Original Article

Efficacy of pcDNA-Alp1 DNA vaccine against *Streptococcus agalactiae* in Nile tilapia (*Oreochromis niloticus*)

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Abstract

Streptococcosis caused by *Streptococcus agalactiae* and *S. iniae* is one of the most important bacterial diseases in Nile tilapia. To prevent Streptococcus infection, various forms of vaccine including inactivated bacterial vaccines and DNA vaccines have been developed. In this study, a plasmid containing the alpha-like protein1 (*alp1*) gene was constructed. Samples of 120 Nile tilapia (*Oreochromis niloticus*) from a provincial fish farm in Thailand were divided into four equal groups: 1) the control, injected with phosphate-buffered saline (PBS), 2) injected with pcDNA3.1 (+), 3) injected with pcDNA-Alp1 and 4) injected with inactivated Streptococcus vaccine. At four weeks after vaccination, the fish were challenged with 10^8 CFU/mL *S. agalactiae* using intraperitoneal injection. The relative percent survival (RPS) rates for pcDNA3.1 (+), pcDNA-Alp1 and inactivated *S. agalactiae* vaccine were 26.6%, 51.4% and 46.6%, respectively. Notably, the efficacy of pcDNA-Alp1 was equivalent to the inactivated *S. agalactiae* vaccine. The results demonstrated that pcDNA-Alp1 vaccine could provide protection to tilapia against *S. agalactiae* infection.

Keywords: DNA vaccine, Nile tilapia, streptococcosis, *S. agalactiae*

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Introduction

Nile tilapia (*O. niloticus*) is one of the important cultured fish species in Thailand and Southeast Asia. The tilapia intensive culture system and fish rearing in public reservoirs such as rivers and dams make this species more susceptible to various infectious diseases. Among the different viruses, parasites and bacterial infections, *S. agalactiae* is one of the most important bacterial diseases that affect tilapia culture in Thailand (Suanyuk et al., 2008; Rodkhum et al., 2011; Kayansamruaj et al., 2014; Suwannasang et al., 2014). In Thailand, the serotypes Ia and III of *S. agalactiae* have been isolated from infected tilapia (Kayansamruaj et al., 2014; Suwannasang et al., 2014; Kannika et al., 2017).

While antibiotics have been used for the control of bacterial infection in fish farms, their usage has been limited due to growing concerns of antibiotic resistance (Heppell and Davis, 2000). As such, alternative strategies, including vaccines and biosecurity using disinfectants, have been implemented to control bacterial diseases in fish aquaculture (Mon-on et al., 2018). Vaccines have been investigated to combat fish bacterial diseases and most of the available fish vaccines are either live-attenuated or inactivated vaccines. For example, a live-attenuated viral vaccine against infectious salmon anemia virus and bacterial vaccines against *Aeromonas salmonicida* and *Vibrio anguillarum* have been proven as tools to reduce the economic impact of these pathogens in the salmon industry (Toranzo et al., 2005). An inactivated *S. agalactiae* has been reported in tilapia. In the past decade, DNA and subunit vaccines have gained special interest in aquaculture due to progress in molecular technologies and genetic engineering (Heppell and Davis, 2000; Gillund et al., 2008; Kurath, 2008; Tonheim et al., 2008).

The aim of this study was to develop a DNA vaccine containing the *alp1* gene of *S. agalactiae*. The efficacy of pcDNA-Alp1 was evaluated following *S. agalactiae* infection in Nile tilapia.

Materials and Methods

***Animals*:** Samples of 150 Nile tilapia (*O. niloticus*; 65±15 g) were acquired from a local fish farm in Ang-thong province, Thailand. The fish were maintained in a laboratory for 2 weeks in fiberglass tanks at a water temperature of 28-30°C. The fish were fed twice daily (total of 2% bodyweight) on commercial feed. Five tilapia were randomly selected for bacterial isolation on tryptic soy agar (TSA) plate to screen for *S. agalactiae* infection. The animal use protocol was approved by the Kasetsart University Animal Use Committee under permit number ACKU02759.

In silico bioinformatics analysis: The Nile tilapia *alp1* nucleotide sequence was amplified from a DNA sample isolated from *S. agalactiae* (serotype Ia)-infected fish in Thailand. The polymerase chain reaction (PCR) product was submitted for sequencing and aligned with *S. agalactiae* 515 (serotype Ia) alpha-like protein1 in the GenBank database (accession number: AY345596.1). To compare amino acid sequence, the *alp1* nucleotide sequence was translated by Translator online server (http://www.fr33.net/translator.php), and then aligned with *S. agalactiae* alpha-like protein1 (GenBank accession number: AAR08144.1) using the protein alignment program (http://web.expasy.org/sim/). Additionally, the antigenic epitopes and predicted domain structures were analyzed using Kolaskar and Tongaonkar (Kolaskar and Tongaonkar, 1990) (http://imed.med.ucm.es/Tools/antigenic.html) and the InterPro software package (https://www.ebi.ac.uk/interpro), respectively.

Construction of pcDNA-Alp1: To produce DNA vaccine, the *S. agalactiae* alpha-like protein1 gene (*alp1*) was amplified using specific primers consisting of the forward primer alp-F: 5'- GGATCCATGTTTAGAAG GTCTAAA-3' and the reverse primer alp-R: 5'- CTCGAGCTATGGTTTTGGTGTCACAT-3'. The PCR condition was initial denaturation at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 58°C for 30 s, extension at 72°C for 1 min and final extension at 72°C for 10 min. The PCR product (approximate size of 1,000 bp) was examined on 1% agarose gel and further purified using a nucleic extraction kit (GeneJet™, Fermentas, Loughborough, UK). The PCR product was then ligated into plasmid vector pcDNA3.1 (+) (Invitrogen, Carlsbad, CA, USA) using T4 DNA ligase (Promega, Madison, WI, USA). This plasmid was later called pcDNA-Alp1 and was transformed into the *Escherichia coli* strain DH5α. To collect pcDNA-Alp1 for DNA vaccine preparation, plasmid was isolated from the *E. coli* strain DH5α using a GeneJet™ plasmid miniprep kit (ThermoFisher, Waltham, MA, USA).

Vaccination and challenge study: Subsamples of 120 fish (average 65±15 g) were equally divided into four groups: 1) control group, 2) pcDNA3.1 (+), 3) pcDNA-Alp1 and 4) inactivated *S. agalactiae* vaccine. The control fish were intramuscularly injected with 0.1 ml PBS (pH 7.4). The fish in group 2 were intramuscularly injected with 0.1 mL of 100 µg/mL of plasmid pcDNA3.1 (+). The fish in group 3 were intramuscularly injected with 0.1 ml of 100 µg/mL of pcDNA-Alp1. The fish in group 4 were intraperitoneally injected with 0.05 ml inactivated *S. agalactiae* vaccine (AquaVac® Strep Sa1: Merck, Boxmeer, the Netherlands). The efficacy of the DNA vaccines was demonstrated by challenging with the *S. agalactiae* serotype III KU-VET01 isolated from moribund Nile tilapia from Ubon-Ratchathani province in 2015. This bacterium was cultured on tryptic soy broth (TSB, Difco Laboratories, Sparks, MD, USA) at 30°C for 24 hr with gentle shaking at 225 rpm. After 24 hr, the bacterial suspension was centrifuged at 3,000×g at 25°C for 15 min. Subsequently, the pellets were washed twice in 0.85% sterile saline solution and adjusted to a concentration equal to OD 0.6 (A= 600 nm) using spectrophotometry (BlueStar Series UV/Vis spectrometer, LabTech, Inc, Hopkinton, MA, USA). The bacterial suspension was ten-fold serially diluted and spread on TSA to determine bacterial count. The OD 0.6 was equal to 10^8 CFU/ml *S. agalactiae* which is the LD50 dose according to the previous experiment conducted in our laboratory. At 28 days post vaccination, all fish were challenged using an intraperitoneal injection of 0.1 ml bacterial suspension. The mortality rate was recorded daily until 28 days post challenge. Dead and moribund fish were removed

twice daily and were processed on TSA plates for *S. agalactiae* isolation from the brain, anterior kidney and blood.

***Statistical analysis*:** The Kaplan-Meier log rank test was used to analyze survival. Statistical differences were tested as significant at P<0.05 level using Duncan's new multiple range test.

Results

The alpha-like protein1 (*alp1*) was amplified from *S. agalactiae* isolated from the infected fish. The amplified product was cloned into pcDNA3.1 (+) and then transformed into *E. coli*. To examine whether or not *alp1* was inserted into pcDNA3.1 (+), plasmid pcDNA-Alp1 was digested with the enzymes BamHI and XhoI. An approximate sequence size of 927 bp was detected on agarose gel (Fig. 1). The analysis of nucleotide sequencing indicated that the pcDNA-Alp1 sequence contained 927 bp (GenBank accession number: KX685456) with 79% sequence similarity to *alp1* of human-isolated *S. agalactiae* (GenBank accession number: AY345596.1) and 98% similarity to the hypothetical protein of *S. agalactiae* isolated from fish (GenBank accession number: CP011325.1) (Supplemental Fig. 1). In addition, the results showed that the pcDNA-Alp1 amino acid sequence shared 73.8% similarity with the site of the Gram-positive signal peptide at position 13-43, N-terminus site at position 41-234 and antigenic site for antibody recognition at position 240-300 (Supplemental Fig. 2).

Both full lengths of pcDNA-Alp1 and Alp1 from human-isolated *S. agalactiae* (GenBank accession number: AAR08144) were examined (Supplemental Table 1); the results revealed that both pcDNA-Alp1 and human *S. agalactiae* Alp1 contained 15 epitopes. However, the human *S. agalactiae* Alp1 had three additional epitopes at the C terminal sequence.

The efficacy of pcDNA-Alp1 was demonstrated in *S. Agalactiae*-challenged fish. At 3 days post challenge, clinical signs were observed of Streptococcus infection including anorexia, exophthalmia, corneal opacity and multiple abscesses on the skin and base of the caudal fin (Figs. 2A, 2B, 2C). Severe clinical signs were observed in the control and pcDNA3.1 (+) groups. The Gram-positive cocci with bacterial morphology similar to *S. agalactiae* were isolated from the anterior kidney of all infected challenged fish (Figs. 2D, 2E). Regarding the efficacy of the DNA vaccine, at two days post challenge, mortality was observed in PBS, pcDNA3.1 (+), and pcDNA-Alp1, whereas mortality in the fish vaccinated with inactivated *S. agalactiae* vaccine commenced at five days post challenge (Fig. 3). The cumulative mortality continued until 6 to 11 days post challenge and remained steady until the end of the experiment. The relative percentage survival (RPS) rates for pcDNA3.1 (+), pcDNA-Alp1 and inactivated *S. agalactiae* vaccine were 26.6%, 51.4% and 46.6%, respectively. The statistical analysis of RPS rate in the four experimental groups revealed that both pcDNA-Alp1 and commercial inactivated vaccines showed higher percentage of survival than the control group (p<0.05), but insignificant difference in the percentage of survival from the pcDNA3.1 (+) group.

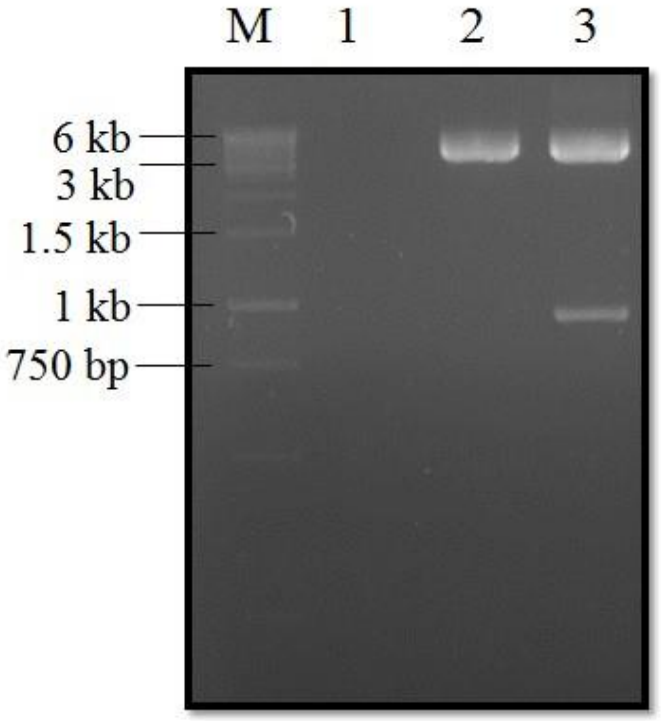

Figure 1 **Construction of pcDNA-Alp1.** The sequence (927 bp) of *alp1* of *S. agalactiae* was cloned into pcDNA3.1 (+). The insert of pcDNA-Alp1 was confirmed using restriction enzymes BamHI and XhoI digestion. M = DNA marker, lane 1 = negative control, lane 2 = pcDNA3.1 (+) and lane 3 = pcDNA-Alp1.

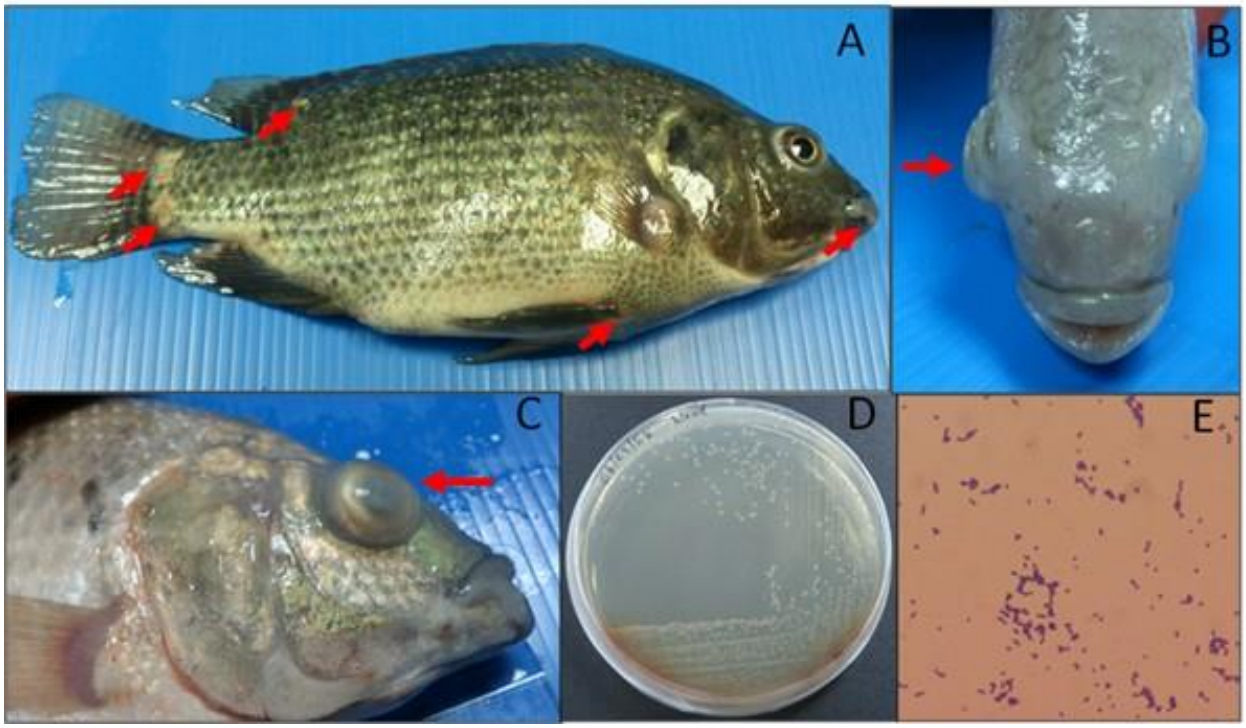

Figure 2 **Clinical signs and re-isolation of *S. agalactiae* in challenged tilapia.** Multiple abscesses (arrow) and dark skin in challenge fish (A). Unilateral exophthalmos and corneal opacity (B, C). Small pinpoint colony of *S. agalactiae* isolated from moribund fish (D). Gram staining of bacteria isolated from challenged fish showing Gram-positive cocci (E).

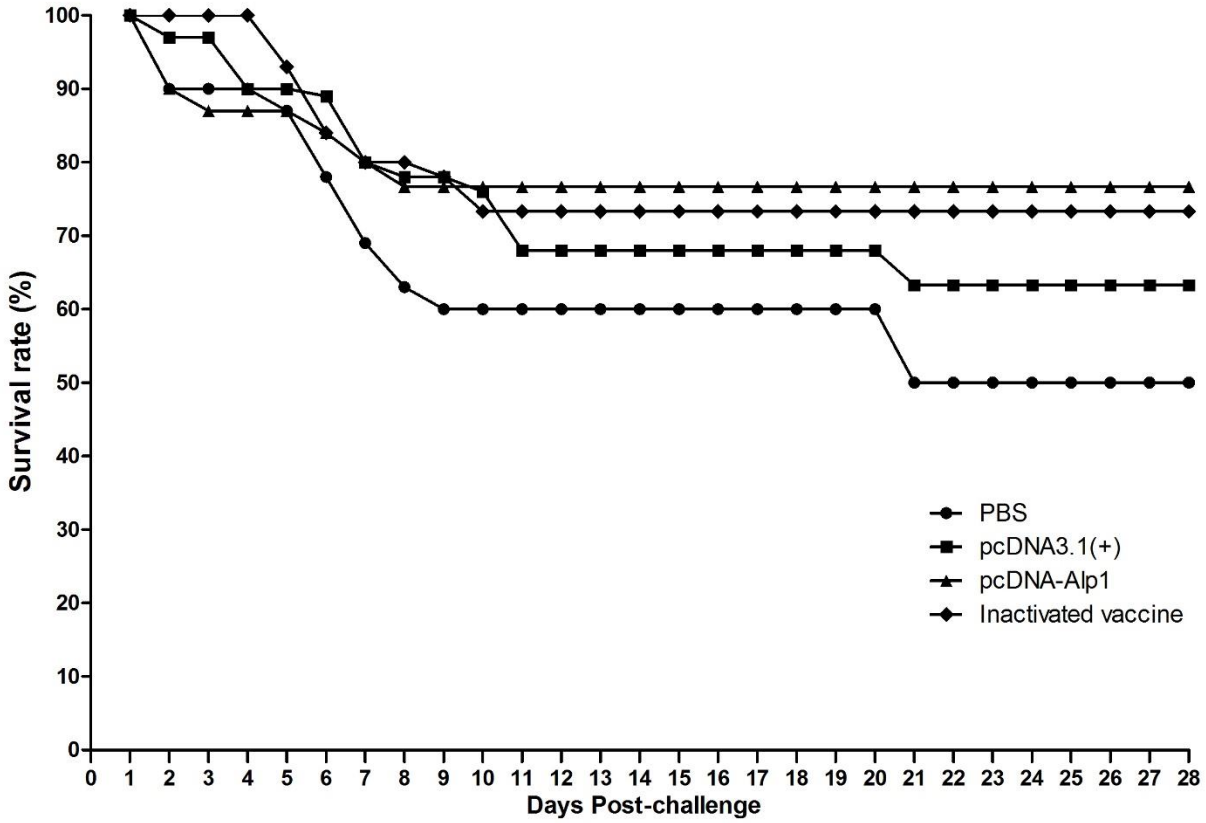

Figure 3 **Survival rate of Nile tilapia post challenged with virulent *S. agalactiae*.** The survival rate was daily recorded until 28 days post challenge. The fish (30 fish/group) were divided into four groups: PBS, pcDNA3.1 (+), pcDNA-Alp1 and inactivated *S. agalactiae* vaccine. All fish were challenged with 10^8 CFU/ml of *S. agalactiae* serotype III.

Discussion

Over the last decade, research into DNA vaccines has resulted in some being commercialized against various bacterial and viral diseases in aquaculture. The concept of a DNA vaccine is based on the administration of the plasmid inserted with one or more of the pathogen's genes. The advantages of DNA vaccine are diverse immunological responses through activation of humoral and cellular immunity, ease of storage, and economical feasibility of production (Heppell and Davis, 2000; Lorenzen et al., 2002; Gillund et al., 2008; Kurath, 2008; Tonheim et al., 2008). The efficacy of DNA vaccines has been determined in many fish models such as the Sia10 protein of *S. iniae* in Turbot (*Scophthalmus maximus*) and Japanese flounder (*Paralichthys olivaceus*) (Sun et al., 2010; Sun et al., 2012). The DNA vaccines containing the glycoprotein of viral hemorrhagic septicemia virus (VHSV) and infectious haematopoietic necrosis virus (IHNV) were evaluated in rainbow trout (*Oncorrhyncus mykiss*) (Lorenzen et al., 2002). Interestingly, the relative percentage of survival reached 86% in rainbow trout receiving the IHNV DNA vaccine after injection challenge (Garver et al., 2005).

The surface protein antigens are some of the candidates for good immunological response in the design of an effective DNA vaccine. Suggested candidate antigens for *S. agalactiae* include the surface proteins, Cβ, R4/Rib, and the alpha-like proteins (Alps), Alp1 and Alp2 (Kvam et al., 2011). Indeed, the alpha-like proteins are a family of highly repetitive surface proteins of *S. agalactiae* which can elicit appropriate protective immunity (Lindahl et al., 2005; Maeland et al., 2004). Analysis of the nucleotide and deduced amino acid sequences of Alp from different GBS serotypes revealed that the Alp was conserved among the isolates (Lachenauer et al., 2000). The current study developed a DNA vaccine against *S. agalactiae* for Nile tilapia. The surface protein Alp1 of *S. agalactiae* was cloned into plasmid pcDNA3.1 (+), which contained CMV enhancer promoter, BGH polyadenylation signal and transcription termination sequence for enhanced mRNA stability, SV40 origin for replication and used as a DNA vaccine. Alp1 is one of the surface proteins found in *S. agalactiae* serotypes Ia and serotype III (Creti et al., 2004). From the sequence analysis, pcDNA-Alp1 contained the signal peptides at amino acid sequence position 13-43, which resembles the first 41 amino acid sequences of human *S. agalactiae* (Michel et al., 1992). Signal peptides have been used for protein transport outside of bacterial cells (von Heijne and Abrahmsèn, 1989; Michel et al., 1992). The antigenic epitope prediction of pcDNA-Alp1 revealed that the pcDNA-Alp1 contained 15 antigenic epitopes while human *S. agalactiae* Alp1 contained 18 antigenic epitopes. Indeed, five epitopes, KFGAASVLIGISFLG, ELEKLLVLSVPD, TTVPQGTPVSD, ITDLVKI and GSKGVPTVVG, contained hydrophilic and electrically charged amino acids that have been shown to be highly immunogenic in mice and humans (ATASSI, 1984; Kolaskar and Tongaonkar, 1990; Rubinstein et al., 2008).

In this study, the RPS in pcDNA-Alp1-vaccinated fish was 26.6%, while the fish receiving the commercial inactivated vaccine and pcDNA3.1 (+) showed RPS rates of 51.4% and 46.6%, respectively. The appearance of clinical signs and the re-isolation of Gram-positive cocci bacteria confirmed that all challenged fish were susceptible to *S. agalactiae* infection (Suanyuk et al., 2008; Suwannasang et al., 2014). Although the immunological responses were not investigated in the current study, both cellular and humoral immunity could be responsible for the improved survival in both inactivated commercial vaccine and pcDNA-Alp1-vaccinated fish. The activation of cells in the immune system and antibody production were reported in response to DNA vaccination (Gillund et al., 2008; Tonheim et al., 2008). In turbot, elevated antibody production was recorded in fish that received *S. iniae* pSia10 vaccine (Sun et al., 2010). Moreover, activation of the cellular immune responses (including elevated interleukin 1-beta (IL-1β), interleukin 6 (IL-6) and IFN-gamma production) was demonstrated in DNA-vaccinated fish (Heppell and Davis, 2000; Kato et al., 2015). It is interesting to note that the fish receiving pcDNA3.1 (+) alone had a higher survival rate than the control group. As the plasmid backbone contains CpG motifs, which could be recognized by the host innate pattern recognition receptor, such recognition may be responsible for the increased survival in pcDNA3.1 (+) alone (Heppell and Davis, 2000; Kojima et al., 2002; Tonheim et al., 2008; Kato et al., 2015).

Recommended benefits of DNA vaccine include the potential of implementation of multiple antigens in a single vaccine, no reversion to virulence and easy production once the prototype is developed (Kurath, 2008; Tonheim et al., 2008). Although some concerns regarding the use of DNA vaccine include plasmid persistence and potential integration of foreign DNA into the host genome, there is no report of these issues in the current DNA vaccines. The present study showed that pcDNA-Alp1 improved the survival rate against Streptococcal infection in Nile tilapia. The application of this vaccine to protect *S. agalactiae* could be an alternative strategy to reduce the potentially substantial economic impact of streptococcosis and to reduce improper antibiotic usage in tilapia farming.

Supplemental Table 1 **Antigenic epitope prediction of pcDNA-Alp1.** The antigenic epitopes were analyzed using Kolaskar and Tongaonkar[9]. Antigenic epitopes of tilapia pcDNA-Alp1 (A), and human *S. agalactiae* (AAR08144.1) (B).

pcDNA-Alp1 (A)

n	Start Position	Sequence	End Position
1	25	KFGAASVLIGFSFLGG	40
2	50	EESIVAASTIPGS	62
3	79	AYIDLYDVKLGKIDPLQLIVLEQ	101
4	103	FTAKYVFR	110
5	115	YYGDVSQL	122
6	139	GLPHVKT	145
7	148	QIDIVSVALTI	158
8	185	RTEVLTG	191
9	210	DSKIVEV	216
10	218	ELEKLLVLSVPD	229
11	240	TTVPQGTPVSD	250
12	253	ITDLVKI	259
13	262	GSKGVPTVVG	271
14	280	GDHKVTVEVTY	290
15	296	DTVEVTVH	303

AAR08144.1 (B)

n	Start Position	Sequence	End Position
1	25	KFGAASVLIGISFLG	39
2	49	STDTVFAAEVISGS	62
3	64	ATLNSALVKN	73
4	79	AYIDIYD	85
5	92	DPLNLIVLTP	101
6	116	IFTSVNQ	122
7	149	QIDIVSLVTTV	159
8	186	RKDVLSKIEVIK	197
9	211	DNKIVEV	217
10	219	ELEKLLVLPVPD	230
11	241	TTVPQGTPVSD	251
12	254	ITDLVKI	260
13	263	GSKGVPTVVG	272
14	281	GDHKVTVDVTY	291
15	297	DTVEVTVHVTPKPVPD	312
16	323	TTVPQGTPVSD	333
17	336	ITDLVKI	342
18	345	GSKGVPTVVG	354

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AY345596.1      ATGTTTAGAAGGTCTAAAAATAACAGTTATGATACTTCACAGACGAAACAACGGTTTTCA
pcDNA-Alp       ATGTTTAGAAGGTCTAAAAATAACAGTTATGATACTTCACAGACGAAACAACGGTTTTCA
                ************************************************************

AY345596.1      ATTAAGAAGTTCAAGTTTGGTGCAGCTTCTGTACTAATTGGTATTAGTTTTTTAGGAGGT
pcDNA-Alp       ATTAAGAAGTTCAAGTTTGGTGCAGCTTCTGTACTAATTGGTCTTAGTTTTTTGGGTGGG
                ****************************************** ********** ** **

AY345596.1      TTTACTCAAGGGCAATTTAATATTTCTACAGATACTGTGTTTGCAGCTGAGGTGATTTCA
pcDNA-Alp       GTTACACAAGGTAATCTTAATATTTTTGAAGAGTCAATAGTTGCTGCATCTACAATTCCA
                 **** *****  *  ********* *  ***  *  *  **** **       *** **

AY345596.1      GGAAGTGCTGCTACATTAAATTCCGCTTTAGTAAAAAATGTATCTGGTGGAAAAGCGTAT
pcDNA-Alp       GGGAGTGCAGCGACCTTAAATACAAGCATCACTAAAAATATACAAAACGGAAATGCTTAC
                ** ***** ** ** ****** *     *    ****** **      ***** ** **

AY345596.1      ATAGATATATATGATGTTAAAAATGGAAAAATAGATCCTTTAAACTTAATTGTTTTAACC
pcDNA-Alp       ATAGATTTATATGATGTAAAGTTAGGTAAAATAGATCCATTACAATTAATTGTTTTAGAA
                ****** ********** **    ** *********** *** * ************

AY345596.1      CCTTCTAATTATTCAGCAAACTATTATATAAAACAAGGTGGAAGGATTTTCACGAGTGTT
pcDNA-Alp       CAA---GGTTTTACAGCAAAGTATGTTTTTAGACAAGGTACTAAATACTATGGGGATGTT
                *       ** * ******* ***  * * * *******   *     *    *  ****

AY345596.1      AATCAACTTCAAACACCAGGTACAGCTACTATTACGTACAACATCCTTGATGAAAATGGA
pcDNA-Alp       TCTCAGTTGCAGAGTACAGGAAGGGCTAGTCTTACCTATAATATATTTGGTGAAGATGGA
                  ***  * ** *   **** *  **** * **** ** ** **  *** **** *****

AY345596.1      AATCCTTATACTAAAAGTGATGGTCAAATAGATATTGTAAGTCTTGTAACAACAGTATAT
pcDNA-Alp       CTACCACATGTAAAGACTGATGGACAAATTGATATAGTTAGTGTTGCTTTAACTATTTAT
                   **  **   ** * ****** ***** ***** ** *** ***    ***  * ***

AY345596.1      GATACTACAGAATTAAGGAATAATATCAACAAAGTAATTGAAAATGCAAATGATCCTAAA
pcDNA-Alp       GATTCAACAACCTTGAGGGATAAGATTGAAGAAGTTAGAACGAATGCAAACGATCCTAAG
                *** * ***   ** *** **** **  *  **** *     ******** ********
AY345596.1      TGGAGCGATGATAGTCGAAAAGATGTACTGAGCAAGATAGAAGTTATAAAAAATGATATT
pcDNA-Alp       TGGACGGAAGAAAGTCGTACTGAGGTTTTAACAGGATTAGATACAATTAAGACAGATATT
                ****  ** ** ***** *  ** **  * *      ****    ** ** *  ******

AY345596.1      GATAATAATCCAAAAACTCAATCTGATATTGATAATAAAATTGTTGAGGTTAATGAATTA
pcDNA-Alp       GATAATAATCCTAAGACGCAAACAGATATTGATAGTAAAATTGTTGAGGTTAATGAATTA
                *********** ** ** *** * ********** ************************

AY345596.1      GAGAAATTGTTAGTATTACCAGTACCGGATAAAGATAAATATGATCCAACAGGAGGGGAA
pcDNA-Alp       GAGAAATTGTTAGTATTGTCAGTACCGGATAAAGATAAATATGATCCAACAGGAGGGGAA
                *****************  ****************************************

AY345596.1      ACAACAGTACCCCAAGGGACACCAGTTTCAGATAAAGAAATCACAGACTTAGTTAAGATT
pcDNA-Alp       ACAACAGTACCCCAAGGGACACCAGTTTCAGATAAAGAAATCACAGACTTAGTTAAGATT
                ************************************************************

AY345596.1      CCAGATGGCTCAAAAGGGGTTCCGACAGTTGTTGGTGATCGTCCAGATACTAACGTTCCT
pcDNA-Alp       CCAGATGGCTCAAAAGGGGTTCCGACAGTTGTTGGTGAACGTCCAGATACTAACGTTCCT
                ************************************ **********************

AY345596.1      GGAGATCATAAAGTAACGGTTGATGTAACTTATCCAGATGGAACAAAGGATACAGTAGAA
pcDNA-Alp       GGAGATCATAAAGTAACGGTAGAAGTAACGTATCCAGATGGAACAAAGGATACAGTAGAA
                ******************** ** ***** ******************************

AY345596.1      GTAACGGTTCATGTGACACCAAAACCAGTACCGGATAAAGATAAATATGATCCAACAGGA
pcDNA-Alp       GTAACGGTTCATGTGACACCAAAACCATAG------------------------------
                ***************************

AY345596.1      GGGGAAACAACAGTACCCCAAGGGACACCAGTTTCAGATAAAGAAATCACAGACTTAGTT
pcDNA-Alp       ------------------------------------------------------------


AY345596.1      AAGATTCCAGATGGCTCAAAAGGGGTTCCGACAGTTGTTGGTGATCGTCCAGATACTAAC
pcDNA-Alp       ------------------------------------------------------------


AY345596.1      GTTCCTGGAGATCATAAAGTAACGGTTGATGTAACTTATCCAGATGGAACAAAGGATACA
pcDNA-Alp       ------------------------------------------------------------


AY345596.1      GTAGAAGTAACGGTTCATGTGACACCAAAACCATAG
pcDNA-Alp       -----------------------------------
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Supplemental Figure 1 **Nucleotide sequence alignment.** The nucleotide sequences of pcDNA-Alp1 (GenBank accession number: KX685456) and *alp1* gene from human *S. agalactiae* (GenBank accession number: AY345596.1) were compared.

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pcDNA-Alp1   MFRRSKNNSYDTSQTKQRFSIKKFKFGAASVLIGLSFLGGVTQGNLNIFEESIVAASTIP        60
AAR08114.1   MFRRSKNNSYDTSQTKQRFSIKKFKFGAASVLIGISFLGGFTQGQFNISTDTVFAAEVIS        60
             ************************************:*****.***::**  :::.**..*.
pcDNA-Alp1   GSAATLNTSITKNIQNGNAYIDLYDVKLGKIDPLQLIVLEQG-FTAKYVFRQGTKYYGDV        119
AAR08114.1   GSAATLNSALVKNVSGGKAYIDIYDVKNGKIDPLNLIVLTPSNYSANYYIKQGGRIFTSV        120
             *******:::.**:..*:****:**** ******:****  . ::*:* ::** : : .*
pcDNA-Alp1   SQLQSTGRASLTYNIFGEDGLPHVKTDGQIDIVSVALTIYDSTTLRDKIEEVRTNANDPK        179
AAR08114.1   NQLQTPGTATITYNILDENGNPYTKSDGQIDIVSLVTTVYDTTELRNNINKVIENANDPK        180
             .***:.* *::****:.*:* *:.*:********:. *:**:* **::*::*  ******
pcDNA-Alp1   WTEESRTEVLTGLDTIKTDIDNNPKTQTDIDSKIVEVNELEKLLVLSVPDKDKYDPTGGE        239
AAR08114.1   WSDDSRKDVLSKIEVIKNDIDNNPKTQSDIDNKIVEVNELEKLLVLPVPDKDKYDPTGGE        240
             *:::**.:**: ::.**.*********:***.**************.**************
pcDNA-Alp1   TTVPQGTPVSDKEITDLVKIPDGSKGVPTVVG----------------------------        271
AAR08114.1   TTVPQGTPVSDKEITDLVKIPDGSKGVPTVVGDRPDTNVPGDHKVTVDVTYPDGTKDTVE        300
             ********************************
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Supplemental Figure 2 **Amino acid sequence alignment between translated pcDNA-Alp1 and alpha-like protein1 from human *S. agalactiae*.** The translated amino acid sequences of pcDNA-Alp1 and alpha-like protein1 from human *S. agalactiae* (GenBank accession number: AAR08144.1) were aligned. Symbols: (*) identical amino acid, (:) conservative substitution and (.) semi-conservative substitution. The signal peptides are located at amino acid position 13-43, the N-terminal of protein at position 41-234, and the antigenic epitope at position 240-300.

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References

ATASSI MZ 1984. Antigenic structures of proteins. Eur J Biochem. 145(1): 1-20.

Creti R, Fabretti F, Orefici G and von Hunolstein C 2004. Multiplex PCR assay for direct identification of group B streptococcal alpha-protein-like protein genes. J Clin Microbiol. 42(3): 1326-1329.

Garver KA, LaPatra SE and Kurath G 2005. Efficacy of an infectious hematopoietic necrosis (IHN) virus DNA vaccine in Chinook *Oncorhynchus tshawytscha* and sockeye *O. nerka* salmon. Dis Aquat Organ. 64(1): 13-22.

Gillund F, Dalmo R, Tonheim TC, Seternes T and Myhr AI 2008. DNA vaccination in aquaculture—expert judgments of impacts on environment and fish health. Aquaculture. 284(1): 25-34.

Heppell J and Davis HL 2000. Application of DNA vaccine technology to aquaculture. Adv Drug Del Rev. 43(1): 29-43.

Kato G, Yamashita K, Kondo H and Hirono I 2015. Protective efficacy and immune responses induced by a DNA vaccine encoding codon-optimized PPA1 against *Photobacterium damselae* subsp. piscicida in Japanese flounder. Vaccine. 33(8): 1040-1045.

Kannika K, Pisuttharachai D, Srisapoome P, Wongtavatchai J, Kondo H, Hirono I and Areechon N 2017. Molecular serotyping, virulence gene profiling and pathogenicity of Streptococcus agalactiae isolated from tilapia farms in Thailand by multiplex PCR. J App Microbiol. 122(6): 1497-1507.

Kayansamruaj P, Pirarat N, Katagiri T, Hirono I and Rodkhum C 2014. Molecular characterization and virulence gene profiling of pathogenic *Streptococcus agalactiae* populations from tilapia (*Oreochromis* sp.) farms in Thailand. J Vet Diagn Invest. 26(4): 488-495.

Kojima Y, Xin KQ, Ooki T, Hamajima K, Oikawa T, Shinoda K, Ozaki T, Hoshino Y, Jounai N and Nakazawa M 2002. Adjuvant effect of multi-CpG motifs on an HIV-1 DNA vaccine. Vaccine. 20(23): 2857-2865.

Kolaskar A and Tongaonkar PC 1990. A semi-empirical method for prediction of antigenic determinants on protein antigens. FEBS Lett. 276(1-2): 172-174.

Kurath G 2008. Biotechnology and DNA vaccines for aquatic animals. Revue scientifique et technique-Office international des épizooties. 27(1): 175.

Kvam AI, Mavenyengwa RT, Radtke A and Maeland JA 2011. *Streptococcus agalactiae* alpha-like protein 1 possesses both cross-reacting and Alp1-specific epitopes. Clin Vaccine Immunol. 18(8): 1365-1370.

Lachenauer CS, Creti R, Michel JL and Madoff LC 2000. Mosaicism in the alpha-like protein genes of group B streptococci. Proceedings of the National Academy of Sciences. 97(17): 9630-9635.

Lindahl G, Stålhammar-Carlemalm M and Areschoug T 2005. Surface proteins of *Streptococcus agalactiae* and related proteins in other bacterial pathogens. Clin Microbiol Rev. 18(1): 102-127.

Lorenzen N, Lorenzen E, Einer-Jensen K and LaPatra SE 2002. Immunity induced shortly after DNA vaccination of rainbow trout against rhabdoviruses protects against heterologous virus but not against bacterial pathogens. Dev Comp Immunol. 26(2): 173-179.

Maeland JA, Bevanger L and Lyng RV 2004. Antigenic determinants of alpha-like proteins of *Streptococcus agalactiae.* Clin Diagn Lab Immunol. 11(6): 1035-1039.

Michel JL, Madoff LC, Olson K, Kling DE, Kasper DL and Ausubel FM 1992. Large, identical, tandem repeating units in the C protein alpha antigen gene, bca, of group B streptococci. Proceedings of the National Academy of Sciences. 89(21): 10060-10064.

Mon-on N, Surachetpong W, Mongkolsuk S and Sirikanchana K 2018. Roles of water quality and disinfectant application on inactivation of fish

pathogenic *Streptococcus agalactiae* with povidone iodine, quaternary ammonium compounds and glutaraldehyde. J Fish Dis. https://doi.org/10.1111/jfd.12776.

Rodkhum C, Kayansamruaj P, Pirarat N, Zhou W, Liu Y and Chen GH 2011. Effect of water temperature on susceptibility to *Streptococcus agalactiae* serotype Ia infection in Nile tilapia (*Oreochromis niloticus*). Thai J Vet Med. 41(3): 309.

Rubinstein ND, Mayrose I, Halperin D, Yekutieli D, Gershoni JM and Pupko T 2008. Computational characterization of B-cell epitopes. Mol Immunol. 45(12): 3477-3489.

Suanyuk N, Kong F, Ko D, Gilbert GL and Supamattaya K 2008. Occurrence of rare genotypes of *Streptococcus agalactiae* in cultured red tilapia *Oreochromis* sp. and Nile tilapia *O. niloticus* in Thailand—Relationship to human isolates? Aquaculture. 284(1): 35-40.

Sun Y, Hu YH, Liu CS and Sun L 2010. Construction and analysis of an experimental *Streptococcus iniae* DNA vaccine. Vaccine. 28(23): 3905-3912.

Sun Y, Zhang M, Liu CS, Qiu R and Sun L 2012. A divalent DNA vaccine based on Sia10 and OmpU induces cross protection against *Streptococcus iniae* and *Vibrio anguillarum* in Japanese flounder. Fish Shellfish Immunol. 32(6): 1216-1222.

Suwannasang A, Dangwetngam M, Issaro A, Phromkunthong W and Suanyuk N 2014. Pathological manifestations and immune responses of serotypes Ia and III *Streptococcus agalactiae* infections in Nile tilapia (*Oreochromis niloticus*). Songklanakarin J Sci Tech Accepted.

Tonheim TC, Bøgwald J and Dalmo RA.2008. What happens to the DNA vaccine in fish? A review of current knowledge. Fish Shellfish Immunol. 25(1): 1-18.

Toranzo AE, Magariños B and Romalde JL 2005. A review of the main bacterial fish diseases in mariculture systems. Aquaculture. 246(1): 37-61.

von Heijne G and Abrahmsèn L 1989. Species-specific variation in signal peptide design Implications for protein secretion in foreign hosts. FEBS Lett. 244(2): 439-446.

บทคัดย่อ

ประสิทธิภาพของวัคซีนดีเอนเอ (pcDNA-Alp1) สำหรับป้องกันโรคสเตรปโตคอคโคซิสในปลานิล

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ปลานิลเป็นปลาที่มีความสำคัญทางเศรษฐกิจทั่วโลก รวมทั้งภูมิภาคเอเชียตะวันออกเฉียงใต้และประเทศไทย โดยประเทศไทยมีการเลี้ยงปลานิลเพื่อการบริโภคภายในประเทศและส่งออกจำนวนหนึ่ง การเลี้ยงปลานิลในประเทศมีหลายรูปแบบ ทั้งการเลี้ยงในบ่อ เลี้ยงแบบกระชัง และในแหล่งน้ำตามธรรมชาติ แต่การเลี้ยงปลานิลยังพบปัญหาเชื้อก่อโรคที่มีทั้ง เชื้อรา ไวรัส และแบคทีเรีย ซึ่งแบคทีเรียที่เป็นปัญหาและก่อให้เกิดความเสียหายในการเพาะเลี้ยงปลานิลมากที่สุด คือ เชื้อ *Streptococcus agalactiae* ซึ่งเป็นสาเหตุของโรคสเตรปโตคอคโคซิส การศึกษาครั้งนี้เริ่มจากการสร้างวัคซีนดีเอนเอที่มียีนของโปรตีน Alp1 ซึ่งเป็นโปรตีนบนผิวเซลล์ของ *S. agalactiae* ทำการทดลองโดยแบ่งปลานิลออกเป็น 4 กลุ่มการทดลองดังนี้ 1. กลุ่มควบคุมฉีดสารละลายเกลือ (Phosphate buffer saline: PBS) 2. กลุ่มฉีดพลาสมิดเปล่า pcDNA 3.1 (+) ซึ่งไม่มีชิ้นส่วนของยีนสร้างโปรตีน Alp1 3. กลุ่มฉีดวัคซีนดีเอนเอ pcDNA-Alp1 และ 4. กลุ่มฉีดวัคซีนเชื้อตายสำหรับเชื้อ *S. agalactiae* ใช้เวลากระตุ้นระบบภูมิคุ้มกัน 4 สัปดาห์ หลังจากนั้นทดสอบความต้านทานเชื้อ *S. agalactiae* ที่ความเข้มข้น 10^8 CFU สังเกตอาการเป็นเวลา 4 สัปดาห์ ทำการเก็บปลาตายหรือป่วยรุนแรงจากตู้เลี้ยงปลา และเพาะแยกเชื้อ การทดลองพบว่า ปลากลุ่มที่ 2, 3 และ 4 มีค่าความสัมพันธ์อัตราการรอดตาย 26.6%, 51.4% และ 46.6% ตามลำดับ จากการเพาะเชื้อแบคทีเรียและย้อมสีแกรมพบว่าปลาทั้งหมดติดเชื้อ *S. Agalactiae* การศึกษาครั้งนี้แสดงให้เห็นว่า โปรตีน Alp1 จาก *S. agalactiae* ทำหน้าที่ในการเป็นแอนติเจนได้ดี สามารถกระตุ้นระบบภูมิคุ้มกันในปลานิลเพื่อป้องกันการติดเชื้อ *S. agalactiae* โดยอัตราการตายสะสมพบน้อยที่สุดเมื่อเปรียบเทียบกับกลุ่มทดลองอื่น

คำสำคัญ: ปลานิล วัคซีนดีเอนเอ โรคสเตรปโตคอคโคซิส เชื้อสเตรปโตคอคโคซิส อากาแลคติเอ

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