Current Challenges of *Streptococcus* Infection and Effective Molecular, Cellular, and Environmental Control Methods in Aquaculture

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Several bacterial etiological agents of streptococcal disease have been associated with fish mortality and serious global economic loss. Bacterial identification based on biochemical, molecular, and phenotypic methods has been routinely used, along with assessment of morphological analyses. Among these, the molecular method of 16S rRNA sequencing is reliable, but presently, advanced genomics are preferred over other traditional identification methodologies. This review highlights the geographical variation in strains, their relatedness, as well as the complexity of diagnosis, pathogenesis, and various control methods of streptococcal infections. Several limitations, from diagnosis to control, have been reported, which make prevention and containment of streptococcal disease difficult. In this review, we discuss the challenges in diagnosis, pathogenesis, and control methods and suggest appropriate molecular (comparative genomics), cellular, and environmental solutions from among the best available possibilities.

Keywords: antimicrobial, aquaculture, geography, sequencing, *Streptococcus*

INTRODUCTION

Aquaculture is among the fastest growing businesses in the food production industry (Bondad-Reantaso et al., 2005), and streptococcal infections have caused significant economic losses in the aquaculture industry (Austin and Austin, 2007; Toranzo et al., 2005).

Various bacterial agents cause streptococcosis; *Streptococcus parauberis*, *Streptococcus iniae*, *Streptococcus agalactiae*, and *Streptococcus dysgalactiae* are the prominent species regardless of geographical region (Agnew and Barnes, 2007; Nho et al., 2009; 2013; Toranzo et al., 2005; Vendrell et al., 2006). Fish are an important food source, but have always been at risk of acquiring *Streptococcus* infections owing to continuous exposure and ubiquitous global presence of various bacterial strains and species. Currently, there are several methods for detecting and identifying *Streptococcus* spp., depending upon the phenotypic characteristics, species, and strains of bacteria.

Control of streptococcus infection mainly relies on the use of antimicrobial compounds, vaccinations, and environmental strategies (Cheng et al., 2010; Darwish and Hobbs, 2005; Hastein et al., 2005; Sommerset et al., 2005; Woo and Park, 2014), of which vaccines and antimicrobial compounds have been ineffective for various reasons (Agnew and Barnes, 2007; Park et al., 2009; Shoemaker et al., 2001; Toranzo et al., 2005). Environmental strategies have been used to control fish infections in their natural and artificial habitats by several methods (Holmer, 2010). In this review, we discuss

the current status and challenges in diagnosis, pathogenesis, and control of streptococcal disease in fish and we suggest effective control strategies.

WORLDWIDE DISTRIBUTION AND RISK FACTORS FOR STREPTOCOCCAL DISEASE

Streptococcal disease occurs in all continents (Americas, Asia, Europe, Africa, and Australia) (Table 1 and Fig. 1). Thousands of *Streptococcus* species (*S. parauberis, S. iniae, S. agalactiae, Lactococcus garvieae, S. dysgalactiae,* and *Vagococcus salmoninarum*) have been reported in different parts of the world (Abdelsalam et al., 2013; Agnew and Barnes,

Table 1. Streptococcus bacterial agents and detailed information of affected fish species, locations, hosts, and clinical criteria

| Species	Host	Fish species	Clinical Criteria	Geographical Location
Streptococcus iniae	Fish, Human	Hybrid striped bass, Nile tilapia, Hybrid tilapia, Rainbow trout, Red drum, Rabbitfish, Sea bass, Olive flounder, Barramundi, Wild fish	Hemorrhage, exophthalmia, abdominal distension, ascites, lesions (liver, kidney, spleen, and intestine)	Canada, Americas, Bahrain, Israel, Thailand, China, Japan, Singapore, Taiwan, Korea
Streptococcus parauberis	Fish, Cow	Olive flounder, Rainbow trout, Cultured turbot, Hybrid striped bass	Chronic wasting syndrome, hemorrhagic septicemia, exophthalmia, meningitis with abnormal swimming	Israel, Italy, Japan, Spain, USA, China, Iran, Korea, Malaysia, India
Streptococcus agalactiae	Fish, Cow, Human, Chickens, Camels, Dogs, Horses, Cats, Frogs, Hamsters, Monkeys	Nile tilapia, Barcoo grunter, Golden pompano, Giant Queensland grouper, Ya-fish, Silver pomfret	Erratic swimming, appetite, lethargy, uncoordinated movements, exophthalmia (uni- or bi-lateral), intraocular hemorrhage, opaqueness of cornea, ascites	Europe, Turkey, China, Indonesia, Malaysia, Japan, Korea, Vietnam, Philippines, Americas
Lactococcus garvieae	Fish, Cow, Human, Cat, Dog, Water buffalo	Rainbow trout, Yellowtail, Tilapia, Japanese eel, Grey mullet, Black rockfish, Catfish, Wild wrasse, Giant fresh water prawn, Olive flounder, Amberjack, kingfish	Melanosis, lethargy, erratic swimming, disorientation, fins, exophthalmia (uni- or bi-lateral), swollen abdomens, anal prolapses, hemorrhages (periorbital, perianal, buccal regions)	Turkey, Australia, South Africa, England, Portugal France, Balkans, Israel, Korea
Streptococcus dysgalactiae	Fish, Calves, Lamb, Human, Sheep, Dogs, Pig, Lamb, Cats	White spotted snapper, Kingfish, Grey mullet, Cobia, Hybrid red tilapia, Pompano, Basket mullet, Pompano, Golden pomfret, Amur sturgeon, Nile tilapia, Yellow tail, Amber-jack	Abnormal swimming, loss of orientation, exophthalmia	Brazil, Indonesia, Malaysia, Taiwan, China, Japan
Vagococcus salmoninarum	Fish	Rainbow trout, Atlantic salmon, Brown trout	Loss of equilibrium, exophthalmia, melanosis, bleeding (jaw, eye, mouth, abdomen, fins, and anus), necropsy, transparent fluid accumulation, fibrinous deposits (heart, liver, spleen)	France, Italy, Spain
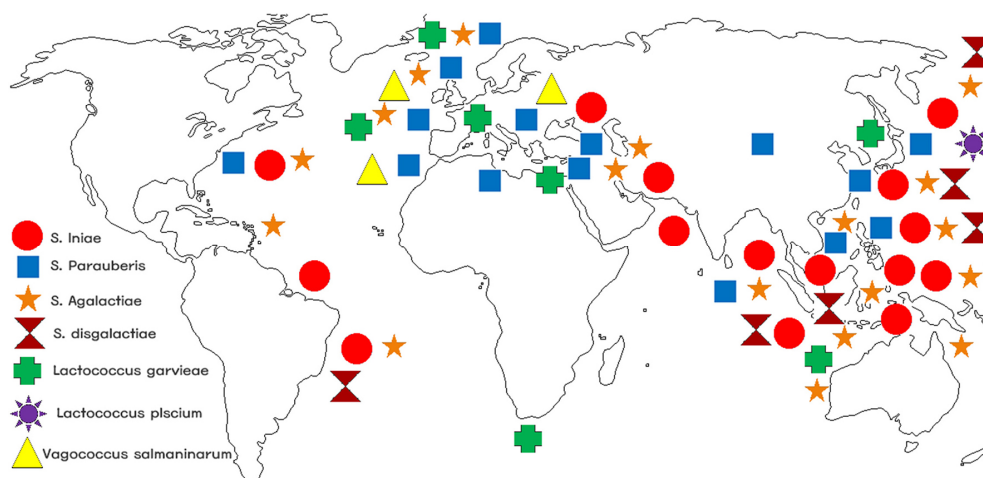
Fig. 1. Phylogeography of major fish pathogens belonging to *Streptococcus* species, *S. iniae* (red circle), *S. parauberis* (blue square), *S. dysgalactiae* (brown inverted box), *S. agalactiae* (orange star), *Lactococcus garvieae* (green plus sign), *Vagococcus salmoninarum* (yellow triangle), and *Lactococcus piscium* (violet rising sun). Distribution pattern shows the presence of these bacterial isolates over the continents.

2007; Baeck et al., 2006; Carson et al., 1993; Chang and Plumb, 1996; Diler et al., 2002; Li et al., 2015; Nho et al., 2013; Pereira et al., 2010; Ruiz-Zarzuela et al., 2005) (Fig. 1). Streptococcosis is a multifactorial disease in fish, depending on host variety, age, immune status, type of pathogen (species and strain), and environmental conditions (Ghittino et al., 1999; Ravelo et al., 2001; Vendrell et al., 2006).

CURRENT CHALLENGES IN STREPTOCOCCOSIS DIAGNOSIS

Currently, the immediate and inexpensive diagnosis of streptococcosis in infected fish is difficult as fish exhibit similar clinical symptoms regardless of the etiological agent (Table 1 and Fig. 1) (Baeck et al., 2006; Muzquiz et al., 1999). Various issues of diagnosis are discussed below.

Clinical phenotype is the primary signature of bacterial infections that depends on various factors, so it is difficult to understand the precise cause of infection. A study of tilapia fish showed that clinical phenotypes and degree of lesions depend on several factors such as *S. agalactiae* strain variations, their infectious dose, water conditions, temperature, and handling procedures (Chang and Plumb, 1996). Due to the complexity of bacteria and their interrelations, microbiologists occasionally misidentify or cannot identify isolates (Lau et al., 2006). Asymptomatic fish serve as a pathogen reservoir and pose challenges for correct identification due to the absence of clinical signs (Bromage et al., 1999). Presently, diagnosis of subclinical infections in fish is a major concern.

Bacterial identification methods based on culture, morphology, or biochemical reactions are time- and resource-consuming. Some pathogen databases (RAPID Strep strip, VITEX systems, API 20E STREP, Rapid Strep 32 and ATB Expression System) are incomplete or incorrect, and result in improper identification of bacteria (Dodson et al., 1999; Facklam et al., 2005; Lau et al., 2006). Additionally, other challenges for accurate identification include the mixed nature of the aquaculture environment, low numbers of biological samples, or unknown tissue location in carriers (Klesius et al., 2006). Identification of *S. agalactiae* based on biochemical features (i.e. capacity to hydrolyze hippurate) or phenotypic characteristics (acidification of tagatose, ribose, and sucrose) are not effective due to high levels of biochemical heterogeneity among strains (Ravelo et al., 2001).

Molecular methods are based on several candidate genes (Table 2), that have been well characterized for diversity, including 16S rRNA, heat-shock genes (*groESL*), and tRNA gene intergenic spacer regions (ITSs) (Clarridge et al., 2001; Teng et al., 2002). Comparative studies have discussed various methodologies and found molecular methods to be most effective for bacterial identification (Bosshard et al., 2006). *S. dysgalactiae* (GCSD) was recently identified as a fish-specific pathogen based on 16S rRNA, *sodA*, and *tuf* gene sequence analysis (Abdelsalam et al., 2013). Phenotypic criteria failed to differentiate between genealogically distinct *L. garvieae* and *Lactococcus lactis* strains, so correct identification was determined by molecular methods (Klijn et al., 1991; Vendrell et al., 2006). However, some limitations are associated with diversity experiments. For example, closely related species could not be distinguished by 16S rRNA in a study by the Mitis group at the NHS (Nielsen et al., 2009). Moreover, the taxonomic position of several aquatic isolates is still unclear (Austin and Austin, 2007).

Another molecular method, multilocus sequence typing (MLST; analyses of multiple genetic loci or housekeeping genes) is considered the "gold standard" of typing for many bacterial species (Maiden, 2006; Jolly et al., 2012). However, insufficient resolution among very closely related bacteria can be a problem (Achtman, 2008).

Table 2. Candidate genes used for differentiation and diagnosis of various Streptococcus bacterial agents

Candidate gene	References
Manganese-dependent superoxide dismutase gene (*sodA*)	Kitten et al., 2012; Poyart et al., 2000
Heat shock protein (*groESL*)	Hung et al., 2013; Teng et al., 2014
Ribosomal protein (*rpoB*)	Drancourt et al., 2013
Recombination and repair protein (*recN*)	Hung et al., 2013
Repair protein *recN*	Glazunova et al., 2013
Lactate oxidase gene (*lctO*)	Zlotkin et al., 1998
rRNA	Clarridge et al., 2002
RNA polymerase	Drancourt et al., 2004
D-alanine-D-alanine ligase	Garnier et al., 1997
b-subunit of the elongation factor	Picard et al., 2004
Polysaccharide capsules gene (*cps*)	Lowe et al., 2007
Invasion associated gene (*iag*)	Rajagopal, 2009
Surface immunogenic protein (*sip*)	Springman et al., 2009
C5a peptidase (*scp*)	Springman et al., 2009
Serine protease (*csp*)	Springman et al., 2009
tRNA gene intergenic spacer region (ITS)	Tung et al., 2007

CURRENT CHALLENGES IN ELUCIDATING STREPTOCOCCOSIS PATHOGENESIS

The pathogenesis of streptococcosis depends upon several factors that vary with fish species and bacterial species and isolates. Further details of virulence and pathogenicity of streptococcosis are given below.

Genetic virulence depends on several factors; for example, *S. iniae* virulence is associated with a unique genetic profile (Fuller et al., 2001). Comparison of 17 geographically different strains of *L. garvieae* based on genetic homogeneity vs. serological data showed that pathogen diversity is related to virulence factors (Barnes and Ellis, 2004). Another study showed that the lactococcal bacterial population presented a clonal structure in endemic regions, while in sporadic regions, it displayed a high genetic heterogeneity (Eyngor et al., 2004). Virulence experiments have shown that capsulated *L. garvieae* strains are more virulent than non-capsulated strains in rainbow trout (Barnes et al., 2002). Virulence varies with bacterial isolates within the same species in *S. dysgalactiae* (Abdelsalam et al., 2010). Currently, the potential for *Streptococcus* species to cross interspecies barriers and cause disease in other hosts is poorly understood.

Many streptococcal species are multi-host pathogens. Humans constantly face the risk of infection due to close interactions with the fish industry (Abdelsalam et al., 2010). *S. iniae*, *S. agalactiae*, *L. garvieae*, and *S. dysgalactiae* are human pathogens, and thus are a major threat to public health. *S. iniae* can cause bacteremic cellulitis, septic arthritis, meningitis, and endocarditis (Agnew and Barnes, 2007; Al-Harbi, 2011; Facklam et al., 2005; Lau et al., 2006; Weinstein et al., 1997), while *S. agalactiae* can cause meningitis and pneumonia in humans (Brimil et al., 2006; Johri et al., 2006).

CURRENT CHALLENGES FOR CONTROLLING STREPTOCOCCOSIS

Disease control using antimicrobials, vaccination, and environmental strategies are used extensively; however, some are associated with various downstream challenges that are mentioned in Fig. 2 and discussed in detail below.

Streptococcal diseases in fish initially affect the skin, fins, gills, and external organs. Thus, controlling infections externally through liquid disinfecting agents that can dissolve in water very easily (copper sulfate and formalin) are a good option, however, these agents cause hazardous environmental side effects.

Antibiotic resistance genes are frequently transferred among bacterial species, leading to resistant *Streptococcus* isolates and imposing a lasting risk to public health (Park et al., 2009). The selection for resistance among dense populations and drug residues in farmed fish are major concerns associated with human health; moreover, elimination of therapeutic compounds depends on several factors that can affect the fish metabolic rates such as dose rate, route of administration, water temperature, and type of isolate (Agnew and Barnes, 2007). *V. salmoninarum* is resistant to
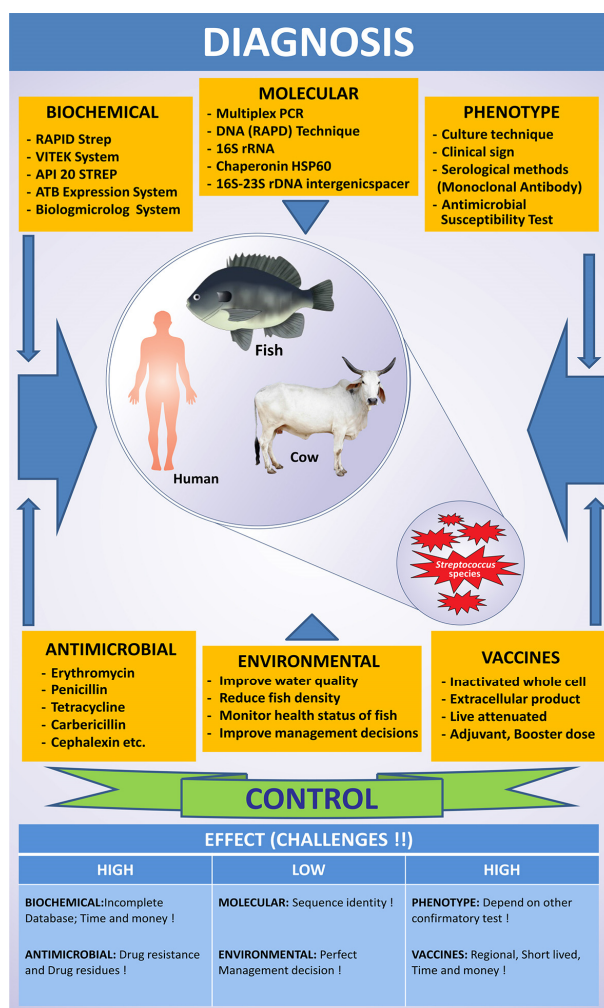

Fig. 2. Schematic diagrams of diagnosis, control, and present challenges of *Streptococcus* disease. Bottom of the diagram shows the effect of these challenges.

most antibiotics registered for aquaculture use in the European Union (Ruiz-Zarzuela et al., 2005). Studies show that the long-term use of antibiotics in olive flounder fish may have generated a higher degree of antibiotic resistance in *S. parauberis* than in *S. iniae* (Park et al., 2009). Antibiotic sensitivity and resistance vary by geographical region in *L. garvieae* (Diler et al., 2002; Ravelo et al., 2001; Vendrell et al., 2006).

The capacity of bacteria to bypass any phagocyte activity and oxidative killing of host cells is a very important step utilized for vaccination strategies (Buchanan et al., 2008). Pathogenicity of streptococcal bacteria depends on their capability to survive in host immune cells. The bacteria induce internal apoptosis while avoiding killing host cells to establish infections (Woo and Park, 2014; Zlotkin et al., 2003). The pathogenesis of *S. iniae* infection is still not fully understood as it depends upon various virulence factors and multistep processes (Zlotkin et al., 2003). Many wide-

spread vaccination programs have been unsuccessful for various reasons such as regional or short-lived effects due to limited knowledge of immunity and virulence factors in fish (Agnew and Barnes, 2007). One of the recent challenges in streptococcal disease is the histological infection of various organs (liver, spleen, kidney, brain, etc.). Thus, vaccination methods will not be effective unless they provoke a systemic immune response (Li et al., 2015). Additionally, complete knowledge of molecular and cellular processes involved in disease progression is crucial to understand various downstream complications. Vaccine preparation and optimizations solely depend on an understanding of many biological processes such as bacterial morphology, biochemical assays (serotype), pathogenicity (virulence), and immunogenicity. However, understanding molecular and cellular processes for long-term protection and cost effectiveness will be crucial to promote the widespread use of vaccines.

The environment can modulate the innate immune system in fish, so any intensive culture systems immediately make fish susceptible to infection and provide a further source for the spread of infection (Magnadottir, 2006). Studies show that increased fish density and other stress factors can elicit harmful effects in fish (Eldar et al., 1995; Shoemaker et al., 2000; 2001). A system for improving water quality and monitoring fish health is another parameter that can decrease the chances of infection, as deteriorating water quality promotes the rapid spread of bacteria and mortality (Eldar et al., 1995). Various additional strategies, such as reducing fish density by using effective physical barriers (netting), and removing moribund fish are also considered to be effective steps (Shoemaker et al., 2000; 2001).

EFFECTIVE CONTROL OF *STREPTOCOCCUS*

Effective control systems can be established through coordination and complete knowledge of the fishery industry, fish molecular and cellular biology, ecological conditions, bacterial molecular and cellular biology, and appropriate management. However, based on various studies, it is also clear that due to the unavailability of any effective and universal vaccines or antibiotics for fish diseases, environmental protective measures may be the best strategies for controlling streptococcal disease (Fig. 3).

Environmental control is most ideal as it is inexpensive, easily monitored, and is not associated with any side effects (Figs. 2 and 3). Here, we briefly discuss the important parameters that should be addressed for effective environmental control.

Studies have suggested that virulence factors play an important role in pathogenesis and disease (Barnett et al., 2015; Rajagopal, 2009). Thus, basic and advanced research at the cellular level can provide more knowledge of biological processes involved in fish resistance against streptococcal diseases. These studies should focus on understanding innate and adaptive responses through cellular (macrophages, T cell and B cell markers) and humoral (various immunoglobulin classes, complement factors, cytokines) pathways. Studies have shown that several factors are involved in the
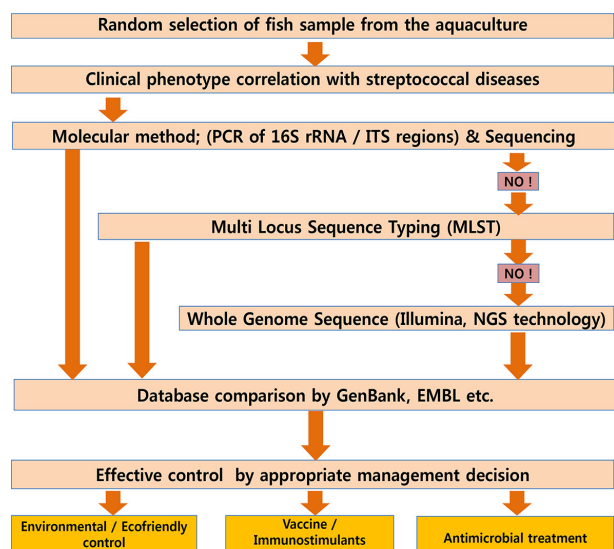

Fig. 3. Schematic diagram of effective control strategy of *Streptococcus* infection in fish.

regulation of Group B streptococci (GBS) disease pathogenesis, including pore-forming toxins and several adherence and immune evasion factors, which are reviewed in detail in Table 1 of Rajagopal, 2009. Additionally, signal transduction systems (STSs) are also important drug targets for effective disease control (Barrett et al., 1998; Rajagopal, 2009). Several *S. agalactiae* factors, such as polysaccharide capsule, hemolysin, superoxide dismutase, and D-alanylated lipoteichoic acid, hold major importance for virulence (Lindahl et al., 2005). Various surface proteins of *S. agalactiae* participate in important functions during disease progression, thus they are also considered important vaccine candidates (Carey-Ann et al., 2003; Lindahl et al., 2005).

Many immunostimulants can stimulate pathogen-associated molecular patterns (PAMPS), which are part of fish immune systems as pathogen recognition receptors (PRRs) and participate in maintaining innate immune protection for fish (Chettri et al., 2011).

Recently, dietary intake of bacterial components, polysaccharides, animal-derived nutrients, plant extracts, nutritional factors, and cytokines has been reported to be an effective method for immunostimulation in fish (Sakai, 1999; Villegas et al., 2006). Further advanced cellular studies of immunostimulants are required for understanding various downstream cellular activities such as hemotaxis, respiratory burst, phagocytosis, and lysozymes to establish the most effective stimulants for fish. Additionally, the development of a range of assays such as immunohistochemistry, immunocytochemistry, flow-cytometry, and gene expression technology combined with *in vivo* challenge studies can improve our understanding in a more advanced way and help design effective vaccines or therapeutic agents for fish diseases.

Knowledge of genetic virulence factors is important for understanding disease mechanisms and progression. Unfor-

tunately, studies have been limited to rat and mouse models for various streptococci so far. The molecular basis of virulence was described in detail by Bennedsen et al. in 2011 (Bennedsen et al., 2011; Springman, 2009). Virulence factors vary with the streptococci strain and host since host/external environments are also responsible for variable expression of virulent genes.

Some important genetic virulence factors like polysaccharide capsules gene (*cps*), invasion associated gene (*iag*), surface immunogenic protein (*sip*), C5a peptidase (*scp*), serine protease (*csp*), and many surface proteins have been studied extensively in various streptococcus species (Table 2) (Lowe, 2007; Rajagopal, 2009; Springman, 2009). Additional factors regulating expression of virulence genes, like STSs, membrane associated sensor histidine kinase (HK), and toxins can also be targeted for disease pathogenesis (Lowe, 2007; Rajagopal, 2009; Springman, 2009). Genomic comparison of virulent and non-virulent *S. agalactiae* showed differences among isolates that infect fish, humans, cattle, and sea mammals; moreover, several host (fish) genes translate proteins that are responsible for adaptation in the aquatic environment (Delannoy et al., 2016). Isolates from one host are not necessarily pathogenic to other hosts or to different strains of the same fish (Nishiki et al., 2010).

The recent introduction of 'next-generation' sequencing technology has brought a revolution in bacterial research as many bacterial genomes and antimicrobial resistance and virulence genes are available for analysis (Chain et al., 2009; Kwong et al., 2015; Medini et al., 2008). Currently, the use of whole-genome sequencing (WGS) for typing any bacterial agent is possible in a cost effective and timely manner (Kwong et al., 2015). The Genus *Streptococcus* comprises diverse bacterial species that emerged through the course of evolution with various known and unknown evolutionarily important factors distributed worldwide. These evolutionary genetic, environmental, biochemical, and physiological factors shape streptococcus at the phenotype and genotype levels. Among them, genetic factors such as genomic variation through addition, reduction, and gene transfer play a crucial role for appropriately measuring exponential growth of diversified streptococcus genomes. Therefore, a future study should examine geographically distributed strains as they develop specific variations under different environmental conditions, which can affect their functional properties, and ultimately their virulence.

Choosing conserved genes (e.g., *16S rRNA, ITS*) over other genes relies on the fact that any variation observed can be directly correlated to the physiology and virulence properties of an organism (Nho et al., 2013) and for understanding evolutionary relationships and disease epidemiology (Dobrindt and Hacker, 2001; Sakala et al., 2002; Wren, 2000). Additionally, a well-defined 16S rRNA and ITS database for sequence comparisons suggested that molecular techniques are appropriate for both taxonomic and identification purposes (Mishra et al., 2017; Mora et al., 2003; Yoon et al., 2017). Advanced genomics refers to high-throughput genetic technologies (based on DNA or RNA nucleotides) and their evaluation through comparative,

functional, or environmental parameters (Gao et al., 2014). Comparative genomics through chemotaxonomic, DNA-DNA hybridization, and 16S rRNA sequencing approaches should be further evaluated occasionally through advanced computational technologies for establishing the evolutionary relationship among strains.

In this review, based on the NCBI database, we selected and analyzed the 16S rRNA gene in 51 representative worldwide strains of streptococcus species (*S. parauberis, S. iniae, S. agalactiae, L. garvieae, S. dysgalactiae,* and *V. salmoninarum*) (Table 3 and Supplementary Table 1). Our phylogenetic analysis demonstrates the interrelation of various strains and suggests that the 16S rRNA gene can easily segregate various strains based on genetic variations (Fig. 4 and Supplementary Fig. 1). The evolutionary history was inferred using the Neighbor-Joining method and MEGA7. Earlier studies of streptococci microevolution have transferred to knowledge about the therapeutic developments (Gao et al., 2014). Although our phylogenetic dendrograms clarify strain diversity according to 16S rRNA, evaluating other genetic factors related to virulence or adaptation will further elucidate pathogenic and evolutionary mechanisms.

Fully developed fishery management systems according to current challenges (diagnosis, pathogenesis, and control mechanism) are required. Understanding fish management at the local level is useful for the aquaculture industry (Aquilera et al., 2015). We therefore suggest an effective aquaculture management module that utilizes a scientific approach to reconcile complex data of various cellular, molecular, and environmental approaches, and monitoring programs generated through research surveys.

Our study has important implications for the epidemiology of streptococcosis in fish, provides important information about the current scenario and challenges in the fish industry, and suggests joint molecular (for diagnosis) and cellular (for control) strategies along with environmental control methods as appropriate (Tables 1, 2 and Figs. 1-4). The above integrated strategies should focus on a worldwide sampling network for appropriate evolutionary monitoring through comparative genomics, then combine this information with ongoing aquaculture management systems.

CONCLUSION AND FUTURE PROSPECTS

Streptococcal infectious diseases, along with complicated control mechanisms, have contributed to a considerable decrease in fish production. This review highlights the current status of *Streptococcus* bacterial diagnosis, pathogenesis, hazardous effects of the host fish, and the overall negative economic impacts on the fish industry. In the absence of extensive knowledge about virulence, new emerging strains, treatments, and control of streptococcal disease, streptococcosis is becoming difficult to control. Based on the information presented in this review, we recommend early diagnosis using molecular methods and improved cellular research along with environmental control through appropriate managerial decisions considering the present scenario.

Table 3. Epidemiological specification (accession number, strain, and geographical location) of Streptococcus bacterial agents based on 16S rRNA gene sequences from the NCBI database

| Species	Accession number	Strain	Geographical region/country
Streptococcus iniae	DQ985468.1	CGX	China
	KY781829.1	HNM-1	China
	KJ162337.1	Ab130920	China
	KF815728.1	WZMH110819	China
	KF555592.1	NS1-2011	Thailand
	KC748467.1	FC0924	China
	KM209199.1	SK10-S	Indonesia
	AB593340.1	Feb-45	Japan
Streptococcus parauberis	AY942573.1	LMG 14376	Finland
	FJ009631.1	JJI51	Korea
	JQ780604.1	partial sequence	Israel
	KC836715.1	RU37-6	China
	AF284579.2	SAP 99	Italy
	KP137361.1	F21	Turkey
	KP137342.1	F57	Turkey
	KP240952.1	CIFT MFB 10119(2)	India
	KC699192.1	CNM465_12	Spain
Streptococcus agalactiae	LC071815.1	JCM 5671	Japan
	AB596948.1	JCM 5671	Japan
	DQ303183.1	ATCC 13813	Canada
	AB002479.1	ATCC 13813- NCTC 8181	Japan
	NR_117503.1	ATCC 13813	USA
	NR_115728.1	ATCC 13813	USA
	GU360730.1	ATCC 13813	Netherlands
	KT869025.1	SAG	Malaysia
	KY635952.1	S29	Brazil
	KY635949.1	S73	Brazil
Streptococcus dysgalactiae	AB002485.1	ATCC 43078	Japan
	AB002500.1	isolate L32	Japan
	AB002509.1	isolate L9	Japan
	NR_027517.1	ATCC 43078	USA
	DQ232540.1	CIP 105120	France
	JN639380.1	CCUG 7977A	Denmark
	JN639434.1	SK1333	Denmark
	JN639432.1	CCUG 36637	Denmark
	JN639410.1	CCUG 48101	Denmark
	AB002484.1	ATCC 27957	Japan
	AY121361.1	ATCC 12394	China
	AF015928.1	ATCC 27957	USA
	AJ314611.1	AC-2074	Germany
	AJ314609.1	AC-2713	Germany
	AJ314610.1	AC-2832	Germany
Lactococcus garvieae	LC145570.1	JCM 12256	Japan
	KF111340.1	TRF1	USA
	HM536980.1	PW1537	China
	KX671996.1	FJ6	Iran
	KF849271.1	SI-IRI	Iran
	AF352164.1	FLG4	China
	KM659863.1	Fish 10/10 LKF	South Africa
Vagococcus salmoninarum	AM490375.1	JIP 20-00	France
	AM490374.1	JIP 27-01(2)	France
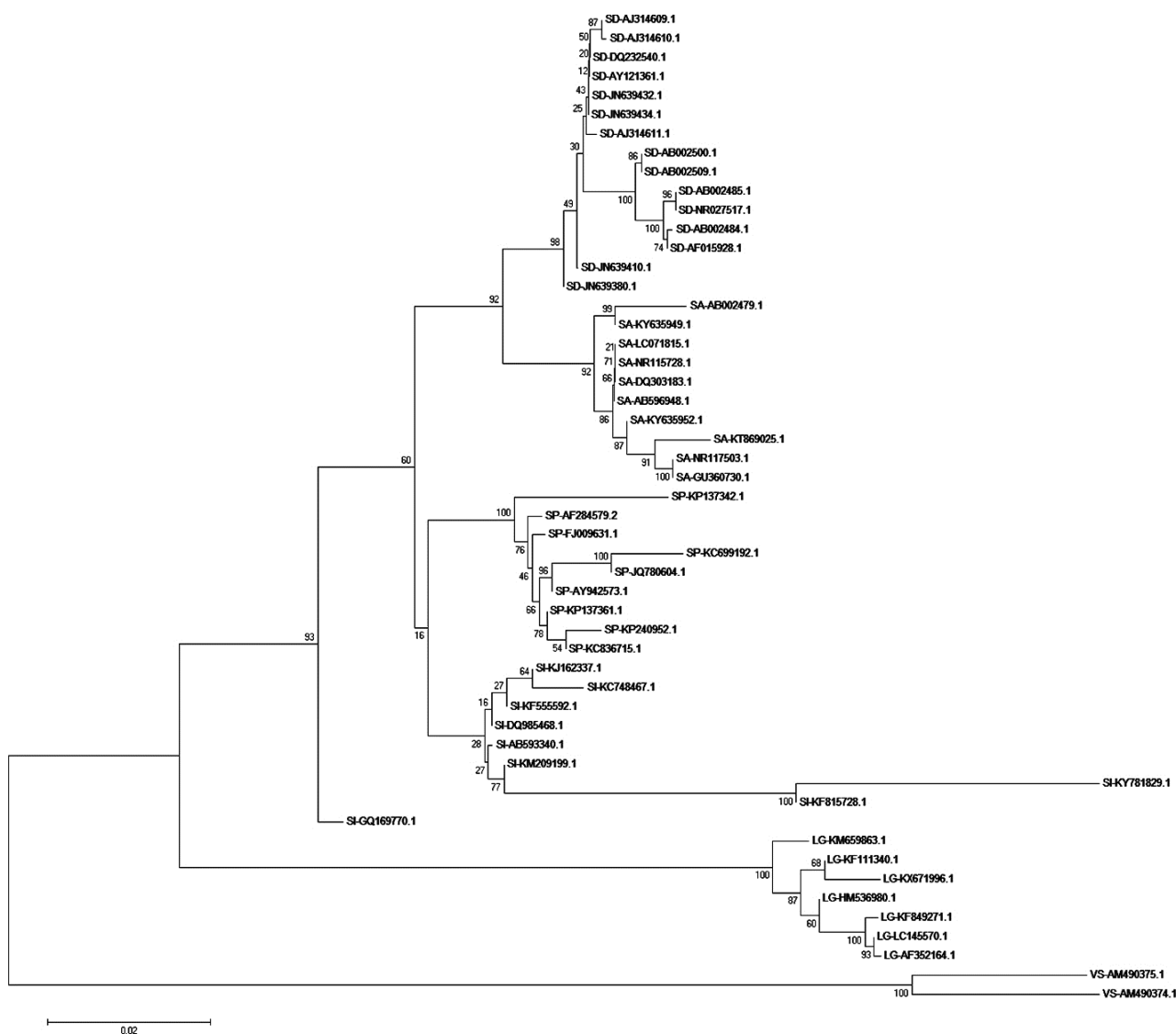
Fig. 4. Phylogenetic analysis of total 51 bacterial strains of *Streptococcus* species (*S. parauberis, S. iniae, S. agalactiae, Lactococcus garvieae, S. dysgalactiae, and Vagococcus salmoninarum*) causing streptococcal diseases based on 16S rDNA sequences from the NCBI database. The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 0.54759876 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) is shown next to the branches.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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