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Phenotypic and genotypic characterization of *Streptococcus agalactiae* isolated from hybrid tilapia (*Oreochromis niloticus* × *O. aureus*)

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ABSTRACT

Outbreaks of streptococcal infection in cultured hybrid tilapia in Saudi Arabia occurred annually since 1992, causing significant economic losses in the aquaculture industry. *Streptococcus agalactiae* (Group B *Streptococcus*, GBS), the etiological agent of streptococcosis in fish, is an important bacterial fish pathogen causing significant morbidity and mortality of cultured and wild fish worldwide. Outbreaks of *S. agalactiae* in cultured tilapia occurred each year from 2000 through 2015, and mortality rate ranged from 40–80% during the summer months, when water temperatures above 28 °C, in combination with high fish stocking density and poor water quality. The most frequent clinical signs of affected fish are anorexia, swimming abnormalities, curved body shape, melanosis, exophthalmia, corneal opacity and petechial hemorrhage in the skin. The phenotypic, biochemical, genotypic and antibiotic susceptibility among *S. agalactiae* isolates recovered consistently from brain, kidney, liver and spleen of diseased hybrid tilapia (*Oreochromis niloticus* × *Oreochromis aureus*) was characterized. Isolates were Gram-positive cocci, catalase-negative, oxidase-negative, non-haemolytic and was serotyped to group antigen. Analysis of all the sixteen isolated strains by the Vitek 2 Compact system, characterized the bacteria as 99% similarity to *S. agalactiae*. Further, phylogenetic analysis of the 16S rDNA gene sequence revealed that the tilapia isolates were showed highly homologous (ranged from 98.2% to 100%) with *S. agalactiae* strain CIP 82.43 type (Genbank accession number NR 117503). All of the 16 bacterial isolates have exhibited similar phenotypic, biochemical, genotypic and antibiotic sensitivity profile. In addition, the findings in this study also indicate that *S. agalactiae* is one of the causative agents of disease outbreaks during the period from 2000 to 2015 in hybrid tilapia, which may change the previous understanding. Therefore, the results of the present study indicated and confirmed that both *S. iniae* and *S. agalactiae* are become the major bacterial pathogenic species responsible for infection tilapia in Saudi Arabia.

Statement of relevance: Streptococcus agalactiae (GBS) have been associated with disease outbreaks among a variety of wild and cultured freshwater and marine fish species worldwide including tilapia. This is the first report describing the phenotypic and molecular characterization of a non-haemolytic group B *Streptococcus agalactiae* (AH2) isolated from diseased hybrid tilapia (*Oreochromis niloticus* × *Oreochromis aureus*) in Saudi Arabia.

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1. Introduction

Streptococcosis is a septicaemic disease affecting wide range of both wild and captive populations of freshwater, brackish-water, and marine fish species throughout the world (Austin and Austin, 2013). The disease has become one of the most serious aquatic pathogens in the last decade causing significant economic losses in the global aquaculture industry. Among the streptococci (the group B streptococcal fish pathogen) *Streptococcus agalactiae* (GBS) have been associated with disease outbreaks among a variety of wild and cultured freshwater, estuarine and marine fish species throughout the world (Abuseliana et al., 2010; Amal et al., 2012; Barato et al., 2015; Bowater et al., 2012; Duremdez et al., 2004; Evans et al., 2002; Geng et al., 2011; Hernandez et al., 2009; Liu et al., 2014; Lusiastuti et al., 2014; Mian et al., 2009; Suanyuk et al., 2008; Ye et al., 2011). Furthermore, GBS organisms have been reported from aquatic mammals, both captive and wild bottlenose dolphins, *Tursiops truncatus* (Zappulli et al., 2005; Evans et al., 2006), cultured bullfrogs, *Rana catesbeiana* (Amborski et al., 1983) and saltwater crocodiles, *Crocodylus porosus* (Bishop et al., 2007). This bacterium is not only causing infectious diseases to aquatic animals, but also associated with diseases in humans (Chaiwarith et al., 2011; Lambertsen et al., 2010; Skoff et al., 2009) and terrestrial mammals such as cattle, dogs and cats (Brochet et al., 2006; Sørensen et al., 2010).

Outbreaks of *S. agalactiae* infection in tilapia have been spread to certain regions of the world, including Brazil (Mian et al., 2009; Salvador et al., 2005), China (Chen et al., 2012; Li et al., 2014; Wang et al., 2013; Ye et al., 2011), Colombia (Barato et al., 2015; Hernandez et al., 2009), Indonesia (Lusiastuti et al., 2014), Malaysia (Abuseliana et al., 2010; Musa et al., 2009) and Thailand (Jantrakajorn et al., 2014; Suanyuk et al., 2008; Kayansamruaj et al., 2014).

A non-haemolytic group B *Streptococcus agalactiae* is an emerging pathogen that causes significant disease and mortality in a variety of

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freshwater and marine fish species including golden shiners, *Notemigonus crysoleucas* (Robinson and Meyer, 1966), wild gulf menhaden, *Brevoortia patronus* (Goode), hardhead sea catfish, *Arius felis* L., striped mullet, *Mugil cephalus* L., pinfish, *Lagodon rhomboides* L., Atlantic croaker, *Micropogonias undulatus* L., spot, *Leiostomus xanthurus* (Lacepede), stingray, *Dasyatis* sp., and silver weakfish, *Cynoscion nothus* (Holbrook) (Plumb et al., 1974), *bullminnows*, *Fundulus grandis* (Baird and Girard) (Rasheed and Plumb, 1984), striped mullet, *Mugil cephalus* L., bluefish *Pomatomus saltatrix*, and striped bass *Morone saxatilis* (Baya et al., 1990), Nile tilapia *Oreochromis niloticus* (Li et al., 2014; Lusiastuti et al., 2014; Mian et al., 2009; Salvador et al., 2005; Wang et al., 2013), *Oreochromis* sp. (Barato et al., 2015), cultured ya-fish *Schizothorax prenanti* (Geng et al., 2011), wild giant Queensland grouper, *Epinephelus lanceolatus* (Bloch), and other wild marine fishes in northern Queensland (Bowater et al., 2012), farmed *Litopenaeus vannamei* (Hasson et al., 2009), and barcoo grunter *Scortum barcoo* (Liu et al., 2014). Furthermore, non-haemolytic *S. agalactiae* has also been reported from dead wild bottlenose dolphins, *Tursiops truncatus* (Evans et al., 2006).

Tilapia (*Oreochromis* spp.) is one of the most important fresh water species cultured in Saudi Arabia (Siddiqui and Al-Harbi, 1995), has frequently been suffered from outbreaks of streptococcicosis every year since 1992, during the summer months, when water temperatures above 28 °C, that leads to significant economic losses to the aquaculture industry (Al-Harbi, 1994, 2011). Although, the first confirmed streptococcal infection in cultured hybrid tilapia (*Oreochromis niloticus* × *O. aurea*) in Saudi Arabia was reported in 1994 (Al-Harbi, 1994, 2011), this is the first report on the isolation and identification of a non-haemolytic group B *Streptococcus agalactiae* from cultured hybrid tilapia. The disease leads to significant morbidity and mortality with acute or chronic infections that are frequently associated with high fish stocking density and poor water quality, showing typical clinical signs of streptococcal infection.

From 1992 to the present, the Aquatic Animal Health Research Laboratory (AAHRL), King Abdulaziz City for Science and Technology, Riyadh, Saudi Arabia diagnosed 36 cases of streptococcal infection in cultured hybrid tilapia (*O. niloticus* × *O. aurea*). The objective of the present study was to investigate the phenotypic, biochemical, genotypic and antibiotic susceptibility characterization among a non-haemolytic group B *S. agalactiae* isolates recovered from natural diseased hybrid tilapia.

2. Materials and methods

2.1. Sample collection

During the outbreak of streptococcosis in cultured hybrid tilapia (*O. niloticus* × *O. aureus*) over the period 1992–2015, moribund fish were collected from the major tilapia cultivation area in central region of Saudi Arabia. Diseased tilapia showing clinical signs including, anorexia, swimming abnormalities, melanosis, exophthalmia, corneal opacity and petechial hemorrhage in the skin were submitted to the Aquatic Animal Health Research Laboratory (AAHRL), King Abdulaziz City for Science and Technology, Riyadh, Saudi Arabia for routine disease surveillance and diagnostic purposes. Bacterial cultures were isolated by streaking samples of brain, kidney, liver and spleen of moribund hybrid tilapia on tryptone soya agar (TSA; Oxoid) at 30 °C for 48 h. Stock suspensions of all isolates were maintained frozen at −80 °C in tryptone soya broth (TSB; Oxoid), supplemented with 10% glycerol until used. For each farm, data was collected on the type of culture system used, water quality parameters, mortality rate record, weight of tilapia infected, strains code, date of isolation, organ and geographical organ were recorded when possible.

2.2. Isolation and maintenance of bacterial strains

A total of 36 archived isolates of *Streptococcus* species strains were used in this study. Bacterial isolates were initially identified as *Streptococcus* species on the basis of standard phenotypic testing criteria (Gram-stain, motility and oxidase and catalase activity). It was initially thought that these fish were infected by *S. iniae*. The bacterial strains were routinely aerobically grown on tryptone soya agar (TSA; Oxoid) at 30 °C for 48 h. Stock suspensions of all isolates were maintained frozen at −80 °C in tryptone soya broth (TSB; Oxoid), supplemented with 10% glycerol until used. An *S. iniae* (AH1) isolated previously from outbreak of streptococcosis in cultured hybrid tilapia (*O. niloticus* × *O. aureus*) (Al-Harbi, 1994) and an *S. agalactiae* isolated previously from moribund silver pomfret (*Pampus argenteus*) (Duremdez et al., 2004) were also included.

2.3. Phenotypic characterization

Bacterial isolates were morphologically, physiologically and biochemically characterized by using conventional plate and tube tests. Tests included colonial morphology, Gram-stain, motility, haemolysis on 5% sheep blood agar, cytochrome oxidase test, catalase test, esculin hydrolysis on bile esculin slants and H_2S production in TSI slants and growth capacity on MacConkey media. The bacterial isolates were further characterized phenotypically by an automated bacterial identification system (Vitek 2 Compact, bioMérieux, Inc. Hazelwood, MO, USA) using the gram-positive (GP) identification card following the manufacturer's instructions. Results were compared with the analytical profile index of the system. All isolates were serogrouped with Slidex Strepto kit for grouping of β-haemolytic streptococci groups A, B, C, D, F and G according to the manufacturer's instructions (BioMerieux, France). In addition, the ability the bacterial strains to grow at different temperature (10, 15, 20, 25, 30, 35, 37, 40 and 42 °C), and sodium concentrations (5and 6.5%) over a period of 10 days on tryptone soya broth (TSB; Oxoid) were determined. The turbidity of the inoculated media was evaluated by the naked eye.

2.4. Antimicrobial susceptibility

The antimicrobial susceptibility testing was performed by Vitek 2 automated system using AST-ST01 card (BioMerieux) according to the manufacturer's instructions with the following antimicrobials: ampicillin (0.25–16 μg/mL^{-1}), benzylpenicillin (0.06–8 μg/mL^{-1}), cefotaxime (0.12–8 μg/mL^{-1}), ceftriaxone (0.12–8 μg/mL^{-1}), clindamycin (0.25–1 μg/mL^{-1}), erythromycin (0.12–8 μg/mL^{-1}), levofloxacin (0.25–16 μg/mL^{-1}), linezolid (2–8 μg/mL^{-1}), tetracycline (0.25–16 μg/mL^{-1}), trimethoprim/sulfamethoxazol 10 (0.5/9.5 μg/mL^{-1}) to 320 (16/304 μg/mL^{-1}), and vancomycin (0.12–8 μg/mL^{-1}) including testing for inducible clindamycin resistance. Briefly, the bacterial strains were harvested after incubation for 24 h on tryptone soya agar (TSA; Oxoid) at 30 °C and suspended in 3 ml of 0.45% sterilized saline and adjusted to a turbidity of 0.5 McFarland standards with Vitek Densichek (BioMerieux). The bacterial suspension was used to fill the antimicrobial susceptibility testing AST-ST01 card, which was then inserted into the incubator-reader of the VITEK 2 system. The minimum inhibitory concentration (MIC) for each antimicrobial was also evaluated by the Vitek 2 system.

2.5. Molecular characterization

2.5.1. DNA extraction

Genomic DNA was extracted from each of the 36 isolates strains described above. Briefly, individual strains were streaked onto tryptone soya agar (TSA; Oxoid) and grown aerobically at 30 °C for 48 h to assess the purity. A single colony was used to inoculate 5 ml of tryptone soya broth (TSB; Oxoid), and cultures were incubated for 24 h at 30 °C, and the cells were harvested by centrifugation at 5000 ×*g* for 10 min at 4 °C. The supernatant was discarded carefully, and the bacterial cells were washed three times with Tris-EDTA (TE) buffer. The bacterial cells were then lysed and DNA was extracted using a QIAamp DNA Mini Kit (Qiagen, Valencia, CA, USA), following the manufacturer's instructions. The concentration and quality of the purified DNA samples were measured by spectrophotometer (GeneQuant, Ge Healthcare).

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