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Vibrio cholerae: A Causal Agent for White Feces Syndrome in Freshwater Cultured Whiteleg Shrimp (*Penaeus vannamei*)

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Key words: Penaeus vannamei, white feces syndrome, *Vibrio cholerae*, pathogenicity, antibiotic susceptibility.

Abstract

Whiteleg shrimp *Penaeus vannamei* is an important commercial shrimp species cultivated in China and many other countries worldwide. Bacteriosis is a major economic problem that inhibits the farming of this species in fresh water. White feces syndrome is an emerging epidemic in freshwater cultured *P. vannamei* and has caused significant economic damage. Only scarce information is available on *Vibrio cholerae* as a possible causal agent for this disease. In this study, a virulent strain BB31 was isolated from diseased *P. vannamei* suffering from white feces syndrome, and identified as a *V. cholerae* isolate through phylogenetic analysis and phenotypic characteristics. A phylogenetic tree was constructed to examine the relationship of isolate BB31 to other *V. cholerae* isolates. Three genes encoding hemolysin, outer membrane protein, and cholera toxin transcriptional activator were present in the BB31 isolate confirming its potential pathogenicity. In addition, isolate BB31 is known to have developed resistance to penicillin, sulfonamides and cephalosporin antibiotics. This was demonstrated when screened against a range of common antibiotics for aquaculture and veterinary use. To the best of our knowledge, this is the first report of white feces syndrome caused by *V. cholerae* in freshwater farmed *P. vannamei.*

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Introduction

Whiteleg shrimp *Penaeus vannamei* has become an important commercial shrimp species worldwide and especially in China. With the rapid development of cultivation techniques, *P. vannamei* has become extremely profitable for farmers (Xie & Gan, 2000). However, bacterial infections have become a major economic problem in *P. vannamei* freshwater farming. For example, *Aeromonas schubertii* has been recognized as a potential pathogen for freshwater cultured *P. vannamei*, causing widespread outbreaks of red body disease (Cao et al., 2015). Bacteriosis in general should be given more attention to enable further sustainable commercial development of this industry.

White feces syndrome has caused significant economic damage in the cultured shrimp industry in China, Indonesia, Malaysia, Thailand, Vietnam and other countries in Southeast Asia. Thus, more attention should be paid to the pathogen of this disease. Gregarines and *Vibrio*s have been reported to be involved in the white feces syndrome (Limsuwan, 2010; Sriurairatana et al., 2014). However, another study has revealed that gregarine parasites are found in only 2% of all sampled diseased and healthy shrimps (Somboon et al., 2012). It thus appears that this disease is probably caused by *Vibrio*s. So far, several *Vibrio* pathogens such as *Vibrio parahaemolyticus*, *Vibrio fluvialis, Vibrio alginolyticus* and *Vibrio mimicus* have been isolated from white feces syndrome-infected *P. vannamei* (Limsuwan, 2010). Little information is available on disease induced by *Vibrio cholerae*.

In this paper, a virulent *V. cholerae* strain was isolated from diseased *P. vannamei* suffering from white feces syndrome in Xiaoshan, Zhejiang China in July 2014, and its phenotypic characterization, taxonomic position, virulent genes, and antibiotic susceptibility were examined. As far as we know, this is the first report of *V. cholerae*-induced white feces syndrome in freshwater farmed *P. vannamei.*

Materials and methods

Whiteleg shrimp samples. Thirty diseased freshwater cultured *P. vannamei* averaging 8.23±0.55 g suffering from white feces syndrome were taken from a whiteleg shrimp farm in Xiaoshan, Zhejiang China in July 2014. The farm had 110 acres of ponds with whiteleg shrimp stocked at an initial rearing density of 55,000 juveniles per acre. Water quality during the disease outbreak was pH 8.56, 7.6 mg/L of dissolved oxygen, 0.80 mg/L of total ammonia and 0.20 mg/L of nitrite. This was the farm's first disease outbreak and it could not be controlled despite the use of chlorine dioxide. Diseased samples were placed in sterile bags, kept in ice, and transported to the laboratory.

Isolation of Bacteria. Each sampled diseased *P. vannamei* was externally disinfected with 75% alcohol, and dissected. Before conducting a careful microscopic examination for gill and intestine parasites, a 0.2 g hepatopancreas sample of each shrimp was cut and streaked onto thiosulfate citrate bile salts sucrose (TCBS) agar plates (Shanghai Huakang Science & Technology Development Co., Ltd.). After incubation for 18-24h at 28^0C, the dominant uniform isolates were purified by streaking and re-streaking onto nutrient agar (NA) plates. Pure isolates of the dominant colonies were supplemented with 15% glycerol and stored at -80^0C. A representative of the dominant isolate, isolate BB31, was characterized further in the present study.

Identification of isolate BB31

Molecular identification. The extraction of genomic DNA from isolate BB31, as well as PCR amplification and sequencing of its 16S rRNA gene were performed according to our previous study (Cao et al., 2015). The near complete 16S rRNA gene sequence was assembled using MegAlign, Editseq and Seqman software. A search was performed in the National Center for Biotechnology Information (NCBI) database for sequence homology using the Basic Local Alignment Search Tool (BLAST) program. A phylogenetic tree from the near complete 16S rRNA gene sequence of the isolate and its homologous sequences was constructed using the neighbor-joining method.

Phenotypic identification. Isolate BB31 was identified phenotypically by API 20E system recommended by Topic Popovic et al. (2007). Isolate BB31 was grown on NA plates (Sinopharm Chemical Reagent Co., Ltd.) at 28^0C for 24h, and a bacterial suspension was then used to inoculate the API 20E test strips (Biomerieux, France)

following manufacturer's instructions. The plate was incubated at 37^0C and observed after 18h and checked against the API identification index and database. The strain ATCC14035 of *V. cholerae* was used as the control.

Bacterial virulence assay. Bacterial virulence was examined by experimentally infecting healthy freshwater cultured shrimps. One hundred and eighty healthy shrimps (av. weight 7.22±0.06 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 shrimps were maintained in 6 replicate aquaria (30 shrimp per aquarium) supplied with 100 L aerated filtered farm water at 28^0C for 14 days to acclimate. Prior to the bacterial virulence assay, isolate BB31 was inoculated into 100 mL nutrient broth (NB), incubated at 28^0C and shaken at 150 rpm for 24 hours. Colony forming units were counted after a 10-fold serial dilution in sterile distilled water. Three replicates of 30 healthy shrimp were challenged by feeding basal diet that contained the isolate BB31 at a concentration of 1.0×10^8 CFU/g as recommended by Zhou et al. (2003). Another three replicates of 30 healthy shrimps exposed to the same experimental conditions and fed only basal diet remained unchallenged and served as control. The daily feeding amount was 2g for each group. The experimental shrimps were kept at 28^0C and observed daily for 18 days without any water change. Any dead shrimps were immediately removed and sampled to re-isolate the challenge organism, and thereby confirm that mortality was attributable to BB31.

Virulence gene assay. The genomic DNA was extracted from pure cultures of isolate BB31 using a genomic DNA extraction kit following manufacturer's instructions (Shanghai Sangon Biological Engineering Technology & Services Co., Ltd.). The virulence genes, including the cholera toxin (*ctxA*) gene, outer membrane protein (*ompU*) gene, hemolysin (*hlyA*) gene, cholera toxin transcriptional activitor (*toxR*) gene, and zonula occludens toxin (*zot*) gene, were respectively amplified by PCR using specific gene primers as recommended by Saravanan et al. (2007). *Escherichia coli* DH5α was used as a control. The specific primers for virulence gene amplification are listed in Table 1. The PCR products were electrophoresed on 1% agarose gel and visualized via ultraviolet trans-illumination. Their partial sequences were assembled using MegAlign, Editseq and Seqman software with a Power Macintosh computer. Searches of the NCBI database were carried out using the BLAST program. The phylogenetic tree from partial sequences of these virulence genes and their homologous sequences was further constructed using neighbor-joining method.

Antimicrobial susceptibility assay. The antibiotic sensitivity of isolate BB31was assayed on NA plates using the Kirby-Bauer disk diffusion method as recommended by Jones et al. (2001). Fourteen 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.

Table 1. Specific virulence gene primers developed by Saravanan et al. (2007) for PCR amplification.

Virulence gene	*Primer (5'→3')*	*Sequence length (bp)*
ctxA	Forward: CGGGCAGATTCTAGACCTCCTG Reverse: CGATGATCTTGGAGCATTCCCAC	564
ompU	Forward: ACGCTGACGGAATCAACCAAAG Reverse: GCGGAAGTTTGGCTTGAAGTAG	869
hlyA	Forward: GGCAAACAGCGAAACAAATACC Reverse: CTCAGCGGGCTAATACGGTTTA	738
toxR	Forward: ATGTTCGGATTAGGACAC Reverse: TACTCACACACTTTGATGGC	883
zot	Forward: TCGCTTAACGATGGCGCGTTTT Reverse: AACCCCGTTTCACTTCTACCCA	947

Results

Identification of the isolate. A dominant isolate BB31 was isolated from the diseased freshwater farmed shrimps and identified as *V. cholerae* by molecular and phenotypic methods. The near complete 16S rRNA gene sequence (1.5 kb) was submitted to GenBank database with an accession no. KF446244. The similarity between its 16S rRNA gene sequence and other *V. cholerae* isolates in the GenBank database is 99%. The phylogenetic tree confirms it as a *V. cholerae* strain (Figure 1). This is also demonstrated by its phenotypic features (Table 2) with an identity of 100% in comparison with the type strain. No parasites were detected in the diseased shrimps from which isolate BB31 was obtained.

Table 2. Phenotypic characterization of the BB31 isolate and the type strain ATCC14035 of *V.cholerae*

Tests	*Reaction*	
	BB31	*ATCC14035*
Arginine dihydrolase	R^-	R^-
Cytochrome oxidase	R^+	R^+
β-Galactosidase	R^+	R^+
Gelatinase	R^+	R^+
Lysine decarboxylase	R^-	R^-
Ornithine decarboxylase	R^+	R^+
Tryptophan deaminase	R^-	R^-
Urease	R^-	R^-
Citrate utilization	R^+	R^+
Acetoin production	R^+	R^+
Indole production	R^+	R^+
H_2S production	R^-	R^-
Arabinose fermentation	R^-	R^-
Amygdalin fermentation	R^-	R^-
Glucose fermentation	R^+	R^+
Inositol fermentation	R^-	R^-
Mannitol fermentation	R^+	R^+
Melibiose fermentation	R^-	R^-
Rhamnose fermentation	R^-	R^-
Sucrose fermentation	R^+	R^+
Sorbitol fermentation	R^-	R^-

R^+: positive reaction; R^-: negative reaction.

The pathogenicity of isolate BB31 was determined in an experimental challenge. 73.33% of the challenged shrimps died and mortality gradually increased over time after the challenge. The dead shrimps exhibited swollen hepatopancreas and white fecal strings, similar to those seen in the original diseased shrimps (Figure 2). The re-isolated bacteria from the challenged dead shrimps were also identified phenotypically and molecularly as BB31. No clinical signs or mortality were noted in the control shrimps.

Virulence genes of the isolate. The specific PCR amplification demonstrates the presence of the virulent *ompU*, *hlyA,* and *toxR* genes in the BB31 isolate (Figure 3). Only three specific virulence gene (*ompU*, *hlyA* and *toxR*) fragments were obtained from the BB31 isolate using a pair of *ompU*-specific primers, *hlyA*-specific primers and *toxR*-specific primers, respectively. No virulence gene fragments were present with the control DH5α strain. The partial sequences of the BB31 isolate's *ompU*, *hlyA* and *toxR* genes were submitted to GenBank database with a respective accession no. KF498633, KF498632 and KF498634. Similarities between the sequences of its *ompU*, *hlyA* and *toxR* genes and those of *Vibrio* strains in the GenBank database are 96%~99%. The

phylogenetic trees further prove the presence of these three virulence genes in the BB31 isolate (Figures 4-6).

Antibiotic sensitivity of the isolate. The antibiotic sensitivity of isolate BB31 is shown in Table 3. The data indicate that isolate BB31 is sensitive to enrofloxacin, gentamicin, neomycin, norfloxacin, ofloxacin, tetracycline, intermediately sensitive to streptomycin, and resistant to ampicillin, cefatrizine, cefuroxime, ceftazidime, cefotaxime, cotrimoxazole, and sulfametoxydiazine. This suggests that isolate BB31 has developed resistance to penicillin and sulfonamide antibiotics for both veterinary and aquacultural use, as well as cephalosporin drugs for veterinary use only.

Discussion

V. cholerae is regarded as an important bacterial pathogen that results in emerging zoonotic diseases in all over the world (Khamesipour et al., 2014). It has been well documented to cause enteritis in *Fugu obscurus*, hemorrhage disease in *Misgurnus anguillicaudatus*, septicemia in *Carassius auratus gibelio*, and ascites disease in *Siniperca chuatsi* (Basilewsky) (Yang et al., 2006; Bing et al., 2009; Qin et al., 2013; Cao et al., 2013). However, there is little definitive information on *V. cholerae* pathogens whichcause the emerging white feces syndrome in freshwater farmed *P. vannamei*. In this study, we demonstrated the pathogenicity of *V. cholerae* BB31, which supports Khamesipour et al. (2014). We also characterized the phenotype, taxonomic position, and antibiotic susceptibility of *V. cholerae* BB31. To our knowledge, this is the first report of *V. cholerae* as a causal agent for white feces syndrome in freshwater cultured *P. vannamei*.

Toxic factors often play a major role in the pathogenicity of shrimp *Vibrios* (Goarant et al., 2000), involving a number of enterotoxins and hemolysins (Baffone et al., 2005). Virulence genes such as *ompU, hly* and *toxR* are most important epidemic markers and contribute to the virulence of *V. cholerae* (Saravanan et al., 2007). Isolates of *V. cholerae* containing *ompU, hly,* and *toxR* genes are recognized to be virulent (Cao et al., 2013). In the present study, *ompU, hly,* and *toxR* genes have been confirmed in the BB31 isolate (Figures 3-6). This explains its pathogenicity in freshwater cultured healthy *P. Vannamei* with a mortality of 73.33%, similar to the natural mortality of 60% to 70% in the shrimp farming region. Apart from the pathogenicity of isolate BB31, there may be other factors which contribute to the development of white feces syndrome such as: over intensification of stocking density and pollutant concentration, poor health status, misuse of contaminated feed, and lack of effective water disinfection.

In addition, detection of virulent genes in the BB31 isolate is considered a potential danger for consumers, suggesting a potential risk for public health. Although most *V. cholerae* strains causing human disease cannot be linked definitively to aquatic animals, attention should be paid to the toxin producing *V. cholerae* isolate from aquatic animals for its potential for zoonoses. Accordingly, proper cooking, storage, and re-heating of shrimps before eating are recommended safety measures for preventing *V. cholerae* infection in humans. Freshwater shrimps should be eaten with caution due to potential contamination with *Vibrio* pathogen (Jiang 2014).

Antibiotic resistance of freshwater *V. cholerae* has developed due to intensive use of antimicrobials in aquaculture farms (Noorlis et al., 2011), resulting in important public health problems that directly relate to disease management and control (Ansari & Raissay, 2010). In our study, the BB31 isolate showed resistance to ampicillin, cotrimoxazole, sulfametoxydiazine in the shrimp cultivation region (Table 3). This might be a consequence of long-term use of these fishery antibiotics by shrimp farmers in controlling pathogenic *Vibrio* populations in their ponds (Noriega-Orzco et al., 2007). The BB31 isolate is also found to be resistant to cephalosporin drugs that are not permitted for use in shrimp farms (Table 3). This could be the result of the possible transfer of plasmids from other cephalosporin-resistant bacteria into isolate BB31 via transformation, conjuction and/or other mobile elements (Raissy et al., 2012).

Although some effective controls for vibriosis in shrimps have been suggested (Graslund & Bengtsson, 2001; Lyle-Fritch et al., 2006), *P. vannamei* with white feces syndrome due to *V. cholerae* may be treated with antibiotics that the BB31 isolate is

susceptible to. Besides the rational use of antibiotics, preventative measures should also be taken to reduce the chance of disease outbreak. These include proper pond preparation, moderate stocking density, stable phytoplankton bloom, good water quality, less feed waste, and routine monitoring of *Vibrio* population (Lekshmy et al., 2012). However, it is a fact that antibiotics can increase virulence of pathogens and promote transference of antibiotic resistance to both shrimp and human pathogens (Moriarty, 1999). Thus, the use of potential probiotics should be considered as a substitute for antibiotics (FAO, 2005; Liu et al., 2010).

In conclusion, the present study has found a *V. cholerae* isolate to be a causal agent for white feces syndrome in freshwater cultured *P. vannamei*. The presence of pathogenicity and virulence genes supports the conclusion that this isolate is an emerging threat in the outbreak of white feces syndrome in *P. vannamei* freshwater farming.

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