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Review Article

Streptococcus dysgalactiae: An emerging pathogen of fishes and mammals

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Abstract *Streptococcus dysgalactiae* subsp. *dysgalactiae* (GCSD) has gained special interest of aquatic health experts throughout the past few years due to its interesting veterinary and public health importance. Increasing records of GCSD infections in farmed fishes have been documented through diverse worldwide aquatic habitats in Japan, China, Malaysia, Indonesia, Taiwan and Brazil. Despite the intraspecies/interspecies dynamic spread of fish GCSD, yet, the genetic basis of its virulence remains unknown. This gap in knowledge is the main reason behind inability to develop a competent vaccine to control the disease in aquatic animals. However, the authors have concluded that the virulence of GCSD is mainly based on its cell surface properties such as high hemagglutination and hydrophobic properties which determine the main adhesive/invasive pathogenic mechanism of the pathogen where GCSD isolates were able to adhere to and invade fish epithelial cell line *in vitro*. Most recently, the molecular pathogenesis investigations have revealed that, serum opacity factor [SOF], superantigen and streptolysin S genes might be the most important virulence factors that have contributed to the swift propagation of streptococcal infection among aquatic and mammalian species. In conclusion, the current research based review has emphasized the current knowledge gap in epidemiology and control of fish GCSD. To bridge this current gap, a swift future development of high tech/accurate molecular research is highly needed to better understand the pathogenic mechanisms of GCSD.

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Contents

1. Introduction

Fish streptococosis was first reported in Japanese cultured rainbow trout by 1957 [1]. Since then, fish streptococosis has become a major problem of wild and cultured fish worldwide [2,3]. At least six different streptococcus species are considered potential fish pathogens: *Lactococcus garvieae*, *Lactococcus piscium*, *Streptococcus iniae*, *Streptococcus agalactiae*, *Streptococcus parauberis* and *Vagococcus salmoninarum* [4]. *Lactococcus garvieae* infection is considered the most serious disease affecting farmed yellowtail *Seriola quinqueradiata* and amberjack *Seriola dumerili* in Japan [5,6]. The successful application of commercial *L. garvieae* vaccines has resulted in remarkable decline in fish losses [7].

Unexpectedly, a similar infection has been discovered among vaccinated and non-vaccinated farmed amberjack/yellowtail in Japanese fish farms with catastrophic mortalities [8]. The α-hemolytic Lancefield group C *Streptococcus dysgalactiae* subsp. *dysgalactiae* [GCSD] was identified as the causative agent [8]. GCSD causes a syndrome that is characterized by systemic multifocal inflammatory reaction, microabscessiations, severe septicemia, and high mortality rates with pathognomonic necrotic ulcers at the caudal peduncle region [8–11]. Epidemiologically, GCSD has been reported to be the main cause behind several mammalian infections such as streptococcal mastitis/endometritis in domestic mammals with some records of skin lesions, meningitis and bacteremia in humans [12–15].

2. Taxonomy

The genus Streptococci was first described by Rosenbach [16] and its taxonomic analyses had been recently reviewed by Facklam [17]. The most important contributions to the classification of streptococci were offered by Shottmuller [18] who had used blood agar to differentiate beta-hemolytic from non hemolytic strains. Lancefield [19] reported specific technique that had been used for decades to demonstrate specific carbohydrate ''group'' antigens associated with the beta-hemolytic strains.

Sherman [20] classified streptococci into four categories; pyogenic, viridans, lactic and enterococci. *S. dysgalactiae* is the only pyogenic streptococcus that is not beta-hemolytic [17]. *S. dysgalactiae* was first described by Diernhofer [21], and subsequently classified as a group-C streptococcus [GCS] by Lancefield [19]. Prior to the 1980s, *S. dysgalactiae* (Diernhofer) were removed from the nomenclature of the Approved Lists of Bacterial Names [22]. The reason for this omission is not known, but it may have happened because no type strain was designated before 1980. *S. dysgalactiae* was later recognized as an official species in 1983 [23]. Since *S. dysgalactiae* was the oldest officially recognized species, all of the genetically similar β-haemolytic *Streptococcus equisimilis* (GCS, GGS of human origin and GLS), and the alpha hemolytic *S. dysgalactiae* were assembled under its stated *S. dysgalactiae* nomenclature [24].

Using the serotyping and biotyping techniques, Devriese [25] had distinguished five ecovars of streptococci; the bovine *S. dysgalactiae* ecovar, the human *S. equisimilis* ecovar, the animal *S. equisimilis* ecovar, the human GGS ecovar and the GLS ecovar. Based on electrophoresis of cell wall proteins and physiological tests, Vandamme et al. [26] proposed the name *S. dysgalactiae* subsp. *dysgalactiae* for strains of animal origin that belong to GCS and GLS. These strains were also reported to be α-, β- or non-haemolytic and did not exhibit streptokinase activity on human plasminogen or proteolytic activity on human fibrin. Vandamme et al. [26] further, proposed the name *S. dysgalactiae* subsp. *equisimilis* for human isolates that belong to GCS and GGS. These investigated β-haemolytic strains exhibited streptokinase activity on human plasminogen as well as proteolytic activity on human fibrin [27]. Later investigations have indicated that *S. dysgalactiae* is classified by a combination of phenotypic and genotypic characteristics into four types: GCS α-hemolytic *S. dysgalactiae* subsp. *dysgalactiae* [GCSD], GCS β-hemolytic *S. dysgalactiae* subsp. *equisimilis*, GGS β-hemolytic *S. dysgalactiae* subsp. *equisimilis*, and GLS β-hemolytic *S. dysgalactiae* subsp. *equisimilis* [28].

GCSD was recently identified as fish specific pathogen based on 16S rDNA sequence analysis, *sodA* gene and *tuf* gene sequence analysis, biased sinusoidal field gel electrophoresis and Lancefield grouping. The biochemical characteristics (a-hemolytic and streptokinase activities) of the fish isolates were different from those of the typical reference of mammalian strains *S. dysgalactiae* subsp. *dysgalactiae* and *S. dysgalactiae* subsp. *equisimilis* [8,29–31].

3. Morphochemical characteristics

The fish specific isolates of α-hemolytic GCS *S. dysgalactiae* subsp. *dysgalactiae* [GCSD] were Gram positive cocci arranged in long chains, catalase negative, oxidase positive and auto-aggregated in saline [8,32], while the mammalian isolates of α-hemolytic GCS *S. dysgalactiae* subsp. *dysgalactiae* were Gram-positive cocci or oval cells arranged in short- to medium-length chains, catalase negative, and oxidase positive

[23]. The optimal temperature for growth of fish and mammalian isolates is 37 °C. Growth does not occur at 10 or 45 °C or in 6.5% NaCl or in 40% bile, bacitracin, or at pH 9.6 [8,23]. Fish specific isolates exhibit α-hemolysis on cattle and sheep blood agar, however the hemolysis pattern on sheep blood agar is mostly changed to β-hemolysis type after prolonged incubation [8], or incubation at 4 °C [32].

In mammalian isolates, acid is formed from the utilization of glucose, lactose, maltose, sucrose, tagatose and trehalose. The biochemical profiles of these isolates are relying on the existence of alkaline phosphatase, leucine arylamidase, acid phosphatase, β-glucuronidase, and α-glucosidase. Differently, fish isolates produce acid from the utilization of ribose, amygdaline, sucrose and trehalose. Similarly, fish specific isolates exhibit specific biochemical profile that is based on the existence of the same enzymes with the exception of naphthol-AS-BI-phosphohydrolase. Fish strains GCSD isolated from Amur sturgeon *Acipenser schrenckii* could utilize lactose and arabinose [32]. Arginine is the direct source of ammonia in both fish and mammalian types [8,10,29]. Both fish and mammalian types exhibit low pathogenicity to mice [8,23].

4. Host susceptibility

GCSD is mainly associated with endometritis, subclinical/clinical mastitis, subcutaneous cellulitis, and toxic shock-like syndrome in bovine [33–35]. The same pathogen is reported as a cause of bacteremia, meningeo-encephalitis and mastitis in sheep [36–38]. GCSD was also associated with umbilical infection in lambs [36], and suppurative polyarthritis in calves [39]; goats [40]; and lamb [41]. GCSD is also associated with neonatal mortalities in puppies [42]. GCSD causes granulomatous ulceration of the anus of pubertal male pigs [43]. GCSD naturally exists on skin lesions, reproductive tract discharges and tonsils of healthy cows/sheep [14,41]. GCSD occasionally causes lower limb cellulitis, meningitis, and bacteremia in humans [12,14,27,44].

In 2002, the first emerging outbreak caused by α-hemolytic GCSD among Japanese cultured fish populations was reported. Clinically, the infected yellowtail and amberjack exhibited a typical form of necrosis in their caudal peduncles and high mortality rates [8,9,29,30]. This pathogen has been isolated from kingfish *Seriola lalandi* in Japan; grey mullet *Mugil cephalus*, basket mullet *Liza alata*, and cobia *Rachycentron canadum* in Taiwan; hybrid red tilapia *Oreochromis* sp. in Indonesia; pompano *Trachinotus blochii* and white spotted snapper *Lutjanus stellatus* in Malaysia; pompano *T. blochii* in China [9–11,45]; Amur sturgeon *Acipenser schrenckii* [32] and golden pomfret *Trachinotus ovatus* in China [46]; Nile tilapia *Oreochromis niloticus* in Brazil [47]. These isolates were characterized by high phenotypic homogeneity irrespective of the countries from where the strains were collected. The majority of isolates was found to be resistant to oxytetracycline and possessed the *tetM* gene. From public health perspective, Koh et al. [15] has reported that GCSD is the causal agent of ascending upper limb cellulitis in human engaged in cleaning fish and hence may be considered an emerging zoonotic agent.

The Japanese fish GCSD isolates were biochemically/genetically homogenous, while remarkable genetic differences from mammalian isolate were reported [31]. To differentiate fish isolates from mammalian isolates, primer sets targeting the soda and sof-FD genes were established [30,48,49]. Based on BSFGE typing by *Apa*I macro-restriction, the fish isolates – including the Japanese, Taiwanese, and Chinese isolates – could be grouped into one main cluster at a 70% similarity level. However, the macro-restriction genotypes of some isolates were apparently distinct from those of the main cluster [10].

5. Media optimization and molecular detection

Abdelsalam et al. [9] was successful in isolation of GCSD from brain, kidney, spleen and caudal peduncle lesions of some cultured Japanese fishes. Authors used Todd-Hewitt agar containing 30 μg mL^{-1} of Congo red dye [TH-CR]. On TH-CR media the GCSD colonies appeared orange in color while those of *L. garvieae* appeared white. This difference in color is mainly attributed to the excellent Congo red binding ability of GCSD. An optimized TH-CR agar supplemented with 5 mg L^{-1} of oxolinic acid was developed to minimize bacterial contaminants and increase isolation performance (Fig. 1) (Abdelsalam, unpublished data). Oxolinic acid has been used as a selective additive in many bacterial media for isolation streptococci [50] and Lancefield group C streptococci [51]. Oxolinic acid has inhibitory effect towards flora, staphylococci and Gram-negative bacteria [52].

Determination of the *sodA*, *spegg*, *tuf* and *sagA* genes sequence is surely one of the most efficient/accurate tools for identification of GCSD, but is still rather expensive to be used as a routine diagnostic screening method [10,11]. The term clone is used in isolates that are indistinguishable in genotype at which the most likely explanation is a common ancestor. In contrary to the popularity of DNA based identification/typing methods are only gradually implemented in molecular typing of the pathogen. Ideally, every typing method should be validated using multiple strains from different geographical regions and disease episodes. Pulsed-field gel electrophoresis [PFGE] targets the whole genome of the bacterium and is widely used as typing method for investigating genetic relationships between isolates. The whole bacterial DNA is digested by a specific restriction enzyme [usually *Sma*I and *Apa*I for GCSD] to obtain a relatively small amount of fragments,

Figure. 1 Pure GCSD colonies on TH-CR agar optimized medium supplemented with 30 μg mL^{-1} of Congo red and 5 mg L^{-1} oxolinic acid after 30 h of incubation at 37 °C compared to impure colonies on TH agar medium without oxolinic acid and Congo red dye supplements.

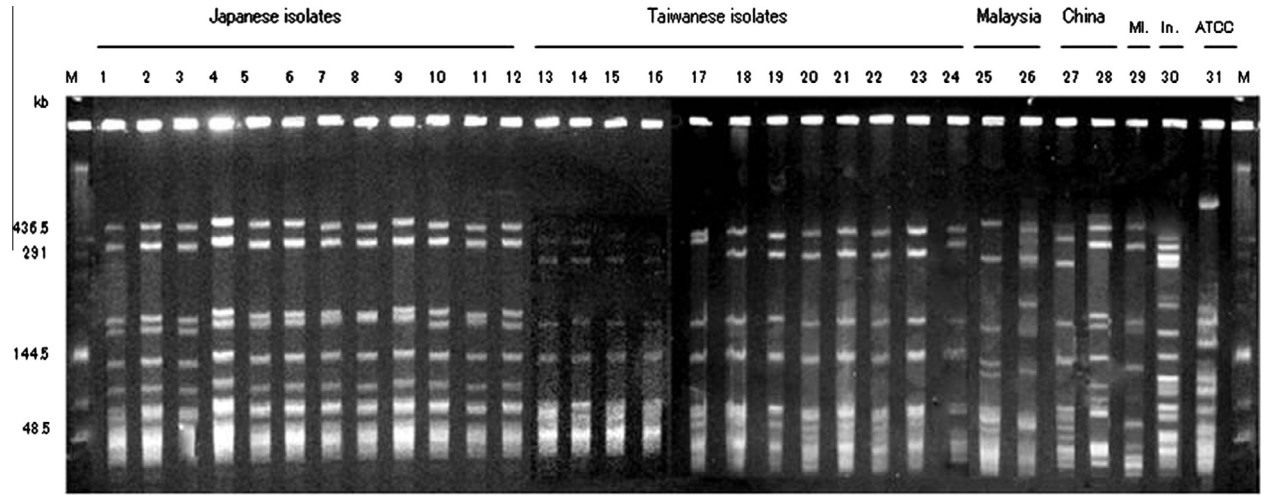

Figure. 2 Biased sinusoidal field gel electrophoresis (BSFGE) *Sma* macrorestriction profiles of *Streptococcus dysgalactiae* isolates from fish lanes (1–30). Lane M, λ DNA ladder marker; Japanese isolates lanes 1–12, Taiwanese isolates lanes 13–24; Malaysian isolates lane 25–26 and 29; Chinese isolates lanes 27–28; Indonesian isolate lane 30; *S. dysgalactiae* subsp. *dysgalactiae* ATCC43078 lane 31.

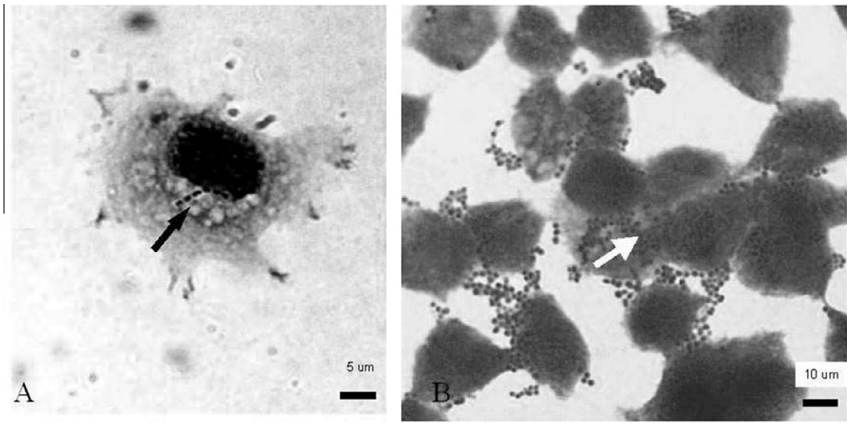

Figure. 3 Microscopic appearances (arrows) of *S. dysgalactiae* adhered to EPC and CHSE-214. Both cell line exposed to 10^7 CFU/well and stained by Giemsa (1000×). (A) A black arrows showed cells of *S. dysgalactiae* adhered to CHSE-214. (B) A white arrows showed cells of *S. dysgalactiae* adhered to EPC.

which are separated in a specific gel electrophoresis apparatus that periodically switches the direction of the electrical current, allowing the separation of large DNA fragments [10]. Random genetic events such as deletions, insertions, and point mutations sometimes alter the restriction sites and affect the restriction pattern of a strain. PFGE has a high discriminatory power that can be enhanced by the choice of an appropriate restriction endonuclease, and it is most useful in epidemiological investigations [53].

Using the discriminatory power of the Biased Sinusoidal Field Gel Electrophoresis (BSFGE), *Sma* macrorestriction enzyme was able to differentiate some geographically diverse GCSD isolates that were originally collected from Japan, Taiwan, Malaysia, China and Indonesia based on difference in DNA banding patterns (Fig. 2) [Abdelsalam, unpublished data]. *Apa*I restriction enzyme has been also employed by the Abdelsalam et al. [10] to differentiate some geographically diverse GCSD isolates.

6. Virulence

The search for a competent vaccine against GCSD is hindered by the lack of knowledge about its pathogenesis and virulence determinants. Thus a comprehensive research on virulence determinants of GCSD should be adopted in future research. The virulence of GCSD is mainly based on its cell surface properties such as high hemagglutination and hydrophobic properties which determine the main adhesion and invasive pathogenic mechanism of the pathogen [45]. This has been confirmed by Abdelsalam et al. [45], who have indicated that GCSD isolates were able to adhere to and invade fish epithelial cell line (EPC: Epithelial papiloma of Carp and CHSE-214: Chinook salmon embryo) cultured *in vitro* (Fig. 3).

Despite the intraspecies/interspecies dynamic spread of fish GCSD, yet, the genetic basis of its virulence remains unknown. Superantigen and streptolysin S genes might be the most important virulence factors that have contributed to the swift

propagation of streptococcal infection. PCR amplification and subsequent sequence analysis revealed that all strains isolated from moribund fish harbored the streptolysin S structural gene (*saga*) [11]. On the contrary, GCSD fish isolates were PCR negative for *emm*, *speA*, *speB*, *speC*, *speM*, *smeZ*, and *ssa*. However, the size of streptococcal pyrogenic exotoxin G [*spegg*] locus, a superantigen in positive fish and swine strains was variable [11]. Interestingly, Abdelsalam et al. [11] have detected an insertion sequence (IS981SC) within the open reading frame [ORF] of *spegg* locus of Japanese GCSD fish strains. Further, they found a unique hybrid insertion element (IS981SC-IS1161) that has been inserted into the ORF of *spegg* locus of two fish GCSD strains collected from Malaysia [11].

Serum opacity factor [SOF] was detected in GCSD isolated from fish [48]. The SOF obtained from fish isolates, named SOF-FD, consists of a putative signal sequence, a large N-terminal opacification domain, a putative fibronectin-binding domain, and an LPXTG Gram-positive anchor motif. The structure of SOF-FD is similar to that of the SOF in *S. pyogenes* [48,49]. The substrate of SOF is a high density lipoprotein [HDL] which has anti inflammation activity [49]. The SOF binds to HDL and then disrupts its structure, which may contribute to the virulence of streptococci. All GCSD strains isolated from fish have serum opacification activity and sof-FD genes with highly homogeneous sequences [48]. In fish isolates, various opacification activities were observed, although their SOF-FD amino acid sequences were identical [48].

In conclusion, comprehensive pathophysiological, biochemical and molecular research is highly needed to better understand the pathogenic mechanisms of GCSD and to overcome the knowledge gap in epidemiology and control of fish GCSD related diseases.

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