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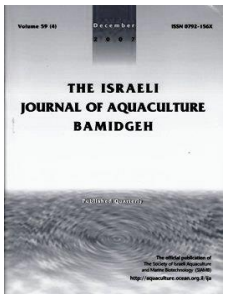

Shewanella algae: an Emerging Pathogen of Black Spot Disease in Freshwater-Cultured Whiteleg Shrimp (*Penaeus vannamei*)

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Abstract

Black spot disease causes significant economic losses in whiteleg shrimp *Penaeus vannamei* yet only limited information is available on *Shewanella algae* as a bacterial pathogen of this disease. In this study, a virulent strain, temporarily named SFH3, was isolated from diseased freshwater-cultured *P. vannamei* suffering from black spot disease, identified phenotypically and molecularly as *S. algae*. A phylogenetic tree was constructed to examine the taxonomic position and relatedness of isolate SFH3 to other *S. algae* isolates. When screened against a range of common veterinary antibiotics, isolate SFH3 is apparently susceptible to aminoglycosides and quinolones antibiotics in aquaculture. To the best of our knowledge, this is the first report of *S. algae* as a causal agent of black spot disease in live freshwater-cultured *P. vannamei.*

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Introduction

The whiteleg shrimp *Penaeus vannamei* is an important shrimp which is widely cultivated in Central and South America, East and South-East Asia, the Middle East, Africa, and USA (Benzie, 2009). In China, the whiteleg shrimp industry has grown rapidly with a total output of over 1.67 million tons in 2016 (Ministry of Agriculture of China, 2017). However, under intensive culture, this industry has been seriously affected by black spot, a disease which should be examined more closely to ascertain its development (Limonta et al., 2012). Studies have revealed that this disease is caused by several bacterial pathogens such as *Pseudomonas* sp. (Khan et al., 1984), *Vibrio* sp., and *Alteromonas* sp. (Cipriani et al., 1980). Very little has been reported on *Shewanella algae* as a causal agent of this disease.

In August 2017, there was an outbreak of black spot disease in whiteleg shrimp cultured in freshwater ponds of Lianyungang, Jiangsu province, China. The disease is highly infectious and lethal, causing over 50% mortality. Diseased whiteleg shrimp suffered mainly from symptoms of black spots on shells.

In the present paper, we isolated and identified an *S. algae* pathogen as a causative agent for this disease and determined its taxonomy and antibiotic susceptibility. To our knowledge, this is the first report of *S. algae* as an emerging pathogen of black spot disease in live freshwater-cultured whiteleg shrimp.

Materials and Methods

Whiteleg shrimp samples

Fifteen diseased whiteleg shrimp (av. weight 12.61±0.68 g) were taken from infected ponds of a shrimp farm in Lianyungang, Jiangsu China in August 2017. The farm has 10 acres of ponds with whiteleg shrimp stocked at an initial rearing density of 60,000 juveniles per acre. The water quality during the disease outbreak was: pH 8.26, dissolved oxygen 6.72 mg/L, total ammonia 0.21 mg/L, and nitrite 0.40 mg/L. Diseased samples were placed in sterile bags, kept in ice and transported to the laboratory.

Isolation of Bacteria

Each sampled diseased whiteleg shrimp was externally disinfected with 75% alcohol and then dissected. Before studying parasites and viruses, samples from hepatopancreas of diseased shrimp was cut and streaked onto nutrient agar (NA) plates (Sinopharm Chemical Reagent Co., Ltd.). After incubation for 18-24h at 28^0C, the dominant uniform isolates were purified by streaking and re-streaking onto NA plates. Pure isolates of the dominant colonies were stored at -80^0C supplemented with 15% glycerol. A representative of the dominant isolates, isolate SFH3, was further characterized.

Identification of the isolate

Molecular identification

Extraction of genomic DNA from isolate SFH3, as well as PCR amplification and sequencing of its 16S rRNA gene were performed according to Li 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 SFH3 was identified phenotypically by API 20E system recommended by Topic Popovic et al. (2007) where the isolate SFH3 was cultured on NA plates (Sinopharm Chemical Reagent Co., Ltd.) at 28^0C for 24h, and the bacterial suspension was then used to inoculate the Analytical Profile Index (API 20E) test strip (Biomerieux, France) following the manufacturer's instruction. The test strip was incubated at 37^0C and observed after 18h for checking against the API identification index. Information related to *Shewanella algae* previously reported (Yang et al., 2009) serves as a reference.

Bacterial virulence assay

Bacterial virulence was examined by experimentally infecting healthy freshwater-cultured whiteleg shrimp. One hundred and fifty healthy shrimp (average weight 18.71±0.42 g) were obtained from a shrimp farm in Shanghai 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 acclimated in ten replicate aquaria (each stocked with fifteen shrimp) supplied with 50 L of aerated filtered farming water at 28^0C for 14 days. Prior to the bacterial virulence assay isolate SFH3 was inoculated onto NA plate, incubated at 28^0C for 24h, and washed with normal saline and placed in a sterile tube. Its cell density was determined by counting colony forming units after a ten-fold serial dilution in sterile distilled water. Two replicates of fifteen healthy shrimp were challenged by muscular injection with 0.1 mL of the isolate SFH3 at concentrations of 5.0×10^3 CFU/mL to 5.0×10^6 CFU/mL. Another two replicates of fifteen healthy shrimp exposed to the same experimental conditions were injected intramuscularly with 0.1 mL of normal saline and served as control. The experimental shrimp were kept at 28^0C and observed daily for five days without feeding and water change. Any dead shrimp were immediately removed and sampled to re-isolate and confirm if mortality was caused specifically by the challenge isolate. The mean lethal dose (LD_{50}) value was calculated according to the graphical probit method as recommended by Ogbuagu & Iwuchukwu (2014).

Antimicrobial susceptibility assay

The antibiotic sensitivity of isolate SFH3 was assayed on NA plates using the Kirby-Bauer disk diffusion method described by Jones et al. (2001). Thirty antibiotic discs were acquired from Hangzhou Binhe 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 isolate

A dominant isolate SFH3 was isolated from the diseased freshwater-farmed shrimp and identified as *S. algae* by molecular and phenotypic methods. Its near complete 16S rRNA gene sequence (1.4 kb) was submitted to GenBank database with an accession no. MG738264. The similarity between its 16S rRNA gene sequence and other *S. algae* isolates in the GenBank database was 99%. The phylogenetic tree confirmed it as an *S. algae* strain (Figure 1). This is also demonstrated by its phenotypic features (Table 1) with an identity of 100% in comparison with the reference strain. No parasites and viruses were detected in the diseased whiteleg shrimp from which the isolate SFH3 was obtained.

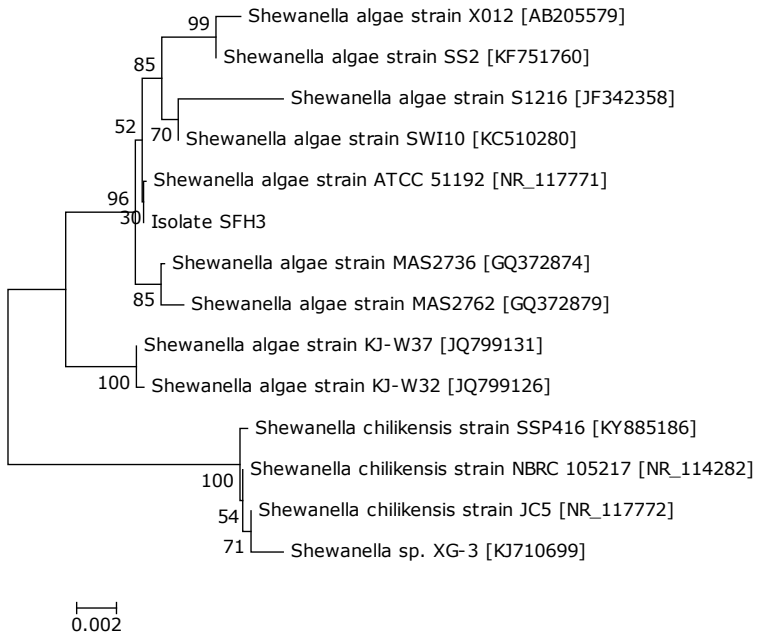

Figure 1. The 16S rRNA phylogenetic tree of 13 known bacteria and the SFH3 isolate constructed using neighbor-joining method. The bootstrap values (%) are shown beside the clades, accession numbers are indicated beside the name of strains, and scale bars represent distance values.

Table 1. Phenotypic characterization of the SFH3 isolate.

Tests	*Reaction*	
	SFH3	*S. algae*[a]
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.
[a]The reference strain's data are in accordance with those previously reported (Yang et al., 2009).

Isolate SFH3 was virulent in whiteleg shrimp with a LD_{50} value of 2.65×10^5 CFU/mL (Table 2). The infected shrimp exhibited similar clinical signs to those seen in the originally diseased shrimp (Figure 2). When shrimp were challenged with a concentration of 5.0×10^6 CFU/mL, acute mortality was observed. The isolate SFH3 could be re-isolated from experimentally dead shrimp. No clinical signs or mortality were noted in the control shrimp.

Table 2. Cumulative mortality of experimental whiteleg shrimp infected by the isolate SFH3.

Group	*Concentration (CFU/mL)*	*Shrimp no.*	*Dead shrimp no. on day after challenge*					*Average cumulative mortality (%)*	*LD_{50} value (CFU/mL)*
			1	*2*	*3*	*4*	*5*		
Control	0	15	0	0	0	0	0	0	2.65×10^5
		15	0	0	0	0	0		
Treatment 1	5.0×10^3	15	0	1	2	0	0	20.0	
		15	0	1	1	1	0		
Treatment 2	5.0×10^4	15	0	1	2	1	1	33.3	
		15	0	2	2	1	0		
Treatment 3	5.0×10^5	15	5	1	0	0	2	56.7	
		15	4	2	1	2	0		
Treatment 4	5.0×10^6	15	9	4	1	1	0	100.0	
		15	8	3	2	2	0		

Figure 2. Gross signs of affected shrimp in the disease outbreak region.

Antibiotic susceptibility of the isolate

The antibiotic susceptibility of isolate SFH3 is shown in Table 3. The data indicate that isolate SFH3 is sensitive to ciprofloxacin, enrofloxacin, gentamycin, kanamycin, neomycin, netilmicin, norfloxacin, ofloxacin, streptomycin, intermediately sensitive to polymyxin B, resistant to doxycycline, florfenicol, sulfamethoxazole and other tested antibiotics. This suggests that isolate SFH3 developed resistance to amphenicols, tetracyclines and sulfonamides antibiotics for aquaculture use.

Table 3. Susceptibility of the SFH3 isolate to antibiotics.

Antibiotics	*Content (µg/disc)*	*Inhibition zone diameter (mm)*
Ampicillin	10	6.38±1.24^{R}
Amoxicillin	10	5.75±0.35^{R}
Chloramphenicol	30	0±0^{R}
Cefamezin	30	0±0^{R}
Cefotaxime	30	0±0^{R}
Ceftazidime	30	0±0^{R}
Cefuroxim	30	0±0^{R}
Ciprofloxacin	5	24.55±0.78^{S}
Doxycycline*	30	8.88±0.53^{R}
Enrofloxacin*	10	24.75±2.47^{S}
Erythromycin	15	0±0^{R}
Florfenicol*	75	0±0^{R}
Furazolidone	300	8.93±0.81^{R}
Furadantin	300	0±0^{R}
Gentamycin	10	21.50±2.83^{S}
Kanamycin	30	18.28±1.10^{S}
Lincomycin	2	0±0^{R}
Neomycin*	30	23.75±1.06^{S}
Netilmicin	30	25.30±1.13^{S}
Norfloxacin	10	22.25±1.77^{S}
Ofloxacin	5	23.73±1.03^{S}
Oxacillin	1	0±0^{R}
Piperacillin	100	0±0^{R}
Polymyxin B	300	10.20±0.28^{I}
Rifampicin	5	0±0^{R}
Streptomycin	300	21.50±0.71^{S}
Sulfamethoxazole*	300	7.58±1.52^{R}
Tetracycline	30	9.00±0^{R}
Trimethoprim	5	0±0^{R}
Vancomycin	30	0±0^{R}

^{S}Susceptible;
^{I}Intermediately susceptible;
^{R}Resistant.
*Veterinary medicine for aquaculture use.

Discussion

The association of *Shewanella* species in aquaculture has been documented with massive mortality reported in *Scinenops ocellata*, *Carrasius auratus* (Altun et al., 2014), *Sciaenops ocellatus* (Zhang et al., 2013), *Babylonia* (Li et al., 2015) and *Cynoglossus semilaevis* (Han et al., 2017). However, there is limited information on *S. algae* isolates as potential pathogens for freshwater-cultured *P. vannamei*. In this study, we characterized the phenotype, taxonomic position, and antibiotic susceptibility of *S. algae* SFH3.

Multiple virulence factors are involved in the pathogenesis of *Shewanella* infections, including enzymatic activity, cytotoxin secretion, adhesion ability, lipopolysaccharide, and the presence of siderophores (Paździor, 2016). Diseases caused by *Shewanella* species in aquaculture are usually associated with the production of these virulent factors. In the present study, *S. algae* SFH3 caused LD_{50} mortality in healthy *P. vannamei* when challenged with a concentration of 2.65×10^5 CFU/mL. This further demonstrates the potential threat of the SFH3 isolate to whiteleg shrimp farming. Apart from the virulence of the SFH3 isolate, there may be other secondary factors that induce this infection in *P. vannamei*, such as high breeding densities and shell damage (Yang et al., 1992). These should also be raised as concerns.

Antibiotic resistance in *S. algae* has been reported in aquaculture as a result of wide use of antibiotics. High resistance of an *S. algae* pathogen from diseased *C. semilaevis* to aminoglycosides and tetracyclines antibiotics has been reported (Han et al. 2017). In our study *S. algae* SFH3 also exhibited resistance to tetracyclines, in addition to amphenicols and sulfonamides antimicrobials used in the shrimp farming regions which suggests that the outbreak of this disease may have resulted from abuse of these antibiotics.

In conclusion, for the first time the present study reports an *S. algae* isolate as an emerging pathogen of black spot disease in freshwater-cultured *P. vannamei*. The pathogenicity and multiple drug resistance of the SFH3 isolate indicate that this infection is an emerging threat to whiteleg shrimp farming.

Acknowledgments

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