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Protein profiles and cross antigenicity analysis for outer surface proteins of two *streptococcus species*, isolated from hybrid red tilapia (*Oreochromis* spp.)

Muhammad Rahmatullah[1], Hassan Mohd Daud[2], Sabri Mohd Yusoff[3], MY Ina-Salwany[*4]

[1]*Faculty of Agriculture, Universiti Putra Malaysia, UPM Serdang, Selangor, Malaysia*

[2]*Faculty of Veterinary Medicine, Universiti Putra Malaysia, UPM Serdang, Selangor, Malaysia and Institute of Bioscience, Universiti Putra Malaysia, UPM Serdang, Selangor, Malaysia*

[3]*Faculty of Veterinary Medicine, Universiti Putra Malaysia, UPM Serdang, Selangor, Malaysia*

[4]*Faculty of Agriculture, Universiti Putra Malaysia, UPM Serdang, Selangor, Malaysia and Institute of Bioscience, Universiti Putra Malaysia, UPM Serdang, Selangor, Malaysia*

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Abstract

Streptococcosis is one of the most devastating diseases for hybrid red tilapia *(Oreochromis* spp.*). Streptococcus iniae* and *Streptococcus agalactiae* are considered as the most prominent etiological agents that cause streptococcosis in fish, consequently resultant high mortality and contribute big losses towards the farmer's economy. Outer surface proteins (OSPs) play an important role to induce protective immunity against pathogens. To study antigenicity of *S. iniae* and *S. agalactiae* proteins, OSPs of these two bacteria were extracted by sodium lauryl-sarcosinate method and analyzed by SDS PAGE. Protein profiling revealed 40 and 52 kDa for *S. iniae,* while only 52 kDa was observed for *S. agalactiae* as major proteins. Our results in Western blotting demonstrated that rabbit anti-serum of *S. iniae* could able to detect the antigenic proteins for *S. iniae* and *S. agalactiae* in both of the homologous and heterologous reactions. Therefore, antigenic gene encoding 52 kDa OSP of *S. iniae* was PCR-amplified and successfully cloned in pET32Ek/LIC vector. The cloned sequence was identified as 1305 bp nucleotide sequence of sip gene coding for polypeptides of 434 amino acid residues with 99% similarity to other surface immunogenic gene (sip) of *S. agalactiae* in GenBank. Based on predicted 20 antigenic sites, it can be assumed that sip gene of *S. iniae* should play a great role in bacterial antigenicity which can be used as an efficient streptococcal vaccine development for tilapia farming.

[*] **Corresponding Author:** MY Ina-Salwany ✉ salwany@upm.edu.my

Introduction

Outer surface proteins (OSPs) of pathogenic gram positive bacteria have the potentiality to be used as a vaccine candidate because OSPs are the interface between host and pathogens, and located in the surface of bacteria. Besides, it can easily be recognized as foreign substances by the immune system of the hosts (Schulz, 2002). Some OSPs can induce specific antibodies against pathogens (Bricknell *et al.*, 1999; Rahman *et al.*, 2000; Bader, 2004) inhibit the bacterial colonization in hosts (Vazquez-Juarez, 2004), and induce cell-mediated immunity (Zhang *et al.*, 2007). OSPs from different pathogenic bacteria, such as *Aeromonas hydrophila* (Rahman *et al.*, 2000), *Aeromonas salmonicida* (Bricknell *et al.*, 1999), *Vibrio parahaemolyticus* (Mao, 2007), *Vibrio alginolyticus* (Qian, 2007), *Vibrio harveyi* (Zhang *et al.*, 2007; Li *et al.*, 2008), and *Edwardsiella tarda* (Kawai *et al.*, 2004; Tang *et al.*, 2010), have been reported as effective protective antigens. Some OSPs mixtures extracted from one pathogenic bacteria, have been proved to provide cross protection for fish against several other bacterial species (Hirst *et al.*, 1994; Fang *et al.*, 2000), suggesting that different species of pathogenic bacteria may share some similar or conserved OSP antigens, which indicates that such OSPs from different pathogens can be used in developing vaccine for simultaneously preventing the diseases caused by different pathogenic bacteria.

In order to minimize the occurrence of streptococcal disease, potential screening of outer surface protein for vaccine development is highly recommended. Therefore, numerous studies had been conducted to find out the antigenic proteins of *S. agalactiae* to reveal the potential vaccine components (Gunnar *et al.,* 2005). *Streptococcus agalactiae* contains hundreds of protein coding genes, both immunogenic and non-immunogenic. Among those proteins Cα protein, Rib protein, Cβ or Beta protein, Surface Immunogenic Protein (sip) and C5a Peptidase (ScpB) have antigenic character and can confer protection against pathogens (Hughes *et al.,* 2002 and Gunnar *et al.,* 2005).

Outer surface protein of Gram positive bacteria plays a significant role to generate pathogenesis in fish (William and Olaf 1999; Gunnar *et al.,* 2005; Baiano and Barnes 2009). Among the surface protein of *S. agalactiae*, Cα is not related to the β protein but similar to Rib protein which was confirmed by N-terminal sequencing (Lachenauer *et al.,* 2000; Gunnar *et al.,* 2005). However, Alpha and Rib proteins do not cross react immunologically. In addition, it was revealed that the Rib and α protein are related in term of structure and considered as unique family of outer membrane protein of *Streptococcus agalactiae* (Michel *et al.,* 1992; Stålhammar-Carlemalm *et al.,* 1993). The other important antigenic surface protein is Sip protein. This protein was also found in the genome of *Streptococcus pneumonia* and *S. pyogenes* (Brodeur *et al.,* 2000; Gunnar *et al.,* 2005).

On the other hand, very little knowledge is available today about the surface protein of *Streptococcus iniae*. To date only few *S. iniae* pathogenic factors have been identified in the context of fish virulence: the capsular polysaccharide which involves in the resistance of phagocytes (Locke *et al.,* 2007a and Lowe *et al.,* 2007), the cytolysin streptolysin S which involves to host cell injury (Fuller *et al.,* 2002; Locke *et al.,* 2007b), phosphoglucomutase, which is essential for cell wall rigidity and cationic antimicrobial peptides resistance (Buchanan *et al.,* 2005), hydrophobic membrane protein, MtsB and surface exposed protein Enolase (ENO) (Membrebe *et al.,* 2016).

To treat streptococcosis, there are several commercial vaccines available in the market such as formalin killed *S. agalactiae* (Department of agriculture, USA), AQUAVAC® Strep Sa vaccine from attenuated *S. agalactiae* (Merck Animal Health Company, USA), Aquavac™ Garvetil™ vaccine against *Lactococcus* and *S. iniae* (Intervet/Schering-Plough Animal Health) and NORVAX® STREP Si vaccine, the monovalent vaccine carrying an inactivated *S. iniae* strain (Merck Animal Health Company, USA). However, most of the commercial vaccines are species specific which means limited to one specific strain only.

To our knowledge, there is no report regarding the availability of vaccine that can confer cross protection both *S. agalactiae* and *S. iniae* infection in hybrid red tilapia. Therefore, the present study aimed at evaluating the cross-antigenicity of OSPs of local *S. iniae* and *S. agalactiae* isolated from hybrid red tilapia (*Oreochromis* spp.). Furthermore, the identified antigenic gene and its molecular characteristics were analyzed to determine its potential as effective vaccine in preventing streptococcal infection.

Materials and methods

Bacterial Strains

The pathogenic *Streptococcus spp.*; *Streptococcus agalactiae*, Millud II and *Streptococcus iniae,* TSK_2 used in this study were previously isolated from diseased hybrid red tilapia from Kenyir Lake, Terengganu, and tilapia cages in Rawang Selangor, Malaysia in 2014. Samples were picked from the eyes, kidneys, brains and livers, of the diseased fish for bacterial characterization. They were cultured directly onto Mueller-Hinton (Oxoid, Hampshire, United Kingdom) supplemented with 5% of horse blood agar and incubated at 37 °C for 24 h. A fresh colony was picked and sub-cultured in blood agar for 24 h at 30 ^{0}C to get pure colony and preserved in glycerol stock. Then the glycerol stock was kept in -80 °C until further use. All isolates were identified by cultural and biochemical tests and characterized by 16S rRNA and lactate oxidase *(lcto)* gene analysis.

Extraction of Outer Surface Proteins (OSPs) of S. iniae, TSK_2 and S. agalactiae, Millud II

The extraction of Outer Surface Proteins (OSPs) of *S. iniae* and *S. agalactiae* were done following Gunnar *et al.* (2005) with minor modification. These bacterial strains were grown in tryptic soya agar (TSA, Difco) for 24h at 30^{0}C and then bacterial colonies were transferred into 5L tryptic soya broth (TSB, Difco) for 24 h at 30^{0}C with mild agitation. After that, the bacterial cultures in broth were centrifuged at 5000×g for 10 minutes at 4^{0}C to get the pellets. Then the pellets were washed three times with 25ml phosphate buffered saline (PBS) and finally it was re-suspended in 5ml PBS. 12.5ml EDTA (0.2M) and 3.75 ml lysozyme (10mg/ml) were supplemented into the mixture to lyse the cell wall of bacteria and incubated at 37^0 C for 1 h. After incubation, the suspension was sonicated on ice with the ultrasound power of 8 amplitude micron for 1 h, operated for 20 s and stopped for 10 s in a cycle by using SONIPREP 150 (MSE). The supernatant was accumulated by running a centrifuge for 20 minutes at 10,000×g at 4^0 C and the unbroken cells and cellular debris was discarded. To get the pellet, the collected supernatant was ultra-centrifuged by using Optima XPN-100 ultracentrifuge (BECKMAN COULTER, USA) at 100,000×g at 4^0 C for 2 h. Then pellet was collected and re-suspended with 2ml of 1% (w/v) N-Lauroylsarcosine Sodium Salt (Sigma-Aldrich, USA) followed by incubation at normal temperature for 2 h. Then the suspension was further centrifuged at 100,000×g for 2h at 4^{0}C to get the pellet again and resuspended with 100 µl of PBS. Finally, BCA Protein Assay Kit (Thermo Scientific, USA) was used to determine the concentration of OSPs.

Rabbit hyper immune sera preparation against S. agalactiae and S. iniae

In order to reveal the conserved characteristics of the OSPs of *Streptococcus iniae* and *Streptococcus agalactiae,* four white New Zealand rabbits were immunized subcutaneously with formalin killed whole cell proteins of *S. agalactiae* and *S. iniae* (2.47×10^8 CFU/ml) at 700µl (emulsified with 300 µl of Freund's adjuvant) of each according to the standard protocols. Primary immunization was carried out on day 0; the second and third immunization was at day 14 and 21 respectively. On the day of 28th serum of the respective rabbits was collected and stored at -20^o C until used.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

To reveal the Outer Surface Proteins (OSPs) profiles of *S. iniae* and *S. agalactiae*, SDS-PAGE gels with 12% (w/v) resolving gel and 4% (w/v) stacking gel solution were prepared. The extracted protein was mixed with loading buffer (Thermo Scientific, USA) at the ratio of 1:1 and boiled at 100^0 C for 8 minutes and electrophoresed by using Mini-Protein® II

Electrophoresis Cell (BIO-RAD, USA) in running buffer (0.025M Tris, 0.192M glycine and 0.1% SDS) at 100 V for 150 minutes. A low range molecular weight standard (Promega, USA) was used to compare the molecular masses of the test samples. Finally, the gel was stained with 0.025% Coomassie Blue followed by de-staining for 5h. The size of protein bands were detected and validated using website http://www.gelanalyzer.com/.

Western Blot

The polypeptides were transferred into 0.45 µm nitrocellulose membrane according to the methods of Towbin *et al.* (1979). To perform immunoblotting, the membrane was subjected to 10 minutes washes in PBS/Tween-20, pH 7.2, three times. Non-specific bindings were blocked through incubation for 1 h at 37° C in PBS containing 1% bovine serum albumin (BSA) and 0.5% (v/v) Tween-20. Then, the membrane was washed again three times before incubation at 37° C for 2 h in blocking buffer containing the respective antiserum (rabbit anti-sera of *S. iniae* and *S. agalactiae*) with dilution of 100 times. The membrane was then put for incubation incorporating horseradish-peroxidase labelled anti-rabbit immunoglobulin (IgG), (Promega, USA) diluted in PBS with dilution of 1000 times for 2 h at 37^0 C with a light shaking. The membrane was washed for 3 times of 10 minutes each and submerged with Tris-buffered saline at 37^0 C for the duration of 5 minutes with a light agitation. Eventually, membrane was covered with DAB Tetrahydrochloride (USA) until the band was appeared and washed with distilled water. The antigenic OSPs proteins were localized and validated using website http://www.gelanalyzer.com/.

PCR amplification of Surface immunogenic protein gene of Streptococcus iniae

Primers

In order to amplify the target gene of interest, primers were designed based on the surface immunogenic gene of *S. iniae* strain TSK_2 following the published sequence of *S. agalactiae*, accession number AF151357. Physical constant and self-complimentary analysis of the primers was done through website: www.basic.northwestern.edu/biotools/oligocalc.html. The designed forward and reverse primer sequences were sip-F (5'-GACGACGACAAGATGAAAATGAATAAA-3') and sip-R (5'GAGGAGAAGCCCGGTTATTTGTTAAA-3') respectively. The primers were designed to generate product with vector cohesive overhangs (underlined) for efficient cloning onto pET-32 Ek/LIC vector (Novagen, Germany). Finally, primers were synthesized by First Base Laboratories, Malaysia.

PCR amplification of the target gene

The extracted DNA of *S. iniae* strain TSK_2 was used as template in this amplification. Reagents used for the amplification of the target gene, Sip are as shown in Table 1. A Mastercycler (Eppendorf, Germany) was used to perform polymerase chain reaction in 35 cycles with initial denaturation at 95°C for 3 minutes, denaturation at 95° C for 1 minute, annealing at 51° C for 1 minute, extension at 72° C for 1 minute and 30 seconds, final extension at 72° C for 10 minutes.

Table 1. Prepared reagents for 50µl volume for each reaction for PCR.

Component	Volume per reaction
10X *pfu* buffer with $MgSO_4$	5µl
dNTP mix (10 µM)	1µl
Forward primer, sip-F (20 µM)	1µl
Reverse primer, sip-R (20 µM)	1µl
Pfu DNA polymerase, 2.5 U/µl	0.5µl
DNA template (10 ng/µl)	2µl
PCR grade water	39.5µl
Total volume per tube	50µl

In order to detect the target amplicons, 1% agarose gel electrophoresis was run in 1× tris–boric acid (borate)–EDTA (TBE) for 1 h at 90 V and finally stained with ethidium bromide to make it visible. An ultraviolet transilluminator (Fisher Scientific, Pittsburgh) was used to observe the gel. After that the target bands were excised and purified by Wizard SV Gel and PCR Clean-UP System (Promega, USA). The eluted purified PCR product was stored at -20° C until further use.

Construction of recombinant plasmid

To generate compatible overhangs on the target gene, the purified PCR product was treated with T4 DNA polymerase.

Ligation of target gene with pET-32Ek/LIC vector (Novagen, USA) was performed before transforming into cloning host, *E-coli* Top10 (Invitrogen, USA). Transformed Aid Bacterial Transformation kit (Promega, USA) was used to transform recombinant plasmid into host *E-coli* Top10 for efficient transformation. The successful clones were cultured in LB broth for plasmid extraction. To screen the successful transformed clones, purified plasmids were subjected to PCR colony screening and Restriction Enzyme analysis.

Nucleotide sequence accession number

The accession number for sip gene of *S. iniae* strain TSK-2 in the GenBank is KT898957.

Results

Outer Surface Protein (OSP) Profiles of Streptococcus iniae, TSK_2 *and Streptococcus agalactiae,* Millud II

SDS-PAGE analysis of the OSPs of *S. iniae,* TSK_2 revealed two major and two minor protein bands. The molecular weight of polypeptide bands ranged between 40 to 62 kDa. The major polypeptide bands were 40 and 52 kDa and the minor bands were observed at 48 and 62 kDa (Fig. 1). On the other hand, analysis of the OSPs of *S. agalactiae,* Millud II discovered one major and four minor protein bands. The range of the molecular weight of those polypeptides bands were between 30 to 75kDa. The only one observed major band is 52kDa and the minor bands are 30, 37 and 75 kDa (Fig. 2).

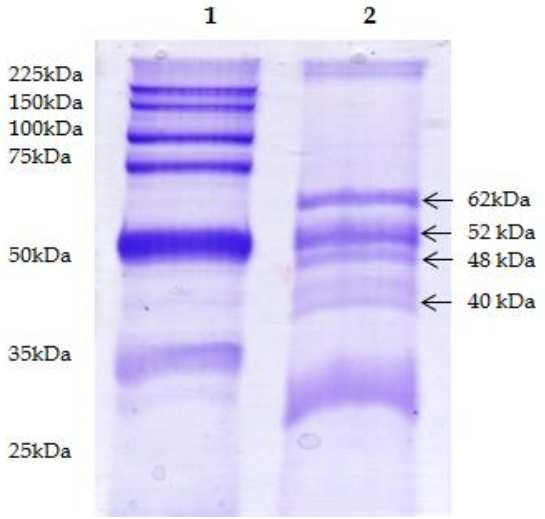

Fig. 1. SDS-PAGE profiles of OSPs of *S. iniae* strain TSK-2 stained by Coomassie blue. Lane 1; Broad Range Protein Molecular Weight Markers (Promega, USA), Lane 2; *S. iniae* strain (TSK_2).

Antigenic analysis for Outer Surface Protein (OSP) of S. iniae, TSK_2 *and S. agalactiae,* Millud II

Homologous immunoblot of OSPs of *Streptococcus iniae* revealed three band of antigenic proteins; 40, 52 and 62 kDa (Fig. 3). On the contrary, OSPs of *S. iniae*, reacted with hyper-immune serum of *S. agalactiae* could not able to detect any antigenic protein (Fig. 4). Similarly, homologous immunoblot of OSPs of *S. agalactiae* revealed one major (52 kDa) and two minor (75 kDa and 110 kDa) antigenic proteins (Fig. 5). Our observation revealed antiserum of *S. iniae* could able to detect all the major (52kDa) and minor antigenic proteins (37 kDa, 43 kDa, 75 kDa, 110 kDa) of *S. agalactiae* (Fig. 3.9). The results from Fig. 3 and 5 showed that 52 kDa is the most prominent protein band in both *S. iniae* and *S. agalactiae* whereas only *S. iniae* (52 kDa) has the cross-antigenic property (Fig. 4 and 6).

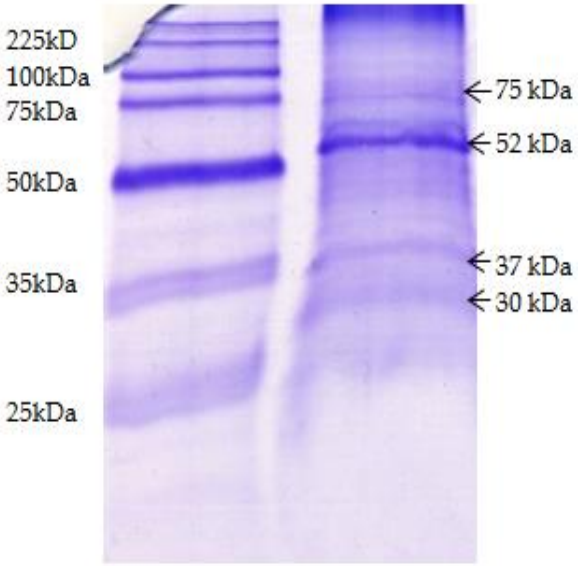

Fig. 2. SDS-PAGE profiles of OSPs of *S. agalactiae* strain Millud II stained by Coomassie blue. Lane1; Broad Range Protein Molecular Weight Markers (Promega, USA), Lane 2; *S. agalactiae* strain (Millud-II).

Molecular cloning and sequencing of 52 kDa encoding surface immunogenic gene of Streptococcus iniae

PCR amplification with designed primers confirmed surface immunogenic gene (sip) in *S. iniae* which is approximately 1305bp long (Fig. 7). The PCR products were purified and successfully cloned into ligation independent pET32Ek/LIC vectors.

The positive clones were selected and screened by colony PCR to reveal the band of approximately 1305bp. The restriction enzyme analysis (RE) of the purified plasmids pET32 Ek/LIC-sip with Kpn I and Xho I successfully cleaved the recombinant plasmids to produce two major bands, the vector pET32Ek/LIC; 5.9kbp and insert ~ 1305bp (Fig. 8). Analysis of restriction enzymes confirmed that the clones carried the target gene as an insert.

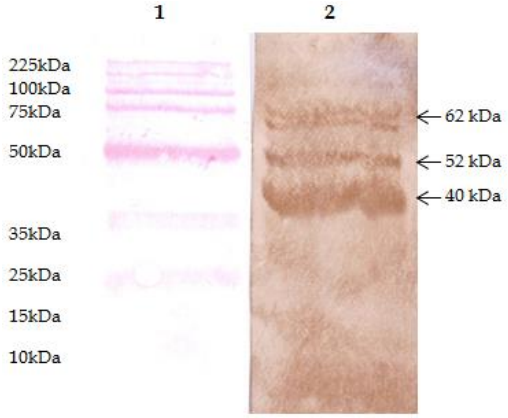

Fig. 3. Immunoblot profiles of the Outer Surface Proteins of *S. iniae*, reacted with hyper-immune serum raised from rabbit against whole-cell proteins of *S. iniae*. Lane 1; Broad Range Protein Molecular Weight Markers (Promega, USA), Lane 2; OSPs of *S. iniae* strain (TSK_2).

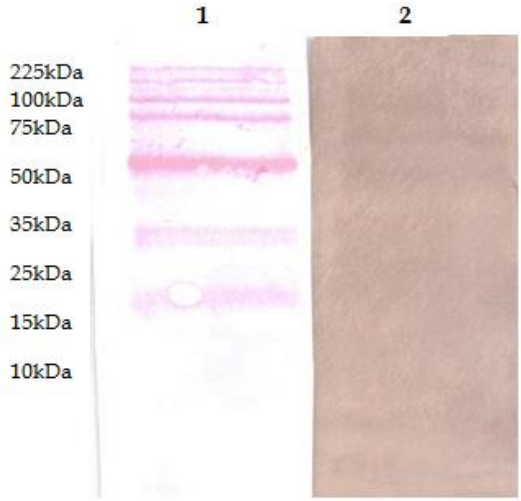

Fig. 4. Immunoblot profiles of the Outer Surface Proteins of *S. iniae*, reacted with hyper-immune serum raised from rabbit against whole-cell proteins of *S. agalactiae*. Lane 1: Broad Range Protein Molecular Weight Markers (Promega, USA), Lane 2: OSPs of *S. iniae* strain (TSK_2). No protein bands were observed in this heterologous reaction.

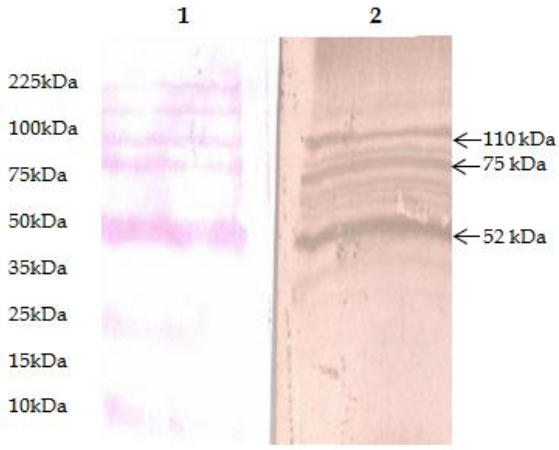

Fig. 5. Immunoblot profiles of the outer surface proteins of *S. agalactiae*, reacted with hyper-immune serum raised from rabbit against whole-cell proteins of *S. agalactiae*.Lane 1: Broad Range Protein Molecular Weight Markers (Promega, USA), Lane 2: OSPs of *S. agalactiae* strain (Millud II).

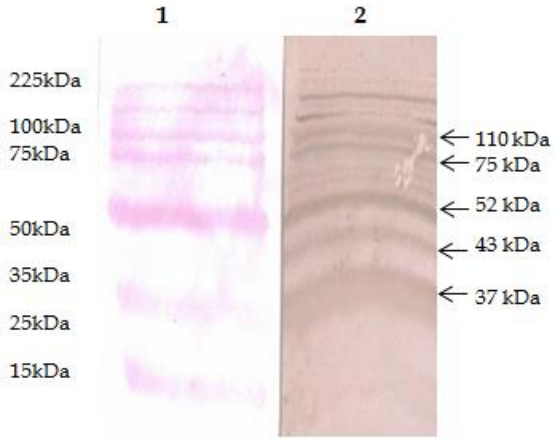

Fig. 6. Immunoblot profiles of the outer surface proteins of *S. agalactiae*, reacted with hyper immune serum raised from rabbit against whole-cell proteins of *S. iniae*. Lane 1: Broad Range Protein Molecular Weight Markers (Promega, USA), Lane 2: OSPs of *S. agalactiae* strain (Millud II).

Bioinformatics analysis

DNA sequencing of recombinant pET32-Ek/LIC plasmid revealed 1305 bp nucleotide sequence which is 99% similar with surface immunogenic gene (sip) of *S. agalactiae* by nBLAST analysis (Table 2). The sequence encodes 434 amino acid residues with 20 antigenic determinants (Fig. 9). The average antigenic propensity for this protein is 1.0319 (Fig. 10).

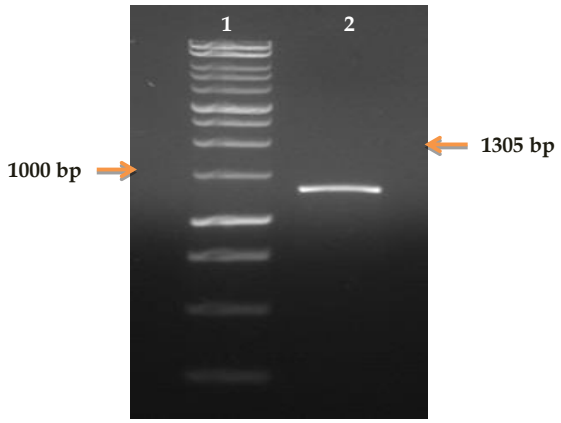

Fig. 7. Agarose gel electrophoresis of PCR amplification of the surface immunogenic gene of *S. iniae*. Lane 1: 1kb DNA ladder; Lane 2: PCR product of *Streptococcus iniae* isolate, TSK_2 strain.

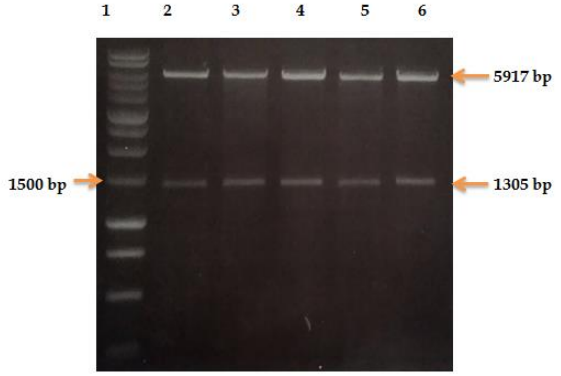

Fig. 8. Restriction enzyme analysis of the positive recombinant clones containing Sip gene as an insert.
Lane 1: 1 kb DNA ladder (Fermentas)
Lane 2 to 6: The upper bands showing the vectors and the lower bands are the Sip gene inserts cleaved from the vectors.

Table 2. nBLAST result of sequenced pET-32 Ek/LIC plasmid.

Organism	Accession No	Query Cover (%)	Similarity (%)
S. agalactiae strain GB00059 Sip (sip) gene, sip-4 allele, complete cds	DQ914270.1	100	99
S. agalactiae strain COH1 group B streptococcal surface immunogenic protein (sip) gene, complete cds	AF151358.1	100	99
S. agalactiae Sip (sip) gene, complete cds	HQ878436.1	99	99
*Streptococcus dysgalactiae*Sd-Sip gene, complete cds	AB769377.1	25	70
Streptococcus pyogenes MGAS8232	AE009949.1	25	70

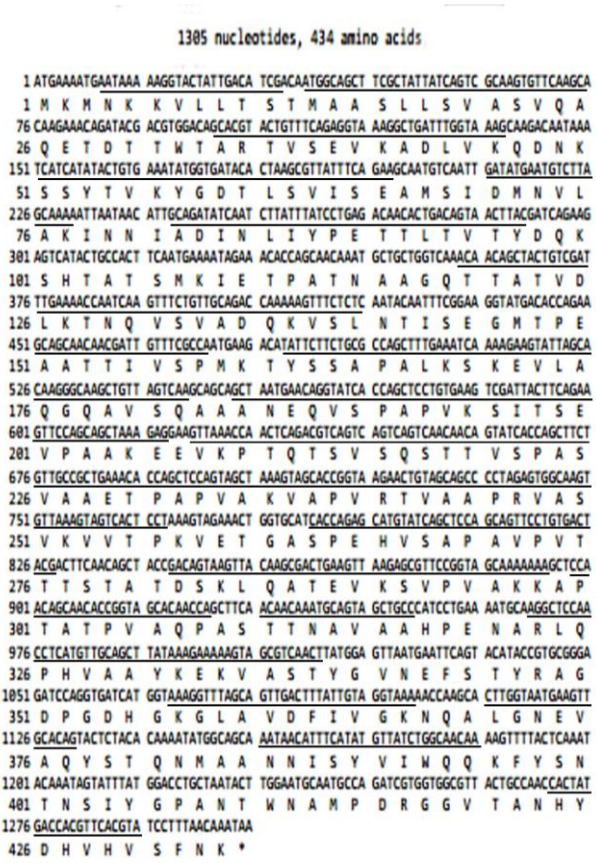

Fig. 9. Full length nucleotide sequence of surface immunogenic gene (sip) of *Streptococcus iniae* (Accession number: KT898957) with antigenic sites underlined.

Discussion

To develop an effective vaccine, antigenic study against the target disease is very important. The OSPs were selected to study the possible vaccine candidate due to its location that attaches with peptidoglycan layers and play vital role to functioning in the bacterial defense mechanisms. In this study, two isolates were selected to study cross protection ability and analyzed by SDS-PAGE, western blotting and immunodetection techniques. The OSPs profiles of *S. iniae* clearly showed 48 and 62 kDa as minor proteins whereas 40 and 52 kDa are major proteins with greater intensity. A similar result has been revealed in M like *S. iniae* protein at mass of 57.5 kDa. In contrast, *S. agalactiae* OSPs profiles discovered only one major band which is 52 kDa and three minor bands including 30, 37 and 75 kDa. Both of these strains revealed 52 kDa as major immunogenic OSPs in western blotting.

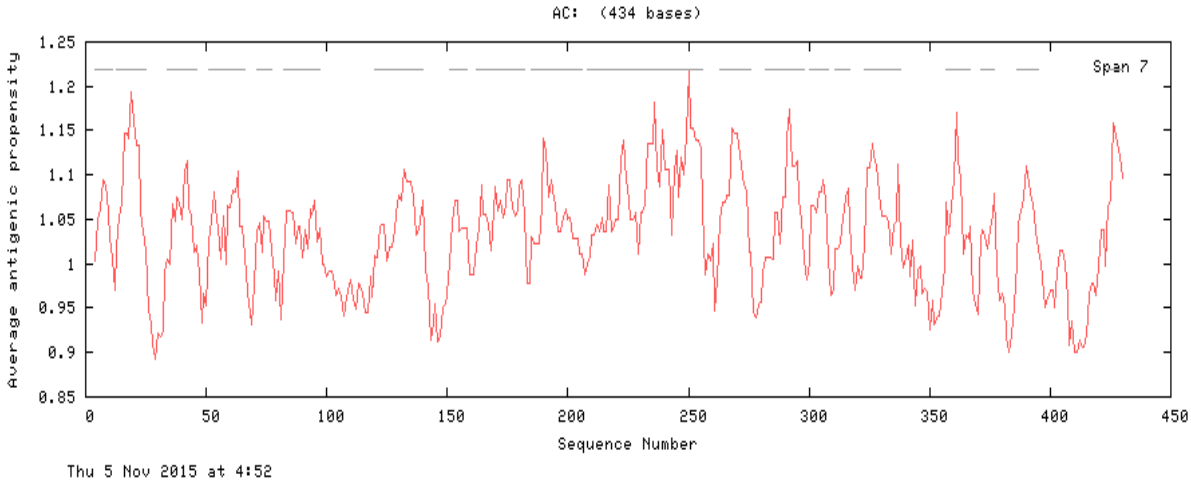

Fig. 10. Antigenic plots for amino acid sequence of *Streptococcus iniae* sip gene.

Fig. 11. Multiple sequence alignments of *S. iniae* (sip) with those of homologous sip gene from other *Streptococcus sp*. Identical amino acid residues presented are boxed in black and the location of dissimilar residues are framed in white. Used accession numbers for alignment analysis of *S. agalactiae*, *S. salivarius* and *S. dysgalactiae* are HQ878436, AM901444 and AB769377 respectively.

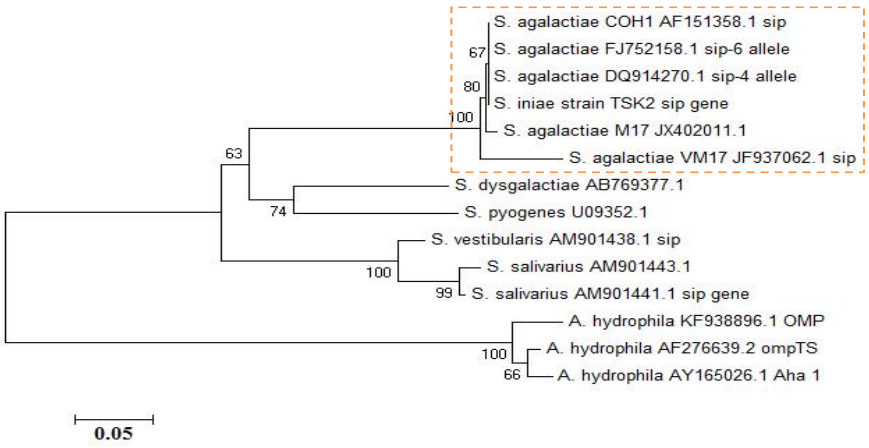

Fig. 12. Phylogenetic tree of *S. iniae* strain TSK_2 sip gene with other *Streptococcus sp*. and some other *Aeromonas sp*. was constructed by the neighbor-joining method and plotted with MEGA 6. Branch support values (10,000 bootstraps) for nodes were indicated. The scale indicates the amount of genetic changes with the length of branch in the horizontal dimension.

Like this protein a band with an approximate molecular mass of 53 kDa which corresponded to the Sip protein was identified with a Sip-specific MAb in every GBS strain tested (Brodeur *et al.,* 2000). A recent study conducted by Nur-Nazifah *et al.* (2014) regarding immunoblot profiles of the OSPs reacted with hyper-immune serum raised from rabbit against whole-cell proteins of *S. agalactiae* discovered 50 kDa antigenic proteins. In addition, a proteomic analysis to identify the major OSPs of S. agalactiae showed a major protein of 46 kDa (Hughes *et al.,* 2002). However, immunoblotting of OSPs for S. iniae, and *S. agalactiae* reacted with hyper-immune serum raised from rabbit against whole-cell proteins of *S. iniae* successfully determined the presence of 52 kDa protein but rabbit antiserum of *S. agalactiae* was unable to detect any antigenic protein in S. iniae. On the basis of this result we choose 52 kDa protein of S. iniae as a vaccine candidate which might confer cross protection against multiple serotypes of *Streptococcus* spp.

The sequence encoding 52 kDa protein of *S. iniae* was amplified based on designed primers and successfully cloned into pET32_Ek/LIC vector. The recombinant plasmid was confirmed to carry the targeted insert which produce 1305 bp of outer surface protein gene (Sip) of *S. iniae* by colony PCR using gene specific primers (sip-F and sip-R) followed by restriction enzyme analysis and plasmid DNA sequencing. This is the first confirmation that sip gene exist in outer surface of *S. iniae*. The identified sip gene sequence of *S. iniae* has twenty antigenic determinants and higher antigenic propensity, approximately 1.0319 which suggest that *S. iniae*-sip gene is highly immunogenic. The BLASTn analysis showed that the surface immunogenic protein (sip) gene from *S. iniae* strain (TSK_2) have the high similarities with the other *Streptococcus species* (Table 2), depicting that it is highly conserved among the *Streptococcus species*. Many previous studies showed that sip genes are considered as highly antigenic which has the potentiality to be a vaccine candidate (Brodeur *et al.*, 2000 and Nishiki *et al.,* 2014).

Furthermore, Group B Streptococcal surface proteins such as α, β and Rib proteins also can induce protective immunity (Bevanger *et al.,* 1979; Lancefield, *et al.,* 1975 and Stålhammar-Carlemalm *et al.,* 1993). Among those surface proteins, the α and Rib proteins are structurally related and are members of the family of streptococcal surface proteins (Michel *et al.,* 1992 and Wästfelt *et al.,* 1996). It was reported that the α protein was found to be present in almost 50% of all clinical isolates (Ferrieri *et al.,* 1997).

Rib protein is capable to elicit protective immunity and is expressed by most strain of type III serotype of GBS. Immunologically protein Rib is unrelated to the α and β proteins, but shares several properties with α protein (Stålhammar-Carlemalm *et al.,* 1993). The α proteins have been reported to occur in forms ranging in size 63 to 200 kDa. However, sequence analysis confirmed that the Sip protein does not have any homology with the α and Rib proteins (Jerlström PG *et al.,* 1996 and Wästfelt, M *et al*., 1996).

Phylogenetic analysis of *S. iniae* sip gene shows that most of the bootstrap values were more than 70% which indicates the strong evidence that the sequences to the right of the node clusters are highly similar to the exclusion of any other. In addition, it was also depicted that *S. iniae* strain TSK-2 sip gene are more similar to *S. agalactiae* sip compared to other Streptococcal strains.

In summary, it is identified that 52 kDa of *S. iniae* and *S. agalactiae* are most prominent immunogenic polypeptide bands in immunoblotting reaction. But in terms of cross antigenicity, outer surface protein 52 kDa of *S. iniae* is more antigenic compared to 52 kDa of *S. agalactiae* because antiserum of *S. agalactiae* could not able to detect any antigenic protein in *S. iniae*. Therefore, it is revealed that outer surface protein of *S. iniae* (52 kDa) could be used as a vaccine candidate that might protect multiple serotypes of Streptococcal infection in aquaculture farms.

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