한국어병학회지 제28권 제1호 (2015)
J. Fish Pathol., 28(1) : 9~15

www.ksfp.org
pISSN 1226-0819, eISSN 2233-5412
http://dx.doi.org/10.7847/jfp.2015.28.1.009

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from *Streptococcus iniae* shows potential as a subunit vaccine against various streptococcal species

Min Sun Kim, Seung Hyuk Choi and Ki Hong Kim†

Department of Aquatic Life Medicine, Pukyong National University, Pusan 608-737, Korea

The potential of *Streptococcus iniae* glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an antigen for a subunit vaccine was investigated using a zebrafish model. The recombinant *S. iniae* GAPDH was purified using His-tag column chromatography, and antisera against the recombinant GAPDH (rGAPDH) were produced by intraperitoneal immunization of rats. By immunization with *S. iniae* rGAPDH, the survival rates of zebrafish against an *S. iniae* challenge increased, suggesting that GAPDH would be an antigen capable of inducing protective immune responses in fish. Furthermore, we demonstrated using Western blotting, that the antisera against rGAPDH of *S. iniae* had cross-reactivity with GAPDH from *Streptococcus parauberis* and *Lactococcus garviae*, which are also culprits of streptococcosis in cultured fish in Korea. These results suggest that *S. iniae* GAPDH may be used as an antigen for the development of a subunit vaccine against streptococcosis caused by diverse cocci in cultured fish.

Key words: *Streptococcus iniae*, GAPDH, Subunit vaccine, Cross-reactivity

A Gram-positive coccus, *Streptococcus iniae*, is a notorious pathogen to aquaculture world-widely, and is responsible for significant economic losses in the fish farm industry. Moreover, zoonotic infection of *S. iniae* in human have been reported from several countries (Weinstein *et al*., 1997; Goh *et al*., 1998; Lau *et al*., 2003, 2006; Koh *et al*., 2004). In Korea, *S. iniae* has been a cause of substantial losses in cultured fish along with *S. parauberis* and *Lactococcus garvieae.* Since antibiotic therapy of farmed fish infected with streptococci gives unsatisfactory results, development of an effective vaccine is inevitably needed to control coccal diseases in cultured fish. Although efficacious vaccine has been developed to induce protection against strptococcosis caused by *S. iniae* with formalin-inactivated bacterins or modified bacterins containing extracellular products (Eldar *et al*., 1997; Klesius *et al*. 1999, 2000, 2002, 2006), their efficacy against different serotypes has not been proved.

Streptococcal capsular polysaccharides are a principal target of protective immunity. However, in general, T cell-independent polysaccharide antigens are poorly immunogenic and are also induces poor memory immune responses. Furthermore, there are some concerns that epizootics can be occurred by serotypes which are not used in the vaccines. In fact, recently, it has been shown that following a 5-year routine vaccination program against *S. iniae* in rainbow trout farms of Israel, a novel serotype, capable of producing generalized bacterial meningitis, has emerged (Bachrach *et al*., 2001).

†Corresponding author: Ki Hong Kim
Tel: +82-51-51-629-5943; Fax: +82-51-51-629-5938
E-mail: khkim@pknu.ac.kr

Protein-based vaccines have the potential advantages of being antigenically conserved across capsular types, comparatively inexpensive to produce by recombinant DNA techniques, and able to induce memory responses which are long-lasting and can be boosted by revaccination. Moreover, antigenic proteins that show little sequence variation in diverse coccal species will likely be superior antigens for the development of a broadly effective vaccine against streptococcosis caused by diverse cocci.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) has been considered a cytoplasmic enzyme in the glycolytic pathway. However, recent reports indicated that various prokaryotic and eukaryotic organisms have cell surface-associating GAPDH, and the vaccine potential of GAPDH has been demonstrated in diverse pathogenic organisms, such as *Streptococcus pneumoniae* (Ling *et al*., 2004), *Edwardsiella tarda* (Kawai *et al*., 2004; Liu *et al*., 2005), and *Schistosoma mansoni* (Goudot-Crozel *et al*., 1989; Argiro *et al*., 2000; El Ridi *et al*., 2001). In the present study, we have analyzed the potential of *S. iniae* GAPDH as a subunit vaccine candidate against streptococcosis.

Materials and Methods

Bacteria and culture conditions

Streptococcus iniae JSL0208, isolated in 1999 from moribund olive flounder (*Paralichthys olivaceus*) in a natural outbreak of streptococcosis on a commercial farm of Korea and used as the strain for streptococcal vaccine in Korea (Cho *et al*., 2006a, b), was kindly provided by the National Fisheries Research & Development Institute, Korea. *Streptococcus parauberis* (KCTC 3651) and *Lactococcus garvieae* (ATCC 49156) were purchased from the Korean Collection for Type Cultures (KCTC) and from the American Type Culture Collection (ATCC), respectively. They were cultured at 37°C overnight in brain heart infusion broth (BHI; Difco) supplemented with 1.0% NaCl. *Escherichia coli* BL21 (DE3) was used as the host strain for recombinant pET28a (Novagen) expression plasmid and cultured at 37°C on Luria-Bertani (LB, Difco) agar containing kanamycin. Expression of the His-tagged fusion protein was induced with 0.1 mM IPTG after reaching an OD_{600} of 1.0, and growth continued at 37°C for 4 h.

Cloning and sequencing GAPDH gene of *S. iniae*, *S. parauberis* and *L. garviae*

Total genomic DNA of each *S. iniae, S. parauberis,* and *L. garvieae* was extracted using AccuPrep® genomic DNA extraction kit according to the manufacturer's instructions (Bioneer, Korea). To amplify GAPDH open reading frame (ORF) of *S. iniae* and *S. parauberis*, 1 set of PCR primers was designed based on the previously recorded GAPDH gene sequences of *S. iniae* (GenBank accession No. AF 421902) and *S. parauberis* (GenBank accession No. AF421901). To amplify GAPDH gene of *L. garviae*, degenerate PCR primers (forward primer; 5'-ATGGTAGTTAAAGTTGGTATTAACGG-3', reverse primer; 5'-TTATTTAGCRATTTTTGCRAAG-3') were designed based on the conserved sequences found at either N- or C-terminal region of GAPDH genes reported previously from various streptococci. The extracted DNA (100 ng) was used in a 20 μl of PCR reaction containing 10 pmoles of each primer and 0.5 U of Taq DNA polymerase (Takara). The reaction was carried out 30 cycles at 95°C for 30 sec, 50°C for 30 sec and 72°C for 40 sec, with an initial denaturation at 95°C for 30 sec using an automated thermal cycler (iCycler, BioRad). The amplified PCR products were cloned into a pGEM-T vector (Promega). Recombinant plasmids containing insert of correct size were screened and the selected clones were purified using an Accuprep® Plasmid Extraction Kit (Bioneer) for sequencing analysis. Sequencing reaction was carried out using BigDye Terminator Ready Reaction Mix (Applied Biosystems) and the sequences were analyzed with an Automated DNA

Sequencer (ABI Prism 377, Applied Biosystems).

Expression and purification of GAPDH

PCR was utilized to amplify the ORF of *S. iniae* GAPDH gene using the forward (5'-CGCGAATTC-ATGGTAGTTAAAGTTGG-3') and reverse primer (5'-CGCGTCGACTTATTTAGCRATTTTTG-3') containing a *EcoR*I and a *Sal*I restriction site (underlined), respectively. The resulting fragment was cloned into pGEM-T vector and then subcloned into the *EcoR*I and *Sal*I sites of the pET28a vector. The integrity of insert DNA was verified by sequence analysis. The His-tagged fusion protein was purified by chromatography under native conditions on Ni-nitrilotriacetic acid resin according to the manufacturer's protocols (Novagen). Protein purity was monitored by SDS-polyacrylamide gel electrophoresis, stained with Coomassie Brilliant Blue, and the protein concentration was determined using the BCA protein assay (Sigma).

Immunization of rats

Specific-pathogen-free male Wistar rats (4 weeks old) were used for the immunization experiment. Three rats were immunized by intraperitoneal injection of 125 µg the recombinant GAPDH emulsified with an equal volume of Freund's complete adjuvant (FCA, Sigma), and boosted with the injection of a same dose of the GAPDH emulsified with Freund's incomplete adjuvant (FIA, Sigma) two weeks later. Three rats of the control group were intraperitoneally injected with PBS and FCA mixture at the first injection, and boosted with PBS and FIA mixture. In the negative control group, 3 rats were injected with PBS only as the above immunization scheme. On 2 weeks post-boost immunization, all rats were bled to obtain serum. All sera were stored at -80°C until analysis.

Immunization and challenge of zebrafish

Zebrafish *Danio rerio* (approximately 3 cm in body length) were obtained from a local aquarium and maintained at 26°C in dechlorinated tap water. Fish were fed at least twice a day with commercially available dry flake food and bit food, and were acclimated for more than two weeks before experiments. Prior to immunization and challenge infection, zebrafish were anesthetized with MS222 (Sigma). In trial I and trial II, fish were divided into three experimental groups (30 fish/group), and were i.p. injected with 10 µl of following formulations. Fish in group 1 received PBS alone, group 2 received Montanide ISA 70 (Montanide, Seppic) emulsified in same volume of PBS, and group 3 received 5 µg of recombinant GAPDH plus Montanide. All the fish were boost-injected with the same formulations two weeks after the first injection. Two weeks after the boost immunization, fish of each group were challenged by 10 µl (3.2×10^2 cells of *S. iniae*/fish) of the bacterial suspension. All dead fish were necropsied, and eyes & internal organ samples were streaked on BHI agar to confirm the presence of *S.iniae*.

Western Blot

Each cell lysate of *S. iniae*, *S. parauberis*, *L. garvieae* was solubilized in SDS-PAGE loading buffer (2% SDS, 14.4 mM β-mercaptoethanol, 25% glycerol, 0.1% bromophenol blue, 60 mM Tris-HCl, pH 6.8), boiled for 5 min and fractionated on a 10% SDS-PAGE gel. Proteins were transferred to nitrocellulose membrane. The membrane was blocked with blocking solution (3% bovine serum albumin in TBS; 150 mM NaCl, 10 mM Tris-HCl, pH 7.5) for 2 h at room temperature (RT), washed with TTBS (0.05% Tween 20 in TBS, pH 7.5) and incubated with diluted rat sera (1:500 or 1:1000) for 2 h at RT. The membranes were washed three times with TTBS and incubated with alkaline phophatase conjugated goat anti-rat IgG (1:2000, Santa Cruz Biotechnology Inc., USA) for 2 h at RT. After washing off unbound secondary antibody, the specific antigen-bound antibody was visualized with NBT-BCIP substrate buffer (Sigma).

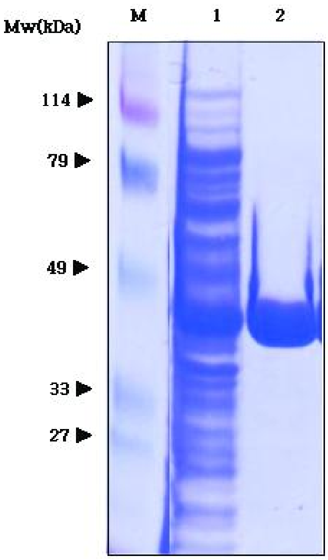

Fig. 1. SDS-PAGE analysis of recombinant GAPDH purification. M, prestained protein marker (Pierce); Lane 1, Supernatant of induced bacterial lysate; Lane 2, Recombinant GAPDH protein purified by a Ni-NTA His-Bind® Resin (Novagen) open column.

Results

Cloning and production of recombinant GAPDH of *S. iniae*

Nucleotides sequence of *S. iniae* GAPDH showed 89% and 82% similarity with that of *S. parauberis* and *L. garvieae* (GeneBank accession number FJ 524849), respectively. Deduced amino acids sequence of *S. iniae* GAPDH showed 92% and 83% similarity with that of *S. parauberis* and *L. garvieae*, respectively.

The recombinant *S. iniae* GAPDH was massively expressed in the cytoplasm of *E. coli* by induction with IPTG. After purification with the His-tag column chromatography, a single massive band of polypeptide corresponding to the MW of recombinant His-tagged GAPDH was confirmed by SDS–PAGE (Fig. 1).

Immunization and challenge of zebrafish

Fish immunized with rGAPDH plus montanide showed the highest survival rates (30% and 33.3%) against *S. iniae* challenge in both trial I and II (Fig. 2). Fish injected with montanide alone also showed higher survival rates (50% and 63.3%) than fish in the control group (both 96.7%) in both trial I and II.

Cross-reactivity of antisera

The rat antisera against the recombinant GAPDH recognized not only *S. iniae* GAPDH but also *S. parauberis* and *L. garvieae* GAPDHs when whole-cell ly-

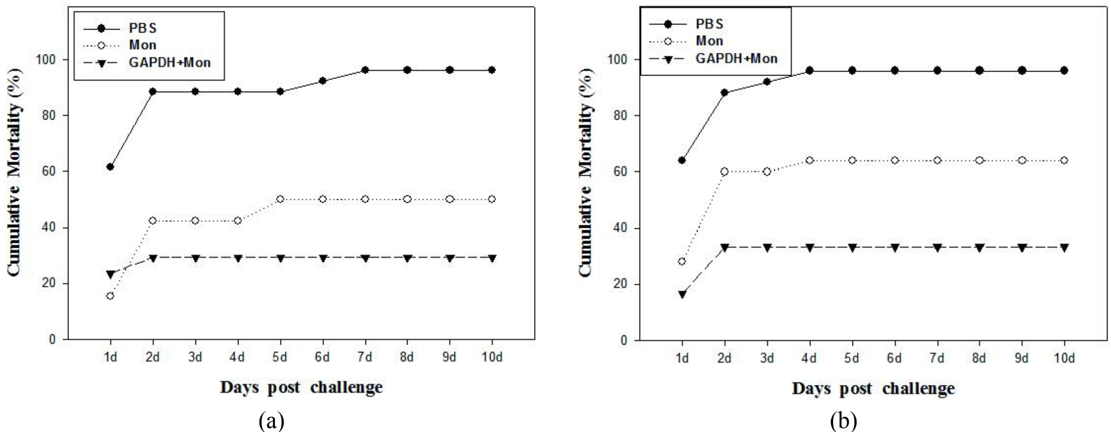

Fig. 2. Cumulative mortality of zebrafish challenged with *Streptococcus iniae* after immunization. In both trial I (a) and trial II (b), zebrafish were immunized with the recombinant GAPDH of *S. iniae* mixed with Montanide ISA 70 VG (GAPDH+Mon). As controls, fish were injected with Phosphate buffered saline (PBS) or Montanide (Mon) alone. Each fish in each group was challenged by i.p. injection of *S.iniae* two weeks after booster injection.

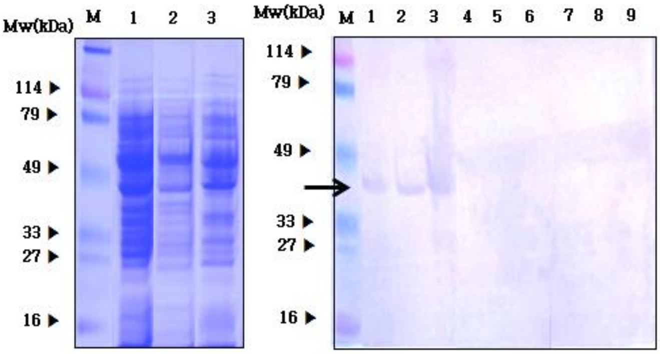

Fig. 3. SDS-PAGE (a) and Western blot (b) analysis of recombinant GAPDH protein immunized-rat serum against lysates of *Lactococcus garviae* (a-Lane 1), *Streptococcus parauberis* (a-Lane 2), and *S. iniae* (a-Lane 3). M, prestained protein marker (Pierce); Lane 1, 4, 7, *L. garvieae* lysate; Lane 2, 5, 8, *S. parauberis* lysate; Lane 3, 6, 9; *S. iniae* lysate. The primary antibody in Lane 1, 2, 3 was recombinant GAPDH injected immune serum, in Lane 4, 5, 6 was PBS injected control serum and in Lane 7, 8, 9 was adjuvant alone injected control serum.

sates were probed in Western blots (Fig. 3). The rat sera of control and negative control groups showed no reactivity.

Discussion

In this study, we have demonstrated that *Streptococcus iniae* GAPDH is a potential target for vaccine development. It has been reported that streptococcal GAPDH contributes to the microorganism's invasiveness by its ability to bind to various host proteins such as plasmin(ogen), lysozyme, fibronectin, myosin, actin, and transferrin (Lottenberg *et al*., 1992; Pancholi and Fischetti, 1992, 1993; Modun and Williams, 1999). Furthermore, the presence of a cell wall associated GAPDH has been demonstrated in pathogenic mammalian streptococci, including *S. pyogenes*, *S. equisimilis*, *S. agalactiae* and *S. pneumoniae* (Lottenberg *et al*., 1992; Pancholi and Fischetti, 1992; Gase *et al*., 1996; Kolberg and Sletten, 1996; Hughes *et al*., 2002). The protective effects of recombinant GAPDH immunization against streptococcal infections in mammals have been demonstrated, such as *S. pneumonia* using mouse as a experimental animal (Ling *et al*., 2004) and *S. suis* in pigs (Okwumabua and Chinnapapakkagari, 2005).

In fish streptococcosis, although Shin *et al*. (2007) reported that GAPDH might be a virulent and protective antigen for *S. iniae*, there has been no report on the in vivo protective effect of streptococcal GAPDH immunization. In the present study, the survival rates of zebrafish against *S. iniae* challenge were increased by immunization with *S. iniae* rGAPDH, which suggests that GAPDH would be an antigen capable of inducing protective immune responses in fish against streptococcosis as in mammals. The usefulness of zebrafish as an experimental model for streptococcosis including *S. iniae* was well demonstrated (Neely *et al*., 2002; Miller and Neely, 2004). However, because of their small size, the routine serological analysis was not possible. Further studies using cultured fish should be conducted to know the adaptive immune responses mediated by GAPDH immunization.

As three species of cocci – *S. iniae*, *S. parauberis*, and *Lactococcus garviae* – are the culprits of streptococcosis in cultured fish in Korea, to elicit cross-protective immune responses against the three cocci, protein antigens conserved among the three cocci species would be favorable. GAPDHs are well conserved proteins in eubacteria and eukaryotes (Figge *et al*., 1999), and, in this study, GAPDHs of the 3 cocci species exhibited a high degree of homology. In the present study, we have demonstrated that the antisera against recombinant GAPDH of *S. iniae* have cross-reactivity against GAPDH of *S. parauberis* and *L. garviae*. This result suggested that *S. iniae* GAPDH may be used as an antigen for development of subunit vaccine against streptococcosis caused by diverse cocci in cultured fish.

Acknowledgments

This research was a part of the project titled 'Development of Fish Vaccines and Human Resource Training', funded by the Ministry of Oceans and Fisheries, Korea.

References

Argiro, L., Kohlstadt, S., Henri, S., Dessein, H., Matabiau, V., Paris, P., Bourgois, A. and Dessein, A.J. : Identification of a candidate vaccine peptide on the 37 kDa *Schistosoma mansoni* GAPDH. Vaccine, 18: 2039-2048, 2000.

Bachrach, G., Zlotkin, A., Hurvitz, A., Evans, D.L. and Eldar, A.: Recovery of *Streptococcus iniae* from diseased fish previously vaccinated with a *Streptococcus* vaccine. Appl. Environ. Microbiol., 67: 3756-3758, 2001.

Cho, M.Y., Lee, J.S., Lee, D.C., Choi, H.J. and Kim, J.W.: Immune response of olive flounder, *Paralichthys olivaceus against β-hemolytic Streptococcus iniae* formalin-killed cells. J. Fish Pathol., 19: 73-82, 2006a. (In Korean with English Abstract)

Cho, M.Y., Lee, D.C., Lee, J.S., Do, J.W., Kim, M.S., Choi, M.Y., Kim, Y.C., Kang, B.K., Yoon, Y.D. and Kim, J.W.: Stability and efficacy of formalin-killed *Streptococcus iniae* vaccine for olive flounder, *Paralichthys olivaceus*. J. Fish Pathol., 19: 165-172, 2006b. (In Korean with English Abstract)

El Ridi, R., Shoemaker, C.B., Farouk, F., El Sherif, N.H. and Afifi, A.: Human T- and B-cell responses to *Schistosoma mansoni* recombinant glyceraldehyde 3-phosphate dehydrogenase correlate with resistance to reinfection with *S. mansoni* or *Schistosoma haematobium* after chemotherapy. Infect. Immun., 69: 237-244, 2001.

Eldar, A., Horovitcz, A. and Bercovier, H.: Development and efficacy of a vaccine against *Streptococcus iniae* infection in farmed rainbow trout. Vet. Immunol. Immunopathol., 56: 175-183, 1997.

Figge, R.M., Schubert, M., Brinkmann, H. and Cerff, R.: Glyceraldehyde-3-phosphate dehydrogenase gene diversity in eubacteria and eukaryotes: evidence for intra- and inter-kingdom gene transfer. Mol. Biol. Evol., 16: 429-440, 1999.

Gase, K., Gase, A., Schirmer, H. and Malke, H.: Cloning, sequencing and functional overexpression of the *Streptococcus equisimilis* H46A gapC gene encoding a glyceraldehyde-3-phosphate dehydrogenase that also functions as a plasmin(ogen)-binding protein. Purification and biochemical characterization of the protein. Eur. J. Biochem., 239: 42-51, 1996.

Goh, S.H., Driedger, D., Gillett, S., Low, D.E., Hemmingsen, S.M., Amos, M., Chan, D., Lovgren, M., Willey, B.M., Shaw, C. and Smith, J.A.: *Streptococcus iniae*, a human and animal pathogen: Specific identification by the chaperonin 60 gene identification method. J. Clin. Microbiol., 36, 2164-2166, 1998.

Goudot-Crozel, V., Caillol, D., Djabali, M. and Dessein, A.J. : The major parasite surface antigen associated with human resistance to schistosomiasis is a 37 kDa glyceraldehyde-3-phosphate dehydrogenase. J. Exp. Med., 170: 2065-2080, 1989.

Hughes, M.J.G., Moore, J.C., Lane, J.D., Wilson, R., Pribul, P.K., Younes, Z.N., Dobson, R.J., Everest, P., Reason, A.J., Redfern, J.M., Greer, F.M., Paxton, T., Panico, M., Morris, H.R., Feldman, R.G. and Santangelo, J.D.: Identification of major outer surface proteins of *Streptococcus agalactiae*. Infect. Immun., 70: 1254-1259, 2002.

Kawai, K., Liu, Y., Onishi, K. and Oshima, S.: A conserved 37 kDa outer membrane protein of *Edwardsiella tarda* is an effective vaccine candidate. Vaccine, 22: 3411-3418, 2004.

Klesius, P.H., Shoemaker, C.A. and Evans, J.J.: Efficacy of a killed *Streptococcus iniae* vaccine in tilapia (*Oreochromis niloticus*). Bull. Eur. Assoc. Fish Pathol., 19, 39-41, 1999.

Klesius, P.H., Shoemaker, C.A. and Evans, J.J.: Efficacy of single and combined *Streptococcus iniae* isolate vaccine administered by intraperitoneal and intramuscular routes in tilapia (*Oreochromis niloticus*). Aquaculture, 188: 237-246, 2000.

Klesius, P.H., Shoemaker, C.A. and Evans, J.J.: *Streptococcus iniae* vaccine. U.S. patent No. 6,379,677B1, 2002.

Klesius, P.H., Evans, J.J., Shoemaker, C.A. and Pasnik, D.J.: A vaccination and challenge model using calcein marked fish. Fish Shellfish Immunol., 20: 20-28, 2006.

Koh, T.H., Kurup, A. and Chen, J.: *Streptococcus iniae* discitis in Singapore. Emerg. Infect. Dis., 10: 1694-1696, 2004.

Kolberg, J. and Sletten, K.: Monoclonal antibodies that recognize a common pneumococcal protein with similarities to streptococcal group A surface glyceraldehyde-3-phosphate dehydrogenase. Infect. Immun., 64: 3544-3547, 1996.

Lau, S.K.P., Woo, P.C.Y., Tse, H., Leung, K.W. and Yuen, K.Y.: Invasive *Streptococcus iniae* infections outside North America. J. Clin. Microbiol., 41: 1004-1009, 2003.

Lau, S.K.P., Woo, P.C.Y., Luk, W.K., Fung, A.M.Y., Hui, W.T., Fong, A.H.C., Chow, C.W., Wong, S.S. Y. and Yuen, K.Y.: Clinical isolates of *Streptococcus iniae* from Asia are more mucoid and β-hemolytic than those from North America. Diagn. Microbiol. Infect. Dis., 54: 177-181, 2006.

Ling, E., Feldman, G., Portnoi, M., Dagan, R., Overweg, K., Mulholland, F., Chalifa-Caspi, V., Wells, J. and Mizrachi-Nebenzahl, Y.: Glycolytic enzymes associated with the cell surface of *Streptococcus pneumoniae* are antigenic in humans and elicit protective immune responses in the mouse. Clin. Exp. Immunol., 138: 290-298, 2004.

Liu, Y., Oshima, S., Kurohara, K., Ohnishi, K. and Kawai, K.: Vaccine efficacy of recombinant GAPDH of *Edwardsiella tarda* against edwardsiellosis. Microbiol. Immunol., 49: 605-612, 2005.

Lottenberg, R., Broder, C.C., Boyle, M.D.P., Kain, S.J., Schroeder, B.L. and Curtiss, R. III.: Cloning, sequence analysis and expression in *Escherichia coli* of a streptococcal plasmin receptor. J. Bacteriol., 174: 5204-5210, 1992.

Miller, J.D. and Neely, M.N.: Zebrafish as a model host for streptococcal pathogenesis. Acta. Trop., 91: 53-68, 2004.

Modun, B. and Williams, P.: The staphylococcal transferrin-binding protein is a cell wall glyceraldehyde-3-phosphate dehydrogenase. Infect. Immun., 67: 1086-1092, 1999.

Neely, M., Pfeifer, J. and Caparon, M.G.: Streptococcus-zebrafish model of bacterial pathogenesis. Infect. Immun., 70: 3904-3914, 2002.

Okwumabua, O. and Chinnapapakkagari, S.: Identification of the gene encoding a 38-kilodalton immunogenic and protective antigen of *Streptococcus suis*. Clin. Diagn. Lab. Immunol., 12: 484-490, 2005.

Pancholi, V. and Fischetti, V.A.: A major surface protein on group A streptococci is a glyceraldehyde-3-phosphate dehydrogenase with multiple binding activity. J. Exp. Med., 176: 415-426, 1992.

Pancholi, V. and Fischetti, V.A.: Glyceraldehyde-3-phosphate dehydrogenase on the surface of group A streptococci is also an ADP-ribosylating enzyme. Proc. Natl. Acad. Sci. USA, 90: 8154-8158, 1993.

Shin, G.W., Palaksha, K.J., Kim, Y.R., Nho, S.W., Kim, S., Heo, G.J., Park, S.C. and Jung, T.S.: Application of immunoproteomics in developing a Streptococcus iniae vaccine for olive flounder (*Paralichthys olivaceus*). J. Chromatogr. B Analyt. Technol. Biomed. Life Sci., 84: 315-322, 2007.

Weinstein, M.R., Litt, M., Kertesz, D.A., Wyper, P., Rose, D., Coulter, M., McGeer, A., Facklam, R., Ostach, C., Willey, B.M., Borczyk, A. and Low, D. E.: Invasive infections due to a fish pathogen, *Streptococcus iniae*. *S. iniae* Study Group. N. Engl. J. Med., 337: 589-594, 1997.

Manuscript Received : Nov 5, 2014
Revised : Apr 7, 2015
Accepted : Apr 9, 2015