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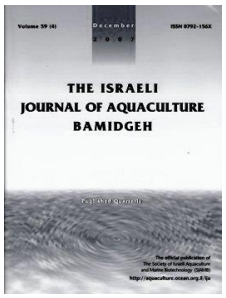

Aeromonas schubertii: a Potential Pathogen for Freshwater Cultured Whiteleg Shrimp, *Penaeus vannamei*

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Abstract

Bacteriosis has become a major global economic problem in freshwater farmed whiteleg shrimp *Penaeus vannamei*. However, limited information is available on the incidence of *Aeromonas schubertii* infections in freshwater cultured *P. vannamei*. Red body disease, an epidemic frequently associated with *P. vannamei* freshwater farming, occurred in a *P. vannamei* farm in Shanghai China in June 2013. A pathogenic strain of *A. schubertii* (isolate HS1) was isolated from diseased freshwater cultured *P. vannamei* suffering from red body disease, and identified through phylogenetic analysis and phenotypic characteristics. A phylogenetic tree was constructed to examine the relatedness of isolate HS1 with other *A. schubertii* isolates. In addition, isolate HS1 showed no signs developing antibiotic resistance when screened against a range of common antibiotics used in aquaculture. To the best of our knowledge, this is the first report of *A. schubertii* infection in freshwater farmed *P. vannamei.*

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Introduction

Whiteleg shrimp *Penaeus vannamei* is widely distributed and cultivated in Hawaii, South Carolina, Texas, China, Thailand, Vietnam, Brazil, Mexico, Venezuela, Peru and Puerto Rico (Briggs et al., 2004; Wakida-Kusunoki et al., 2011). With the rapid development of farming techniques, *P. vannamei* has been successfully cultured in freshwater in China since 2000 (Ding et al., 2014). It has become the most important commercial shrimp species in China and has been extremely profitable financially (Funge-Smith et al., 2004). However, bacterial disease has become a major economic problem in *P. vannamei* freshwater farming (Wang et al., 2013). *Proteus penneri* has been recognized as an important and destructive pathogen for freshwater cultured *P. vannamei*, causing widespread outbreaks of red body disease (Cao et al., 2014). More focus on bacteriosis is needed to improve the development of a sustainable *P. vannamei* freshwater farming industry.

Red body disease causes significant economic losses in the cultured shrimp industry in China, India, the Philippines and other countries in Southeast and East Asia (Alapide-Tendencia and Dureza, 1997; Zheng et al., 2011). Thus, more attention should be paid to this pathogen. Several bacterial pathogens such as *Vibrio alginolyticus*, *Vibrio parahaemolyticus* and *Proteus penneri* have been reported to cause red body disease of farmed *P. vannamei* (Zhou et al., 2003; Fan et al., 2006; Chen et al., 2012a; Cao et al., 2014). However, no information is available on the occurrence of *A. schubertii* infections in freshwater farmed shrimp.

In this study, a pathogenic strain of *A. schubertii* was isolated from freshwater cultured *P. vannamei* suffering from red body disease in Shanghai China in June 2013. The aim of this study was to characterize the phenotype, taxonomic position, and antibiotic sensitivity of this strain. As far as we know, this is the first report of infection by *A.schubertii* in freshwater farmed *P. vannamei*

Materials and Methods

Whiteleg shrimp samples. The first disease outbreak involving 26 freshwater cultured *P. vannamei* (average weight 7.6±0.6 g) suffering from red body disease, characterized by the reddening of the shrimp body as described by Chen et al. (2012a) occurred at a 400 acre farm of ponds with whiteleg shrimp stocked at an initial rearing density of 60,000 juvenile shrimps per acre. The water quality parameters during the disease outbreak were: pH 8.73, 7.8 mg/l of dissolved oxygen, 0.30 mg/l of total ammonia, and 0.25 mg/l of nitrite. The disease could not be controlled although providone-iodine, and chlorine dioxide were used. Diseased samples were placed in sterile bags, kept in ice and transported to the laboratory.

Isolation of Bacteria. Diseased *P. vannamei* were externally disinfected with 75% alcohol, dissected and sampled for casual organisms. Before conducting a careful microscopic examination for gill parasites, a 0.2 g hepatopancreas sample of each shrimp was cut and streaked onto nutrient agar (NA) plates (Sinopharm Chemical Reagent Co., Ltd.) as described by Cao et al. (2014). After incubation for 24-48h at 28^0C, the most common uniform isolates were purified by streaking and re-streaking onto NA plates. Pure isolates of these colonies were stored at -80^0C supplemented with 15% glycerol. A representative of these colonies, isolate HS1, was characterized further in the present study.

Phenotypic identification. Isolate HS1 was identified phenotypically by conventional methods described by Dong and Cai (2001). Isolate HS1 was grown on NA plates (Sinopharm Chemical Reagent Co., Ltd.) at 28^0C for 24h, and a bacterial suspension was then used to inoculate biochemical tubes (Hanghzhou Tianhe Microorganism Reagent Co. Ltd., China) to test for motility, Voges-Proskauer reaction, malonate utilization, indole production, H_2S production, arginine dihydrolase, gelatinase production, lysine decarboxylase, ornithine decarboxylase, and urease activities, acid production from D-arabitol, D-glucose, D-mannitol, D-mannose, dulcitol, erythritol, L-arabinose, lactose, and maltose. The tubes were incubated and observed according to the manufacturer's instructions. Information relating to *A. schubertii* in *General manual of systematic and determinative bacteriology* served as a reference (Dong and Cai, 2001).

Bacterial virulence assay. Bacterial virulence was examined by experimentally infecting healthy freshwater cultured shrimp. Two hundred and forty healthy shrimp (average weight 7.1±0.6 g) were obtained from Pinghu Aquaculture Co., Ltd. in Zhejiang China. Their health status was assessed according to the guidelines recommended by the Marine Products Export Development Authority & Network of Aquaculture Centers in Asia-Pacific (2003). The shrimp were maintained in 6 replicate aquaria (40 shrimps per aquarium) containing 100 l aerated filtered farm water at 28^0C for 14 days to acclimate. Prior to the bacterial virulence assay, isolate HS1 was inoculated into 100 ml nutrient broth (NB) according to Cao et al. (2010), incubated at 28^0C and shaken at 200 rpm for 24h. Colony forming units were counted after a 10-fold serial dilution in sterile distilled water. Three replicates of 40 healthy shrimps were challenged by immersion in 100 l of water containing the isolate HS1 at a concentration of 5.0×10^6 CFU/ml. Another three replicates of 40 healthy shrimps exposed to the same experimental conditions remained unchallenged and served as control. The experimental shrimps were kept at 28^0C and observed daily for 7 days without feed or water change. Dead shrimp were immediately removed and sampled to re-isolate and confirm if mortality was caused specifically by the challenge isolate.

Antibiotic sensitivity assay. The antibiotic sensitivity of isolate HS1 was assayed on NA plates using the Kirby-Bauer disk diffusion method as recommended by Jones et al. (2001). Nine fishery antibiotic discs were acquired from Hangzhou Tianhe Microorganism Reagent Co., Ltd. The zones of inhibition were measured after a 24h incubation period at 28^0C. The antibiotic susceptibility was determined according to the manufacturer's guidelines.

Results

Identification of the pathogenic isolate. A strain, HS1 of bacteria was isolated from the diseased freshwater farmed shrimp and identified by molecular and phenotypic methods as *A. schubertii.* Its near complete 16S rRNA gene sequence (1450 nucleotides) was submitted to GenBank database with the accession no. KF307773. The similarity between the 16S rRNA gene sequence of the HS1 isolate and other *A. schubertii* isolates in the GenBank database was 99%. The phylogenetic tree, constructed according to the neighbor-joining method confirmed that the isolate HS1 was an *A. schubertii* strain (Figure 1). This was again confirmed by the phenotypic features as *A. schubertii* (Table 1) with 100% identity compared to the type strain. No parasites were detected in the diseased shrimps from which isolate HS1 was obtained.

Figure 1. A 16S rRNA gene tree of 16 known bacteria and the HS1 isolate constructed using the neighbor-joining method. The bootstrap values (%) are shown besides the clades, accession numbers are indicated beside the names of strains, and scale bars represent distance values.

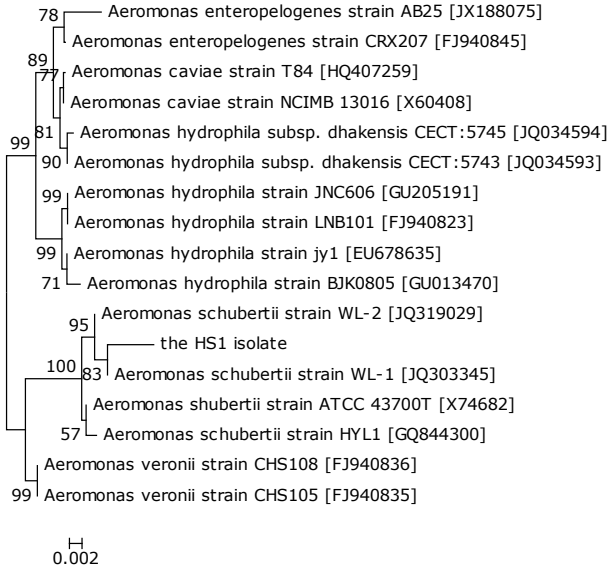

Table 1. Phenotypic characterization of isolate HS1.

Tests	*Reaction*	
	HS1	*A. schubertii*[a]
Motility	R^+	R^+
Voges-Proskauer	R^-	R^-
Malonate utilization	R^-	R^-
Indole production	R^-	R^-
H_2S production	R^-	R^-
Gelatinase production	R^+	R^+
Arginine dihydrolase	R^+	R^+
Lysine decarboxylase	R^+	R^+
Ornithine decarboxylase	R^-	R^-
Urease	R^-	R^-
Acid production from		
D-arabitol	R^-	R^-
D-glucose	R^+	R^+
D-mannitol	R^-	R^-
D-mannose	R^+	R^+
Dulcitol	R^-	R^-
Erythritol	R^-	R^-
L-arabinose	R^-	R^-
Lactose	R^-	R^-
Maltose	R^+	R^+

[a]The reference strain's data are in accordance with those previously reported (Dong and Cai, 2001); [+]positive reaction; [-]negative reaction.

Figure 2. Pathological symptoms of the cultured shrimps suffering from red body disease. Arrows indicate diseased shrimps.

Isolate HS1 was found to be pathogenic in an experimental challenge. The death of shrimp began one day after the challenge and increased gradually over time. 96% of the shrimps challenged with isolate HS1 died and exhibited a reddening of their body, similar to that seen in the originally diseased shrimps (Figure 2).

In addition, the re-isolated bacteria from dead shrimp were identified phenotypically and molecularly as HS1. No clinical signs or mortality were noted in the control shrimps.

Antibiotic sensitivity. The antibiotic sensitivity of isolate HS1 is shown in Table 2. The data indicate that isolate HS1 is sensitive to enrofloxacin, norfloxacin, ofloxacin, gentamicin, tetracycline, neomycin, trimethoprim-sulfamethoxazole, streptomycin, and intermediately sensitive to sulfamethoxydiazine. This suggests that isolate HS1 is not resistant to these antibiotics.

Table 2. Susceptibility of isolate HS1 to antibiotics.

Antibiotics	*Content (μg/disc)*	*Inhibition zone diameter (mm)*
Enrofloxacin	5	25.80 ± 0.47^S
Norfloxacin	10	25.15 ± 0.10^S
Ofloxacin	5	27.28 ± 0.27^S
Gentamicin	10	20.75 ± 0.35^S
Sulfamethoxydiazine	5	14.08 ± 0.60^I
Tetracycline	30	30.25 ± 0.35^S
Neomycin	30	23.90 ± 1.68^S
Trimethoprim-sulfamethoxazole	23.7/1.25	29.00 ± 1.41^S
Streptomycin	10	19.16 ± 1.38^S

Data are presented as the mean ± standard deviation; [S]Sensitive; [I]Intermediately sensitive.

Discussion

In aquaculture, massive mortality due to *A. scubertii* infection in *Channa maculata* (Lacepède), (Chen et al., 2012b), *Garra rufa* (Yu et al., 2009), *Ophiocephalus argus* (Cantor) (Liu and Li, 2012), *Oncorhynchus mykiss* Walbaum (Akaylı et al., 2011) and *Puntius ticto* (Hamilton) (Hossian et al., 2011), has been well documented. However, there is no definitive information on isolation of *A. schubertii* in cultured shrimps (Oxley at al., 2002). In this study, we characterized the phenotype, taxonomic position, and antibiotic susceptibility of *A. schubertii* HS1. To our knowledge, this is the first report of *A. schubertii* pathogen in freshwater farmed *P. vannamei*.

Aeromonas spp. produce numerous pathogenic effects including the secretion of extracellular enzymes, cytotonic enterotoxins, and hemolysins (Carnahan et al., 1989; Daskalov, 2006). Infection by *A. schubertii* is usually associated with the production of components such as hemolysin, elastase, lipase, and lecithinase (Chen et al., 2012b). In the present study, the HS1 isolate was found to be pathogenic to healthy *P. vannamei* leading to 96% mortality. This further demonstrates the potential threat of *A. schubertii* to shrimp aquaculture. Apart from the virulence of the HS1 isolate, there may be other secondary factors that are conducive to red body disease development in shrimps such

as over intensification of shrimp culture and the use of contaminated feed; these should also be raised as concerns.

Although the HS1 isolate in our study was susceptible to almost all the common antibiotics used in the shrimp farming regions, suggesting that the outbreak of this disease may not be related to the abuse of antibiotics, antibiotic resistance in *A. schubertii* has been reported elsewhere as a result of the wide use of antibiotics. Eight *A. Schubertii* isolates from diseased *Ophiocephalus argus* (Cantor) were found to be resistant to ampicillin, bacitracin and rifampicin (Liu and Li, 2012), an *A. Schubertii* isolate from diseased *Garra rufa* developed resistance to ampicillin and sulfamethoxazole (Yu et al., 2009).

In conclusion, this study is the first to report an *A. schubertii* infection in freshwater cultured *P. vannamei*. The pathogenicity of the HS1 isolate supports the conclusion that infection by *A. scubertii* is an emerging threat in whiteleg shrimp farming.

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References

Akaylı T., Çanak Ö., Başaran B., 2011. A study on *Aeromonas schubertii* infection in rainbow trout (*Oncorhynchus mykiss* Walbaum, 1792). *Res. J.Biol. Sci.,* 4(1):99-106.

Alapide-Tendencia E.V., Dureza L.A., 1997. Isolation of *Vibrio* spp. from *Penaeus monodon* (Fabricius) with red disease syndrome. *Aquaculture,* 154(2): 107–114.

Briggs M., Funge-Smith S., Subasinghe R., Phillips M., 2004. Introductions and movement of *Penaeus vannamei* and *Penaeus stlirostris* in Asia and the Pacific. *FAO Fisheries Technical Paper,* 1-12pp.

Cao H., He S., Lu L., Hou L., 2010. Characterization and phylogenetic analysis of the bitrichous pathogenic *Aeromonas hydrophila* isolated from diseased Siberian sturgen (*Acipenser baerii*). *Isr. J. Aquacult. - Bamidgeh,* 62(3): 181-188.

Cao H., He S., Lu L., Yang X., **Chen B.,** 2014. Identification of a *Proteus penneri* isolate as the causal agent for the red body disease of the cultured white shrimp *Penaeus vannamei* and its control with *Bdellovibrio bacteriovorus*. *Antonie van Leeuwenhoek J. Microbiol.,* 105:423-430.

Carnahan A.M., Marii M.A., Fanning G.R., Pass M.A., Joseph S.W., 1989. Characterization of *Aeromonas schubertii* strains recently isolated from traumatic wound infections. *J. Clin. Microbiol.,* 27(8): 1826-1830.

Chen J., Zhu N., Kong L., Ding X., Zheng T., Du J., 2012a. Molecular characteristics and antimicrobial sensitivity of bacterial pathogen from the outbreak of *Litopenaeus vannamei* red-body disease. *J. Fisheries of China,* 36(12): 1891-1900.

Chen Y.F., Liang R.S., Zhuo X.L., Wu X.T., Zou J.X., 2012b. Isolation and characterization of *Aeromonas schubertii* from diseased snakehead, *Channa maculata* (Lacepède). *J. Fish Dis.,* 35(6): 421-430.

Daskalov H., 2006. The importance of *Aeromonas hydrophila* in food safety. *Food Control,* 17:474-483.

Ding S., Wang F., Dong S., **Li Y.,** 2014. Comparison of the respiratory metabolism of juvenile *Litopenaeus vannamei* cultured in seawater and freshwater. *J Ocean Univ China (Oceanic and Coastal Sea Research),* 13: 331-337.

Dong X.Z., Cai. M.Y., 2001. *General manual of systematic and determinative bacteriology.* Science Press, Beijing, 117pp.

Fan J.F., Li W.Z., Zang H.M., Wang B., Song L., 2006. The pathogen of red body disease in *Litopenaeus vannamei*. *Acta Hydrobiologica Sinica,* 30(6): 742-746.

Funge-Smith S., Briggs M., Subasinghe R., 2004. Is white shrimp (*Penaeus vannamei*) a threat to Asian shrimp culture? *FAO Aquaculture Newsletter,* 30: 19-23.

Hossian M.M.M., Rahman M.A., Mondal S., Shadat Mondal A.S.M., Chowdhury M.B.R., 2011. Isolation of some emergent bacterial pathogens recovered from capture and culture fisheries in Bangladesh. *Bangladesh Res. Pub. J.,* 6(1): 77-90.
Jones R.N., Ballow C.H., Biedenbach D.J., 2001. Multi-laboratory assessment of the linezolid spectrum of activity using the Kirby-Bauer disk diffusion method: Report of the Zyvox® antimicrobial potency study (ZAPS) in the United States. *Diagn. Micr. Infec. Dis.,* 40:59-66.
Liu C.,Li K., Wang Q., Chang O., Liang H., Wang F., Pan D., Shi C., Wu S., 2012. Isolation, identification and characterization of *Aeromonas schubertii* from hybrid snakehead (*Channa maculata*♀ × *C. argus*♂). *J. Fish. China,* 36(7): 1119-1125.
Liu J.Y., Li A.H., 2012. First case of *Aeromonas schubertii* infection in the freshwater cultured snakehead fish, *Ophiocephalus argus* (Cantor), in China. *J. Fish Dis.,* 35(5): 335-342.
Marine Products Export Development Authority, Network of Aquaculture Centres in Asia-Pacific, 2003. Shrimp Health Management Extension Manual. MPEDA house, Cochin, India. pp23.
Oxley A.P.A., Shipton W., Owens L., McKay D., 2002. Bacterial flora from the gut of the wild and cultured banana prawn, *Penaeus merguiensis*. *J. Appl. Microbiol.,* 93: 214–223.
Wakida-Kusunoki A.T., Amador-del Angel L.E., Alejandro P.C., Brahms C.Q., 2011. Presence of Pacific white shrimp *Litopenaeus vannamei* (Boone, 1931) in the Southern Gulf of Mexico. *Aquatic Invasions,* 6(Suppl): S139-S142.
Wang L., Chen Y., Huang H., Huang Z., Chen H., Shao Z., 2013. Isolation and identification of *Vibrio campbellii* as a bacterial pathogen for luminous vibriosis of *Litopenaeus vannamei*. *Aquacult. Res.,* DOI: 10.1111/are.12191.
Yu H., He Z., Yan Y., Yang G., Hu J., Zhou M., 2009. Identification of *Plesiomonas shigelloides* and *Aeromonas schubertii* from doctor fish (*Garra rufa*) and antibiotic sensitivity. *China Animal Health Inspection,* 26(7): 37-39.
Zheng T., Kong L., Zhu N., 2011. Epidemiology and control strategy of red body disease of *Penaeus vannamei* in Zhejiang, China. *Sci. Fish Farming,* 12: 48-49.
Zhou Y., Zhang B., Chen X., Qian J., 2003. Preliminary studies on the red body disease in *Penaeus vannamei*. *Marine Sci.,* 27(5): 61-65.