Journal of Advanced Research (2015) 6, 233–238

Cairo University

Journal of Advanced Research

SHORT COMMUNICATION

# Genetic diversity of geographically distinct *Streptococcus dysgalactiae* isolates from fish

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ARTICLE INFO

*Article history:*
Received 14 September 2013
Received in revised form 13 November 2013
Accepted 5 December 2013
Available online 12 December 2013

*Keywords:*
*Streptococcus dysgalactiae*
Epidemiology
*tuf* gene sequencing
BSFGE

ABSTRACT

*Streptococcus dysgalactiae* is an emerging pathogen of fish. Clinically, infection is characterized by the development of necrotic lesions at the caudal peduncle of infected fishes. The pathogen has been recently isolated from different fish species in many countries. Twenty *S. dysgalactiae* isolates collected from Japan, Taiwan, Malaysia and Indonesia were molecularly characterized by biased sinusoidal field gel electrophoresis (BSFGE) using SmaI enzyme, and *tuf* gene sequencing analysis. DNA sequencing of ten *S. dysgalactiae* revealed no genetic variation in the *tuf* amplicons, except for three strains. The restriction patterns of chromosomal DNA measured by BSFGE were differentiated into six distinct types and one subtype among collected strains. To our knowledge, this report gives the first snapshot of *S. dysgalactiae* isolates collected from different countries that are localized geographically and differed on a multinational level. This genetic unrelatedness among different isolates might suggest a high recombination rate and low genetic stability.


## Introduction

*Streptococcus* infection of fishes has become a major problem affecting a variety of wild caught and cultured fish throughout the world. *Lactococcus garvieae* infection was the most serious disease affecting primarily farmed amberjack *Seriola dumerili* and yellowtail *S.* quinqueradiata in Japan. After the successful application of commercial formalin killed oral/injectable vaccines against *L. garvieae* [1], the economic damage caused by *L. garvieae* has been decreased. However, the vaccinated and unvaccinated farmed fishes exhibited comparable clinical signs of *L. garvieae* infection such as high mortality with severe necrotic lesions at their caudal peduncles [2,3]. The α-hemolytic *Streptococcus dysgalactiae* of Lancefield group C was identified as the causative agent of these epizootics [2]. Mortality was attributed to the lethal effects of severe bacterial septicemia and systemic granulomatous inflammatory disease [4].

*S. dysgalactiae* has been isolated from kingfish *S. lalandi*, yellowtail *S. quinqueradiata* and amberjack *S. dumerili* in Japan, cobia *Rachycentron canadum*, basket mullet *Liza alata* and gray mullet *Mugil cephalus* in Taiwan, golden pomfret *Trachinotus ovatus*, amur sturgeon *Acipenser schrenckii*, Siberian sturgeon *Acipenser baerii*, grass carp *Ctenopharyngodon idella*, crucian carp *Carassius carassius*, Soiny mullet *L. haematocheila* and pompano *Trachinotus blochii* in China, hybrid

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Peer review under responsibility of Cairo University.

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http://dx.doi.org/10.1016/j.jare.2013.12.003

red tilapia *Oreochromis* sp. in Indonesia, white spotted snapper *Lutjanus stellatus* and pompano *T. blochii* in Malaysia, Nile tilapia *O. niloticus* in Brazil, and rainbow trout *Oncorhynchus mykiss* in Iran [5–12]. Outside of the aquatic arena, *S. dysgalactiae* is considered as a main causative agent of bovine mastitis [13,14], ovine suppurative polyarthritis [15], bacteremia and ascending upper limb cellulitis in humans engaged in cleaning fish [16–18]. Eventually, the growing numbers of reports involving the clinical/pathological implementations of *S. dysgalactiae* are highly suggestive of a critically expanding importance of such pathogen.

Despite its clinical importance, just a few studies involving the fish *S. dysgalactiae* have been published till now [8,11,19,20]. Thus, little information is available about the outbreaks and epidemiology of such pathogen in farmed fish. Molecular typing methods permit typing of strains of the same bacterial species that appear indistinguishable by conventional methods, such as antibiogram or serotyping. Pulsed-field gel electrophoresis (PFGE) is considered as a gold standard typing method [21]. The bacterial whole genome is investigated by PFGE to assess genetic relationships among bacterial isolates. PFGE is useful in studying a short-term as well as a long-term epidemiological follow-up [21]. Biased sinusoidal field gel electrophoresis (BSFGE) is a modified PFGE [8]. Other molecular method, such as the sequencing of *tuf* gene has also been allowed the analysis of intraspecies sequence variations that reached up to 2.6% in streptococci [22].

The most prevalent molecular assays applied for genetic analysis of fish pathogen *S. dysgalactiae* are sequencing of housekeeping genes [5,7,8,11,20,23], PFGE and BSFGE profiles [2,8,11]. In this study, BSFGE analysis of *Sma*I was employed to establish distinct genetic profiles for *S. dysgalactiae* strains collected from a variety of moribund fishes and geographical areas. In addition, the partial sequencing of *tuf* gene and the phylogeny of the obtained sequences were investigated to evaluate the applicability of these techniques in future epidemiological studies.

## Material and methods

### *Bacterial isolates*

Twenty clinical *S. dysgalactiae* isolates were used in the current study. All *S. dysgalactiae* isolates were isolated from lesions in the caudal peduncle or the kidney of moribund fishes. Geographic origin and fish species from which *S. dysgalactiae* isolates were retrieved are shown in Table 1. The reference strain *S. dysgalactiae* subsp. *dysgalactiae* ATCC43078 was included for comparative purpose.

### *Growth conditions and DNA extraction*

All *S. dysgalactiae* isolates were aerobically grown on Todd Hewitt agar (THA; Difco, Sparks, MD, USA) plates and incubated at 37 °C for 24 h. Stock cultures were maintained frozen at −80 °C in Todd-Hewitt broth (Difco, Sparks, MD, USA). Lancefield serotyping C [24] was confirmed by using PASTOREX Strep (Bio-Rad, Marnes-la-Coquette, France). The identification of the *S. dysgalactiae* isolates was performed by using API 20 STREPT® (bioMerieux, Marcy-l'Etoile, France). Genomic DNA was performed from bacterial colonies by using a DNAzol® reagent (Invitrogen, Carlsbad, USA) according to the manufacturer's protocol.

### *PCR identification and partial sequences of tuf gene*

Internal fragment of the *tuf* gene was amplified using primers set designed from ATCC43078 (AF276263);

*tuf*1: 5′-GTAGTTGCTTCAACAGACGG-3′ and *tuf*2: 5′-GGCGATTGGGTGGATCAACTC-3′ that yield 795-bp. Generally, the PCR mixture was subjected on a thermal cycler to the following program; a denaturation at 95 °C for 4 min followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 51 °C for 30 s, extension at 72 °C for 90 s, and a final extension at 72 °C for 10 min. The amplified fragment of *tuf* gene of thirteen *S. dysgalactiae* isolates was then sequenced according to the method reported by Abdelsalam et al. [8]. Briefly, the amplified products of thirteen isolates were directly ligated into the plasmid pGEM-T Easy vector (Promega, Madison, WI, USA), and the recombinant plasmid was introduced into *Escherichia coli* DH5α according to the manufacture's protocol. Plasmid DNA was purified by using the QIAprep Spin Miniprep kit (Qiagen, Germantown, MD, USA). Sequencing reactions were performed by using the GenomeLab DTCS Quick Start Kit (Beckman Coulter, Fullerton, CA, USA) with the oligonucleotide primers SP6 (5-ATTTAGGTGACACTATAGAA-3) and T7 (5-TAATACGACTCACTATAGGG-3). The PCR products were loaded into the CEQ 8000 Genetic Analysis System (Beckman Coulter), and the nucleotide sequence was determined. The nucleotide sequences were analyzed by using BioEdit version 7.0 [25]. The phylogenetic analysis was then carried out by the neighbor joining method using MEGA version 5 [26].

### *Biased sinusoidal field gel electrophoresis (BSFGE)*

The restriction enzyme-digested chromosomal DNA was analyzed by BSFGE [8,18]. *S. dysgalactiae* isolates were cultured on THA at 37 °C for 24 h, and the preparation of genomic DNA and DNA digestion with a restriction *Sma*I enzyme was carried out according to the previously described method [8]. Briefly, plugs prepared from the isolates were treated sequentially with 1 mL of lysis buffer, pH 8.0 (0.1 M EDTA with 0.05% lauroylsarcosine) containing 5 mg $mL^{-1}$ lysozyme. After incubation at 37 °C for 3 h with gentle shaking, the plugs were replaced in 1 mL of proteinase solution (30 units $mL^{-1}$ proteinase K in 0.1 M EDTA with 1% sodium dodecyl sulfate), and incubated at 55 °C over night with gentle shaking. The incubated plugs were washed 6 times in 2.5 mL TE buffer and stored in TE buffer at 4 °C until the DNA digestion was performed using restriction enzyme. Macrorestriction fragment digested with *Sma*I was separated using 1% agarose horizontal gel by the BSFGE system (Genofield; ATTO, Tokyo, Japan). After gel electrophoresis, the gel was stained and visualized under UV light. The macrorestriction patterns were visually analyzed.

### *BSFGE pattern analysis*

The trial version of phoretix 1D software (TotalLab Ltd, Newcastle upon Tyne, United Kingdom) was used to analyzed