academicJournals

Vol. 8(16), pp. 1722-1729, 16 April, 2014
DOI: 10.5897/AJMR2014.6659
Article Number: 90E401B44055
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Massive mortality associated with *Streptococcus iniae* infection in cage-cultured red drum (*Sciaenops ocellatus*) in Eastern China

Francis Pius Mmanda, Suming Zhou, Jiting Zhang, Xiaoye Zheng, Shuwei An and Guoliang Wang*

Key Laboratory of the Ministry of Education for Applied Marine Biotechnology, School of Marine Science, Ningbo University, Ningbo 315211, China.

Received 24 January, 2014; Accepted 24 March, 2014

In August, 2011, an enzootic disease characterized by hemorrhage throughout body surface, enlarged spleen and kidney occurred in cage farmed red drum fish, in Dongtou, Zhejiang of China. The diseased fish weighed between 100 to 200 g, and the cumulative mortality within 60 days was higher than 70%. Several bacterial isolates that exhibited the same phenotypic traits and biochemical characteristics were isolated from the lesions of diseased fish. According to the results obtained from biochemical tests and sequence analysis of the 16S rDNA, the disease's pathogen (strain WZMH110819) was identified as *Streptococcus iniae*. In the challenge trials, the LD_{50} value of the clinical bacterial isolate WZMH110819 was 9.65×10^6 CFU per fish. Moreover, bath exposure or oral administration by *Streptococcus iniae* also caused a considerable number of deaths in fish. Antibiotic susceptibility tests showed that strain WZMH110819 was sensitive to most of the antibiotics including ampicillin, erythromycin and gentamicin *in vitro*. This finding has provided a basis for the control and prevention of further outbreaks of this enzootic disease in red drum farms.

Key words: *Streptococcus iniae*, *Sciaenops ocellatus*, biochemical characterization, 16S rDNA analysis.

INTRODUCTION

Streptococcal infections in fish have become an increasingly important health problem in modern intensive aquaculture (Eldar et al., 1995a). The main pathogenic species responsible for these streptococcal infections include *Streptococcus iniae*, *Streptococcus agalactiae*, *Streptococcus parauberis* and *Streptococcus dysgalactiae* (Eldar et al., 1995a; Domenech et al., 1996; Bercovier et al., 1997; Nomoto et al., 2004). *S. iniae* was firstly isolated from the subcutaneous abscesses in Amazon freshwater dolphins *Inia geoffrensis* (L.) in aquariums (Pier and Madin, 1976), and then large epidemic diseases in fish associated with this pathogen occurred. The most significant clinical signs of *S. iniae* infections in fish are septicemia and meningoencephalitis which are quite similar to signs caused by other streptococcal pathogens of fish (Eldar et al., 1994,

*Corresponding author. E-mail: wangguoliang@nbu.edu.cn.

1995a, 1995b; Eldar and Ghittino, 1999). More importantly, humans can also be infected by *S. iniae* and a number of human cases have been reported in North America and Asia (Weinstein et al., 1997; Lau et al., 2003; Koh et al., 2004; Lau et al., 2006).

The red drum (*Sciaenops ocellatus*) is one of the most important economic fish species and it was introduced in China in 1991 (Shen et al., 2005). In a few decades, bacterial infections in red drum have become an increasingly important health problem because of the modern intensive aquaculture. *Streptococcosis* is one of the most common bacterial diseases in cultured red drum worldwide (Eldar et al., 1999; Colorni et al., 2002; Shen et al., 2005). Recently, an infectious disease outbreak with high mortality occurred in cage farmed red drum, causing severe economic impact on fisheries in Dongtou, Zhejiang province, China.

The aim of the present study was to isolate, identify and characterize the pathogenic bacteria of enzootic disease in red drum as well as to determine some potential drug candidates. The results obtained upon completion of this research can be taken from the laboratory into the field as a guideline for assisting red drum cage-cultured fish farmers for the control and the prevention of further streptococcal disease outbreak.

MATERIALS AND METHODS

Bacterial isolation and biochemical characterization

In August, 2011, an infectious fish disease occurred among the red drum cage farmed fish in Dongtou, Zhejiang province China. More than 80 moribund fish with an approximate weight of 100 to 200 g were subjected to bacteriological examination. Several pure colonies of bacterial isolates (Strain WZMH110819) from liver, spleen and head kidney tissues of the diseased fish were recovered on Brain Heart Infusion Agar (BHIA; HuanKai, China) supplemented with 50% distilled sterile seawater. The sampling was conducted over a conservative two days period of high mortality rate. For the isolation of the etiological pathogen, tissues of liver, spleen and kidney from affected fish were streaked across the Brain Heart Infusion Agar (BHIA; HuanKai, China) supplemented with 50% distilled sterile seawater. The inoculated plates were incubated at 28°C for 24 h. Single colonies from plates with virtually pure culture growth were re-streaked onto the same media to obtain pure isolates. All pure isolates were cultured in liquid BHI and then add 20% glycerol for preservation at -80°C.

Bacterial identification was conducted using a number of biochemical and phenotypical tests by the API20 STREP kits (BioMerieux, France), according to the manufacturer's instructions. For the detection of hemolysis, the bacterial isolate was cultured overnight on trypticase soy agar supplemented with 5% defibrinated sheep blood (Toocle co., Hangzhou, China) at 28°C. The destruction of red blood cells (zones of beta-hemolysis) in the blood agar was detected prior incubation. The gram staining procedure for the bacterium followed the standard protocol.

16S rDNA amplification and sequence analysis

Total genomic DNA was extracted using a genomic DNA extraction Kit (Takara). The conserved primers used for the amplification of 16S rDNA were reported previously (Edward and Ewing, 1986): 8F 5'-AGAGTTTGATCCTGGCTCAG-3' and 1542R 5'-AAGGAGGTGATCCAGCCGCA-3'. The PCR mixture contained bacterial DNA, PCR buffer (10 mM Tris-HCl, pH 8.8, 50 mM KCl, 2 mM $MgCl_2$, 0.08% Nonidet P40), a 200 µM concentration of each deoxynucleoside triphosphate, 0.1 µM of each primer and 1.5 U of Taq polymerase (Takara). The thermocycling parameter used for this conserved primer set was 30 cycles of 94°C for 1 min, 56°C for 1 min and 72°C for 2 min, and a final extension at 72°C for 5 min in an automated thermal cycler (PTC-100, Bio-RadTM, USA).

The amplification products were gel-purified using PCR purification kit (Omega) and sequenced using the same primers as for PCR. Sequence was aligned using Clustal X version 2.1 followed by refinement by eye. Phylogenetic analysis of the 16S rDNA sequence was performed with Molecular Evolutionary Genetics Analysis (MEGA) software version 5.05 with the two-parameter of Kimura model for DNA based on the Neighbour Joining (N to J).

Experimental infection trials

Healthy red drum fish (102.3±15.5 g) were divided into 10 groups (n=10 for each group) and then were acclimatized in tanks (400 l) for 7 days at 30 ± 2°C. Before experimental infection, three fish selected randomly were subjected to bacteriological examination for confirming the fish not to be infected with bacteria. To determine the virulence of the bacterial isolate WZMH110819, four groups were injected intraperitoneally with the bacterium suspension at the doses of 1.93×10^6, 1.93×10^7, 1.93×10^8 and 1.93×10^9 CFU per fish respectively and one group of fish was injected intraperitoneally with PBS for negative control. The fish mortalities were recorded daily for 14 days after the challenge, and the LD_{50} value was calculated by using the Reed-Muench method (Reed and Muench, 1938).

To investigate the infection routes of this bacterial pathogen, fish were also exposed to bacterium through oral or bath exposure. For oral administration, two group of normal fish (n=10) were challenged by oral admission of 1 ml of bacterial suspension at the doses of 1.93×10^6 and 1.93×10^8 CFU per fish, respectively. For bath exposure, normal fish or fish undergoing epidermal scarification were removed from their tanks and placed into a container of seawater containing 1.93×10^7 CFU/ml bacteria. After an exposure period of 30 min, the fish were removed and placed into their respective tank. Controls used for every method of challenge were subjected to identical handling procedures without being exposed to the isolated bacterium. Bacterial concentrations administered in the artificial experimental trials were evaluated using the indirect viable cell counts method as previously described (Todar, 2009). Briefly, a bacterial suspension was made from bacterial isolate grown on Brain Heart Infusion Agar (BHIA; HuanKai, China) for 24 h at 28°C were serially tenfold diluted with sterile phosphate-buffered saline to 10^8. Each dilution was then plated to BHIA to determine cells in each dilution. Each viable unit grows and forms of a colony were counted prior incubation.

Antimicrobial susceptibility testing

The antimicrobial susceptibility of the bacterial isolate WZMH110819 was determined using the standard Kirby-Bauer method (Bauer et al., 1966). Briefly, a suspension (0.1 ml) of the bacterial strain WZMH110819 (diluted to a turbidity equivalent of a Macfarland No. 0.5 standard solution) was spread onto Mueller-Hinton agar and antimicrobial agent paper discs (Hangzhou Tianhe Microorganism Reagent) were then added to the surface of the agar.

Figure 1. Diseased red drum fish showing (A) enlarged kidney and spleen, (B) cornea opacity or POP eye, (C) hemorrhages on the base of fins and (D) dorsal and tail fin erosion.

RESULTS

Bacterial isolation from diseased fish

Diseased fish exhibited emaciation, lethargy, abnormal swimming, exophthalmia and hemorrhages on the base of fins. Internally, congestive kidney and spleen were observed in diseased fish (Figure 1). Multiple small bacterial colonies (strain WZMH110819) were isolated from different lesions of the diseased red drum.

Phenotypic traits and biochemical characteristics of the bacterial isolates

These bacterial isolates formed whitish colonies that were smooth around the edges across the BHIA plates and all strains were positive for β-hemolysis on blood agar plate. These isolates were all Gram-positive streptococcus-shaped bacteria in optical microscope. Moreover, all bacterial isolates and reference *S. iniae* showed the same biochemical characteristics by using the API 20 STREP kits (Table 1).

16S rDNA sequence analysis

The universal 16S rDNA primers set used in this study yielded the expected 1541 bp amplicon for bacterial strain WZMH110819. Sequence analysis showed that bacterial strain WZMH110819 showed 100% similarity with the reference *S. iniae* strain ATCC 29178^T, but showed 98% similarity with *S. parasuis* and 97% similarity with *S. agalactiae* and *S. dysgalactiae,* respectively. The phylogenetic analysis based on the 16S rDNA sequence (accession numbers: KF815728) also showed that the bacterial strain WZMH110819 clustered most clearly with *S. iniae* strain ATCC 29178^T (Figure 2).

Result of experimental infections

In challenge trails, the bacterial isolate WZMH110819 exhibited moderate virulence to the red drum with an intra-peritoneal LD_{50} value of 9.65×10^6 CFU per fish. Depending on the amount of doses challenged, mortality rate ranged from 20 to 100% was recorded in our study (Figure 3). The cumulative mortality of each intraperitoneal

Table 1. Biochemical Profile of strain WZMH110819 and references *S.iniae*

API 20 STREP test	Strain *WZMH110819*	*S. iniae*ATCC 29178[a]	*S. iniae*SO-2[b]
Oxidase	-	-	N.A
Vogus-proskauer	-	-	-
H_2S production	-	-	N.A
Indole production	-	-	N.A
Citrate utilization	-	-	N.A
β-Galactosidase	+	+	N.A
Arginine dihydrolase	+	+	+
Lysine decarboxylase	-	-	N.A
Ornithine Decarboxylase	-	-	N.A
Tryptophane deaminase	+	+	N.A
Urase	-	-	-
Gelatinase	-	-	N.A
Glucose	+	+	+
Mannitol	+	+	+
Inositol	-	-	N.A
D-sorbitol	-	-	N.A
Rhamnose	-	-	N.A
Sucrose	+	-	+
Melibiose	-	-	-
Amygdalin	-	-	N.A
L-arabinose	-	-	N.A

+, Positive; -, negative; [a]data was from Pier and Madin 1976; [b]data was from Shen et al., 2005; N.A, Not available.

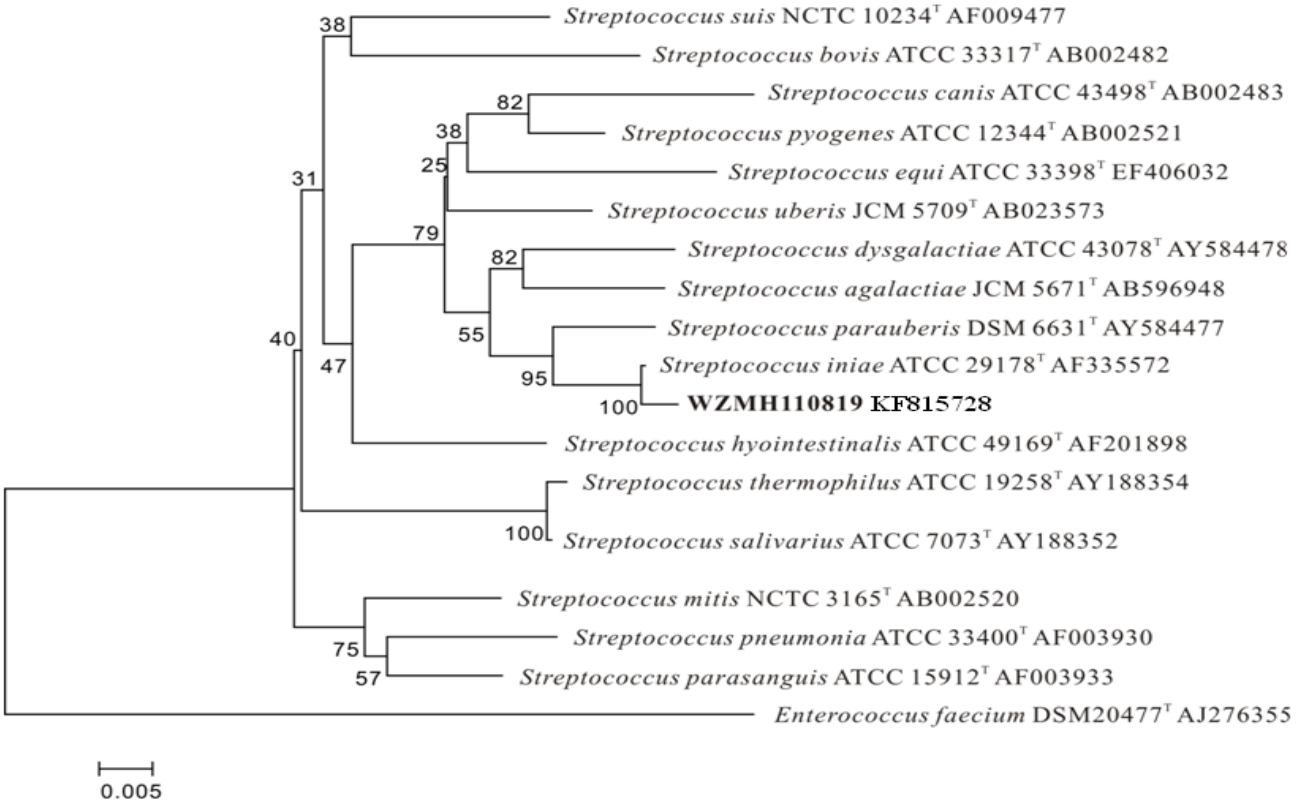

Figure 2. Neighbor-joining phylogenetic tree of 16S rDNA sequences of bacterial isolate WZMH110819, reference *S. iniae* and the most closely related species of streptococci.

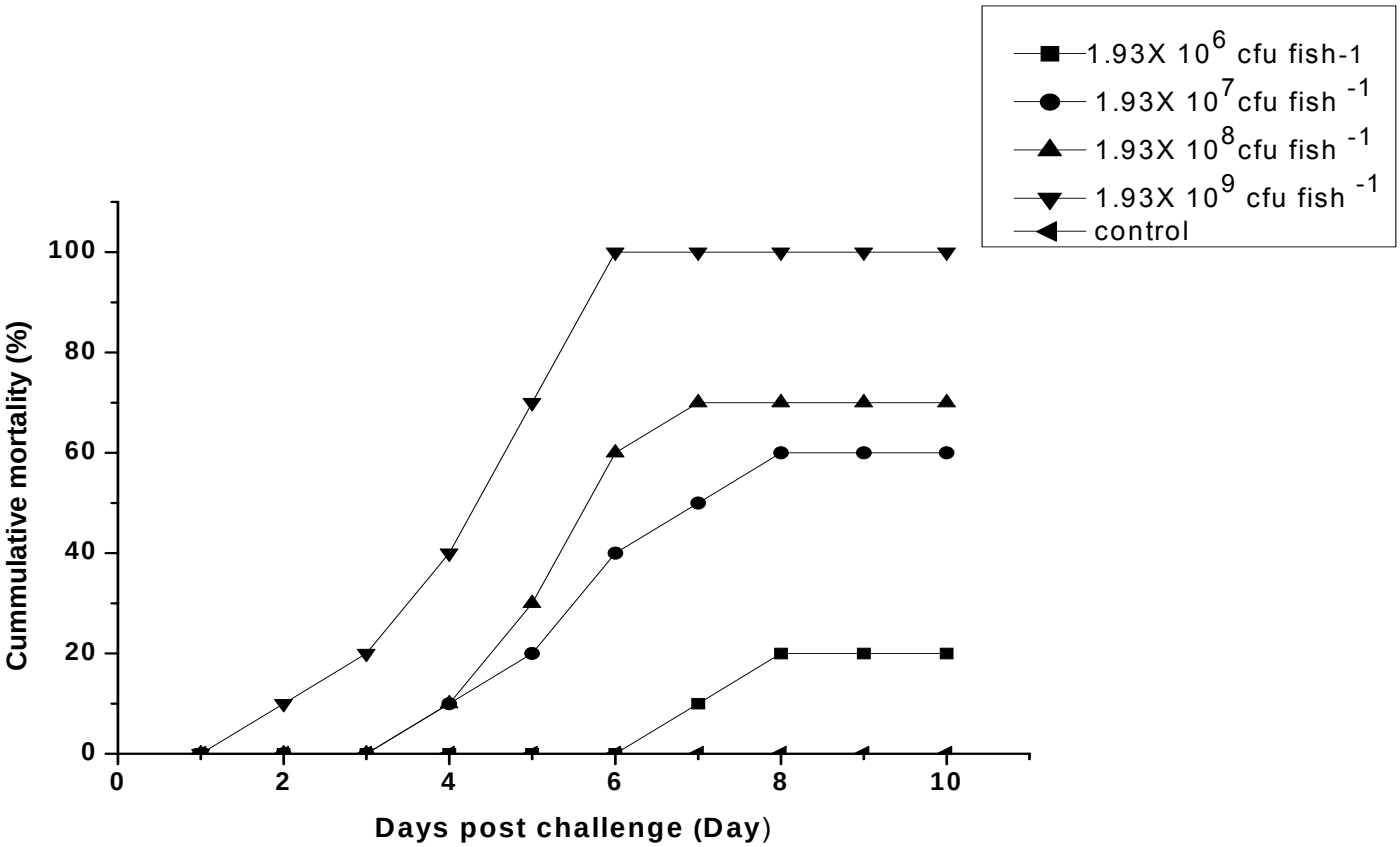

Figure 3. Mortality rate of red drum intraperitoneally injected with various concentration of S. iniae isloate WZMH110819.

challenge group or negative control is showed in Figure 4. Bath exposure with the bacteria isolate WZMH110819 after skin trauma resulted in 40% mortality for 1.93×10^7 CFU per fish, in contrast to 0.0 % mortality for 1.93×10^7 CFU per fish with normal pathogen exposure. For oral administration, the mortality was 10.0% for 1.93×10^6 CFU per fish and 40% for 1.93×10^8 CFU per fish (Figure 4).

The typical clinical signs of the moribund fish in experimental trails were hemorrhages on the base of fins, lethargy, abnormal swimming and enlarged and congestive visceral organs which were similar to naturally infected fish.

Antimicrobial susceptibility results

Antimicrobial susceptibility results showed that the bacterial isolate WZMH110819 was susceptible to 19 of 20 antibiotics tested in present study, including ampicillin, chlorophenicol, erythromycin, cefazolin, piperacilin, cefoperazone, ceftzaidime, sultamicillin, cefepime, streptomycin, kanamycin, amikacin, gentamicin, tetracycline, doxycycline, norfloxacin, ciprofloxacin, imipenem and trobramycin, but showed resistance to SMZ+TMP (Table 2).

DISCUSSION

Red drum is one of most important economic fish species mainly farmed in Zhejiang and Fujian provinces, China. During 2011 to 2012, massive mortality in cage cultured red drum occurred in Dongtou fish farms, Wenzhou, Zhejiang province. The outbreak of the disease occurred in breeding fish which were almost one year of age (approximately 100 to 300 g) when the water temperature was up to 29°C. However, the large yellow croaker, another important economic native fish cultured in the same coastal region, was not affected by this epidemic disease. The diseased or moribund red drum exhibited skin lesions, exophthalmia, disorientation and hemorrhage around the tail and anal fin. Moreover, there were some gram-positive streptococcus-like bacteria observed in the tissue smears.

The bacterial strain isolated from the diseased fish was G. positive, cocci-chain and oxidase-negative. On the basis of these results and those of the API20 STREP kits (BioMerieux, France) listed in Table 1, the isolate was identified as *S. iniae*. The bioche-mical characters of the *S. iniae* strain WZMH110819 were similar to those results reported previously by other researchers (Pier and Madin, 1976; Shen et al., 2005; Zhou et al., 2008).

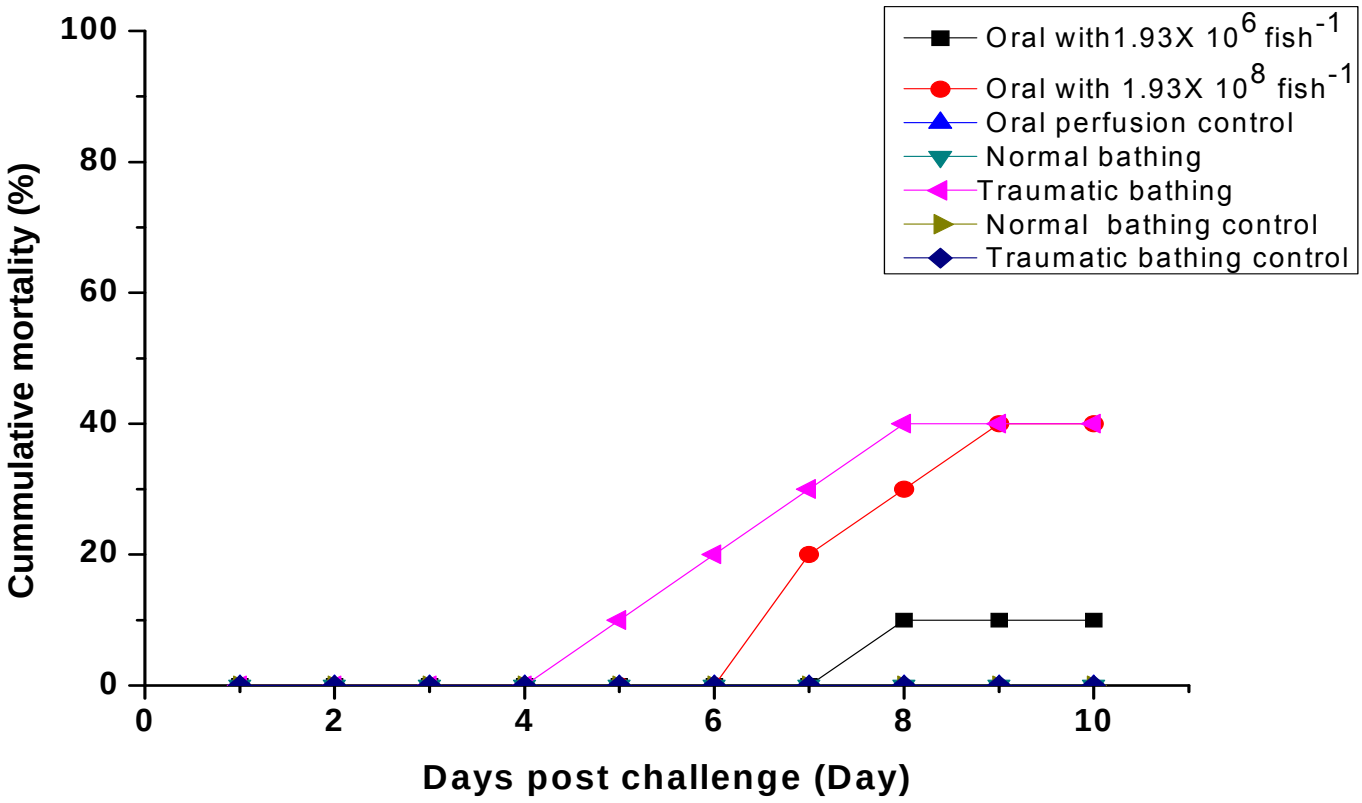

Figure 4. Mortality rate of Red drum exposed to bacterial strain WZMH110819 in deploys of natural infection trials via bath treatment or oral administration.

Table 2. Results of disc diffusion drugs susceptibility testing

Name of antibiotic	Paper content (μg/disc)	Inhibitory zone diameter (mm)	Sensitivity
Ampicillin	10	30	+
Chloramphenicol	30	25	+
Erythromycin	15	30	+
Cefazolin	30	23	+
Piperacilin	100	22	+
Cefoperazone	75	25	+
Ceftzaidime	30	23	+
Sultamicillin	10/10	25	+
Cefepime	30	31	+
Streptomycin	10	24	+
Kanamycin	30	28	+
Amikacin	30	25	+
Gentamicin	10	25	+
Tetracycline	30	26	+
SMZ+TMP	23.75	10	-
Doxycycline	30	26	+
Norfloxacin	10	28	+
Ciprofloxacin	5	32	+
Imipenem	10	43	+
Trobramycin	10	19	+

+, Sensitive (S); -, resistance (R).

Meanwhile, this bacterium had ability hydrolyze arginine, suggesting that it may share the same serotype (serotype I) that have been previously reported by other researchers (Pier and Madin, 1976; Shen et al., 2005; Zhou et al., 2008).

Infections associated with *S. iniae* in red drum in Zhejiang province has been reported early in 2005 (Shen et al., 2005). However, the epidemic reported by Shen occurred from September to December and the cumulative mortality was 20 to30%. Moreover, the *S. iniae* isolates seemed of low or moderate virulence with the intra-peritoneal LD_{50} value of 4.18×10^8CFU per fish and 1.19×10^7CFU per fish (100-120 g), respectively, according to the report (Shen et al., 2005). In the present study, the epidemic broke out from July to September, and the cumulative mortality was up to 70%. Experimental infection trials showed that the clinical isolate WZMH110819 exhibited moderate virulence to the red drum with an intra-peritoneal LD_{50} value of 9.65×10^6 CFU per fish (102.3±15.5 g) in the current study. This result suggested that the *S. iniae* strain WZMH110819 might be more virulent than the strains isolated by Shen et al. (2005).

Previously, the study reported that epidermal scarification did not appeared to enhance the ability of *S. iniae* to enter the barramundi and increase mortalities over traumatized fish (Bromage and Owens, 2002). However, it showed that the mortalities of the red drum were up to 40% in bath exposure group undergoing epidermal scarification in the current study, compared to normal bath exposure or oral exposure.

Oral administration and bath exposure experiments, which indicated that the red drum were more likely to be infected with *S. iniae* via skin trauma. The average stocking density of red drum fish in Dongtou cage farms were about 20 to 25 fish per m^3. High stocking density or close contact between the fish themselves may damage the integrity of their epidermal and protective mucosal layers, consequently predispose them to *S. iniae* infection (Shoemaker et al., 2001). Thus, the frequent outbreaks of this enzootic disease in this area may be due to high stocking rates and feeding the affected fish meal (Zhou et al., 2008).

Several investigators have reported that some *S. iniae* strains exhibited resistance to several commonly used antimicrobial drugs. Shen et al. (2005) has reported that the *S. iniae* isolates from red drum fish developed some resistance to gentamycin, lincomycin and norfloxacin. Similarly, Tran et al. (2013) has reported that *S. iniae* isolated from in diseased barramundi (*Lates calcarifer*) cultured in Vietnam was sensitive to several antibiotics including norfloxacin, ciprofloxacin, sulphamethoxazol / trimethoprim, ampicillin, erythromycin and doxycycline but was resistant to gentamicin, and streptomycin. In present study, the bacterial isolate WZMH110819 was sensitive to most of antibiotics tested including gentamicin and norfloxacin *in vitro*, but showed resistance to sulfamethoxazole compound (SMZ+TMP). This result suggests that antibiotics therapy would be effective in treating fish affected by this pathogen.

The results of the present study demonstrates that the massive mortality in red drum in Dongtou fish farms was due to infection with *S. iniae*, and the epidermal scarification might enhance the pathogens ability to enter the fish and increase mortalities. This important finding would suggest that reduced handling and decreased the stocking density might be an important means of preventing problems caused by this pathogen.

Conflict of Interests

The author(s) have not declared any conflict of interests.

ACKNOWLEDGEMENTS

The research was supported by Program for Changjiang Scholars and Innovative Research Team in University (IRT0734), Ningbo Municipal Natural Science Foundation (2009A610116), Ningbo Municipal Innovative Research Team (2013B82012 and K. C. Wong Magna Fund in Ningbo University.

REFERENCES

Bauer AW, Kirby WM, Sherris JC, Turck M (1966). Antibiotic susceptibility testing by a standardized single disk method. Am. J. Clin. Pathol. 45(4):493-496.

Bercovier H, Ghittino C, Eldar A (1997). Immunization with bacterial antigens: infections with streptococci and related organisms. In: Gudding R, Lillehaugg R, Midtlying PJ, Brown F (Eds.), Fish Vaccinology. Dev. Biol. Stand. Karger, Basel, Switzerland, pp.153-160.

Bromage ES, Owens L (2002). Infection of barramundi *Lates calcarifer* with *Streptococcus iniae*: Effect of different routes of exposure. *Diseases of Aquatic Organisms*, *52*:199-205.

Colorni A, Diamant A, Eldar A, Kvitt H, Zlotkin A (2002). *Streptococcus iniae* infections in Red Sea cage-cultured and wild fishes. Dis. Aquat. Org. 49:165-170.

Domenech A, Fernandez-Garayzabal JF, Pasqual C, Garcia JA, Cutuli MT, Moreno MA, Collins MD, Dominguez L (1996). Streptococcosis in cultured turbot, *Scophthalmus maximus* (L.), associated with *Streptococcus parauberis*. J. Fish Dis. 19:33-38.

Edward PR, Ewing WH (1986). Identification of *Enterobacteriaceae* 4th ed. Elsevier Science Publishing Co. Inc., New York.

Eldar A, Bejerano Y, Bercovier H (1994). *Streptococcus shiloi* and *Streptococcus difficile*, two new streptococcal species causing a meningo-ecephalitis in fish. *Curr. Microbiol.* 28:139-143.

Eldar A, Bejerano Y, Livoff A, Horovitez A, Bercovier H (1995a). Experimental streptococcal meningo-encephalitis in cultured fish. Vet. Microbiol. 43:33-40.

Eldar A, Frelier PF, Assenta L, Varner PW, Lawhon S, Bercovier H (1995b). *Streptococcus shiloi*, the name for an agent causing septicemic infection in fish, is a junior synonym of *Streptococcus iniae*. Int. J. Syst. Bacteriol. 45:840-842.

Eldar A, Ghittino C (1999). *Lactococcus garvieae* and *Streptococcus iniae* infections in rainbow trout (*Oncorhynchus mykiss*): Similar, but different diseases. Dis. Aquat. Org. 36:227-231.

Eldar A, Perl S, Frelier PF, Bercovier H (1999). Red drum *Sciaenops ocellatus* mortalities associated with *Streptococcus iniae* infection. Dis. Aquat. Org. 36:121-127.

Koh TH, Kurup A, Chen J (2004). *Streptococcus iniae* discitis in Singapore.

Emerg. infect. Dis. 10:1694-1696.
Lau SKP, Woo PCY, Luk WK, Fung AMY, Hui WT, Fong AHC, Chow CW, Wong SSY, Yuen KY (2006). Clinical isolates of *Streptococcus iniae* from Asia are more mucoid and beta-hemolytic than those from North America. Diagn. Microbiol. Infect. Dis. 54:177.
Lau SKP, Woo PCY, Tse H, Leung KW, Wong SSY, Yuen KY (2003). Invasive *Streptococcus iniae* infections outside North America. J. Clin. Microbiol. 41:1004-1109.
Nomoto R, Munasinghe LI, Jin DH, Shimahara Y, Yasuda H, Nakamura A, Misawa N, Itami T, Yoshida T (2004). Lancefield group C *Streptococcus dysgalactiae* infection responsible for fish mortalities in Japan. J. Fish Dis. 27:679-686.
Pier GB, Madin SH (1976). *Streptococcus iniae*, a beta-hemolytic *Streptococcus* isolated from Amazon freshwater dolphin, *Inia geoffrensis.* Int. J. Syst. Bacteriol. 26:545-553.
Reed LJ, Muench H (1938). A simple method of estimating fifty percent endpoints. Am. J. Hyg. 27:493-497.
Shen ZH, Qian D, Xu WJ, Gu JH, Shao JZ (2005). Isolation, identification and pathogenicity of Streptococcus iniae isolated from red drum *Sciaenops ocellatus*. Acta Hydrobiol. Sin. 29:678-683.
Shoemaker CA, Klesius PH, Evans JJ (2001). Prevalence of *Streptococcus iniae* in tilapia, hybrid striped bass, and channel cartfish on commercial fish farms in the United States. Am. J. Vet. Res. 62:174-177.
Todar T (2009). Todar's online textbook of bacteriology. The growth of bacterial populations (page 2) (http://textbookof bacteriology.net/growth_2.html).
Tran VH, Dang HQV, Huu DN, Wergeland HI (2013). Experimental *Streptococcus iniae* infection in barramundi (*Lates calcarifer*) cultured in Vietnam. Int. J. Aquat. Sci. 4(1):3-12.
Weinstein M, Litt M, Kertesz D, Wyper P, Rose D, Coulter M, McGreer A, Facklam R, Ostach C, Willey B, Borczyk A, Low D (1997). Invasive infections due to a fish pathogen, *Streptococcus iniae*. N. Engl. J. Med. 9:589-594.
Zhou SM, Xie MQ, Zhu XQ, Ma Y, Tan LZ, Li AX (2008). Identification and genetic characterization of *Streptococcus iniae* strains isolated from diseased fish in China. J. Fish. Dis. 31:869-875.