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# Isolation and characterization of *Edwardsiella ictaluri* strains as pathogens from diseased yellow catfish *Pelteobagrus fulvidraco* (Richardson) cultured in China

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## Abstract

Yellow catfish *Pelteobagrus fulvidraco* (Richardson) is a commercially important fish generally distributed in Southeast Asian countries. The well-known aetiological agent of enteric septicaemia of catfish, *Edwardsiella ictaluri*, was isolated from diseased yellow catfish *P. fulvidraco* (Richardson) reared at two commercial fisheries in China. The economic losses due to the high mortalities (about 50%) caused by this bacterium have been increasing annually. The affected fish presented two different, typical symptoms: pale gills, slight exophthalmia and a 'hole in the head', and haemorrhage on the opercula, in the skin under the jaw, creating a 'hole under the jaw'. These diseases were found frequently in cultured yellow catfish throughout China. The isolates from both outbreaks were all Gram negative, facultatively anaerobic and short rod. Morphological and biochemical tests and phylogenetic analysis based on the 16S rDNA sequences all strongly indicated that these yellow catfish isolates were highly identical to the known *E. ictaluri*. In addition, the isolates possessed the typical plasmid profile of *E. ictaluri*. Experimental infection assays were conducted and pathogenicity (by an intraperitoneal injection) was demonstrated in yellow catfish and channel catfish *Ictalurus punctatus*. The results showed that yellow catfish isolates were quite conservative phenotypically and genetically, and were able to cause two different, typical symptoms in this fish under unknown conditions and mechanism.

**Keywords:** isolation, characterization, *Edwardsiella ictaluri*, yellow catfish

## Introduction

*Edwardsiella ictaluri*, a Gram-negative, rod-shaped, oxidase-negative, peritrichous, fermentative bacterium, has been reported almost exclusively as an obligate pathogen of ictalurids fish and causes enteric septicaemia of catfish (ESC), especially in channel catfish *Ictalurus punctatus* (Hawke, Mc-Whorter, Steigerwalt & Brenner 1981). In the United States, enteric septicaemia of catfish (ESC) was recognized as the most prevalent disease affecting farm-raised channel catfish (Hawke 1979), costing approximately 50 million dollars in losses annually (Mitchell 1997). Some other ictalurids (i.e. the ictaluridae family) fish could be infected by *E. ictaluri* under natural conditions, such as blue catfish *Ictalurus furcatus*, white catfish *Ictalurus catus* (Newton, Bird, Blevins, Wilt & Wolfe 1988) and brown bullhead *Ictalurus nebulosus* (Iwanowicz, Griffin, Cartwright & Blazer 2006).

Isolation of *E. ictaluri* from non-ictalurids has been reported in natural disease outbreaks in green knife fish *Eigenmannia virescens* (Kent & Lyons 1982), danio *Danio devario* (Blazer, Shotts & Waltman 1985; Waltman, Shotts & Blazer 1985; Petrie-Hanson, Romano, Mackey, Khosravi, Hohn & Boyle 2007), walking catfish *Clarias batrachus* L. (Kasornchandra, Rogers & Plumb 1987), rosy barb *Puntius conchonius* (Humphrey, Lancaster, Gudkovs & McDonald 1986), rainbow trout *Oncorhynchus mykiss* (Keskin, Secer, Izgur, Turkyilmaz & Mkakosya 2004), freshwater catfish *Pangasius hypophthalmus* (Sauvage) (Crumlish, Dung, Turnbull, Ngoc & Ferguson 2002), Tra catfish *Pangasianodon hypophthalmus* (Sauvage) (Truong, Areechon, Srisapoome & Mahasawasde 2007), harle-

ququin tetra *Rosbara heteromorpha* (Reid & Boyle 1989) and tadpole madtoms *Noturus gyrinus* (Klesius, Lovy, Evans, Washhuta & Arias 2003). Additionally, other species, such as chinook salmon *Oncorhynchus tshawytscha*, tilapia *Sarotherodon aureus* and European catfish *Silurus gianis*, have been infected experimentally with *E. ictaluri* (Plumb & Sanchez 1983; Plumb & Huge 1987; Baxa, Groff, Wishkovsky & Hdrick 1990), but natural outbreaks in these species have not been reported. Actually, Plumb and Sanchez (1983) reported that a number of warmwater species, including largemouth bass *Micropterus salmoides*, golden shiner *Notemigonus crysoleucas* and bighead carp *Aristichthys nobilis*, were highly resistant to *E. ictaluri* experimental intraperitoneal infections. White sturgeon *Acipenser transmontanus*, striped bass *Morone saxatilis* and rainbow trout *Oncorhynchus mykiss* were resistant to *E. ictaluri* in experimental immersion exposures as well (Baxa *et al.*1990).

Yellow catfish *Pelteobagrus fulvidraco* is a species of order Siluriformes, family Bagridae, genus *Pelteobagrus*, placed in the same order with channel catfish, but in a different family. Yellow catfish is a commercially important fish generally distributed and cultured in Southeast Asian countries and especially in China. However, the problem of bacterial diseases related to this fish has drawn little attention. The present report documents the first isolation of *E. ictaluri* strains from the moribund cultured yellow catfish with different clinical symptoms and reared at two different farms in China. The organisms from the two sources were proved to be identical and pathogenic to yellow catfish, channel catfish fingerlings by an experimental intraperitoneal injection.

## Materials and methods

### Diseased yellow catfish and characterization

Epizootics occurred in adult yellow catfish (body weight 100 – 130 g approximately) cultured in earthen ponds of two commercial fisheries in Wuhan (located in the middle of China) and Huzhou (located in eastern China) during August and September in 2007 and 2008 respectively. During the outbreaks, the water temperatures were approximately 25–27 °C.

In the two areas, the behavioural signs of affected fish included reduced feeding activity, lethargy in response, listless swimming on the surface with a 'head up–tail down' posture, sometimes spiralling or occasional rapid swimming, usually followed by death. Clinical signs of the diseased fish in Huzhou included ulcerated haemorrhages on the oral area, on the opercula, at the base of all fins and in the skin under the jaw, even creating a 'hole-under-the-jaw' condition (Fig. 1b). Upon necropsy, numerous petechial haemorrhages were found on the liver, intestines and adipose tissue, and accumulation of ascites in the abdomen, whereas clinical signs of the affected fish from Wuhan included pale gills, slight exophthalmia, with the most serious symptom being an open necrotic lesion in the skull between the eyes, namely a 'hole in the head' (Fig. 1a). Five fish showing typical clinical signs representative of each outbreak were collected and sent to the laboratory alive for further examination in plastic transportation bags with oxygen supply.

### Bacterial isolation

Using aseptic techniques, samples taken from the brain, kidney and liver of the moribund yellow catfish were streaked on brain–heart infusion (BHI, BD, Franklin Lakes, NJ, USA) agar plates and incubated at 26 °C for 48 h. Colonies on the plates were pure and single colonies were selected and re-streaked on the same media. Six bacterial strains were collected from the different moribund fish: two strains (HSN-1 and HSS-1) from Wuhan and four strains (HSA-1, HSA-2, HSX-1 and HSX-2) from Huzhou. HSN-1 and HSS-1 were isolated from the brain and the kidney

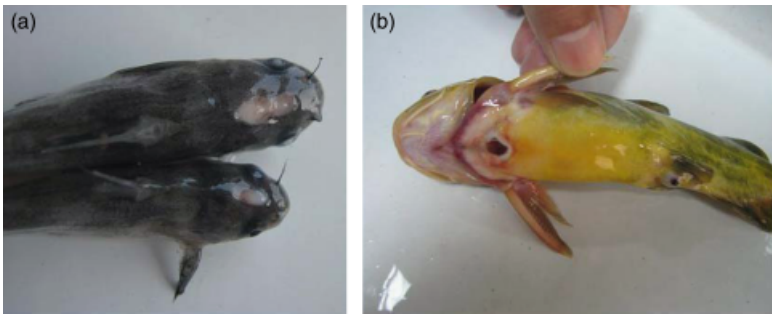


**Figure 1** Yellow catfish affected by *Edwardsiella ictaluri*, showing the most typical external clinical signs of the disease: a 'hole in the head' found in Wuhan (a) and a 'hole under the jaw' found in Huzhou (b).

respectively; HSA-1, HSA-2, HSX-1 and HSX-2 all were isolated from the liver. All isolates were maintained separately in BHI (BD) broth with 15% glycerol at − 80 °C.

### Morphological studies

Isolates were inoculated on BHI (BD) agar overnight at 26 °C; colony morphology was observed directly and using light microscopy. Cell morphology was examined using scanning electron microscopy (Hitachi, Japan) as described previously (Goldstein, Newbury, Joy, Lyman, Echlin, Lifshin, Sawyer & Michael 2003). Cell mobility was determined in wet mounts using a phase-contrast microscopy (Olympus, Shinjuku-ku, Tokyo, Japan) examination (× 1000) and in semisolid glucose motility deep cultures (Walters & Plumb 1978).

### Biochemical analysis

Biochemical tests were performed at 30 °C unless otherwise specified. Before being tested, the isolates were subcultured twice overnight in BHI (BD) broth at 30 °C. For cultural and morphological examinations, Gram staining, oxidation/fermentation (O/F) reaction, indole and hydrogen sulphide ($H_2S$) production were investigated using conventional methods. The growth on MacConkey (BD) agar was tested for 7 days. Catalase activity was determined by transferring fresh colonies from BHI agar to a drop of 5% (v/v) $H_2O_2$ (SCRC, Shanghai, China) in a slide glass. Oxidase activity was determined using 1% (w/v) dimethyl ρ-phenylenediamine chloride. The optimum growth temperature for each isolate was tested in BHI broth after incubation at 4, 10, 15, 20, 25, 30, 37 and 42 °C for 15 days. Growth in the presence of 1.0%, 2.0% and 3.0% (w/v) of NaCl was determined in BHI broth until growth was observed or otherwise at least for 15 days. Hydrolysis of esculin and gelatine was detected using the method described previously (Hsu, Shotts & Waltman 1985). Reduction in 1% (w/v) nitrate was determined as described previously after incubation in BHI broth with 1% nitrate for 7 days. Acid formation from carbohydrates was tested using a basal medium containing 2.0 g tryptone, 5.0 g NaCl, 0.2 g $K_2HPO_4$, 6.0 g agar, 10.0 g carbohydrate and 1000 mL distilled water, adjusting to pH 7.0. Bromothymol blue was used as an indicator.

### Enzymatic activity profiles

Enzyme production of the isolates was determined using the API ZYM kit (API ZYM 25200, bioMérieux, Marcy l'Etoile, France) according to the manufacturer's instructions.

### Antimicrobial susceptibility testing

The susceptibility patterns of bacteria isolates to 25 antimicrobial agents (Oxoid, Mercers Row, Cambridge, UK) including ampicillin (10 μg), bacitracin (0.04 IU), cephalothin V (30 μg), chloramphenicol (30 μg), ciprofloxacin (5 μg), clarithromycin (15 μg), doxycycline (30 μg), enoxacin (10 μg), erythromycin (15 μg), florfenicol (30 μg), furadantin (300 μg), furazolidone (300 μg), gentamicin (10 μg), kanamycin (30 μg), neomycin (30 μg), norfloxacin (10 μg), ofloxacin (5 μg), oxytetracycline (30 μg), piperacillin (100 μg), rifampicin (5 μg), spectinomycin (100 μg), streptomycin (10 μg), sulphamethoxazole/trimethoprim (23.75/1.25 μg), tetracycline (30 μg) and tobramycin (10 μg) were tested and determined using the standard method of Kirby–Bauer (Bauer, Kirby, Sherris & Turck 1966) on Mueller–Hinton agar (MHA, BD) and incubated for 48 h at 26 °C. The *Escherichia coli* reference strain ATCC 25922 was used as quality control (Miller, Walker, Baya, Clemens, Coles, Hawke, Henricson, Hsu, Mathers, Oaks, Papapetropoulou & Reimschuessel 2003), but incubation occurred for 24 h at 37 °C. Results were interpreted as susceptible, mid-susceptible or resistant, based on zone diameters of inhibition, including the diameter of the disc (mm) (Liu, Li, Ji & Yang 2009).

### Molecular analysis

All isolates were incubated into 5 mL of BHI broth separately for 24 h at 30 °C. Genomic DNA was extracted using the silica gel film genomic DNA extraction kit (SBS, Shanghai, China) according to the manufacturer's protocol. DNA was stored at − 20 °C until use. The PCR reaction contained 0.5 U of Taq polymerase (TaKaRa, Shimogyo-ku, Kyoto, Japan), 5 μL of 10 × PCR buffer, 3 μL of 25 mM $MgCl_2$, 2 μL of each 10 mM dNTP (TaKaRa), 1 μL of each 10 mM primer and 500 ng of template DNA, in a final volume made up to 50 μL with sterile double-distilled water. Two sets of universal primers U8f: 5′-AGAG TTGATCATGGCTCAG-3′ and U1492r: 5′-GGTTCAC TTGTTACGACTT-3′ (Weisburg, Barns, Pelletier & Lane 1991) were used to amplify the 16S rRNA gene, which was synthesized by Invitrogen (Shanghai, China). The amplifications were carried out in a thermal cycler (BioRad, Alfred Nobel Drive, Hercules, CA, USA) with the following parameters: an initial dena-

 

turation step of 94 °C for 3 min; 35 serial cycles of 94 °C for 1 min, 56 °C for 30 sec and extension at 72 °C for 90 sec; and a final extension step of 72 °C for 10 min. A negative control (no template DNA) was included in the PCR. The PCR products were analysed by 1% (w/v) agarose gel (containing ethidium bromide) electrophoresis in 1% Tris–acetic acid–EDTA buffer. Gels were visualized and photographed under UV illumination.

The PCR products were purified and cloned into pMD18-T (TaKaRa) in order to transform *E. coli* (DH5α) competent cells. The positive clones were sequenced by Bigdye-Terminator in Unigene (Shanghai, China). The 16S rRNA sequence obtained were BLAST (Altschul, Gish, Miller, Myers & Lipman 1990) in GeneBank database and analysed by the Ribosomal Database Project (RDP-II) (Cole, Chai, Farris, Wang, Julam, McGarrel, Garrity & Tiedje 2005). Previously published 16S rDNA sequences of related organisms were obtained from the GeneBank database. The nucleotide sequences were aligned using the CLUSTAL X program, version 1.8 (Thompson, Gibson, Plewniak, Jeanmougin & Higgins 1997). Genetic distances were obtained using Kimura's two-parameter model (Kimura 1980) and evolutionary trees were constructed using the neighbour-joining method (Saitou & Nei 1987) with MEGA 3 program (Kumar, Tamura & Nei 2004) through 1000-replicate bootstrap analysis. GeneBank Accession numbers of the nucleotide sequences used for the phylogenetic analysis are shown in Fig. 2.

## Plasmid profile analysis

All isolates were incubated in 5 mL of BHI broth overnight at 30 °C and 1.5 mL aliquots were centrifuged at 17 000 *g* for 1 min. Then plasmid DNA of isolates was extracted using the GenExtract™ plasmid DNA extraction kit (DouPson, Beijing, China) according to the manufacturer's protocol. Plasmid DNA extracts were analysed by 0.7% (w/v) agarose gel (containing ethidium bromide) electrophoresis in 1% Tris–acetic acid–EDTA buffer. A supercoiled DNA ladder marker (TaKaRa) was used as a plasmid DNA molecular marker. Gels were visualized and photographed under UV illumination.

## Pathogenicity test

In order to test the pathogenic potential of *E. ictaluri* isolates, two strains HSN-1 and HSA-1 were selected for the experimental infection of healthy yellow catfish or channel catfish. Bacteria isolates were cultured on BHI agar under 30 °C overnight and made into bacterial suspensions with sterile phosphate-buffered saline (PBS) buffer. Yellow catfish with a mean weight of 12.5 ± 1.2 g and channel catfish with a mean weight of 10.5 ± 0.3 g were used in the infection trials.

For the HSN-1 isolate, two species of fish, yellow catfish and channel catfish, were challenged. Each species of fish had three groups (eight fish in each group), and each fish was injected intraperitoneally with 0.2 mL of bacterial suspension containing $5.8 \times 10^7$, $5.8 \times 10^6$ or $5.8 \times 10^5$ CFUmL$^{-1}$ in PBS. In the control group, each fish injected with 0.2 mL of PBS was kept under the same conditions. For the HSA-1 isolate, only yellow catfish, which were divided into five groups (each consisting of seven fish), were used. Each fish was injected intraperitoneally with 0.2 mL of bacterial suspension containing from $1.0 \times 10^8$ to $1.0 \times 10^4$ CFUmL$^{-1}$ in PBS, and seven fish were injected with 0.2 mL of PBS as a control. All assays were conducted in 100 L aquaria with

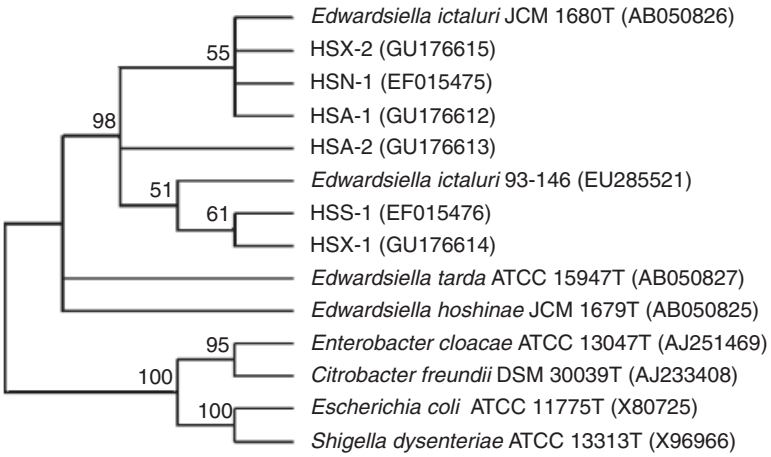


**Figure 2** Phylogenetic relationships between yellow catfish *Edwardsiella ictaluri* isolates and other related bacteria based on 16S rRNA gene sequences. The phylogenetic tree was generated using the neighbour-joining method. The numbers indicate bootstrap values 1000. GeneBank accession numbers are given in parentheses.

50 L still water at a temperature ranging from 25 to 27 °C, with aeration. The water was exchanged half with aeration water once in 2 days. Clinical signs and mortality were recorded daily for 14 days post challenge.

## Results

### Morphologic, phenotypic and biochemical characteristics

Colonies of isolates on the BHI agar plate were usually opaque, smooth, circular and slightly convex, with a diameter of 0.7–1.0 mm. All isolates were found to be phenotypically homogeneous and formed a small** (change ok??) rod shape, with a width and a length of 0.9–1.0 and 2.0–3.0 μm respectively.

The biochemical characteristics of isolates are summarized in Table 1 and compared with the known *E. ictaluri* and *E. tarda* strains. All isolates in our research yielded identical biochemical test results. They were Gram-negative, catalase-positive and oxidase-negative. Motility was observed at 25 °C but not at 37 °C. All could grow at 10, 15, 20, 25, 30 and 37 °C but failed to grow at 4 °C. All could grow on MacConkey agar and were not tolerant of > 2% NaCl. Gas was produced from glucose by isolates at 25 °C but not at 37 °C. All isolates were positive for nitrate reduction, urease and phenylalanine ammonialyase and negative for $H_2S$, indole, MR, VP, dihydrolysis of arginine, hydrolysis of esculin and gelatine, ornithine decarboxylase, lysine decarboxylase or utilization of malonate, citrate and acetate. All produced acid from alantin, D-galactose, D-glucose, D-maltose, D-ribose and mannitose but did not produce acid from D-cellobiose, D-mannitol, D-melezitose, D-raffinose, D-sorbitol, esculin, galactitol, inositol, lactose, laetrile, L-arabinose, melibiose, rhamnose, salicin, starch, sucrose, trehalose and xylose. In addition, all strains produced acid weakly from glycerol.

### Enzyme production profile

In the API ZYM test, six strains presented identical enzymatic properties. All showed positive reactions

**Table 1** Characteristics of yellow catfish *Edwardsiella ictaluri* isolates compared with known *Edwardsiella ictaluri* and *Edwardsiella tarda* isolates

| Characteristic | Six isolates | *Edwardsiella ictaluri* ATCC33202* | *Edwardsiella* tarda ATCC15947† | Characteristic | Six isolates | *Edwardsiellaictaluri* ATCC33202* | *Edwardsiella* tarda ATCC15947† |
|---|---|---|---|---|---|---|---|
| Gram stain | 0 | − | − | Urease | 0 | − | − |
| Growth at 4 °C | 0 | − | − | Phe deaminase | 0 | − | − |
| Growth at 37 °C | 6 | + | + | Acid production from | | | |
| Motility at 25 °C | 6 | + | + | Amygdalin | 0 | − | − |
| Motility at 37 °C | 0 | − | + | Arabinose | 0 | − | − |
| Gas from glucose at 25 °C | 6 | + | + | Dulcitol | 0 | − | − |
| Gas from glucose at 37 °C | 0 | − | + | Galactose | 0 | − | + |
| Maconkey agar | 6 | + | + | Glucose | 6 | + | + |
| Oxidase | 0 | − | − | Inositol | 0 | − | − |
| Catalase | 6 | + | + | Lactose | 0 | − | − |
| Oxidation/fermentation | 6/6 | +/+ | +/+ | Maltose | 6 | + | + |
| Nitrate reduction | 6 | + | + | Mannitol | 0 | − | − |
| Gelatine hydrolysis | 0 | − | − | Mannose | 6 | + | + |
| Esculin hydrolysis | 0 | − | − | Mellibiose | 0 | − | − |
| Malonate | 0 | − | − | Raffinose | 0 | − | − |
| Simmons citrate | 0 | − | − | Rhamnose | 0 | − | − |
| $H_2S$ production | 0 | − | + | Ribose | 6 | + | + |
| Indole test | 0 | − | + | Salicin | 0 | − | − |
| Methyl red | 0 | − | + | Sorbitol | 0 | − | − |
| Voges–Proskauer test | 0 | − | − | Starch | 0 | − | − |
| Lysine decarboxylase | 6 | + | + | Sucrose | 0 | − | − |
| Ornithine decarboxylase | 6 | + | + | Trehalose | 0 | − | − |
| Arginine double hydrolase | 0 | − | − | Xylose | 0 | − | − |

*Combined results from Hawke *et al.*1981, Waltman *et al.* (1986) and Holt and Noel (1994).

†Combined results from Amandi, Hiu, Rohovec and Fryer (1982) and Holt and Noel (1994). +, 90% or more of strains positive; −, 90% or more strains negative; ND, not determined.

**Table 2** Enzymatic profiles of yellow catfish *Edwardsiella ictaluri* isolates

| Biochemical test | Two isolates from Wuhan* | Four isolates from Huzhou |
|---|---|---|
| Alkaline phosphatase | + | + |
| Esterase (C4) | + | + |
| Esterase lipase (C8) | + | + |
| Lipase (C14) | − | − |
| Leucine arylamidase | + | + |
| Valine arylamidase | + | + |
| Cystine arylamidase | − | − |
| Trypsin | − | − |
| Chymotrypsin | + | + |
| Acid phosphatase | + | + |
| Naphthol-AS-BI-phosphohdrolase | + | + |
| α-galactosidase | − | − |
| β-galactosidase | − | − |
| β-glucuronidase | − | − |
| α-glucosidase | − | − |
| β-glucosidase | − | − |
| *N*-acetyl-β-glucosaminidase | + | + |
| α-mannosidase | − | − |
| α-fucosidase | − | − |

*+, positive; −, negative.

for the following enzymes: alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohdrolase and *N*-acetyl-β-glucosaminidase, and negative reactions for lipase (C14), cystine arylamidase, trypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, α-mannosidase and α-fucosidase (Table 2).

## 16rDNA sequences analysis

Nucleotide sequences of 16S rRNA genes of six isolates were determined. The GeneBank Accession Numbers were EF015475, EF015476, GU176612, GU176613, GU176614 and GU176615 respectively. They had almost identical 16S rRNA sequences (99.9% similarity), implying that they were members of one species. A phylogenetic tree was constructed and is shown in Fig. 2. They formed a single cluster with the *E. ictaluri* type stain JCM1680 and *E. ictaluri* 93-146 (GeneBank Accession no. AB050826, EU285521 respectively).

## Plasmid profile

Plasmid DNA was prepared from the infected yellow catfish isolates and is shown in Fig. 3. Each of the isolates was found to harbour two plasmids. By interpolation from the reference plasmid molecular marker, the estimated size of the two plasmids in each of the isolates was about 4100 and 5600 base pairs (bp).

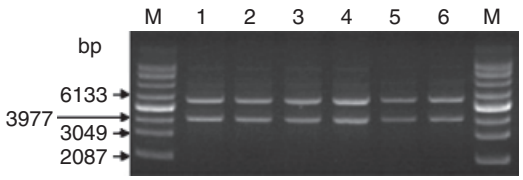


**Figure 3** Plasmid profiles of yellow catfish *Edwardsiella ictaluri* isolates M, supercoiled DNA ladder marker; 1, HSN-1; 2, HSS-1; 3, HSA-1; 4, HSA-2; 5, HSX-1; and 6, HSX-2.

**Table 3** Antimicrobial susceptibility testing of yellow catfish *Edwardsiella ictaluri* isolates

| Antimicrobial agents | Two isolates from Wuhan* | Four isolates from Huzhou |
|---|---|---|
| Acetylspiramycin | R | R |
| Ampicillin | R | R |
| Bacitracin | R | R |
| Cephalothin V | S | S |
| Chloramphenicol | S | S |
| Ciprofloxacin | S | S |
| Clarithromycin | R | R |
| Clindamycin | R | R |
| Doxycycline | S | S |
| Enoxacin | S | S |
| Erythromycin | M | R |
| Florfenicol | S | S |
| Fosfomycin | S | S |
| Furadantin | S | S |
| Gentamicin | S | S |
| Kanamycin | S | S |
| Neomycin | S | S |
| Norfloxacin | S | S |
| Ofloxacin | S | S |
| Oxytetracycline | S | S |
| Piperacillin | R | S |
| Rifampicin | R | M |
| Spectinomycin | R | R |
| Streptomycin | M | M |
| Sulphamethoxazole/trimethoprim | R | S |
| Tetracycline | S | S |
| Tobramycin | S | S |

*R, resistant; S, susceptible; M, medium susceptible.

## Antimicrobial susceptibility result

The susceptibility pattern of the isolates from 25 antibacterial agents is shown in Table 3. All isolates were

susceptible to cephalothin V, ciprofloxacin, doxycycline, enoxacin, florfenicol, furadantin, furazolidone, neomycin, norfloxacin, ofloxacin, oxytetracycline and tetracycline. Additionally, isolates from Wuhan were susceptible to chloramphenicol, gentamicin, kanamycin and tobramycin, while Huzhou isolates were susceptible to ampicillin, piperacillin and sulphamethoxazole/trimethoprim. Wuhan isolates showed medium susceptibility to erythromycin and streptomycin, while Huzhou isolates showed medium susceptibility to chloramphenicol, gentamicin, kanamycin and rifampicin. According to the result, the appropriate drug was recommended to the fisheries, resulting in the successful control of the outbreaks. This test was carried out not only for this purpose; the drug resistance profile of isolates also reflects the characteristics of this species.

### Pathogenicity

In challenge trials, the results showed that *E. ictaluri* isolates HSN-1 and HSA-1 were virulent for yellow catfish and channel catfish fingerlings. For the HSN-1 isolate, clinical signs of affected yellow catfish included haemorrhages on the oral area, at the base of the pectoral fin and accumulation of ascites in the abdomen. Mortality occurred at the rates of 75%, 100% and 100% via the low, medium and high doses respectively. For channel catfish, ulcer or perforation on pars cephalica, haemorrhages in the oral area and on the base of the pectoral fin, and accumulation of ascites in the abdomen were observed. Mortality occurred at the rates of 0%, 50% and 62.5% via the low, medium and high doses respectively. For the HSA-1 isolate, the symptoms of infected fish were identical to those of the naturally infected fish, and the $LD_{50}$ value in yellow catfish was determined to be $0.2 \times 10^{4.7}$ CFU mL$^{-1}$from the dose–response relationships using the improved Karber method. A pure culture of *E. ictaluri* was re-isolated and identified from the organs (brain, liver and kidney) of the dying fish. No control fingerlings developed clinical signs or died and no bacteria were re-isolated from control fish.

## Discussion

Yellow catfish production in China has increased in recent years. The abundance of freshwater, the climate and the consumption market have led to increased cultivation of yellow catfish. However, it appears that the higher the increase in fish production, the higher the risk of disease outbreak. The main reason may be the high density of cultured fish in the intensive system. The waste from unconsumed feed, excretion and poor pond management lead to the occurrence of fish disease. Among the diseases found commonly in yellow catfish, bacterial infection is worth discussing and considering due to the high mortality rate and the loss of income (Deng, Luo, Tan, Qiu & Chen 2008).

In the present study, morphological, biochemical and molecular assays confirmed that the yellow catfish isolates were *E. ictaluri*, the same species as that found from other species of aquatic animals by others (Hawke 1979; Hawke *et al.* 1981; Waltman, Shotts & Hsu 1986). The six strains, isolated from two regions, had identical physiological and biochemical characteristics, 16S rRNA gene sequences, plasmid pattern and enzymatic profile, with a few different antimicrobial susceptibility patterns.

*E. ictaluri* was placed in the genus *Edwardsiella* within the family *Enterobacterieae* based on biochemical characterization and DNA–DNA homology (Hawke *et al.* 1981). Biochemically, *E. ictaluri* has been described as a very homogenous organism (Plumb & Vinitnantharat 1989) and shares many common features with another member of this genus, *E. tarda*, except for some different characteristics including the production of indole, the production of $H_2S$ in SIM media and motility at 37 °C (Waltman, Shotts & Hsu 1986). The yellow catfish isolates were negative for the production of indole or $H_2S$, and motility was observed at 25 °C and not at 37 °C, which was the same as in *E. ictaluri*, but not *E. tarda*.

According to 16S rDNA sequence analysis, the isolates had 16S rDNA gene sequences (99% similarity) that were almost identical to those of the validated *E. ictaluri* species. The phylogenetic tree of 16S rDNA showed that the isolates formed a single cluster and were closely related to the type strain of *E. ictaluri* (Fig. 2). Furthermore, plasmid profile analysis has been applied to identify the isolates of *E. ictaluri* rapidly. Several studies indicated that most *E. ictaluri* isolates, regardless of origin, harboured homologous cryptic plasmids of similar, but not identical sizes that are genetically related (Lobb & Rhoades 1987; Speyerer & Boyle 1987; Newton *et al.* 1988; Reid & Boyle 1989; Lobb, Ghaffari, Hayman & Thompson 1993). These plasmid classes include a 4.0–4.7 kb plasmid and a 5.6 kb plasmid (Reger, Mockler & Miller 1993; Fernandez, Pittman-Cooley & Thune 2001). This suggests that these plasmids are extremely stable and

 

highly conserved in almost all *E. ictaluri* isolates (Abbott & Janda 2006), indicating that the plasmid profile is valuable for the identification of *E. ictaluri*. In contrast to the small-size plasmids detected in *E. ictaluri* strains, much larger extrachromosomal elements have been isolated from *E. tarda* strains (Abbott & Janda 2006). Plasmids in the *E. tarda* species range in molecular mass from 2 to 120 MDa, and plasmid carriage appears to vary significantly from strain to strain (Janda, Abbott, Kroske-Bystrom, Cheung, Powers, Kokka & Tamura 1991). In our studies, the estimated size of the two plasmids in each of the isolates was 4.1 and 5.6 kb, which was similar to other *E. ictaluri* strains as discussed above.

The owner of the fishery had suffered a severe loss of up to 30–40% pond fish in the outbreaks. In compliance with our suggestion, the owner treated the fish in some of the affected ponds by oral administration of oxytetracycline, and within 2 weeks, there were no longer any deaths of fish. Interestingly, the antimicrobial susceptibility pattern of yellow catfish isolates in the present study was quite similar to that recorded by other researchers (Stock & Wiedemann 2001; McGinnis, Gaunt, Santucci, Simmons & Endris 2003; Dung, Haesebrouck, Tuan, Sorgeloos, Baele & Decostere 2008), indicating that *E. ictaluri* possesses a relatively stable antimicrobial susceptibility pattern regardless of its geographical source.

In channel catfish, *E. ictaluri* can cause acute septicaemia or a chronic open lesion between the frontal bones of the skull, posterior to or between the eyes (Plumb 1993). Concerning the pathogenesis, *E. ictaluri* can infect fish by two different routes. Waterborne bacteria can invade the olfactory organ via the nasal opening of the fish and migrate into the olfactory nerve and then into the brain (Miyazaki & Plumb 1985; Shotts, Blazer & Waltman 1986). The infection spreads from the meninges to the skull and skin, thus creating a 'hole-in-the-head' condition. *E. ictaluri* can also be ingested and can enter the blood through the intestine and result in septicaemia (Shotts, Blazer & Waltman 1986). By this route, the bacteria apparently colonize capillarities in the dermis, causing necrosis and haemorrhage.

In the present study, *E. ictaluri* was found to cause diseases in the yellow catfish population naturally and caused two kinds of clinical signs, 'hole-under-the-jaw' and 'hole-in-the-head' symptoms, which were similar to those of the affected channel catfish (Plumb 1993), except that severe haemorrhage occurs in the skin under the jaw, creating a 'hole under the jaw' in yellow catfish.

The experimental infection trials using yellow catfish and channel catfish fingerlings all clearly demonstrated the pathogenic potential of the *E. ictaluri* isolates, which could develop acute septicaemia under experimental conditions. The 'hole-under-the-jaw' symptom was only reproduced slightly in a few yellow catfish, but not in channel catfish. And the pathogenic mechanism was worth studying clearly. Nevertheless, the 'hole-in-the-head' symptom was a chronic disease caused by many reasons, and could not be replicated in our challenge experiments.

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