

***Aeromonas hydrophila*, one reason causing the death of freshwater crayfish *Procambarus clarkii* (Girard, 1852)**

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Abstract

Procambarus clarkii crayfish has been developed as an excellent animal model to study the characteristics and pathogenesis of white spot syndrome virus and the host defense mechanisms of crustacean. However, due to pathogen infections of crayfish in Chinese farms, research based on this model has been slowed down. Using triplex polymerase chain reaction, we found the virulence *Aeromonas hydrophila* was one reason that caused the death of crayfish. The isolated AH-3 produced 100% mortalities with 3-24h after inoculation of 1×10^6 colony-forming units of live cells per crayfish at 20 °C.

Keywords: White spot syndrome virus, *Aeromonas hydrophila*, *Procambarus clarkii*, Triplex polymerase chain reaction

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Introduction

The gamma-proteobacteriacea *Aeromonas* spp. is widely distributed and can be isolated from soil, fresh water, and seawater (Albert *et al.*, 2000). Aeromonads are food- and water-borne bacteria and are considered to be zoonotic human pathogens that can cause severe diarrhea, dysentery, and bacteremia (Blair *et al.*, 1999; Trower *et al.*, 2000). Now, the number of published species of the *Aeromonas* spp is 25 (Figueras *et al.*, 2011). Among them, *A. hydrophila*, *A. sobria* and *A. caviae*, are the most commonly known species to cause diseases in human or animals (Hu *et al.*, 2012). *A. hydrophila* has also been identified as the dominant infectious agent of “fish-bacterial-septicaemia” in the freshwater cultured cyprinid fishes, including of crucian carp *Carassius carassius*, Wuchang bream *Megalobrama amblycephala*, silver carp *Hypophthalmichthys molitrix*, bighead carp *Aristichthys nobilis*, common carp *Cyprinus carpio* and grass carp *Ctenopharyngodon idellus* in the southeast China in the 1990s. The “fish-bacterial-septicaemia” occurs from April to November, especially in summer. The cumulative mortality may reach 80 to 100%, (Chen and Lu, 1991). A number of virulence factors have been identified in *A. hydrophila*, namely, flagella, pili and adhesions (Quinn *et al.*, 1993; Pepe *et al.*, 1996; Kirov *et al.*, 2004), O-antigens and capsules (Martinez *et al.*, 1995; Zhang *et al.*, 2002; Zhang *et al.*, 2003), S-layers (Dooley and Trust, 1988), exotoxins such as haemolysins and

enterotoxin (Santos *et al.*, 1988; Howard *et al.*, 1996), and many extracellular proteins including amylase, chitinase, elastase, aerolysin, nuclease, gelatinase, lecithinase, lipase and protease (Pemberton *et al.*, 1997). Several virulence-associated genes have been targeted for the detection of potentially pathogenic aeromonads by PCR assays (Nam and Joh, 2007; Balakrishna *et al.*, 2010).

Red swamp crayfish *Procambarus clarkii*, a wild species of China, often used experimentally to examine the pathogenesis of white spot syndrome virus (WSSV) and the immune system of crustacean (Wei *et al.*, 1998; Zeng and Lu, 2009). Due to increased domestic and export demand, *P. clarkii* is now cultured widely in China. However, the pathogens such as bacteria, fungi and viruses have been reported in the cultured crayfish (Wang *et al.*, 2007; Chen *et al.*, 2009; Zhou *et al.*, 2011). The main reason for the outbreak disease in crayfish is WSSV and there are also a few papers mentioning bacterial pathogen such as *A. hydrophila* and *Vibrio parahaemolyticus* being the causative agents. Having been infected with pathogens in the wild field, crayfish is hard to feed in laboratory. Thus, the researches projectsoften got into trouble as crayfish usually die before experiment.

In the present work, a multiplex PCR assay was reported for the detection of virulence-associated *aer*, aerolysin gene and *ahp*, serine-protease gene and a species-specific region of 16S rDNA of *A. hydrophila* in the dead crayfish in

laboratory. The results obtained were confirmed by 16S rDNA gene sequence analysis.

Materials and methods

Procambarus clarkii crayfish, ranging in weight from 20 to 30 g were purchased from the market in Yantai of China, and maintained in aerated freshwater about 20°C in the laboratory. The plastic containers and other instruments used to hold crayfish were sterilized before experiment. They were dissected to detect the WSSV and *A. hydrophila* as soon as they were dead. Meanwhile, the asymptomatic crayfish lived in the laboratory for more than one week was used to study the histopathology and virulence of *A. hydrophila*.

Touching the haemolymph of the crayfish with an inoculating loop, the bacteria were inoculated on the tryptic soy agar (TSA) and incubated at 28°C for one day. The bacterial colony on the

TSA plate was boiled with a water bath in 20µl water for 10 min and centrifuged at 12000×g for 5min. The supernatant was used as a template for PCR reaction. The information of primers was listed in Table 1. Triplex-PCR assays were performed with reaction mixtures (final 20µl) containing 1µl of aerF (10µM), 1µl of aerR (10µM), 1µl of ahpF (10µM), 1µl of ahpR (10µM), 0.5µl of 16SrDNAF (10µM), 0.5µl of 16SrDNAR (10µM), 2µl of 10×buffer, 1.2µl of MgCl₂ (25mM), 0.5µl of dNTP (10mM), 9.9µl PCR-grade water, 0.4µl (2U) of Taq DNA polymerase and 1µl of template. PCR was carried out by the following conditions: pre-denaturation at 94°C for 5min, 35 cycles of 94°C for 30s, 60°C for 30s, 72°C for 30s, then a further 10min at 72°C for final extension. The PCR products were analyzed on 1.5% agarose gel to check the virulent *A. hydrophila*.

Table 1: Primers for multiplex PCR amplification of *Aeromonas hydrophila*.

Primer	Sequence (5'-3')	Product size (bp)	Target gene	Reference
aerF	ATGACGGCGGTTGCGAGGGTT	561	aerolysin	Wang <i>et al.</i> , 2008
aerR	TGTGGTTCCAGTTCGGGCGATTGT			
ahpF	CTCCTACTCCAGCGTCGGC	128	serine-protease	Wang <i>et al.</i> , 2008
ahpR	GATCGTCGGTGCGGTTGT			
16SrDNAF	GAAAGGTTGATGCCTAATACGTA	686	16S rDNA	Chu and Lu, 2005
16SrDNAR	CGTGCTGGCAACAAAGGACAG			
vp28F	TGAGGTTGGATCAGGCTAC	302	VP28 of WSSV	Our laboratory
vp28R	TGAAGGAGGAGGTGTTGG			

Two strains confirmed with three correct bands by triplex-PCR were chosen randomly to be amplified with primer pair 16SrDNAF and 16SrDNAR again. The products were inserted into the pUCm-T vector (Sangon) and sequenced by Shanghai Sangon Biological Engineering Technology &

Services Co.,Ltd.

Gill tissue (100 mg) from individual crayfish was homogenized in 0.4 ml Proteinase K buffer (10 mM Tris-HCl Ph 7.8, 5 mM EDTA, 0.5% SDS) in a sterile 1.5ml microfuge tube using a disposable tissue grinding pestle followed by the addition of 1 µl 20 mg

ml⁻¹ Proteinase K and overnight incubation at 55°C. Total DNA was then extracted using a standard phenol-chloroform method, precipitated under ethanol overnight at -20°C and recovered by centrifugation at 15000×g for 10 min at 4°C. The DNA pellet was dissolved in 30 µl TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA) for PCR with the primer pair vp28F and vp28R (Table 1) to detect the WSSV. The PCR program was performed as follows: 94 °C for 5min, followed by 35 cycles of 94 °C for 30s, 56 °C for 30s, 72 °C for 30s, then a further 10min at 72 °C for final extension. The PCR products were analyzed on 1.5% agarose gel to identify the WSSV.

The *A. hydrophila* strain AH-3 identified in this paper was cultured overnight in tryptic soy broth (TSB) at 28°C. The bacteria cell were washed three times with sterile 0.01mM phosphate buffered saline (PBS) (8 g NaCl, 0.2 g KCl, 1.44 g Na₂HPO₄, 0.24 g K₂HPO₄, adjusted pH to 7.4, added dH₂O to 1 L) by centrifugation at 5000×g for 5min at room temperature. Cell concentration was adjusted and verified by viable plate counts and was recorded by colony-forming units (CFU) per milliliter. The test crayfish samples were injected intramuscularly with 0.1ml of the bacterial suspension. The control crayfish individuals were injected with PBS and handled similarly.

The histopathological studies of crayfish infected by *A. hydrophila* strain AH-3 were compared to the crayfish receiving injection of PBS. The tested crayfish samples were injected

with live bacterial cells of strain AH-3 at doses of 1×10⁶CFU in 0.1 ml per crayfish. Meanwhile, the control crayfish ones were injected with the same volume of sterile PBS. The crayfish were sampled from each trial at 12h after injection. All samples were fixed in Bouin's fluid and dyed by H&E staining.

Results

The triplex-PCR was optimized using the PCR conditions as indicated in material and methods. The bacterium confirmed with three correct bands was identified to be virulent *A. hydrophila* (Fig.1). The sequences obtained from the two delegates were compared with the sequences in the database GenBank (<http://ncbi.nlm.nih.gov>) by using the BLASTN program. The nucleotide identifies between these two samples and the 16S r DNA of *A. hydrophila* (GenBank accession no. AB698740) are both 99%. They belong to *A. hydrophila*.

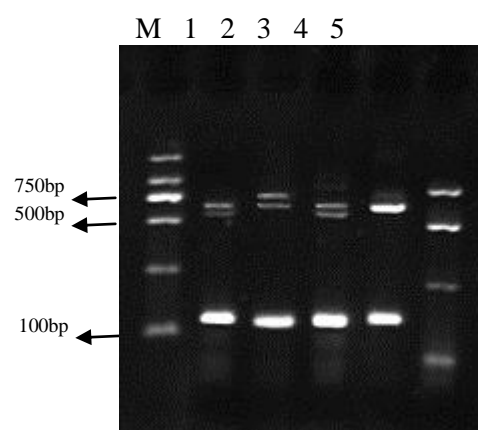


Figure 1: Results of triplex-PCR. Lane M: DL2000 DNA marker; lanes 1 and 3: the virulent *Aeromonas hydrophila*; lane 2,4 and 5: other bacterial strains.

A 302bp gene fragment was amplified

from the genomic DNA of the crayfish with primer pair vp28F and vp28R which suggests that the crayfish has been infected with WSSV (Fig. 2).

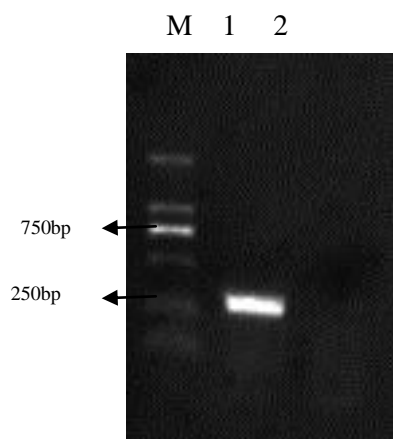


Figure 2: Results of WSSV detection. Lane M: DL2000 DNA marker; lane 1: WSSV infected crayfish; lane 2: uninfected crayfish.

The experiment was repeated twice. All of the crayfish died within one week after they arrived in the laboratory. The virulent *A. hydrophila* strains were found in all 12 crayfish in the first group and only one of them was verified to be infected with WSSV. Similar results were obtained for the second group. All of the 15 crayfish were found to be infected with virulent *A. hydrophila* and the WSSV was detected in only two of them.

The isolated AH-3 caused 100% mortalities within 3-24h after

inoculation of 1×10^6 CFU of live cells per crayfish at 20 °C. It was a highly virulent pathogen. No death was observed in the control group. *A. hydrophila* was re-isolated in pure culture after haemolymph from dead crayfish was streaked onto TSA. Therefore, *A. hydrophila* AH-3 has been chosen for further pathogenesis studies to illuminate how this bacterium can be so harmful to the crayfish.

The crayfish samples have been injected with AH-3 became moribund within 4-6h post-injection, whereas all crayfish have been received an injection with PBS were alive after 12h injection. These crayfish were sacrificed for histopathological examinations. Crayfish injected with AH-3 showed extensive necrotic lesions with pyknotic nuclei in various tissues including heart, gills and interstitial tissue of hepatopancreas, the haemolymph circulatory system and massive haemocytes were infiltrated and aggregated in the haemal sinuses with the appearance of many pyknotic nuclei (Fig. 3a-c, arrows). No pathological changes have been observed in the control crayfish injected with PBS (Fig. 4).

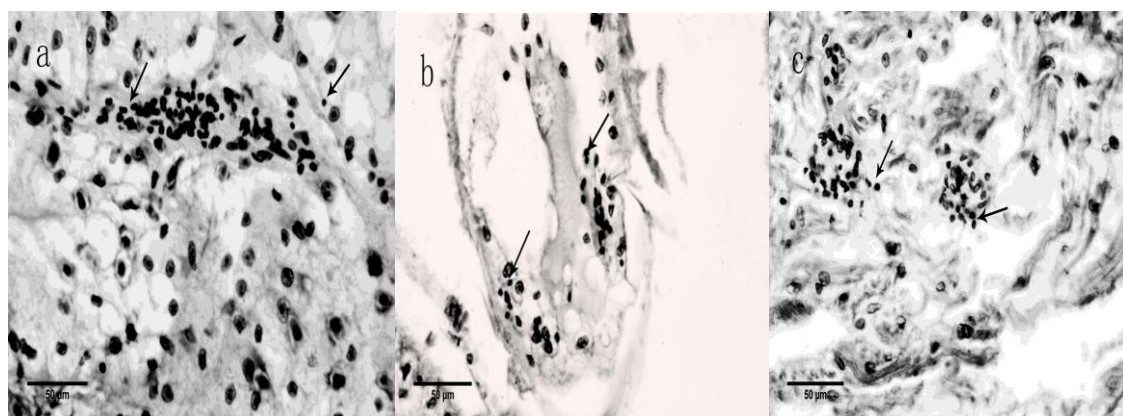


Figure 3: Tissue sections of crayfish injected with *Aeromonas hydrophila* AH-3. The hepatopancreas (a) showing dead cells with pyknotic nuclei (arrows) scattered in hemal sinus as well as in hemal canals (c) of the gill lamellae. Heart showing obviously infiltrated and aggregated hemocytes and necrotized tissue in the myocardium.

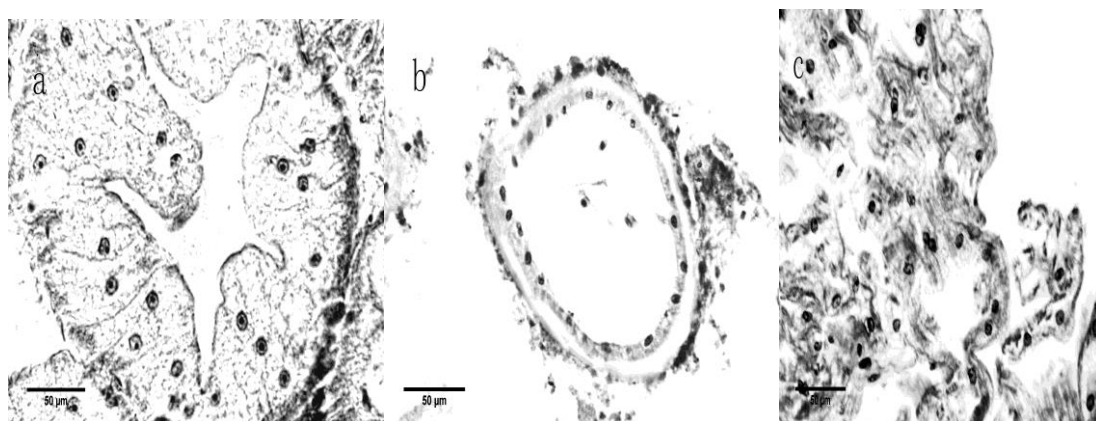


Figure 4: Control crayfish injected with PBS. No pathological changes in any tissue including hepatopancreas, gill and heart could be observed.

Discussion

A mixed bacterial population, including *Pseudomonas*, *Aeromonas*, *Acinetobacter*, *Flavobacterium*, *Vibrio*, *Micrococcus* and *Staphylococcus*, can be isolated from the apparently healthy crayfish species such as *P. clarkii* (Edgerton *et al.*, 2002). It is believed that the aquatic crustaceans can tolerate bacteria in the haemolymph with no apparent detrimental effects. The disease is likely to occur only when the host is stressed by unfavorable environmental conditions. *A. hydrophila* is abundant in water fauna, the crayfish may get an opportunistic

infection whereby virulent strains invade the haemocoel through the oral route or wounds, proliferate in the hemolymph and then multiply in all tissues. As a result, the immune system of crayfish was destroyed by cell death with pyknotic nuclei and karyorhexis, consequently causing death of crayfish after infection.

The virulent *A. hydrophila* produces a series of virulence factors. The aerolysin gene and serine-protease gene were often used as targets to detect the virulent *A. hydrophila* by PCR (Nam and Joh, 2007; Singh *et al.*, 2008; Balakrishna *et al.*, 2010; Li *et al.*, 2011).

Besides these two kinds of genes, haemolysin (*hlyA*), cytolytic aerolysin (*Aero*), glycerophospholipid-cholesterol acyl transferase (*GCAT*), cytotoxic enterotoxin (*alt*), lipase (*lip*), glycerophospholipid: cholesterol acyltransferase (*gcaT*), DNases (*exu*), elastase (*ahyB*) and the structural gene flagellin (*fla*) were often used as virulence genes to detect the virulent *Aeromonas* spp (Chu and Lu, 2005; Balakrishna *et al.*, 2010; Nawaz *et al.*, 2010; Li *et al.*, 2011). The specificity of species-specific primer pair 16SrDNAF and 16SrDNAR had been confirmed by Chu and Lu (2005). Only a 686bp fragment can be amplified from the genomic DNA of *A. hydrophila*, but not any other *Aeromonas* spp. The triplex-PCR performed with these three pairs of primers in this study was efficient to identify the virulent *A. hydrophila* in the crayfish.

The WSSV overshadows all other crustacean disease agents and becomes one of the most devastating viruses to penaeid shrimp (Hossain *et al.*, 2001). A major outbreak of WSSV infection in 1993 resulted in a 70% reduction in shrimp production in China (Cen, 1998; Zhan and Wang, 1998). The WSSV was first reported in *P. clarkii* farmed in China in 2007 (Wang *et al.*, 2007) and now white spot disease (WSD) has become a major problem for the crayfish-farming industry (Ding *et al.*, 2008; Xu *et al.*, 2008; Liang *et al.*, 2010). There were only a few reports regarding the bacterial pathogens including *A. hydrophila* and *V. parahaemolyticus* (Chen *et al.*, 2009; Zhou *et al.*, 2011). Besides having been

cultured widely in China as an important economic species, the *P. clarkii* has also been developed as an excellent animal model to examine the pathogenesis of WSSV and the host defense mechanisms of shrimp (Wei *et al.*, 1998; Zhu and Lu, 2001; Du *et al.*, 2006, 2007; Li *et al.*, 2006; Zeng and Lu, 2009). However, crayfish often died after arriving in laboratory environment, causing the slow-down of research based on this animal. There was no paper describing the pathogen responsible for the mortality.

From the results of triplex-PCR, the virulent *A. hydrophila* has been identified in each of the dead crayfish in this paper. Meanwhile, the WSSV was only detected in a few of them. The virulent *A. hydrophila* should be one reason to cause the death of freshwater crayfish *P. clarkii*. A highly virulent *A. hydrophila* produced 90-100% mortalities within 6-15h after inoculation of 4.5×10^5 - 2.5×10^6 CFU of live cells per crayfish was also detected in the freshwater crayfish *Pacifastacus leniusculus* (Jiravanichpaisal *et al.*, 2009). In general, besides the infection of WSSV, we should pay more attention to the infection of bacterial pathogen, especially the virulent *A. hydrophila* in crayfish.

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