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Expression, genetic localization and phylogenic analysis of *NAPlr* in piscine *Streptococcus dysgalactiae* subspecies *dysgalactiae* isolates and their patterns of adherence

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ABSTRACT

Streptococcus dysgalactiae, the long recognized mammalian pathogen, has currently received a major concern regarding fish bacterial infection. Adhesion to host epithelial cells and the presence of wall-associated plasminogen binding proteins are prerequisites to Streptococcus infection. This is the first study of the occurrence of nephritis-associated plasminogen-binding receptor (*NAPlr*) and α-*enolase* genes in piscine *S. dysgalactiae* subspecies *dysgalactiae* (SDSD) isolates. Further characterization of surface localized *NAPlr* of fish SDSD revealed a similar immune-reactive band of 43 KDa as that from porcine *S. dysgalactiae* subsp. *equisimilis* (SDSE). The phylogenetic analysis revealed that *NAPlr* of fish SDSD is more associated with those of mammalian SDSE and *Streptococcus pyogenes* rather than of other streptococci. Our findings warrant public attention to the possible implication of these virulence genes in dissemination of SDSD to different tissues of infected hosts and to get advantage to new niches. The SDSD adherence patterns were also studied to better understand their pathogenicity.

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The patterns of adherence of SDSD on two different cell lines showed a different pattern of adherence. Such difference gives an insight about the variance in host susceptibility to infection.

Introduction

Streptococcus dysgalactiae was discovered by Diernhofer in 1932 [1], and officially recognized as a new species in 1983 [2]. *S. dysgalactiae* was subdivided into two genetically similar subspecies: the animal subspecies *dysgalactiae* (belongs to Lancefield group C (GCS)) and human subspecies *equisimilis* (belongs to GCS or GGS or GLS) [3]. The α-hemolytic *S. dysgalactiae* subsp. *dysgalactiae* (SDSD) is a strict animal pathogen of pyrogenic streptococcus [4]. SDSD is responsible for diverse problems such as mastitis, toxic shock like syndrome, subcutaneous cellulitis in cows [5], extensive fibrinous pleurisy in ewes [6], suppurative polyarthritis in lambs [7], neonatal mortalities in dogs [8], severe septicemia in fish [9], and bacteremia and meningitis in immunocompromised individuals [10,11]. SDSD is potentially considered as an emerging zoonotic agent since it is implicated in cutaneous cellulitis in humans engaged either in cleaning fish [12] or handling livestock [13].

SDSD has been associated with high mortalities in Kingfish (*Seriola lalandi*), amberjack (*S. dumerili*) and yellowtail (*S. quinqueradiata*) in Japan [9,14–17], Nile tilapia (*Oreochromis niloticus*) in Brazil [18], Amur sturgeon (*Acipenser schrenckii*), the Siberian sturgeon (*A. baerii*), golden pomfret (*Trachinotus ovatus*), Soiny mullet (*Liza haematocheila*) grass carp (*Ctenopharyngodon idella*), crucian carp (*Carassius carassius*) and pompano (*Trachinotus blochii*) in China [19–22]. It has been recovered from cobia (*Rachycentron canadum*), basket mullet (*Liza alata*) and grey mullet (*Mugil cephalus*) in Taiwan, hybrid red tilapia (*Oreochromis* sp.) in Indonesia, white spotted snapper (*Lutjanus stellatus*) and pompano (*T. blochii*) in Malaysia [9,16,17,23], and rainbow trout (*Oncorhynchus mykiss*) in Iran [24]. The infected fish revealed systemic pyrogranulomatous inflammation with a severe necrotic lesion in their caudal peduncles [25]. Despite its clinical significance, the complete sequence revelation and virulence characterization are generally unknown for SDSD. Fish SDSD was found to possess some virulence factors such as streptolysin S structural gene (*sag*A), streptococcal pyrogenic exotoxin G gene (*spegg*) and serum opacity factor (*SOF-FD*) [17,26]. Fish SDSD strongly adheres to and invades fish epithelial cell line as Epithelial Papiloma of Carp (EPC) *in vitro* [14]. However, the adherence patterns and the surface structures implicated in adhesion are still uncovered. The M/M-like proteins (*emm*), surface dehydrogenase (*SDH*) and α-*enolase* are the most important wall-associated plasminogen-binding proteins of pathogenic streptococci [27]. The ability of pathogenic streptococci to bind host plasminogen system empowers their invasiveness through utilizing the fibrinolytic activity of plasmin and promoting the adherence of streptococci to host cells [27]. Plasminogen-binding glycoproteins, such as α-*enolase* and *SDH*, are generally found in the cytosolic compartment and are transported to the bacterial cell wall by a yet unknown mechanism that comprised moonlighting functions [28–30]. The surface protein *SDH* displays ADP-ribosylating activities and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) [31], and has been recognized as a potential nephritogenic protein under the name nephritis-associated plasminogen-binding receptor (*NAPlr*) [32]. Streptococcal cell wall α-*enolase* is associated with streptococcal infection and post-streptococcal autoimmune disease in human [28,30].

Hence, *NAPlr* and α-*enolase* genes are important virulence factors in *Streptococcus pyogenes* [33,34], *S. agalactiae* [35], *S. iniae* [30], and *S. pneumoniae* [28,29] due to its contribution to the establishment of infections and colonization by bacterial pathogens [27,36]. This is the first study to investigate the occurrence of *gapdh*/*naplr*/*sdh* and α-*enolase* genes in piscine isolates of SDSD. We also investigated the adherence patterns of selected SDSD strains to EPC and CHSE-214 (Chinook salmon embryo) cell lines *in vitro*.

Material and methods

Bacterial isolates

Twenty-three bacterial isolates were used in this study. The α-hemolytic SDSD isolates ($n = 18$) were recovered from moribund fishes obtained from various fish farms in Japan ($n = 9$; three from king fish, three from amberjack and three from yellowtail), Taiwan ($n = 5$; three from grey mullet, one from cobia and one from basket mullet), Malaysia ($n = 2$; one from pompano and one from snapper), China ($n = 1$; one from pompano) and Indonesia ($n = 1$; one from tilapia). For comparative purpose, β-hemolytic *S. dysgalactiae* subsp. *equisimilis* (SDSE) isolates ($n = 5$) were collected from pigs with endocarditis (Kumamoto meat inspection office in Japan).

DNA extraction

The pure stock isolates were stored in Todd-Hewitt broth (THB; Difco, Sparks, MD, USA) at −80 °C. All isolates were cultured aerobically on Todd Hewitt agar (THA; Difco, Sparks, MD, USA), and on 5% sheep blood agar (Columbia agar base; Becton Dickinson, Cockeysville, MD, USA), and then incubated at 37 °C for 24 h. Genomic DNA was extracted from cultivated strains using a DNAzol® reagent (Invitrogen, Carlsbad, USA) [37]. The fish SDSD isolates were discriminated from pig SDSE isolates by using *sodA* gene primers specific for fish SDSD detection. PCR was performed as described previously [37].

PCR detection of virulence genes

PCR amplification of *emm* was performed using specific primer pairs; A: (5′-TATTAGCTTAGAAAATTAA-3′) and B: (5′-GCAAGTTCTTCAGCTTGTTT-3′) as described previously by Zhao et al., [38]. To amplify a 963-bp fragment of *NAPlr*; the specific primer pairs of Plr 1: 5′-GTTAAAGTTGGTATTAACGGT-3′, and Plr 2: 5′-TTGAGCAGTGTAAGACATTTC-3′ were designed based on nephritis

associated plasminogen receptor gene of SDSE (GenBank accession number AB217852). PCR was performed with the following parameters: an initial denaturation cycle at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 s, primer annealing at 52 °C for 30 s, elongation at 72 °C for 50 s, and a final cycle at 72 °C for 10 min. To amplify a 1308-bp fragment of *α-enolase*; the primer pairs of Eno1: 5′-ATGTCAATTATTACTGATGT-3′, and Eno2: 5′-CTAT TTTTTTAAGTTATAGA-3′ were designed based on *α-enolase* gene of SDSE (AP012976). The thermal scheme of PCR was performed as described for the *NAPlr* gene, except that the primer annealing was adjusted at 50 °C and the primer extension was set for 1 min.

Cloning and sequencing of NAPlr and α-enolase

The *NAPlr* and *α-enolase* genes were sequenced according to Abdelsalam et al. [17]. The amplified products were cloned into pGEM-T easy vector (Promega, Madison, WI, USA), and the recombinant plasmid was introduced into *Escherichia coli* DH5α. The QIAprep Spin Miniprep kit (Qiagen, Germantown, MD, USA) was used to purify the plasmid DNA. Sequencing reactions were performed by using the oligonucleotide primers SP6 (5-ATTTAGGTGACACTATAGAA-3) and T7 (5-TAATACGACTCACTATAGGG-3) with the GenomeLab DTCS Quick Start Kit (Beckman Coulter, Fullerton, CA, USA). The samples were then loaded into the CEQ 8000 Genetic Analysis System (Beckman Coulter) and the nucleotide sequence was determined. The nucleotide sequences were analyzed by using BioEdit version 7.0 [39]. The phylogenetic analysis was performed by the neighbor joining method using MEGA version 5 [40]. The nucleotide sequences of the *NAPlr* and *α-enolase* genes were submitted to the DNA Data Bank of Japan (DDBJ) nucleotide sequence database.

Surface protein extraction

Bacterial surface proteins were extracted according to the protocol described by Fujino et al. [32] with some modifications. Briefly, bacteria were inoculated onto Todd Hewitt agar and the culture was incubated for 16 h at 37 °C. Then, bacterial colonies were harvested from the surface of the grow medium/agar plates by loops and were suspended in phosphate-buffered saline (PBS, pH 7.5) in a tube. The bacterial cells were then centrifuged at 10,000*g* for 20 min. The bacterial cell pellet was then resuspended in PBS. Bacterial cell pellets were washed three times with sterile PBS, and surface proteins were extracted using sodium dodecyl sulfate (SDS; Bio-Rad, Hercules, CA, USA, 30 mg wet weight of bacteria per 100 µl of 0.2% SDS) for 1 h at 4 °C. Extraction mixture was centrifuged and supernatant protein samples were recovered. The SDS extract of bacterial surface proteins was filtered consecutively through 0.45-µm (Millex-HV, Millipore) and 0.22-µm (Millex-GX, Millipore) sterile Millipore filters to remove bacteria. Protein concentration was determined using Bradford assay kit (Bio-Rad, Hercules, CA, USA).

Production of anti-NAPlr monoclonal antibody

Anti-*NAPlr* monoclonal antibody (mAb) was produced as previously described [32]. Briefly, the specific pathogen-free BALB/c mice were injected intraperitoneal (IP) with 100 mg recombinant NAPlr emulsified in Freund's complete adjuvant. Three weeks later, the mice were given a booster immunization with 100 mg of recombinant Plr emulsified in Freund's incomplete adjuvant. Thirty days later, the mice were injected intraperitoneally with 100 mg of recombinant PH in PBS. After 3 days, the mice were sacrificed and their spleens removed. The splenocytes were fused with P3U1 myeloma cells. Hybridoma cultures that secreted anti-Plr antibody were cloned by limiting dilution and the resulting monoclonal antibodies (mAbs) were rescreened to determine the specificity and reactivity with Plr.). The gained Anti-*NAPlr* mAb from hybridoma cultured cells was evaluated by ELISA using r*NAPlr*. *All Institutional and National Guidelines for the care and use of animals were followed.*

Western blots for NAPlr

Protein extract (10 µg protein/lane) of three SDSD isolates (12–06, KNH07808, T11358) and another (5 µg protein/lane) of three SDSE isolates (PAGU656, PAGU706, PAGU707) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) on 12.5% polyacrylamide gels (SuperSep 12.5%. Wako Pure Chemical, Osaka, Japan), and then transferred to PVDF (Millipore, Bedford, MA, USA) using a semi-dry blotter (ATTO Bioscience, Tokyo, Japan). SDS–PAGE "wide range" (200–6.5 kDa) molecular weight standard was purchased from Sigma. *NAPlr* was identified by the use of the previously prepared anti-*NAPlr* mAb combined with peroxidase-labeled anti-mouse IgG (American Qualex, San Clemente, CA, USA) and ECL Advance Western Blotting Detection Kit (GE Healthcare, Buckinghamshire, UK). Blot of *E. coli* was included as a negative control. *NAPlr* expression in each strain was quantified based on the strength of the luminescence of the mAb – specific band with the densitometry system (Atto).

Adherence pattern of SDSD

This assay was performed according to the method described by Duary et al. [41] with some modifications. Briefly, a sterile 12 mm diameter glass cover-slip coated with poly-L-lysine (NeuVitro, El Monte, CA, USA) was placed in each well of the 24-well tissue culture plate (Costar, Corning, Inc., NY, USA) and the wells were seeded with EPC or CHSE-214 cells. The seeded cells of the EPC or CHSE-214 were grown in Leibovitz-15 (L-15) medium (Gibco Invitrogen, USA) containing 10% (v/v) fetal bovine serum and penicillin (5 µg/ml; Sigma–Aldrich Inc., USA), and incubated at 25 °C and 18 °C respectively, in 5% CO_2, and inspected daily until they attained semi-Confluency (2×10^5 cells/well). The SDSD isolates (12–06, KNH07808, T11358) were incubated in THB overnight at 37 °C to midlogarithmic phase (10^8 CFU/ml), and then centrifuged at 2190*g* for 30 min. Pellets were washed twice with phosphate-buffered saline (PBS; pH 7.2), and the cell concentration/counts were adjusted to approximately 10^8 CFU/ml. 100 µl of the bacterial suspension was inoculated to the wells containing EPC and CHSE-214 cells (final bacterial cell concentration in the wells was approximately 10^7 CFU/ml) and the culture plates were incubated for 30 min at 25 °C and 18 °C for EPC and CHSE-214,

Table 1 The α-hemolytic fish SDSD and β-hemolytic pig SDSE strains used in this study.

	No.	Isolates	Source	Country	Hemolysis	*NAPlr*[a]	*eno*[b]	*emm*[c]
Fish SDSD strains	1	12-06	Amberjack	Japan	α	+	+	–
	2	Kdys0412	Amberjack	Japan	α	+	+	–
	3	Kdys0429	Amberjack	Japan	α	+	+	–
	4	Kdys0728	Yellowtail	Japan	α	+	+	–
	5	Kdys0719	Yellowtail	Japan	α	+	+	–
	6	Kdys0707	Yellowtail	Japan	α	+	+	–
	7	KNH07808	King fish	Japan	α	+	+	–
	8	KNH07901	King fish	Japan	α	+	+	–
	9	KNH07902	King fish	Japan	α	+	+	–
	10	95980	Mullet	Taiwan	α	+	+	–
	11	95921	Mullet	Taiwan	α	+	+	–
	12	95900	Mullet	Taiwan	α	+	+	–
	13	951003	Basket mullet	Taiwan	α	+	+	–
	14	AOD-96086-K	Cobia	Taiwan	α	+	+	–
	15	PF880	Pompano	Malaysia	α	+	+	–
	16	PP1564	Pompano	China	α	+	+	–
	17	WSSN1609	Snapper	Malaysia	α	+	+	–
	18	T11358	Tilapia	Indonesia	α	+	+	–
Pig SDSE strains	19	PAGU656	Pig	Japan	β	+	+	+[d]
	20	PAGU657	Pig	Japan	β	+	+	–
	21	PAGU699	Pig	Japan	β	+	+	–
	22	PAGU706	Pig	Japan	β	+	+	+[d]
	23	PAGU707	Pig	Japan	β	+	+	+[d]

[a] *NAPlr*: Nephritis associated plasminogen receptor.
[b] *eno*: α-enolase gene.
[c] *emm*: M protein gene.
[d] The sequences of *emm* locus of positive SDSE isolates not determined.

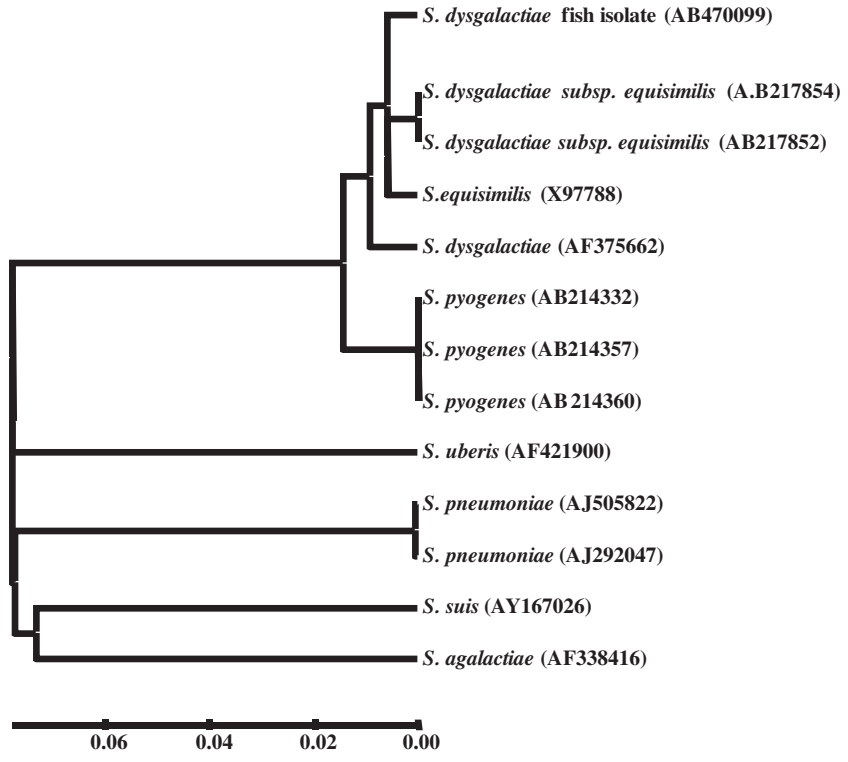

Fig. 1 Phylogenetic tree of *NAPlr* of fish SDSD and related species of the genus *Streptococcus*.

respectively. The monolayers were then carefully washed several times with L-15 medium to remove non-adherent bacteria by gentle pippeting. The cells were then fixed with 70% methanol for 10 min and fixed cells were stained with 10% Giemsa

stock solution for 2 h. Finally, the glass cover-slips were thoroughly washed with PBS and mounted onto glass slides before being examined by light microscope and photographed. The bacterial adherence patterns were categorized according to the following criteria: localized-like-adherence (LAL), when the bacteria adhered to the cell surface, forming loose clusters; localized adherence (LA), when the bacteria adhered to the cell surface as tight clusters; diffuse adherence (DA), when the bacteria adhered diffuse to the cell surface; and aggregative adherence (AA), when the bacteria adhered to the cell surface and to the cover slip in a stacked-brick pattern [42]. The adherence rate was expressed as the number of adhering bacteria per 50 cells of EPC or CHSE-214. The results were expressed as a weak adherence ($\leqslant$100 adherent bacteria), moderate adherence (100–200 adherent bacteria) and strong adherence ($\geqslant$200 adherent bacteria) [43].

Nucleotide sequence accession numbers

The nucleotide sequences determined in this study were submitted to the DDBJ nucleotide sequence database. The accession numbers of sequenced *Gapdh*/*sdh*/*naplr* and *α-enolase* genes are AB470099 and AB758245, respectively.

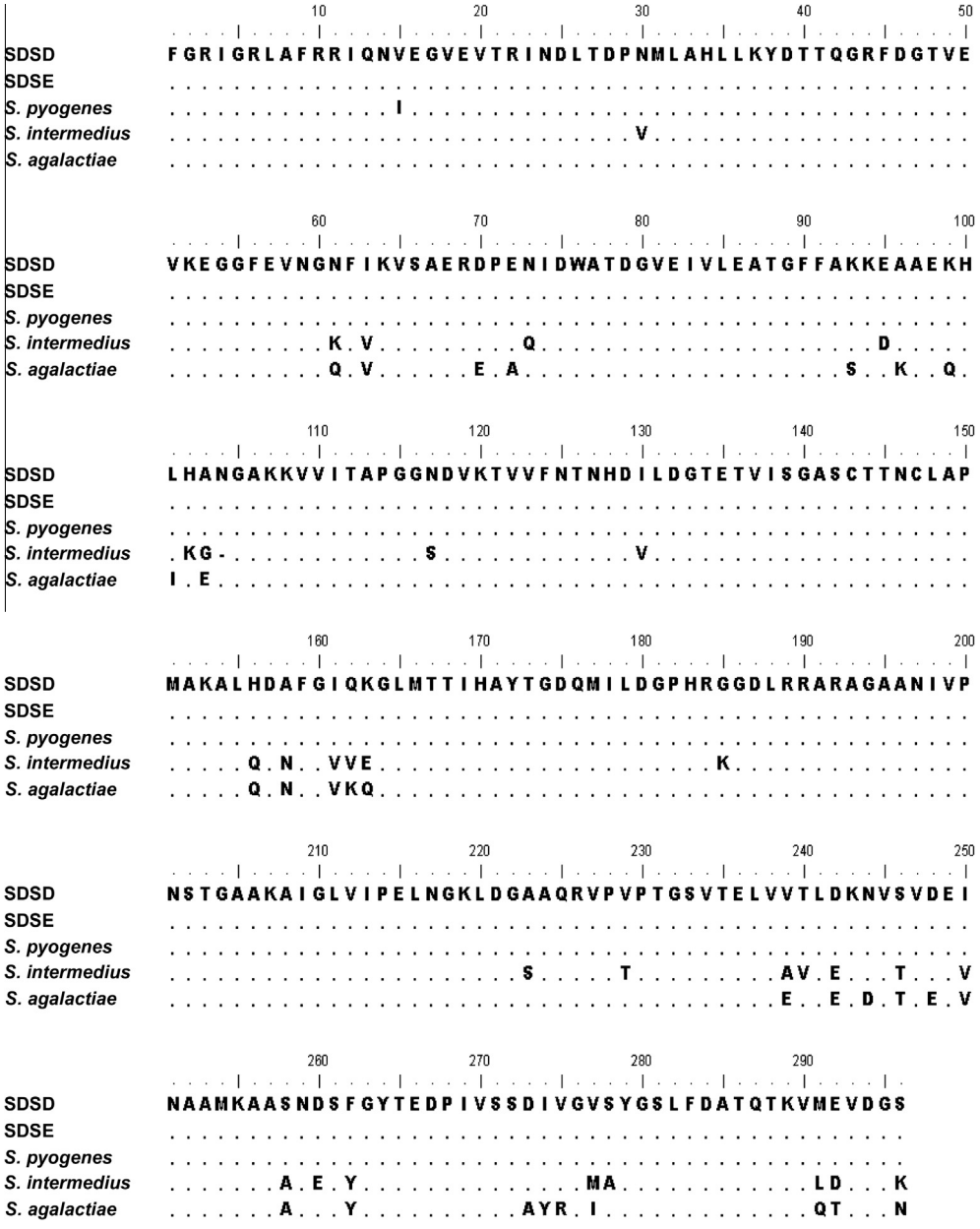

Fig. 2 Alignment of the deduced amino acid sequences of the NAPlr from fish SDSD (accession No. AB470099), *S. pyogenes* (accession No. AB088214), and SDSE (accession No. AB217852), *S. intermedius* (accession No. NC022244) and *S. agalactiae* (accession No. AB221040). The dots represent identical residues. NAPlr from SDSD shares 100%, 99%, 91% and 90% identity with its homologous from SDSE, *S. pyogenes*, *S. agalactiae* and *S. intermedius* respectively. The numbering of the residues is indicated above the amino acids.

Results

Occurrence of emm, NAPlr and α-enolase genes

All fish SDSD isolates were PCR negative for *emm*. However, three SDSE isolates (PAGU656, PAGU706, PAGU707) were PCR positive for *emm*. All SDSD and SDSE isolates contained homologous segments of *NAPlr* and *α-enolase* (Table 1). The PCR products of distinct strains were of the expected size, 963 bp and 1308 bp, respectively.

Nucleotide sequence analyses of NAPlr

The *NAPlr* gene of SDSD collected from diseased fish was sequenced under the GenBank accession number AB470099. The *NAPlr* gene obtained from SDSD strain (T11358) was 963 bp long. The *NAPlr* was found to have 99% similarity to *NAPlr* (AB217852) of SDSE and 97% similarity to *NAPlr* (AB214357) of *S. pyogenes*, and has one ORF encoding 336 amino acids. Therefore, phylogenetic analysis revealed that *NAPlr* of piscine SDSD isolate was related to that of SDSE and *S. pyogenes* and separated from other *gapdh*/*sdh*/*naplr* clusters of other streptococci (Fig. 1). The deduced amino acid sequence of fish SDSD *NAPlr* was identical to the previous investigated nephritogenic strains of SDSE and *S. pyogenes* (Fig. 2).

Nucleotide sequence analyses of α-enolase

The *α-enolase* gene of SDSD from diseased fish was sequenced under the GenBank accession number AB758245. The *α-enolase* locus obtained from fish SDSD strain (KNH07808) was 1308 bp long. The *α-enolase* was found to have 99% similar to that of SDSE (AP011114), 97% similarity to *S. pyogenes* (EF362410), and 91% similarity to *S. iniae* (KF460454), and has one ORF encoding 435 amino acids. Therefore, phylogenetic analysis revealed that *α-enolase* of fish SDSD isolate was related to that of SDSE and *S. pyogenes* and separated from other *α-enolase* clusters of other streptococci (Fig. 3).

Western blots

The presence of *NAPlr* in the cell wall was analyzed by Western blotting using anti-*NAPlr* mAb. As expected, a 43-kDa band corresponds to the molecular weight of *NAPlr* of SDSD was clearly detected (Fig. 4). All fish isolates of SDSD and pig isolates of SDSE expressed a similar 43-kDa *NAPlr* band (Fig. 4).

Cell adherence pattern

Fish SDSD has a localized adherence pattern (Fig. 5) characterized by the presence of one chain of bacterial cells attached to the surface of CHSE-214 at a focal point. On the other hand, fish SDSD has an aggregated adherence pattern (Fig. 5) characterized by clumps or clusters of bacterial cells on the EPC cells. SDSD were also attached to the surfaces of the cultured EPC and to exposed areas of the glass slide around the EPC cells. EPC and CHSE-214 cells that were infected with SDSD showed cytoplasmic vacuoles.

Tested 12-06, KNH07808 and T11358 isolates were categorized as strongly adhesive ($\geqslant$200 adherent bacteria) on EPC culture, but weak adhesive ($\leqslant$100 adherent bacteria) on CHSE-214 culture.

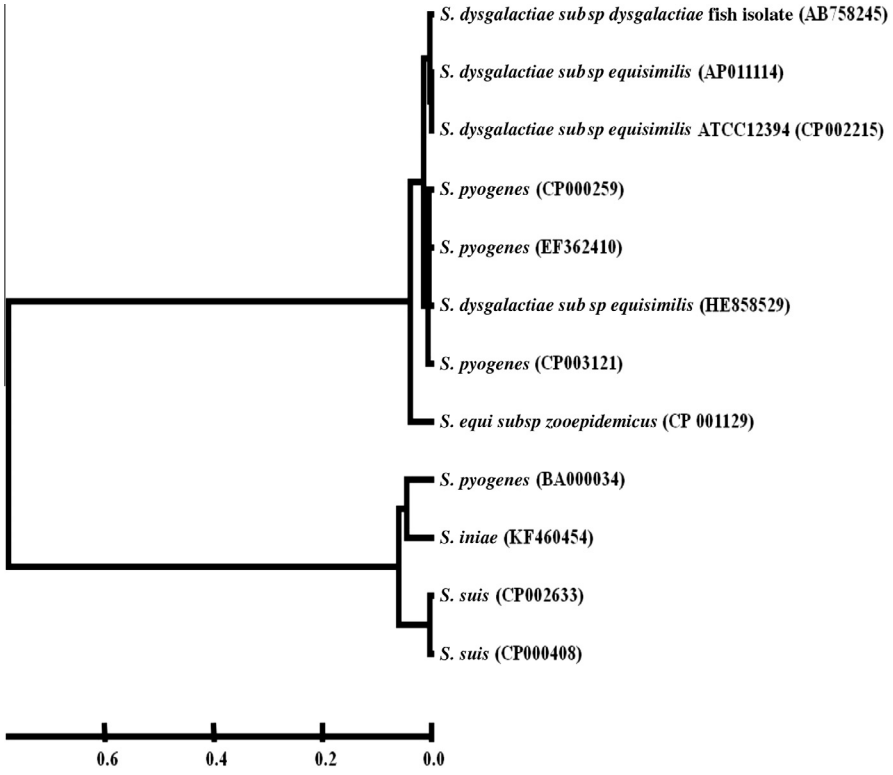

Fig. 3 Phylogenetic tree of *enolase* of fish SDSD and related species of the genus *Streptococcus*.

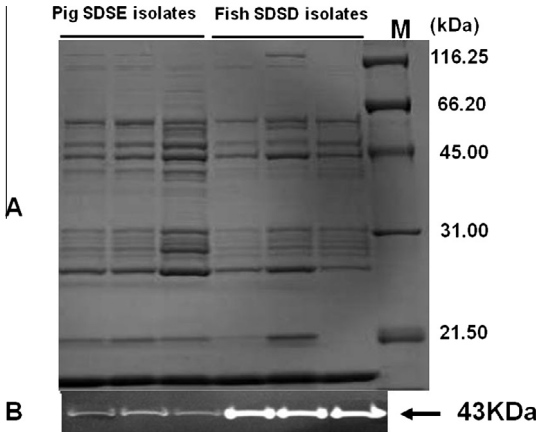

Fig. 4 Expression of NAPlr. (A) Protein extracts of fish SDSD (10 μg protein/lane) and pig SDSE (5 μg protein/lane) were separated by 10% polyacrylamide SDS–PAGE and subjected to Western blotting. The similar densities of the 43-kDa NAPlr protein bands and other protein bands on the same blot indicate that the samples contain equal amounts of proteins. (B) Western blot analysis of NAPlr of three isolates of fish SDSD (12-06, KNH07808, T11358) and three isolates of pig SDSE (PAGU656, PAGU706, PAGU707) probed with anti-NAPlr mAb.

Discussion

S. dysgalactiae was found in human and porcine β-hemolytic SDSE isolates and in piscine, bovine, and porcine α-hemolytic SDSD isolates [17,37]. Recently, SDSD infection has been observed in farmed fish resulting in severely necrotizing caudal peduncles [23,25]. SDSD caused either an opportunistic infection in immunocompromised patients [12,13,44], or invasive infection in individuals handling livestock and seafood [10,11]. Pathogenesis and clinical signs of different Streptococcus species appear highly similar among a variety of infected hosts. This means that similar virulence traits may exist [45]. However, little is known about the virulence factors of fish SDSD when compared with other streptococci.

Pathogenic streptococci can use host plasminogen for adherence to cell surfaces, dissemination in the body, and protects against immune defense [27,34]. This complex pathogenic scenario reveals the complicated adaptation of streptococci in invading their host environments. Streptococci harbor a broad variety of different plasminogen binding and activation mechanisms. The M/M like protein, *gapdh*/*sdh*/*naplr* and α-*enolase* have been described as proteins associated with virulence in several pathogenic bacteria [27–31,34]. In this study, fish SDSD strains were found to be PCR negative for *emm* gene. This indicates either the absence of this gene within the investigated isolates or the isolates possess gene variants that could not be detected by *S. pyogenes*-based primers used in this study. On the other hand, three SDSE isolates were positive for the presence of the *emm* gene. These findings concur with previous investigations that proved the presence of *emm* gene in clinical isolates of β-hemolytic SDSE, but not in bovine SDSD [45].

The present study also confirms the presence of *NAPlr* and α-*enolase* genes in all examined fish SDSD and pig SDSE isolates using their specific primers. These findings go parallel with previous reports that detected the presence of *GAPDH* and α-*enolase* in bovine SDSD [45]. Interestingly, the sequenced fragments of *NAPlr* and α-*enolase* genes revealed 99% similarity with those of SDSE. Moreover, the partial predicted amino acid sequence of *NAPlr* of fish SDSD shows no difference from that of SDSE. Most of amino acid variants observed in fish SDSD are structurally relevant and functionally compatible with their corresponding substitution residues in other isolates (e.g. the replacement of non-polar Valine (V) amino acid residue with non-polar Isoleucine (I) at 16, and substitution of I with V at positions 63, 130, 161 and 250). These results agree with Madureira et al. [35]. Recently, *gapdh*/*sdh*/*naplr* and α-*enolase* genes play multiple roles in virulence of pathogenic streptococci such as adhesions, helping the bacteria escape detection by neutrophils, and allowing the evasion of the complement system [33,34,36]. It has been also reported that *gapdh*/*sdh*/*naplr* induces clot formation, disrupts intracellular signaling in the host, promotes bacterial adherence to host cells, and binding to various host proteins, including plasmin, actin, fibronectin and myosin [27,31–35]. The recent studies have provided definitive proof that *NAPlr* is a potent nephritogenic antigen [31]. *NAPlr* gene was thought to be a factor leading to the pathogenesis of acute post-streptococcal glomerulonephritis (*APSGN*). Kim et al. [30] proposed that α-*enolase* might facilitate the invasion and dissemination of *S. iniae* in infected fish. Our findings signify that α-hemolytic fish SDSD isolates carried homologous genes that may be responsible for pathogenesis and virulence of SDSE and *S. pyogenes*. Consequently, α-hemolytic fish SDSD isolates should not be neglected as putative infectious disease

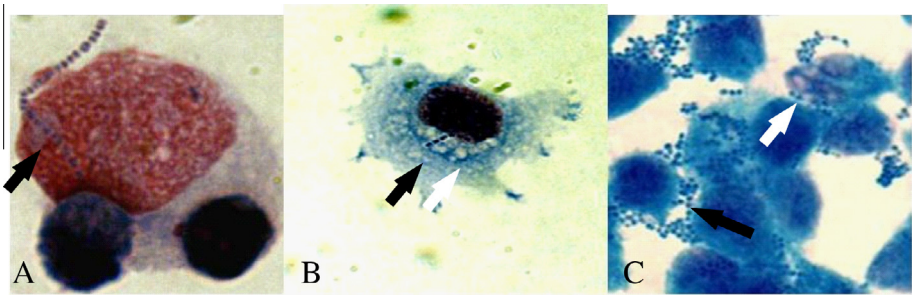

Fig. 5 Microscopic appearances of SDSD adhered to: (A) CHSE-214; (B) CHSE-214; and (C) EPC. Both cell lines were exposed to 10^7 CFU/well and stained with Giemsa (X1000). Black arrows showed cells of SDSD adhered to CHSE-214 and EPC. The white arrow showed cytoblasmic vacuoles.

agents in mammals and humans. Further studies are needed to clarify the role of *NAPlr* and *α-enolase* in the pathogenesis of SDSD among cultured fish.

It has been postulated that the portal of entry of SDSD into fish is mainly through the skin rather than the oral route. Therefore, adherence of streptococci to epithelial cells was tested since adherence capacity is correlated with the pathogenesis of streptococci. Here the adherence of tested isolates to cell lines, CHSE-214 and EPC cells, was generally varied. Fish SDSD was previously found to adhere strongly to EPC due to the high hydrophobic character of SDSD [14]. In this study, the isolates that adhered strongly to EPC cells were the same ones that adhered weakly to CHSE-214 cells. This variation may occur due to either different cell lines were used or it was associated with the host susceptibility to bacterial infection. The SDSD with the same surface hydrophobic property might employ different mechanisms in adherence upon growth on different cell lines.

Conclusions

In conclusion, this is the first study on molecular characterization of *NAPlr* and *α-enolase* – as two virulence-related genes – in fish SDSD isolates. Our finding clearly demonstrates the immune-reactive similarity of *NAPlr* protein as that from SDSE. With more conserved nature, the phylogenetic analysis proved that *NAPlr* of fish SDSD is more related to SDSE and *S. pyogenes* and separated from other *gapdh*/*sdh*/*naplr* clusters of other streptococci.

Conflict of interest

The authors have declared no conflict of interest.

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