bectoral fin (arrows); (B), haemorrhages of genital papilae and at the base of anal fin (arrows)

