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RESEARCH ARTICLE

Comparative evaluation of 16S rRNA gene in world-wide strains of *Streptococcus iniae* and *Streptococcus parauberis* for early diagnostic marker

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Abstract Two bacterial etiological agents of the disease, *Streptococcus iniae* and *Streptococcus parauberis* has been associated with fish mortalities and heavy economic loss in all over the world. Bacterial identification based on 16S rRNA sequencing is very fast, accurate and reliable in comparison to other traditional phenotype methodologies. In this study, we investigate the usefulness of this method for diagnosis and identification of Streptococcus species. We have selected 61 phylogeographic strains of Streptococcus (34 strains of *S. iniae* and 27 strains of *S. parauberis*) and designed the universal primer against the identified most hypervariable region of the 16S rRNA gene. Our universal primer able to identify any geographical strains and offers a useful and fast alternative in a clinical laboratory under routine conditions. Based on our studies, we have developed an algorithm for appropriate control of *S. iniae* and *S. parauberis* disease. We suggested the phenotype observation along with universal primer combination to detect any kind of infection or carriers at early stages.

Keywords Phylogenetic tree · Geography · Sequencing · *Streptococcus iniae* · *Streptococcus parauberis*

Introduction

Streptococcus species is gram-positive coccus that causes streptococcosis (septicemia disease) affecting both captive and wild populations of fresh water and marine fish species throughout the world. First Streptococcal infections in fish were reported to rainbow trout (*Oncorhynchus mykiss*) in Japan in 1958 (Hoshina et al. 1958). Fish farming one of major growing industry, facing problems of controlling streptococcal infection in cultured fish populations (Agnew and Barnes 2007; Austin and Austin 2007; Baeck et al. 2006; Shoemaker et al. 2001; Toranzo et al. 2005). Various bacterial agents caused streptococcosis such as *Lactococcus garvieae, Streptococcus parauberis, Streptococcus iniae, Streptococcus agalactiae* and *Streptococcus difficilis* (Nho et al. 2009; Toranzo et al. 2005). Out of above, two main etiologic agents *S. iniae* and *S. parauberis* found predominantly regardless of geographical regions.

Streptococcus parauberis was first reported from turbot (*Scophthalmus maximus*) cultured in Spain (Domeénech et al. 1996). Important fish species infected by *S. parauberis* were Olive flounder, Rainbow trout, cultured Turbot and Hybrid striped bass (Domeénech et al. 1996; Hoshina

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et al. 1958; Shoemaker et al. 2001). Apart from fish, *S. parauberis* also infect the dairy cow (Williams et al. 1990).

Another bacteria *S. iniae* has been also associated with disease outbreaks of different freshwater and marine fish species (Agnew and Barnes 2007). It is first isolated in 1976 from a subcutaneous abscess of a captive freshwater dolphin *Inia geoffrensis* (Pier and Madin 1976). It infects various fish species like, hybrid striped bass, Nile tilapia, hybrid tilapia, rainbow trout, red drum, rabbitfish, sea bass, flounder, barramundi and other fishes (Agnew and Barnes 2007; Al-Harbi 2011; Cheng et al. 2010; Klesius et al. 2006; Zlotkin et al. 1998). In addition to fish, *S. iniae* is also known to be an opportunistic human pathogen, which is threat to public health by causing bacteremic cellulitis, meningitis, endocarditis, and septic arthritis (Agnew and Barnes 2007; Al-Harbi 2011; Facklam et al. 2005; Lau et al. 2006; Sun et al. 2007; Weinstein et al. 1997). Both the bacterial agents showed that complex clinical symptoms during disease, which also vary with fish species (Table 1). Phylogeographically, *S. iniae* and *S. parauberis* were present in all over continents (Americas, Asia, Europe, Australia and Africa). The particulars of both bacterial agents (fish species, clinical symptoms and geographical region) were given in Table 1. The identification of bacterial infection was always challenging because of variation on phenotype characteristics due to effect of ecology, environment and host itself. Currently, there were several detection methods available for proper diagnostic and identification for *S. iniae* and *S. parauberis*. These detection methodologies varies from biochemical, molecular to immunological categories. The biochemical methods were

Table 1 Streptoccocus bacterial agents and detail information of affected fishes, locations, host and clinical criteria with references

Particular	*Streptoccoccu iniae*	*Streptoccoccu parauberis*	References
Fish	Hybrid striped bass, Nile tilapia, Hybrid tilapia, Rainbow trout, Rabbitfish, Sea bass, Japanese flounder, Barramundi, Red drum	Olive flounder; Rainbow trout Cultured turbot spin Hybrid striped bass	Cho et al. (2008), Shoemaker et al. (2001) and Agnew and Barnes (2007)
Geographical location	Canada, USA, Brazil, Caribbean, Bahrain, Israel, Iran, Singapore, Thailand, China, Japan, Korea	USA, Brazil, Bahrain, Israel, Tunisia, Spain, Norway, Iran, India, Thailand, Malasia,China, Japan, Taiwan, Korea	Baeck et al. (2006), Nho et al. (2013), and Agnew and Barnes (2007)
Host	Fish, Human	Fish, Cow	Lau et al. (2006), Weinstein et al. (1997), and Williams et al. (1990)
Clinical Criteria	Haemorrhage, Exophthalmia, Abdominal distension, Lesions	Haemorrhage, Exophthalmia, Abdominal distension, Ascites, Lesions	Nho et al. (2009), Kanai et al. (2009), and Agnew and Barnes (2007)

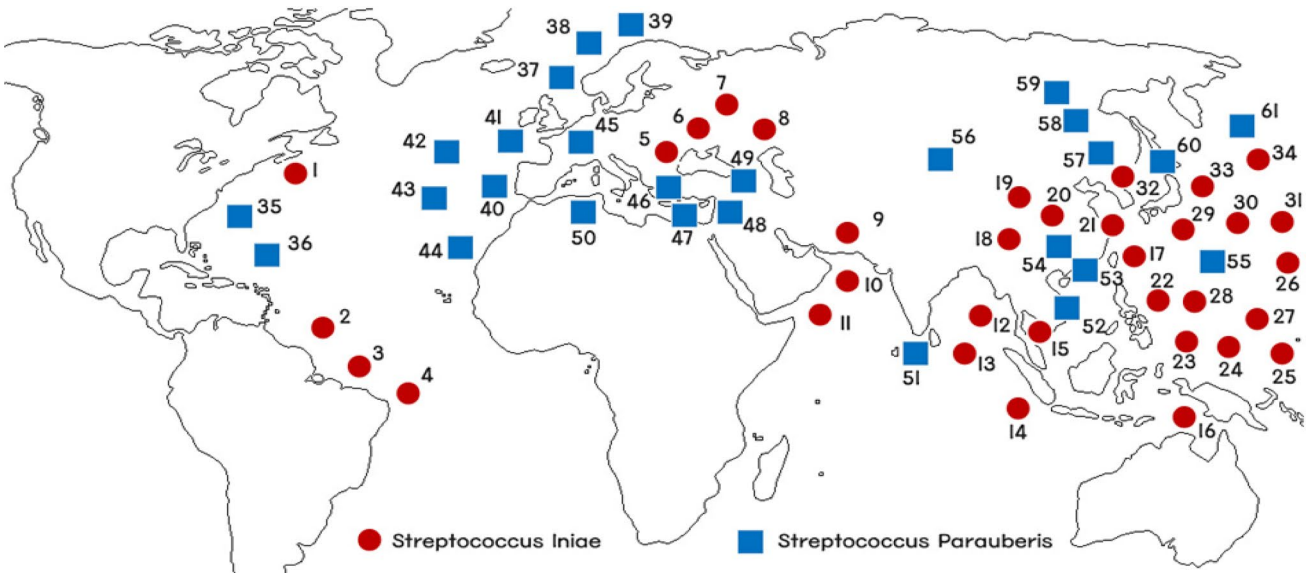


Fig. 1 Phylogeography study of bacterial agents (*Streptococcus iniae* and *Streptococcus parauberis)* of streptococcal diseases. *Red color circle* from 1 to 34 represent 34 strains of *S. iniae* and *blue color square* from 35 to 61 represents 27 strains of *S. parauberis*

Table 2 Data on 34 strains of *Streptococcus iniae* and 27 strains of *Streptococcus parauberis* studied for phylogenetic and phylogeography analysis

ID	Bacterium species	Strain	Base pair	Geographical location	GenBank Accession No.
1	*Streptococcus iniae*	ATCC 29178	1466	Canada	DQ303187.1
2	*Streptococcus iniae*	ZO7	839	Brazil	KJ561074.1
3	*Streptococcus iniae*	81FG	862	Brazil	KJ561076.1
4	*Streptococcus iniae*	SI25-06	702	Brazil	FJ803997.1
5	*Streptococcus iniae*	Dan 1	1490	Israel	AF335573.1
6	*Streptococcus iniae*	s41	1399	Israel	AY260834.1
7	*Streptococcus iniae*	maris rubric 041110	1114	Israel	JQ780607.1
8	*Streptococcus iniae*	ATCC 29178	1536	Israel	NR_025148.1
9	*Streptococcus iniae*	SF2	1384	Iran	FJ870987.1
10	*Streptococcus iniae*	L2B	223	Iran	KC708485.1
11	*Streptococcus iniae*	2010-LG3	1399	Iran	HM055572.1
12	*Streptococcus iniae*	NS45-2011	1137	Thiland	KF555596.1
13	*Streptococcus iniae*	JW9	1141	Thiland	GQ338315.1
14	*Streptococcus iniae*	TSK-2	1399	Malasia	KT722586.1
15	*Streptococcus iniae*	DM4279/05	513	Singapore	DQ193526.1
16	*Streptococcus iniae*	SIJW 1	516	Indonesia	KJ408270.1
17	*Streptococcus iniae*	SCCF5L	1486	Taiwan	AY762259.1
18	*Streptococcus iniae*	CGX	1447	China	DQ985468.1
19	*Streptococcus iniae*	WZMH110819	1541	China	KF815728.1
20	*Streptococcus iniae*	Ab131025	1404	China	KJ162338.1
21	*Streptococcus iniae*	HRS12718K	1513	China	KF264475.1
22	*Streptococcus iniae*	SF1	501	China	GQ891547.1
23	*Streptococcus iniae*	CMS004	1465	China	EU620577.1
24	*Streptococcus iniae*	TSG004	1399	China	KF826094.3
25	*Streptococcus iniae*	FC0924	1399	China	KC748467.1
26	*Streptococcus iniae*	YG1	1399	China	GQ169798.1
27	*Streptococcus iniae*	DGX01	1399	China	HM053435.1
28	*Streptococcus iniae*	YZ-1	1513	China	JQ990158.1
29	*Streptococcus iniae*	TOS08	1509	China	KP729644.2
30	*Streptococcus iniae*	GZSR-3	1403	China	KJ847243.1
31	*Streptococcus iniae*	ZS2013711-2	1399	China	KJ183030.1
32	*Streptococcus iniae*	FPS-76	1463	Korea	JQ945262.1
33	*Streptococcus iniae*	I1	1493	Japan	AB470235.1
34	*Streptococcus iniae*	Feb-45	1438	Japan	AB593340.1
35	*Streptococcus parauberis*	RP25	1419	USA	JQ988835.1
36	*Streptococcus parauberis*	DSM 6631	1471	USA	AY584477.1
37	*Streptococcus parauberis*	LMG 14376	1459	Finland	AY942573.1
38	*Streptococcus parauberis*	332	1485	Finland	AY942572.1
39	*Streptococcus parauberis*	Mast_7	680	Finland	JQ953671.1
40	*Streptococcus parauberis*	83C-LHICA	803	Spain	JN630846.1
41	*Streptococcus parauberis*	DSM 6632	803	Spain	JN630844.1
42	*Streptococcus parauberis*	CNM472_12	1001	Spain	KC699197.1
43	*Streptococcus parauberis*	3AA4	323	Spain	DQ313166.1
44	*Streptococcus parauberis*	ATCC 19436	803	Spain	JN630842.1
45	*Streptococcus parauberis*	SAP 99	1541	Italey	AF284579.2
46	*Streptococcus parauberis*	H32	950	Israel	EF204350.1
47	*Streptococcus parauberis*	D104	764	Israel	EF204342.1
48	*Streptococcus parauberis*	maris rubric 250409	1114	Israel	JQ780604.1
49	*Streptococcus parauberis*	F21	1331	Turkey	KP137361.1

Table 2 (continued)

ID	Bacterium species	Strain	Base pair	Geographical location	GenBank Accession No.
50	*Streptococcus parauberis*	BMG 149	1407	Tunisia	EU081009.1
51	*Streptococcus parauberis*	CIFT MFB10119-2	1432	India	KP240952.1
52	*Streptococcus parauberis*	DT8	926	Vietnam	GQ247721.1
53	*Streptococcus parauberis*	PLB-2	1045	China	KT825567.1
54	*Streptococcus parauberis*	8-7	1013	China	KJ477389.1
55	*Streptococcus parauberis*	RU37-6	1340	China	KC836715.1
56	*Streptococcus parauberis*	IMAU11138	1403	Mangolia	KR858792.1
57	*Streptococcus parauberis*	JJI51	1510	South Korea	FJ009631.1
58	*Streptococcus parauberis*	EP8MR-1	924	South Korea	KF193975.1
59	*Streptococcus parauberis*	FPS-93	1003	South Korea	JQ945267.1
60	*Streptococcus parauberis*	FPS-58	1009	South Korea	JQ945266.1
61	*Streptococcus parauberis*	FPS-10	887	South Korea	JQ945265.1

depending upon phenotype characteristics of species and strains of bacteria and varied from plate cultivation, biochemical techniques, enzyme reactions, phenotypic analysis and whole cell fatty acid analysis (Bosshard et al. 2006; Cheng et al. 2010; Dodson et al. 1999; Klesius et al. 2006; Shoemaker et al. 2000). However, above techniques were time-consuming, cross reactive and pass through various downstream challenges like absence of proper-catalogued database for comparison, mixed nature of aquaculture environment, fewer amounts of biological samples or some time unknown tissue locations of infectious agents, which always make the decision difficult (Klesius et al. 2006). In molecular methodologies, candidate gene sequencing method is very appropriate for diagnosis and identification among bacterial isolates (Goh et al. 1998; Petti 2007; Poyart et al. 1998). Moreover, 16S rRNA gene sequencing method used preferable for over other candidate gene methods (Al-Harbi 2011; Austin and Austin 2007). Study shows that, 16S rDNA sequencing has become the reference method for bacterial taxonomy and identification due to its presence in almost all bacteria, relatively short of size (approximately ~1500 bp) as well as conserved in nature (Baker et al. 2003; Janda and Abbott 2007; Petti et al. 2005; Van de Peer et al. 1996). It also seems to be of interest in bacteria that remained unidentified or mis-identified by phenotypic methods (Drancourt et al. 2000; Petti et al. 2005). There are well defined Public database and software packages (GenBank, Nucleotide Sequence Database at European Molecular Biology Laboratory, DNA Data bank of Japan), Ribosomal Database project (RDP II), SmartGene IDNS, MicroSeq and Ribosomal Differentiation of Medical Microorganisms (RI-DOM) available for rapid phylogenetically analysis, due to vast number of bacterial 16S rDNA sequences deposited regularly (Bosshard et al. 2006; Woo et al. 2008; Maidak et al. 2000; Cole et al. 2007; Patel et al. 2000; Harmsen et al. 2003). The databases of RDP-II and SmartGene IDNS contain sequences downloaded from GenBank, whereas all sequences in the databases of RIDOM and MicroSeq were obtained by sequencing the 16S rDNA genes of bacterial strains of culture collections (Woo et al. 2008). Although, 16S rRNA sequencing method is not full proof of diagnosis due to various limitations attached to it, but many amendments and recommendations make it always preferential choice over other traditional methodologies (Drancourt et al. 2000; Mignard and Flandrois 2006; Stackebrandt and Goebel 1994).

Importantly, the existence of different types of *Streptococcus* species and strains of *S. iniae* and *S. parauberis* emphasizes the difficulties of definitive identification based on phenotypic traits alone (Fig. 1; Table 2). Continuously growing fish farming via uncontrolled exchange of fishes across various countries, creates problem of controlling various infections, which not only affect the health of human beings but also impart negative on economy (Shoemaker et al. 2000; Toranzo et al. 2005). Presently aquaculture is one of big industry, adopting strategies for maximum economic benefit with the culture of huge numbers of maximum varieties of fishes in bigger culture system (artificial or natural conditions). However, due to various risk factors, fishes were always facing chances of *streptococcal* infection, so there is urgent requirement to develop cheap and rapid method which helps in monitoring even at large sample size, regardless of any biological materials in any mixed clinical phenotype at first instance. The objective of this study was to review the use of 16S rDNA sequences to develop a rapid and cheapest method to detect and identify *S. iniae* and *S. parauberis*, in aquaculture industry thereby providing an improved diagnostic technique for this major fish pathogen.

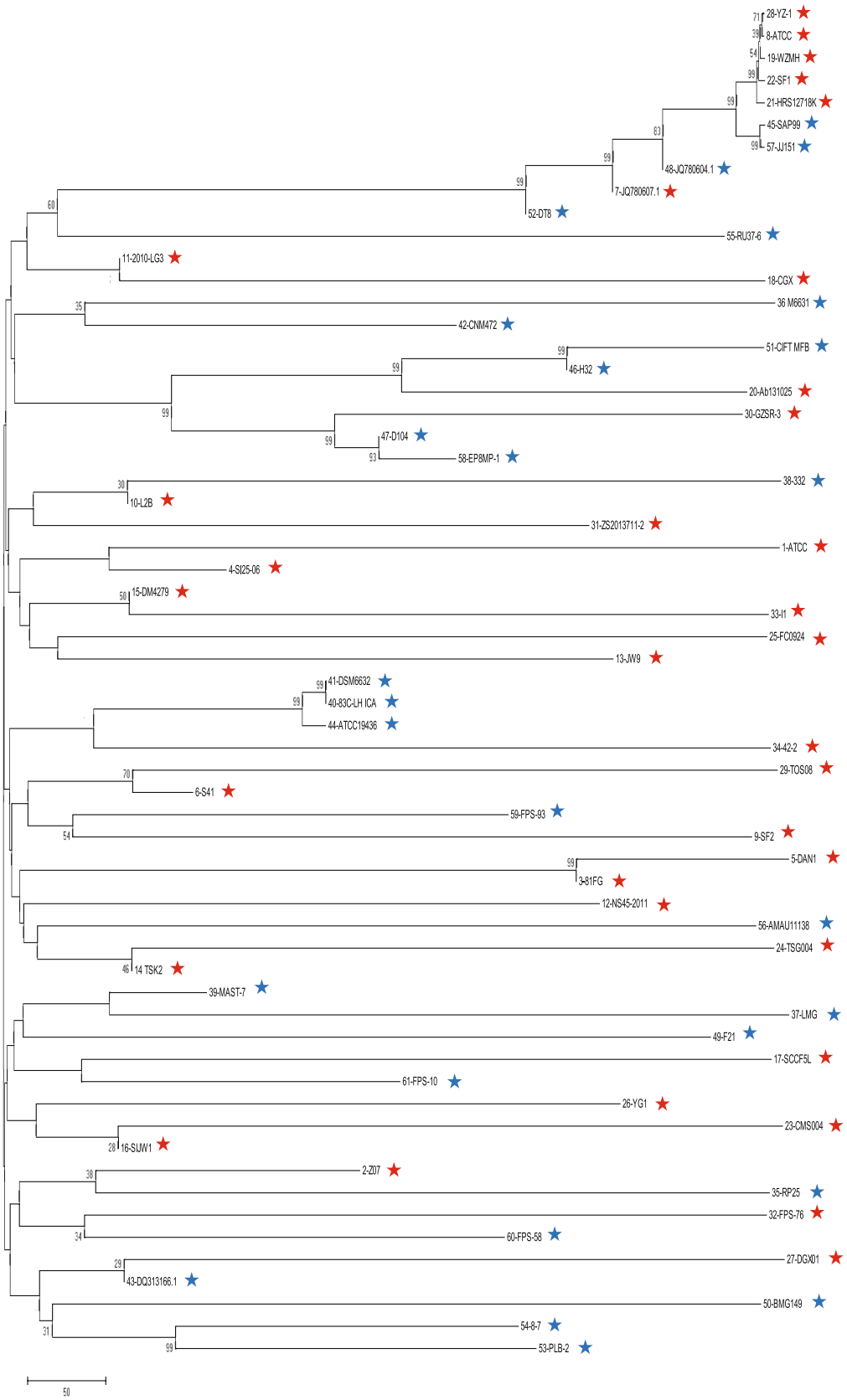
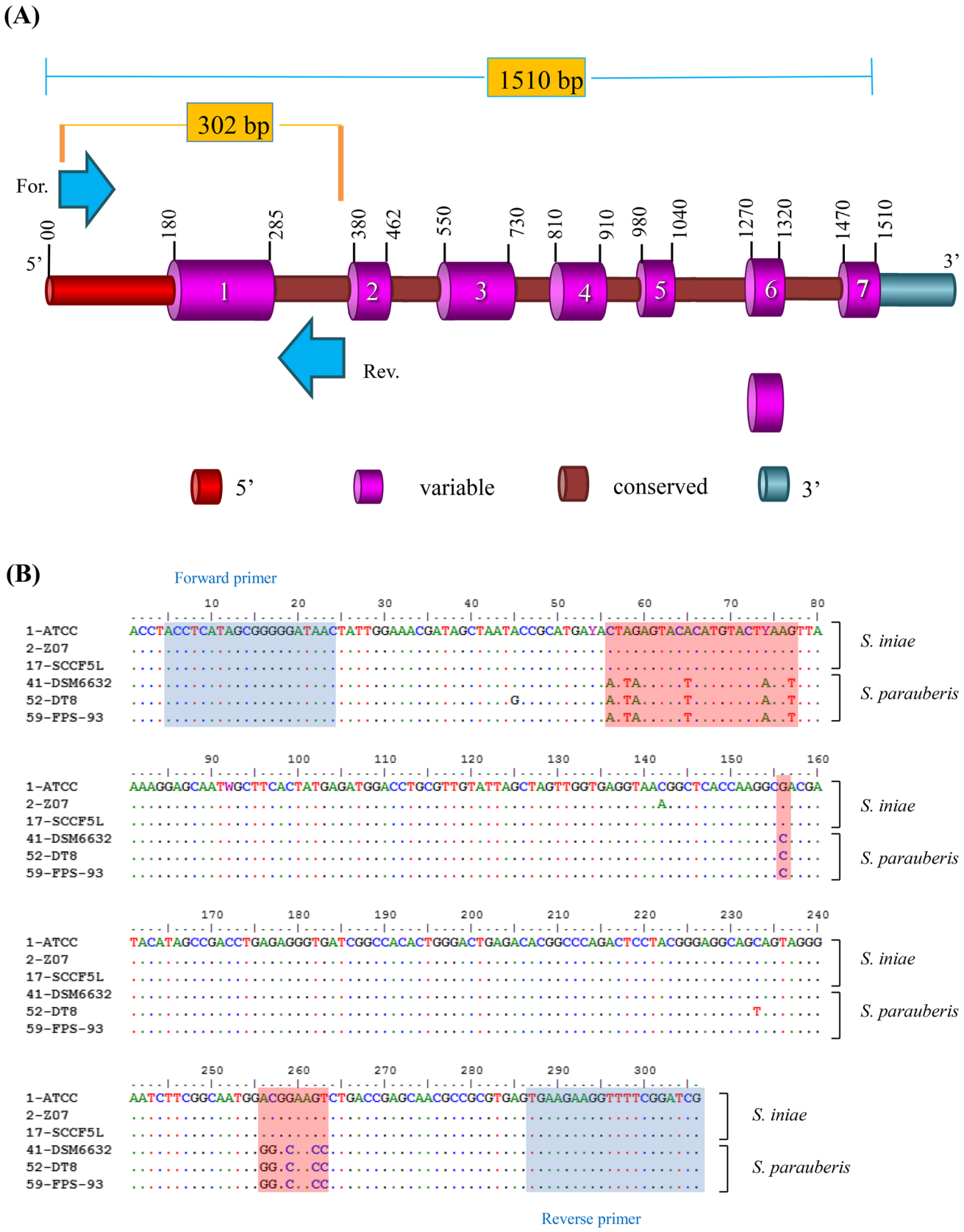


Fig. 2 Phylogenetic analysis of total 61 bacterial strains of *Streptococcus iniae* and *Streptococcus parauberis*. *Red color stars* represent 34 strains of *S. Iniae* and *blue color stars* represent 27 strains of *S. parauberis*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7
5'
variable
conserved
3'
(B)
Forward primer
1-ATCC
2-Z07
17-SCCF5L
41-DSM6632
52-DT8
59-FPS-93
S. iniae
S. parauberis
Reverse primer

◂**Fig. 3** **a** Structure of *16S rRNA* gene under analysis (conserved and variable regions were indicated). Figure highlighted the amplified region of 302 base pair in *16SrRNA* gene. **b** Schematic representation of conserved and variable regions and forward and reverse primer binding sites of *16S rRNA* gene. Three representative strains of *S. Iniae* and *S. parauberis* were respectively selected to demonstrate the *blue box* (primer binding region) and *red box* (variable region of *S. parauberis*) in the amplified region of gene

Materials and methods

Streptococcus iniae and *Streptococcus parauberis* isolates and geographical distribution

A total 61 bacterial strain (34 *S. iniae* and 27 *S. parauberis*) were selected from NCBI database belonging to various geographical regions of wormld (Table 2). The phylogeography analysis demonstrate bacterial distributions in the entire continent of earth (Table 2; Fig. 1).

Multiple sequence alignment and identification of variable and conserved regions

A total 61 bacterial strain (34 *S. iniae* and 27 *S. parauberis*) sequences were downloading from NCBI database. These sequences were aligned in BIOEDIT to find out the variation in sequences. We used BLAST (Basic Local Alignment Search Tool) searches in GenBank database for multiple sequence alignment of 61 strains of *S. iniae* and *S. parauberis*, and found the most hyper-variable region in 16S rRNA gene (Supplementary Fig. 1).

Phylogenetic analysis

Construction of a phylogenetic tree were performed using MEGA6 program via FASTA format (Tamura et al. 2013). The phylogenetic trees of *S. iniae* and *S. parauberis* together and separately constructed on the basis of Neighbor-joining method. Bootstrap evaluation of the branching patterns was analyzed with 1000 replications. The phylogenetic trees of *S. iniae* (from ID 1 to 34; red star mark) and *S. parauberis* (from ID 35 to 61; blue star mark) demonstrated in Fig. 2.

Primer design

Universal primer designed to be complementary to the conserved regions of the groups (61 bacterial strains) present in Table 2 (Fig. 3a, b; Supplementary Fig. 1). The transcripts were assessed by PCR using the primer sets of: 5′-ACCTCATAGCGGGGGATAAC-3′ and 5′-CGATCCGAAAACCTTCTTCA-3′.

Streptococcal bacterial strain assessment

The two bacterial strains of *S. iniae* (SI-BS9) and *S. parauberis* (SPOF3K) were investigated for validation of primers. These two strains were obtained from microbiology department of Pusan National University, Busan, Korea. Genomic DNA (gDNA) extraction was conducted using the KIT (Qiagen) and primer pairs were tested against gDNA extracted from above two strains.

Primers were validated for temperature and concentration and standerised at annealing temperatures of between 54 and 58 °C. Cycling parameters included initial denaturing for 3 min at 95 °C; 35 cycles of 1 min at 94 °C, 1 min at 57 °C and 2 min at 72 °C. The amplification size is 302 base pair. A single and sharp peak was detected in each primer pair, which indicates that the primers amplify one specific product in PCR (Fig. 4a–d). No dimers derived from primer sets were detected. Further, PCR products were analyzed quantitatively using the Image-J program (Fig. 4b, d) and demonstrate the quality of primer set.

16S rDNA quantification by real time PCR

The gram positive Streptococcus bacterial strains *S. iniae* and *S. parauberis* was subjected to quantitative Real Time PCR assay (Supplementary Fig. 2A, B).

Sensitivity of primer

The PCR has shown that prime can easily detect *S. iniae* and *S. parauberis* at 1 picogram of DNA concentrations respectively (Fig. 4a–d). The quantitative Real time PCR has shown that prime can easily detect *S. iniae* and *S. parauberis* at 0.1 pg of DNA concentrations respectively (Supplementary Fig. 2A, B).

Results and discussion

We have designed universal primer for the most variable region of 16S rRNA gene, of Streptococcus species (*iniae* and *parabauris*). Since, this primer was designed on the basis of 16S rDNA sequence variation information about all geographically important 61 strains, so it may act as effective “universal primer” which can be used on worldwide strains for diagnosis and identification purpose. The geographical representation of strains highlighted the importance of study for making single diagnosis marker for cosmopolitan *S. iniae* and *S. parauberis* bacterial agents (Table 2; Figs. 1, 2). Many study shows that genetic

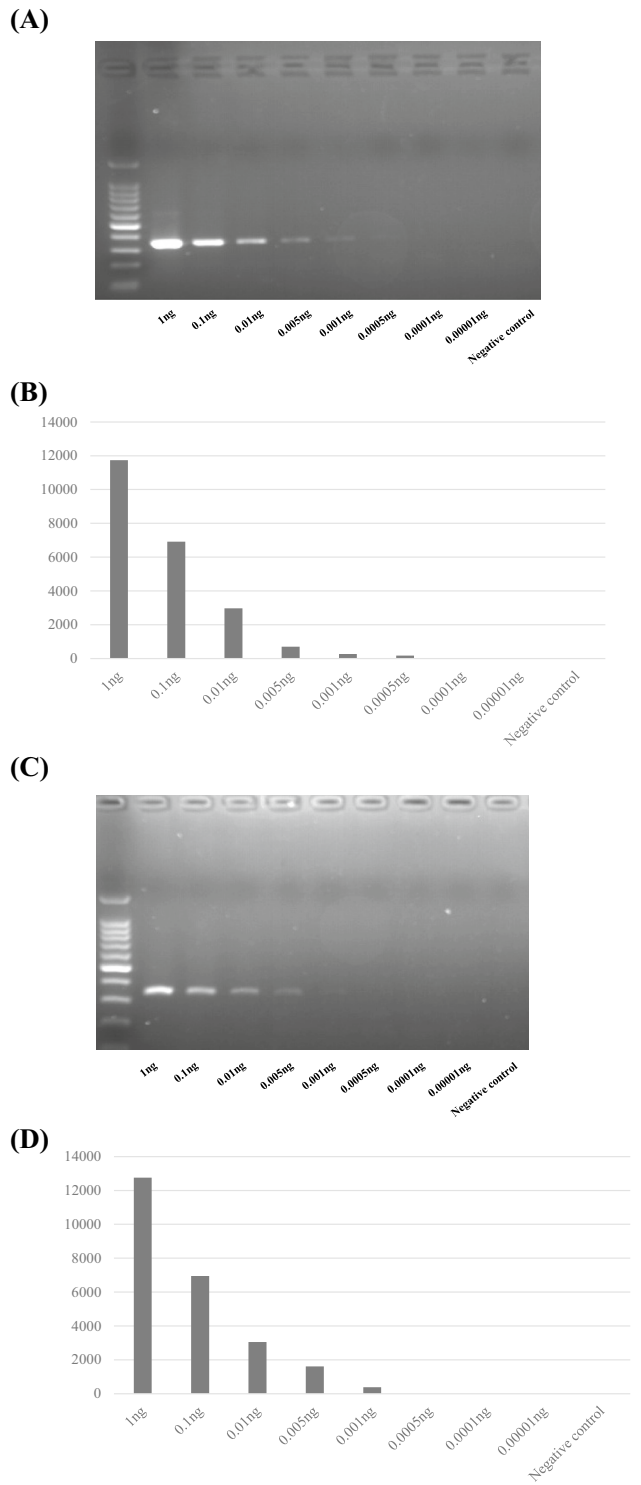

Fig. 4 **a** PCR amplification of *Streptococcus iniae* genetic material (gDNA) with the universal primer at concentration from 1, 0.1, 0.05, 0.01, 0.005, 0.001, 0.0005, 0.0001, 0.00001 to 0 ng respectively, **b** PCR products were analyzed quantitatively using the Image-J program. The *X-axis* of the *bar graph* indicates concentrate of *Streptococcus iniae* gDNA samples and the *Y-axis* of the *bar graph* indicates the relative amplification levels. **c** PCR amplification of *Streptococcus parauberis* genetic material (gDNA) with the universal primer at concentration from 1, 0.1, 0.05, 0.01, 0.005, 0.001, 0.0005, 0.0001, 0.00001 to 0 ng respectively, **d** PCR products were analyzed quantitatively using the Image-J program. The *X-axis* of the *bar graph* indicates concentrate of *Streptococcus parauberis* gDNA samples and the *Y-axis* of the *bar graph* indicates the relative amplification levels

variations in any strains arise from gene mutations, insertions, deletions and/or genetic noise. These variations may reflect an evolutionary trend or may suggest that there is a relationship between serotype and virulence. So, our universal primer will be able to bring out the information of variation in various strains or even in new emerging strains arises due to the course of evolution as it is specifically designed for geographically distinct strains of *S. iniae* and *S. parauberis* (Fig. 1; Table 2). Another uniqueness of universal primer was its short product size which can be sequenced fast and cheap way. One of the benefit of choosing the strain-specific conserved gene (16S rRNA) over other candidate genes is thought to its representation for the physiological and virulence properties of an organism. This study will also be helpful for understanding evolutionary relationships and disease epidemiology like other earlier studies (Dobrindt and Hacker 2001; Wren 2000). Additional benefit of this primer is its sensitivity at a low amount of host DNA (1 and 0.01 pg of gDNA via PCR and Real Time PCR respectively) (Fig. 4a–d; Supplementary Fig. 2A, B). Comparison of various earlier important study of 16S rRNA gene primers based on bacterial agents, product size, biological experiment, suitability and cost effectiveness shows an important diagnostic and identification aspects of our study (Table 3). The universal primer may be able to amplify streptococcal bacterial species or strains, geographically presented in any part of the world so, caution should be taken while used. Various epidemiology studies suggest the presence of specific clinical phenotype in specific geographical regions in specific fishes, infected with *S. iniae* and *S. parauberis* diseases (Table 1). That's why, firstly we have to notice previous data onto incidence of *S. iniae* and *S. parauberis* disease, in specific fishes and regions then apply the diagnosis with universal primer. The positive result itself not gives guarantee of bacterial infection (*S. iniae* and *S. parauberis*) but actually indicate the presence of specific variation in other bacterial species too. So, above result should be taken together with other epidemiological parameters for judging the possibilities of streptococcal infections (*S. iniae* and *S. parauberis*) in the fishes. For this reason, we have developed a proper parameter and algorithm for proper diagnosis on the basis of our universal primer (Fig. 5a, b).

Present challenges in diagnosis of Streptococcus

Fishes were always on risk for acquiring Streptococcus infections, which were sometimes face problems of

identification with clinical laboratories due to under-diagnosis or un-identified or as a mis-identified (Lau et al. 2006). Evidence suggested that fish able to carry the pathogen asymptomatically (not show symptoms of disease), which may serve as a pathogen reservoir of future infections (Bromage et al. 1999; Eldar et al. 1995; Zlotkin et al. 1998). Moreover, standard bacteriological methods were not adequate for identification of many strains or sometimes even in new emerging fish pathogen. There were several reasons either it is not listed in the databases (RAPID Strep strip, VITEX systems, API 20E STREP, Rapid Strep 32, API CH 50, or the ATB Expression Systems) or downstream application failure for various reasons (Dodson et al. 1999; Facklam et al. 2005; Lau et al. 2006). Overall, making together diagnosis and identification more complicated. Comparative studies discuss in detail about various methodologies, their diagnosis capabilities and identified molecular methods as one of the important categories (Bosshard et al. 2006). Therefore, final detection cannot be determined without the support of molecular methods (genetic data).

Diagnosis based on molecular methods depends on pathogenic molecules related to candidate genes. Currently, it is difficult to show pathogenicity in all species of *Streptococcus* as it variate and depends on several factors which can change even from strain to strain in same species. Proper, understanding of pathogenicity of many species and strains were very important in molecular studies for deciding appropriate candidate gene as a diagnostic purpose. However, still research warranted to address this issue. Earlier research shows that pathogenicity of *streptococcal* species depends on the ability of bacteria to survive in host immune cells and induce their apoptosis by avoidance of host killing and for the establishment of infection (Woo and Park 2014; Zlotkin et al. 2003). In *S. iniae* pathogenicity depends on various virulence factors, which still needs to understand (Agnew and Barnes 2007; Zlotkin et al. 2003). In the molecular diagnosis, earlier studies of specific PCR on the other candidate genes like, 16S rRNA gene, the 16S–23S rRNA gene intergenic spacer region, the chaperonin HSP60 and the lactate oxidase gene (lctO) have been developed for rapid identification (Berridge et al. 1998; Goh et al. 1998; Mata et al. 2004; Zlotkin et al. 1998). However, candidate genes were varying from bacteria to bacteria, so this method has limitations in identification, whereas 16S rRNA gene make lead being conserved in nature. Although, diagnosis based on 16S rRNA gene also shows some difficulties in identification of bacteria such as the resolution of sequencing concerns sequence similarities or identity for the other *Streptococcus* groups (Janda and Abbott 2007; Petti 2007). A study reported that 16S rRNA gene sequence data not able to distinguish between recently diverged species (Janda and Abbott 2007).

Effective diagnosis of *S. iniae* and *S. parauberis*

A study shows that Fish industry results in an estimated loss of US $150 million annually (Shoemaker et al. 2001). So, immediate and rapid diagnosis of fish with our universal primer must alarm the fish producers at very advanced stage. Our universal primer diagnosis based on 16S rRNA may be used as both taxonomic purpose and conventional diagnostic purpose, as in addition to rapid, it is sensitive

Table 3 Comparison of various important study of 16S rRNA gene primers based on bacterial agents product size, biological experiment, their suitability and cost effectiveness with references

Bacterial agent	Base Pair	Experiment	Most suitable	Cost	References
Pathogen bacteria	58	PCR, qPCR, NGS, Sequencing	Diagnosis	Lower	Chakravorty et al. (2007)
Pathogen bacteria	204	PCR, qPCR, NGS, Sequencing	Diagnosis	Lower	Chakravorty et al. (2007)
Streptococcus mutants	282	PCR, qPCR, NGS, Sequencing	Diagnosis and Identification	Lower	Rupf et al. (1999)
Streptococcus iniae and *Streptococcus parauberis*	302	PCR, qPCR, NGS, Sequencing	Diagnosis and Identification	Lower	In this study
Bacteria and Archaea	~	PCR, qPCR, NGS, Sequencing	Diagnosis and Identification	Middle	Takahashi et al. (2014)
Bacteria	466	PCR, qPCR, NGS, Sequencing	Diagnosis and Identification	Middle	Muyzer et al. (1993)
Bacteria	466	PCR, qPCR, NGS, Sequencing	Diagnosis and Identification	Middle	Caporaso et al. (2011)
Enterococcus	762/598	PCR, Sequencing	Diagnosis and Identification	Middle	Xu et al. (2004)
Streptococcus species	1320	PCR, Sequencing	Identification	Higher	Bentley and Leigh (1995)
Enterococcus	1343	PCR, Sequencing	Identification	Higher	Woo et al. (2001)
Bacteria	1503	PCR, Sequencing	Identification	Higher	Reysenbach et al. (1994)
Bacteria	1503	PCR, Sequencing	Identification	Higher	Edwards et al. (1989)

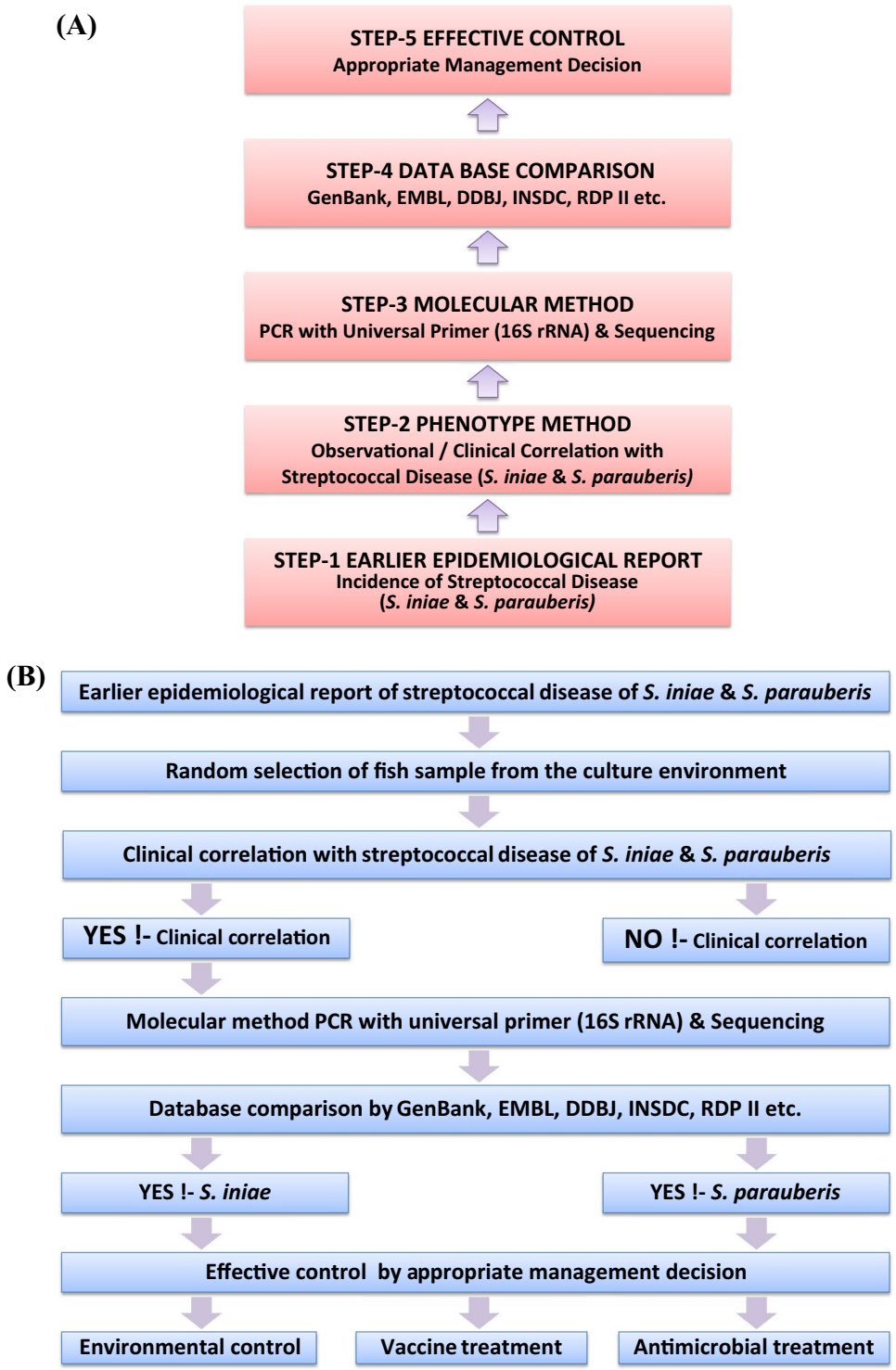

Fig. 5 **a** Algorithm for the identification of streptococcal diseases caused by *S. iniae* and *S. parauberis*. **b** Effective and appropriate control strategy for the streptococcal diseases caused by *S. iniae* and *S. parauberis*. Effective control strategy involved many steps and finally dependent on appropriate management decision

and specific to two main bacterial agents. Importantly, this universal primer can be used for multiplex PCR with another disease marker's in mixed sample type. We have demonstrated in Fig. 3b, that there is internal variation (pink box) within primer range which can segregate the *S. iniae* and *S. parauberis* strains by multiplex. The protective measurement such as high quality of water, good health management system were considered as the best protective measures for disease control (Agnew and Barnes 2007). Continuous monitoring of phenotype of fish by random selection and immediate diagnosis of fish with a universal primer of 16S rRNA by matching the variation with the standard database will be the effective way of early

diagnosis, which can lead to further appropriate control strategy (Fig. 5a).

Combined phylogenetic tree (*S. iniae* and *S. parauberis*) demonstrated the interrelation of both species in Fig. 2. Our phylogeography and phylogenetic study together show differentiation for geographical and serological strains, which provide useful information on epidemiology and evolutionary perspective of the *S. iniae* and *S. parauberis* strains. Recent advancement of gene sequencing and NGS technology help us to analyze single to huge biological samples in cost-effective way by restricting the target gDNA size (Table 3). In line of all above fact this study, offer new and rapid insights into *S. iniae* and *S. parauberis* specific potential diagnostic strategies through PCR, qPCR, Sequencing based on genetic analysis. Although, our *in silico* analysis demonstrated the validity of our universal primer in worldwide isolates (Supplementary Fig. 1), however large sample size indeed help us to validate more appropriately. Based on all above discussed fact and complications, our early diagnosis algorithm (Fig. 5b) will work as effective and proper tool for identification of *S. iniae* and *S. parauberis* infected fishes.

Conclusion

In conclusion, our universal primer is able to identify *S. iniae* and *S. parauberis* in rapid and cost effective manner along with high sensitivity and specificity, then previously reported primers. Our study highlighted the current scenario of *S. iniae* and *S. parauberis* diagnosis system. Based on our suggestion, improved management decisions on early diagnosis through combined clinical phenotype and 16S rRNA sequencing method, were the appropriate lead, which will be able to counter balances the negative economic impact caused by *S. iniae* and *S. parauberis* disease in aquaculture production systems.

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Compliance with ethical standards

Conflict of interest Anshuman Mishra, Gyu-Hwi Nam, Jeong-An Gim, Minji Seong, Yunjeong Choe, Hee-Eun Lee, Ara Jo, Suhkmann Kim, Do-Hyung Kim, Hee-Jae Cha, Ho Young Kang, Yung Hyun Choi, and Heui-Soo Kim declare no conflict of interest on the contents of the manuscript.

Research involving animal rights Pusan National University guidelines for the care and use of animals were followed.

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