American Journal of Biochemistry and Biotechnology

Original Research Paper

Isolation, Identification and Genomic Analysis of *Plesiomonas shigelloides* Isolated from Diseased *Percocypris pingi* (Tchang, 1930)

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Article history
Received: 25-10-2017
Revised: 11-12-2017
Accepted: 20-12-2017

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Abstract: Recently, the outbreak of a serious infectious disease of unknown etiology was noted in *Percocypris pingi* (Tchang, 1930) farms in Yunnan province. Due to currently limited information, we aimed to identify the pathogen isolates, determine the susceptibility of the isolates, evaluate the pathogenicity and analyze the genome of the representative strain. Ten strains of Gram-negative rods were isolated from diseased *P. pingi* and the isolates were identified as *Plesiomonas shigelloides* based on biochemical characteristics, 16S rRNA gene sequencing and species-specific PCR detection. The results of susceptibility analysis showed that two selected strains LS1 and LL2 were resistant to ampicillin, penicillin G, trimethoprim/sulfamethoxazole, tetracycline, enrofloxacin, nalidixic acid and enoxacin. A virulence assay indicated that the pathogen was virulent to zebrafish. Genomic analysis revealed that the LS1 isolate was closely related to strain GN7, which was isolated from animal farms. To the best of our knowledge, this is the first report of *P. shigelloides* as a pathogen of *P. pingi*. This study will provide a rational framework for exploration of epidemiological analysis of *P. shigelloides* in fish diseases and would further benefit conservation of the species.

Keywords: *Plesiomonas shigelloides*, *Percocypris pingi* (Tchang, 1930), Identification, Genomic Analysis

Introduction

Plesiomonas shigelloides is a Gram-negative, motile, non-spore-forming, oxidase-positive and rod-shaped bacterium belonging to the Enterobacteriaceae family (Brenner *et al*., 2005). *P. shigelloides* is widely distributed in the aquatic environment even under cold climatic conditions (Salerno *et al*., 2010). Thus far, *P. shigelloides* has been isolated from streams, lakes, estuarine waters, mammals, crustaceans, mollusks, reptiles, amphibians and fish (Alexander *et al*., 2016). *P. shigelloides* is an emerging enteric pathogen that is linked to intestinal and extra-intestinal infections in humans (González-Rey *et al*., 2011). It is recognized as a potential fish pathogen and has been isolated from red hybrid tilapia (Nadirah *et al*., 2012). In China, *P. shigelloides* is the pathogen of grass carp (*Ctenopharyngodon idella*), sturgeon (*Huso huso×Acipenser ruthenus*), gibel carp (*Carassius auratus gibelio*), black carp (*Mylopharyngodon piceus*) and tilapia (*Oreochromis niloticus*).

Percocypris pingi (Tchang, 1930), an endemic and commercially important species only found in the upper

reaches of the Yangtze River in China, is a benthic fish that is typically found in rivers with torrential flow. The population of this fish decreased intensely due to overfishing, dam construction, habitat deterioration and water pollution, becoming endangered in the past decade. In May 2015, this species has been listed as Endangered (EN) in the Red List of China's Vertebrates (Li *et al*., 2016). Aquaculture may counteract this decline and improve biodiversity by sustaining healthy fish populations (Pan *et al*., 2016). Therefore, further research is essential to maintain sufficient production of *P. pingi* to meet fishing demands for conservation.

Recently, artificial propagation of *P. pingi* has been conducted in Sichuan and Yunnan provinces in China to restore their population and the hatchery-reared fish have been released into the river by several institutions (Li *et al*., 2016). Unfortunately, bacterial diseases are one of the major obstacles to the expansion of *P. pingi* farming. In 2016, an outbreak caused by *P. shigelloides* occurred in farmed *P. pingi* in China. However, the information about *P. pingi* disease is limited. In this study, we have described the isolation, identification and genomic analysis of *P. shigelloides* from diseased *P. pingi*. The aim of this study is to provide a theoretical foundation for further understanding of the epidemiology of *P. shigelloides* strains and ensure effective prevention and control measures.

Materials and Methods

Sample Collection

In August 2016, a disease outbreak in *P. pingi* (average body weight 45 gram) were obtained from a commercial source, which were cultured in a recirculating system in Lijiang city, Yunnan province, China. The culture water temperature was maintained at 24±1°C. In the early stage of the disease, several fish began to show clinical signs including anorexia, caudal fin and skin ulceration and ascites (accumulation of abdominal fluid). Four days later, mortalities started to occur in this farm. Moribund fish were sampled and kidney tissues were incubated on Brain Heart Infusion (BHI, Difco Laboratories, Detroit, MI, USA) agar for bacterial isolation.

Bacterial Isolation

Tissue samples were aseptically collected from the kidney of moribund *P. pingi* (N=10) and incubated on BHI agar. Plates were incubated at 28°C for 24 h. The bacterial colonies were re-streaked on BHI agar to obtain a pure culture. For long-term preservation the bacterial isolates were stored at -80°C in sterile BHI containing 15% glycerol. In this study, a total of ten strains were isolated and two of them, were selected for further study.

Molecular Characterization

Genomic DNA from ten isolates was extracted using the EasyPure Genomic DNA Kit (Transgen Biotech, Beijing, China) in accordance with the manufacturer's instructions. A partial 16S rRNA gene sequence was amplified as described previously (Zhang *et al*., 2011). Briefly, universal PCR primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-TACGGMTACCTTGTTACGACTT-3′) were used for amplification of the 16S rRNA gene. In addition, primers PS23FW3 (5′-CTCCGAATACCGTAGAGTGCTATC C-3′) and PS23RV3 (5′-CTCCCCTACCCAATAACACCTAAA-3′) were used for detection of the 23S rRNA gene of *P. shigelloides* (González-Rey *et al*., 2000).

Biochemical Characteristics

Of the ten isolates, two (strains LS1 and LL2) were identified based on biochemical characteristics, which were examined by API 20E system (bioMèrieux, Marcy l'Etoile, France) as reported previously (Krovacek *et al*., 2000) and by API strips (Chen *et al*., 2013). Hemolytic activity was assayed by spot inoculation of these strains on 5% sheep erythrocytes in BHI agar and erythrocyte lysis was recorded after 24 h of incubation at 37°C.

Pathogenicity Test

In this study, zebrafish (*Danio rerio*) was used as the target fish to evaluate the pathogenicity of strain LS1 (Saralahti and Rämet, 2015). The isolate LS1 was used for the experimental infection of healthy zebrafish to assess their pathogenic potential. A total of 300 healthy zebrafish were randomly divided into seven groups (20 per group) and then allowed to acclimatize at 28°C for 7 d prior to challenge. Challenge assays were performed in triplicate. Isolate LS1 was cultured in BHI broth overnight, centrifuged and collected. The bacteria were resuspended in sterile PBS buffer at six different doses. Experimental groups were intraperitoneally inoculated with 20 μL of cultured cells at doses of 3×10^8, 3×10^7, 3×10^6 and 3×10^5 CFU/mL, respectively. The zebrafish in the control group were injected with the same volume of PBS buffer. Clinical signs and mortality were recorded daily for 14 d. In addition, bacteria were isolated from the moribund zebrafish and were detected by PCR assay based on the 23S rRNA gene of *P. shigelloides*. The statistical analysis was calculated using SPSS software (version 22.0) and the survival curve was performed by Origin software (version 9.0).

Antimicrobial Susceptibility Testing

In order to select the antimicrobial agents for treatment and define the antibiotic pattern of the isolates, antibiotic

susceptibility of bacterial isolates was determined by the Kirby-Bauer disc-diffusion method. *Escherichia coli* ATCC25922 strain was used as control in the antimicrobial susceptibility tests. The sensitivity and resistance of the isolates and the zone diameter interpretive standards were determined in accordance with the CLSI (Clinical and Laboratory Standards Institute) criteria for animal isolates (CLSI, 2012). The susceptibility patterns of strains LS1 and LL2 against 24 antimicrobial agents (Oxoid, Mercers Row, Cambridge, UK), including acetylspiramycin, erythromycin, midecamycin, penicillin G, ampicillin, cefazolin, ceftiofur, ceftriaxone, ceftazidime, ceftriaxone, tetracycline, doxycycline, enrofloxacin, nalidixic acid, enoxacin, tobramycin, streptomycin, spectinomycin, kanamycin, gentamicin, chloramphenicol, florfenicol, vancomycin and trimethoprim/sulfamethoxazole, were studied.

Genome Sequencing, Assembly And Analysis

The genome of the strain LS1 was sequenced using the Illumina HiSeq 4000 sequencing platform at Total Genomics Solution (TGS, Shenzhen, China). The SOAPdenovo (version 2.04) and GapCloser (version 1.12) software were used to assemble about 404 M sequencing reads and obtain a genome of 3,866,061 bp that resulted in an estimated average 100-fold coverage of the genome. The reads length was 150 bp and the Q20 and Q30 clean data were 97.37% and 92.79%, respectively. Reads assembly with the SOAPdenovo (version 2.04) and GapCloser (version 1.12) software yielded 51 scaffolds and 53 contigs. The genome was then screened using GeneMarks (version 4.6b) software, revealing 3,445 genes. The functions of encoded genes were annotated using the Virulence Factor of Pathogenic Bacteria Database (VFDB, www.mgc.ac.cn/VFs/main.htm) and the Antibiotic Resistance Genes Database (ARDB, http://ardb.cbcb.umd.edu/).

Phage detection was performed using the publicly available Phage Search Tool (PHAST) (http://phast.wishartlab.com/). Single nucleotide polymorphisms (SNPs) were detected using the MUMmer software (version 3.22) (Kurtz *et al*., 2004), the query sequence was aligned with the reference sequence (GenBank accession no. GCA_900087055.1). The variation sites between the query and reference sequences were identified and filtered preliminarily to detect potential SNP sites. The sequences with the length of 100 bp at both sides of SNP in the reference sequence were extracted and aligned with assembly results to verify SNP sites using BLAT. If the length of the aligned sequence was shorter than 101 bp, the SNP was considered incredible and would be removed; if the extracted sequence could be aligned with the assembly results several times, the SNP was considered to be located in the repeat region and would be removed. BLAST, TRF (Version: 4.04) and Repeatmasker software (Version: 3.2.9) were used to predict SNPs in the repeat regions. The credible SNPs can be obtained by filtering those located in the repeat regions.

For comparative analysis, genome sequences of 3 reference *P. shigelloides* strains were selected, including those collected from water at an animal farm (GN7), from humans (302-73) and the NCTC 10360 strain as the type strain. The phylogenetic tree was constructed using the array of SNPs obtained from the strain LS1 and the reference strains. For each bacterium, all SNPs were connected with a similar order and the sequences with the same length were obtained as an input file in FASTA format. Then, the phylogenetic tree was constructed by TreeBeST (Nandi *et al*., 2010) using the method of PHYML and by setting bootstraps as 1,000.

Results

Isolation and Identification

Ten isolates were isolated from ten diseased *P. pingi*. They were cultured, purified and identified as *P. shigelloides* by PCR detection based on 23S rRNA gene and 16S rRNA gene sequencing (González-Rey *et al*., 2000; Marathe *et al*., 2016). Sequence analysis revealed that the 16S rRNA gene sequences for the ten isolates displayed 100% identity to the corresponding gene of *P. shigelloides* (NCTC 10360). The representative strains LS1 and LL2 were selected for further identification. Biochemical characteristics of the two strains were consistent with those of *P. shigelloides* (Table 1). The strains LS1 and LL2 did not show hemolysis on plates containing 5% sheep blood agar and the lactose reaction result was negative for the two isolates (Table 1). The results revealed that strains LS1 and LL2 were identified as *P. shigelloides*.

Pathogenicity to Zebrafish

In the challenge trials, the isolate LS1 was pathogenic to zebrafish. Infected zebrafish began to die at 16 h post-inoculation with a high dose and the most of the mortality occurred within 72 h (Fig. 1). The average cumulative mortalities of zebrafish were 90%, 50%, 30% and 18% at 3×10^8, 3×10^7, 3×10^6 and 3×10^5 CFU/mL within 7 days after challenge, respectively (Fig. 1). The moribund infected zebrafish showed clinical signs of hemorrhages at the base of the fins, fin ulceration, ascetic fluid and enlargement of the liver and gall bladder. Pure cultures of *P. shigelloides* were re-isolated from the kidneys of moribund fish. No clinical signs or bacteria were detected in control fish. Therefore, the isolate LS1 was a potential pathogen for fish.

Table 1: Biochemical characteristics of strains LS1 and LL2 isolated from diseased *P. pingi*

	Reaction		Reference
Tests	LS1	LL2	*P. shigelloides**
β-galactosidase	+	+	+
Arginine dihydrolase	+	+	+
Lysine decarboxylase	+	+	+
Ornithine decarboxylase	+	+	+
Citrate utilization	–	–	–
H_2S production	–	–	–
Urease	–	–	–
Tryptophan deaminase	–	–	–
Indole production	+	+	+
V-P reaction	–	–	–
Gelatin	–	–	–
Glucose	+	+	+
Mannitol	–	–	–
Inositol	+	+	+
Sorbitol	–	–	–
Rhamnose	–	–	–
Sucrose	–	–	–
Melibiose	–	–	–
Amygdalin	–	–	–
L-arabinose	–	–	–
Oxidase	+	+	+
Lactose	–	–	–

Notes: +, positive; –, negative
*Desicription in the Bergey's manual of systematic bacteriology (9th)

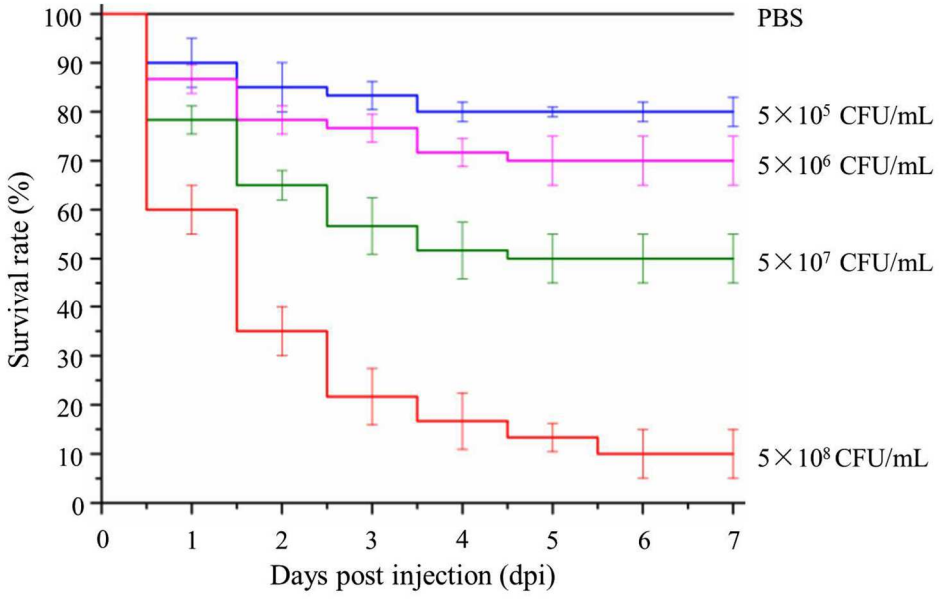

Fig. 1: Survival curve of zebrafish infected with *P. shigelloides*

Antimicrobial Susceptibility

Antibiotic susceptibility tests indicated that LS1 and LL2 isolates were resistant to ampicillin, penicillin G, trimethoprim/sulfamethoxazole, tetracycline, enrofloxacin, nalidixic acid and enoxacin; mid-resistant to acetylspiramycin; and susceptible to other antibiotics (Table 2). These results indicated that strains LS1 and LL2 were multi-drug resistant strains.

Genome Features and Genomic Analysis

Genome sequence was assemble and contained a total of 3,866,061 bp. The whole genome contains 3,445 genes that account for 83.27% of the genome. The ARDB annotated 3 genes conferring resistance to tetracycline (*tetA*), trimethoprim (*dfra*) and bacitracin (*baca*). Strain LS1 contained 10 prophage sequences with a total length of 190,791 bp, including 2 intact prophages and 8 incomplete prophages. The draft genome sequences for *P. shigelloides* LS1 have been deposited at GenBank under accession no. MUNJ00000000.

Compared to the reference strain NCTC 10360, strains LS1, 302-73 and GN7 contained 65,685, 65,997 and 66,859 SNPs, respectively. For the strain LS1, approximately 87.8% of the SNPs within coding sequences resulted in synonymous changes at the protein level and 18.0% of the total SNPs were intergenic mutants. In the phylogenetic tree, strains LS1 and GN7 were grouped into one evolutionary branch (Fig. 2). The results revealed that strain LS1 was closely related to strain GN7 at the genome level, which was isolated from water at an animal farm.

Table 2: Susceptibility of strains LS1 and LL2 to 24 antimicrobial agents

		Susceptibility	
Antimicrobial agents	Content (μg)	LS1	LL2
Acetylspiramycin	30	I	I
Erythromycin	15	S	S
Midecamycin	30	S	S
Penicillin G	10 U	R	R
Ampicillin	10	R	R
Cefazolin	30	S	S
Ceftiofur	30	S	S
Ceftriaxone	30	S	S
Ceftazidime	30	S	S
Cefoperazone	75	S	S
Tetracycline	30	R	R
Doxycycline	30	S	S
Enrofloxacin	5	R	R
Nalidixic acid	30	R	R
Enoxacin	10	R	R
Tobramycin	10	S	S
Streptomycin	10	S	S
Spectinomycin	100	S	S
Kanamycin	30	S	S
Gentamicin	10	S	S
Chloramphenicol	30	S	S
Florfenicol	30	S	S
Vancomycin	30	S	S
Trimethoprim/Sulfamethoxazole	300	R	R

Notes: S, sensitivity; I, intermediate resistance; R, resistance. U, unit

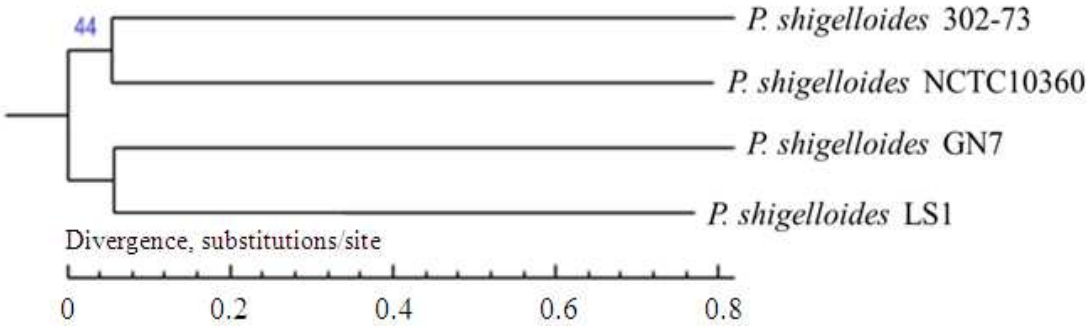

Fig. 2: The phylogenetic tree of *P. shigelloides* based on SNPs

Discussion

In this study, ten bacterial strains with identical morphology were isolated from diseased *P. pingi* cultured in Yunnan province, China. LS1 and LL2, two of the ten isolates, were identified as *P. shigelloides*, according to general biochemical characteristics and 16S rRNA gene sequencing. The 16S rRNA sequencing analysis also indicated a very close relationship with *P. shigelloides* (100%, for GenBank accession No. LT575468). Furthermore, species-specific PCR confirmed that the isolates were *P. shigelloides* (González-Rey *et al*., 2000). The strain LS1 caused a high mortality at dose $\geq 3\times10^7$ CFU/mL, indicating that isolate LS1 was strongly virulent to zebrafish. The results suggested that *P. shigelloides* was an important pathogen of *P. pingi*. For the purpose of conserving the population of *P. pingi*, more studies should be carried out on diseases caused by *P. shigelloides*.

Plesiomonas shigelloides, an emerging pathogen of humans and animals, is widespread in the aquatic environment. They have been isolated from various different animals, such as shellfish, freshwater fish, snakes, dogs, goats, cats, swine and monkeys (González-Rey *et al*., 2011). Interestingly, not all farmed fishes were infected with *P. shigelloides*, however, this bacterium is a common source of disease in grass carp, tilapia, Chinese giant salamander (*Andrias davidianus*) and black carp in China. To the best of our knowledge, this is the first report on *P. shigelloides* as a pathogen of *P. pingi*. The present results showed that *P. shigelloides* as an important pathogen to *P. pingi*. And the *P. pingi* is susceptible to *P. shigelloides* infection, resulting in high mortality. In the present study, two weeks after the initial onset of *P. shigelloides* infection, mortality levels had reached 50%.

The results of antibacterial drug sensitivity tests suggested that the two selected isolates of *P. shigelloides* were sensitive to most of the antibiotics, but resistant to ampicillin, tetracycline and trimethoprim/sulfamethoxazole. Furthermore, isolate LS1 contained the tetracycline resistance gene *tetA* and the trimethoprim/sulfamethoxazole resistance gene *dfra* according to ARDB annotated. Importantly, these results were similar to those for isolates from Asian arowana (*Scleropages formosus*) in Korea (Jun *et al*., 2011). *P. shigelloides* is mostly resistant to penicillin and ampicillin, whereas it is usually sensitive to the third-generation cephalosporin as reported previously (Brenden *et al*., 1988). Similar to the report by Brenden *et al*. (1988), strains LS1 and LL2 were resistant to penicillin and ampicillin, but were sensitive to ceftriaxone, ceftazidime and cefoperazone.

In this study, control of *P. shigelloides* infection in *P. pingi* was based on treatment with doxycycline, so that after one week, the mortality of *P. pingi* was effectively controlled and was less than 5%. In addition, water is the major source of contamination with *P. shigelloides* and an important vehicle for the spread of this species (Kim *et al*., 2015). Therefore, it is important to control the abundance of *P. shigelloides* in water for prevent *P. shigelloides* infection in *P. pingi*.

In the present study, we confirmed the isolation, identification and genomic analysis of *P. shigelloides* isolated from diseased *P. pingi* reared to restore their population in China. We found that *P. shigelloides* was pathogenic to zebrafish and it possessed 3 antibiotic-resistant genes in the genome sequence. Furthermore, to investigate the phylogenetic relationships among these four strains, we used the neighbor-joining method to construct a phylogenetic tree using the SNPs. Strains GN7, LS1 and NCTC10360 belong to the same evolutionary clade and strain LS1 was found to be closely related to strain GN7, which was isolated from animal farm. The results indicated that *P. shigelloides* strains from an animal could be transmitted to fish. Given that *P. pingi* is a near endangered cyprinid species, the results would be provide some effective prevention and control strategies for the farm of *P. pingi* and would further benefit conservation of the species.

Acknowledgement

The authors thank Ph.D. Xujie Zhang for his adivce relating to the analysis of biochemical characteristics.

Funding Information

This study was supported by the Project (2017FB01) of State Key Laboratory of Freshwater Ecology and Biotechnology, the Project (2016NSG01) of State key laboratory of water resources and hydropower engineering science (Wuhan University); the National Natural Science Foundation of China (Grant No. 51439006) and the Project (2016A001) of Hubei Key Laboratory of Regional Development and Environmental Response (Hubei University).

Author's Contributions

Lei Pan and Shuiyi Liu: Contributed to the planning and implementation of this study as well as interpretation of article preparation and drafted the manuscript.

Xuwei Cheng and Yiting Tao: Coordinated the data-analysis and contributed to the writing of the manuscript.

Tao Yang, Peipei Li, Zhengxiang Wang and Dongguo Shao: Designed the research plan and participated in the experiments.

Defeng Zhang: Contributed to the planning and implementation of research work, and revising the article.

Ethics

Animal experiments were carried out according to animal welfare standards and were approved by the Ethical Committee for Animal Experiments of the Pearl River Fisheries Research Institute, Chinese Academy of Fishery Sciences. All animal experiments were in compliance with the guidelines of the Animal Welfare Council of China.

Conflict of Interest

The authors declare that they have no competing interests. The corresponding author affirms that all of the authors have read and approved the manuscript.

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