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First description of a novel *Weissella* species as an opportunistic pathogen for rainbow trout *Oncorhynchus mykiss* (Walbaum) in China

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ABSTRACT

Six strains of Gram-positive, catalase-negative, non-motile, irregular short rod-shaped *Weissella* bacteria, with width and length of 0.5–0.6 and 1.2–2.7 μm were isolated from diseased rainbow trout *Oncorhynchus mykiss* (Walbaum) in winter of 2007 at a commercial fishery in Jingmen, Hubei province, China. The diseased rainbow trout exhibited hemorrhage in eyes, anal region, intestine and abdomen wall, petechia of liver, some fish with hydrocele in stomach. Six isolates had identical biochemical reactions, phylogenetic analysis of 16S rDNA sequences, amplified ribosomal DNA restriction analysis (ARDRA), enzymatic profile analysis and antimicrobial susceptibility results, indicating as a single clonal outbreak. But all were different from any other validated twelve *Weissella* species in the term of physiological and biochemical characters. It is indicated that isolates are phylogenetically closer to *Weissella halotolerans*, *Weissella viridescens* and *Weissella minor* on 16S rDNA phylogenetic analysis result, than to *W. halotolerans* and *W. viridescens* on the result of ARDRA study and enzymatic profile analysis. Antimicrobial susceptibility testing was used to scan effective drugs for the therapy of this disease. Experimental infection assays with one isolate were conducted and pathogenicity (by intraperitoneal injection) was demonstrated in rainbow trout *O. mykiss* (Walbaum) and crucian carp (*Carassius auratus gibelio*) fingerlings. Because no *Weissella* was detected in fish feedstuffs and pond water, the source of this pathogen remains unknown, and *Weissella* isolates were regarded as an opportunistic pathogen for rainbow trout. This is the first report of *Weissella* strains which can cause disease of cultured fish in the world.

1. Introduction

The *Weissella* species are Gram-positive, catalase-negative, non-spore-forming, heterofermentative, non-motile, irregular or coccoid rod-shaped organisms (Collins et al., 1993). Members of the genus *Weissella* have been isolated from a variety of sources, such as fresh vegetables, fermented silage (Dellaglio and Torriani, 1986; Ennahar et al., 2003; Wang and Nishinno, 2008), meat or meat products (Milbourne, 1983; Santos et al., 2005; Koort et al., 2006), desert spring and desert plants (Holzapfel and van Wyk, 1982), sugar cane, carrot juice, row milk and sewage (Hammes and Vogel, 1995), milking machine slime (Kandler et al., 1983), soil (Magnusson et al., 2002), fermented sausages (Collins et al., 1993), Korean kimchi (Choi et al., 2002; Lee et al., 2002; Kim and Chun, 2005), Malaysian foods (Leisner et al., 1999; Björkroth et al., 2002) or fermented mescal of *Agave salmiana* (Escalante-Minakata et al., 2008). But only *Weissella confusa* and *Weissella cibaria* have been described from human or animal clinical samples. *W. confusa* have been isolated from human feces (Green et al., 1990, 1991; Walter et al., 2001), peritoneal

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fluids and abdominal walls of patients (Riebel and Washington, 1990), and blood cultures of patients developed infective endocarditis (Flaherty et al., 2003; Shin et al., 2007) or bacteremia (Olano et al., 2001). Vela et al. (2003) have described a systemic infection caused by *W. confusa* in Primate (*Cercopithecus mona*). *W. cibaria* have been isolated from intestinal contents of a healthy human (Wang et al., 2008) and the feces of healthy dogs (De Graef et al., 2005). Björkroth et al. (2002) have isolated *W. cibaria* and *W. confusa* from necropsy specimens of a dog and the ear of a dog with otitis. Yet, *Weissella* spp. reports associated with fish and fish products were rare. *Weissella hellenica* was isolated from the intestinal contents of flounder (*Paralichthys olivaceus*) (Cai et al., 1998). *Weissella thailandensis* was described from fermented fish (pla-ra, pla-com) in Thailand (Tanasupawat et al., 2000). *W. confusa* was isolated from intestines of adult farmed seabass (*Lates calcarifer*) (Sirirat et al., 2008) and traditional smoked and sun-dried fish products of the Eastern Himalayas (Thapa et al., 2006). So far, no any *Weissella* species was reported as a pathogen of fish.

In the present study, a novel *Weissella* species, isolated from diseased rainbow trout *Oncorhynchus mykiss* (Walbaum) in China, was characterized. This is the first case report of *Weissella* strains associated with fish disease in the world.

2. Materials and methods

2.1. Diseased rainbow trout and characterization

From December 2007 to February 2008, a severe sequential outbreak occurred in a commercial fishery of rainbow trout *O. mykiss* (Walbaum) in Jingmen, Hubei province, China. The fish were cultured in concrete ponds with constant flowing water coming from the deep of a reservoir. During the epidemic outbreak, a loss of 40% was suffered. The major symptoms of the diseased fish were hemorrhages in eyes, anal region, intestines and abdominal wall and petechia in liver, and hydrocele in stomach of some fish.

Four adult fish with typical clinical signs were collected from the affected ponds and sent alive to the laboratory in 4 h in plastic transportation bags, with oxygen supply. Two diseased fish were sampled in the second diagnosis one month later. Water temperature was 15–16 °C when samples were collected.

2.2. Bacteria isolation

Using aseptic techniques, samples taken from blood, kidney, liver and brain of moribund rainbow trout (*O. mykiss*) were streaked on brain heart infusion (BHI, BD, USA) agar plates and incubated at 20 °C for 48 h. All colonies on some plates were identical in morphology and considered as pure culture. Single colonies were selected and re-streaked on the same media. All isolates were maintained separately in BHI broth with 15% glycerol at −80 °C. Strains JZ-1L, HZ-L-2, RT-1L, RT-2L were isolated from the liver of diseased fish, HZ-K from kidney and RT-2Br from brain. But no isolates was recovered from blood cultures. Two reference strains, *Weissella viridescens* ATCC 12706^T (^{T}, type strain) and *Weissella halotolerans* ATCC 35410^T obtained from American Type Culture Collection, were used as control in contrast experiments.

2.3. Morphological studies

Isolates were inoculated on de Man Rogossa Sharpe (MRS, BD, USA) agar overnight at optimum temperature 30 °C, colony morphology was observed directly and by light microscopy. Cell morphology was examined by scanning electron microscopy. Cell mobility and gliding movement were assessed by phase-contrast microscopy examination (1000×) using colonies in MRS broth.

2.4. Physiological and biochemical characterization

Before being tested, the strains were subcultured twice overnight in MRS broth at 30 °C. Determination of Gram reactions was performed using the KOH method of Gregesen (1978). Catalase activity was determined by transferring fresh colonies from MRS agar to a drop of 5% (v/v) H_2O_2 in slide glass. Oxidase activity was determined using 1% (w/v) dimethyl ρ-phenylenediamine chloride. Motility was detected by the appearance of stab cultures in soft agar (Whittenbury, 1963). The growth of isolates at different temperatures was tested in MRS broth after incubation at 4, 10, 15, 20, 30, 37, and 42 °C at least for 15 d. Growth in the presence of 2.0, 4.0, 5.0, 6.0, 6.5, 8.0, 10.0% of NaCl was determined in MRS broth and incubated at 30 °C until growth was observed or otherwise at least for 15 d. The acid tolerance was observed in MRS broth at pH 3.0 after incubation at 30 °C for 90 min. Growth on MacConkey agar was tested at 30 °C for 7 days. Production of dextran (slime) from sucrose was observed on MRS agar in which glucose had been replaced by 5% sucrose (Hitchener et al., 1982). The gas (CO_2) production from glucose was determined using Poly Peptone Yeast Extract Medium (PY) soft agar in which 3% glucose and 0.2% Tween 80 were contained. Hydrolysis of esculin, arginine, and starch were detected by the methods described previously (Tanasupawat et al., 1992). Reduction of 1% (w/v) nitrate in MRS broth and production of NH_3 from arginine was determined as described previously after incubation for 7 days (Leisner et al., 1994). Acid formation from carbohydrates was tested by the basal medium according to Garvie (1984), containing 10.0 g tryptone, 2.5 g yeast extract, 0.1 g Tween 80, and 1000 mL distilled water, adjusting to pH 6.8. Bromcresol purple was used as an indicator.

2.5. Enzymatic activity profiles

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

2.6. Antimicrobial susceptibility testing

The susceptibility pattern of bacteria isolates to 20 antimicrobial agents (Oxoid, UK) such as ampicillin (10 μg), bacitracin (0.04 IU), cephalothin V (30 μg), chloramphenicol (30 μg), clarithromycin (15 μg), doxycycline (30 μg), erythromycin (15 μg), furadantin (300 μg), gentamicin (10 μg), kanamycin (30 μg), neomycin (30 μg), norfloxacin (10 μg), ofloxacin (5 μg), piperacillin (100 μg), rifampicin (5 μg), streptomycin (10 μg), sulfamethoxazole (23.75 μg)/trimethoprim (1.25 μg), tetracycline (30 μg), tobramycin (10 μg), vancomycin (30 μg) were tested and determined by using the standard method of Kirby and Bauer (Bauer et al., 1966) on Mueller Hinton (MH, BD, USA) agar.

2.7. Molecular analysis

All isolates were incubated into 5 mL of MRS broth respectively and allowed to grow overnight at 30 °C. The bacteria were washed twice with sterile phosphate buffer (pH 7.4), and genomic DNA were extracted using Silica gel film Genomic DNA extraction kit (SBS, Shanghai) 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, Japan); 5 μL of 10× PCR buffer; 3 μL of 25 mM $MgCl_2$; 2 μL of each 10 mM dNTP (TaKaRa, Japan); 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′-AGAGTTGATCATGGCTCAG-3′ and U1492r: 5′-GGTTCACTTGTTACGACTT-3′ (Weisburg et al., 1991), were used to amplify 16S rRNA gene. The *Weissella* genus-specific primers used were WeiF: 5′-CGTGGGAAACCTACCTCTTA-3′ and WeiR: 5′-CCCTCAAACATCTAGCAC-3′ by producing 725-bp PCR fragments only from *Weissella* species (Jang et al., 2002). The amplifications were carried out in a thermal cycler (BioRad, USA) with the following parameters: an initial denaturation step of 94 °C for 3 min; 35 serial cycles of 94 °C for 1 min, universal primers annealed at 56 °C for 30 s, 72 °C for 90 s; and a final extention step of 72 °C for 10 min. And *Weissella* genus-specific primers annealed at 61 °C and extended at 72 °C for 1 min each cycles. A negative control (no template DNA) and two positive controls of *W. viridescens* ATCC 12706^T and *W. halotolerans* ATCC 35410^T were included in the PCR. The PCR products were analyzed by 1% (w/v) agarose gel (containing ethidium bromide)