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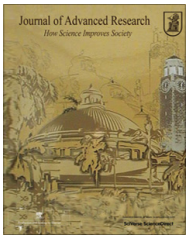

SHORT COMMUNICATION

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

M. Abdelsalam [a,*], A.E. Eissa [a,b], S.-C. Chen [c,d]

[a] Department of Fish Diseases and Management, Faculty of Veterinary Medicine, Cairo University, Giza, Egypt
[b] Departments of Poultry and Fish Diseases, Faculty of Veterinary Medicine, Tripoli University, Tripoli, Libya
[c] Graduate Institute of Animal Vaccine Technology, National Pingtung University of Science and Technology, Pingtung, Taiwan
[d] Department of Veterinary Medicine, National Pingtung University of Science and Technology, Pingtung, Taiwan

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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*

* Corresponding author. Tel.: +20 2 1122671243/35720399; fax: +20 2 35725240/35710305.
E-mail address: m.abdelsalam@staff.cu.edu.eg (M. Abdelsalam).

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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 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.

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Table 1 The α-hemolytic Lancefield group C *Streptococcus dysgalactiae* isolates used in this study.

No.	Isolate	Source	Country	Year of isolation	*tuf* accession no.	BSFGE profiles
1	Kdys0716	Amberjack	Japan	2007	AB755610	A
2	Kdys0717	Amberjack	Japan	2007	AB755611	A
3	Kdys0719	Yellowtail	Japan	2007	AB755612	A
4	KU070202	Amberjack	Japan	2007	AB755613	A
5	OT073284	Amberjack	Japan	2007	AB755614	A
6	OT061202	Amberjack	Japan	2006	ND[a]	A
7	TS041207	Amberjack	Japan	2004	AB755615	A
8	KNH07807	King fish	Japan	2007	AB755616	A
9	KNH07903	King fish	Japan	2007	AB755617	A
10	94455	Gray mullet	Taiwan	2005	ND[a]	B
11	94485	Gray mullet	Taiwan	2005	ND[a]	B
12	95542	Gray mullet	Taiwan	2007	ND[a]	B
13	95900	Gray mullet	Taiwan	2007	AB755618	B
14	95921	Gray mullet	Taiwan	2007	AB755619	B
15	95980	Gray mullet	Taiwan	2007	AB755620	B
16	95985	Gray mullet	Taiwan	2007	AB755621	C
17	PF880	Pompano	Malaysia	2003	AB755622	D
18	T11358	Tilapia	Indonesia	2004	ND[a]	E
19	PP1398	Pompano	Malaysia	2005	ND[a]	F
20	WSSN1609	Snapper	Malaysia	2004	ND[a]	G
21	ATCC43078	Cow			AF276263	ND[a]

[a] ND: Not determined.

BSFGE pattern analysis

The trial version of phoretix 1D software (TotalLab Ltd, Newcastle upon Tyne, United Kingdom) was used to analyzed bands of BSFGE. The automatic band detection was performed with a minimum slope of 100 and a noise reduction of 13. Bands were manually approved and matched to construct an absent/present binary matrix. A dendrogram was constructed by Unweighted Pair Group Method with Arithmetic Mean (UPGMA). Interpretation of BSFGE patterns was based on the criteria of Tenover et al. [27] Briefly, strains presenting one- to three-band differences and a similarity of $>85\%$ upon dendrogram analysis were considered to represent PFGE pattern subtypes, while more than three DNA band variations and a similarity of $<85\%$ at dendrogram analysis were considered to represent different BSFGE types.

Nucleotide sequence accession numbers

The nucleotide sequences determined throughout this study were submitted to the DNA Data Bank of Japan (DDBJ) nucleotide sequence database. The given accession numbers are shown in Table 1.

Results

*Partial sequences of **tuf** gene*

All isolates reacted positively to the *tuf* gene primers that were designed from *S. dysgalactiae* subsp. *dysgalactiae* ATCC43078. A single amplification product with the expected size of 795-bp was obtained from all the examined isolates (Fig. 1). The *tuf* gene sequences of thirteen isolates collected from different fish species and countries were submitted to the GenBank sequence database (Table 1). Ten isolates, collected from Taiwan, Japan and Malaysia, were identical (100% sequence identity). However, three isolates (Kdy0719, TS041207 and KNH07903 collected from Japan), have a single nucleotide differed from that of the other isolates. On the other hand, the determined sequence of the reference strain ATCC43078 differed from the fish *S. dysgalactiae* isolates sequences by 5–6 nucleotides. The phylogenetic tree generated based on the *tuf* gene sequences of *S. dysgalactiae* isolates from fish and other related *Streptococcus* species is shown in Fig. 2. Such phylogenetic tree is obviously revealing that all sequenced fish *S. dysgalactiae* isolates belonged to only one cluster, and they were separated from other related *Streptococcus* species.

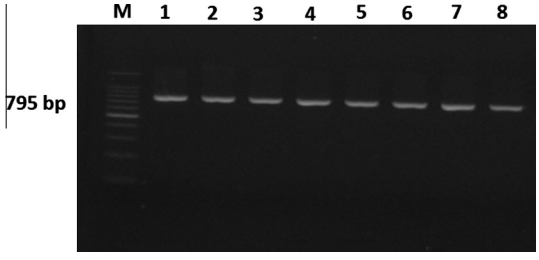

Fig. 1 Amplification of the *tuf* locus extracted from fish *S. dysgalactiae* isolates and *S. dysgalactiae* ATCC43078 yielded 795-bp when the primer pairs tuf1 and tuf2 were used. Lane M, marker; lane 1, *S. dysgalactiae* ATCC43078; lanes 2 and 3 Japanese fish isolates *S. dysgalactiae*; lanes 4 and 5, Taiwanese fish isolates *S. dysgalactiae*; lanes 6 and 7, Malaysian fish isolates *S. dysgalactiae*; and lane 8, Indonesian fish isolate *S. dysgalactiae*.

Biased sinusoidal field gel electrophoresis (BSFGE)

All the fish *S. dysgalactiae* isolates were typeable using BSFGE. Remarkably, the macrorestriction patterns were

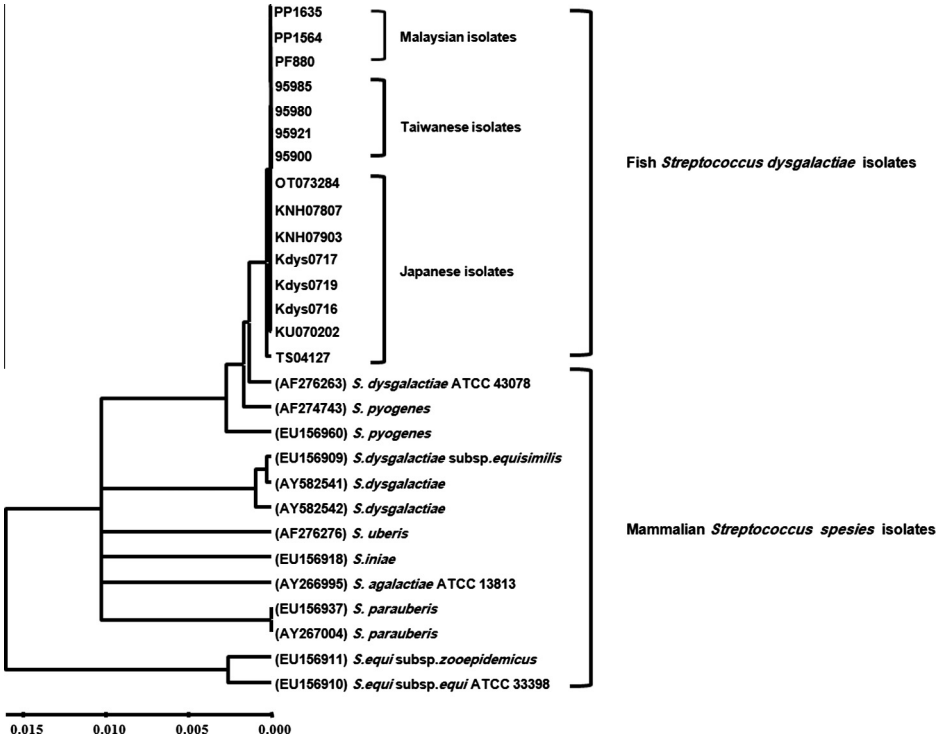

Fig. 2 Phylogenetic tree generated based on the comparative analysis of the *tuf* gene sequences, showing the relationship among the fish strains of *S. dysgalactiae* and related species of the genus Streptococcus.

superbly conserved between fish *S. dysgalactiae* isolates collected from Japan and Taiwan. Generally, analysis of these patterns allowed the differentiation of isolates into six types and one subtype as shown in Fig. 3a. The computer-generated dendrogram revealed that fingerprint variations obtained by digestion with *Sma*I could classify all isolates into three distinct clusters at a 64% similarity level as shown in Fig. 3b. All the macrorestriction patterns of Japanese isolates are indistinguishable from each other representing type (A) with 100% similarity. All Taiwanese isolates are indistinguishable from each other representing type (B) with 100% similarity, except for strain 95985 representing subtype (C) that is showing 90% similarity with other Taiwanese isolates. Both Japanese isolates and Taiwanese isolates could be grouped at 78% similarity. In contrast, Malaysian isolates (PF880 and PP1398) presented two different types D and F with 72% similarity and they grouped with Japanese and Taiwanese isolates at 65% similarity level. The Indonesian isolate (T11358) and

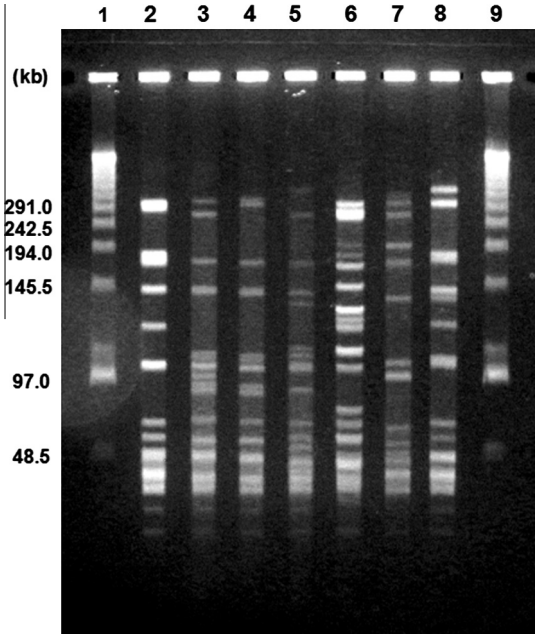

Fig. 3a The macrorestriction fragment profiles of DNAs digested with *Sma*I in seven representative isolates of *S. dysgalactiae* collected from fish. Lanes 1 and 9, marker DNA; Lane 2, KNH07807 (Japan); lane 3, 951003 (Taiwan); lane 4, 95985 (Taiwan); lane 5, PF880 (Malaysia); lane 6, T11358 (Indonesia); lane 7, PP1398 (Malaysia); lane 8, WSSN1609 (Malaysia).

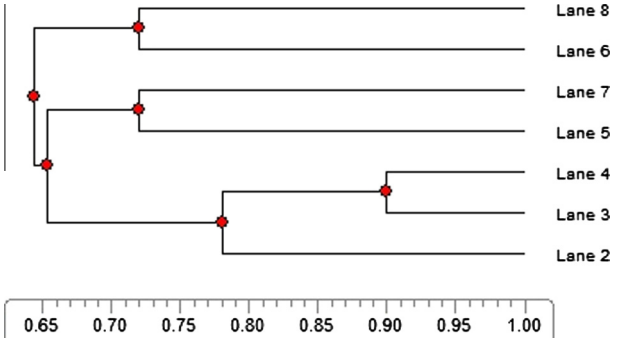

Fig. 3b Dendrogram constructed for BSFGE analysis using the UPGMA method with Phoretix 1D trial version.

Malaysian isolate (WSSN1609) presented two different types; E and G with 72% similarity.

Discussion

Fish *S. dysgalactiae* isolates have been considered as homogenous taxon on the basis of the conventional phenotypic methods that included colonial characteristics, binding to Congo Red dye, biochemical/physiological features and Lancefield serological test [2,8,19,20,28–30]. Therefore, the use of sensitive molecular methods is necessary to assess the strain heterogeneity within this fish pathogen. In the current study, analysis of fish *S. dysgalactiae* isolates by using restriction endonuclease of *Sma*I and partial sequencing *tuf* revealed new insight into the identification and epidemiology of *S. dysgalactiae*.

Several studies have been performed on the molecular identification of the genus *Streptococcus* by using the sequencing method targeting some housekeeping genes [5,7,8,11,20,23,31,32]. The elongation factor (*tuf*) gene is concerned in protein biosynthesis that facilitates the elongation of polypeptides from the ribosome and aminoacyl-tRNA throughout translation. It is universally distributed and in most Gram-positive bacteria just one *tuf* gene has been found. The *tuf* sequences of streptococci usually provide additional discrimination power than 16S rDNA sequences and may enable identification at the species level of even most closely related streptococcal species; therefore it is ideally fitted to phylogenetic studies [22,33,34]. The sequencing of the *tuf* gene was performed to match different isolates collected from geographically distinct areas. A 100% sequence identity was determined among *S. dysgalactiae* isolates irrespective of their country of origin, aside from three isolates. Thus, the phylogenetic analysis demonstrated that fish *S. dysgalactiae* isolates belonged to one cluster and distinct from other *Streptococcus* species.

Interestingly, *S. dysgalactiae* of fish origin appeared to be more related to *S. pyogenes* rather than *S. dysgalactiae* subsp. *equisimilis*. Therefore, these results suggested that *tuf* gene analysis could be a valid tool for identifying *S. dysgalactiae* subsp. *dysgalactiae* to the subspecies level. Our results suggested that *tuf* gene analysis also could be a valid tool for inferring relationships among closely related bacterial species. However, the lack of *tuf* sequence variations in *S. dysgalactiae* isolates collected from moribund fishes showed its inadequacy for any intraspecific relationship analysis (e.g., as a typing tool at the strain level).

On the other hand, PFGE is considered to be superior for interpreting inter-strain relationships among enterococci [35]. The most common method used for typing streptococci consists of the restriction of genomic DNA with *Sma*I endonuclease, followed by PFGE analysis [11,36]. In previous studies, the *Sma*I analysis was performed for the genotypic comparison characterization between Japanese fish and mammalian isolates of *S. dysgalactiae* [19], and genetic characterization of *S. dysgalactiae* recovered from Nile tilapia in Brazil [11]. In this study, all fish *S. dysgalactiae* isolates were belonged to the major types-classified based on the macrorestriction patterns obtained by digestion with *Sma*. We were particularly interested to determine whether there were localized geographical strains clustering or whether *S. dysgalactiae* was clonally related on a multinational level. All Japanese isolates were indistinguishable and presented type A. The results obtained in this study supported those obtained in previous publication by Nomoto et al. [19] and Nishiki et al. [20] who demonstrated that the Japanese isolates of *S. dysgalactiae* were clonally related to each other. All Taiwanese isolates (except 95985) were indistinguishable and presented type B and apparently differed from other isolates, including the Japanese, Indonesian, and Malaysian isolates. Thus, we can conclude that *S. dysgalactiae* isolates collected from different countries are localized geographically and differed on a multinational level. Our results contradict our previous assumptions [8] in which the fingerprint variations obtained by digestion with *Apa*I of *S. dysgalactiae* isolates could be related to each other at the multinational level irrespective of their country of origin as well as the fish species. This may *indicate that Apa*I digestion might be less discriminatory than *Sma*I digestion for closely related isolates. The present finding is in agreement with that of Phuektes et al. [37].

The *S. dysgalactiae* isolates evaluated during this study represented a genetically different population with an obvious relationship between geographical origin and genotype that is similar to that found in other streptococci fish pathogens [38,39]. This finding will have important implications in determining *S. dysgalactiae* global epidemiology and ultimately impact the future vaccine development as well as vaccination policies.

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