Original Article

Streptococcus agalactiae serotype Ib, an emerging pathogen affecting climbing perch (*Anabas testudineus*) and Günther's walking catfish (*Clarias macrocephalus*) polycultured in southern Thailand

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Abstract

Streptococcus agalactiae (Group B *Streptococcus*; GBS) causes serious damage to fish aquaculture worldwide. The present study reports *S. agalactiae* infection in climbing perch (*Anabas testudineus*) and Günther's walking catfish (*Clarias macrocephalus*) polycultured in southern Thailand between 2011 and 2015. During the disease outbreak, a mortality rate of 10-40% was observed, affecting climbing perch and Günther's walking catfish weighing from 60-150 g and 30-90 g, respectively. The infected fish exhibited a variety of symptoms typical of streptococcosis, including lethargy, exophthalmia, corneal opacity, ascites, haemorrhage and erratic swimming. One hundred and twenty six isolates from the infected fish were identified as *S. agalactiae* serotype Ib by biochemical, serological, as well as molecular analyses. The *S. agalactiae* isolates from the present study were completely sensitive to chloramphenicol, erythromycin, lincomycin and oxytetracycline, but resistant to oxolinic acid and sulfamethoxazole/trimethoprim. Investigation into virulence-associated genes (*bca*, *bac*, *scpB*, *lmb* and GBSi1) indicated that the *S. agalactiae* isolates from climbing perch and Günther's walking catfish contained only *bca*, which differed from *S. agalactiae* previously isolated from infected tilapia. The *S. agalactiae* isolates from the present study were found to be strongly virulent for climbing perch with 80-100% mortality within 7 days following intraperitoneal (i.p.) injection with *S. agalactiae* at a concentration of 10^7 CFU/ml. Major histopathological changes of naturally infected climbing perch revealed diffuse haemorrhage in several organs. To our knowledge, this is the first isolation of *S. agalactiae* serotype Ib from climbing perch and Günther's walking catfish polycultured in southern Thailand.

Keywords: *Anabas testudineus*, *Clarias macrocephalus*, *Streptococcus agalactiae* serotype Ib, pathology

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Introduction

Climbing perch, *Anabas testudineus* (Perciformes: Anabantidae), have become an economically important food fish in Thailand, Malaysia and the Philippines (Chotipuntu and Avakul, 2010). This brown or dark greenish-brown fish, native to Southeast Asia, is highly tolerant of very turbid and brackish water conditions. An accessory breathing organ enables this fish to survive out of water for several days (Hitchcock, 2008). Nowadays, the polyculture of climbing perch and Günther's walking catfish, *Clarias macrocephalus* (Cypriniformes: Clariidae), is common in southern Thailand because catfish are bottom feeders which can feed on leftover food. Further, *Clarias* catfish can be cultivated in high densities, even in circumstances where limited water quality and quantity prevent the culture of other species (Wattanutchariya, 1982). However, the culture of climbing perch and Günther's walking catfish under stress conditions may lead to serious outbreaks of infectious diseases in fish farms. The diseases to which climbing perch and *Clarias* catfish are prone include motile *Aeromonas* septicemia (Angka et al., 1995), tail and fin rot disease (Rahman et al., 2010), enteric septicemia of catfish (Suanyuk et al., 2014) and streptococcosis (Dung and Duy, 2013).

Streptococcosis has caused severe losses in cultured fish worldwide. The major causative agents responsible for streptococcosis in fish are *Streptococcus iniae*, *S. dysgalactiae*, *S. parauberis*, and *S. agalactiae* (Group B *Streptococcus*; GBS). In Thailand, streptococcosis was reported first in sand goby (*Oxyeleotris marmoratus*) cultured in central Thailand (Kasornchan et al., 1986). Since then, the disease has been reported in Asian seabass (*Lates calcarifer*) cultured in southern Thailand (Direkbusarakom and Donayadol, 1987; Wanman et al., 2005) and tilapia (*Oreochromis* spp.) cultured throughout the country (Suanyuk et al., 2008; Suanyuk et al., 2010; Jantrakajorn et al., 2014). *S. agalactiae* is the most common agent of invasive infections in cultured and wild warm-water fish, causing ascites, exophthalmia, haemorrhagic septicemia and meningitis (Evans et al., 2002; Duremdez et al., 2004; Suanyuk et al., 2008; Zamri-Saad et al., 2010; Abuseliana et al., 2011; Suwannasang et al., 2014). Only *S. agalactiae* serotypes Ia and III have been reported causing serious damage in tilapia farming in Thailand (Suanyuk et al., 2008; Rodkhum et al., 2012; Suwannasang et al., 2014; Dangwetngam et al., 2016). Recent reports have suggested that *S. agalactiae* is an emerging pathogen for white shrimp (*Litopenaeus vannamei*) cultured in Latin America (Hasson et al., 2009), and ya fish (*Schizothorax prenanti*) and barcoo grunter (*Scortum barcoo*) cultured in China (Geng et al., 2012; Liu et al., 2014). While little is known about diseases of climbing perch and Günther's walking catfish, the present study reports on streptococcosis caused by *S. agalactiae* serotype Ib infection in climbing perch and Günther's walking catfish polycultured in Thailand.

Materials and Methods

Fish epizootics: The study reported in this paper investigated an outbreak of streptococcosis which occurred in southern Thailand between 2011 and 2015. An infection chronology and a list of fish hosts collected for bacterial isolation are summarized in Table 1. The first case occurred in October 2011 in a stock of climbing perch and Günther's walking catfish polycultured in earthen ponds in Nakhon Si Thammarat province. Subsequent cases included both climbing perch and Günther's walking catfish cultured in the same area. Clinical signs of the infected fish were recorded and plankton diversity and water quality, i.e. salinity, alkalinity and pH, were analyzed by standard methods.

Table 1 Host and sampling area for *S. agalactiae* isolation

Date	Origin (No. of farm)	Host (No. of fish)	No. of isolates	Culture system
20 Oct 2011	Sichon, Nakhon Si Thammarat (1)	Climbing perch (12) Günther's walking catfish (4)	41 13	Earthen pond
26 Oct 2011	Sichon, Nakhon Si Thammarat (2)	Climbing perch (15) Günther's walking catfish (1)	21 1	Earthen pond
6 Aug 2012	Sichon, Nakhon Si Thammarat (3)	Climbing perch (14)	38	Earthen pond
29 May 2015	Sichon, Nakhon Si Thammarat (1)	Climbing perch (3) Günther's walking catfish (1)	9 3	Earthen pond

Isolation of pathogenic bacteria: Samples from the liver, kidney and brain of infected fish were collected using aseptic techniques. The samples were streaked onto tryptic soy agar (TSA: Difco) and incubated at 30°C for 24-48 h. After incubation, single presumptive colonies selected from pure or dominant colonies on TSA were picked for primary testing by Gram staining and catalase activity. One hundred and twenty six purified bacterial isolates of gram-positive cocci and catalase negative were inoculated into tryptic soy broth (TSB: Difco) supplemented with 15% glycerol, and stored at -70°C.

Biochemical and serological characterization: Twenty purified bacterial isolates, including selected isolates from each commercial fish farm, were characterized biochemically using standard biochemical methods and the API20STREP system (bioMérieux). The bacterial isolates were further characterized for their haemolytic reaction on blood agar base (Merck) supplemented with 5% defibrinated sheep blood. The Lancefield serogroup and serotype of the bacterial isolates were determined using Slidex Strepto Plus (bioMérieux) and group B streptococci typing antisera (Denka Seiken), respectively. The biochemical and serological characterization were quality controlled and validated using two known isolates, *S. agalactiae* PSU-KSAAHRC-ST81 serotype Ia isolated from infected tilapia cultured in Nakhon Si Thammarat province and *S. agalactiae* PSU-KSAAHRC-ST87 serotype III isolated from infected tilapia cultured in

Songkhla province, southern Thailand (Suanyuk et al., 2008). The bacteria were classified into genus and species using the APILAB PLUS program (bioMérieux) and the method described in Bergey's Manual of Systematic Bacteriology (Hardie, 1986).

Polymerase chain reaction (PCR): PCR analysis was used to confirm the bacterial genus and species. Total nucleic acid was extracted from 126 bacterial isolates from infected fish using the method of Berridge et al. (1998). Oligonucleotide primers targeting the *16S rRNA* gene of *S. agalactiae* (Martinez et al., 2001) were used in this study. PCR amplification was carried out according to the method described by Suanyuk et al. (2008) using *S. agalactiae* PSU-KSAAHRC-ST81 and *S. agalactiae* PSU-KSAAHRC-ST87 isolates as positive control strains.

16s rDNA sequencing and phylogenetic analysis: *S. agalactiae* isolate PSU-KSAAHRC-298, originally isolated from naturally infected climbing perch, was chosen for *16s rDNA* sequencing and phylogenetic analysis because it produced high mortality in experimental climbing perch. The forward primer 20F and reverse primer 1500R described by Weisburg et al. (1991) were used to amplify the *16S* ribosomal genes. PCR amplification was performed according to the method described by Suanyuk et al. (2014). The PCR amplified product was purified with a gel extraction kit (Qiagen) and sequenced using PCR primers 20F and 1500R.

The sequences generated were used to search the Entrez database for homologous sequences using the Basic Local Alignment Search Tool (BLAST, http://www.ncbi.nlm.nih.gov). Twenty-eight homologous *Streptococcus* sequences were chosen for phylogenetic analysis using the clustalW application of the Molecular Evolutionary Genetic Analysis (MEGA) package. The analysis included *Lactococcus garvieae* as an out-group species. An un-rooted evolutionary tree was inferred using the neighbor-joining tree algorithm and the resultant tree topologies were evaluated by bootstrap analysis based on 1000 replicates.

Comparative analysis of virulence-associated genes of S. agalactiae: Eight *S. agalactiae* selected isolates (6 isolates from climbing perch and 2 isolates from Günther's walking catfish) from each commercial fish farm from the present study were chosen for the detection of virulence-associated genes in comparison with *S. agalactiae* PSU-KSAAHRC-ST81 serotype Ia and *S. agalactiae* PSU-KSAAHRC-ST87 serotype III isolated from infected tilapia from a previous study (Suanyuk et al., 2008). Genomic DNA was extracted from *S. agalactiae* isolates using the method described by Granlund et al. (2001). These genomic DNAs were used as templates in PCR reactions containing primers specific to virulence-associated genes in *S. agalactiae*. These genes included surface protein genes, *bca* and *scpB* (Dmitriev et al., 1999), *bac* (Dmitriev et al., 2002), *lmb* (Jain et al., 2012) and the group II intron, GBSi1 (Bidet et al., 2003). The *bca* gene encodes Cα protein which participates in the invasion of epithelial cells (Baron et al., 2007) and *bac* encodes Cβ protein that binds to the Fc region of human IgA and participates in bacterial resistance to mucosal immune defense mechanisms (Berner et al., 2002). The *lmb* gene encodes the laminin binding protein which mediates the attachment of *S. agalactiae* to human laminin, and is essential for bacterial colonization of damaged epithelium (Spellerberg et al., 1999). The *scpB* gene which encodes C5a peptidase cleaves C5a and binds fibronectin (Beckmann et al., 2002) and the group II intron, GBSi1, is located downstream from the *scpB* gene (Granlund et al., 2001; Al Safadi et al., 2010).

PCR amplification was carried out in an MJ Mini™ thermal cycler (Bio-Rad) using 7.5 µL of RBC Blue Tag Mastermix (RBC Bioscience), 0.5 µL of 10 µM of each primer, 5 µL of DNA template and 1.5 µL of distilled water. For the *bca*, *scpB*, *bac* and GBSi1 genes, the thermal cycle was initial denaturation at 94°C for 4 min; 35 cycles of 94°C for 40 seconds, 55°C for 1 min, and 72°C for 1 min; and a final extension at 72°C for 5 min. For the *lmb* gene, the thermal cycle was initial denaturation at 94°C for 1 min; 35 cycles of 94°C for 1 min, 46°C for 1 min, and 72°C for 2 min; and a final extension at 72°C for 3 min. The reaction products were separated by 1.5% agarose gel electrophoresis in TBE buffer (90 mM Tris, 90 mM Borate and 2 mM ethlylenediaminetetraacetic acid, pH 8.0) alongside a 100 bp DNA ladder (Genedirex).

Antibiotic susceptibility testing: Antibiotic susceptibility patterns of the 20 *S. agalactiae* isolates selected from each commercial fish farm were determined using the modified disc diffusion method (Clinical and Laboratory Standards Institute, 2012) on Mueller-Hinton agar (Merck) plates supplemented with 5% sheep blood. Antibiotic discs (Oxoid) including approved and unapproved antibiotics for treatment of bacterial diseases in aquaculture were used in this study. Antibiotic discs containing each of the following antibiotics: oxolinic acid (2 µg), sulfamethoxazole/trimethoprim (25 µg), erythromycin (15 µg), chloramphenicol (30 µg), lincomycin (2 µg), oxytetracycline (30 µg), norfloxacin (10 µg), ampicillin (10 µg) and ciprofloxacin (5 µg) were used in this study. The plates were incubated at 30°C for 24-48 h and results were interpreted as resistant, intermediate or susceptible by measurement of the diameter of the inhibition zone according to the interpretive standards of the Clinical and Laboratory Standards Institute (2012). The *Staphylococcus aureus* ATCC25923 reference strain was used as a control.

Infectivity trials: Healthy climbing perch with an average weight of approximately 3 g were obtained from a commercial fish hatchery in Nakhon Si Thammarat province, southern Thailand. The fish were cultured to experimental size (24.25±3.84 g) in three-ton fiber glass tanks with aeration. During the rearing period, the fish were fed twice daily to satiation on commercial fish feed. A sample of the experimental fish was examined to be *Streptococcus* spp.-free prior to use in the infectivity trials.

Pathogenicity of the 20 *S. agalactiae* isolates obtained from each commercial fish farm was determined using a method modified from Wanman et al. (2005). The experiment was conducted in twenty-

one 45x60x45 cm fiber glass tanks containing 60 L dechlorinated water with aeration. Five climbing perch were infected per bacterial isolate by intraperitoneal (i.p.) injection with 0.1 ml of bacterial suspension in PBS (pH, 7.4) containing 10^7 CFU/ml. The control group was similarly inoculated with sterile PBS. Tissue samples of moribund fish were aseptically collected and streaked onto TSA and incubated at 30°C for 24-48 h. The bacterial cultures were identified by API20STREP to confirm the cause of death. Mortality was monitored daily for 14 days. The pathogenicity level of each *S. agalactiae* isolate was assessed and labeled as follows: strongly virulent (mortality of at least 4 fish); virulent (mortality of 2-3 fish); weakly virulent (mortality of 1 fish) and avirulent (no mortality).

Histopathological study: Tissue samples from the liver, kidney, spleen, heart, brain and eye of the infected climbing perch obtained from commercial fish farms were collected and examined for histopathological study using the standard method of Humason (1979). Briefly, the infected tissues were fixed in 10% formalin. After fixation, each sample was cut into small pieces and embedded in paraffin. Histological sections were made in about 3-5 µm and stained with haematoxylin and eosin (H&E). Prepared slides were examined under a light microscope (Olympus) for histopathological changes.

Results

Fish epizootics: Large numbers of gram-positive cocci were isolated from the infected climbing perch and Günther's walking catfish (Fig. 1). During sample collection, a mortality rate of 10-40% was observed, affecting climbing perch and Günther's walking catfish weighing from 60-150 g and 30-90 g, respectively. The infected climbing perch exhibited lethargy, erratic swimming, exophthalmia, corneal opacity, fin erosion, and haemorrhages of the skin, fin base and anus. Internally, haemorrhages of internal organs, pale liver, splenomegaly and accumulated fluid in the peritoneal cavity were observed. External clinical symptoms exhibited by the infected Günther's walking catfish included body discoloration, skin lesions and pale liver. The culture water contained *Microcystis* spp., *Oscillatoria* spp., and *Scenedesmus* spp. Water quality during the disease outbreak was as follows: salinity, 0%; alkalinity, 86.0±39.89 (41-117) mg/l; and pH, 6.25±0.36 (5.85-6.55).

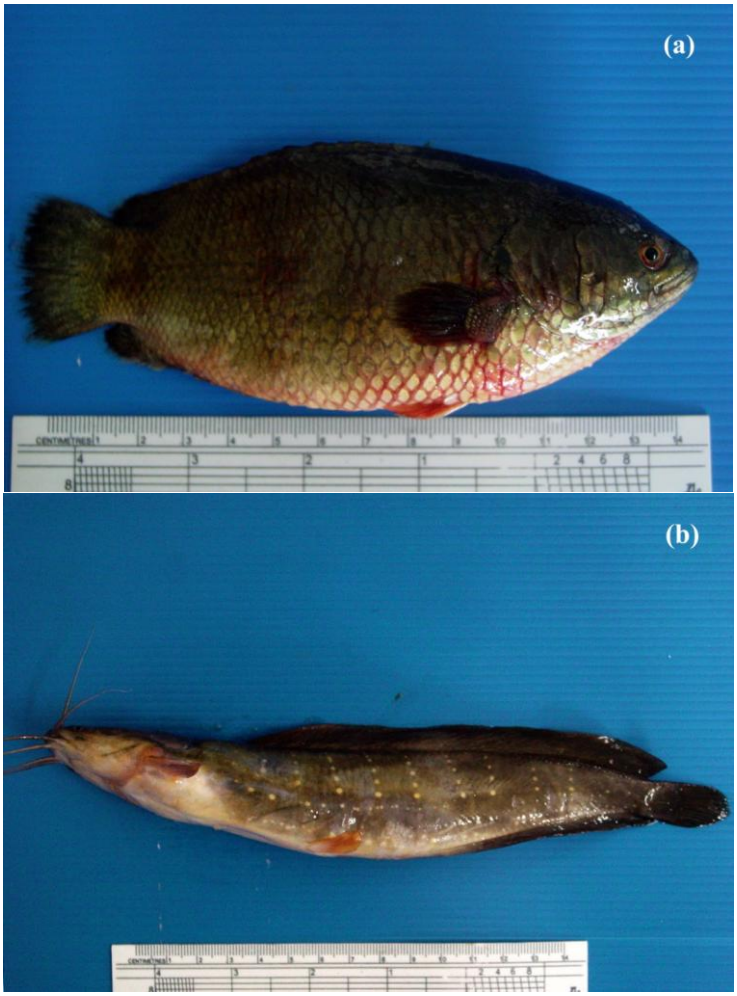


Figure 1 Gross pathology of climbing perch and Günther's walking catfish infected with GBS serotype Ib. (a) Typical external clinical signs of climbing perch naturally infected by *S. agalactiae* serotype Ib showing fin erosion, haemorrhaging of skin and abdominal distension. (b) Typical external clinical sign of Günther's walking catfish infected by *S. agalactiae* serotype Ib showing skin abrasion.

Biochemical and serological characterization: Sixteen bacterial isolates from the infected climbing perch and four bacterial isolates from the infected Günther's walking catfish were identified as *S. agalactiae*. These bacteria were gram-positive cocci in chain, 1-2 mm in diameter, catalase negative, oxidase negative, and γ-haemolysis on the 5% sheep blood agar. All isolates obtained from the infected climbing perch and Günther's walking catfish displayed similar biochemical characteristics (Table 2). The Slidex Strepto Plus kit and group B streptococci typing antisera kit successfully classified the bacterial isolates into group B *Streptococcus* and serotype Ib, respectively. The API20STREP, Slidex Strepto Plus and group B streptococci typing antisera kits successfully identified *S. agalactiae* PSU-KSAAHRC-ST81 serotype Ia and *S. agalactiae* PSU-KSAAHRC-ST87 serotype III reference strains.

Table 2 Host and sampling area for *S. agalactiae* isolation

Test	Climbing perch (n=16)	Günther's walking catfish (n=4)	Test	Climbing perch (n=16)	Günther's walking catfish (n=4)
Gram staining reaction	+	+	Alkaline phosphatase	+	+
Cell morphology	Cocci	Cocci	Leucine aminopeptidase	+	+
Catalase	-	-	Arginine dihydrolase	+	+
Oxidase	-	-	Acid production from		
Serogroup	B	B	- Ribose	-	-
Serotype	Ib	Ib	- Arabinose	-	-
Haemolysis	Y	Y	- Mannitol	-	-
VP test	+	+	- Sorbital	-	-
Hippurate	+	+	- Lactose	-	-
Esculin	-	-	- Trehalose	-	-
Pyrrolidonyl arylamidase	-	-	- Inulin	-	-
α-Galactosidase	-	-	- Raffinose	-	-
β-Glucuronidase	+	+	- Starch	-	-
β-Galactosidase	-	-	- Glycogen	-	-

PCR and phylogenetic analysis: The identification of *S. agalactiae* was confirmed by the PCR and phylogenetic analysis. The PCR analysis showed the amplification of a 220 bp band specific to *16s rRNA* of *S. agalactiae*. This specific band was detected for all *S. agalactiae* isolated from the infected climbing perch and Günther's walking catfish from the present study including the *S. agalactiae* reference strains (Fig. 2).

Detailed phylogenetic analysis based on the *16s rDNA* sequences of the *S. agalactiae* PSU-KSAAHRC-298 is shown in Fig. 3. The phylogenetic tree clustered the *S. agalactiae* PSU-KSAAHRC-298 strain with four *S. agalactiae* isolates stored in Genbank with accession numbers FO393392, CP007482, HF952106 and HG939456.

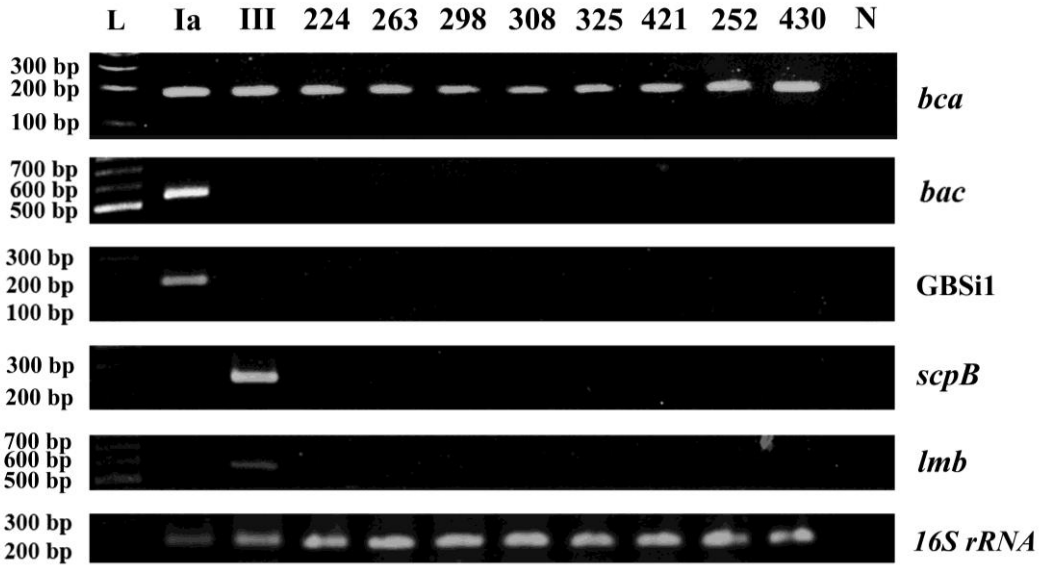


Figure 2 Identification of *S. agalactiae* virulence-associated genes. Genes involved in *S. agalactiae* virulence were detected by PCR assay to determine their occurrence in *S. agalactiae* isolates from climbing perch and Günther's walking catfish in comparison with *S. agalactiae* isolates from tilapia. L, 100 bp DNA ladder; Ia, *S. agalactiae* serotype Ia isolated from infected tilapia; III, *S. agalactiae* serotype III isolated from infected tilapia; 224, 263, 298, 308, 325 and 421, *S. agalactiae* serotype Ib isolated from infected climbing perch; 252 and 430, *S. agalactiae* serotype Ib isolated from Günther's walking catfish; N, Negative control (DDW).

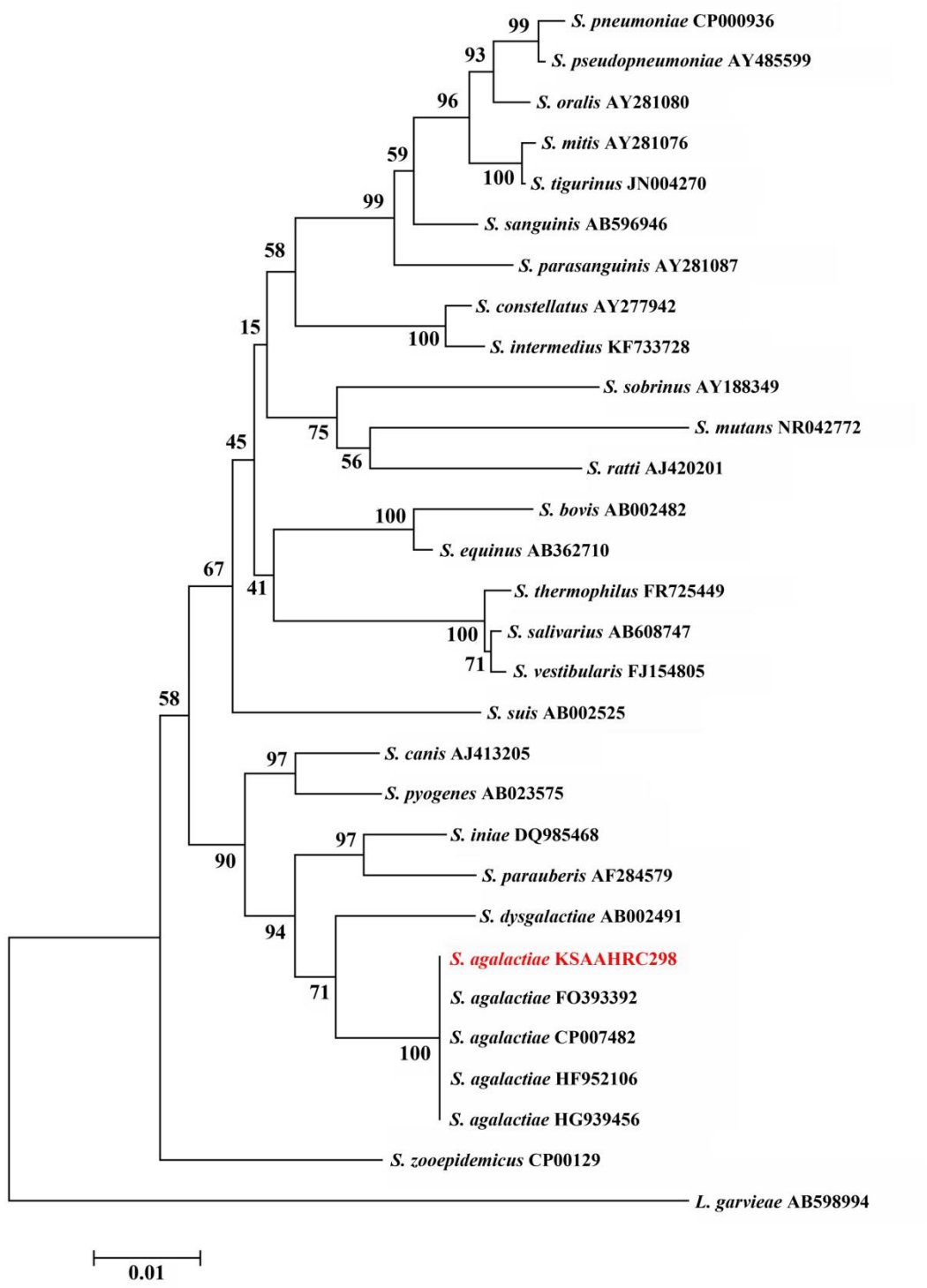


Figure 3 Phylogenetic tree based on *16s rDNA* sequences of *S. agalactiae* from infected climbing perch from the present study *S. agalactiae* KSAAHRC298 and homologous sequences in the Genbank database. The accession numbers are written beside the name of the strains, and the scale bars represent distance values.

Comparative analysis of virulence-associated genes of S. agalactiae: The PCR assay successfully detected the *bca*, *bac* and GBSi1 genes from *S. agalactiae* serotype Ia and the *bca*, *scpB* and *lmb* genes from *S. agalactiae* serotype III isolates from infected tilapia (Fig. 2). The climbing perch and Günther's walking catfish isolates of *S. agalactiae* produced a strong PCR product only in the reactions for the *bca* gene (Fig. 2). The *bac*, GBSi1, *scpB* and *lmb* genes, however, were absent in all reactions containing the climbing perch and Günther's walking catfish DNA.

Antibiotic sensitivity test: All the *S. agalactiae* isolates obtained from the infected climbing perch and Günther's walking catfish displayed a similar antibiotic susceptibility pattern. They were completely sensitive to four of the nine tested antibiotics, chloramphenicol, erythromycin, lincomycin and oxytetracycline. The *S. agalactiae* isolates were, however, resistant to oxolinic acid and sulfamethoxazole/trimethoprim (Fig. 4).

Infectivity trials: There was no mortality observed in the climbing perch i.p. injected with sterile PBS. All the *S. agalactiae* isolates were found strongly virulent for the climbing perch. The climbing perch inoculated by i.p. injection with all the *S. agalactiae* isolates exhibited 80-100% mortality within 7 days. During experimental infection, the infected fish displayed lethargy, darkening of the skin pigment, eye opacity, serpentine movement, haemorrhaging anus and meningitis.

Histopathological study: Histopathological changes in the climbing perch infected with *S. agalactiae* were observed in several organs including the brain, eye, heart, kidney, spleen and liver. The major histopathological finding was diffuse haemorrhage which was found in several organs of the infected fish including the outer layers of the brain (Fig. 5a), the bulbus arteriosus and the heart (Figs. 5b and 5c), the eye (Fig. 5d), the kidney (Fig. 5e), the spleen (Fig. 5f) and the liver (Fig. 5g).

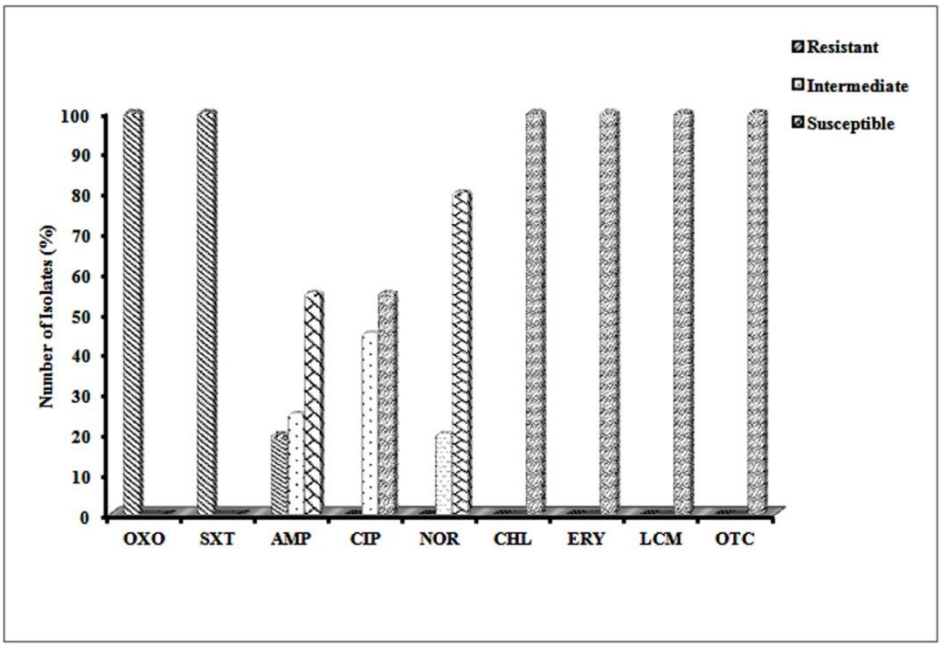
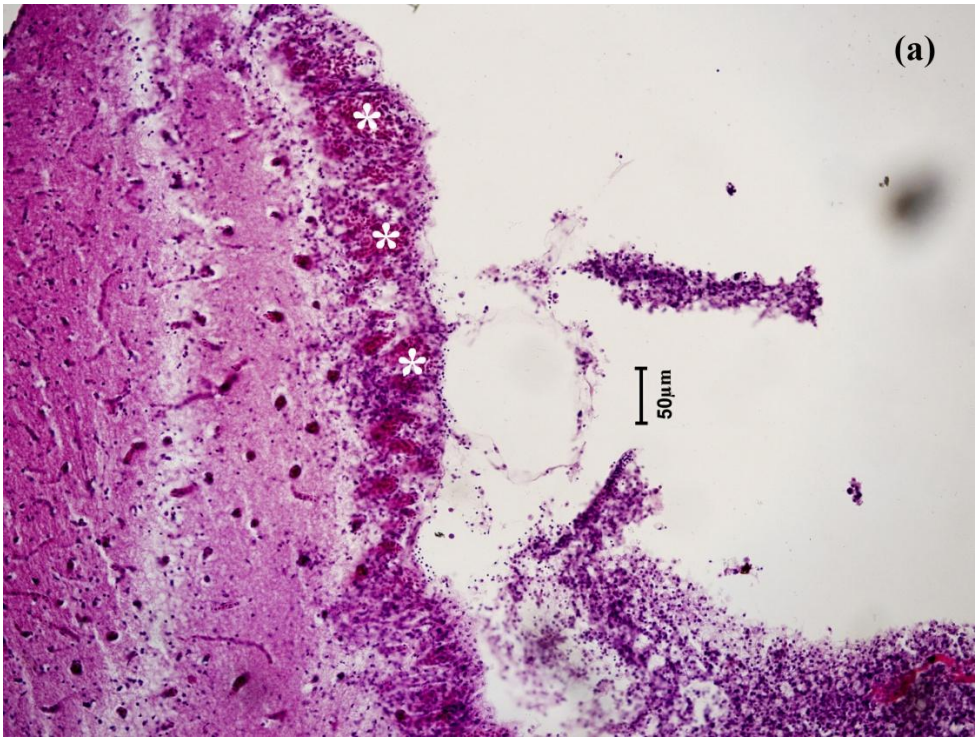
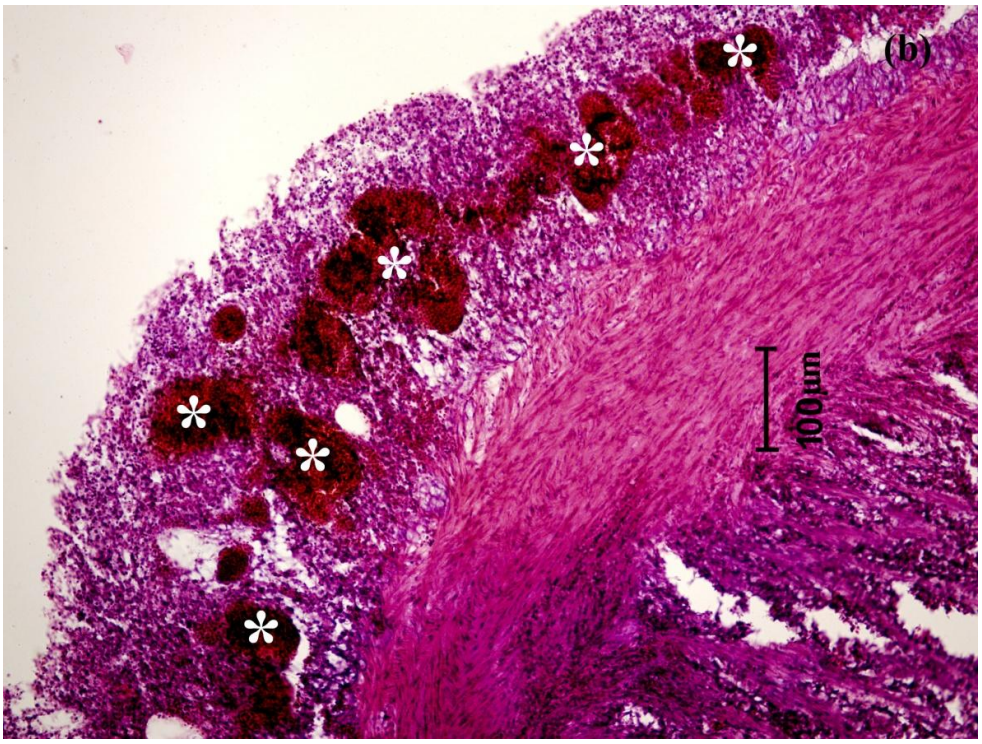
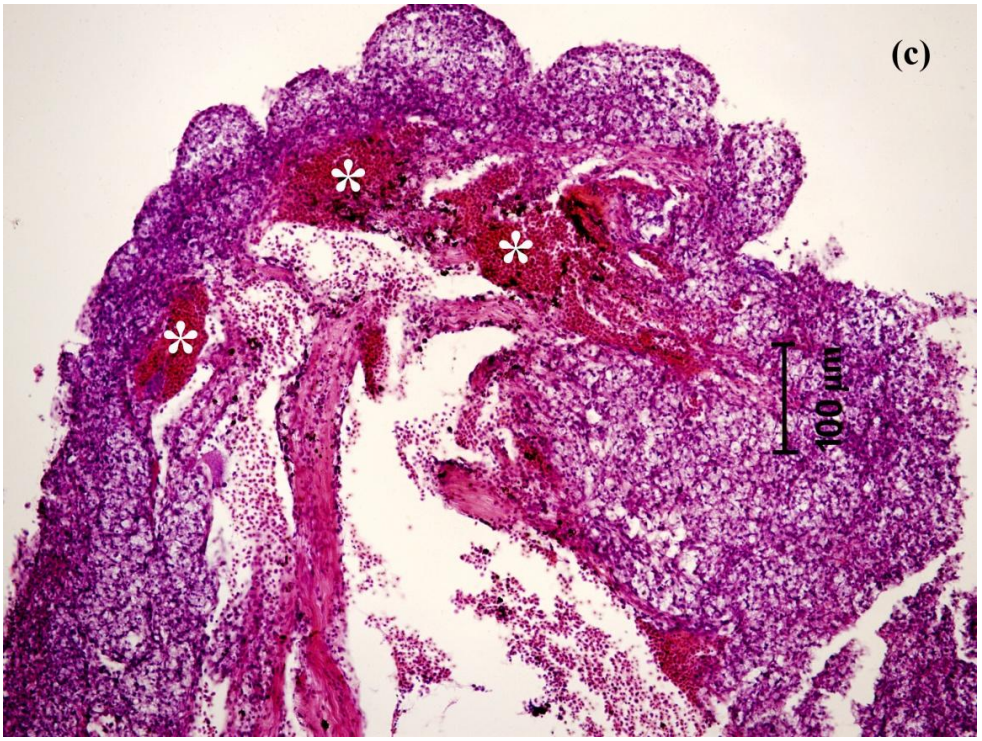


Figure 4 Antibiotic susceptibility of *S. agalactiae* isolated from infected climbing perch and Günther's walking catfish. OXO, oxolinic acid; SXT, sulfamethoxazole/trimethoprim; AMP, ampicillin; CIP, ciprofloxacin; NOR, norfloxacin; CHL, chloramphenicol; ERY, erythromycin; LCM, lincomycin; OTC, oxytetracycline.




(b)
100µm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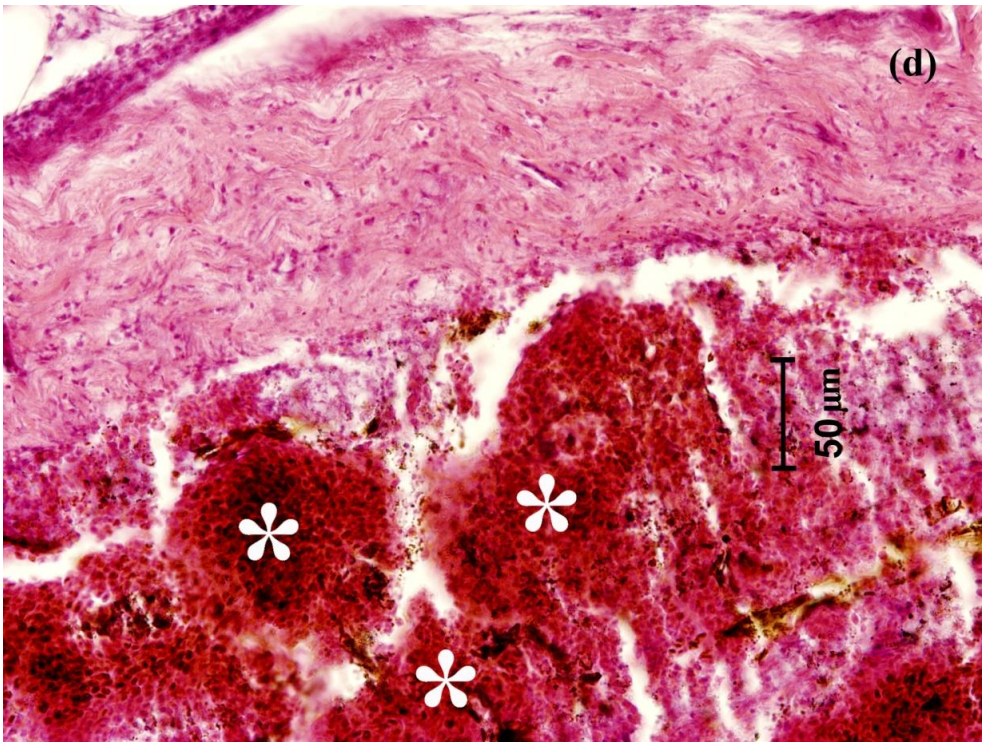
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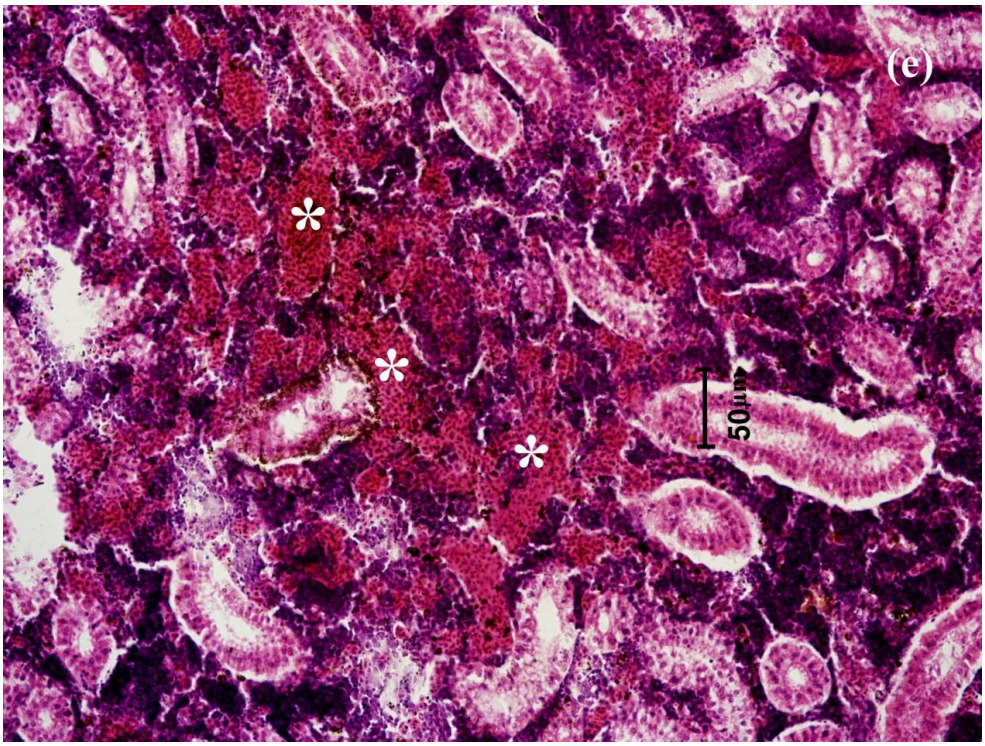
(e)
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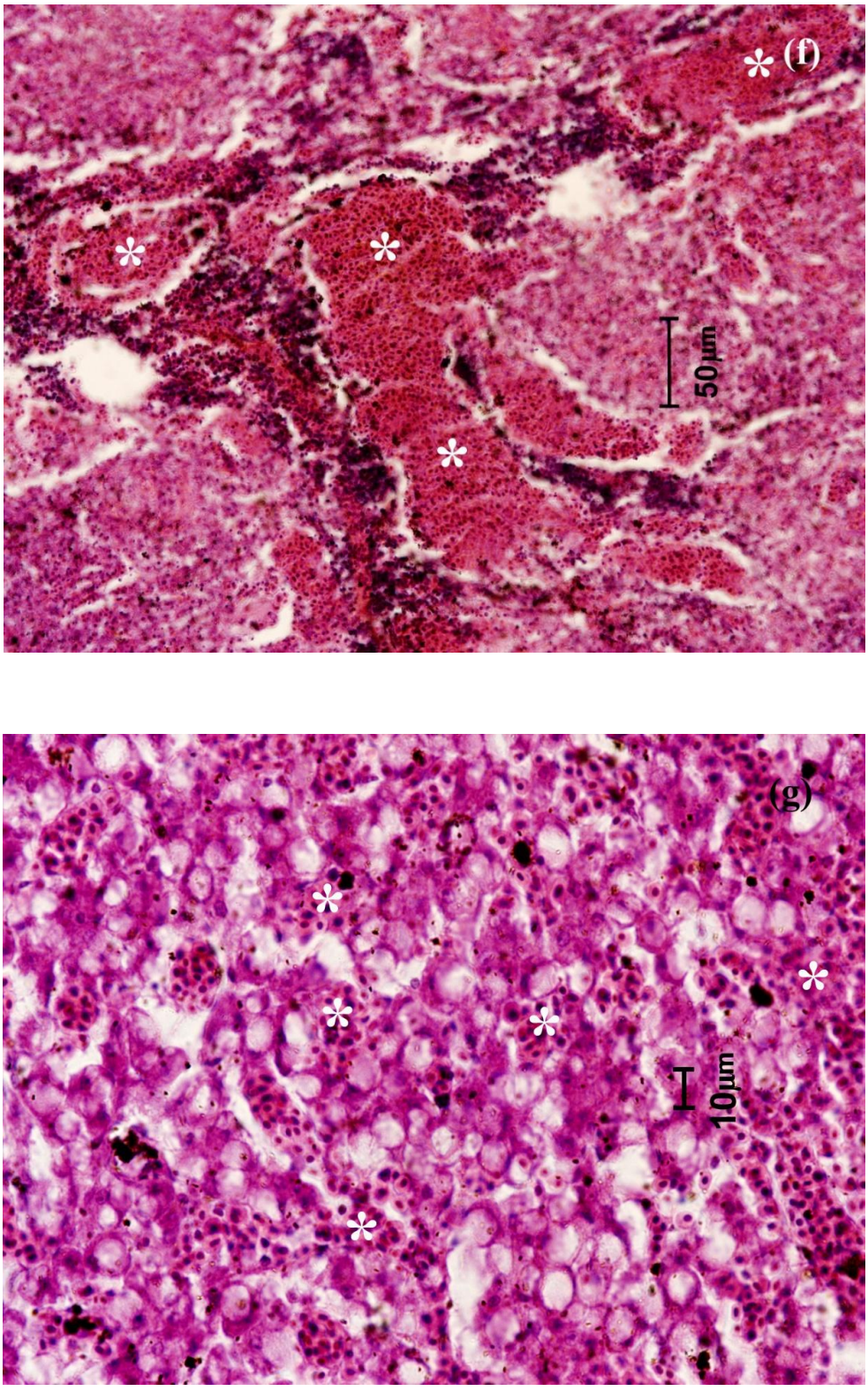

Figure 5 Histological sections showing diffuse haemorrhage (asterisk) at the (a) outer layer of brain, (b) bulbus arteriosus, (c) heart, (d) eye, (e) kidney, (f) spleen and (g) liver from infected climbing perch

Discussion

S. agalactiae is an emerging bacterial pathogen in aquatic animals with recent reports on *S. agalactiae* infection in white shrimp, ya fish and barcoo grunter (Hasson et al., 2009; Geng et al., 2012; Liu et al., 2014). In 2011, mortality of climbing perch and Günther's walking catfish polycultured in earthen ponds with typical signs of streptococcosis occurred in a fish farm in Nakhon Si Thammarat, southern Thailand. Similar cases were recorded in other farms in the same area in 2012 and 2015. The classification of these isolates from infected climbing perch and Günther's walking catfish was achieved using the method described in Bergey's Manual of Systematic Bacteriology. The results showed that these isolates were biochemically similar to *S. difficilis* [*S. difficile* (sic) the species epithet was corrected by Euzéby (1988)] isolates from diseased fish

with meningoencephalitis in Israel (Eldar et al., 1994). However, recent studies have demonstrated *S. difficilis* to be a group B, type Ib *Streptococcus* with a whole-cell protein electrophoretic profile indistinguishable from *S. agalactiae* and a biochemical reactivity similar to that observed for other *S. agalactiae* type Ib isolated from fish and frogs (Vandamme et al., 1997). Furthermore, the genetic similarity of the 16S-23S rDNA intergenic spacer sequence (Berridge et al., 2001) and the 16S rRNA, *gyrB*, *sodA*, *gyrA* and *parC* gene sequences (Kawamura et al., 2005) suggest that *S. difficilis* and *S. agalactiae* are synonymous.

At present, 10 different *S. agalactiae* serotypes (Ia, Ib and II to IX) have been described based on the composition of the capsular polysaccharide (Slotved et al., 2007). Among the *S. agalactiae* isolates, Ia, Ib and III have been reported as the cause of disease in fish (Vandamme et al., 1997; Evans et al., 2008; Suanyuk et al., 2008; Suwannasang et al., 2014). The present study indicated that the *S. agalactiae* isolates from infected climbing perch and Günther's walking catfish in Thailand belonged to the capsular polysaccharide antigen types Ib. There have been a number of reports on increases in *S. agalactiae* serotype Ib infection in fish published recently, in which *S. agalactiae* serotype Ib was isolated from wild giant Queensland grouper (*Epinephelus lanceolatus*) in Australia (Bowater et al., 2012), tilapia in Honduras, Colombia, Costa Rica and Belgium, and rosy barb (*Puntius conchonius*) and golden ram (*Mikrogeophagus ramirezi*) in Australia (Delannoy et al., 2013) and tilapia in China (Li et al., 2013). In Thailand, only *S. agalactiae* serotypes Ia and III have been reported in cultured fish (Suanyuk et al., 2008; Rodkhum et al., 2011; Suwannasang et al., 2014; Dangwetngam et al., 2016). Therefore, this is the first report on streptococcosis caused by *S. agalactiae* serotype Ib in polycultured climbing perch and Günther's walking catfish. The different *S. agalactiae* serotypes isolated from the infected fish from the present study supported the findings of a previous study (Suanyuk et al., 2008) that the *S. agalactiae* outbreaks in cultured fish throughout the country were not all related, suggesting that the *S. agalactiae* isolates from climbing perch and Günther's walking catfish polycultured in Thailand may have originated from different sources.

Recently, multilocus sequence typing (MLST) and genotyping have identified four subpopulations of *S. agalactiae* isolates in aquatic mammals and fish. One of these four subpopulations consists of non-haemolytic *S. agalactiae* serotype Ib isolated from fish belonging to ST260 and ST261, which has never been identified in humans, and none of these isolates contained any of the surface protein genes or mobile genetic elements that were investigated (Delannoy et al., 2013). The presence of the *bca* gene from our study suggests that the *S. agalactiae* serotype Ib isolates from climbing perch and Günther's walking catfish in this study may not belong to any of these four subpopulations. Further study by MLST and genotyping of these *S. agalactiae* isolates may provide a deeper understanding of possible phylogenetic relationships among fish, aquatic animal, and human *S. agalactiae* serotype Ib isolates.

S. agalactiae demonstrates several virulence factors, resulting in different clinical symptoms during the progression of disease in fish. A number of virulence-associated genes from *S. agalactiae* isolates from fish have previously been identified (Suanyuk et al., 2008; Delannoy et al., 2013; Kayansamruaj et al., 2014). In this study, the PCR assay successfully detected the *bca* gene in all the *S. agalactiae* isolates, including isolates from both climbing perch and Günther's walking catfish as well as from tilapia isolates from the previous study (Suanyuk et al., 2008), suggesting that *bca* may play a major role in *S. agalactiae* pathogenesis in fish. Moreover, the absence of the *bac*, GBSi1, *scpB* and *lmb* genes in the *S. agalactiae* isolates from climbing perch and Günther's walking catfish indicates that these genes are not involved in the pathogenesis of *S. agalactiae* serotype Ib in fish. Furthermore, the presence of the *bca*, *bac* and GBSi1 genes in the *S. agalactiae* serotype Ia and the *bca* gene in the *S. agalactiae* serotype III isolates from tilapia was similar to those detected in the *S. agalactiae* serotypes Ia and III isolates from infected tilapia cultured in Thailand and Vietnam, suggesting that the *S. agalactiae* serotypes Ia and III from our study may possibly be the same subpopulations as the *S. agalactiae* reported by Delannoy et al. (2013). Additionally, the presence of the *bca* and *bac* genes in the *S. agalactiae* serotype Ia and the *bca* gene in the *S. agalactiae* serotypes Ib and III isolates from this study supports the finding of Delannoy et al. (2013) that the *bac* gene is always found in association with the *bca* gene but the *bca* gene can be present in the absence of the *bac* gene.

The *scpB* and *lmb* genes encode C5a-peptidase and laminin binding protein, respectively (Granlund et al., 2001). In this study, the *scpB* and *lmb* genes were detected only in the *S. agalactiae* serotype III isolates from tilapia suggesting that these genes may be involved in the pathogenesis of *S. agalactiae* serotype III in fish. This result is consistent with a previous report from Kayansamruaj et al. (2014) where the *scpB* and *lmb* genes were detected in *S. agalactiae* serotype III but not in *S. agalactiae* serotype Ia isolates from fish. The *scpB* and *lmb* genes were detected in all human-derived streptococcal strains but only 20 to 39% was found in animal-derived streptococcal strains (Franken et al., 2001; Dmitriev et al., 2002). The *scpB-lmb* intergenic region is a hot-spot for integration of two mobile genetic elements, IS*1548* or GBSi1 (Al Safadi et al., 2010). In this study, no *scpB*, *lmb* and GBSi1 genes were detected in the *S. agalactiae* serotype Ib, whereas only GBSi1 was detected in the *S. agalactiae* serotype Ia and the *scpB* and *lmb* genes were detected in the *S. agalactiae* serotype III without GBSi1. Investigation into the genomic organization of the *scpB-lmb* intergenic region of *S. agalactiae* isolates from fish is needed to understand the role of *S. agalactiae* binding and the invasion of host surfaces.

The antibiotic susceptibility testing indicated that the *S. agalactiae* serotype Ib isolates from the present study were sensitive to chloramphenicol, erythromycin, lincomycin and oxytetracycline, but resistant to oxolinic acid and sulfamethoxazole/trimethoprim. Similar results have been reported in *S. agalactiae* isolates from infected cultured silver pomfret (*Pampus argenteus*) in Kuwait

(Duremdez et al., 2004) and *S. agalactiae* serotypes Ia and III isolates from infected tilapia cultured in Thailand (Dangwetngam et al., 2016). Resistance to oxolinic acid, a quinolone derivative, is primarily due to alteration in drug targets, alteration in drug permeation and plasmid-mediated resistance (Hooper, 2003). Resistance to sulfamethoxazole/trimethoprim, also called co-trimoxazole, is mediated by the permeability barrier and/or efflux pumps, naturally insensitive target enzymes, regulational changes in the target enzymes, mutational or recombinational changes in the target enzymes and acquired resistance by drug-resistant target enzymes (Huovinen, 2001). To date, other antibiotics such as enrofloxacin and amoxicillin are commonly used in Thai aquaculture. Therefore, the antibiotic susceptibility for these drugs is interesting and deserves to be further studied.

The climbing perch experimentally infected with *S. agalactiae* serotype Ib isolates exhibited 80-100% mortality within 7 days, indicating that the *S. agalactiae* from the present study was pathogenic to fish. Gross pathological and histopathological study supports the finding that naturally infected fish exhibited several clinical signs and histopathological changes. Similar clinical signs and histopathological changes in infected fish, typical of streptococcosis caused by *S. agalactiae* were observed in seabream (*Sparus auratus*) and mullet (*Liza klunzingeri*) (Evans et al., 2002), silver pomfret (Duremdez et al., 2004) and tilapia (Suanyuk et al., 2008; Zamri-Saad et al., 2010; Abuseliana et al., 2011; Suwannasang et al., 2014) infected with *S. agalactiae*.

During the disease outbreak, it was observed that the mortality of climbing perch happened before that of Günther's walking catfish (fish farmer, personal communication, 2011). In this study, however, the experimental infection of *S. agalactiae* isolates in Günther's walking catfish was not examined. Therefore, the possible transmission of *S. agalactiae* from climbing perch to Günther's walking catfish needs to be investigated. Additionally, it should be noted that environmental factors may influence the fish diseases. Previously, the virulence of *S. agalactiae* isolates from tilapia showed positive correlation with water temperature (Rodkhum et al., 2011). Further study of the epizootiology of *S. agalactiae* serotype Ib may provide more insight into the pathogenicity of *S. agalactiae* serotype Ib infection in climbing perch and Günther's walking catfish.

In summary, the *S. agalactiae* serotype Ib isolates from the present study caused severe infectious disease in climbing perch and Günther's walking catfish. The detection of virulence-associated genes indicated that this bacterium harbored the *bca* gene and had a different genetic relationship from *S. agalactiae* isolates from other fish and aquatic animals. This is the first report on *S. agalactiae* serotype Ib infection in polycultured climbing perch and Günther's walking catfish.

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บทคัดย่อ

แบคทีเรีย *Streptococcus agalactiae* ซีโรไทป์ Ib เชื้อโรคอุบัติใหม่ในปลาหมอไทย (*Anabas testudineus*) และปลาดุกอุย (*Clarias macrocephalus*) ที่เลี้ยงร่วมกันในภาคใต้ของประเทศไทย

ชุติมา คลิ้งขลิบ นเรศ ช่วนยุก*

แบคทีเรีย *Streptococcus agalactiae* (Group B *Streptococcus*; GBS) สร้างความเสียหายต่อการเพาะเลี้ยงปลาทั่วโลก การศึกษาครั้งนี้รายงานการติดเชื้อแบคทีเรีย *S. agalactiae* ในปลาหมอไทย (*Anabas testudineus*) และปลาดุกอุย (*Clarias macrocephalus*) ที่เลี้ยงร่วมกันในภาคใต้ของประเทศไทย ระหว่างปี พ.ศ. 2554 ถึงปี พ.ศ. 2558 ระหว่างการระบาดของโรค พบอัตราการตาย 10-40 เปอร์เซ็นต์ในปลาหมอไทยน้ำหนัก 60-150 กรัมและปลาดุกอุยน้ำหนัก 30-90 กรัม โดยปลาที่ติดเชื้อแสดงอาการของโรคสเตรปโตคอคโคซีสหลายแบบ ได้แก่ เซื่องซึม ตาโปน ตาขุ่น น้ำขังในช่องท้อง เลือดออก และว่ายน้ำผิดปกติ การศึกษาครั้งนี้สามารถแยกแบคทีเรียจากปลาป่วยจำนวน 126 ไอโซเลต และจำแนกชนิดได้เป็นแบคทีเรีย *S. agalactiae* ซีโรไทป์ Ib โดยอาศัยคุณสมบัติทางชีวเคมี ซีรั่มวิทยา และการวิเคราะห์ทางอณูชีวโมเลกุล แบคทีเรีย *S. agalactiae* ที่แยกได้ในครั้งนี้มีความไวต่อยาปฏิชีวนะคลอแรมเฟนิคอล อิริโทรมัยซิน ลินโคมัยซิน และออกซีเตตร้าซัยคลิน แต่ดื้อต่อยาปฏิชีวนะออกโซลินิค แอซิด และซัลฟาเมทอกซาโซล/ไตรเมโทพริม การตรวจสอบยีนที่เกี่ยวข้องกับความรุนแรง (*bca*, *bac*, *scpB*, *lmb* และ GBSi1) ของแบคทีเรีย *S. agalactiae* ที่แยกได้จากปลาหมอไทยและปลาดุกอุย พบเพียงยีน *bca* ซึ่งแตกต่างจากแบคทีเรีย *S. agalactiae* ที่แยกได้จากปลานิลป่วย การศึกษาความรุนแรงของแบคทีเรีย *S. agalactiae* ที่แยกได้จากการศึกษาครั้งนี้ในปลาหมอไทยโดยวิธีการฉีดแบคทีเรียความเข้มข้น 10^7 ซีเอฟยู/มล. พบว่า แบคทีเรียมีความรุนแรงสูง ทำให้ปลาหมอไทยตาย 80-100 เปอร์เซ็นต์ภายในระยะเวลา 7 วัน การศึกษาการเปลี่ยนแปลงทางพยาธิสภาพของปลาหมอไทยป่วยที่เลี้ยงในฟาร์มพบปลาป่วยมีเลือดออกกระจายในหลายอวัยวะ การศึกษาครั้งนี้นับเป็นรายงานครั้งแรกของการแยกแบคทีเรีย *S. agalactiae* ซีโรไทป์ Ib จากปลาหมอไทยและปลาดุกอุยที่เลี้ยงร่วมกันในภาคใต้ของประเทศไทย

คำสำคัญ: *Anabas testudineus Clarias macrocephalus Streptococcus agalactiae* ซีโรไทป์ Ib พยาธิวิทยา

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