Outbreak of Severe Infection Caused by *Pseudomonas putida* in Cultured Hybrid Catfish *Silurus asotus*♀ × *Silurus meridionalis*♂

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Pseudomonas putida is a rod-shaped, aerobic, Gram-negative bacterium commonly found in natural sources of water, soil and the surfaces of living organisms (Bouallègue *et al*. 2004). *P. putida* is commonly considered an important opportunistic human pathogen that can lead to sepsis and bacteraemia (Fujita *et al*. 1998, Korcova *et al*. 2005, Yoshino *et al*. 2011, Liu *et al*. 2014). However, the role of this bacterium in aquatic animal diseases is less clear. It has been reported as an opportunistic pathogen that infects some fish species. For instance, *P. putida* was considered to be the cause of hemorrhagic ascites of ayu (Wakabayashi *et al*. 1996), an epizootic in yellowtail (Kusuda and Toyoshima 1976), skin ulcer in rainbow trout (Altinok *et al*. 2006) and disease in oyster toadfish (Smolowitz *et al*. 1998) and large yellow croaker (Shen *et al*. 2008).

Hybrid catfish *Silurus asotus*♀ × *Silurus meridionalis*♂ is one of the most numerous catfish species in China. Because of its good meat quality and wide consumer acceptance, this species has become an important foodfish in China. However, the rapid expansion and intensification of hybrid catfish aquaculture have led to a series of problems, including the occurrence of various diseases that have limited sustainable development of the industry.

In the summer of 2014, a very serious infectious disease broke out on a freshwater hybrid catfish farm in Sichuan Province, China. Almost all ponds on the hybrid catfish farm were affected. The main clinical signs of disease were listlessness, loss of appetite, skin discoloration and obvious deep dermal ulcers with necrosis of the underlying musculature on the flank, head or caudal peduncle. During the disease outbreak, water temperature ranged from 25 to 28 C. Diseased fish often died within several days and cumulative mortality reached 70 percent.

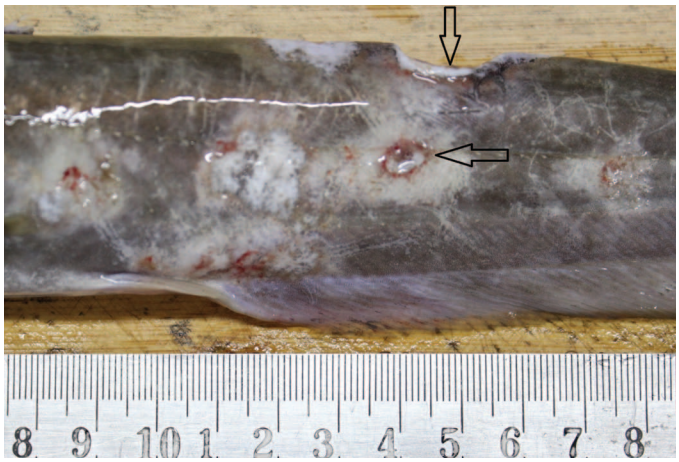

FIGURE 1. *Mutifocal skin ulcers appeared on the body, with marked skin discoloration and hemorrhaging around the ulcers*

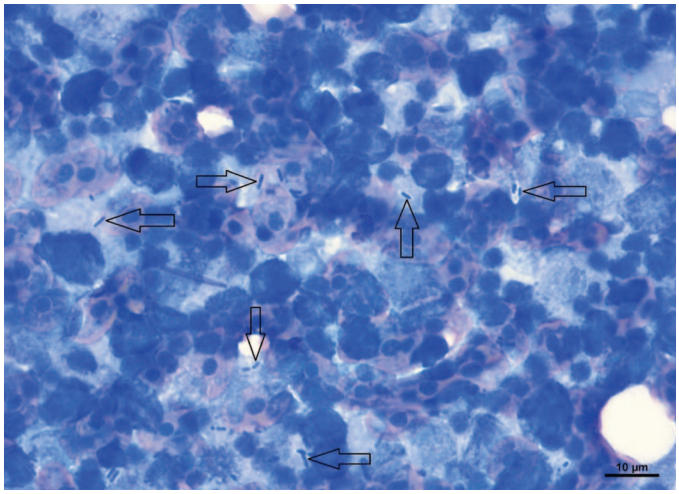

FIGURE 2. *Rod-shaped bacteria were found in kidney imprints with Diff-quick staining.*

We conducted a study to definitively identify the pathogen of this disease and to evaluate the pathogenicity of the pathogen. This is the first report of *P. putida* as a pathogen of hybrid catfish.

Materials and Methods

Bacterial isolation. Fifty diseased and moribund hybrid catfish with typical clinical signs of disease were collected from affected ponds for bacterial isolation. Specimens were transferred alive to the laboratory in plastic bags provided with oxygen. Sterile swabs of the kidney and spleen were made during necropsy for routine bacteriology from each fish. Bacteria were grown on blood agar at 28 C for 24-48 h. Dominant isolates were purified by streaking and re-streaking on the same type of agar plates. Pure-stock isolates were stored at -80 C in sterile balanced salt solution (0.8 percent NaCl, 0.11 percent K_2SO_4, 0.135 percent NaH_2PO_4, 0.005 percent $NaHCO_3$; pH 7.2) supplemented with 15 percent glycerol.

Bacterial identification. After incubation for 24 h at 28 C on blood agar, colonies were characterized using Gram-stain and oxidase, catalase, motility tests, and standard biochemical tests according to standard methods. Identification to species level was performed using the 16S rRNA gene and the gryB gene sequence analysis. Both genes were amplified by polymerase chain reaction (PCR). The universal primers 27F (5-AGAGTTTGATCCTGGCTCAG-3) and 1492R (5-GGCTACCTTGTTACGACTT-3), specific for 16S rRNA gene of

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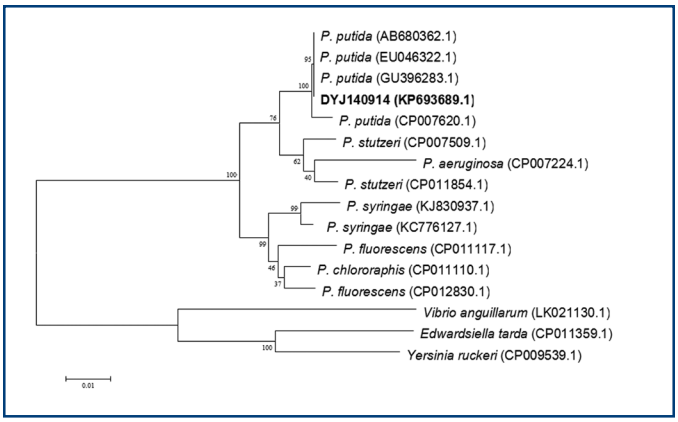

FIGURE 3. *The phylogenetic tree of* P. putida*, based on 16S rDNA sequences, was generated by using the neighbor joining method, maximum composite likelihood, and 1000 bootstrap replicates. Bootstrap values (percent) are shown besides the clades, accession numbers are written besides the name of strains, and scale bars represent distance values.*

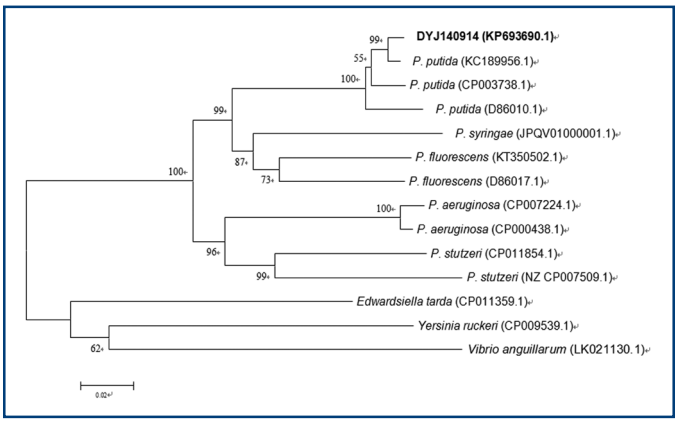

FIGURE 4. *The phylogenetic tree of* P. putida*, based on gyrB gene sequences, was generated by using the neighbor joining method, maximum composite likelihood, 1000 bootstrap replicates. Bootstrap values (percent) are shown besides the clades, accession numbers are written besides the name of strains, and scale bars represent distance values.*

bacteria (Weisburg *et al*. 1991), were used to amplify the 16S ribosomal gene (Jensen *et al*. 2002). The universal primers reported in a previous work (Yamamoto and Harayama 1995) for the amplification of partial gyrB genes from *Escherichia coli*, *Pseudomonas putida*, and *Bacillus subtilis* were used to amplify the gyrB gene. These primers were used to yield an expected approximately 1.5-kb segment of the 16S rRNA gene, and an expected approximately 1.2-kb segment of the gyrB gene. The PCR products were purified using the Gel DNA Purification Kit (TaKaRa, Dalian, China). Sequencing of PCR products was performed using a 3730 DNA sequencer (Shanghai Invitrogen Biotechnology Co., Ltd., Shanghai, China). The obtained 16S rDNA sequences and the gyrB gene sequences were aligned to related sequences of bacteria in GenBank using the BLAST program. The aligned 16S rDNA sequences and the gyrB gene sequences of the related species were retrieved from the National Center for Biotechnology Institution's (NCBI) nucleotide database. Phylogenetic and distance analysis of the aligned sequences was performed using the program MEGA 4.1 (Tamura *et al*. 2007). The resulting unrooted tree topologies were evaluated using the bootstrap analysis of the neighbor-joining method based on 1000 re-samplings.

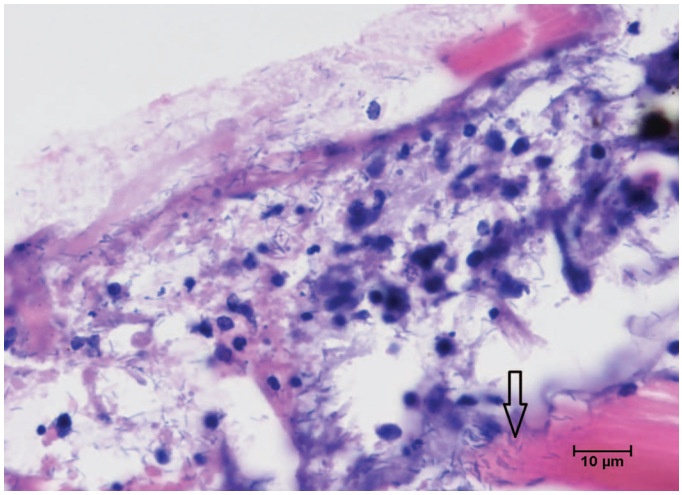

FIGURE 5. *Marked locally extensive integumental and skeletal necrosis, with exposed tissues heavily colonized by rod-shaped bacteria. Arrow shows invasive bacteria in muscle.*

Infectivity experiments. To test the pathogenicity of *P. putida* obtained from hybrid catfish, one strain was selected for experimental infection. Six groups (20 fish in each group) of healthy, 200-300 g hybrid catfish were acclimated in separate 1-m^3 tanks at 25-27 C for 7 d prior to bacterial challenges. Catfish were challenged with 0.1 mL bacterial suspensions of the strain in 0.8 percent NaCl solution at approximately 3.4×10^8, 3.4×10^7, 3.4×10^6, 3.4×10^5, 3.4×10^4 CFU/mL for each group via intraperitoneal injection. The control group received an intraperitoneal injection of the same volume of sterile 0.8 percent NaCl solution. Clinical signs and mortality were recorded daily for 14 days after the challenge. Re-isolation and identification of the bacteria from culture of kidneys and spleens from moribund experimental fish were performed on blood agar plates. All animal challenges were conducted following IACUC-approved protocols of Sichuan Agricultural University.

Histopathology. Tissues for histopathology were collected and fixed immediately in 10 percent neutral buffered formalin (1:10 ratio of tissue to fixative). Tissues included gill, brain, eye, kidney, spleen, heart, liver, nares, stomach, intestine and body wall. Bone and cartilage (cranium, eye, body wall, and gill) were decalcified by immersion for 24 hours in a commercially available decalcification fluid. After decalcification, tissues were trimmed into cassettes, dehydrated in graded ethanol solutions, cleared in xylene, and embedded in paraffin wax. Tissues were cut to 5 µm and stained with hematoxylin and eosin.

Antimicrobial susceptibility testing. Antibiotic susceptibilities of isolated strains were determined using the disc diffusion method and criteria specified by the National Committee for Clinical Laboratory Standards (CLSI 2006). Briefly, 5 mL of BHI broth were inoculated with one loop of culture. After incubation for 24 h at 28 C, the suspension obtained was uniformly spread onto the surface of dry Mueller-Hinton agar (Oxoid, Basingstoke, UK) plates containing 5 percent defibrinated sheep's blood using broth-impregnated swabs. The inoculum concentration was approximately 1.0×10^8 cfu/mL. Disks (Hangzhou Taihe Microbiological Reagent, Hangzhou, China) of nine antimicrobial agents were used. Plates were incubated with antibiotic-impregnated discs at 28 C for 24 h and inhibition of the bacteria by the antimicrobial drugs was scored. The sensitivity and resistance of each isolate were determined following the manufacturer's instructions.

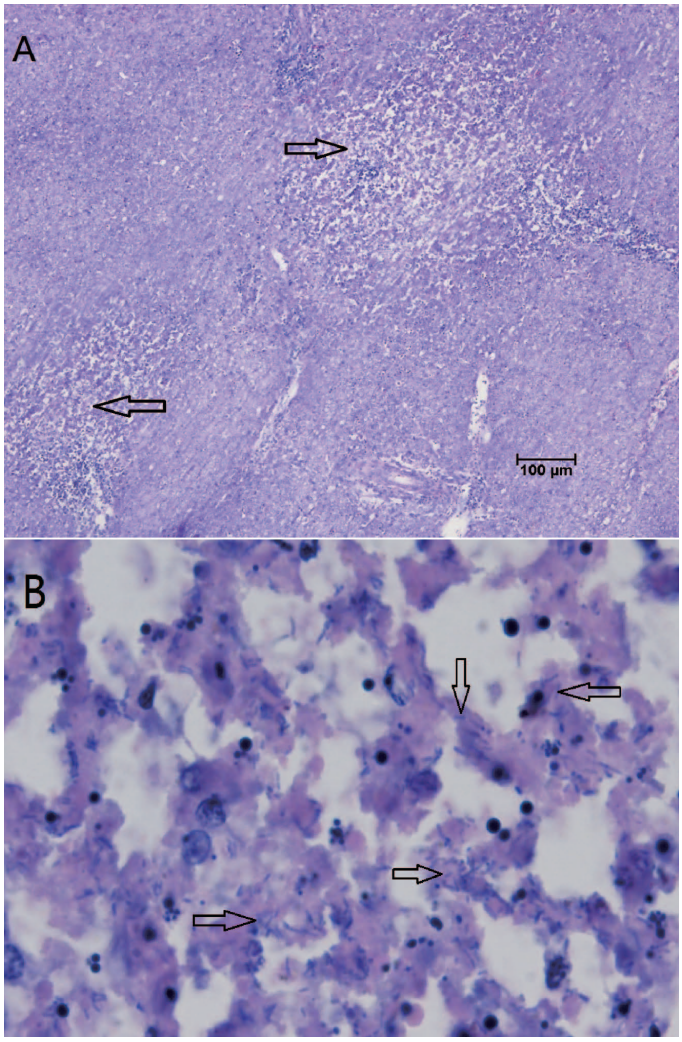

FIGURE 6. *Necrotizing hepatitis with rod-shaped bacteria, with multifocal are marked. Multifocal necrosis in the liver (A) and the magnification of the foci (B). Hepatic cells were markedly necrotic, with chromatin condensed, karyoclasis and massive rod-shaped bacteria. Arrows in figure A showed the necrotic foci and Arrows in B show the invasive bacteria.*

TABLE 1. CHARACTERISTICS OF *P. PUTIDA* ISOLATES FROM HYBRID CATFISH COMPARED WITH PUBLISHED *P. PUTIDA* ISOLATES.

Items	*DYJ140914-DYJ140917*	P. putida[a]	P. putida[b]
Arginine dihydrolase	+	+	+
Ornithine decarboxylase	+	n.a.	+
Lysine decarboxylase	+	n.a.	+
Cytochrome oxidase	+	+	n.a.
Urease	+	+	+
Indole test	+	+	n.a.
H2S	+	+	+
Glucose fermentation	+	+	+
Citrates	+	n.a.	+
Malonate	+	n.a.	+
Sucrose	+	+	+
Sorbitol	+	+	+
Melibiose	+	n.a.	+
Mannose	+	+	n.a.
Mannitol	+	+	+
Maltose	+	+	+
Arabinose	+	+	+
Nitrate	+	n.a.	+
MR test	+	n.a.	n.a.
VP test	+	n.a.	n.a.
Xylose	+	n.a.	+
Rhamnose	+	+	+
Inositol	+	n.a.	+
Galactose	+	n.a.	n.a.
Gelatin liquefaction	+	n.a.	+

[a, b] *Phenotypic characteristics of* P. putida *obtained from Altinok* et al. *(2006) and Shen* et al *(2008).*

n.a.: not applicable. This test was not done for reference strain.

RESULTS

Necropsy findings. Diseased fish had remarkable multifocal whitish skin discoloration and deep dermal skin ulcers on the dorsal fin, tail fin, caudal peduncle, peri-eye, and flank (Fig. 1). The skin of the ulcers was obviously necrotic, revealing the underlying musculature. The edge of ulcer appeared congested. Internal organs including liver, spleen, and kidney were moderately enlarged. The stomach and intestine were empty. Rod-shaped bacteria were easily found in the kidney imprint (Fig. 2).

Bacterial isolation and identification. More than twenty colonies grew on each plate in all samples after incubation at 28 C for 24 h on blood agar. However, only one type of colony was dominant on each plate. These were faint yellow in color, translucent, round, convex and 1.0~2.2 mm in diameter. Four isolates were obtained from different samples and numbered DYJ140914, DYJ140915, DYJ140916 and DYJ140917. All isolates had the same basic physiological and biochemical characteristics, indicating characteristics of *P. putida*. The bacteria were cytochrome oxidase, arginine dihydrolase, and urease positive; fermentation of glucose, utilization of citrates and arabinose, reduction of nitrate; ornithine decarboxylase, and lysine decarboxylase negative; a lack of indole and H_2S production; and non-utilization of malonate, sucrose, sorbitol and melibiose (Table 1).

For similar physiological and biochemical characteristics, one isolate (DYJ140914) was chosen to be identified with 16S rRNA gene sequences and gyrB gene sequences analysis. The nearly full-length 16S rRNA gene sequences of DYJ140914 were amplified and compared with the related 16S rRNA sequences of bacteria in GenBank. It was submitted to NCBI and subsequently assigned NCBI accession number KP693689.1. A phylogenetic tree was constructed based on the 16S rDNA sequences of the isolate and the homologous sequences of other strains of *P. putida* (Fig. 3). The isolate investigated in this study, together with the other three strains, formed a tight cluster with 99.9 percent sequence similarities.

The partial gyrB gene sequences (1.2 kbp) of the isolate were amplified and sequenced. It was submitted to NCBI and subsequently assigned NCBI accession numbers KP693690.1. A phylogenetic tree

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TABLE 2. The sensitivity of bacterial isolates to various antimicrobial agents.

Antibiotics	STANDARD (mm) Resistant (R)	Intermediate (I)	Sensitive (S)	Diameter of inhibition zone (mm)	Sensitivity
Neomycin	≤12	13~16	≥17	13	I
Doxycycline	≤12	13~15	≥16	20	S
Florfenicol	≤12	13~17	≥18	0	R
Norfloxacin	≤12	13~16	≥17	22	S
Sinomin	≤12	13~16	≥17	0	R
Cefoxitin	≤14	15~17	≥18	0	R
Azithromycin	≤13	14~17	≥18	0	R
Levofloxacin	≤13	14~16	≥17	24	S
Rifarnpin	≤16	17~19	≥20	13	R

was constructed based on the gyrB gene sequences of the isolate (DYJ140914) and the homologous sequences of other strains of *P. putida* (Fig. 4). The isolate investigated in this study together with *P. putida* (KC189956.1) formed a tight cluster with 99.0 percent sequence similarities. On the basis of morphology, biochemical and physiological characteristics, and 16S rDNA and gyrB gene sequences analysis results, the isolate was identified as *P. putida*.

Histopathology. All fish had multi-tissue and systematic marked lesions, especially in the liver and on the skin. Some moderate changes could also be seen in the spleen, kidney, heart and intestines. Skin tissue sections indicated marked necrotic dermal dermatitis with massive infiltration of bacteria (Fig. 5). All tissue sections of liver indicated necrotizing hepatitis with marked, multi-focal necrosis and numerous rod-shaped bacteria (Fig. 6). Diseased fish also had moderate splenitis, interstitial nephritis and endocarditis, and mild gastroenteritis. Rod-shaped bacteria were also easily found in the spleen, interstitial kidney, and sometimes in the heart and gastrointestinal tract. No significant morphological changes were found in the brain or gills.

Pathogenicity. The challenge experiment indicated that strain DYJ140914 was lethal to hybrid catfish. Affected fish showed similar clinical signs of disease as naturally infected hybrid catfish. On the first day after challenge, affected fish displayed only less swimming and anorexia. The first fish died after the second day post-challenge and none died after day seven. Dead fish showed obvious multifocal round areas of skin discoloration and cutaneous hemorrhage at the edge of lesions. Some had obvious dermal ulcers. The bacterial strain could be re-isolated as pure colonies from the kidney and spleen of all challenged hybrid catfish, and the re-isolated strain was also strongly virulent to healthy channel catfish. No mortality or visible changes were observed in the control group.

Antimicrobial susceptibility testing. Isolates were sensitive to doxycycline, norfloxacin, levofloxacin; had intermediate susceptibility to neomycin; and were resistant to florfenicol, sinomin, cefoxitin, azithromycin, and rifarnpin (Table 2). On the basis of the sensitivity results, doxycycline, norfloxacin, levofloxacin are recommended to producers to control outbreaks of this disease.

Discussion

Ulcerative dermatitis can be found frequently in catfish. However, disease outbreaks in cultured catfish have been reported without a definitive etiology. *Vibrio mimicus* can infect freshwater catfish and lead to skin ulcers (Geng *et al*. 2014, Zhang *et al*. 2014). Skin erosions or ulcers in southern catfish were caused by *Edwardsialla ictaluri* (Geng *et al*. 2013) or *Aeromonas caviae* (Ji *et al*. 2008). *Aeromonas caviae*-like bacterium were responsible for ulcerative disease in Indian catfish *Clarias batrachus* (Thomas *et al*. 2013). In this study, four bacterial isolates were obtained from diseased hybrid catfish and these were confirmed to be *P. putida* by morphological, biochemical, and molecular assays. To our knowledge, this is the first report of *P. putida* infection in hybrid catfish. Further studies are needed to evaluate the possibility that *P. putida* is a common pathogenic bacterium and can infect other freshwater catfish.

Pseudomonas aeruginosa and *Pseudomonas fluorescens* are considered to be opportunistic pathogens in aquaculture (Doménech *et al*. 1999, Palleroni 2010, Thomas *et al*. 2014). However, other species of the genus may also induce serious infection. For instance, *P. luteola* could infect rainbow trout (Altinok *et al*. 2006), *P. anguilliseptica* infect farmed trout and salmon (Wiklund and Lonnstrom 1994), and *P. alcaligenes* infect Chinese sturgeon (Xu *et al*. 2015). *Pseudomonas putida* is not a common pathogen in aquaculture, but it can infect fish. It causes bloody ascites in ayu *Plecoglossus altivelis* (Wakabayashi *et al*. 1996), large abscesses in farmed yellowtail (Kusuda and Toyoshima 1976), and skin ulcers in rainbow trout (Altinok *et al*. 2006).

Here we described clinical findings, histopathology and biochemical properties of *P. putida*, which can naturally infect hybrid catfish, causing severe mortality. *Pseudomonas* spp., especially *P. putida*, can result in mortality of fish in stressed and non-stressed environments (Smolowitz *et al*. 1998). The results support the idea that *P. putida* may be as an opportunistic pathogen in intensive aquaculture, but more proof is needed to demonstrate definitively.

The results of the antibacterial drug sensitivity tests showed that all *P. putida* isolates from hybrid catfish were sensitive to only three antibiotics (doxycycline, norfloxacin and levofloxacin) and were resistant to most antibiotics tested. Compared to other bacteria, *Pseudomonas* spp. or closely related species had fast growth, versatile adaptation to different conditions, and can establish resistance to stresses, including antibiotics. *Pseudomonas* spp. showed the strongest potential to be an environmental reservoir of antibiotic resistance mechanisms (Meireles *et al*. 2013). Furthermore, the

use of antibiotics is associated with the development of antibiotic-resistant pathogens and environmental deterioration. Thus, antibiotic treatment is not recommended to control infection by *P. putida* and the development of rapid diagnostic methods and vaccines are warranted for early detection and prevention of *P. putida* infections.

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Notes

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