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Molecular characterization of *Streptococcus agalactiae* in diseased farmed tilapia in China

Defeng Zhang [a,b,d], Aihua Li [a,*], Yujuan Guo [c], Qianqian Zhang [a,b], Xuenian Chen [c], Xiaoning Gong [a]

[a] State Key Laboratory of Freshwater Ecology and Biotechnology, Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan 430072, PR China
[b] University of Chinese Academy of Sciences, Beijing 100049, PR China
[c] College of life sciences, Zhaoqing university, Zhaoqing 526061, China
[d] Pearl River Fisheries Research Institute, Chinese Academy of Fishery Sciences, Guangzhou 510380, PR China

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ABSTRACT

A severe outbreak of streptococcosis in cultured tilapia (*Oreochromis niloticus*) caused by *Streptococcus agalactiae* (Group B streptococcus, GBS) has occurred annually in Southern China during the past years, causing significant economic losses. Little is known about the genetic characteristics of the tilapia *S. agalactiae* that is prevalent in these areas. A total of 51 GBS isolates from tilapia were collected from 27 farms located in distinct geographic regions. The genetic characteristics of GBS were analyzed by MLST, MLVA, serotyping, and PCR screening of mobile genetic elements, genetic marker, and virulence-related genes. The results showed that all of *S. agalactiae* tilapia isolates have the genotype, Ia-ST7-*bac*-*bca*-*fbsA*-*sip*-*cfb*-IS*1381*-IS*861*-GBSil-IS*Sag2*, which suggests a low level of genetic diversity and sharing of a recent and similar origin and a low level of genetic differentiation under similar environment selective pressures. Compared with the control, significant differences were detected between the tilapia strains and the *S. agalactiae* strains of human and bovine origin. Phage typing successfully differentiated all 51 tilapia GBS isolates into two distinct molecular types (type A and B). Interestingly, the phage type of the tilapia GBS isolates was also observed to shift from type A to type B, with the year 2010 as the turning point. Type B GBS isolates are currently prevalent in tilapia populations in China, and should thus be the target strains to detect and develop prophylactic–therapeutic measures. The genetic data obtained in the present study will be helpful in the determination of the epidemiology of tilapia streptococcosis internationally.

1. Introduction

Streptococcus agalactiae (also known as Group B streptococcus or GBS) is an important bacterial pathogen for humans and mammals, and for a number of cultured fish species, such as seabream, rainbow trout, channel catfish, hybrid striped bass, and tilapia (Abuseliana et al., 2011; Ye et al., 2011). China, the largest tilapia producer in the world (Amal and Zamri-Saad, 2011), has suffered from a continuously severe and extensive outbreak of streptococcosis in cultured tilapia for years, particularly in the south provinces including Guangdong, Guangxi, Hainan, and Fujian, for years, causing very serious economic losses yearly since 2009. The cumulative mortality were 30–80% (Chen et al., 2012a, 2012b), and some ponds suffered from total crop failure because of this disease. Furthermore no chemotherapeutic or immunological measures have been developed to prevent or control this disease effectively so far. The causative agent has been identified in many studies as *S. agalactiae* (Chen et al., 2012b; Suanyuk et al., 2008; Ye et al., 2011). However, despite the significant impact of *S. agalactiae* as an infectious agent, few reports on the genetic characterization of clinical tilapia GBS isolates circulating in China exist, and few epidemiological studies on the effects of this bacterium on infected fish have been published. The genetic diversity study is of interest not only to understand the epidemiology of this bacterial pathogen in the tilapia population, but also to enable the development of appropriate and effective vaccine(s) against tilapia streptococcosis caused by *S. agalactiae*.

The characterization of *S. agalactiae* strains from human and mammal sources has been studied using a broad range of methods, such as molecular serotyping, random amplified polymorphic DNA, amplified fragment length polymorphism (AFLP), enterobacterial repetitive intergenic consensus, pulsed field gel electrophoresis, multilocus sequence typing (MLST), and multiple-locus variant-repeat assay (MLVA) (Chen et al., 2012b; Delannoy et al., 2013; Evans et al., 2008; Hernández et al., 2009; Imperi et al., 2010; Olivares-Fuster et al., 2008; Pereira et al., 2010; Suanyuk et al., 2008; Ye et al., 2011). Recently, phage typing was used in epidemiological investigations because lysogeny was specific to each intraspecific lineage of GBS strain (Salloum et al., 2011). In addition, numerous mobile genetic elements (MGEs), including six insertion sequences (IS) and a group

* Corresponding author. Tel./fax: +86 27 68780053.
E-mail address: liaihua@ihb.ac.cn (A. Li).

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II intron, were employed to detect the diversity of GBS isolates. *S. agalactiae* strains isolated from animals possess properties distinct from human isolates (Franken et al., 2001; Lindahl et al., 2005), and previous studies failed to discriminate the GBS strains of fish origin. Thus, more detailed studies on tilapia isolates, at least from the epidemiological standpoint, is necessary.

In the present study, we genetically characterized 51 *S. agalactiae* isolates collected from several epizootics of streptococcosis in different tilapia farms in China over a six-year period. The aim of the study was to gain insights into the molecular genetic characterization, epidemiology, and possible temporal and spatial genotype variations of the infectious tilapia streptococci prevalent in the epidemic area of China, as well as to provide comparable genetic data for different laboratories. For this purpose, the tilapia GBS isolates were analyzed for genetic markers or virulence-related genes (*scpB*, C5a peptidase gene; *lmb*, laminin-binding surface protein gene; *bca*, C-α protein gene; *bac*, C-β protein gene; *cfb*, CAMP factor, *fbsA*, fibrinogen-binding protein gene, and *sip*, surface immunogenic protein gene) (Lin et al., 2011; Liu et al., 2012; Wang et al., 2012) by MLST, MLVA, molecular serotyping, phage typing, and PCR screening.

2. Material and method

2.1. Bacterial strains

Fifty-one strains of *S. agalactiae* were used in this study (Table 1). The GBS isolates were recovered from the liver, spleen, or brain of diseased tilapia (*Oreochromis niloticus*). The tilapias were taken from 27 farms in 18 distinct geographical regions in the southern provinces of China from 2007 to 2012. The body weight of diseased fish ranged from 8 g (farm G1) to approximately 600 g (farm Q). The water temperature during disease outbreaks was all higher than 25 °C. Diseased fish showed typical clinical signs, such as exophthalmia, corneal opacity, swimming abnormalities, and hemorrhagic ulcers inside the operculum and at the base of the pectoral. For isolation of bacteria, using aseptic techniques, samples taken from blood, kidney, liver and brain of moribund tilapia were streaked onto blood agar and Brain heart infusion (BHI) agar. Plates were incubated at 28 °C for 24–48 h, single colony was sub-cultured on BHI plate at 28 °C for 24 h to obtain pure isolate. A total of 51 isolates were recovered from the diseased tilapia collected from 27 ponds of 18 farms (outbreaks), and then stored at −80 °C for later analysis. All of the isolates were identified by morphological observation, biochemical and molecular identification. A series of biochemical tests were done and the characteristics of these isolates coincided with those of *S. agalactiae* described by Ye et al. (2011). Molecular identification was achieved by sequence analysis of 16S rRNA gene of each strain. BLAST analysis revealed that the 16S rRNA gene sequences of these isolates were highly homologous to that of *Streptococcus agalactiae* strain (99.9%–100%). No any other bacteria were identified from these samples.

Two bovine GBS strains, C1271 and C918, were used as control for identification and for genetic analysis.

2.2. MLST and MLVA

Genomic DNA was extracted from the isolates using a TIANamp Bacteria DNA Kit (TIANGEN BIOTECH CO., China). MLST was performed by sequencing seven housekeeping genes (*adhP*, *pheS*, *atr*, *glnA*, *sdhA*, *glcK*, and *tkt*) according to Jones et al. (2003). The PCR amplification conditions were as follows: initial denaturation at 94 °C for 5 min, 30 cycles of denaturation at 94 °C for 30 s, annealing at 53 °C for 30 s, and extension at 72 °C for 1 min; and a final extension at 72 °C for 10 min. The PCR products were cloned and

Table 1
Information of *S. agalactiae* isolates from tilapia and dairy cow in this study.

Farm ID (Outbreak)	Host※	Age (months)	Fish pond ID	No. of isolate*	Collection time	Geographical location
1	Tilapia	3	A	1	2007-07	Fujian province
1	Tilapia	3	B	1	2007-07	Fujian province
2	Tilapia	4	C	1	2008-08	Qionghai city, Hainan province
3	Tilapia	4	D	1	2009-08	Zhaoqing city, Guangdong province
3	Tilapia	5	E1	3	2010-08	Zhaoqing city, Guangdong province
3	Tilapia	5	E2	1	2010-08	Zhaoqing city, Guangdong province
4	Tilapia	5	F	2	2010-08	Xinqiao town, Zhaoqing city, Guangdong province
5	Tilapia	1	G1	2	2010-08	Baizhu town, Zhaoqing city, Guangdong province
5	Tilapia	5	G2	2	2010-08	Baizhu town, Zhaoqing city, Guangdong province
5	Tilapia	5	G3	2	2010-08	Baizhu town, Zhaoqing city, Guangdong province
5	Tilapia	5	G4	2	2010-08	Baizhu town, Zhaoqing city, Guangdong province
6	Tilapia	5	H	2	2010-08	Liantang town, Zhaoqing city, Guangdong province
7	Tilapia	4	I	1	2010-07	Wenchang city, Hainan province
7	Tilapia	4	J	1	2010-07	Wenchang city, Hainan province
8	Tilapia	4	K	1	2011-07	Maoming city, Guangdong province
9	Tilapia	4	L	1	2011-07	Yinhai area, Beihai city, Guangxi province
10	Tilapia	5	M	1	2011-08	Hepu area, Beihai city, Guangxi province
11	Tilapia	5	N	1	2011-08	Xingning area, Nanning city, Guangxi province
12	Tilapia	5	O	1	2011-08	Liuzhou city, Guangxi province
13	Tilapia	5	P	1	2011-08	Bobai area, Yulin city, Guangxi province
14	Tilapia	6	Q	1	2011-09	Qinzhou city, Guangxi province
15	Tilapia	5	R1	8	2012-08	Gaoyao area, Zhaoqing city, Guangdong province
15	Tilapia	5	R2	10	2012-08	Gaoyao area, Zhaoqing city, Guangdong province
16	Tilapia	3	S1	1	2012-07	Zhuhai city, Guangdong province
16	Tilapia	3	S2	1	2012-07	Zhuhai city, Guangdong province
17	Tilapia	2	T	1	2012-06	Qionghai city, Hainan province
18	Tilapia	2	U	1	2012-06	Wanning city, Hainan province
	Dairy cow			1	2012-07	Inner Mongolia
	Dairy cow			1	2009-09	Inner Mongolia

※ Only one tilapia (from farm C) was hybrid tilapia (*Oreochromis aureus* × *Oreochromis niloticus*), the others were Nile tilapia (*Oreochromis niloticus*).
* Different isolates were recovered from different fish which were taken from the same or different outbreaks.