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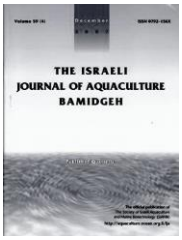

Providencia alcalifaciens: a Causal Agent of Red Leg Disease in Freshwater-Cultured Whiteleg Shrimp *Penaeus vannamei*

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Keywords: *Providencia alcalifaciens*; red leg disease; pathogen; *Penaeus vannamei*.

Abstract

Red leg disease causes significant economic losses in whiteleg shrimp *Penaeus vannamei*. Yet only scarce information is available on *Providencia alcalifaciens* as a bacterial pathogen of this disease. In this study, a virulent strain, temporarily named P3, was isolated from diseased freshwater-cultured *P. vannamei* suffering from red leg disease, identified phenotypically and molecularly as *P. alcalifaciens*. A phylogenetic tree was constructed to examine the taxonomic position and relatedness of isolate P3 to other *P. alcalifaciens* isolates. When screened against a range of common veterinary antibiotics, isolate P3 exhibited susceptibility to aminoglycosides and quinolones antibiotics in aquaculture. To the best of our knowledge, this is the first report of *P. alcalifaciens* as a causal agent of red leg disease in freshwater-cultured *P. vannamei.*

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Introduction

Whiteleg shrimp *Penaeus vannamei* is widely cultivated in Central and South America, USA, East and South-East Asia, Middle East, and Africa (Benzie, 2009), which accounts for 75% of the global shrimp products (Zhou, 2016). However, under intensive culture, this industry has been seriously affected by bacterial diseases. These should be given more consideration to ascertain the future development and sustainability. Recent studies have revealed that *Providencia* species have been reported to cause mortality in American alligators *Alligator mississippiensis* (Camus, 2002), Indian major carp *Labeo rohita* (Ramcumar et al., 2013; Ramkumar et al., 2013), silver carp *Hypophthalmichthys molitrix* (Bejerano et al., 1979), soft-shelled turtle *Pelodiscus sinensis* (Fan et al., 2001). However, very little has been reported on *Providencia alcalifaciens* as a potential pathogen for whiteleg shrimp.

Red leg disease, characterized by reddened periopods and pleopods (Sudheesh & Xu, 2001), is found worldwide in penaeid shrimp (Aguirre-Guzmán et al., 2004). This disease is highly infectious and lethal, generally causing over 90% mortality (Xu et al., 1994; Ministry of Agriculture of China, 2016). In the present study, we isolated and identified a *P. alcalifaciens* pathogen as a causative agent of this disease and determined its taxonomy and antibiotic susceptibility. To our knowledge, this is the first report of *P. alcalifaciens* as an emerging pathogen of red leg disease in freshwater-cultured whiteleg shrimp.

Materials and methods

Whiteleg shrimp samples

Sixteen diseased freshwater-cultured whiteleg shrimp averaging 7.73±0.44 g were sampled from infected ponds of a shrimp farm in Cixi, Zhejiang China in August 2017. The farm had 1,600 acres of ponds with juvenile whiteleg shrimp stocked at an initial rearing density of 80,000 juveniles per acre. Water quality during the disease outbreak was pH 8.24, dissolved oxygen 6.70 mg/L, total ammonia 0.15 mg/L , and nitrite 0.01 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 dissected. Before conducting careful examination of parasites and viruses, samples from hepatopancreas of diseased shrimp were 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 P3, was characterized further in the present study.

Identification of the isolate

Molecular identification

The extraction of genomic DNA from isolate P3, as well as PCR amplification and sequencing of its 16S rRNA gene were performed according to Wang et al. (2014). 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 neighbor-joining method.

Phenotypic identification

Isolate P3 was identified phenotypically by API 20E system recommended by Topic Popovic et al. (2007) where the isolate P3 was grown 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 instructions. The test strip was incubated at 37^0C and observed after 18h for checking against the API identification index. The type strain ATCC 9886 of *P. alcalifaciens* was used as the control.

Bacterial virulence assay

Bacterial virulence was examined by experimentally infecting healthy freshwater-reared whiteleg shrimp. One hundred and fifty healthy shrimp (average weight 6.53±0.61 g) were obtained from Baishazhou Fishery Co., Ltd. in Wuhan China. Their health status was assessed according to the guidelines recommended by 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 P3 was inoculated onto NA plate, incubated at 28^0C for 24h, and washed with normal saline into 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 P3 at concentrations of 5.0×10^4 CFU/mL to 5.0×10^7 CFU/mL. Another two replicates of fifteen healthy shrimp exposed to the same experimental conditions and injected intramuscularly 0.1 mL of normal saline that served as control. The experimental shrimp were kept at 28^0C and observed daily for six days without feeding and water change. Any dead shrimp were immediately removed and sampled to re-isolate and confirm if the mortality was caused specifically by the challenge isolate. The mean lethal dose (LD_{50}) value was calculated using graphical probit method as recommended by Ogbuagu & Iwuchukwu (2014).

Antimicrobial susceptibility assay

The antibiotic sensitivity of isolate P3 was assayed on NA plates using Kirby-Bauer disk diffusion method as described by Jones et al. (2001). Twenty-six 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 P3 was isolated from the diseased freshwater-farmed shrimp and identified as *P. alcalifaciens* by molecular and phenotypic methods. Its near complete 16S rRNA gene sequence (1.4 kb) was submitted to GenBank database with an accession number MH161805. The similarity between its 16S rRNA gene sequence and other *P. alcalifaciens* isolates in the GenBank database is 99%. The phylogenetic tree confirms it as a *P. alcalifaciens* 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 P3 was obtained.

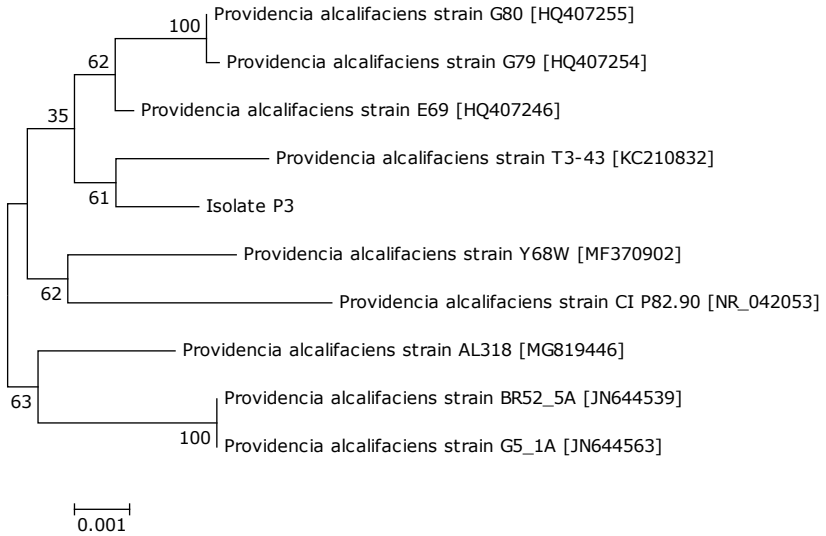

Figure 1. The 16S rRNA phylogenetic tree of 9 known bacteria and the P3 isolate constructed using neighbor-joining method. The bootstrap values (%) are shown besides the clades, accession numbers are indicated beside the name of strains, and scale bars represent distance values.

Table 1. Phenotypic characterization of the P3 isolate.

Tests	*Reaction*	
	P3	*ATCC 9886*
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^-
Adonitol 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.

Isolate P3 was virulent to whiteleg shrimp with a LD_{50} value of 2.45×10^5 CFU/mL (Table 2). The infected shrimp exhibited similar red leg signs to those seen in the originally diseased shrimp (Figure 2). When shrimp were challenged with a concentration of 5.0×10^7 CFU/mL, acute mortality was observed. In addition, the isolate P3 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 P3 isolate.

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*	*6*		
Control	0	15	0	0	0	0	0	0	0	2.45×10^5
		15	0	0	0	0	0	0		
T1	5.0×10^4	15	0	0	2	1	1	0	23.3	
		15	0	0	2	0	1	0		
T2	5.0×10^5	15	4	2	0	0	0	2	53.3	
		15	3	3	1	0	1	0		
T3	5.0×10^6	15	7	5	1	0	2	0	96.7	
		15	7	4	2	1	0	0		
T4	5.0×10^7	15	9	4	2	0	0	0	100	
		15	9	5	1	0	0	0		

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

Antibiotic susceptibility of the isolate

The antibiotic susceptibility of isolate P3 is shown in Table 3. The data indicate that isolate P3 is sensitive to ciprofloxacin, enrofloxacin, furadantin, furazolidone, gentamycin, neomycin, netilmycin, norfloxacin, ofloxacin, streptomycin, tobramycin, and resistant to other tested antibiotics. This suggests that isolate P3 has developed multiple resistance to chloramphenicols, sulfonamides, and tetracyclines in aquaculture.

Table 3. Susceptibility of the P3 isolate to antibiotics.

Antibiotics	*Content (μg/disc)*	*Inhibition zone diameter (mm)*
Ampicillin	10	0 ± 0^{R}
Amoxicillin	10	0 ± 0^{R}
Chloramphenicol	30	9.17 ± 0.76^{R}
Cefazolin	30	0 ± 0^{R}
Cefotaxime	30	0 ± 0^{R}
Ceftazidine	30	0 ± 0^{R}
Cefuroxime	30	0 ± 0^{R}
Ciprofloxacin	5	34.41 ± 3.41^{S}
Cotrimoxazole*	25	0 ± 0^{R}
Doxycycline*	30	9.33 ± 0.76^{R}
Enrofloxacin*	5	31.38 ± 2.17^{S}
Erythromycin	15	12.48 ± 0.60^{R}
Florfenicol*	30	0 ± 0^{R}
Furadantin	300	25.11 ± 1.12^{S}
Furazolidone	300	20.37 ± 0.66^{S}
Gentamycin	10	19.28 ± 0.84^{S}
Lincomycin	2	8.62 ± 0.45^{R}
Neomycin*	30	23.08 ± 1.03^{S}
Netilmycin	30	24.35 ± 0.71^{S}
Norfloxacin	10	25.63 ± 0.54^{S}
Ofloxacin	5	33.09 ± 1.01^{S}
Penicillin	10	0 ± 0^{R}
Streptomycin	10	23.03 ± 0.58^{S}
Sulfamonomethoxine	5	7.80 ± 0.34^{R}
Tetracycline	30	10.64 ± 0.41^{R}
Tobramycin	10	18.51 ± 0.67^{S}

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

Discussion

There is little documentation regarding the connection between *P. alcalifaciens* and shrimp diseases. In this study, we characterized the phenotype, taxonomic position, and antibiotic susceptibility of *P. alcalifaciens* P3 from red leg disease-infected *P. vannamei*. This demonstrates the emergence of *P. alcalifaciens* as a novel pathogen for whiteleg shrimp.

P. alcalifaciens is a Gram-negative invasive pathogen (Guth & Perrella, 1996) that has caused gastroenteritis and diarrhea in humans (Janda et al., 1998; Yoh et al., 2005). In the present study, *P. alcalifaciens* P3 attained LD_{50} mortality value in healthy *P. vannamei* when challenged with a concentration of 2.45×10^{5} CFU/mL. This further poses the potential threat of the P3 isolate to whiteleg shrimp farming and should be raised as a concern. Various virulence factors are involved in the pathogenicity of *P. alcalifaciens*, such as invasion of cells (Magalhães et al., 1996), production of cytolethal distending toxins (Shima et al., 2012). The pathogenesis of *P. alcalifaciens*-induced red leg disease could probably be associated with the production of these virulent factors and systemic bacterial distribution via hemolymph (Soto-Rodriguez et al., 2010).

The development of antimicrobial resistance in *Providencia* pathogens is a matter of concern (Chander et al., 2006). In a survey of antibiotic susceptibility of pathogenic *Providencia* in aquaculture, it was found that 21% of the 139 isolates of *Providencia* were resistant to sulfafurazole, tetracycline, and sulfamethoxazole with trimethoprim used by local farmer for disease treatment (Benedict & Shilton, 2016). *P. alcalifaciens* P3 in our

study also exhibited resistance to tetracyclines and sulfonamides antimicrobials in the shrimp farming regions, suggesting that the outbreak of this disease may have resulted from abuse of antibiotics (Benedict & Shilton, 2016).

In conclusion, this is the first study to describe a *P. alcalifaciens* isolate from red leg disease-infected *P. vannamei*. The pathogenicity and multiple antibiotic resistance of the isolate support this infection as an emerging threat in the whiteleg shrimp farming.

Acknowledgments

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References

Aguirre-Guzmán G., Ruíz H.M., Ascencio R.F., 2004. A review of extracellular virulence product of *Vibrio* species important in diseases of cultivated shrimp. *Aquaculture Research,* 35: 1395-1404.

Bejerano Y., Sarig S., Horne M.T., Roberts R.J., 1979. Mass mortalities in silver carp, *Hypophthalmichthys molitrix* (Valenciennes) associated with bacterial infection following handling. *Journal of Fish Diseases,* 2(1): 49-56.

Benedict S., Shilton C.M., 2016. *Providencia rettgeri* septicaemia in farmed crocodiles. *Microbiology Australia,* 37: 114-116.

Benzie J.A.H., 2009. Use and exchange of genetic resources of penaeid shrimps for food and aquaculture. *Reviews in Aquaculture,* 1: 232-250.

Camus A.C., 2002. *Providencia rettgeri*-associated septicemia and meningoencephalitis in juvenile farmed American alligators *Alligator mississippiensis*. *Journal of Aquatic Animal Health,* 14: 149-153.

Chander Y., Goyal S.M., Gupta S.C., 2006. Antimicrobial resistance of *Providencia* spp. isolated from animal manure. *Veterinary Journal,* 172: 188-191.

Fan H., Huang G., Guo A., Lu C., 2001. The viscera abscess of soft-shelled turtle caused by *Providencia rettgeri. Journal of Nanjing Agricultural University,* 24(4): 71-74.

Guth B.E.C., Perrella E., 1996. Prevalence of invasive ability and other virulence-associated characteristics in *Providencia alcalifaciens* strains isolated in São Paulo, Brazil. *Journal of Medical Microbiology,* 45: 459-462.

Janda J.M., Abbott S.L., Woodward D., Khashe S., 1998. Invasion of HEp-2 and other eukaryotic cell lines by *Providenciae*: further evidence supporting the role of *Providencia alcalifaciens* in bacterial gastroenteritis. *Current Microbiology,* 37: 159-165.

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. *Diagnostic Microbiology and Infectious Disease,* 40:59-66.

Marine Products Export Development Authority, Network of Aquaculture Centres in Asia-Pacific, 2003. Shrimp Health Management Extension Manual. MPEDA house, Cochin, India. pp23.

Magalhães V., Leal N.C., Melo V.M., Sobreira M., Magalhães M., 1996. Invasion of Hela cells by *Providencia alcalifaciens* presumably is plasmid-encoded. *Memórias do Instituto Oswaldo Cruz,* 91(6):767-768.

Ministry of Agriculture of China, 2016. Epidemic Report of Major Aquatic Animal Diseases of China. Beijing: China Agriculture Press, p4.

Ogbuagu D.H., Iwuchukwu E.I., 2014. Evaluation of the toxicity of three hair shampoos on the catfish (*Clarias gariepinus*) fingerlings. *Applied Ecology & Environmental Sciences,* 2(3):86-89.

Ramcumar R., Ravi M., Jayaseelan C., Abdul Rahuman A., Anandhi M., Rajthilak C., Perumal P., 2013. Description of *Providencia vermicola* isolated from diseased Indian major carp, *Labeo rohita* (Hamilton, 1822). *Aquaculture,* 420-421:193-197.

Ramkumar R., Anandhi M., Rajthilak C., Natarajan T., Perumal P., 2013. Studies on ulcerative diseases caused by *Providencia stuartii* bacteria in Indian major carp, *Labeo rohita* (Ham.). *International Journal of Innovative Research in Science, Engineering and Technology,* 2(10):5283-5289.

Shima A., Hinenoya A., Asakura M., Sugimoto N., Tsukamoto T., Ito H., Nagita A., Faruque S.M., Yamasaki S., 2012. Molecular characteristics of cytolethal distending toxin produced by *Providencia alcalifaciens* strains isolated from patients with diarrhea. *Infection and Immunity,* 80(4): 1323-1332.

Soto-Rodriguez S.A., Gomez-Gil B., Lozano R., 2010. 'Bright-red' syndrome in Pacific white shrimp *Litopenaeus vannamei* is caused by *Vibrio harveyi*. *Diseases of Aquatic Organisms,* 92:11-19.

Sudheesh P.S., Xu H., 2001. Pathogenicity of *Vibrio parahaemolyticus* in tiger prawn *Penaeus monodon* Fabricius: possible role of extracellular proteases. *Aquaculture,* 196: 37-46.

Topic Popovic N., Coz-Rakovac R., Strunjak-Perovic I., 2007. Commercial phenotypic tests (API 20E) in a diagnosis of fish bacteria: a review. *Veterinarni Medicina,* 52(2): 49-53.

Wang X., Wang J., Hao H., Qiu L., Liu H., Chen S., Dang R., Yang Z., 2014. Pathogenic *Providencia alcalifaciens* strain that causes fatal hemorrhagic pneumonia in piglets. *Current Microbiol,* 68: 278-284.

Xu B., Xu H., Ji W., Shi J., 1994. Pathogens and pathogenicity to *Penaeus orientalis* Kishinouye. *Acta Microbiologica Sinica,* 13: 297-304.

Yoh M., Matsuyama J., Ohnishi M., Takagi K., Miyagi H., Mori K., Park K., Ono T., Honda T., 2005. Importance of *Providencia* species as a major cause of travellers' diarrhoea. *Journal of Medical Microbiology,* 54: 1077-1082.

Zhou J., 2016. Development path and technological change of shrimp aquaculture in China. *Chinese Agricultural Science Bulletin,* 32(8): 22-29.