Research paper

Selection and identification of Singapore grouper iridovirus vaccine candidate antigens using bioinformatics and DNA vaccination

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A B S T R A C T

In this study, we described a rapid and efficient method which integrated the bioinformatic prediction and DNA vaccine technology to identify vaccine candidates against Singapore grouper iridovirus (SGIV). The 162 previously defined open reading frames (ORFs) of SGIV were subjected to extensive sequence similarity searches, as well as motif, cellular location, and domain prediction. Based on our analysis, 13 genes were chosen and cloned into the eukaryotic expression vector pcDNA 3.1. \textit{In vitro} and \textit{in vivo} expression of these DNA vaccine constructs was examined in \textit{Epinephelus achoa} spleen cells (EAGS) and immunized fish by Western blot and RT-PCR analysis, respectively. Three weeks after the second booster, immunized fish were challenged with SGIV and the level of protection and survival was assessed. Fish vaccinated with plasmid DNA encoding viral ORF072, ORF039 and ORF036 (designated as pcDNA-72, pcDNA-39 and pcDNA-36, respectively) exhibited 66.7%, 66.7% and 58.3% relative percent survival rates, respectively, in comparison with the control fish. These three DNA vaccines induced innate immune responses, raising significantly high level of Mx expression relative to the fish vaccinated with the empty plasmid at 3 days post-vaccination. Furthermore, recombinant protein from ORF072 was also used to immunize another set of fish and similar protective effect was obtained. Taken together, our results validated the applicability of bioinformatics in genome mining, resulting in the identification of three protective antigens. The promising results obtained in the present study have prompted further testing to improve the immunogenicity of these potential DNA vaccines.

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1. Introduction

Grouper, \textit{Epinephelus} spp., a major species being maricultured in China and Southeast Asian countries, are high value and commercially important food fish. However, recently intensive aquaculture is still encountering increasing difficulties, especially the infectious diseases caused by virus, bacteria and parasites. Iridoviruses have been identified as one of the most important pathogens of grouper culture in the last decade (Chou et al., 1998; Lu et al., 2005; Qin et al., 2003). Singapore grouper iridovirus (SGIV), a pathogenic iridovirus, firstly isolated from diseased grouper, has been characterized as a novel species of \textit{Ranavirus}, the family of \textit{Iridoviridae} (Qin et al., 2001, 2003). The virus caused more than 90% mortality in grouper and sea bass (Qin et al., 2003). However, despite the high mortality caused by the virus, no effective treatment is available.
It is generally accepted that vaccination is a most cost-efficient way of controlling infectious diseases in fish. SGIV inactivated vaccine has been found to be highly efficient in mounting protection in the immunized fish (unpublished data). However, the use of whole-killed virus vaccine has its own limitations, which include poor induction of cell-mediated immunity and the increased risk of environmental exposure to viral products (Davis and McCluskie, 1999). Moreover, fish generally needed a relatively high dose to achieve protection compared with terrestrial animals, the cost of producing vaccines based on inactivated viruses is usually too high to make this strategy economically viable (Sommerset et al., 2005). New generation vaccine, such as recombinant subunit vaccine and DNA vaccine, has gained considerable attention as an alternative approach to traditional vaccines. In order to develop these types of innovative vaccines, there is an urgent need to identify of protective antigens. To date, efforts to identify specific vaccine candidates against iridovirus are limited and no protective antigens have been identified in SGIV.

DNA-based vaccination, which involves administration of a plasmid vector mediating expression of pathogen-derived genes in the vaccinated-animal, has appeared to be a promising method for immunization since the first demonstration by Wolff et al. (1990). DNA vaccine has been shown to be highly efficient against fish viruses, such as infectious hematopoietic necrosis virus (IHNV) and viral hemorrhagic septicemia virus (VHSV) (Lorenzen and LaPatra, 2005; Tonheim et al., 2008). Since it is easy and rapid to clone and modify genes in plasmid expression vectors, DNA vaccine offers an excellent opportunity to quickly discover new antigens and handle the antigenicity of the protein at the sequence level without requiring the lengthy and laborious protein expression and purification (Yero et al., 2007). In addition, DNA vaccine has other advantages, which include the process of antigen production by host cells after DNA vaccination mimics the production of antigens during a natural infection and the induction of both humoral and cellular immune response (Barry and Johnston, 1997; Donnelly et al., 2000). Here, we describe development and application of a rapid and efficient method which integrates the bioinformatic prediction and DNA-based immunization to identify vaccine candidates against SGIV. With this approach, we have identified three antigens which provided significant protection against SGIV infection.

2. Materials and methods

2.1. Bioinformatic analysis and gene selection

To date, the complete genome and proteome of SGIV has been determined (Song et al., 2004, 2006). A further screening for coding capacity of the grouper iridovirus genome was carried out using comparative genomic tools and defined 140 authentic open reading frames (ORFs) in SGIV (Eaton et al., 2007). Based on a minimum ORF size of 140 amino acids (aa) and literature information, 111 ORFs were selected for the following analysis.

Firstly, sequence similarity searches were conducted by running BLAST analyses to identify two classes of ORFs (Altschul et al., 1990). The class of ORFs coding for known cytoplasmic and nuclear functions was not further investigated, whereas the class for others was selected for further analyses. A second screening step aimed at identifying putative proteins with signals for extracellular exposure (either surface anchored or secreted) was applied. Cellular location predictions, for each ORFs, were carried out as follows: prediction of presence and location of signal peptide in the N-terminal of an ORF, using the program SignalP (Nielsen et al., 1997); prediction of membrane-spanning regions, using the program DAS-TMfilter (Cserzo et al., 2002), TMHMM (Krogh et al., 2001) and Tmpred (Hofmann and Stoffel, 1993). Meanwhile, SWISS-PROT, PROSITE, SMART, MOTIFS, as well as InterPro, Pfam, and Blocks databases, were used to predict features typical of surface-associated proteins such as transmembrane domains, leader peptides, homologies to known surface proteins, membrane anchoring motifs and host cell binding domains such as RGD motif.

2.2. Plasmid construction and purification

For the construction of SGIV DNA vaccines, selected genes were amplified by polymerase chain reaction (PCR) from genomic DNA using specific primers (Table S1). To improve translational efficiency, a Kozak sequence, ACCATGG, was incorporated around the start codon at the 5′ end of the gene (Kozak, 1986). The reactions were carried out by using PrimeSTAR HS DNA Polymerase (TaKaRa, Japan) with varying cycle numbers according to the Taq manufacturer’s recommendations. The resultant PCR products were then cloned into pcDNA 3.1 (Invitrogen, USA). The identities of the inserted genes were confirmed by DNA sequencing, and the resulting plasmids were termed according to the cloned gene name, such as pcDNA-16. Plasmid DNA was purified using the PureLink™ Hipure Plasmid Maxiprep kit (Invitrogen) with pyrogen-free materials and suspended in phosphate-buffered saline (PBS). The concentration was then appropriately adjusted to fit the immunization protocol.

Genes selected for prokaryotic expression were amplified from the virus genome by PCR. A fragment encoding 354 residues (62aa–415aa) of ORF072 was amplified using primers 5′-TCG GAT CCT TCG GGC AAC AGT TTG CCG-3′ and 5′-GCC GAA TTC AAA TGG TTA CAG TGA TGC TAG CG-3′ and ligated into the prokaryotic vector pRSET-A (Invitrogen). Constructs were confirmed by sequencing and the resulting plasmid was named as VP72.

2.3. In vitro and in vivo expression of the DNA vaccines

To test the in vitro expression of the DNA vaccines, cultured fish cells were transfected with the constructs to which a polyclonal antibody was available in our laboratory. Briefly, Epinephelus akena spleen cells (EACS) cultured in 6-well plates for 16–18 h were transiently transfected with purified plasmids, using Lipofectamine 2000 Reagent (Invitrogen) according to the manufacturer’s instructions. After incubation for 48–72 h, the cells were
harvested and directly resuspended in sodium dodecyl sulfate (SDS) sample buffer. Protein was separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto a PVDF membrane using standard techniques (Huang et al., 2008). The membrane was reacted first with the corresponding polyclonal antibodies, followed by horseradish peroxidase (HRP)–conjugated goat-antimouse IgG (Pierce, USA). Development was performed with the DAB-HRP Color Development Kit (TIANGEN, China).

To investigate whether the DNA vaccines expressed in the immunized fish, muscle tissues were sampled from fish at 3 days post-vaccination (d.p.v.). Tissue samples of 1 cm × 1 cm were taken from 4 fish to cover the area of injection and pulverized together to powder in liquid nitrogen. Total RNA was extracted from the samples using Trizol Reagent (Invitrogen) according to the manufacturer’s recommendations. Five micrograms of RNA were treated with DNase I (Promega, USA) to remove any genomic DNA traces that might interfere with the PCR reactions and then used to obtain cDNA using the ReverTra Ace-α-Kit (Toyobo, Japan). The cDNA was subsequently amplified by PCR in a 25 μl reaction volume using gene-specific forward and reverse primers (Table S1).

2.4. Recombinant protein expression and purification

Escherichia coli BL21 (DE3) competent cells were transformed with the constructs and expression of recombinant proteins was induced by incubation in LB-ampicillin medium supplemented with 1 mM IPTG. Protein was produced as inclusion bodies and the recombinant protein used for immunization was purified by centrifuge-based method. Briefly, the inclusion body pellet was washed (50 mM Tris buffer, pH 8.0, containing 0.1 M NaCl, 1 mM EDTA and 1% Triton X-100) and centrifuged (15 min at 12,000 × g at 4 °C). Then the supernatant was decanted and the precipitate washed in 2 M urea for 1–2 h. After washing, the inclusion bodies were recovered by centrifugation at 12,000 × g for 15 min. These semipurified preparations were then used for the following immunization experiment. Protein concentration was determined using the BCA Protein Assay Kit (Biomiga, USA). Protein analysis was performed in SDS-PAGE gels according to Laemmli (1970) and the relative amount of recombinant protein in total was estimated with the densitometry analysis software BandScan. E. coli transformed with pRSET-A vector (termed pRSET) was handled in parallel and the insoluble proteins were purified and used as a negative control in the immunization experiment.

2.5. Immunization and challenge

Grouper (Epinephelus coioides) of approximately 8 g obtained from Daya Bay Aquaculture Centre, Guangdong, China were maintained at a laboratory closed re-circulating dechlorinated-water system at 25 °C with filtered and UV-treated seawater, and fed daily with a commercial diet. Prior to experiments, ten fish were randomly sampled for the examination of SGIV by PCR assay using the specific primers described in the previous study (Ou-yang et al., 2010), and no specific band was detected in all the examined fish (data not shown). Fish were acclimatized to laboratory conditions for 2 weeks before immunization.

To examine the protective efficiencies of DNA vaccines, groups of 300 fish were randomly assigned to one of fifteen groups and then anaesthetized in 100 ppm of tricaine methane sulfonate (MS-222, Sigma). Each fish was injected intramuscularly, immediately anterior and lateral to the dorsal fin (i.e. in the epaxial muscle), with 30 μg of each plasmid on day 0. Two weeks later (day 14), the fish were given a boosting injection with 20 μg of the corresponding plasmids. Control groups were included in the experiment that consisted of fish injected on days 0 and 14 with PBS and pcDNA 3.1 vector, respectively. For recombinant subunit vaccines, two groups of 20 fish each were injected intraperitoneally (IP) with 0.2 ml of vaccine preparation that contained 50 μg recombinant protein formulated in PBS. Control fish were injected with same amount of proteins purified from empty vector transformed bacterial.

On day 21 after the last immunization, each group of fish were IP challenged with 7.0 × 10⁴ TCID₅₀ SGIV per fish. The fish were monitored for clinical signs of disease. Postchallenge fish mortality in each group was recorded, and dead fish were removed daily for 16 days. PCR assays were performed to confirm that the fish were infected with SGIV (data not shown). The relative percent of survival (RPS) was calculated according to the following formula, as described by Johnson et al. (1982): RPS = [1 – (% mortality in vaccinated fish/ % mortality in control fish)] × 100, where pcDNA 3.1/pRSET injected fish were used as control for these calculations.

2.6. Quantitative real-time reverse transcriptase-PCR (qRT-PCR) analysis

Based on the sequences of E. coioides Mx genes (Lin et al., 2006), the primers used for qRT-PCR analysis were designed as follows: for Mx-I, forward primer 5′-CGA AAG TAC GCT GGA CGA GAA-3′ and reverse primer 5′-TGT TGG ATC TGC TCC TTG ACC AT-3′; for Mx-II, forward primer 5′-GCT TCA TCA ACT ACA AGA CCT TGA A-3′ and reverse primer 5′-GCG CTT CCT AAC AGT ATC TTC TAT TT-3′; and for Mx-III, forward primer 5′-GGG AAG GAT AGA AGA CGT AAC GC-3′ and reverse primer 5′-GAG GAA ACA ATC AGC TCC ATT TTA-3′. The primers used to amplify the β-actin gene have been reported previously (Ou-yang et al., 2010). Mx gene expression was conducted in spleen tissue by qRT-PCR. Briefly, the whole spleen was sampled from four fish immediately before vaccination and used as control. Thereafter, spleen was sampled from fish (four at each group) vaccinated with pcDNA-36, pcDNA-39, pcDNA-72, pcDNA 3.1 and PBS 3 d.p.v. RNA extraction and cDNA synthesis was performed as described in Section 2.3.

qRT-PCR assays were performed using the LightCycler 480 system (Roche, Switzerland). Briefly, real-time PCR was carried out with the specific primers. The standard cycling conditions were 95 °C for 1 min, followed by 45 cycles of 95 °C for 5 s, 60 °C for 10 s and 72 °C for 15 s. All samples were analyzed in three duplications and the β-actin gene was used as a general housekeeping gene to normalize target genes. Fish injected with PBS were used as
negative control and served as the calibrator group, while the increase in expression was calculated relative to this group.

2.7. Statistical analyses

Data are expressed as mean ± standard error (SE). Differences in transcription levels were compared using Student’s t-tests. If significant, the least significant difference test was further used. The chi-square test was used to assess the protective efficacy of the different vaccine preparations in the comparison with the control group. In all cases, differences were considered statistically significant at $p < 0.05$ and the statistical analyses were performed using SPSS 16.0 (SPSS, USA).

3. Results

3.1. Bioinformatics and selection

The bioinformatic analysis of the SGIV coding sequences was conducted to identify genes encoding potential vaccine candidates. For enveloped viruses, the envelope proteins are particularly important because these proteins are the first molecules to interact with the host, and they therefore play critical roles in cell targeting as well as in the triggering of host defenses (Chazal and Gerlier, 2003). So the primary criterion for selection as a putative vaccine candidate was the presence of signal sequences or motifs predictive of expression as secreted or membrane-associated proteins. Using the criteria outlined above, we obtained 13 genes and their predicted sizes ranged from 513 to 3156 bp, as shown in Table 1.

3.2. In vitro and in vivo expression of the DNA vaccines

To confirm the ability of the DNA vaccine candidate constructs to express the viral genes in fish cells, cultured EAGS cells were transfected with the constructs of pcDNA-16, pcDNA-19, pcDNA-38, pcDNA-72, pcDNA-88, and pcDNA-90, respectively, and the protein expression was identified in transfected cell lysates by Western blot analysis (Fig. 1). Specific bands were observed at the predicted sizes (46.2, 36.8, 19.0, 50.5, 54.0 and 43.5 kDa) with respective antibodies. No specific band was observed in cells transfected with pcDNA 3.1 vector (data not shown).

After confirmation of expression in EAGS cells, the plasmid constructs were injected into grouper and the in vivo expression of plasmids containing viral genes was studied at transcriptional level on DNase I-treated RNA extracted from muscle of injected fish. Specific products of expected sizes were amplified for each construct (Fig. 2). No band was detected in muscle tissue of control fish immunized with pcDNA 3.1 vector (data not shown).

3.3. Plasmid DNA immunization and challenge

To examine the protective efficacy of the DNA vaccines, the vaccinated and control fish were artificially challenged with $7.0 \times 10^4$ TCID$_{50}$ virus per fish and monitored for cumulative mortality. Significant protection was observed with a cumulative percent mortality (CPM) of 25%, 20% and 20% in pcDNA-36, pcDNA-39 and pcDNA-72 vaccinated fish, respectively. Based on these data, the protection efficacies, in terms of RPS, of pcDNA-36, pcDNA-39, and pcDNA-72 were, respectively, 58.3%, 66.7% and 66.7% in comparison with the control of pcDNA 3.1 (Table 2). Other ORFs did not provide significant protection.

3.4. DNA vaccines induced the Mx expression in vivo

Since DNA vaccines of pcDNA-36, pcDNA-39, and pcDNA-72 exhibited a much higher level of immunoprotection, we thus further compared the effects of the DNA vaccines on the induction of Mx expression in the spleen tissue. Our results showed that immunization with these DNA vaccines significantly stimulated the expression of all the three Mx isoforms (Fig. 3). The induction profiles induced by pcDNA-36 and pcDNA-39 vaccination were similar, while the highest expression level was observed in the pcDNA-72 immunized fish.

Fig. 1. Western blot analysis of DNA vaccine constructs expressed in transfected fish cells. The major products of the protein synthesis from pcDNA-16 (lane 1), pcDNA-19 (lane 2), pcDNA-38 (lane 3), pcDNA-72 (lane 4), pcDNA-88 (lane 5) and pcDNA-90 (lane 6) correspond to polypeptides of 46.2, 36.8, 19.0, 50.5, 54.0 and 43.5 kDa, well in accordance with the coding capacity of the respective cloned sequences.

Fig. 2. Expression of DNA vaccines in muscle tissues of vaccinated fish. Lanes 1–13 shown the PCR products obtained using primers specific for pcDNA-16, pcDNA-19, pcDNA-26, pcDNA-36, pcDNA-38, pcDNA-39, pcDNA-59, pcDNA-72, pcDNA-83, pcDNA-87, pcDNA-88, pcDNA-90 and pcDNA-101, respectively.
Table 1
Selected open reading frames (ORFs) used for antigenicity studies.

<table>
<thead>
<tr>
<th>Selected ORFs</th>
<th>Accession no.</th>
<th>Length (aa)</th>
<th>TMHMM</th>
<th>Tmpred</th>
<th>Signal sequence</th>
<th>Conserved domain or signature</th>
<th>Predicted function</th>
</tr>
</thead>
<tbody>
<tr>
<td>ORF016</td>
<td>YP_164111.1</td>
<td>413</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>LTXXQ motif</td>
<td></td>
</tr>
<tr>
<td>ORF019</td>
<td>YP_164114.1</td>
<td>342</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>Poxvirus motif</td>
<td></td>
</tr>
<tr>
<td>ORF026</td>
<td>YP_164121.1</td>
<td>566</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>Acetyl-CoA hydrolase</td>
<td></td>
</tr>
<tr>
<td>ORF036</td>
<td>YP_164131.1</td>
<td>329</td>
<td>None</td>
<td>None</td>
<td>1–18</td>
<td>CaaX motif</td>
<td></td>
</tr>
<tr>
<td>ORF038</td>
<td>YP_164133.1</td>
<td>170</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>RGD motif</td>
<td></td>
</tr>
<tr>
<td>ORF039</td>
<td>YP_164134.1</td>
<td>1051</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>Protein kinase domain</td>
<td></td>
</tr>
<tr>
<td>ORF059</td>
<td>YP_164154.1</td>
<td>146</td>
<td>15–37</td>
<td>25–41</td>
<td>None</td>
<td>Major capsid protein</td>
<td></td>
</tr>
<tr>
<td>ORF072</td>
<td>YP_164167.1</td>
<td>463</td>
<td>None</td>
<td>364–386</td>
<td>None</td>
<td>Protease atpase-dependent</td>
<td></td>
</tr>
<tr>
<td>ORF083</td>
<td>YP_164178.1</td>
<td>445</td>
<td>None</td>
<td>87–108</td>
<td>None</td>
<td>Virion membrane protein</td>
<td></td>
</tr>
<tr>
<td>ORF087</td>
<td>YP_164182.1</td>
<td>215</td>
<td>None</td>
<td>None</td>
<td>1–18</td>
<td>Lipid membrane protein</td>
<td></td>
</tr>
<tr>
<td>ORF088</td>
<td>YP_164183.1</td>
<td>506</td>
<td>None</td>
<td>None</td>
<td>191–211</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ORF090</td>
<td>YP_164185.1</td>
<td>313</td>
<td>None</td>
<td>120–142</td>
<td>None</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ORF101</td>
<td>YP_164196.1</td>
<td>313</td>
<td>None</td>
<td>60–81</td>
<td>None</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

3.5. Immunoprotective effect of VP72 as a recombinant subunit vaccine

Since DNA-based immunization identified 3 protective antigens, we examined its immunoprotective potential as a purified recombinant subunit vaccine. For this purpose, the coding region of genes was subcloned into E. coli, and recombinant proteins were semipurified from inclusion bodies. SDS-PAGE analyses indicated that band corresponding to the fusion protein was observed at the expected size (Fig. 4). Virus-encoded genes ORF036 and ORF039 were also cloned and expressed in E. coli with partial and full length forms. But the expression level was too low to get enough protein for immunization, so these two genes were not included in the recombinant subunit vaccine experiment. To examine the vaccine potential of the protein, grouper were vaccinated and subsequently challenged with the pathogenic virus. The fish were monitored for mortality, and the results showed that the cumulative mortalities of pRSET and VP72 were 65% and 30%, respectively, which corresponds to an RPS of 53.8% for VP72-vaccinated fish with significant difference (Fig. 5). This result demonstrated the conferment of protection following recombinant protein and DNA vaccination.

4. Discussion

Although grouper iridovirus has antigens to induce strong immune responses (Qin et al., 2002), so far no specific antigens associated with a protective immune

Table 2
Cumulative mortalities and relative percentage survival (%) of the DNA vaccine immunized groups after experimental challenge with SGIV.

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>Mortalitya</th>
<th>Relative percentage survival</th>
<th>p-Value (if &lt; 0.05)b</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>12/20 (60%)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>pcDNA-3.1</td>
<td>12/20 (60%)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>pcDNA-16</td>
<td>10/20 (50%)</td>
<td>16.7%</td>
<td></td>
</tr>
<tr>
<td>pcDNA-19</td>
<td>9/20 (45%)</td>
<td>25%</td>
<td></td>
</tr>
<tr>
<td>pcDNA-26</td>
<td>9/20 (45%)</td>
<td>25%</td>
<td></td>
</tr>
<tr>
<td>pcDNA-36</td>
<td>5/20 (25%)</td>
<td>58.3%</td>
<td>0.025</td>
</tr>
<tr>
<td>pcDNA-38</td>
<td>14/20 (70%)</td>
<td>−16.7%</td>
<td>0.01</td>
</tr>
<tr>
<td>pcDNA-39</td>
<td>4/20 (20%)</td>
<td>66.7%</td>
<td></td>
</tr>
<tr>
<td>pcDNA-59</td>
<td>13/20 (65%)</td>
<td>−8.3%</td>
<td></td>
</tr>
<tr>
<td>pcDNA-72</td>
<td>4/20 (20%)</td>
<td>66.7%</td>
<td>0.01</td>
</tr>
<tr>
<td>pcDNA-83</td>
<td>8/20 (40%)</td>
<td>33.3%</td>
<td></td>
</tr>
<tr>
<td>pcDNA-87</td>
<td>10/20 (50%)</td>
<td>16.7%</td>
<td></td>
</tr>
<tr>
<td>pcDNA-88</td>
<td>8/20 (40%)</td>
<td>33.3%</td>
<td></td>
</tr>
<tr>
<td>pcDNA-90</td>
<td>9/20 (45%)</td>
<td>25%</td>
<td></td>
</tr>
<tr>
<td>pcDNA-101</td>
<td>11/20 (55%)</td>
<td>8.3%</td>
<td></td>
</tr>
</tbody>
</table>

a Number of mortalities/total number of fish. The percentage mortalities are shown in parenthesis.
b The p-values were calculated by chi-square analysis for each challenge group.
response have been identified. Therefore, identification of immunoprotective proteins is a critical parameter in the development of antigen-based vaccine. In this study, we describe an approach to select and identify DNA vaccine candidates from the SGIV genomic sequence database using bioinformatics. Ultimately, we identified 3 immunogenic proteins, which were demonstrated as protective antigens for the first time.

Successful viral vaccine candidates usually located at the virus surface and accessible to the host. Such surface-exposed or exported proteins could be identified in the genomic sequence database by one or more signature sequence motifs commonly found in known selected/surface-associated proteins. By utilizing this screening approach, we have identified 13 ORFs encoding putative membrane-associated proteins that are potential vaccine candidates. It is worth mentioning that 46% (6/13) of the selected genes have been identified as envelope proteins (Zhou et al., 2011), indicating that this tactic works well in prediction envelope proteins from SGIV genome. Notably, a relatively high proportion of protective antigens were identified with this strategy. Of the 13 antigens analyzed, 3 (~23%) were capable of inducing protective immune response in grouper, suggesting that antigen selection based on bioinformatic analysis is an effective way to identify potential vaccine candidates against SGIV. Channel catfish virus, another enveloped large DNA virus, has been successfully identified 2 protective antigens from 7 selected virus genes based on their associated with membrane structures or being virus-encoded late genes (Nusbaum et al., 2002). Nevertheless, similar strategy has been widely used and shown to be efficacious in identifying protective antigens from bacterial genome (Movahedi and Hampson, 2008; Pizza et al., 2000; Zagursky et al., 2003).

To induce appropriate immune responses and protection, not only promising candidates but also optimized antigen application is of importance. Although requirements may vary with different pathogens, the cellular immunity is usually crucial in mediating protection against virus infection (Seder and Hill, 2000). Actually, previous studies have shown that the specific cellular immune response was mainly responsible in mounting protection against red seabream iridovirus (RSIV) (Caipang et al., 2006a,b). Thus, vaccine against SGIV should induce a potent cell-mediated immunity. DNA-based immunization fulfills this basic requirement. DNA vaccines are known to be more effective than traditional vaccines in stimulating the host cellular immune response (Davis and McCluskie, 1999).
It has been reported that DNA immunization provokes the activation of cell-mediated cytotoxicity against VHSV (Boudinot et al., 2004; Utke et al., 2008) and DNA vaccine induced cellular responses suggest to play an important role in mediating a protective immune response against fish viruses (Cai pang et al., 2006b; Kurath et al., 2006). Vaccination of animals with DNA vaccines requires an effective dose to trigger an efficient and protective immune response (Dunham, 2002). Several studies have shown that the efficiency of DNA immunization depends on several parameters like the expression vector, the delivery route and the dosage that is used (Gurunathan et al., 2000). A major goal of the optimization process is the increase of the antigen amount that is recognized by the immune system. In this study, grouper were given two immunizations with the respective DNA vaccines and three antigens demonstrated a moderate protection against SGIV. In fact, the DNA vaccine trial reported here had been preceded by an additional trial, in which a single immunization with 15 μg of each plasmid was conducted and a low level of protection was observed only in the pcDNA-72 immunized fish (not published data). Other DNA vaccine trials have also shown that boosters enhance the level of protection in mammals (Robinson et al., 1997), chicken (Klotz et al., 2007) and fish (Mikalsen et al., 2005).

There were three isoforms of Mx genes, Mx-I, Mx-II, and Mx-III from grouper, with sequence identity between 65 and 76% at the amino acid level (Lin et al., 2006). In this study, the results demonstrated that all three DNA vaccines were able to up-regulate the expression of Mx genes, which usually used as an indicator of alpha/beta interferon (IFN) system induction. Similar up-regulation of type I IFN-related genes was extensively observed in plasmid DNA vaccinated fish (Leong et al., 2000; Lorenzen et al., 2002; Siwicki et al., 2010). Recently, it has become clear that IFN-α/β represents key molecules in the protective immune response to many virus infections, not only by interfering with replication of intruding virus, but also by paving the way for the subsequent specific responses (Biron, 2001; Tough and Le Bon, 2002). The upregulation of Mx gene by these DNA vaccines may act as accessory molecules in directing efficient specific immune response, as suggested by in rainbow trout vaccinated with DNA vaccines against IHNV or VHSV (Lorenzen et al., 2002).

Among the identified candidates, ORF072 encoded the virus major capsid protein (MCP). MCP is the major protein that produced by iridovirus during progressive infection and can account for up to 45% of the virion protein (Williams, 1996). Hence it is not surprising that the MCP of SGIV is a protective antigen. The immunogenicity of MCP has also been identified in previous studies of other iridotviruses, such as RSIV (Cai pang et al., 2006b) and lymphocystis disease virus (Tian et al., 2008; Zheng et al., 2006). Former study demonstrated that the iridoviral antigenic related proteins are more associated with the virion surface and virus assembly sites (Qin et al., 2002). ORF039 has been identified as a viral envelope protein (Zhou et al., 2011) and a core protein within the Iridoviridae family (Eaton et al., 2007). ORF036 was only found in grouper iridovirus, which predicted to include a signal peptide at its N-terminal and a CaaX prenylation motif at C-terminal. Protein prenylation modification, wherein an isoprenoid tail is attached to the end of a substrate protein, usually helps localize the prenylated protein to cellular membranes and aids in protein–protein interactions (Casey, 1994; Marshall, 1993). We proposed that after cleavage of the signal peptide leaves the protein into the membrane anchored by its C-terminal isoprenoid tail. Obviously, experiments are needed to confirm the membrane location of this protein.

In summary, as demonstrated in this study, combining bioinformatic prediction with DNA vaccine technology allows for efficacious selection of in vivo immunogens. While the bioinformatic strategy results in selection of 13 vaccine candidates, the DNA-based vaccination and challenge method enables to identify 3 protective antigens. Further investigations are in progress to improve these vaccines efficacy.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vetimm.2012.05.021.

References


re-annotating and defining the core set of iridovirus genes. Virol. J. 4, 11.