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CHARACTERIZATION OF PATHOGENIC *AEROMONAS VERONII* BV. *VERONII* ASSOCIATED WITH ULCERATIVE SYNDROME FROM CHINESE LONGSNOUT CATFISH (*LEIOCASSIS LONGIROSTRIS* GÜNTHER)

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ABSTRACT

273 bacterial strains were isolated from 20 Chinese longsnout catfish samples. The biochemical characteristics of all strains conformed to the species description of *Aeromonas veronii* bv. *veronii* on the basis of Vitek GNI+ card. Furthermore, 16S rDNA, *gyrB* and *rpoD* sequences of the representative strain PY50 were sequenced and showed high similarity with *A. veronii* bv. *veronii* in Genbank. Antibiotic-resistance of the representative strain PY50 was assessed by the Kirby-Bauer disk diffusion method, and the results showed it was susceptible and moderately susceptible to 13 and 4 of the 21 antimicrobial agents tested. Extracellular products of strain PY50 contained gelatinase, lecithinase, elastase, most of lipase and lipopolysaccharide. Virulence of strain PY50 and extracellular products to Chinese longsnout catfish were also tested, and LD_{50} were about 3.47×10^4 CFU per fish and 11.22 μg per fish in intraperitoneal injection respectively. This is the first report that *A. veronii* bv. *veronii* was the pathogenic agent of ulcerative syndrome in Chinese longsnout catfish.

Key words: *Aeromonas veronii* bv. *veronii*; ulcerative syndrome; *Leiocassis longirostris* Günther; Identification

INTRODUCTION

Aeromonas spp. are ubiquitous inhabitants of aquatic ecosystems and broadly distributed throughout the world (5). These bacteria have a broad host range, and often have been implicated in the cause of numerous infections, such as humans with diarrhea and fish with hemorrhagic septicemia (6, 20). The strains isolated from the environment do not seem to differ from those isolated from cases of infection with respect to the prevalence of virulence factors (11). However, some studies showed only certain species have displayed clinical importance for aquatic animals and are recognized as potential pathogens causing diseases (10). In recent years, some new aquatic pathogens belong to the genus *Aeromonas* have been reported (18, 24). It is very possible that more pathogens of this genus could be identified in the near future with the recent advances in the aquatic epidemiological research.

Chinese longsnout catfish (*Leiocassis longirostris*

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Günther) is a warm-water and carnivorous freshwater aquaculture species and is high valued because of its good taste in China. Recently, outbreaks of ulcerative syndrome and mass mortalities occurred in China among the cultured Chinese longsnout catfish. The diseased fish had similar clinical signs including open dermal ulcers on the body, lack of feeding, and visible pathological changes on the liver characterized by irregular hemorrhagic blots. In light of the increased incidence of ulcerative syndrome and the economic importance of these epizootic diseases and because of possible public health effects, it is of great importance to further study and characterize the etiologic agents of ulcerative syndrome. Thus, we presumed that the Chinese longsnout catfish might be infected by certain bacterial pathogens and expected to isolate the pathogen from the diseased fish. In the current study, we have described the isolation, characterization, and virulence of the pathogenic agent, *A. veronii* bv. *veronii*, which caused ulcerative syndrome in Chinese longsnout catfish in China.

MATERIALS AND METHODS

Bacterial isolation and identification

The moribund fish were selected from 10 commercial ponds where nearly all the fish had developed similar pathological signs and more than 70% of the animals died at Panyu district in Guangdong province. These fish swam slowly on the surface of the water and had different degrees of deep hemorrhagic ulcers in the body. The diseased fish were sanitized with 75% alcohol and dissected in the laboratory. The muscle tissue of rotten body and liver of diseased fish were cut and homogenized for at least 2-3 min using a homogenizer in alkaline peptonewater (APW) to produce a uniform homogenate. About 50 µl homogenate was transferred and cultured on nutrient agar (Huankai Co Ltd., Guangzhou, China) at 28°C for 48 h. The dominant colonies were then purified by streaking and re-streaking on the same agar. The pure stock isolates were stored in nutrient broth (Huankai Co Ltd., Guangzhou, China) supplemented with 10% glycerol at -70°C. The strains were further biochemically characterized and identified by the Vitek GNI+ card (bioMerieux Vitek, Hazelwood, MO).

The genomic DNA of the representative strain PY50 was extracted from cultivated strains using boiling method (4). Primers based on 16S rDNA, *gyrB* and *rpoD* were designed according to Soler *et al.* (21). Characteristics of primers used for PCR amplification and sequencing of 16S rDNA, *gyrB* and *rpoD* are summarized in Table 1. Part of 16S rDNA, *gyrB* and *rpoD* sequence of the strain PY50 was amplified by PCR and sequenced as described previously (21). The amplified products were sequenced by Shanghai Sangon Biologic Engineering & Technology and Service Co. Ltd., China.

The nucleotide sequences of 16S rDNA, *gyrB* and *rpoD* were independently aligned by the ClustalX program (23). The evolutionary trees were constructed by the neighbour-joining method with the Mega program (12).

Sensitivity of the isolated strain to various antimicrobial agents

The representative strain PY50 of the 273 similar field isolates was tested for its antibiotic-resistance by the Kirby-Bauer disk diffusion method (2). The antibiotics (Oxoid) were tested included ampicillin (10 µg), chloramphenicol (30 µg), chlortetracycline (10 µg), ciprofloxacin (5 µg), , doxycycline hydrochloride (30 µg), enrofloxacin (5 µg), erythromycin (15 µg), gentamycin (10 µg), furazolidone (100 µg), kanamycin (30 µg), nalidixic acid (30 µg), neomycin (10 µg), nitrofurantoin (300 µg), oxolinic acid (2 µg), oxytetracycline (30 µg), streptomycin (25 µg), sulfisoxazole (300 µg), sulphonamide (300 µg), tetracycline (10 µg), trimethoprim (5 µg) and vancomycin (10 µg). All filter-paper discs were obtained from Difco Laboratories. Zones of inhibition were measured after 18 h and again after 48 h of incubation at 28°C. The isolates were classified as sensitive (S), moderately sensitive (M) or resistant (R) on the basis of the size of the zone of bacterial growth inhibition according to the National Committee for Clinical Laboratory Standards (16).

Evaluation of putative virulence factors

The degradation of egg yolk (lecithin), elastin, gelatin, sheep blood (β-haemolysis), skimmed milk (casein) and Tween 20 (lipase) was recorded after incubation at 28°C for up to 4 days according to Orozova *et al.* (19).

Extracellular products (ECPs) were prepared from cellophane overlays on TSA after incubation at 28°C for 48 h (13). The concentrations of protein and lipopolysaccharide (LPS) were determined by the method of Bradford and Keler & Novotny (3, 9). ECPs were stored at -20°C until used.

Virulence of strain PY50 and ECPs for fish

The Chinese longsnout catfish weighing approximately 50±1.47 g were held in tanks (2500 liter) for 2 weeks in order to adapt to laboratory conditions at 25-28°C. All fish were anaesthetized with MS-222 (Sigma, St. Louis, MO, USA) and then injected intraperitoneal injection of 0.1 ml bacterial suspensions (24 h bacterial culture, 10^2-10^7 CFU per fish) or 0.1 mL ECPs (464.2, 215.4, 100.0, 46.4, 21.5 and 10.0 μg protein/ml). The LD_{50} tests were conducted with batches of 20 fish per dose. Sterile PBS was injected into other group of fish as parallel controls. Intraperitoneal injection was repeated in triplicate independently. Mortalities were recorded daily for 2 weeks post injection with bacterial suspension.

The fish injected with bacterial suspension were observed for pathologic signs. Bacteriological analyses of dead fish were carried out in all the cases. Death was considered to be because of inoculated bacteria only if the original strain used for inoculation was re-isolated from the muscle of rotten body of the fish injected with bacterial suspension in pure culture.

RESULTS

Bacterial isolation and taxonomy

Approximately 273 bacterial isolates were observed on the plates in all samples after incubation for 48 h on nutrient agar, and the color were light yellow and straight rod from 20 Chinese longsnout catfish samples. The average diameter of colonies on both plates was 2.5-3.4 mm. Results of all isolates from the Vitek database indicated that the percent probabilities of identification of *A. veronii* bv. *veronii* were 95 to 99%. However, the percent probabilities of identification of *A. hydrophila*, *A. caviae* and *A. sorbia* were only 69 to 83%. So, we tentatively identified the pathogen caused ulcerative syndrome to Chinese longsnout catfish as *A. veronii* bv. *veronii*.

Part of the 16S rDNA, *gyrB* and *rpoD* gene of the representative strain PY50 have been sequenced. All sequence has the highest homology to some *A. veronii* bv. *veronii* strains reported in Genbank with a similarity value of 97% to 100%. The accession numbers of 16S rDNA, *gyrB* and *rpoD* gene were HQ434550, HQ540319 and HQ540320 respectively in GenBank. Aligned with some sequences of the closest strains in Genbank via ClustalX method, the sequences of the representative strain PY50 and some aeromonad strains formed a tight clade as shown in Fig. 1.

Sensitivity of the isolated strain to various antimicrobial agents

The representative strain PY50 was susceptible and moderately susceptible to 13 and 4 of the 21 antimicrobials tested, respectively, as shown in Table 2. The results showed the strain PY50 exhibited 81% of susceptibility to various antimicrobial agents.

Evaluation of putative virulence factors

The phenotypic determination of possible virulence factors showed that the ECPs were ß-haemolytic on sheep blood agar. Gelatinase, lecithinase and elastase were produced, lipase by most. The ECPs contained LPS, and the concentrations of ECPs and LPS were 3.59 mg/ml and 2.37 μg/ml respectively.

Virulence of strain PY50 and ECPs for fish

Injection of bacterial cells of the representative strain PY50 into Chinese longsnout catfish was lethal to the fish, but not to the control groups injected with sterile PBS. The LD_{50} of

the strain PY50 live cells for Chinese longsnout catfish was about 3.47×10^4 CFU per fish in intraperitoneal injection (Table 3). The moribund or dead fish exhibited the same signs as the diseased fish on ulcerative syndrome in the ponds. This strain could be re-isolated as pure colonies from the muscle of rotten body or liver of the moribund fish. Furthermore, the re-isolated strains were high virulent to Chinese longsnout catfish. As the same, the LD_{50} of ECPs from strain PY50 for Chinese longsnout catfish was about 11.22 μg per fish in intraperitoneal injection.

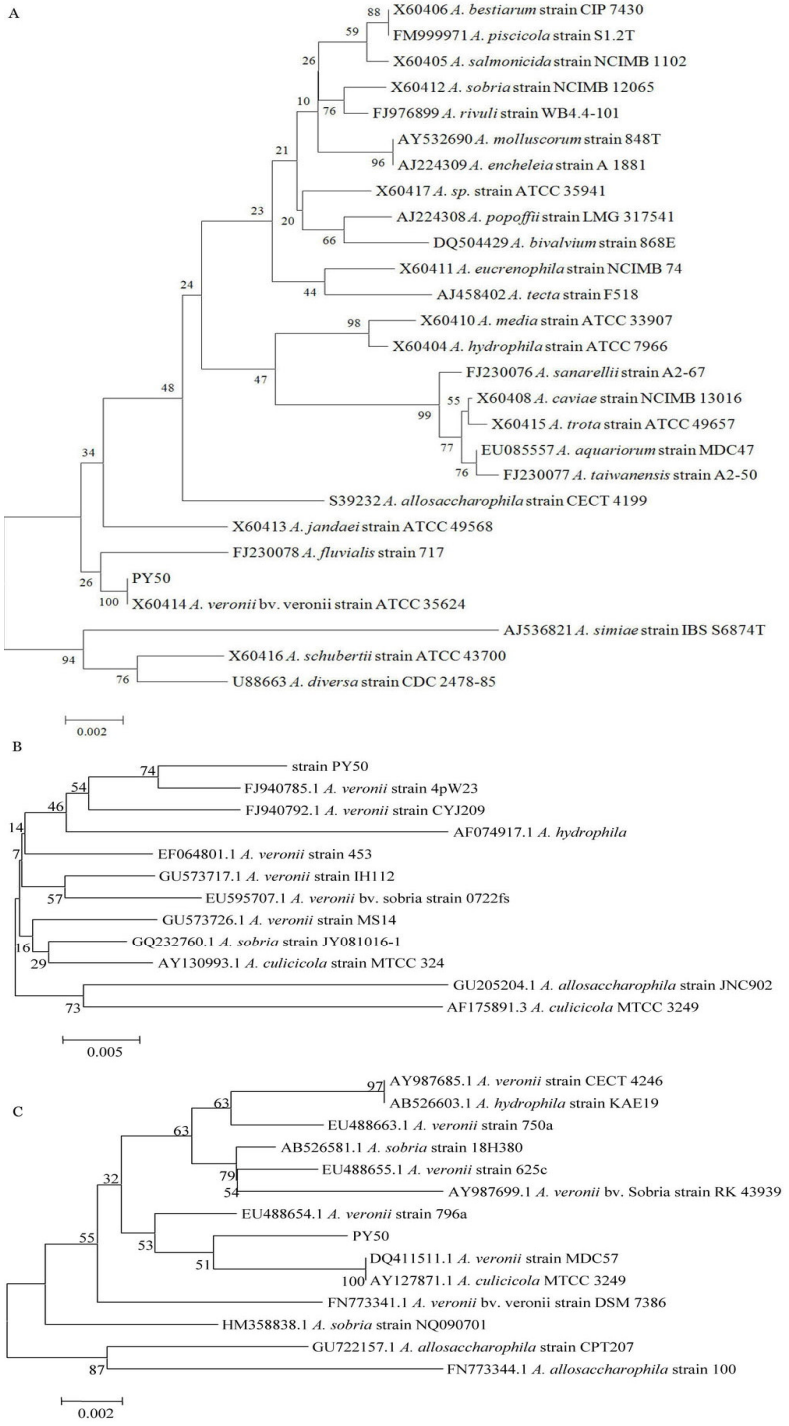

Figure 1. Unrooted phylogenetic trees based on 16S rDNA, *gyrB* and *rpoD* gene sequences, showing relationships in the genus *Aeromonas*. Numbers shown at nodes indicate bootstrap values (percentage of 1000 replicates). A, Unrooted phylogenetic trees based on 16S rDNA; B, Unrooted phylogenetic trees based on *gyrB* gene; C, Unrooted phylogenetic trees based on *rpoD* gene.

Table 1. Primers used for PCR amplification and sequencing of 16S rDNA, *gyrB* and *rpoD* genes

Primer	Sequence (5'→3')	Positions*
16S rDNA		
Unip1	CTAACACATGCAAGTCGAGCGCAAGTCGAGCG	48-68
Unip2	ATGGTGTGACGGGCGGTGTGTA	1402-1423
gyrB		
gyrB 3F	TCCGGCGGTCTGCACGGCGT	334-354
gyrB 14R	TTGTCCGGGTTGTACTCGTC	1464-1444
rpoD		
rpoD 70Fs	ACGACTGACCCGGTACGCATGTA	280-302
rpoD 70Rs	ATAGAAATAACCAGACGTAAGTT	1139-1117

*Positions according to *Escherichia coli* numbering.

Table 2. Sensitivity of strain PY50 to various antimicrobial agents

Antimicrobial	Disc content (μg)	Sensitivity*
Ampicillin	10	R
Chloramphenicol	30	R
Chlortetracycline	10	S
Ciprofloxacin	5	M
Doxycycline hydrochloride	30	S
Enrofloxacin	5	R
Erythromycin	15	S
Gentamycin	10	M
Furazolidone	100	S
Kanamycin	30	R
Nalidixic acid	30	S
Neomycin	10	S
Nitrofurantoin	300	S
Oxolinic acid	2	S
Oxytetracycline	30	S
Streptomycin	25	S
Sulfisoxazole	300	S
Sulphonamide	300	M
Tetracycline	10	M
Trimethoprim	5	S
Vancomycin	10	S

*R, resistance; S, sensitive; M, moderately sensitive

Table 3. Virulence test of bacterial cells of representative strain PY50 injected into Chinese longsnout catfish during two weeks observations

Sample	Dose (CFU or μg per fish)	Mortality (%)	LD_{50} value (CFU or μg per fish)
PBS		0	
Bacterial cells	0.1×10^2	0	3.47×10^4
	0.1×10^3	10	
	0.1×10^4	35	
	0.1×10^5	60	
	0.1×10^6	90	
	0.1×10^7	100	
ECPs	1.00	0	11.22
	2.15	5	
	4.64	20	
	10.00	45	
	21.54	65	
	46.42	100	

DISCUSSION

Despite Abbott *et al.* reported that *Aeromonas* species are often mistakenly identified as *Vibrio* by Vitek identification systems because they share many phenotypic characteristics (1). Some papers reported it seems possible that the Vitek GNI+ card had been useful for species identification within the genus *Aeromonas* (14, 17). All the 273 field isolates obtained from the moribund fish during the outbreak of ulcerative syndrome from Chinese longsnout catfish were tentatively identified as *A. veronii* bv. *veronii* by the Vitek GNI+ card.

Phylogenetic analysis based on the 16S rDNA gene is considered an appropriate tool for the reconstruction of evolutionary history and phylogenetic relationships of bacterial genera and it is universally used (22). In some *Aeromonas*, 16S rDNA gene sequences showed that the genus is composed of a very tight group of species, some of them differing by only a few nucleotides (15). Other genes have therefore been evaluated as tools for the phylogenetic and taxonomic analysis of this genus. *gyrB* and *rpoD* gene sequence have proved to be an excellent molecular chronometer for phylogenetic inference in the genus *Aeromonas* (21, 26). This phylogenetic marker revealed strain groupings consistent with the taxonomy proposed in most previous genetic and phylogenetic studies, particularly in agreement with 16S rDNA sequence analysis. In this study, the molecular identification of the representative strain PY50 was based on 16S rDNA, *gyrB* and *rpoD* sequence were conducted. All sequences of 16S rDNA, *gyrB* and *rpoD* gene from the representative strain PY50 showed high similarity (97% to 100%) with *A. veronii* bv. *veronii*, and fairly low homology with other *Aeromonas*. In summary, the representative strain PY50 was a strain of *A. veronii* bv. *veronii* on the basis of the results described above.

From the in vitro sensitivity assay, the representative strain PY50 was multi-resistant to the most frequently used antimicrobial agents in China during the present study, such as ampicillin, enrofloxacin and kanamycin, but not resistant to most of the antibiotics tested. Joseph *et al*. reported *A. veroni* bv. *veroni* was resistant to ampicillin, but sensitive to enrofloxacin and kanamycin (8). Vila *et al*. reported *A. veronii* was resistant to nalidixic acid and pipemidic acid (25). The results showed *A. veroni* bv. *veroni* was resistant to different antimicrobial agents in different area and country, so the test of antimicrobial resistance should be performed when the bacteria are isolated from the samples, in order to avoid therapeutic failures with the development of serious clinical symptoms and spread of the pathogenic organisms in the environment with secretive and excretive products.

Han *et al*. reported that a *A. veroni* bv. *veroni* strain RY001 was virulent to goldfish with an LD_{50} value of 1.6×10^6 CFU per fish which was much higher comparing to the present strain PY50 in Chinese longsnout catfish for intraperitoneal injection (3.47×10^4 CFU per fish) (7). This difference could be due to different strain or host studied. Many virulence determinants, such as proteases, haemolysins and enterotoxins, have been identified to cause disease in *Aeromonas* (19). The ECPs, such as collagenase were reported as the important virulent factor to *A. veroni* bv. *veronii* (7). In the present study, the ECPs of strain PY50 was lethal to Chinese longsnout catfish and the LD_{50} was 11.22 μg per fish, and the results were in accordance with the above-mentioned study.

In conclusion, we confirmed that representative strain PY50 is a strain of *A. veronii* bv. *veronii* highly pathogenic to Chinese longsnout catfish, and *A. veronii* bv. *veronii* is the pathogen caused outbreaks of ulcerative syndrome in Chinese longsnout catfish.

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