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by Nurrahman Nurrahman

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Streptolysin Encoding Genes *sagC* and *sagD* as Biomarkers of Fish Pathogen *Streptococcus iniae*: An *In Silico* Study

Stalis Norma Ethica[1], Sri Darmawati[1], Sri Sinto Dewi[2], Nurrahman[2], and Ayu Rahmawati Sulistyaningtyas[2*]

[1]Magister Program of Medical Laboratory Science, Universitas Muhammadiyah Semarang,
Jl. Kedungmundu Raya No. 18, Semarang, 50273, Indonesia
[2]Faculty of Nursing and Health Sciences, Universitas Muhammadiyah Semarang,
Jl. Kedungmundu Raya No. 18, Semarang, 50273, Indonesia

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Abstract

Streptococcus iniae has been notorious as a serious tilapia fish pathogen leading to many disease outbreaks in warm water marine aquaculture. An *in silico* investigation about the potential of virulence genes of *S. iniae*, *sagC* and *sagD*, as biomarkers of the bacterial species, has been carried out. The aim was to determine bacterial biomarkers, which are important to facilitate early rapid diagnosis of *S. iniae* streptococcal infection in fish and also in humans. First, specific primers were designed from *sagC* and *sagD* genes of *S. iniae* SF1 genomic DNA using Primer3Plus. Next, in silico PCR (Polymerase Chain Reaction) analysis was carried out using the newly designed primers and 117 genomic DNA of streptococci (all species) retrieved from the database. Primers designed from *sagC* and *sagD* genes (SagCF: '5- TGCTGACCTCCTAAAAGGGC -3' and SagCR: '5- CTATGCGGCGGGTTTAAGGT -3' as well as SagDF: 5'- GCCAATCCAATCCTGTCATGC -3' and SagDR: 5'- TGCAGCTTCCATAACCCCTC -3') could result in a single band of each matching to 558-bp and 590-bp PCR products only from *S. iniae*. From 116 other streptococcal genomes studied using similar primers have resulted in no amplicon bands. A further check showed that the amplicons were truly part of *sagC* and *sagD* genes of *S. iniae*. These results showed that sagC and sagD genes appeared to be biomarkers of *S. iniae*, which are potential to be used to facilitate rapid diagnostic of the pathogenic bacterium.

Keywords: ***Streptococcus iniae, sagC, sagD, in silico PCR***

1. Introduction

Streptococcus iniae has been notorious as the most severe aquatic pathogen in the last decade. Disease outbreaks caused by the bacterium had most likely been occurring for several decades in marine aquaculture leading to high losses in farmed marine in warmer regions. *Streptococcus iniae* is known as the endemic pathogen responsible for the development of streptococcosis in fish. Yet, the bacterial species is also known as a rising pathogen in human for causing infection on soft tissues (Agnew & Barnes, 2007; Fuller et al., 2002; Nho et al., 2009; Rajoo et al., 2015). *Streptococcus iniae* infection in human generally occurs through injuries associated with preparing whole fresh fish for cooking (Baiano & Barnes, 2009).

S. iniae had been isolated from culture of red hybrid (Nile Tilapia *Oreochromis niloticus* × Mozambique Tilapia *O. mossambicus*) in Malaysia, blue tilapia (*Oreochromis aureus*) in Mexico and olive flounders (*Paralichthys olivaceus*) in South Korea. The isolated Gram-positive *S. iniae* appeared punctiform, transparently white, catalase and oxidase negative and produced complete b-hemolysis on blood agar. The isolate was sensitive to tetracycline, vancomycin, and bacitracin but was resistant to streptomycin, ampicillin, penicillin, and erythromycin (Ortega et al., 2018; Rahmatullah et al., 2017; Rajoo et al., 2015).

The current understanding of virulence mechanisms of *Streptococcus iniae* in fish is lacking. Although, it is known that in the case of fish infection by *S. iniae,* the virulence factor is B-hemolysin S (Locke et al., 2007). Hemolysins, or cytolysins, are well-recognized

*Corresponding author.
E-mail: ayurs@unimus.ac.id

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features of many bacterial species, including streptococci, and are generally associated with damage to the membranes of a variety of mammalian cell types. Examples of streptococcal cytolysins include streptolysin O (SLO) and streptolysin S (SLS) of group A streptococci (GAS) as well as the b-hemolysin/cytolysin of group B streptococci (GBS) implicated as a virulence factor in animal models of infection (Le Breton & McIver, 2013).

The pathogen group A Streptococcus (GAS) produces a wide spectrum of infections including necrotizing fasciitis (NF). Streptolysin S (SLS) produces the hallmark b-haemolytic phenotype produced by GAS. The nine-gene GAS locus (*sagA-sagI*) resembling a bacteriocin biosynthetic operon is vital and sufficient for SLS production. Datta et al. (2005) reported that for production of SLS, all of sagA, sagB, sagC, and sagD, are needed. Being members of *S. iniae* SLS-associated gene cluster responsible for streptolysin production, the *sagC* and *sagD* encoded predicted products with significant amino acid identity to corresponding *sag* products of GAS, yet shared no homology with other proteins in the GenBank database (Fuller et al., 2002). Both genes encode two streptolysin associated proteins, SagC and SagD, respectively.

Identification of biomarkers is critical for the diagnosis of fish bacterial infection early during invasion. However, the identification of reliable biomarkers is often hampered by a low concentration of microbes. Shanks et al. (2007) stated that the development of specific PCR assay targeting bacterial DNA as markers is important for the identification and diagnosis of bacterial fish infection with low concentration of bacterium. Various PCR methods designed for *S. iniae* detection had been studied (Zhou et al., 2011). However, none of them targeting *sagC* and *sagD* sequences of *S. iniae*, while it had been revealed the connection between the *sagC* and *sagD* genes with the invasive fish-infection agent (Fuller et al., 2002).

The term *in silico* referred to "computational" experiment conducted virtually using computers complementing both *in vitro* and *in vivo* terms, which refer to works done in living organisms. In genomics area of study, the intention of *in silico* PCR is achieving predicted PCR results (theoretical) using updated sequences of bacterial genome allowing amplification of targeted DNA sequences (Kalendar et al., 2017; Moretti, 2011). In addition, *in silico* PCR is useful for computing ability prediction of primers to amplify selected gene fragments by using up-to-date bacterial genomic DNA sequences retrieved from a database. It allows amplification of specific DNA sequences supporting successful DNA amplification using *in vivo* PCR (Bikandi et al., 2004; Ethica et al., 2013; Ethica, 2014; Kalendar et al., 2017; Priyadharsini et al., 2018).

In silico PCR method has been widely known as capable to be used for investigating genes in *A. baumannii* (an emerging fish pathogen) and its associated species. Moreover, accomplished development of a real-time PCR analysis supported by in silico PCR to amplify signature genes to quantify a group of bacteria that causes sepsis has also been widely used (Bellemain et al., 2010; Gupta et al., 2017). In this investigation, *sagC* and *sagD* genes of *S. iniae* are tested for their potential as DNA biomarkers of the bacterial species. The aim is to seek if they could be used as DNA biomarkers, which are important to facilitate early rapid diagnosis of *S. iniae* streptococcal infection.

2. Material and Methods

Literature study was initially conducted to get information about the most unique phenotypic feature of *S. iniae*, particularly on specific toxins it produces. Sequences of these genes were then retrieved from genbank NCBI (*National Center for Biotechnology Information*) under accession number of NZ_CP010783.1 (*sagC*, Gene ID: 35765491) and NZ_CP010783.1 (*sagD*, Gene ID: 35766625) (Rajoo et al., 2015). DNA sequences of *sagC* gene used as the basis of SagCF-SagDR primer design along with its 3-D protein model are shown in Figure 1 and 2.

Next, the genotype feature related with genes associated with proteins underlining the unique phenotype was targeted. Primers were then designed from internal part of open reading frame of the targeted gene sequences using Primer3Plus (Ethica et al., 2013; 2014; 2019; Untergasser et al., 2007). Two pairs of primers that have the least possibility of hairpin formation, self-complementarity, and dimerization was selected (Ethica et al., 2017). The newly designed primers were used as input for the web-based *in silico* PCR, which was run from *http://insilico.ehu.es/PCR/* using all streptococcal genomes retrieved from its source of database (Bikandi et al., 2004; Ethica et al., 2019; San Millán et al., 2013). A sequence analysis was finally conducted by clicking DNA band appeared as PCR product in program output or using 'Amplify Show Result' tool. This step was needed to clarify if the *in silico* PCR products (amplicons) were specific to *S. iniae* genome and were truly part of signature genes targeted as biomarker.

Streptococcus iniae strain YSFST01-82, complete genome
NCBI Reference Sequence: NZ_CP010783.1
GenBank Graphics

>NZ_CP010783.1:c1242370-1241306 Streptococcus iniae strain YSFST01-82, complete genome
ATGAGATACCAATTAAATAGTAATGTTCGTATTGTGCAATTTGATGATACCTTTTGTTTTAGAAAAGGAT
TATGGGATTTTAATGAAGCTGTATTAGATTTGTCACAAGAACCACAAATGTTAAAAGAAGTTTACCAGCA
AATTGTTGCTGACCTCCTAAAAGGGCTAGTGGTCGATACAGATGATTATGAAAAAGAGTTAGAAGCTGAA
TTATTTGCTAAAGTGATGGAAGTCATGACAGCTCTATATTATAATGATGTACTCATGTTTGAAGATGATT
ACGCCCTTGAAGAAAATGTTATGAAAGTATTGATGGGAAATTTCCGCTTTATGGCCCAAGAAGGCATAC
TAAGAATACGGACCCTGTCTTGTTTATCAGTGACTCTAGTTATGTTAATGACTCAGCAAAGTTACTTGCA
GAACATTTGAATTTGAACCTTGTTGTGGCAAGTGATGAACTAAAAATGCTGATTCAAAAGATTGATGTTA
CCTCTCGTTTAGATGCATTAGAACACCATCGCAATATGAAGCGTTTATCTGGAAAGTTAATGCCTTATCA
AAGTATTGTAGTTTGCCAGGAGCGTTTGAACATAATGATGCTTCGTCATCTTAATGAAATCAGTGTTGCT
TTGAAAAAGCAAATGGTACTTGGTTTTGTAGATGGTCCTTTTATTCATGCTTGTACCTTAAACCCGCCGC
ATAGCGCAGATTTTGATAGTTTAGAAAGAAGGGTTTTAGCTCGTTTGCAAGATCATGCTCTTTATCAGCA
TTTTGCCAGCCAAATGTTGCCAACGACGCAACCAGTTAGTCATTCCTATCTGCCTTTATTGAATATTTTA
ATGAATTTAGTTGTTAGTGAGGCCTTTATTATTGCACAAACAGGAAGTTCCAAATTTGAAGGGCGATTGT
TGAGTATTTATTTGCCAACACTTGAGATTCAGGTTCAAGATATTTTGAAAATGTCCAATTCTCAAACCCA
AGGTGCACTTGCGAAATTAAGGTATGAGGACCAACAAATTTCGACACGAGAAATTGTCAAAAACCTCCTA
AAAGAAGATTCCTAA

Figure 1. Gene sequence of *sagC* used as the basis of SagCF-SagCR primer design

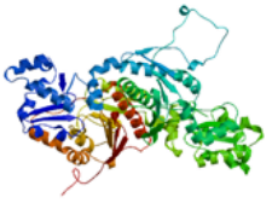

Figure 2. The 3-D structure of V4QUH6_STRIN V4QUH6 Streptolysin S biosynthesis protein *SagC* of *Streptococcus iniae* visualized by SWISS-MODEL (Created on December 9, 2019)

3. Results and Discussion

From literature studies, it is known that *sagC* and *sagD* genes are among the unique genotypic features of virulence bacterium *Streptococcus iniae*, the main cause of streptococcal infection in tilapia fish (Datta et al., 2005). Sequences of these genes (both genes were under accession number of NZ_CP010783.1) were used as the basis of primer design (the genes and their encoded protein are displayed in Figure 1 & 2 respectively). Both 3D structures of *SagC* and *SagD* are displayed as validity test if the genomic DNA sequences used to design primer (*sagC* and *sagD*) produce folded (functional) proteins. Figure 1 and 2 showed that both proteins are folded, and hence functional. This opens possibility to use both *SagC* and *SagD* proteins as bacterial protein markers or to develop vaccines using both proteins as target. DNA sequences of *sagD* gene from NCBI Genbank used as the basis of SagDF-SagDR primer design along with its 3-D protein model are shown in Figure 3 and 4. Using primer3Plus, pairs of primers obtained by using *sagC* and *sagD* DNA sequences as input (Figure 1 and 2 respectively) are listed in Table 1.

In silico PCR using SagCF-SaGR primers and genomic DNA of 117 streptococci resulted in single 558-bp DNA band (Figure 5) belonging to *S. iniae* SF1 or species no 29 (Table 2). That using SagDF-SagDR primers resulted in also a single amplicon sized 590 bp (Figure 5) on *S. iniae* SF1 or species no. 2 according to Streptococci list in Table 2. Using *in silico* PCR run on *http://insilico.ehu.es/PCR/*, the resulted amplicon could be seen on Figure 5 and Figure 6.

In silico PCR conducted in this study was run directly from *http://insilico.ehu.es/PCR/*. Table 2 shows all current Streptococci stored in the program database (by choosing the menu APPLY TO ALL Streptocccus when selecting genomic templates of *in silico* PCR) by the time this study is conducted. Currently, genome sequences of *S. iniae* other than SF1 strain have been released (GenBank accession nos.: BANM00000000, AMOO00000000, and AOCT00000000). Yet, the only representative of *S. iniae* species sequence available in the program is strain SF (fish isolate of serotype I), which is the cause of an epidemic broke out in a fish farm in north China (Cheng et al., 2010). Although not all strains of *S. iniae* could be tested using the current *http://insilico.ehu.es* database, BLAST test showed that among other *S. iniae* strains, the *SagC* and *SagC* are highly conserved (99-100% homology level) in the species group, compared with other *Streptococcus* species (starting from *S. equi* by 84%).

Streptococcus iniae strain YSFST01-82, complete genome
NCBI Reference Sequence: NS_CP010783.1
GenBank Graphics

>NZ_CP010783.1:c1241293-1239935 Streptococcus iniae strain YSFST01-82, complete genome
ATGTTACATTATTACCCGTCCTTTAACCATATTTTAGATGAACTCAAAGGTTTGAGTGGCAATAGAACAG
GGATTTTAAATCAATCACAAGTTCCAGTTTGTAACCATCAACATGATGTGTACTTAAAAAGTGTGACTGG
TCAGATACCTGATTATCACAAACAATATATTGATGAATTGAGTCAAGTTAGCTATCATATTATTGGTTAT
GGCAGTCATTATGAAGAAGCACTTATTAAATATTTAGGTGAGAGTATTGAACGCTATGCAACTATTATTG
CAGGTGATTTGCTTTCAGATCGCATTGTTTACGCTTCTTATAAGGAACTCAGCCAATCCAATCCTGTCAT
GCCATTAGAATATTTACAGGTTTTTACACAGGAACAAATTGATCAGTCGTGCAAACTTCATATGCATATG
TGTGAAAAAATGGTGACTGAAGATGATGTTTTAGGTTGGGTGAAATGCCCCATGTTTTTTGAAGATAAGG
AACTGTATGTGCCTATTCAGATGCTTTGCATTGGCTATAAGCCTAATCGTGAAGTAGGTGAACAGTATGT
TATTCCTGGATTTTCAACTGGTACGGCTTCTCATAAAACACTTGAAGCAGCTATGTGTAATAGTCTAATT
GAATACATTCAAATTGACTCAATGATGCTTAGCTGGCATACCAAAAAAGCTTGTCCAAGAATCATTATTG
ATGATCCAGACATTCAAGCTATTTTGGAAGAAGCAAGACTTGGTAAAGACAGCTTGTATGAGATTATCCC
AATTGACATGACTGTTGGAGAGGACAATCCCCTATATACCTTTGGTATTATCCTTAAAAATAAATATGAA
GAAGGTCCCTACCTTTTATTTGGTGTTCAGGCTGGTTTAGATCCCAAGCATACACTACTAAGAGGGGTTA
TGGAAGCTGCAGCCATTAGTTACAGTTACTATTATAATCTCTTTTATCAAAAGGAATCTTTAGCAAATAT
TGAGAGCGATTCGCCTTTGTTCTTGGACTTAGATAGCAATGTCTTTTATTACGCTCATCCTAAGGATCAA
GAGCATAAATGGAAAGCTTTTGAACCTTTAATTTCAGGTGAGATTTGTCTGAGTGATTTAAAAAATAATG
CAGGTCAAGATAAGAAGGAAAATCTGAAAATTCTATTAGCTTATACTAAAAAAGTGAGTCCTAATGCAGT
ATTTTTAGACATTACACCTCCAGAAGCTTCTGAAAAAGGTTGGTACGTCACACGAGTGTTAACACCTGAA
CTTCTTGAAATGTGTATTCCAGCATTTCCATTTGCTAATCACCCACGAATGAGACAGTTTGGAGGTGTTA
CCAATGAATTTGTCCACCCAATGCCTTAA

Figure 3. Gene sequence of *sagD* used as the basis of SagDF-SagDR primer design

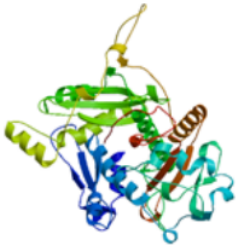

Figure 4. The 3-D structure of V4R003_STRIN V4R003 Streptolysin S biosynthesis protein SagD of *S. iniae* visualized by SWISS-MODEL (Created on December 9, 2019)

Table 1. Primers designed using Primer3Plus from *sagC* and *sagD* gene sequences

Gene Source	Primer Name	Forward Primer	Reverse Primer	Amplicon Size (bp)
sagC	SagCF-SagCR	TGCTGACCTCCTAAAAGGGC	CTATGCGGCGGGTTTAAGGT	558
sagD	SagDF-SagDR	GCCAATCCAATCCTGTCATGC	TGCAGCTTCCATAACCCCTC	590

After carrying out the in silico PCR, it was necessary to check whether amplicon products, which were seen on virtual gel electrophoresis (Figure 5 and 6), were correct based on primer positions. The analysis was carried out easily by clicking amplicon size appeared on the *in silico* PCR result window. New tab was then appeared showing that *in silico* amplicons were truly parts of *sagC* and *sagD*. Results appeared on the new tabs from two PCR runs (for SagCF-SagCR and SagDF-SagDR primer pairs of each) are displayed in Figure 7 and 8.

Infectious diseases are a group as ailments caused by virulence microbes, such as viruses, bacteria, or parasites. Those types of diseases have been major issues worldwide affecting public health and economy of many countries. Investigation on the ideal biomarkers for diagnostics of infectious diseases (with high sensitivity, specificity, and predictive capacity) in the beginning must be focused on detection and identification of the virulence agents (Hwang et al., 2018; Mohan & Harikrishna, 2015). Serious infection outbreaks often present from fish and other foods creating a diagnostic challenge for clinicians. There are not many official guidelines to help medical scientists in determining which biomarkers are useful to diagnose the presence of

Table 2. List of genomic DNA of Streptococcus species used as *in silico* PCR templates

Complete list of Streptococcus strains in the Database of *in silico* PCR	
1 - *Streptococcus agalactiae* 09mas018883	60 - *Streptococcus pneumoniae* R6
2 - *Streptococcus agalactiae* 2-22	61 - *Streptococcus pneumoniae* SPNA45
3 - *Streptococcus agalactiae* 2603V/R	62 - *Streptococcus pneumoniae* ST556
4 - *Streptococcus agalactiae* A909	63 - *Streptococcus pneumoniae* TCH8431/19A
5 - *Streptococcus agalactiae* GD201008-001	64 - *Streptococcus pneumoniae* TIGR4
6 - *Streptococcus agalactiae* ILRI005	65 - *Streptococcus pneumoniae* Taiwan19F-14
7 - *Streptococcus agalactiae* ILRI112	66 - *Streptococcus pneumoniae* gamPNI0373
8 - *Streptococcus agalactiae* NEM316	67 - *Streptococcus pseudopneumoniae* IS7493
9 - *Streptococcus agalactiae* SA20-06	68 - *Streptococcus pyogenes* A20
10 - *Streptococcus anginosus* C1051	69 - *Streptococcus pyogenes* Alab49
11 - *Streptococcus anginosus* C238	70 - *Streptococcus pyogenes* HSC5
12 - *Streptococcus constellatus* subsp. *pharyngis* C1050	71 - *Streptococcus pyogenes* M1 476 DNA
13 - *Streptococcus constellatus* subsp. *pharyngis* C232	72 - *Streptococcus pyogenes* M1 GAS
14 - *Streptococcus constellatus* subsp. *pharyngis* C818	73 - *Streptococcus pyogenes* MGAS10270
15 - *Streptococcus dysgalactiae* subsp. *equisimilis* 167	74 - *Streptococcus pyogenes* MGAS10394
16 - *Streptococcus dysgalactiae* subsp. *equisimilis* AC-2713	75 - *Streptococcus pyogenes* MGAS10750
17 - *Streptococcus dysgalactiae* subsp. *equisimilis* ATCC 12394	76 - *Streptococcus pyogenes* MGAS15252
18 - *Streptococcus dysgalactiae* subsp. *equisimilis* GGS_124	77 - *Streptococcus pyogenes* MGAS1882
19 - *Streptococcus dysgalactiae* subsp. *equisimilis* RE378	78 - *Streptococcus pyogenes* MGAS2096
20 - *Streptococcus equi* subsp. *equi 4047*	79 - *Streptococcus pyogenes* MGAS315
21 - *Streptococcus equi* subsp. *zooepidemicus*	80 - *Streptococcus pyogenes* MGAS5005
22 - *Streptococcus equi* subsp. *zooepidemicus* ATCC 35246	81 - *Streptococcus pyogenes* MGAS6180
23 - *Streptococcus equi* subsp. *zooepidemicus str.* MGCS10565	82 - *Streptococcus pyogenes* MGAS9429
24 - *Streptococcus gallolyticus* UCN84	83 - *Streptococcus pyogenes* NZ131
25 - *Streptococcus gallolyticus* subsp. *gallolyticus* ATCC 43143	84 - *Streptococcus pyogenes* SSI-1
26 - *Streptococcus gallolyticus* subsp. *gallolyticus* ATCC BAA-	85 - *Streptococcus pyogenes* str. Manfredo
27 - *Streptococcus gordonii* str. *Challis* substr. CH1	86 - *Streptococcus pyogenes* strain MGAS8232
28 - *Streptococcus infantarius* subsp. *infantarius* CJ18	87 - *Streptococcus salivarius* 57.I
29 - *Streptococcus iniae* SF1	88 - *Streptococcus salivarius* CCHSS3
30 - *Streptococcus intermedius* B196	89 - *Streptococcus salivarius* JIM8777
31 - *Streptococcus intermedius* C270	90 - *Streptococcus sanguinis* SK36
32 - *Streptococcus intermedius* JTH08	91 - *Streptococcus sp.* I-G2
33 - *Streptococcus lutetiensis* 033	92 - *Streptococcus sp.* I-P16
34 - *Streptococcus macedonicus* ACA-DC 198	93 - *Streptococcus suis* 05ZYH33
35 - *Streptococcus mitis* B6	94 - *Streptococcus suis* 98HAH33
36 - *Streptococcus mutans* GS-5	95 - *Streptococcus suis* A7
37 - *Streptococcus mutans* LJ23	96 - *Streptococcus suis* BM407
38 - *Streptococcus mutans* NN2025	97 - *Streptococcus suis* D12
39 - *Streptococcus mutans* UA159	98 - *Streptococcus suis* D9
40 - *Streptococcus oligofermentans* AS 1.3089	*99* - *Streptococcus suis* GZ1
41 - *Streptococcus oralis* Uo5	100 - *Streptococcus suis* JS14
42 - *Streptococcus parasanguinis* ATCC 15912	101 - *Streptococcus suis* P1/7
43 - *Streptococcus parasanguinis* FW213	102 - *Streptococcus suis* S735
44 - *Streptococcus parauberis* KCTC 11537	103 - *Streptococcus suis* SC070731
45 - *Streptococcus pasteurianus* ATCC 43144	104 - *Streptococcus suis* SC84
46 - *Streptococcus pneumoniae* 670-6B	105 - *Streptococcus suis* SS12
47 - *Streptococcus pneumoniae* 70585	106 - *Streptococcus suis* ST1
48 - *Streptococcus pneumoniae* A026	107 - *Streptococcus suis* ST3
49 - *Streptococcus pneumoniae* AP200	108 - *Streptococcus suis* T15
50 - *Streptococcus pneumoniae* ATCC 700669	109 - *Streptococcus suis* TL13
51 - *Streptococcus pneumoniae* CGSP14	110 - *Streptococcus suis* YB51
52 - *Streptococcus pneumoniae* D39	111 - *Streptococcus thermophilus* CNRZ1066
53 - *Streptococcus pneumoniae* G54	112 - *Streptococcus thermophilus* JIM 8232
54 - *Streptococcus pneumoniae* Hungary19A-6	113 - *Streptococcus thermophilus* LMD-9
55 - *Streptococcus pneumoniae* INV104	114 - *Streptococcus thermophilus* LMG 18311
56 - *Streptococcus pneumoniae* INV200	115 - *Streptococcus thermophilus* MN-ZLW-002
57 - *Streptococcus pneumoniae* JJA	116 - *Streptococcus thermophilus* ND03
58 - *Streptococcus pneumoniae* OXC141	117 - *Streptococcus uberis* 0140J
59 - *Streptococcus pneumoniae* P1031	

Note: Yellow highlighted is genomic sequence of *S. iniae* strain from database used as template of both primer design and *in silico* PCR amplification

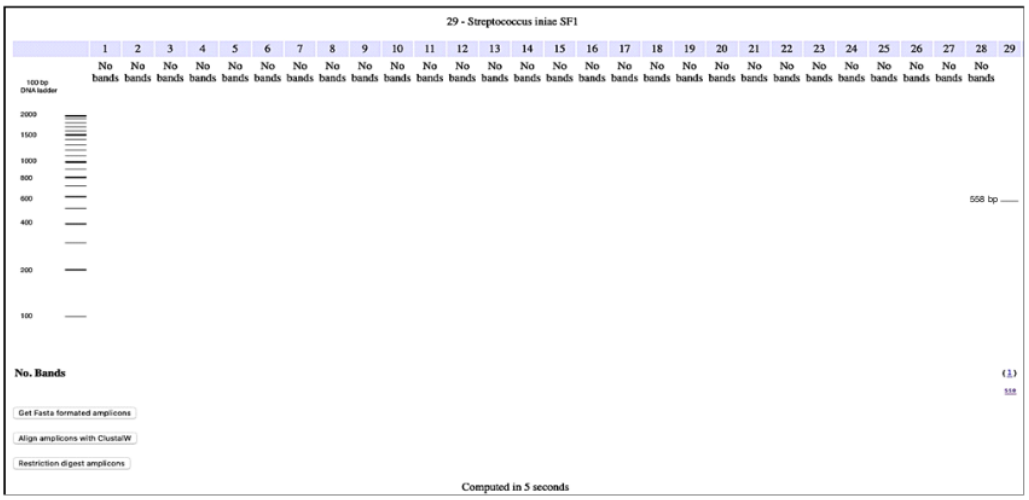

Figure 5. *In silico* PCR using SagCF-SaGR primers and genomic DNA of streptococci resulted in a single 558-bp DNA band belonging to strain no. 29 or *S. iniae* SF1 species

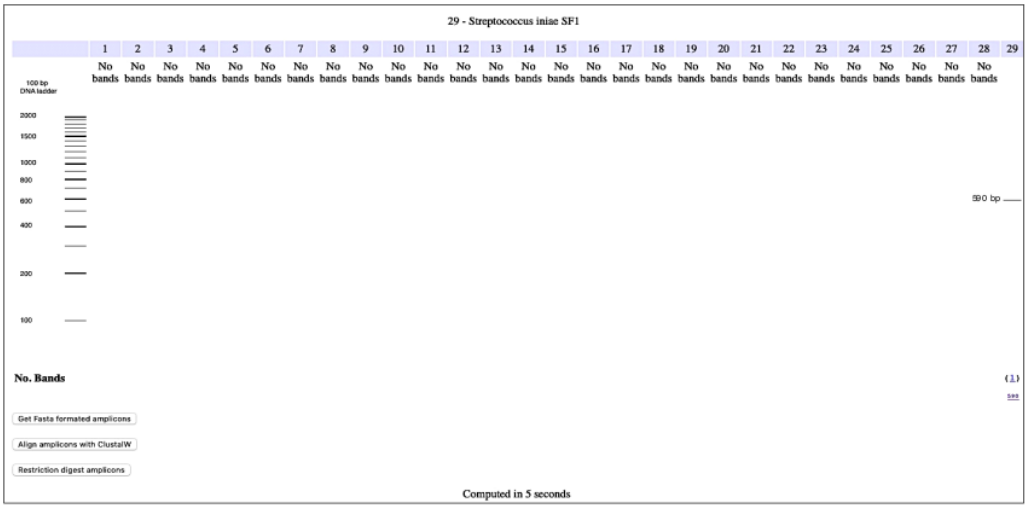

Figure 6. *In silico* PCR using SagDF-SagDR primers resulted in also a single amplicon sized 590 bp on strain no. 29 or *S. iniae* SF1 species

infections by bacteria (Rogers et al., 2011). Yet, the initial step of bacterial biomarker development to detect *S. iniae* had been carried out in this study. It was expected that in case of streptococcal infection outbreaks, the involvement of *S. iniae* could be determined by detecting the presence of their DNA biomarkers.

In silico PCR is a computational polymerase chain reaction allowing amplification of targeted DNA sequences using updated sequenced bacterial genome available in the program database. *In silico* PCR aimed to predict ability of primers to amplify targeted DNA segments, which could support accurate *in vitro* DNA amplification.

In this *in silico* PCR study, *sagC* and *sagD* were predicted to be potential DNA biomarkers for *S. iniae*. This is based on initial literature study (Fuller et al., 2002) and NCBI search showing that both genes encoded predicted products with significant amino acid identity to corresponding sag products of GAS, yet

>NC_021314, from 1246525 to 1247082 (558 bp); Streptococcus iniae SF1
CTATGCGGCGGGTTTAAGGTACAAGCATGAATAAAAGGACCATCTACAAAACCAAGTACCATTTGCTTTT
TCAAAGCAACACTGATTTCATTAAGATGACGAAGCATCATTATGTTCAAACGCTCCTGGCAAACTACAAT
ACTTTGATAAGGCATTAACTTTCCAGATAAACGCTTCATATTGCGATGGTGTTCTAATGCATCTAAACGA
GAGGTAACATCAATCTTTTGAATCAGCATTTTTAGTTCATCACTTGCCACAACAAGGTTCAAATTCAAAT
GTTCTGCAAGTAACTTTGCTGAGTCATTAACATAACTAGAGTCACTGATAAACAAGACAGGGTCCGTATT
CTTAGTATGCCCTTCTTGGGCCATAAAGCGGAAATTTCCCATCAATACTTTCATAACATTTTCTTCAAGG
GCGTAATCATCTTCAAACATGAGTACATCATTATAATATAGAGCTGTCATGACTTCCATCACTTTAGCAA
ATAATTCAGCTTCTAACTCTTTTTCATAATCATCTGTATCGACCACTAGCCCTTTTAGGAGGTCAGCA
streptolysin associated protein SagC [Streptococcus iniae]
NCBI Reference Sequence: YP_008056992

Figure 7. *In silico* PCR amplicons using SagCF-SagCR primers

>NC_021314, from 1245231 to 1245820 (590 bp); Streptococcus iniae SF1
TGCAGCTTCCATAACCCCTCTTAGTAGTGTATGCTTGGGATCTAAACCAGCCTGAACACCAAATAAAAGG
TAGGGACCTTCTTCATATTTATTTTTAAGGATAATACCAAAGGTATATAGGGGATTGTCCTCTCCAACAG
TCATGTCAATTGGGATAATCTCATACAAGCTGTCTTTACCAAGTCTTGCTTCTTCCAAAATAGCTTGAAT
GTCTGGATCATCAATAATGATTCTTGGACAAGCTTTTTTGGTATGCCAGCTAAGCATCATTGAGTCAATT
TGAATGTATTCAATTAGACTATTACACATAGCTGCTTCAAGTGTTTTATGAGAAGCCGTACCAGTTGAAA
ATCCAGGAATAACATACTGTTCACCTACTTCACGATTAGGCTTATAGCCAATGCAAAGCATCTGAATAGG
CACATACAGTTCCTTATCTTCAAAAAACATGGGGCATTTCACCCAACCTAAAACATCATCTTCAGTCACC
ATTTTTTCACACATATGCATATGAAGTTTGCACGACTGATCAATTTGTTCCTGTGTAAAAACCTGTAAAT
ATTCTAATGGCATGACAGGATTGGATTGGC
streptolysin associated protein SagD [Streptococcus iniae]
NCBI Reference Sequence: YP_008056991

Figure 8. *In silico* PCR amplicons using SagDF-SagDR primers

shared no homology with other proteins in the GenBank database. Both genes also showed high homology level (99-100%) among *S. iniae* group of species. To prove the hypothesis, 2 pairs of primers, SagCF-SagCR and SagDF-SagDR, were successfully designed using web-based Primer3Plus software based on *S. iniae sagC* and *sagD* gene sequences.

The first designed specific pair of primers, SagCF: '5- TGCTGACCTCCTAAAAGGGC-3' and SagCR: '5-CTATGCGGCGGGTTTAAGGT-3' were able to selectively amplify internal part of *sagC* gene fragment of only from genomic sequence of bacterial species *S. iniae* SF1, but not those of 116 other streptococci. The second designed specific pair of primers SagDF: 5'- GCCAATCCAATCCTGTCATGC-3' and SagDR: 5'-TGCAGCTTCCATAACCCCTC-3' could also selectively amplify internal part of *sagD* gene fragment of only from genomic sequence of bacterial species *S. iniae* SF1, but not those of 116 other streptococci. The results infer that:

1. The *sagC* and *sagD* are biomarkers for bacterial species, *Streptococcus iniae*, which differentiate it from other streptococci. As bacterial biomarkers, both genes have potential to facilitate early rapid diagnosis of *S. iniae* streptococcal infection;

2. The newly designed primers SagCF-SagCR and SagDF-SagDR are potential to be used as primers to detect the presence of *S. iniae* in a streptococcal samples using *in vitro* PCR.

Results from this work is based on *in silico* or prediction study and are limited using resource available from *http://insilico.ehu.es/PCR*. As soon as the new database is updated, particularly when new genomic DNA sequence of *S. iniae* new strains are added, new *in silico* PCR tests could be consequently conducted using primers designed in this study to test their specificity. However, these results could be used to support successful DNA amplification using an *in vivo* PCR as a way to early detect and identify the causing agent of streptococciasis, thus help in controlling the *S. iniae* infection.

4. Conclusion

Based on *in silico* PCR carried out in this study, *sagC* and *sagD* genes were theoretically proved to be DNA biomarkers of the fish and human pathogen *S. iniae*, which could be used to selectively differentiate the species from other streptococci. The newly

designed primers designed from these genes in this study bear the potential as *in vitro* PCR tools to facilitate early rapid diagnosis of *S. iniae* streptococcal infection.

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