

Yersinia enterocolitica as a cause of septicemia in crucian carp (*Carassius carassius*)

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Abstract

Yersinia enterocolitica is an important pathogen to animals and humans. Thirteen strains of *Y. enterocolitica* were isolated from diseased fish. The partial 16SrDNA gene, five virulence genes, pathogenicity, and drug resistance of *Y. enterocolitica* strain G6029 were studied using molecular biological technology and toxicological method. Results showed that the length of amplified 16SrDNA sequence was 1448bp, and revealed 99% homology with *Y. enterocolitica*. Its GenBank accession number was JX855135. Five virulence genes (*ail*, *ystB*, *yadA*, *virF* and *HPIINT*) were detected, and only *yadA* gene was not seen. Twenty-eight crucian carps that were injected with strain G6029 died within a week, and the mortality was 93.3%, indicating highly pathogenic attribute of this strain. In addition, the strain G6029 was resistant to four antibiotics (sulfafurazole, furazolidone, enrofloxacin and norfloxacin), but it was susceptible to six antibiotics (florfenicol, vibramycin, cefaloridine, ciprofloxacin, streptomycin, and ampicillin). Further study of pathogenicity of *Y. enterocolitica* in teleost fish is suggested.

Keywords: *Yersinia enterocolitica*; Virulence gene; Pathogenicity; *Carassius auratus*

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Introduction

Y. enterocolitica is a widespread member of genus *Yersinia*, which belongs to the phylum *Proteobacteria*, class Gammaproteobacteria, order *Enterobacteriales*, family Enterobacteriaceae (Bottone, 1997). *Y. enterocolitica* is Gram-negative, rod-shaped, non-sporulating, facultatively anaerobic c-proteobacteria. *Y. enterocolitica* is most often transmitted by consumption of contaminated food, unpasteurized or inadequately pasteurized milk, untreated water, or by direct or indirect contact with animals (Fredriksson *et al.*, 2003; Sabina *et al.*, 2011;). It is classified using biochemical characteristics along with serotyping. There are six major biotypes designated as 1A (the non-pathogenic type), and there are pathogenic biotypes 1B, 2, 3, 4, and 5 (Kot *et al.*, 2010).

Y. enterocolitica, a classical enteric pathogen, causes human and animals infections whose symptoms include diarrhea, terminal ileitis, intestinal intussusception, mesenteric lymphadenitis, arthritis, and septicemia (Imoto *et al.*, 2012). Moreover, *Y. enterocolitica* can occasionally be present as a primary skin and soft tissue abscess from direct inoculation and extend to cause regional suppurative adenitis (Menzies, 2010). The primary pathogenic event of *Y. enterocolitica* is colonization of the intestinal tract where most of the

pathologic effects and clinical manifestations occur. Temperature and calcium concentration regulate expression of virulence factors that guide the invading yersinia and allow them to survive and disseminate (Fàbrega and Vila, 2012; Asadishad *et al.*, 2013). The genome sequencing of a representative of the most epidemiologically successful *Y. enterocolitica* subsp. *palaearctica* strain Y11, serotype O:3, biotype 4 has been finished and annotated (Batzilla *et al.*, 2011). Some virulence genes (i.e., *yadA*, *virF*, *ysa*, *inv*, *ail*, and *yst*) located in chromosome or plasmid of pathogenic *Y. enterocolitica* has been widely used to identify pathogenic strains in epidemiological studies (Fredriksson *et al.*, 2006). However, the pathogenesis and epidemiology of *Y. enterocolitica* are complex and remain incompletely understood as most cases are sporadic and reported without an apparent source.

Y. enterocolitica had been isolated from humans, alpine ibex, piglets, sheep, birds, rodents, African green monkeys, and other animals, as well as from the environment, such as water and soil (; Neubauer and Sprague, 2003; Fàbrega and Vila, 2012; Joutsen *et al.*, 2012; Söderqvist *et al.*, 2012; Virtanen *et al.*, 2012; Soto *et al.*, 2013; Virtanen *et al.*, 2014). However, few reports existed in the literature regarding infections caused by this organism in fish (Andrew *et al.*, 2001). Crucian carp (*C. auratus* L.,

abbreviated CC) belongs to the genus of *Carassius* within the family of *Cyprinidae*. Due to its good survival rate, high reproduction rate and good disease resistance, it is widely bred in Eurasia and America. In China, crucian carp is one of the most important freshwater species for Chinese aquaculture and has been found in most provinces (Xiao *et al.*, 2011). In this study, the case of fatal *Y. enterocolitica* infections was reported in crucian carp, and the partial 16SrDNA gene, virulence genes, pathogenicity and antibiotic resistance were studied by molecular biological technology and toxicological method.

Materials and methods

Bacterial isolates

In May, 2012, thirteen crucian carps (body weight 46-68g) were obtained from a local fish farm in Chengdu city, Sichuan Province. The symptoms of these carps included: sluggishness, extensive dermatorrhagia mainly around lower jaw and abdomen, abdominal swelling, and red swelling of the anus. The morbidity and mortality were about 15% and 10%, respectively. Necropsy was performed on these crucian carps within 30 min of death. Typically, the hearts and spleens showed hyperaemia and swelling, the intestines were enlarged with diffuse hemorrhagy. The heart samples of thirteen crucian carps were immediately inoculated in blood plates

under aseptic processing. Afterwards, the samples were subcultured onto general nutrition agar plate and incubated at 28°C for 17h. Single colonies of bacterial strains were picked and further grown and sub-cultured several times to obtain a pure culture. Biochemical characterizations of the strains was performed using API 20E system (Simmons *et al.*, 2014). The isolate was biotyped by special biochemical characterizations, and serotyped by slide agglutination with antisera (O:3, O:9 and O:8).

DNA isolation of bacteria

After 18h cultivation in lysogeny broth (LB) medium at 28°C, bacterial DNA was extracted using genomic extraction kit for bacterium (Axygen, China) according to the manufacturer's instruction. The integrity of DNA was checked using 1.0% (w/v) agarose gel in TBE buffer. DNA was quantified and stored at -20°C until required.

PCR amplification of 16SrDNA gene

The 16SrDNA gene was partially amplified in PCR reactions using the universal primers 27F and 1492R. The 27F primer was 5'-AGA GTT TGA TCC TGG CTC AG-3' and the 1429R primer was 5'-ACG GCT ACC TTG TTA CGA CTT-3'. The PCR reaction mixture included 21µL of ddH₂O, 2µL of bacterial DNA, 2µL of each primer (10

umol), and 23 μ L of rtaq Mix (Takara, Japan) in a 50 μ L final volume. Amplifications were performed at 94°C for 4min, 94°C for 30s, 60°C for 30s, 72°C for 4min (5 cycles); 94°C for 30s, 55°C for 30s, 72°C for 4 min (5 cycles); 94°C for 30s, 50°C for 30s and 72°C for 4 min (30 cycles). PCR products were examined for size and yield using 1.2% (w/v) agarose gel in TBE buffer. The obtained product was sequenced on both strands using an ABI PRISM 3730 automated sequencer (ABI, America).

Phylogenetic analysis

DNA sequences were edited and assembled using the programs DNA Star. A comparison of the 16SrDNA gene sequence of the test strain with the non-redundant collection of sequences was performed using BLAST program (<http://www.ncbi.nlm.nih.gov/BLAST>) (Raja *et al.*, 2015). Multiple sequence alignments were generated using ClustalW. A phylogenetic tree was constructed using neighbor joining method with MEGA5 package. The reliability of the neighbor joining tree was estimated using bootstrap analysis with 1000 replicates.

Amplification of virulence gene

Five virulence genes (ail, yadA, ystB, virF and HPIint) of *Y. enterocolitica* were designed according to relevant sequences in Genbank database and

reference (Thoerner *et al.*, 2003). These primers are shown on Table 1 and synthesized by Shanghai Sangon Biotech Companies. The PCR reaction mixture included 12 μ L of rtaq Mix (Takara, Japan), 1 μ L of bacterial DNA, 11 μ L of ddH₂O, 1 μ L of each primer (10 μ mol) in a 25 μ L final volume. Amplifications were performed with the following PCR parameters: 94°C for 5 min; 94°C for 60s, 48-56°C for 60s, 72°C for 60s (35 cycles); 72°C for 10 min. The optimal annealing temperatures for yadA genes were proposed by gradient PCR, which varied from 40°C to 60°C. PCR products were examined for size and yield using 1.2% (w/v) agarose gel in TBE buffer.

Artificial infection experiment

The pathogenicity of the *Y. enterocolitica* was performed in sixty crucian carps weighing 49-72g. These crucian carps were artificially cultivated in Chengdu city, Sichuan province. Each treatment group had 10 apparently healthy crucian carps randomly allocated, with 2 replicate tanks. A volume of 0.1 mL of bacterial concentration (3×10^8 cfu/mL) was given to each fish by intraperitoneal injection. While, the control groups (30 fishes) were treated in the same way but were inoculated with 0.1 mL of PBS (pH7.4). This challenge lasted for a week and during this time the fishes were normally maintained and not fed. The mortality was given as number of dead

animals /total number of animals tested. Bacteriological analyses of dead crucian carp were carried out in all the cases. Samples were taken from the experimental group and cultured using cefsulodin-irgasan-novobiocin (CIN) agar. Then, the pure culture was identified by biochemical characteristics and PCR. Death was considered caused by inoculated bacteria only if the strain used for inoculation was isolated in pure culture.

Susceptibility testing

Susceptibility of the *isolated* strain to twelve antibiotics was determined by Kirby-Bauer disk diffusion method according to the clinical laboratory standards institute guidelines. The test organism was picked up with sterile loop and suspended in peptone water and incubated at 37°C for 2 hours. Standard 0.5 McFarland saline suspensions of bacteria were used to inoculate Mueller Hinton agar media confluent with a cotton swab. Antibiotic disks were placed on the agar, and incubated at 37°C overnight. The zone of inhibition was measured and interpreted as per the CLSI guidelines. *Escherichia coli* (ATCC25922) was used as quality control strains for the susceptibility testing method.

Results

Bacterial isolates

Samples were taken from the hearts of thirteen crucian carps and inoculated in blood nutrient agar plate and nutrition agar plate. After incubation at 28°C, the thirteen pure cultures were identified. On nutrient agar, single isolates produced smooth transparent colonies with an entire edge. These isolates displayed morphologic characteristics typical of the *Yersinia* appearing as small rod-shaped, Gram-negative coccobacilli. Biochemical characterization identified these isolates as *Y. enterocolitica* (probability 99.9%). For further study, one strain of these isolates was named G6029, and identified to be biotype 1B and serogroup 0:8.

PCR amplification and analysis of 16S rDNA gene

In order to further characterize the isolate, 16SrDNA of strain G6029 was partially sequenced. This 16SrDNA sequence was 1448bp, which showed 99% homology with *Y. enterocolitica*. It was submitted to NCBI, and gained a GenBank accession number (JX855135). A phylogenetic analysis to assess the relationship of strain G6029 with other bacterium was performed (Fig.1). The result revealed the relationships between strain G6029 and *Yersinia*. Strain G6029 belonged to the group of *Y. enterocolitica*, and grouped together with *Y. enterocolitica* (FJ717343), as the closest neighbour.

Strain G6029 was confirmed again to be *Y. enterocolitica* in light of the sequence analysis of 16S rDNA.

Amplification of virulence gene

PCR-based assays were developed for the detection of five virulence genes in strain G6029. The *yadA* and *virF* were plasmid-borne genes, while the *ail*, *ystB* and *HPIint* were chromosomal virulence genes. In the results the *ail*, *ystB*, *virF* and *HPIint* were detected, however, *yadA* gene was not seen after optimizing the experimental conditions of PCR (Fig. 2). Additionally, the lengths of *ail*, *ystB*, *virF* and *HPIint* gene were about 351bp, 146 bp, 561bp and 714bp, respectively, which were in line with the anticipant results.

Artificial infection experiment

In order to investigate the pathogenicity of *Y. enterocolitica* strain G6029, it was injected into abdominal cavity of thirty crucian carps. The symptoms observed in intraperitoneally inoculated fish consisted of inactivity, anorexia, and red swelling of the anus, which accorded with the natural cases observed. The mortality was observed 48 h after inoculation of

bacterial suspension. The time of death ranged from two to seven days after the challenge. A number of 28 crucian carps that had been injected with strain G6029 died within a week, while all normal controls (30 healthy crucian carps) were alive, and had no obvious symptom. The mortality of the crucian carps was 93.3%. *Y. enterocolitica* strains were reisolated using CNI-1 culture medium from all moribund and dead crucian carp after the challenge. *Y. enterocolitica* colonies on CIN formed the characteristic deep red center (bull's eye) with a transparent margin and diameter 2-4 mm.

Susceptibility testing

The antibiotic resistance of *Y. enterocolitica* strain G6029 was screened by Kirby-Bauer disk diffusion method. This stain was resistant to four antibiotics (sulfafurazole, furazolidone, enrofloxacin, and norfloxacin). However, it was insensitive to two antibiotics (erythromycin, and penbritin). In addition, it was susceptible to six antibiotics (florfenicol, vibramycin, cefaloridine, ciprofloxacin, streptomycin, and ampicillin).

Table 1: Primers of virulence genes for *Y. enterocolitica*

Gene	Forward primer 5'-3'	Reverse primer 5'-3'	Tm(°C)	Size(bp)
<i>ail</i>	taa tgt gta cgc tgc gag	gac gtc tta ctt gca ctg	50	351
<i>ystB</i>	gta cat tag gcc aag aga cg	gca aca tac ctc aca aca cc	56	146
<i>yadA</i>	ctt cag ata ctg gtg tgc ctg t	atg cct gac tag agc gat atc c	56	849
<i>virF</i>	ggc aga aca gca gtc aga cat a	ggg gag cat aga gaa tac gtc g	48	561
<i>HPIINT</i>	tgc gcc atg egg tcc atc	ggg gca taa gat tct egg	50	714

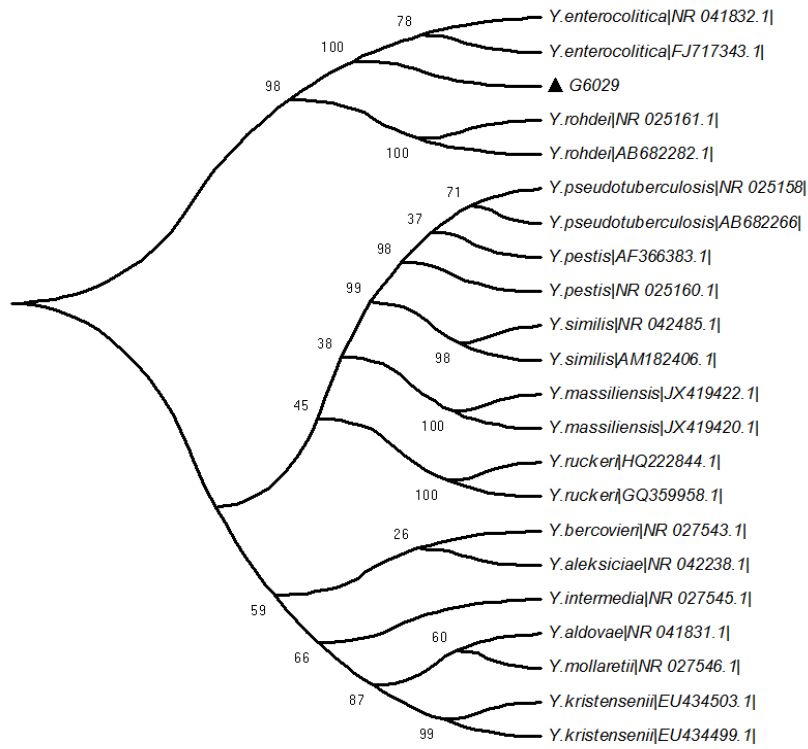


Figure 1: Phylogenetic tree of strain G6029 and other *Yersinia*.

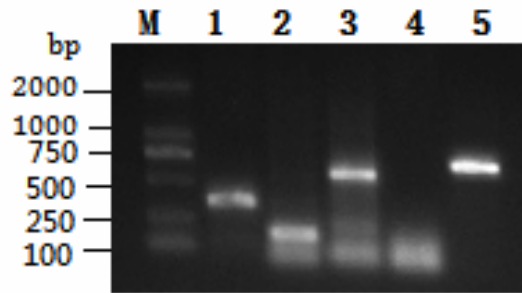


Figure 2: The virulence genes of *Y. enterocolitica* strain G6029
1.ail, 2. ystB, 3. virF, 4. yadA, 5.HPI, M.DNA Marker

Discussion

Y. enterocolitica is a Gram-negative, predominantly extracellularly located pathogen that causes food-borne acute or chronic gastrointestinal diseases in animals and humans. Rapid and reliable isolation and identification of strains of the species *Y. enterocolitica* within the genus *Yersinia* and the differentiation of the pathogenic from the non-pathogenic biotypes are increasingly important. Isolation of pathogenic *Y. enterocolitica* can be done using CIN agar which is useful to expedite the recovery of *Y. enterocolitica* and mVYE agar to differentiate virulent from non-virulent isolates. The characteristic deep red center (“bull’s eye”) with a transparent margin and appearance of *Yersinia* colonies with a diameter 2-4 mm on CIN incubated at 30°C for 24h is important for identification and is due to the presence of mannitol (Fukushima *et al.*, 2011).

Salmonella-Shigella-desoxycholate-calcium chloride (SSDC) agar, and *Y. enterocolitica* chromogenic medium (YeCM) were also used as selective agar media. For enrichment, irgasan-ticarcillin-potassium chlorate (ITC) broth and peptone-sorbitol-bile (PSB) broth were incubated at 25°C for 48h (Premaratne *et al.*, 2012). In this study, *Y. enterocolitica* strains were reisolated from artificial infection experiment using CIN agar that

confirmed the CIN agar was useful for the isolation and identification of *Y. enterocolitica*.

Y. enterocolitica is an important cause of acute gastrointestinal disease and post-infectious complications in humans and animals. *Y. enterocolitica* biotype 1B, serotype O:8 (1B/O:8), is the most pathogenic of the *Yersinia* species because of the presence of high-pathogenicity island and the *Yersinia* virulence plasmid (pYV). There was a pediatric case of *Y. enterocolitica* 1B/O:8 bacteremia and enterocolitis. A 20-month-old girl was admitted to hospital with fever, pharyngitis, and abdominal pain on day 2. Blood culture on admission was positive for *Y. enterocolitica* 1B/O:8 (Ito *et al.*, 2012). In this study, *Y. enterocolitica* strain 6209 was also identified to be biotype 1B and serogroup O:8, and it was highly pathogenic to crucian carps. Fresh fish from France, Great Britain and Portugal were examined for the pathogens *Y. enterocolitica*. It showed that *Y. enterocolitica* was detected from both salmon and trout in Great Britain (Andrew *et al.*, 2001). Many materials about *Yersinia ruckeri* infection in fish has been widely reported (Tobback *et al.*, 2007; Bastardo *et al.*, 2012; Jaafar *et al.*, 2012). However, there is still little information about the *Y. enterocolitica* infection in fish by now.

There are some researches

investigating the distribution of virulence genes in clinical isolates of pathogenic *Y. enterocolitica* (Kumar and Viridi, 2012). It also highlights the importance of addressing genetic and phenotypic variations among closely related bacterial strains, even those belonging to the same bioserotype (Garzetti *et al.*, 2012). Virulence genes can be applied as distinguishing markers and indicators of the potential virulence of *Y. enterocolitica* strains. Plasmid-encoded targets, like *virF* and *yadA* genes, and chromosomally encoded targets, like *ail*, *inv*, *ystB*, and *HPIINT* genes, have also been used in PCR to identify *Y. enterocolitica* strains in clinical, food, and environmental samples (Bhaduri *et al.*, 2005; Thisted and Danielsson, 2005). The virulence of *Y. enterocolitica* can be increased, by acquiring new genes and/or improving the function of essential virulence proteins, resulting in permanently hyper-virulent strains.

The attachment invasion locus (*ail*) gene, located in the chromosome of pathogenic *Y. enterocolitica* strains was the most frequently used targets. The *ail* sequence was highly conserved among the same serotype strains from different sources (Huang *et al.*, 2010). The *ail* and *ystA* genes were detected in all the strains isolated from humans and animals, but only in two strains isolated from food (Falcão *et al.*, 2006). A high correlation has been found between the *ail* gene and

the virulence of *Yersinia*. However, there was a report about two *Y. enterocolitica* biotype 1A strains that are usually nonpathogenic and carry the *ail* gene. The *ail* gene sequences of biotype 1A strains displayed similarity to the bioserotype 1B/O:8 strain 8081 suggesting that *ail*-based detection methods for *Y. enterocolitica* alone are insufficient to detect real pathogenic strains (Sihvonen *et al.*, 2011). A number of 160 isolates of pathogenic *Y. enterocolitica* cultured from stool samples were examined for virulence genes (*inv*, *ail*, *ystA*, *ystB*, *ystC*, *yadA*, *virF*) by PCR. The positive rate of virulence genes tested in 160 isolates was *inv* (100%), *ail* (94%), *ystA* (93%), *ystB* (7.5%), *ystC* (5%), *yadA* (89%) and *virF* (82%), respectively (Zheng *et al.*, 2008).

In this study, five virulence genes (*ail*, *ystB*, *HPIINT*, *yadA*, and *virF*) of *Y. enterocolitica* were analyzed. These genes except *yadA* were all detected, which was basically consistent with the above reports. These data showed that not all pathogenic *Y. enterocolitica* necessarily carry all traditional virulence genes in both plasmids and chromosomes to cause illness. Perhaps, some of them, lacking some traditional virulence genes, contain other unknown virulence markers that interact with each other and play an important role in the diverse pathogenicity of pathogenic *Y. enterocolitica*. The *yadA* gene was

located on the *Yersinia* virulence plasmid and its expression is mainly temperature-regulated occurring at 37°C. However, strain G6029 was cultured in 28°C. So the reason why *yadA* gene had not been seen might be due to the low temperature or possible plasmid loss on subculture and storage. The HPIint (High-Pathogenicity Island) gene is a partial genomic island indispensable for pathogenicity of *Yersinia* and was detected in strain G6029, which indicated strain G6029 might have high pathogenicity. Moreover, the pathogenicity of the *Y. enterocolitica* strain G6029 was proved in crucian carps by artificial infection test. This study showed that this strain had high pathogenicity to crucian carp. Furthermore, it also indicated that this strain might be virulent to other aquatic animals.

In conclusion, thirteen *Y. enterocolitica* strains were isolated from *C. auratus*, and the 16SrDNA, pathogenicity and antibiotic resistance of strain G6029 were studied by molecular biological technology and microbiological method. To the best of our knowledge, it is the first case of *Y. enterocolitica* infection in *C. auratus*. Further investigations should be focused on the pathogenesis of *Y. enterocolitica* in teleosts.

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