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Characterization and identification of streptococci from golden pompano in China

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ABSTRACT: Streptococcal infections cause significant mortality and high economic losses in the fish farm industry worldwide, including in the culture of golden pompano *Trachinotus ovatus* L., a species gaining popularity in China. A total of 9 streptococcal strains were isolated from cage-cultured diseased golden pompano in Beihai, Zhanjing, and Shenzhen, China, between 2012 and 2014. Conventional and rapid identification systems were used to determine that the isolates were *Streptococcus agalactiae*, *S. iniae*, and *S. dysgalactiae* subsp. *dysgalactiae.* All isolates were gram-positive cocci cells in pairs or short-chain, non-motile, catalase negative, α or β hemolytic cocci. The results of multiplex PCR assays and 16S rRNA BLAST analysis also showed that the β hemolytic strains were *S. agalactiae* and *S. iniae* and the α hemolytic strain was *S. dysgalactiae* subsp. *dysgalactiae*, respectively. Pathogenicity assays revealed that *S. agalactiae* (lethal dose [LD_{50}]: 6.38×10^4 CFU ml^{-1}) was more virulent for golden pompano than *S. iniae* (LD_{50}: 1.47×10^7 CFU ml^{-1}) and *S. dysgalactiae* subsp. *dysgalactiae* (LD_{50}: 2.57×10^6 CFU ml^{-1}) when they were challenged by intraperiotoneal (i.p.) injection. The results of antibiotic susceptibility showed that all strains were extremely susceptible to cefradine, erythromycin, and cefotaxime but resistant to gentamicin, penicillin G, novobiocin, neomycin, ciprofloxacin, roxithromycin, furazolidone, enrofloxacin, norfloxacin, kanamycin, ampicillin, tetracycline, and vancomycin This is the first report of a phenomenon of golden pompano coinfection with *S. agalactiae* and *S. iniae*, which will contribute to the diagnosis and prevention of streptococcicosis.

KEY WORDS: *Trachinotus ovatus* · *Streptococcus* · Pathogenicity · Biochemical characterization · Multiplex PCR assay

INTRODUCTION

Streptococcal infections, which have increased in frequency with the intensification of aquaculture, are responsible for significant economic losses in the fish farm industry worldwide. To date, the main pathogenic species of *Streptococcus* responsible for bacterial infections in fish include *S. agalactiae* (Duremdez et al. 2004, Abuseliana et al. 2010, Bowater et al. 2012), *S. iniae* (Colorni et al. 2002, Figueiredo et al. 2012, Vi et al. 2013), *S. parauberis* (Baeck et al. 2006, Haines et al. 2013, Woo & Park 2013), *S. dysgalactiae* (Nomoto et al. 2004, Yang & Li 2009, Netto et al. 2011), *S. phocae* (Gibello et al. 2005, Romalde et al. 2008), and *S. ictaluri* (Shewmaker et al. 2007), which can affect various fish species including rainbow

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trout *Oncorhynchus mykiss* (Walbaum), Atlantic salmon *Salmo salar* L., mullet *Mugil cephalus* L., golden shiner *Notemigonus crysoleucas* (Mitchill), sea bass *Dicentrarchus labrax* L., olive flounder *Paralichthys olivaceus* (Temminck & Schlegel 1846), Nile tilapia *Oreochromis niloticus* L., pinfish *Lagodon rhomboides* L., channel catfish *Ictalurus punctatus* L., and ornamental fish (Yanong & Francis-Floyd 2010). Fish with streptococcosis often exhibit very similar symptoms and clinical signs regardless of the etiological agent, including swimming abnormalities, septicemia, meningoencephalitis, subcutaneous abscesses in the caudal peduncle region, and exophthalmia ('pop-eye') (Bowater et al. 2012, Figueiredo et al. 2012, Haines et al. 2013). Amongst the *Streptococcus* species, *S. agalactiae* and *S. iniae* have been reported more frequently as fish pathogens than other species. However, the growing numbers of reports involving the pathological implementations of *S. dysgalactiae* infection are highly suggestive of the critically increasing importance of this pathogen (Yang & Li 2009, Pan et al. 2009, Abdelsalam et al. 2010, 2015, Netto et al. 2011, Pourgholam et al. 2011, Qi et al. 2013, Costa et al. 2014). No information is available concerning outbreaks of *Streptococcus* in golden pompano *Trachinotus ovatus* L.

Golden pompano is a marine water fish that has become popular in China in recent years because of its rapid growth, good taste, high economic value, and demand from local and overseas markets (Li et al. 2006, Yang 2006). However, disease problems have accompanied the rapid expansion in production of this species. From 2012 to 2014, epizootics with 20 to 25% cumulative mortality occurred in cage-cultured golden pompano, with the larger, fast-growing, and adult fish supplied to the market, showing the disease symptoms in most cases and thus resulting in reduced market prices. Moribund golden pompano from the disease outbreak were sampled in order to isolate bacteria potentially involved in the mortalities. Bacteria were identified using phenotypic and biochemical tests, Lancefield serotyping, and molecular techniques, and their pathogenicity and antibiotic susceptibility were assessed.

MATERIALS AND METHODS

Bacterial isolation

A total of 8 moribund golden pompano (400–500 g) were collected in each of 3 separate golden pompano farms; Tieshan Harbor (Beihai), Donghai Island (Zhanjing), and Daya Bay (Shenzhen) in China between 2012 and 2014. Swabs of brain, liver, spleen, and kidney of diseased fish were sampled aseptically, streaked onto tryptic soy agar with yeast extract (Huankai) with 2.0% NaCl, and cultured at 28°C for 96 h initially. However, subsequent isolation and cultivation was carried out in brain heart infusion agar (BHIA; Huankai) also supplemented with 2.0% NaCl and incubated at 37°C for 24 h. The dominant bacterial isolates were purified by streaking and re-streaking onto fresh media to obtain pure isolates. The isolates were then stored at −80°C in sterile (autoclaved at 100 kPa for 20 min) brain heart infusion (BHI) supplemented with 20% (v/v) glycerol until use.

Biochemical identification

Morphological investigation was performed using the negative-staining method. A range of conventional phenotypic characteristics were studied and biochemical tests performed, including Gram staining; hemolysis (cultured on medium containing 5% sheep blood); oxidase (reaction with dimethyl-*p*-phenylenediamine); catalase (reaction with 10% hydrogen peroxide), and the ability to grow at pH 9.6 and temperatures of 10, 20, 37, and 45°C, in 0, 0.5, 1, 2.5, or 6.5% NaCl and 40% bile (Dong & Cai 2001). Utilization of various sugars (ribose, arabinose, mannitol, sorbitol, lactose, trehalose, inulin, raffinose, amygdaline, and glycogen) was studied using the cystine trypticase agar basal medium (Difco). The isolates were incubated at 37°C, and the results of the conventional tests (Dong & Cai 2001) and the rapid-method test using the API 20 Strep kit (bioMérieux) were assessed after 24 h incubation. The Lancefield streptococcal grouping for the isolates was confirmed using Microgen Strep (HKM) according to the manufacturer's protocol. Reference strains *Streptococcus agalactiae* (verified through API 20 STREP and specific PCR; Amal et al. 2012) from *Trachinotus blochii* (Buller 2004), *S. iniae* ATCC29178, and *S. dysgalactiae* subsp. *dysgalactiae* ATCC43078 (Nomoto et al. 2004) were used as positive controls for comparing the results.

Multiplex PCR assays and phylogenetic analysis

Multiplex PCR assays were used to simultaneously detect *S. agalactiae, S. iniae* and *S. dysgalactiae* subsp. *dysgalactiae*. The stored isolates were cultured in BHI broth (Huankai) at 37°C for 24 h, and

DNA was extracted using a DNeasy kit (Tiangen) with the additional pretreatment in 180 µl of lysis buffer (20 mM Tris–HCl, pH 8.0; 2 mM Na_2-EDTA; 1.2% Triton X-100) containing lysozyme (20 mg ml^{-1}) for 180 min at 37°C and treated with 25 µl of Proteinase K (20 mg ml^{-1}) for 30 min at 56°C according to the manufacturer's protocol and finally eluted with 50 to 200 µl elution buffer. The oligonucleotide primer sets used to identify isolates were derived from published sequences (Table 1). Multiplex PCR was performed in 25 µl reaction mixtures containing 50 ng DNA template (1 µl), 1.5 mM $MgCl_2$, 0.4 mM concentration of each deoxynucleoside triphosphate, 1 U of *Taq* Plus DNA polymerase (TranGen), 0.2 µM each of primers cfb-F/cfb-R, 0.08 µM each of primers tuf-F/tuf-R, and 1.6 µM each of primers Si-F/Si-R, and 1×*Taq Plus* buffer. Amplification was performed in an S1000 Thermal cycler (Bio-Rad) with the following parameters: an initial denaturation step of 94°C for 5 min; 32 serial cycles of a denaturation step of 94°C for 35 s, annealing at 55°C for 40 s, and extension at 72°C for 45 s; and a final extension step of 72°C for 10 min. Multiplex PCR products were analyzed on a 1.5% (w/v) agarose gel containing 1% (w/v) ethidium bromide. DNA bands were visualized under UV transillumination and photographed.

To verify the results of the multiplex PCR and analyze the evolutionary relationship, further molecular identification was performed by amplification of the bacterial isolates' 16S rRNA gene using primers 27F (AGA GTT TGA TCM TGG CTC AG) and 1492R (GGT TAC CTT GTT ACG ACT T) (Lane 1991). The expected size of the 16S rRNA amplicons was approximately 1500 bp. The PCR products were sequenced in both directions, following purification using a TIANgel Midi Purification Kit (TranGen) and cloning into the vector pMD18-T, using a 3730 DNA sequencer (Guangzhou Invitrogen Biotechnology) and assembled with DNAStar software. The sequences were analyzed using the BLASTn algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to verify species. The limit fixed for identification of a bacterial species was 97.5% nucleotide identity. To determine relationships with other *Streptococcus* isolates, sequences were independently aligned using ClustalX 2 (Larkin et al. 2007) and MEGA 6.0 (Tamura et al. 2013). Genetic distances and clustering were determined using Kimura's 2-parameter model (Kimura 1980), and evolutionary trees were reconstructed using the neighbor-joining method (Saitou & Nei 1987). The stability of the relationships was assessed using the bootstrap method with 1000 replicates.

Pathogenicity trials

Golden pompano with an average weight of 50 g, determined to be disease-free by the microbiological methods described previously in the initial isolation from moribund fish, and by multiplex PCR screening, were obtained from a fish farm in Tieshan Harbor of Guangxi Province in China. The fish (n = 300) were initially maintained in 20 fiberglass-reinforced plastic aquaria (diameter 1 m, 100 l capacity) supplied with flow-through seawater and were subjected to 12 h light:12 h dark cycles at 28°C for 7 d. Fish were fed twice daily with commercial fish expanded pellets (Guangdong Yuehai Feed Group), and waste was removed daily. For the pathogenicity trial, a total of 80 fish for each bacterial strain (TOS01, TOS06, and TOS09) were randomly selected and were subdivided into 10 groups per strain (4 treatment groups and 1 control group; 2 replicates per treatment group), with each group containing 8 fish. The representative strains of each *Streptococcus* species (TOS01, TOS06, and TOS09) were thawed and cultured on BHIA at 37°C for 48 h. The number of cells was estimated by plating on BHIA plates after suspending in 0.85% (w/v) saline; suspensions from 10×10^8 to 10×10^5 CFU ml^{-1} were then prepared by serial 10-fold dilution. A small amount (0.1 ml) of each dilution was injected intraperitoneally (i.p.) into 2 groups of 8 fish per bacterial isolate. Sterile normal saline (0.1 ml) was injected i.p. into all fish in the control

Table 1. Primer sequences used for Multiplex PCR amplification of *Streptococcus* spp. and the expected amplicon size

Pathogen	Primer	Nucleotide sequences (5′–3′)	Expected size (bp)	References
S. agalactiae	cfb-F	AAG CGT GTA TTC CAG ATT TCC T	471	Li et al. (2010)
	cfb-R	CAG TAA TCA AGC CCA GCA A		
S. iniae	Si-F	CAC TAA TCC AAA GAG TT	1391	Chang et al. (2009)
	Si-R	TTA GGC GGC TGG CTC CTA A		
S. dysgalactiae	tuf-F	GTA GTT GCT TCA ACA GAC GG	795	Abdelsalam et al. (2015)
subsp. *dysgalactiae*	tuf-R	GGC GAT TGG GTG GAT CAA CTC		

groups. After injection, each group was maintained separately in a 100 l aquarium under the same conditions as described above. Challenged fish were monitored for 14 d, the mortality rate was noted, and survivors were examined every day. Samples of brain and kidney tissue were collected from all dead fish for re-isolation of bacteria. Furthermore, samples from brains of moribund fish were streaked onto BHI media plates and then incubated at 37°C for 24 h. Dominant isolates were purified by streaking using a wire loop onto fresh media and further tested by multiplex PCR as described above.

Antibiotic susceptibility

The susceptibility patterns of the isolates to 29 different antibiotics were determined according to the disc diffusion method using Mueller-Hinton agar (Bauer et al. 1966), and the diameters of the inhibition zones were measured using Vernier calipers. Resistant, intermediate and susceptible phenotype determinations were based on manufacturer guidelines (Tianhe). The strains were inoculated onto Mueller-Hinton agar plates (Tianhe) and allowed to absorb into agar for 10 min, and antibiotic discs (Tianhe) were added after 24 h of incubation at 37°C. Categories and amounts (µg) of antibiotics were as follows: azithromycin (15), levofloxacin (5), gentamicin (10), chloramphenicol (30), penicillin G (10), cefradine (30), novobiocin (30), neomycin (30), gatifloxacin (5), oxymethylpyrimidine (5), ciprofloxacin (5), roxithromycin (15), furazolidone (300), enrofloxacin (15), norfloxacin (10), doxycycline (30), rifampicin (5), sulfamethoxazole (1.25), nitrofurantoin (300), kanamycin (30), ampicillin (10), streptomycin (10), erythromycin (15), clindamycin (30), amoxicillin (10), tetracycline (30), clarithromycin (15), cefotaxime (30), and vancomycin (30).

RESULTS

Phenotypic characterization

In total, 9 streptococcal strains were isolated from diseased golden pompano during 2012 to 2014. Their codes, tissue of isolation, collection date, isolation temperature, and geographical origin are listed in Table 2. General microbiological examinations revealed that the isolates were gram-positive cocci cells in pairs or short chain, non-motile, and showing no catalse activities. Those isolates could grow in media at 20 and 37°C, and at 0, 0.5, 1, and 2.5% NaCl. Hemolytic analysis showed that 8 isolates were β-hemolytic, whereas 1 isolate was α-hemolytic. The β-hemolytic isolates were identified as *Streptococcus agalactiae* (TOS01–TOS05) and *S. iniae* (TOS06–TOS08), and the α-hemolytic isolate was identified as *S. dysgalactiae* subsp. *dysgalactiae* (TOS09) via multiplex PCR. In addition, the phenomenon of polymicrobial infection was observed in that 2 different species, *S. agalactiae* TOS01 and *S. iniae* TOS06, were isolated from the brain of a diseased golden pompano in 2012.

Biochemical characterization of streptococcal isolates

Biochemical analyses revealed complete homogeneity in all *S. agalactiae* (i.e. positive reactions on the Voges-Proskauer, hydrolysis of hippurate, alkaline phosphatase, leucine arylamidase, arginine hydrolysis, ribose, trehalose, and amygdaline tests and negative reactions on the esculin, pyrolidonylamidase, α-galactosidase, β-galactosidase, β-glucuronidase, arabinose, mannitol, sorbitol, lactose, inulin, raffinose, and glycogen tests) and *S. iniae* (i.e. positive reac-

Table 2. Code, tissue of isolation, collection date, isolation temperature, and geographical origin of the streptococcal strains presented in this study. Dates given as mo-yr

Isolate	Bacterial type	Date of isolation	Code of API strep	Accession number	Site of isolation	Stocking density (kg m^{-3})	Fish size (g)	Temp. (°C)	Salinity (‰)
TOS01	B	10-2012	3263014	KF826095	Brain	9.3	450	28	28
TOS02	B	7-2014	3263014	KP729639	Brain	9.6	505	30	29
TOS03	B	7-2014	3263014	KP729640	Kidney	10.8	623	30	27
TOS04	B	7-2014	3263014	KP729641	Spleen	10.5	610	30	27
TOS05	B	7-2014	3263014	KP729642	Spleen	10.3	543	30	28
TOS06	B	10-2012	4563117	KF826094	Brain	9.3	450	28	28
TOS07	B	7-2014	4563117	KP729643	Brain	10	510	30	29
TOS08	B	7-2014	4563117	KP729644	Liver	9.6	461	30	28
TOS09	C	9-2012	0463417	KM077497	Brain	9.7	512	28	27

tions on the esculin, pyrolidonylamidase, alkaline phosphatase, leucine arylamidase, arginine hydrolysis, trehalose, amygdaline, and glycogen tests and negative reactions on the Voges-Proskauer, hydrolysis of hippurate, α-galactosidase, β-galactosidase, β-glucuronidase, ribose, arabinose, mannitol, sorbitol, lactose, inulin, and raffinose tests), which were identical to the reference strain. However, the *S. dysgalactiae* subsp. *dysgalactiae* isolate and the reference strain showed enzyme profiles which were not completely consistent (Table 3). Lactose reaction was positive for *S. dysgalactiae* subsp. *dysgalactiae* isolated in the current study and negative for ATCC43078. This is similar to findings by Nomoto et al. (2004) and Abdelsalam et al. (2010).

Phylogenetic analysis of 16S rRNA sequence

The 16S rRNA sequence of the strains TOS01–TOS09 were determined and submitted to GenBank under accession numbers KF826095, KP729639, KP729640, KP729641, KP729642, KF826094, KP729643, KP729644, KM077497, respectively. BLAST analysis of the 16S rRNA product sequence revealed that the strains TOS01–TOS05 exhibited a similarity of 98–

Table 3. Bacteriological characteristics, using conventional methods and an API 20 STREP kit (bioMérieux), of *Streptococcus* spp. isolated from the golden pompano *Trachinotus ovatus* (present study) and from reference strains; nd: not determined; +: positive; −: negative

Characteristics	Fish isolates (this study)			Reference strains		
	TOS01–TOS05	TOS06–TOS08	TOS09	*S. agalactiae* (*Trachinotus blochii*)[a]	*S. iniae* ATCC29178[b]	*S. dysgalactiae* subsp. *dysgalactiae* ATCC43078[c]
Gram stain	+	+	+	+	+	+
Hemolysis (5% sheep blood agar)	β	β	α	β	β	α
Cell morphology	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci
Catalase	−	−	−	−	−	−
Growth at: 10°C	−	−	−	nd	nd	−
20°C	+	+	+	nd	nd	nd
37°C	+	+	+	+	+	+
45°C	−	−	−	nd	nd	−
NaCl: 0%	+	+	+	nd	nd	nd
0.5%	+	+	+	nd	nd	nd
1%	+	+	+	nd	nd	nd
2.5%	+	+	+	nd	nd	nd
6.5%	−	−	−	nd	nd	−
40% bile	−	−	−	nd	nd	−
pH 9.6	−	−	−	nd	nd	−
Voges-Proskauer	+	−	−	+	−	−
Hydrolysis of hippurate	+	−	−	+	−	−
Esculin	−	+	−	−	+	−
Pyrolidonyl arylamidase	−	+	−	−	+	−
α-Galactosidase	−	−	−	−	−	−
β-Galactosidase	−	−	−	−	−	−
β-Glucuronidase	−	−	+	−	−	+
Alkaline phosphatase	+	+	+	+	+	+
Leucine arylamidase	+	+	+	+	+	+
Arginine hydrolysis	+	+	−	+	+	−
Acid from ribose	+	−	+	+	−	+
Arabinose	−	−	−	−	−	−
Mannitol	−	−	−	−	−	−
Sorbitol	−	−	−	−	−	−
Lactose	−	−	+	−	−	−
Trehalose	+	+	+	+	+	+
Inulin	−	−	−	−	−	−
Raffinose	−	−	−	−	−	−
Amygdaline	+	+	+	nd	+	+
Glycogen	−	+	−	−	+	−
Group antigen (Lancefield group)	B	B	C	B	nd	C

[a]Amal et al. (2012); [b]Buller (2004); [c]Nomoto et al. (2004)

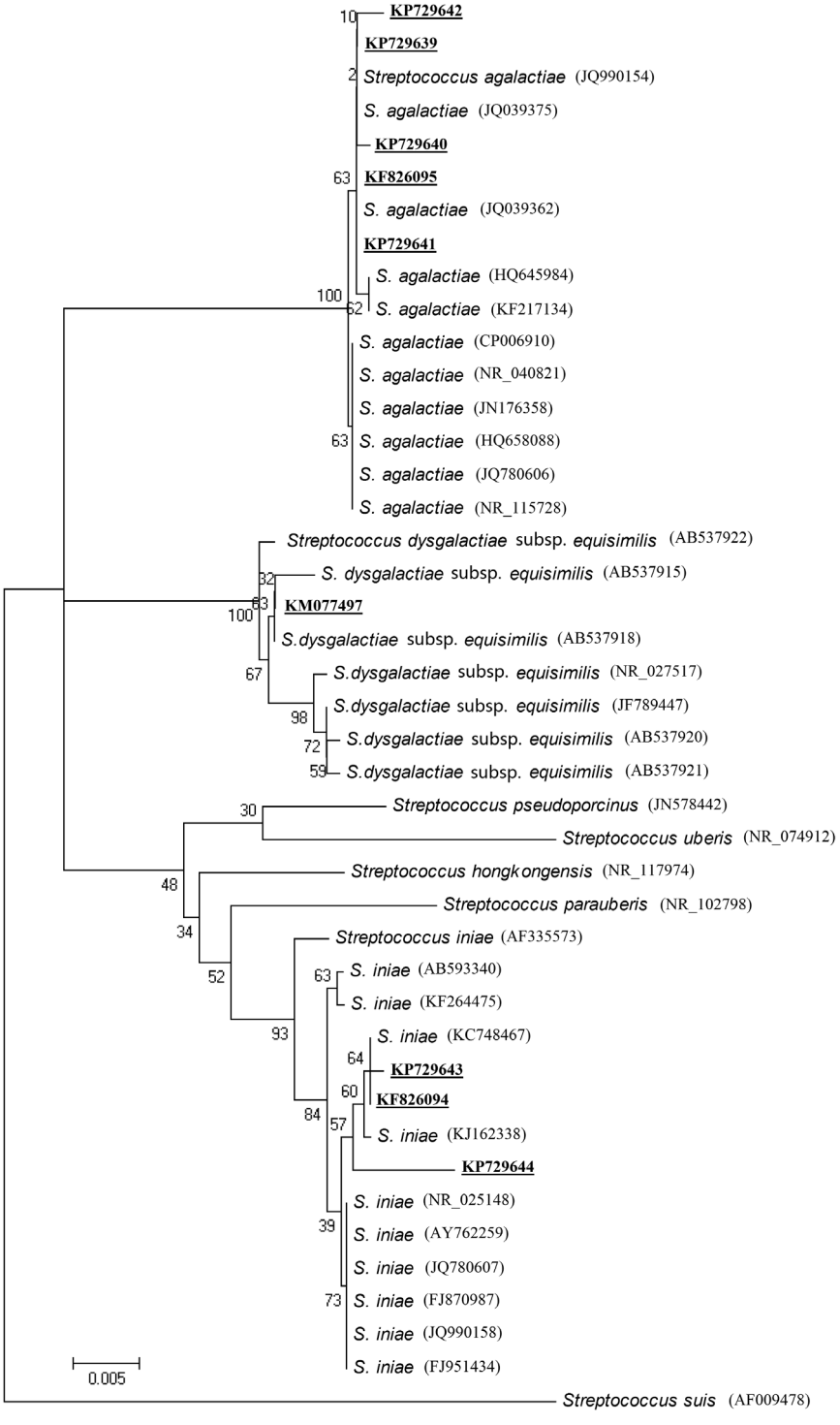

Fig. 1. Phylogenetic tree of golden pompano *Trachinotus ovatus* bacterial isolates (indicated by bold and underline) and other *Streptococcus* species reference strains from GenBank, based on the 16S rRNA gene. Sequence analysis was done using ClustalX 2 (Larkin et al. 2007) and phylogenetic analyses were conducted with MEGA6.0 (Tamura et al. 2013), using the neighbor-joining method with the Kimura 2-parameter model (Kimura 1980). Confidence values displayed as percentages were estimated from bootstrap analysis of 1000 replicates. Accession numbers of sequences are given before the species name. Bar represents 5% sequence diversity

99% to *S. agalactiae* (JQ990154, JQ039375, JQ039362, HQ645984, KF217134) isolated from tilapia. Strains TOS06–TOS08 exhibited the same similarity of 99% to *S. iniae* strain FC0924 (KC748467) and strain AB131025 (KJ162338) isolated from golden pompano and Amur sturgeon *Acipenser schrenckii*. Isolate TOS09 showed 99% homology with *S. dysgalactiae* subsp. *dysgalactiae* strain AB537918 isolated from amberjack *Seriola dumerili*. A phylogenetic tree generated based on the nucleotide sequences of the 16S rRNA gene is shown in Fig. 1. The tree revealed that all *S. agalactiae* strains isolated in the current study clustered together with *S. agalactiae* strains isolated previously from fish and were separated from strains of other origins. The phylogenetic analysis showed that the strains TOS01–TOS05 were clustered together with *S. agalactiae*, TOS06–TOS08 were clustered together with *S. iniae*, and TOS09 was clustered together with *S. dysgalactiae* subsp. *dysgalactiae* (Fig. 1).

Pathogenicity of the representative bacteria

Pathological features of diseased golden pompano are displayed in Fig. 2. Pathogenicity assays revealed that 3 strains (TOS01, TOS06, and TOS09) were virulent for golden pompano when they were challenged by i.p. injection. The lethal dose (LD_{50}) of TOS01 was 6.38×10^4 CFU ml^{-1}, while those of TOS06 and TOS09 were 1.47×10^7 and 2.57×10^6 CFU ml^{-1}, respectively (Table 4). The experimentally infected fish showed obvious similar clinical signs to those of the diseased farm fish,

Fig. 2. Pathological features of diseased golden pompano *Trachinotus ovatus* showing (a) exophthalmia, (b) necrotic lesion on the caudal peduncle, (c) meningitis, (d) gross pathology lesions and opacification of the eye (scale at bottom is in cm), and (e) erratic swimming

such as exophthalmia or opacification of the eye, hemorrhaging at the bases of fins, subcutaneous abscesses in the caudal peduncle region, and erratic swimming. Pure cultures of bacteria were re-isolated from the brain and kidney of moribund fish. As expected, the re-isolated strains were identified as the same species.

Antibiotic susceptibility

All strains were extremely susceptible to cefradine, erythromycin, cefotaxime, but resistant to gentamicin, penicillin G, novobiocin, neomycin, ciprofloxacin, roxithromycin, furazolidone, enrofloxacin, norfloxacin, kanamycin, ampicillin, tetracycline, and vancomycin (Table 5).

Table 4. Total mortality in 2 groups of golden pompano *Trachinotus ovatus* challenged by intraperitoneal injection with 100 µl *Streptococcus* isolate TOS01 (*S. agalactiae*), TOS06 (*S. iniae*), and TOS09 (*S. dysgalactiae* subsp. *dysgalactiae*)

Group	Bacterial conc. (CFU ml^{-1})	No. of fish group^{-1}	No. of mortalities	Total mortality (%)
S. agalactiae	1.5×10^8	8, 8	8, 8	100
	1.5×10^7	8, 8	8, 8	100
	1.5×10^6	8, 8	6, 8	87.5
	1.5×10^5	8, 8	5, 4	56.25
Control	Physiological saline	8, 8	0, 0	0
S. iniae	1.5×10^8	8, 8	7, 8	93.75
	1.5×10^7	8, 8	7, 5	75
	1.5×10^6	8, 8	6, 4	62.5
	1.5×10^5	8, 8	5, 3	50
Control	Physiological saline	8, 8	0, 0	0
S. dysgalactiae subsp. *dysgalactiae*	1.5×10^8	8, 8	8, 8	100
	1.5×10^7	8, 8	7, 7	87.5
	1.5×10^6	8, 8	5, 6	68.75
	1.5×10^5	8, 8	5, 3	50
Control	Physiological saline	8, 8	0, 0	0

DISCUSSION

Streptococcosis has been associated with acute to chronic infections resulting in fish mortalities and has become an increasingly important problem in the aquaculture industry in many countries (Pan et al. 2009, Yang & Li 2009, Abdelsalam et al. 2010, 2015, Netto et al. 2011, Pourgholam et al. 2011, Qi et al. 2013, Costa et al. 2014). *Streptococcus agalactiae, S. iniae*, and *S. dysgalactiae* are responsible for disease in more than 30 species of freshwater, estuarine, and marine fish worldwide (Shoemaker et al. 2015), but this is the first report of streptococcosis in *Trachinotus ovatus*, and also of coinfection by *S. agalactiae* and *S. iniae*.

In previous studies, various water quality factors have been associated with streptococcal outbreaks in fish species, including slow rate of water flow (Amal & Zamri-Saad 2010a), low oxygen levels and increased nitrite (Bunch & Bejerano 1997), and high ammonia and transparency (Amal et al. 2015), with high water temperature in particular showing a strong association with the increased mortality (Al-Marzouk et al. 2005, Amal et al. 2010b, Sepahi et al. 2013). Muzquiz et al. (1999) reported that *S. agalactiae* and *S. iniae* usually result in warm-water streptococcosis presenting high virulence at water temperatures above 17°C. Yuasa et al. (1999) reported that *S. iniae* injected intramuscularly caused chronic and acute infections to white-spotted rabbitfish *Siganus canaliculatus* at 25 and 28–32°C, respectively. *S. agalactiae* was found to be highly virulent to mullet *Liza klunzingeri* at 33°C (Al-Marzouk et al. 2005). The growth rate of *S. dysgalactiae* subsp. *dysgalactiae*, a bacterial species which results in significant economic losses in cultured amberjack *Seriola dumerili* and yellowtail *Seriola quinqueradiata*, was higher at 37°C than at 25°C in broth cultures (Nomoto et al. 2004). In this study, a high mortality rate also occurred in golden pompano farms at high temperatures (>30°C, June to July in southern China) especially in relatively large fish weighing 500 g, which is approximately marketable size. Suzuki et al. (2011) reported that *Streptococcus* has acquired key virulence genes in order to adapt to changing environments (water temperature and fish density) or new hosts. In humans, the transcription of some important virulence factors of *S. agalactiae* increased at 40°C in comparison with 30°C (Mereghetti et al. 2008). The change of *Streptococcus* virulence related to water temperature in fish species requires further study.

Fish with streptococcosis exhibit very similar symptoms and clinical signs regardless of the etiological agent (Abuseliana et al. 2010, Figueiredo et al. 2012, Abdelsalam et al. 2015). In this study, we obtained 9 bacterial isolates from golden pompano exhibiting clinical symptoms of exophthalmia, subcutaneous abscesses in the caudal peduncle region, and erratic swimming. These isolates were phenotypically identified as *S. agalactiae*, *S. iniae*, and *S. dysgalactiae* subsp. *dysgalactiae*. However, the biochemical results obtained from conventional tests and an API 20 Strep kit were not always consistent with those reported for *S. agalactiae* (Evans et al. 2002), *S. iniae* (Zhou et al. 2008), and *S. dysgalactiae* subsp. *dys-*

Table 5. Antibiotic sensitivities of *Streptococcus* spp. isolated from golden pompano *Trachinotus ovatus* during farm mortalities. *: inhibition diameter (mm); R: resistant; I: intermediate; S: susceptible

	Antibiotic (µg)	Sensitivity*		
		TOS01–TOS05	TOS06–TOS08	TSO9
1	Aithromycin (15)	I(17–18)	R(9–10)	I(15)
2	Levofloxacin (5)	I(13–16)	R(7–9)	R(13)
3	Gentamicin (10)	R(7–9)	R(5–7)	R(7)
4	Chloramphenicol (30)	I(13–15)	R(8–10)	I(16)
5	Penicillin G (10)	R(12–13)	R(17)	R(15)
6	Cefradine (30)	S(18–19)	S(18)	S(18)
7	Novobiocin (30)	R(13–14)	R(10–11)	R(15)
8	Neomycin (30)	R(7–11)	R(5–7)	R(13)
9	Gatifloxacin (5)	I(17–21)	R(13)	I(17)
10	Oxymethylpyrimidine (5)	R(0–3)	R(6–7)	I(12)
11	Ciprofloxacin (5)	R(13–14)	R(10)	R(14)
12	Roxithromycin (15)	R(17)	R(10–12)	R(11)
13	Furazolidone (300)	R(6–9)	R(7–10)	R(11)
14	Enrofloxacin (15)	R(12)	R(10)	R(14)
15	Norfloxacin (10)	R(10)	R(8)	R(12)
16	Doxycycline (30)	R(14–16)	R(12)	I(18)
17	Rifampicin (5)	R(14–16)	R(13–14)	I(17)
18	Sulfamethoxazole (1.25)	R(8–9)	R(8–9)	I(15)
19	Nitrofurantoin (300)	I(14–16)	R(8–11)	R(14)
20	Kanamycin (30)	R(8–9)	R(8–9)	R(8)
21	Ampicillin (10)	I(15–17)	R(10–11)	I(16)
22	Streptomycin (10)	R(5–7)	S(10)	I(8)
23	Erythromycin (15)	S(18–20)	S(18–19)	S(18)
24	Clindamycin (30)	I(18–19)	I(18–19)	I(18)
25	Amoxicillin (10)	R(16)	R(13–16)	R(17)
26	Tetracycline (30)	R(16–17)	R(10–11)	R(15)
27	Clarithromycin (15)	S(18–19)	R(10–11)	I(17)
28	Cefotaxime (30)	S(19–21)	S(18–19)	S(21)
29	Vancomycin (30)	R(10–12)	R(10–11)	R(11)

galactiae (Nomoto et al. 2004). In this study, the pyrolidonylamidase and α-galactosidase test for *S. agalactiae* and the β-galactosidase, ribose, and mannitol tests for *S. iniae* were negative but positive for the isolates in the above studies; the lactose reaction for *S. dysgalactiae* subsp. *dysgalactiae* was positive and negative for ATCC43078. These variations may reflect differences in the number of isolates tested, geographical locations, or physiochemical factors (e.g. temperature). On the other hand, the existence of different types of *Streptococcus* species emphasizes the difficulties of definitive identification based on phenotypic traits alone. Therefore, final identification cannot be determined without the support of genetic data. In the present study, the multiplex PCR assay and 16S rRNA sequencing confirmed the phenotypic and biochemical identification

Streptococcus species are not only emerging invasive fish pathogens responsible for major economic losses in warm-water finfish aquaculture worldwide (Shoemaker et al. 2001, Agnew & Barnes 2007), but they should also be considered as an emerging threat to other organisms and to public health due to their zoonotic potential. They have been reported as causing bacterial meningitis in humans (Sun et al. 2007) and endocarditis in swine (Atanassov et al. 2015), and some of the bovine and human strains are able to infect fish and cause meningoencephalitis (Pereira et al. 2010). Streptococcal transmission between fish was identified resulting from direct contact with wild carrier or infected fish (Zlotkin et al. 1998). *Streptococcus* isolated from sturgeon can invade the brain of 2 kinds of carp (LD_{50} within the range of 2.45×10^8 to 5.54×10^8 CFU; Yang & Li 2009). The phylogenetic analysis of 16S rRNA showed that all 9 isolates clustered together with fish strains and separated from strains from other sources; in particular, all *S. agalactiae* isolates in this study appeared to be more related to strains from tilapia than from other species. In China, the problems of *S. agalactiae* infection in tilapia culture have become more and more serious, with high frequency since 2008 (Zhou et al. 2008, Wang et al. 2013). Due to their euryhaline nature, tilapia are often cultured in seawater near culture areas of golden pompano in Guangdong and Guangxi Provinces of China, which may have resulted in streptococcal outbreaks in golden pompano due to bacterial contamination of water in the culture systems.

To date, the vast majority of microbiological studies have been monomicrobial in fish, and yet, the phenomenon of polymicrobial infection in fish is also widespread. In this study, golden pompano were coinfected with 2 strains of *Streptococcus*, *S. agalactiae* and *S. iniae,* with different virulence (LD_{50} of 6.38×10^4 and 1.47×10^7 CFU ml^{-1}, respectively). Recently, several studies have reported the coinfection of *Streptococcus* with other microorganisms, such as *Trichodina* sp. (Evans et al. 2007), *Gyrodactylus niloticus* (Xu et al. 2007), and highly pathogenic porcine reproductive and respiratory syndrome virus (Xu et al. 2010). In those studies, *Streptococcus* was often considered as a secondary infection which increased the pathogenicity of the virus or parasite. The initial virus or parasite infection was thought to potentially suppress the host immune system, facilitating invasion by *Streptococcus*. *S. agalactiae* and *S. iniae* can overcome and then inhibit the host's immune defense system by entering and multiplying within macrophages to induce apoptosis or necrosis of macrophages (Poyart et al. 2001, Zlotkin et al. 2003, Kline et al. 2011). Initial infection with one *Streptococcus* species, capable of inhibiting host responses, may predispose the host to infection with a second bacterial species and could have resulted in the coinfection occurrence of *S. agalactiae* and *S. iniae* in golden pompano.

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