Serotype distribution and antimicrobial susceptibilities of *Streptococcus agalactiae* isolated from infected cultured tilapia (*Oreochromis niloticus*) in Thailand: Nine-year perspective

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*Streptococcus agalactiae* (group B *Streptococcus*, GBS) infection remains a major problem associated with high mortality of cultured tilapia worldwide. The present study reports the serotype distribution and antimicrobial susceptibilities of GBS isolated from infected tilapia cultured in Thailand. One hundred and forty-four GBS isolates were identified by biochemical, serological and molecular analyses. Of these 144 GBS isolates, 126 were serotype Ia and 18 were serotype III. Antimicrobial susceptibilities of the 144 GBS isolates were determined by the disc diffusion method. Most GBS isolates were susceptible to lincomycin, norfloxacin, oxytetracycline, ampicillin, erythromycin and chloramphenicol, but resistant to oxolinic acid, gentamicin, sulfamethoxazole and trimethoprim. However, 17 isolates displayed an oxytetracycline-resistant phenotype and harboured the *tet(M)* gene. The broth microdilution method was used to determine the minimal inhibitory concentrations (MICs) of 17 oxytetracycline-resistant GBS isolates, and then minimal bactericidal concentrations (MBCs) of these isolates were evaluated. Oxytetracycline-resistant isolates were found to be susceptible to ampicillin, lincomycin, norfloxacin, erythromycin and chloramphenicol, with the MIC and MBC ranging from ≤0.125 to 0.5 μg ml⁻¹ and ≤0.125 to 2 μg ml⁻¹, respectively. Moreover, all 17 oxytetracycline-resistant isolates demonstrated resistance to trimethoprim, oxolinic acid, gentamicin, sulfamethoxazole and oxytetracycline, with the MIC and MBC ranging from 16 to ≥128 μg ml⁻¹ and ≥128 μg ml⁻¹, respectively. These findings are useful information for antibiotic usage in fish aquaculture.

INTRODUCTION

Tilapias (*Oreochromis niloticus*) are an economically important food fish in tropical countries. However, the success of tilapia aquaculture may be influenced by the prevalence of bacterial diseases that can cause large losses. The major pathogenic bacteria responsible for mortalities in tilapia include *Aeromonas hydrophila* (Tipmongkolsilp et al., 2012), *Francisella noatunensis* subsp. *orientalis* (Soto et al., 2013), *Flavobacterium columnare* (Dong et al., 2015), *Vibrio vulnificus* (Chen et al., 2006), *Streptococcus iniae* (Suanyuk et al., 2010) and *Streptococcus agalactiae* (group B *Streptococcus*, GBS) (Suanyuk et al., 2008).

Streptococciosis of fish in Thailand was first reported in 1986 in sand goby (*Oxyeleotris mamoratus*) (Kasornchan et al., 1986). Since then, in Thailand, the disease has been reported in Asian sebass (*Lates calcarifer*) and tilapia (Direkbusarakom & Donayado, 1987; Wanan et al., 2005; Kitancharoen et al., 2006; Suanyuk et al., 2008, 2010). Streptococciosis of tilapia caused by GBS and *S. iniae* have been reported (Kitancharoen et al., 2006; Suanyuk et al., 2008, 2010) and to our knowledge, GBS is the most common species isolated from infected tilapia in Thailand.

Abbreviations: GBS, group B *Streptococcus*; MIC, minimal inhibitory concentration; MBC, minimal bactericidal concentration; mPCR, multiplex PCR.

Two supplementary figures are available with the online Supplementary Material.
GBS is an emerging zoonotic agent isolated from terrestrial and aquatic animals (Martinez et al., 2000; Duremdez et al., 2004; Bishop et al., 2007; Evans et al., 2008; Suanyuk et al., 2008; Fischer et al., 2013). In order to treat bacterial diseases in cultured tilapia, oxytetracycline is permitted for use in Thai aquaculture (Baoprasertkul et al., 2012). The consequences of these practices are, however, residues of antibiotics in both cultured organisms and local environments, and could result in resistance of microorganisms to antibiotics. The present study reports the serotype distribution and antimicrobial susceptibility of GBS isolated from infected tilapia cultured in Thailand over the past 9 years. In addition, the susceptibilities of oxytetracycline-resistant GBS isolates to antibiotics and detection of tetracycline resistance genes are also reported in this paper.

**METHODS**

**Bacterial strains.** One hundred and forty-four GBS isolates from infected tilapia obtained from different farms, between 2003 and 2011, were used in this study. Of these 144 GBS isolates, 74 were obtained from a collection previously evaluated by our group (Suanyuk et al., 2008) and 70 were obtained from infected tilapia from the present study (Fig. 1). These bacteria were isolated from the brain, liver, spleen, kidney, eye and ascitic fluid of infected tilapia that were streaked onto tryptic soy agar (TSA; Difco) and incubated at 30 °C for 24–48 h. They were identified as GBS by both standard biochemical methods and multiplex PCR (mpPCR) targeting of the 16S rRNA gene of GBS, the lactate oxidase-encoding gene (lectO) of *S. iniae* and the 16S rRNA gene of *Lactococcus garvieae* (Itasaro et al., 2012). The Lancefield group antigens of GBS isolates were confirmed using the SLIDEX Strepto Plus kit for grouping of streptococci groups A, B, C, D, F and G (bioMérieux). The serotype of GBS was determined using Group B Streptococci Typing antisera (Denka Seiken). The experiments were quality controlled and validated with the GBS DMST17129 and GBS PSU-KSAAHRC-ST81 isolates from infected tilapia cultured in Nakhon Si Thammarat Province (Suanyuk et al., 2008).

**Antibiotic susceptibility testing.** Antibiotic susceptibilities of 144 GBS isolates were determined using the disc diffusion method modified from the Clinical and Laboratory Standards Institute (CLSI, 2012), mainly because the optimal growth conditions for *Streptococcus agalactiae* isolated from tilapia cultured in Thailand were at temperature 30 °C and we also could not acquire *Streptococcus pneumoniae* ATCC 49619 (although recommended by CLSI for control) when we performed the tests. Antibiotic susceptibility tests were performed on Mueller–Hinton agar (MHA; Merck) supplemented with 5 % sheep blood. Antibiotic discs (Oxoid) containing each of the following antibiotics: trimethoprim (5 μg), ampicillin G (10 μg), oxolinic acid (2 μg), gentamicin (10 μg), lincomycin (2 μg), norfloxacin (10 μg), sulfamethoxazole (25 μg), erythromycin (15 μg), oxytetracycline (30 μg) and chloramphenicol (30 μg) were used in this study. These antibiotics were selected primarily based on those approved for treatment of bacterial diseases in aquaculture such as trimethoprim, oxolinic acid and oxytetracycline. Some non-approved antibiotics for aquatic animals such as chloramphenicol were also included in this study. Antibiotic discs were applied aseptically with a maximum of five discs per plate. The plates were incubated aerobically at 30 °C for 24–48 h. The results were recorded as resistant, intermediate or susceptible by measurement of the zone of inhibition diameter according to the interpretive standard of the Clinical and Laboratory Standards Institute (CLSI, 2012). *Staphylococcus aureus* ATCC 29523 was used as a control strain. Only oxytetracycline-resistant isolates were chosen for further study.

**Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC).** The MIC of 17 oxytetracycline-resistant GBS isolates was determined using the standard micro-dilution procedure modified from the Clinical and Laboratory Standards Institute (CLSI, 2012). MIC was performed in Mueller–Hinton broth medium (MHB; Merck), containing antibiotic at final concentrations of 0.125 to 128 μg ml⁻¹. Antibiotics (Sigma) used in this study were trimethoprim, ampicillin G, oxolinic acid, gentamicin, lincomycin, norfloxacin, sulfamethoxazole, erythromycin, oxytetracycline and chloramphenicol; antibiotic-free broth was also used. To prepare standardized inocula, GBS grown in tryptic soy broth (Difco) for 16 h at 30 °C were harvested and resuspended in MHB at a final concentration of approximately 10⁸ c.f.u. ml⁻¹. The GBS suspension was inoculated into each well of 96-well plates to achieve the final volume of MHB containing GBS and standard antibiotic of 200 μl per well. After incubation at 30 °C, the wells having no visually detected bacterial growth and showing no change in absorbance at 600 nm before and after 24 h of application of the test were determined to contain the MIC of the compound.

MBC of 17 oxytetracycline-resistant GBS isolates was determined using the streaking method modified from the Clinical and Laboratory Standards Institute (CLSI, 2012). At the end of 24 h of incubation, the 96-well plates were read for the MIC and then the MBC was determined by sampling all macroscopically clear wells and the first turbid well in the series. The suspension was inoculated onto TSA plates, which were incubated for 24 h at 30 °C.

MIC and MBC experiments were carried out in triplicate and were correlated against the *Staphylococcus aureus* ATCC 25923 control strain.

**Tetracycline resistance (tetR) genes.** Seventeen oxytetracycline-resistant GBS isolates were examined for the presence of *tet* gene by PCR assay. Crude nucleic acid preparations were made following the method described by Berridge et al. (1998). Validated primer sets, obtained from Integrated DNA Technologies and capable of detecting ten *tet* genes including *tet(A), tet(B), tet(D), tet(E), tet(G), tet(M), tet(O), tet(Q), tet(S) and tet(W) as described by Seyfried et al. (2010), were used in this study. All DNA amplification was conducted in 25 μl reaction volumes containing 12.5 μl of 2× PCR mastermix blue (RBC Bioscience), 1.65 μl of a 10 μM stock of each forward and reverse primer, 5 μl of DNA template and 4.2 μl of distilled water. Amplification of target DNA was performed in a thermal cycler (Bio-Rad). Thermal cycling conditions were as follows: initial denaturation at 95 °C for 7 min; 35 cycles of 95 °C for 45 s, annealing from 51–59.2 °C (depending on the primer melting temperatures) for 1 min, and 72 °C for 90 s; and a final extension of 72 °C for 7 min. The PCR analysis was conducted in triplicate and GBS DSM17129 was used as a positive control. The DNA molecular mass marker used was Gendirex OneMark100.

The amplified product obtained from the PCR assay was purified and sequenced on an Applied Biosystems Genetic Analyzer. The Basic Local Alignment Search Tool (BLAST; http://blast.ncbi.nlm.nih.gov/ Blast.cgi) was used to confirm the presence of *tet* genes in the GBS isolate.

**RESULTS AND DISCUSSION**

**Bacterial strains.** Streptococcosis of cultured fish caused by GBS infection results in severe economic losses to fish farmers. In the
present study, 144 bacterial isolates from infected tilapia were identified as GBS based on biochemical and serological characteristics, as well as mPCR analysis. Of these, 126 isolates (87.5 %) from Surat Thani, Nakhon Si Thammarat, Phetchaburi, Ayutthaya, Kanachanaburi, Suphanburi, Nakhon Pathom and Pattani provinces were identified as GBS serotype Ia, and 18 isolates (12.5 %) from Songkhla, Samut Prakan, Phetchaburi and Phatthalung provinces were identified as GBS serotype III (Fig. 1). Based on the composition of the capsular polysaccharide, GBS can be subdivided into 10 different serotypes (Ia, Ib and II to IX) (Slotved et al., 2007). To date, only serotypes Ia, Ib and III have been previously reported in fish (Vandamme et al., 1997; Evans et al., 2008; Suanyuk et al., 2008; Suwannasang et al., 2014). GBS is an emerging zoonotic agent isolated from piscine, dolphin, bovine, crocodile, camel and human sources (Ip et al., 2006; Bishop et al., 2007; Evans et al., 2008; Fischer et al., 2013). Multilocus sequence typing (MLST) of some GBS isolates from fish in Asia, including Thailand, indicated that these belong to sequence type (ST)-7/serotype Ia and ST-283/serosubtype III-4, which are associated with invasive disease of humans in Asia (Evans et al., 2008; Delannoy et al., 2013; Kayansamruaj et al., 2014). Although all GBS isolates from the present study were serotypable using GBS typing antisera, further genotyping by methods such as MLST of these GBS isolates may be useful in understanding potential relationships or differences among human GBS serotype Ia and III isolates (Kong et al., 2002, 2005; Zeng et al., 2006; Zhao et al., 2006; Evans et al., 2008; Delannoy et al., 2013).

Antibacterial susceptibilities

Antibiotic susceptibilities of the 144 GBS isolates are given in Table 1. Most GBS isolates were susceptible to lincomycin, norfloxacin, oxytetracycline, ampicillin, erythromycin, and chloramphenicol but were resistant to oxolinic acid,
gentamicin, sulfamethoxazole and trimethoprim. Resistant phenotypes of the GBS isolates are presented in Fig. 2. Most GBS isolates were shown to have a resistant phenotype of oxolinic acid, gentamicin, sulfamethoxazole and trimethoprim (76.38 %) or a resistant phenotype of oxolinic acid, gentamicin, sulfamethoxazole, trimethoprim and oxytetracycline (11.11 %). Similar resistant phenotypes of GBS were reported in silver pomfret (Pampus argenteus) cultured in Kuwait (Duremdex et al., 2004). Generally, resistance to oxolinic acid, a member of the quinolone family, is primarily mediated by chromosomal mutations in topoisomerase genes and mutations that decrease drug accumulation by reducing uptake or increasing efflux (Miranda et al., 2013), while resistance to gentamicin by GBS is mediated by the production of aminoglycoside-modifying enzymes 6'-acetyltransferase and 2'-phosphotransferase (Buu-Hoi et al., 1990). Sulfamethoxazole and trimethoprim are used in combination as the drug co-trimoxazole (Schmitz

### Table 1. Antibiotic susceptibilities of GBS isolates from infected tilapia

<table>
<thead>
<tr>
<th>Number of isolates</th>
<th>OXO</th>
<th>GEN</th>
<th>SMZ</th>
<th>TMP</th>
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<th>NOR</th>
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<td>18</td>
<td>18</td>
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</table>

Fig. 2. Resistance phenotypes of 144 GBS isolates from infected tilapia cultured in Thailand. Abbreviations: OXO, oxolinic acid; GEN, gentamicin; SMZ, sulfamethoxazole; TMP, trimethoprim; LCM, lincomycin; NOR, norfloxacin; OTC, oxytetracycline.
et al., 2001). Resistance to these antibiotics is mediated by the permeability barrier and/or efflux pumps, naturally insensitive target enzymes, regulational, mutational or recombinational changes in the target enzymes genes and acquired drug-resistance genes (Huuvinen, 2001). Among the 144 GBS isolates from the present study, one GBS serotype III and three GBS serotype Ia isolates exhibited lincomycin and norfloxacin resistance, which is uncommon among GBS isolates from fish. Lincomycin, a member of the lincosamides, prevents protein synthesis by inhibiting peptidyltransferase through binding to several nucleotides of the 23S rRNA gene in the 50S subunit of the bacterial ribosome (Petinaki et al., 2008). Two major mechanisms responsible for lincosamide resistance are alteration of the antibiotic target site and enzymic modification (Petinaki et al., 2008). Norfloxacin is a widely used representative member of the fluoroquinolone family, having a fluorine atom at position 6 and a piperazine ring at position 7 (Sharma et al., 2008). The mechanism of resistance to fluoroquinolones is the same as that to the quinolone family. Further investigation of genes responsible for lincomycin and norfloxacin resistance is required for understanding the mechanisms that render GBS resistant to these antibiotics.

In this study, resistance to oxytetracycline was found in 17 GBS isolates (11.8%). Of these 17 GBS isolates, 15 GBS isolates from Songkhla, Samut Prakan, Phetchaburi and Phatthalung provinces belong to serotype III, and two GBS from Surat Thani and Nakhon Si Thammarat provinces belong to serotype Ia (Table 1). Tetracycline-resistant GBS has been reported in several organisms, including those of piscine (Geng et al., 2012), bovine (Dogan et al., 2005; Jain et al., 2012), camel (Fischer et al., 2013) and human (Boswihi et al., 2012; Dogan et al., 2005) origin. Oxytetracycline, a member of the tetracycline group produced by Streptomyces rimosus, is permitted to be used in Thai aquaculture (Baoprasertkul et al., 2012). This antibiotic is a broad-spectrum agent displaying activities against both Gram-positive and Gram-negative bacteria by inhibiting bacterial protein synthesis preventing the association of aminocyl-tRNA with the bacterial ribosome (Chopra & Roberts, 2001).

### MIC and MBC

The susceptibilities of the 17 oxytetracycline-resistant GBS isolates to 10 antibiotics, expressed as MIC and MBC, are shown in Table 2. These bacteria were susceptible to ampicillin, lincomycin, norfloxacin, erythromycin and chloramphenicol. Ampicillin and chloramphenicol were the most active antibiotics tested, with MIC and MBC of $\leq 0.125 \mu g \text{ml}^{-1}$. The MIC and MBC for lincomycin, norfloxacin and erythromycin ranged from $\leq 0.125$ to 0.5 $\mu g \text{ml}^{-1}$ and 0.5 to 2 $\mu g \text{ml}^{-1}$, respectively. All strains were resistant to trimethoprim, oxolinic acid, gentamicin, sulfamethoxazole and oxytetracycline with the MIC and MBC values ranging from 16 to $\geq 128$ and $\geq 128 \mu g \text{ml}^{-1}$, respectively (Table 2). The results of the broth dilution method for the 17 oxytetracycline-resistant GBS isolates were comparable to those of the disc diffusion method for all antibiotics used in this study, except norfloxacin and lincomycin, with three GBS isolates exhibiting major errors (MICs indicated susceptibility; resistance by disc diffusion method). Possibly, the blood supplement in the culture medium is vulnerable to growth of the GBS, or the blood components may interfere with the antibiotic. However, the correlations between the disc diffusion method and broth dilution method for 10 antibiotics against 17 oxytetracycline-resistant isolates were 97.65% in agreement between methods and 2.35% showing major error. Major errors between methods using fluoroquinolones such as ciprofloxacin and ofloxacin against Enterobacteriaceae (Steward et al., 1999), and clindamycin, the second-generation lincomycin against staphylococci and micrococci (Doern et al., 1981), have been reported previously. In the present study, the finding of increasing incidence of GBS isolates resistant to tetracycline with high MIC and MBC values emphasizes the need to

<table>
<thead>
<tr>
<th>Antibiotic agent</th>
<th>Number of isolates with MIC ($\mu g \text{ml}^{-1}$):</th>
<th>MBC ($\mu g \text{ml}^{-1}$)</th>
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<tr>
<td></td>
<td>$\leq 0.125$</td>
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<tr>
<td>Trimethoprim</td>
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<td>Oxytetracycline</td>
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<tr>
<td>Chloramphenicol</td>
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</table>

Table 2. MIC and MBC values of the 17 oxytetracycline-resistant GBS isolates to various antibiotic agents.
implement preventive measures. Moreover, study on MIC and MBC for the newer tetracyclines such as doxycycline is interesting and deserves further study.

TetR genes

The 17 oxytetracycline-resistant GBS isolates from infected tilapia and the GBS DMST 17129 control strain were determined as positive for the presence of the tet(M) gene. Examples of PCR products generated by a primer set specific to the tet(M) gene of different serotypes of GBS are given in Fig. S1 (available in the online Supplementary Material). No other tetracycline resistance genes were found in oxytetracycline-resistant GBS isolates. Sequencing analysis based on the tet(M) gene indicated 100% similarity to the tet(M) gene sequences of several GBS isolates, including those with GenBank accession numbers CP007572.1, CP007570.1, CP007631.2, HG939456.1, HF952106.1, HF952104.1 and HP930766.1. Details of the sequencing results of the PCR product are given in Fig. S2. Tetracycline resistance is often due to the acquisition of genes encoding proteins involved in the energy-dependent efflux of tetracyclines, protection of the bacterial ribosome from the action of tetracyclines, and enzymic inactivation of tetracycline (Chopra & Roberts, 2001). The current study identified the presence of the tet(M) gene, which encodes ribosomal protection proteins, in the oxytetracycline-resistant GBS. Similar genetic organization has been reported in Streptococcus parauberis isolated from olive flounder (Paralichthys olivaceus) (Park et al., 2009) and Aeromonas sobria collected from Iranian fish farms (Hedayatianfard et al., 2014), where only tet(M) was detected. In this study, we also attempted to detect the presence of 10 tetR genes in some oxytetracycline-sensitive GBS isolates, but none of the other tetR genes could be detected, indicating that tet(M) is the only mechanism for the tetracycline-resistant phenotype of GBS isolates from fish.

The present study indicated that all 17 oxytetracyclineresistant GBS isolates from infected tilapia were resistant to trimethoprim, oxolinic acid, gentamicin and sulfamethoxazole. Multiple drug resistance patterns of GBS have been reported in several organisms, including fish (Duremdez et al., 2004) and humans (Berkowitz et al., 1990; Nagano et al., 2012; Banno et al., 2014). Further characterization of fluoroquinolone and clindamycin resistance to other drugs by molecular methods is needed to confirm that these oxytetracycline-resistant GBS isolates are multi-drug-resistant.

In conclusion, our results demonstrate the serotype distribution and antimicrobial susceptibilities of GBS isolates from cultured tilapia. The finding of oxytetracycline-resistant GBS in the present study indicates the importance of reducing the usage of antibiotics in tilapia aquaculture. In addition, the detection of the tet(M) gene in GBS isolates provides insight into oxytetracycline-resistant GBS isolates from fish and may be useful information in regard to prescribing antibiotic usage in fish aquaculture.

ACKNOWLEDGEMENTS

This work was supported by the Higher Education Research Promotion and National Research University Project of Thailand, Office of the Higher Education Commission.

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