Fish and Shellfish Immunology 94 (2019) 308–317

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Immune response of channel catfish (*Ictalurus punctatus*) against *Ichthyophthirius multifiliis* post vaccination using DNA vaccines encoding immobilization antigens

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ARTICLE INFO

Keywords:
Molecular immune response
Antibody
Survival
Fish parasite
Immune genes
qPCR

ABSTRACT

The channel catfish (*Ictalurus punctatus*) immune response against *Ichthyophthirius multifiliis* (Ich) after vaccination using plasmid DNA vaccines pcDNA3.1-IAg52a and pcDNA3.1-IAg52b, encoding Ich immobilization antigen genes was studied. Parasite infection level, serum anti-Ich antibodies level, fish mortality after theront challenge, and immune-related gene expression were measured. After *in vitro* transfection of walking catfish gill cells (G1b) with both pcDNA3.1-IAg52a and pcDNA3.1-IAg52b, antigens IAG52A and IAG52B were detected. During the vaccination trial, 76-fold increase in the Iag52b gene expression was observed in the vaccinated fish group h4 post vaccination. Administration of DNA vaccines by IM injection induced significant gene up-regulation in the head kidney, including immunoglobulin M (IgM), cluster of differentiation 4 (CD4), major histocompatibility I (MHC I), and T cell receptor α (TcR-α) from h4 to d5 post immunization. Fish vaccinated with DNA vaccines or theronts showed increased gene expression of the cytokine interferon (IFN-γ), complement component 3 (C3), and toll-like receptor-1 (TLR-1). Anti-Ich antibodies were detected in fish received pcDNA3.1-IAg52a, pcDNA3.1-IAg52b and the combination of both vaccines d10 post vaccination. Fish vaccinated with pcDNA3.1-IAg52b showed mild parasite infection level, partial survival (20%) and longer mean day to death (MDD) after theront challenge. By contrast, a heavy parasite load, 0% survival and short MDD were observed in the sham vaccinated control fish that received pcDNA3.1 (plasmid without genes encoding Ich immobilization antigen). Further research is needed to improve DNA vaccines for Ich that can induce strong protective immunity in fish. Suggested studies include improved transfection efficiency, use of appropriate adjuvants and including additional parasite antigen genes in the plasmid.

1. Introduction

The parasite *Ichthyophthirius multifiliis* (Ich) is a severe fish parasite that infects almost all freshwater fish during every growth stage [1,2]. Ichthyophthiriasis causes high mortalities in fishes worldwide and leads to heavy economic loss in aquaculture [3,4]. The life cycle of the parasite includes 3 stages: an infective theront, a parasitic trophont and a reproductive tomont [5].

Currently, the control of Ich parasitism depends largely on the use of chemicals. However, chemotherapy of ichthyophthiriasis has several disadvantages, including poor effectiveness once the parasite penetrates the skin and gill of fish, cost to fish farmers, and concerns for food and environmental safety [6]. Prevention by vaccination is an alternative approach to combat parasitic diseases and has a paramount importance for sustainable aquaculture. Previous studies have demonstrated that channel catfish [7,8], carp [9], tilapia [10], trout [11] and other freshwater fish [12] are able to acquire protection against Ich after immunization with Ich antigens. Antigens from Ich include live theronts, freeze-thawed or fixed theronts and trophonts, subunit components of the parasite such as cilia membrane proteins and purified protein antigens [3,4]. The most promising antigens for immunization are the immobilization antigens (i-antigens), and research has demonstrated that antibodies specific to i-antigens are important for protective immunity [13,14].

To date, the most effective method to immunize fish against Ich is by exposing fish to light infestations or by IP injection using live theronts [3,4]. However, this approach is not practical due to the need for large-scale *in vitro* culture of Ich that is not possible, thus rendering the ability to produce parasite antigen, a significant obstacle for vaccine development [3,4,11,15]. The development of a practical vaccine likely

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https://doi.org/10.1016/j.fsi.2019.08.071
Received 16 July 2019; Accepted 27 August 2019
Available online 28 August 2019
1050-4648/ Published by Elsevier Ltd.

will require the expression of recombinant i-antigens in cells that can be grown in culture. Transgene expression of i-antigen genes imposes a challenge in heterologous systems such as bacteria because Ich uses a nonstandard genetic code [13]. This problem has been addressed by synthesizing and cloning i-antigen gene constructs with codons that are not rejected in *Escherichia coli* [16], or by expressing them in *Tetrahymena thermophila* [17]. Over the past two decades, studies have been conducted to develop antigen–encoding plasmid DNA vaccines. The DNA vaccine has several attractive features: easy to construct and produce, relatively inexpensive, identical production processes for different vaccines, able to produce multivalent vaccines and safe to use as they do not contain pathogenic microorganisms [15,18–20]. DNA vaccines were shown to induce both cellular and humoral immunity [15]. DNA vaccines have been developed and commercialized for protection against fish viral diseases, such as Infectious Hematopoietic Necrosis (IHNV) in Atlantic salmon [18,20]. Research on the development of DNA vaccines against Ich has been conducted in several fish species. Experimental DNA vaccines encoding i-antigens, and a cysteine protease from Ich were tested in rainbow trout (*Oncorhynchus mykiss*) and resulted in an elevation of anti-*I. multifiliis* specific antibody response but did not result in satisfactory protection [15]. An earlier study in channel catfish indicated that immunization with a DNA vaccine encoding synthetic i-antigen genes of Ich induced a specific antibody response [16]; however, this study did not evaluate potential protection conferred by the DNA vaccine. No commercial vaccine is currently available to prevent parasite infections of fish, including Ich. The objective of this study was to evaluate the immune response of channel catfish following vaccination with plasmid DNA vaccines against *I. multifiliis* by determining parasite infection level, measuring serum anti-Ich antibodies, monitoring fish mortality after theront challenge, and evaluating expression of immune-related genes.

2. Materials and methods

2.1. Fish

Channel catfish (*Ictalurus punctatus*) were obtained from stock fish that were reared to experimental size in indoor tanks at the USDA, Aquatic Animal Health Research Unit, Auburn, AL. All fish treatment protocols were approved by Institutional Animal Care and Use Committee at the Aquatic Animal Health Research Unit prior to the trials.

2.2. Parasite and serotype determination

Ichthyophthirius multifiliis (ARS-17) was originally isolated from an infected channel catfish and maintained by serial transmission on catfish held in 37-l glass aquaria as described previously [10]. The ARS-17 isolate was identified as serotype D by theront immobilization assay using specific murine monoclonal antibody G3-61 (provided by Dr. Harry W. Dickerson, University of Georgia) and confirmed by PCR using two specific primer pairs (Table 1) targeting sequences of i-antigen genes IAG52A (GenBank AF324424, bases 1552–2200) and IAG52B (GenBank AF405431, bases 444–918) [16]. The PCR reaction was conducted in 15 μl volume contained 7. 5 μl PCR master mix (Promega, Madison, WI), 0.5 μl of each primer (10 μM), 1 μl of Ich genomic DNA (5 ng/μl) and 5.5 μl of water. The amplification was performed with the following cycling parameters: initiation denaturation at 94 °C for 2 min, followed by 30 cycles of 94 °C for 30s, 54 °C for 1 min, 72 °C for 1 min, and a final extension at 72 °C for 10 min. PCR products were electrophoresed in a 1.5% agarose gel containing SYBR safe DNA gel stain (Invitrogen, Carlsbad, CA) and visualized using Gel Doc™ XR^+ Molecular Imager (BioRad, Hercules, CA). The gel image of the PCR products showed both PCR amplicons had single band with expected sizes for IAG52A and IAG52B (Fig. 1A).

2.3. Water quality

Dissolved oxygen (DO) and temperature were measured using an YSI 85 oxygen meter (Yellow Springs Instrument, Yellow Springs, OH). The pH, hardness, ammonia and nitrite were determined using Hach CEL/890 Advanced Portable Laboratory (Loveland, CO). During the trial, a light: dark period of 12:12 h was maintained and aeration was supplied by air stones. Water quality was monitored throughout the trial to maintain the following parameters: dissolved oxygen 5.3 ± 0.1 mg l^{-1}, pH 7.3 ± 0.1, ammonia 0.15 ± 0.1 mg l^{-1}, hardness 102 ± 7.9 mg l^{-1}, nitrite concentration below detectable level and water temperature 27.5 ± 0.6 °C.

2.4. Theront preparation and parasite challenge

Theronts were cultured and harvested for vaccination (positive control) or fish challenge using published procedures [10]. Briefly, channel catfish heavily infected with mature trophonts were euthanized with 300 mg l^{-1} buffered tricaine methane sulfonate (MS 222), rinsed with water and the skin was gently scraped to dislodge the parasites. Trophonts were harvested, placed in Petri dishes and incubated for 18–24 h at 22–24 °C for producing infective theronts. For intraperitoneal (IP) injection, theronts were harvested by pouring through a sieve with a pore size of 22 μm, concentrated by centrifugation at 100 × *g* for 3 min and suspended in 50% phosphate-buffered saline (PBS, pH 7.2). For Ich challenge, theront numbers were determined using a Sedgewick-Rafter counting chamber.

2.5. Construction of DNA vaccines

Gene sequences encoding 52 kDa immobilization antigens, IAG52A (GenBank AF324424, bases 1330–2737) and IAG52B (GenBank AF405431, bases 71–1454) [16] were modified by replacing the protozoan stop codons UAA and UAG with standard codons CAA and CAG. This change eliminated unwanted stop codons and substituted the preferred codons for expression of i-antigen genes in *E. coli* or fish. The modified gene sequences were sent to GenScript (Piscataway, NJ, USA) for gene synthesis. Gene codons were further optimized for increased protein expression in channel catfish using GenScript's Optimum Gene algorithm. The synthesized genes were then cloned into the vector pcDNA3.1(+)myc-His A between restriction sites EcoRI and Xhol by GenScript. The recombinant vectors were transformed into NovaBlue competent *E. coli* cells (Novagen, Madison, WI), and positive transformants pcDNA3.1-IAg52a and pcDNA3.1-IAg52b were grown in LB broth containing ampicillin. The vector pcDNA3.1 without parasite gene insert served as negative control. Plasmid DNA (pDNA) was isolated from overnight cultures using EndoFree Plasmid Mega Kit (Qiagen) following manufacturer's instructions.

2.6. In vitro transfection of pcDNA3.1-IAg52a(b) in walking catfish gill cells

Lipofectamine 3000 (Thermo Fisher Scientific) was used and transfection conditions were optimized in walking catfish gill cells (G1B, ATCC® CRL-2536) following the manufacturer's instructions. To confirm the desired protein expression *in vitro*, the G1B cells were grown in F–12K medium (Thermo Fisher Scientific, Waltham, MA) in 24-well plate until 70–90% confluent. Transfection of pcDNA3.1-IAg52a, pcDNA3.1-IAg52b or pcDNA3.1 (plasmid without genes encoding Ich immobilization antigen) was performed in monolayer of G1B cells using Lipofectamine 3000 transfection reagent at ratio of 1 μg DNA: 1.5 μl lipofectamine 3000 for 4 h, followed by incubation in F–12K medium. After 24 h, the cells were fixed with 70% ethanol for 15 min at room temperature, washed with PBS, and then blocked using PBS containing 1% bovine serum albumin (BSA) for 30 min at room temperature. Channel catfish serum antibody against Ich (serotype D)

Table 1
Primers and probes used in PCR and qPCR for detecting Ich antigen genes, evaluating expression of immune related genes or determining plasmid vaccine DNA in tissues of channel catfish.

Gene	GenBank accession #	Amplicon size(bp)	Primer code/sequence (5′–3′)
IgM	M27230	142	F/CTGTGCAAAGCGCCCCGAAATC R/CTCCCGCTCGCATCCTTCCATAT
CD4	DQ435302	178	F/CCTCTGCGAACCCATCTTCA R/AGGCAGGTCCAGATTCTTGC
MHC class I (MHC I)	AY008848	117	F/CAGAGGTGGTGTGTCATGCTA R/CCTGGTTGGGTAACGTCTCTC
T cell receptor alpha (TCR-α)	JN097591	79	F/AGAAACGGGAGCCGTCAATTT R/TTGTGAACTGTAAAGCGCGTG
Inteferon (IFN-γ)	BM494266	179	F/CAGCAGTGACTTCAGCCAAA R/GCCTCAGAGTACGCCATCAT
Interleukin1 beta type a (IL-1)	EE993591	219	F/CAGCAGTGACTTCAGCCAAA R/GCCTCAGAGTACGCCATCAT
Complement factor 3 (C3)	BM438508	196	F/AGTTGAATACCGCTGCCAAC R/CTCTCCATGCGCTGAGTACA
Toll-like receptor 1 (TLR-1)	HQ677713	109	F/AGCCAAAGAAATGCCAACTG R/TGAAGTCTCGTTCGTGGTGA
18S rRNA (18s)	AF021880	128	F/GAGAAACGGCTACCACATCC R/GATACGCTCATTCCGATTACAG
i-antigen IAG52A (IAG52A)	AF324424	648	F/GCTGGTGCTTAACCAAATCC R/TTTATTTGCTGGGCAAGGTA
i-antigen IAG52B (IAG52B)	AF405431	474	F/GTAATCCTACAGGTCAGG R/TAGCCTCAAGACCTGATC
pcDNA3.1-IAg52a *(IAg52a)	AF324424	115	F/ACCTGTACACCCTGCCCTCA R/CGGCGATAGCTGTTCCAGCA
pcDNA3.1-IAg52b * (IAg52b)	AF405431	105	F/CCTCTGCCCAGAACCCTGGA R/AGTTTGGGCCAGGCACCATT **P/AGCTGATCGCCAACCCAGACGCAGCT

* Genes encoding Ich antigens were modified and optimized for channel catfish from AF324424 and AF405431.
** FAM-labeled fluorescent probe with 5′ reporter 6FAM–3′ quencher BHQ1.

was diluted (1:50) in PBS, directly added to the fixed G1B cells and kept overnight at 4 °C. The cells were washed with PBS and incubated with mouse anti-catfish immunoglobulin heavy chain monoclonal antibody [21] at 1:100 dilution in PBS for 60 min at room temperature. The cells were then incubated with goat anti-mouse IgG antibody FITC conjugate (Sigma) at 1:1000 dilution for 60 min. After washing with PBS, cells were observed under an Olympus IX70 fluorescence microscope.

2.7. Vaccination and tissue sampling in the vaccine trial

Channel catfish with an average weight of 23.1 ± 3.9 g and length of 15.1 ± 1.0 cm were used in the vaccine trial. Ten catfish were sampled prior to the trial. All fish were negative for Ich infection (*i.e.* no visible attached theronts under microscopy) and no anti-Ich antibody was detected in fish serum prior to the study. Catfish were acclimated for 14 d in flowing dechlorinated municipal water at 24 ± 2 °C prior to study. Fish were then distributed into groups with 27 fish tank^{-1}, 3 tanks group^{-1} and exposed to followings: (1) pcDNA3.1-IAg52a at 10 μg fish^{-1}, (2) pcDNA3.1-IAg52 b at 10 μg fish^{-1}, (3) pcDNA3.1-IAg52a + pcDNA3.1-IAg52 b at 5 μg each fish^{-1} (pcDNA3.1-IAg52ab), (4) live theronts at 15,000 theronts fish^{-1} (positive control) and (5) pcDNA3.1 at 10 μg fish^{-1} (mock-immune control). The DNA vaccines were mixed with lipofectamine 3000, incubated at room temperature for 30 min and adjusted to the desired concentration (100 μg pDNA ml^{-1}) for fish vaccination. Each fish in groups 1–3 and 5–6 was anesthetized with 100 mg L^{-1} buffered MS-222 and then injected with

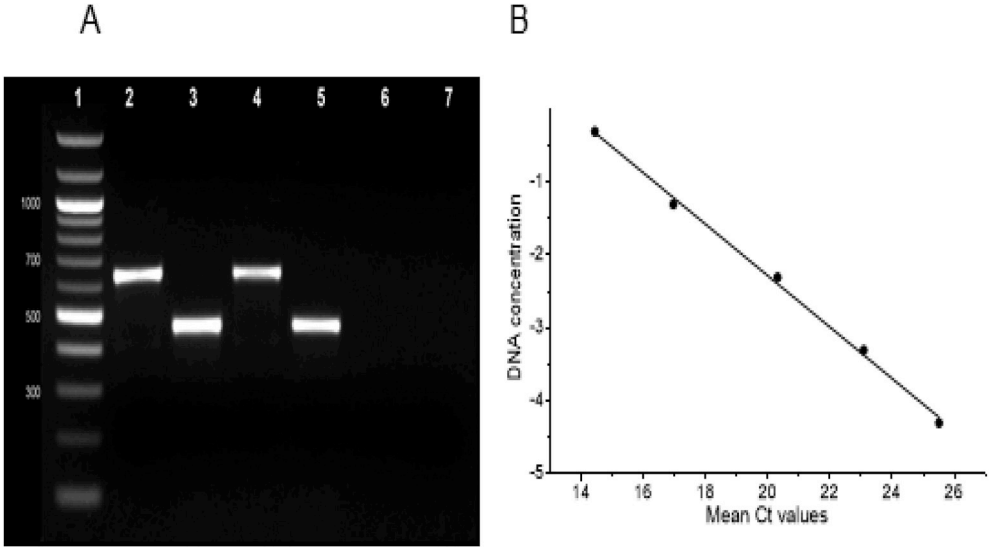

Fig. 1. (A)PCR identification of ARS-17 isolate as *Ichthyophthirius multifiliis* serotype D using two specific primer pairs targeting sequences of i-antigen genes IAg52a and IAg52b: DNA ladder (lane 1), IAg52a (lane 2) and IAg52b (lane 3) of ARS-17 theronts, IAg52a (lane 4) and IAg52b (lane 5) of ARS-17 trophonts, ARS-17 theront DNA without primers (lane 6) and ARS-17 trophont DNA without primers (lane 7). The PCR products are 648 bp for IAg52a and 474 bp for IAg52b. Fig. 1B. Standard curve for quantifying plasmid DNA IAg52b in channel catfish muscle. Mean Ct values (Ct) are plotted against log10 concentrations of plasmid DNA (Y), Y = 4.6222–0.3432Ct (R^2 = 0.9915). Concentrations of plasmid DNA range from 0.5 ng μl^{-1} to 50 fg μl^{-1} in standard curves, a 10-fold serial dilution.

100 μl of DNA vaccines by intramuscular injection (each fish received 10 μg of DNA vaccine). Fish in group 4 were IP injected with 15,000 live theronts per fish as positive control. Group 6 was a mock-immune (pcDNA3.1 at 10 μg fish^{-1}) and unchallenged control in a single tank with 15 fish.

Six fish (two fish per tank) were sampled prior to the vaccination to serve as a non-vaccination control (h0). At h4, d1, d2, d5, d10 and d20 post vaccination, two fish were randomly sampled from each tank. Blood serum was collected to measure antibody against Ich using an enzyme-linked immunosorbent assay (ELISA). The gill, liver, trunk kidney and muscle near the injection site were collected and frozen at −80 °C until extraction of gDNA to quantify vaccine DNA in tissues. The head kidney was collected and immediately immersed in RNAlater (Invitrogen, Carlsbad, CA) for evaluating immune-related gene expression. All head kidney samples were kept at 4 °C overnight and then transferred to – 20 °C until RNA extraction.

2.8. Anti-Ich antibodies in serum of channel catfish

Serum anti-Ich antibodies were measured by ELISA as described previously with modification [21]. An optical density (OD) reading in a sample from vaccinated fish was considered positive when its value was 1.5-fold greater than the OD readings of the control fish. The ELISA titer was the inverse of the highest dilution of serum at which a sample was positive.

2.9. Challenge and parasite infection level

At d21 post vaccination, 15 fish in each tank were challenged by live Ich theronts [10]. Water in each tank was reduced to approximately 10 L and theronts were added to each tank at a dose of 20,000 theronts fish^{-1}. The fish were exposed to theronts for 1h and water flow was resumed in each tank at 0.5 L per min. The mock-immune fish in group 6 were mock-challenged without any theronts. Fish were monitored for Ich infection and mortality for 3 weeks after theront challenge. Five days post theront challenge, the parasite infection levels in each tank were evaluated using a semi-quantitative scale [10]: no infection (0 trophonts fish^{-1}), light infection (< 50 trophonts fish^{-1}), mild infection (50–150 trophonts fish^{-1}) and heavy infection (> 150 trophonts fish^{-1}).

2.10. gDNA, RNA extraction and cDNA synthesis

Fish tissues (gill, liver, head kidney, muscle and trunk kidney) used to quantify vaccine DNA with qPCR were weighed (approximately 20 mg) and macerated with sterilized Kontes disposable pestles in a microcentrifuge tube. Total gDNA in fish tissues was extracted by the DNeasy Tissue kit following the manufacturer's instructions (Qiagen, Valencia, CA, USA) and eluted with a volume of water equal to 10 μl water per mg tissue.

Total RNA from head kidney was extracted using RNeasy Plus Universal Kit (Qiagen) following the manufacturer's protocol. The isolated RNA samples were treated with gDNA eliminator solution to minimize contamination of genomic DNA. Quantity and quality of RNA samples were determined using Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Rockland, DE) and by electrophoresis on 1.0% agarose gel. The first strand cDNAs used for quantitative PCR were synthesized using 2 μg of total RNA, AMV reverse transcriptase, and Oligo-dT primer provided by the cloned AMV first strand cDNA synthesis kit (Invitrogen).

2.11. Primers and quantitative PCR for immune related genes

Eight immune related genes were selected in this study based on published reports of immune genes induced by parasite infestation, including immunoglobulin, immune cell receptor, cytokine, complement factor and toll-like receptor [15,22,23] genes. The gene-specific primers were designed with the aid of Primer3 (http://biotools.umassmed.edu/bioapps/primer3_www.cgi) and are listed in Table 1. Channel catfish 18S ribosomal RNA gene [23] was used as an internal calibrator to normalize cDNA quantities. Primers for immune genes were synthesized by Sigma-Aldrich (St. Louis, MO). Quantitative PCR (qPCR) was performed in triplicate for each cDNA sample in MicroAmp® Optical 96-well reaction plates (Applied Biosystems, Foster City, CA) using Applied Biosystems (ABI)7500 Real-Time PCR System. All qPCR reactions were performed using Platinum® SYBR® Green qPCR SuperMix-UDG with ROX (Invitrogen) in a total volume of 12 μl. The qPCR mixture consisted of 0.5 μl of cDNA (approximately equivalent to 5 ng of RNA), 1 μl of 5 μM gene-specific forward primer, 1 μl of 5 μM gene-specific reverse primer, 6 μl of 2 × SYBR Green SuperMix and 3.5 μl of DEPC-treated water. The qPCR thermal cycling parameters were 50 °C for 2 min, 95 °C for 2 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min.

2.12. Distribution and transcripts of pcDNA3.1-IAg52b in fish tissues

A TaqMan-based qPCR was used to quantify pcDNA3.1-IAg52b in fish tissues. Specific primers and FAM-labeled fluorescent probe were synthesized and targeted the inserted sequence encoding IAg52b (Table 1). The expected product size was 105 bp. The qPCR mixture in a volume of 12 μl consisted of 6 μl of Platinum® Quantitative PCR SuperMix-UDG (Invitrogen, Carlsbad, CA), 1 μl of gDNA from tissue samples, 0.5 μl of 5 μM forward primer, 0.5 μl of 5 μM reverse primer, 0.25 μl FAM-labeled probe and 3.75 μl DEPC-treated water. The qPCR was performed on an Applied Biosystems 7500 Real-Time qPCR station (ABI, Foster City, CA) using following amplification conditions: 50 °C for 2 min, 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Extracted DNA from fish tissues (1 μl) were used as templates in qPCR and the concentration of pDNA in fish tissue was determined via the standard curves. A standard curve was included in each qPCR run using 10-fold serial diluted plasmid pcDNA3.1-IAg52b DNA from 50 fg μl $^{-1}$ to 0.5 ng μl $^{-1}$ as templates. The threshold cycle (Ct) values (x-axis) were plotted against the $\log_{10}$ pDNA concentrations (y-axis). The standard curve revealed a linear correlation between log concentration (con) of pDNA and Ct values (Con = 4.6222–0.3432Ct) with R^2 = 0.9915 (Fig. 1B). The Ct obtained from individual fish tissue samples was interpolated from the standard curve and converted to quantity of pDNA. Since 1 μl of eluted sample was run in qPCR, the concentration of pDNA in each gram of tissue (ng g^{-1}) was equal to pDNA concentration (ng μl $^{-1}$) × eluted volume × 1000/tissue weight (mg). The plasmid DNA at a concentration of 0.0005 ng μl $^{-1}$ was used as endogenous reference for all plates to normalize Ct values [24]. For each plate, a normalized reference (nRef) was calculated using nRef = Ct (0.0005) - mean Ct (0.0005). Normalized Ct (nCt) was then determined for each Ct value using nCt = Ct – nRef. To evaluate the transcripts of pcDNA3.1-IAg52b in fish tissues, qPCR was performed as described in Section 2.11 using cDNA as templates and specific primers and FAM-labeled probes for IAg52b (Table 1).

2.13. Data analysis

The relative transcriptional levels of immune genes were determined by subtracting the cycle threshold (Ct) of the sample by that of the 18S ribosomal RNA, the calibrator, using the formula: ΔCt = Ct (sample) - Ct (calibrator). The relative expression level of specific genes in the immunized fish at certain time point (x) post vaccination was calculated by the formula of $2^{\Delta\Delta Ct}$ [25], where ΔΔCt = ΔCt (time point 0) – ΔCt (time point x). All data were analyzed with SAS software [26]. Mean day to death (MDD) was calculated by the SAS Lifetest procedure (Kaplan-Meier method) post challenge. Serum antibody titers from different immunized groups were analyzed with Duncan's multiple range test. The correlation between concentration and expression of

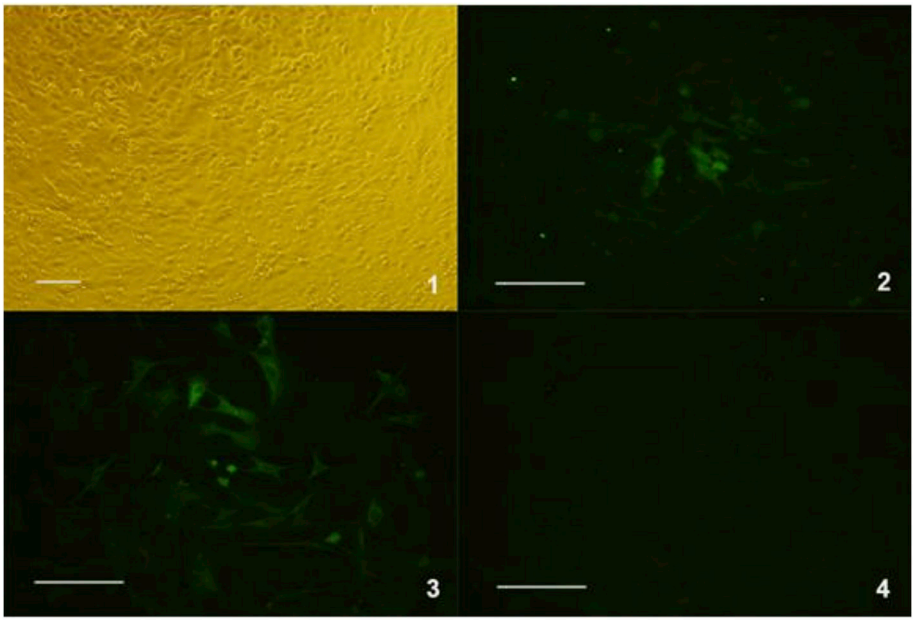

Fig. 2. Expression of i-antigens (IAG) in transfected walking catfish gill (G1b) cells: (1) G1b cell culture, (2) IAG52A positive G1b cells transfected with pcDNA3.1-IAg52a, (3) IAG52B positive G1b cells transfected with pcDNA3.1-IAg52b, and (4) IAG negative G1b cells transfected with pcDNA3.1 vector containing no insert. Immunostaining was conducted using channel catfish serum antibody against Ich (serotype D) as primary antibody, incubated with mouse anti-catfish immunoglobulin heavy chain monoclonal antibody and then goat anti-mouse IgG antibody FITC conjugate. The cells were observed under an Olympus IX70 fluorescence microscope. Scale bar = 100 μM.

vaccine DNA in fish tissue was determined using Pearson correlation. Probabilities of 0.05 or less were considered statistically significant.

3. Results

3.1. Ich antigen expression in transfected fish cells

The IAG52A and IAG52B i-antigens were detected in transfected G1b cells with catfish anti-Ich serum. The positive transfected cells showed bright green fluorescence (Fig. 2), indicating IAG52A and IAG52B i-antigen proteins were expressed in G1b cells. No green fluorescence was observed in G1b cells transfected with pcDNA3.1 vector (Fig. 2).

3.2. Serum antibody against Ich in vaccinated fish

No anti-Ich antibodies were detected in serum of fish sampled prior to vaccination or in mock-immune control fish received pcDNA3.1. Anti-Ich antibodies were detected in fish that received pcDNA3.1-IAg52a, pcDNA3.1-IAg52b and the combination of pcDNA3.1-IAg52a and pcDNA3.1-IAg52 b at d5-d20 post vaccination (Fig. 3). The fish immunized with DNA vaccines showed the highest anti-Ich antibodies at d10 post vaccination. Anti-Ich antibodies were detected in fish vaccinated with live theronts 10 days post vaccination and then increased greatly until d20 post vaccination. Fish vaccinated with live theronts showed much higher levels of serum anti-Ich antibodies than fish vaccinated with DNA vaccines.

3.3. Parasite infection and fish protection

After challenge with Ich theronts at 3 weeks post vaccination, fish that received vaccines showed different parasite infection levels, survival rate and MDD (Table 2). Fish showed mild infection with 51–150 spots $fish^{-1}$ for those vaccinated with pcDNA3.1-IAg52a, pcDNA3.1-IAg52b, or a mixture of both vaccines. Fish sham vaccinated with pcDNA3.1 showed heavy infection with more than 150 spots per fish at d5 post challenge.

All fish vaccinated with DNA vaccines died except fish that received pcDNA3.1-IAg52b. There was 20% fish survival in the group that received pcDNA3.1-IAg52b and MDD was longer in this group as compared to fish vaccinated with the other DNA vaccines (Table 2). MDDs were significantly shorter in the sham vaccinated control (pcDNA3.1 only) than fish vaccinated with any of the pDNA vaccines (pcDNA3.1-IAg52a, pcDNA3.1-IAg52b or pcDNA3.1-IAg52ab). Fish showed heavy infection, 0% survival and a short MDD in the sham vaccinated control. All fish vaccinated with live Ich theronts survived the Ich challenge and displayed a light Ich infection ($\leq$50 spots $fish^{-1}$).

3.4. Distribution and expression of vaccine DNA in fish tissues

No pcDNA3.1-IAg52b were detected in tissues of fish prior to vaccination (h0) or sham vaccinated fish (with pcDNA3.1). Vaccine pDNA was detected in the muscle of fish vaccinated with pcDNA3.1-IAg52b from h4 to d20 post vaccination (Fig. 4). Approximately 275 ng pDNA were detected per gram of muscle from vaccinated fish h4 post vaccination (Fig. 4). The fish maintained high pDNA (13.0–275 ng g^{-1}) levels in muscle from h4 to d2. Then pDNAs were significantly reduced to 0.9 ng g^{-1} at d5 and dropped to 0.1 ng g^{-1} at d10-d20 post vaccination. pDNA was also detected in gill, trunk kidney and head kidney (Fig. 5) but showed lower concentration than muscle. The distribution patterns of pDNA in gill, truck kidney and head kidney were similar to that in muscle. Lower vaccine DNA was detected in liver compared to other tissues (Fig. 4). qPCR also confirmed the presence of pcDNA3.1-IAg52a in muscle, gill and kidney of fish vaccinated with pcDNA3.1-IAg52a. The distribution of pcDNA3.1-IAg52a in tissues was similar with fish received pcDNA3.1-IAg52b (data not shown).

Fish vaccinated with the DNA vaccine exhibited rapid increase in the concentration of pcDNA3.1-IAg52b in head kidney from h4 post vaccination (Fig. 5). The vaccinated fish showed 0.54–1.51 ng of pcDNA3.1-Iag52 b g^{-1} in head kidney from d1 to d5 post vaccination, which was significantly higher than that prior to vaccination (h0). There was a positive correlation between pcDNA3.1-IAg52b concentration and transcript level of IAg52b gene in head kidney (coefficient of correlation 0.8648, $P < 0.01$). The IAg52b transcripts were increased 76-fold h4 post vaccination and then kept at upregulation level from d1 to d20 post vaccination in the vaccinated fish comparing to those before vaccination (Fig. 5).

3.5. Expression of immunoglobulin and immune cell receptor genes

Administration of DNA vaccines by IM injection induced significant IgM gene expression in the head kidney from h4 to d5 (Fig. 6). Fish vaccinated with pcDNA3.1-IAg52b and pcDNA3.1-IAg52ab showed similar pattern of IgM expression as fish vaccinated with live theronts by

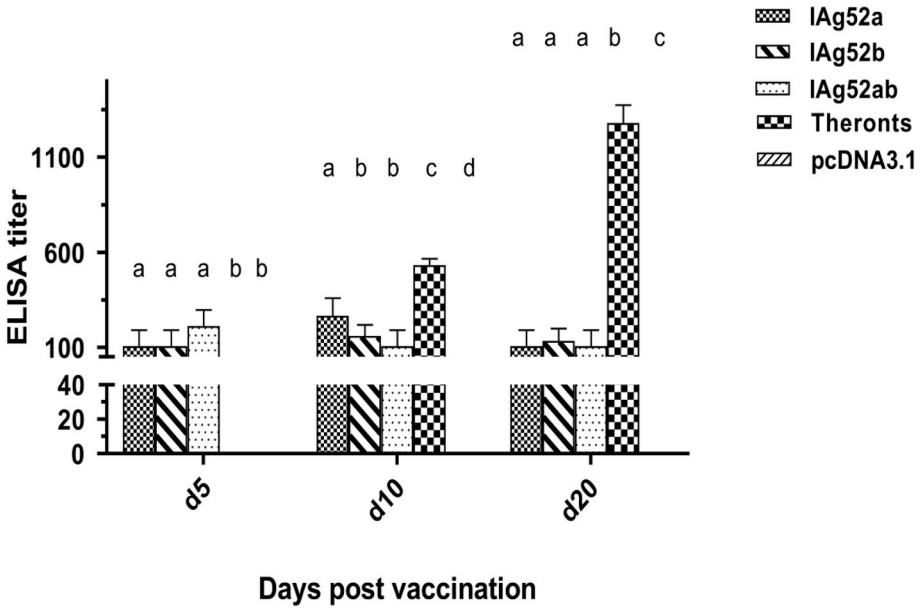

Fig. 3. Antibody titers of enzyme-linked immunosorbent assay (ELISA) against *Ichthyophthirius multifiliis* in fish serum at each time point post vaccination. Within a given time, means (± SD) with different superscript letters on the top of columns are statistically different ($P < 0.05$). IAg52a: fish injected with 10 μg pcDNA3.1-IAg52a fish $^{-1}$; IAg52b: 10 μg pcDNA3.1-IAg52b fish $^{-1}$; IAg52ab: 5 μg pcDNA3.1-IAg52a and 5 μg pcDNA3.1-IAg52b fish $^{-1}$; Therons: live therons at 15,000 therons fish $^{-1}$; pcDNA3.1: mock-immune control.

IP injection. The IgM gene showed an up-regulation in the fish vaccinated with pcDNA3.1-IAg52b, pcDNA3.1-IAg52ab and live therons from h4 to d20 post immunization comparing to fish prior to vaccination (Fig. 6).

The CD4, MHC I, and TcR-α genes showed up-regulation in all fish vaccinated with DNA vaccines or live therons from h4 to d10 post immunization (Fig. 6). Among fish that received DNA vaccines, fish vaccinated with pcDNA3.1-IAg52b increased expression of immune cell receptor genes rapidly at h4 post immunization by 15-fold or higher and then remained up-regulated throughout the trial compared with fish prior to vaccination (Fig. 6). The expressions of CD4, MHC I, and TcR-α genes were similar in fish vaccinated with pcDNA3.1-IAg52b to those of fish vaccinated with live Ich therons.

3.6. Expression of cytokine, complement factor and toll-like receptor genes

Fish vaccinated with DNA vaccines and therons increased expression of the IFN-γ gene rapidly at h4 post immunization by 5-fold or higher and then remained up-regulated until d10 post immunization compared with fish prior to vaccination (Fig. 7). The expression of the IL-1 gene was very different between fish vaccinated with pcDNA3.1-IAg52b and fish vaccinated with live Ich therons. Cytokine IL-1 gene expression rapidly increased 11-fold in pcDNA3.1-IAg52b vaccinated fish at h4 post immunization and then decreased at other sampling timepoints. Fish vaccinated with live Ich therons, however, showed high expression of cytokine IL-1 gene from h4 to d10 post immunization.

The complement component 3 (C3) gene exhibited a rapid increase to 20-fold in fish immunized with pcDNA3.1-IAg52b or live therons at h4 post immunization compared to prior vaccination (Fig. 7). The expression level maintained at least 2-fold higher than prior vaccination until d10 post immunization. The expression of toll-like receptor (TLR) gene increased rapidly and peaked (14-fold) at h4 post immunization in fish immunized with pcDNA3.1-IAg52b compared with fish prior to vaccination. Like the fish vaccinated with live therons, the fish vaccinated with pcDNA3.1-IAg52b showed 2-fold or higher expression until d10 post immunization as compared to before vaccination (Fig. 7).

4. Discussion

The vaccine pDNA was mainly distributed in the muscle tissue near the injection site of the fish after vaccination. The concentration of pDNA was 20–100 fold higher in the muscle tissue than the other tissues at different sampling times. In this study, pDNA was also detected in the gill, head kidney, trunk kidney and liver after vaccination. The change in the concentration of pDNA in the gill and kidney had a pattern similar to that in muscle. The pDNA exhibited the highest concentration in h4 with a gradual reduction from d1-d5 and then dropped to a lower level d10-d20 post vaccination. Although the pDNA concentration was mainly higher near the injection site, its concentration could be comparably detected in different organs. These results indicate that the injected pDNA were able to move beyond the skeletal muscle at the injection site and reached other organs, such as gills, liver and kidney of vaccinated fish possibly through vascular system. In a rainbow trout study [15], the pDNA was detected in the muscle tissue throughout the whole trial after fish were immunized with pDNA

Table 2
Infection level and survival of vaccinated channel catfish following challenge by exposure to therons of *Ichthyophthirius multifiliis* at a dose of 20,000 therons per fish for 1 h and observed for 21 days. Forty-five fish were used for each immunization group.

Immunization groups	Infection level	Number of fish death	Survival (%)	MDD[a] (days)	25–75% MDD[b] (days)
pcDNA3.1-IAg52a	mild[c]	45	0[a]	7.0[a]	6–8
pcDNA3.1-IAg52b	mild	36	20[a]	8.2[b]	8–9
pcDNA3.1-IAg52ab	mild	45	0[a]	7.2[a]	7–8
pcDNA3.1	heavy	45	0[a]	5.7[c]	5–6
Therons	light	0	100[b]	NA	NA
Sham-challenge	no	0	100[b]	NA	NA

[a] Mean day to death (MDD) was calculated with the Lifetest procedure, SAS.
[b] Quartile estimate (lower quartile–upper quartile).
[c] No: no infection; light: light infection ≤ 50 spots fish^{-1}; mild: mild infection with 51–150 spots fish^{-1}; heavy: heavy infection > 150 spots fish^{-1}.

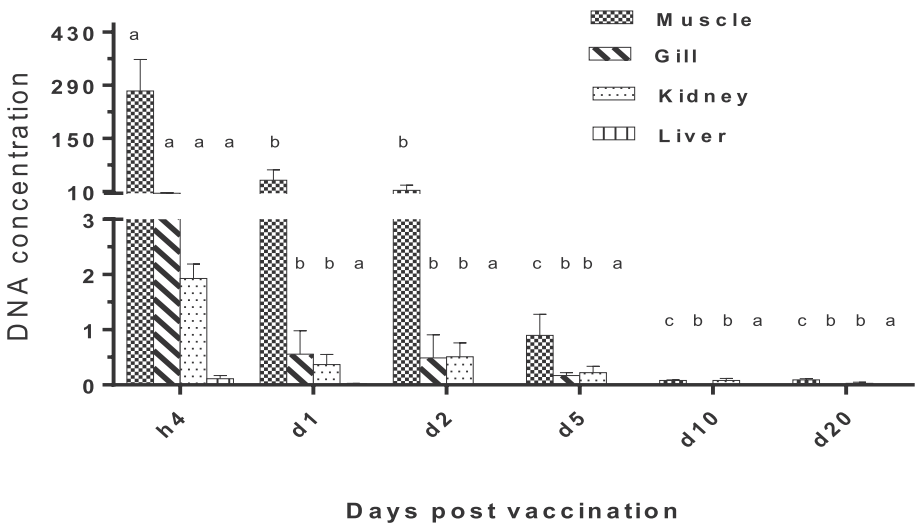

Fig. 4. Concentration of vaccine (pcDNA3.1-IAg52b) in the muscle, gill, trunk kidney and liver of channel catfish (ng g^{-1}). Fish were vaccinated by intramuscular injection with 10 μg pcDNA3.1-IAg52b per fish and tissues were sampled from 6 fish at each time point post vaccination. Within a given tissue, means with different superscript letters on the top of columns are statistically different at different sampling times ($P < 0.05$).

vaccine encoding selected antigens. Tonheim et al. [19] detected pDNA in blood of Atlantic salmon (*Salmo salar*) after IM injection and reported the circulatory system as the route for the tissue distribution of the pDNA after vaccine injection. In other fish species, pDNAs have been detected from sites such as liver, spleen, head-kidney, heart and intestine for some time post injection, with persistence mainly noted at the injection site [27]. The processes of a heterologous gene transcription and translation are carried out by fish cell's own machinery and may be influenced by a variety of factors such as pDNA concentrations, administration volumes and route of administration, age and size of the fish [27]. There was a positive correlation between pDNA concentration and transcription of IAg52b gene in this study. Concentration of pcDNA3.1-IAg52b and up-regulation of IAg52b both peaked in the head kidney h4 post vaccination. The transcript level of IAg52b then decreased following the decrease of pcDNA3.1-IAg52b concentration.

Anti-Ich antibodies increased significantly in fish vaccinated with live Ich theronts as well as those that received DNA vaccines d10 and d20 post vaccination. However, the anti-Ich antibody titer was at least 10-fold lower in fish vaccinated with DNA vaccines than fish vaccinated with live Ich theronts d20 post vaccination, which might explain the low protection in the DNA vaccinated fish. In a rainbow trout vaccination study, fish vaccinated with a mixture of plasmids encoding membrane bound i-antigen IAg52B and cysteine protease ICP2 showed significantly higher antibody than non-vaccinated control at 53 days post vaccination.

Vaccination of catfish with DNA vaccines increased the expression of several markers of the adaptive immune response, including IgM gene, cell receptor genes CD4, MHC I, and TcR-α from h4 to d10 post immunization compared to control fish. There was a similar gene expression pattern on IgM, CD4, MHC I, and TcR-α in catfish vaccinated with DNA vaccines compared to those vaccinated with live Ich theronts from h4 to d10 post vaccination. In a previous study [22], immune genes IgM, CD4, MHC I, and TcR-α were found to exhibit a rapid up-regulation in channel catfish from h4 to d6 post vaccination with live Ich theronts, indicating that different immune cells were actively involved in humoral and cellular immune responses. Sigh et al. [12] also noted a significant up-regulation of IgM and MHC genes by immunization with Ich theronts in rainbow trout. In rainbow trout vaccinated with plasmid DNA vaccines [15], IgM and TcR genes were found to be upregulated from day 32–47 post vaccination. On d20 post vaccination in this study, up-regulation of IgM and cell receptor genes was mainly seen in fish vaccinated with Ich theronts. Fish received pcDNA3.1-IAg52a showed down-regulation in adaptive immune genes at d20 post vaccination.

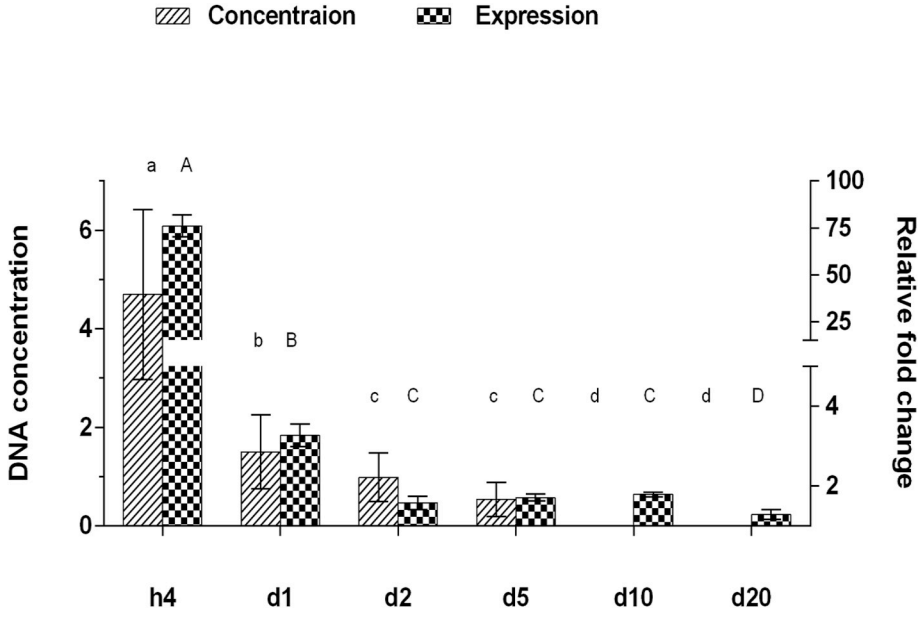

Fig. 5. Comparing concentration and expression of DNA vaccine pcDNA3.1-IAg52b in the head kidney of channel catfish. Fish were vaccinated by intramuscular injection with 10 μg pcDNA3.1-IAg52b per fish and tissues were sampled from 6 fish at each time point post vaccination. The bars at the left at each time point were plotted against left y-axis and represented concentration of pcDNA3.1-Iag52b in the tissue (ng g^{-1} tissue). Mean concentration with different lowercase letters on the top of left bars are statistically different ($P < 0.05$). The bars at the right were plotted against right y-axis, represented transcription and expressed as relative fold changes comparing to fish prior vaccination (h0). Fold changes with different uppercase letters on the top of right bars are statistically different.

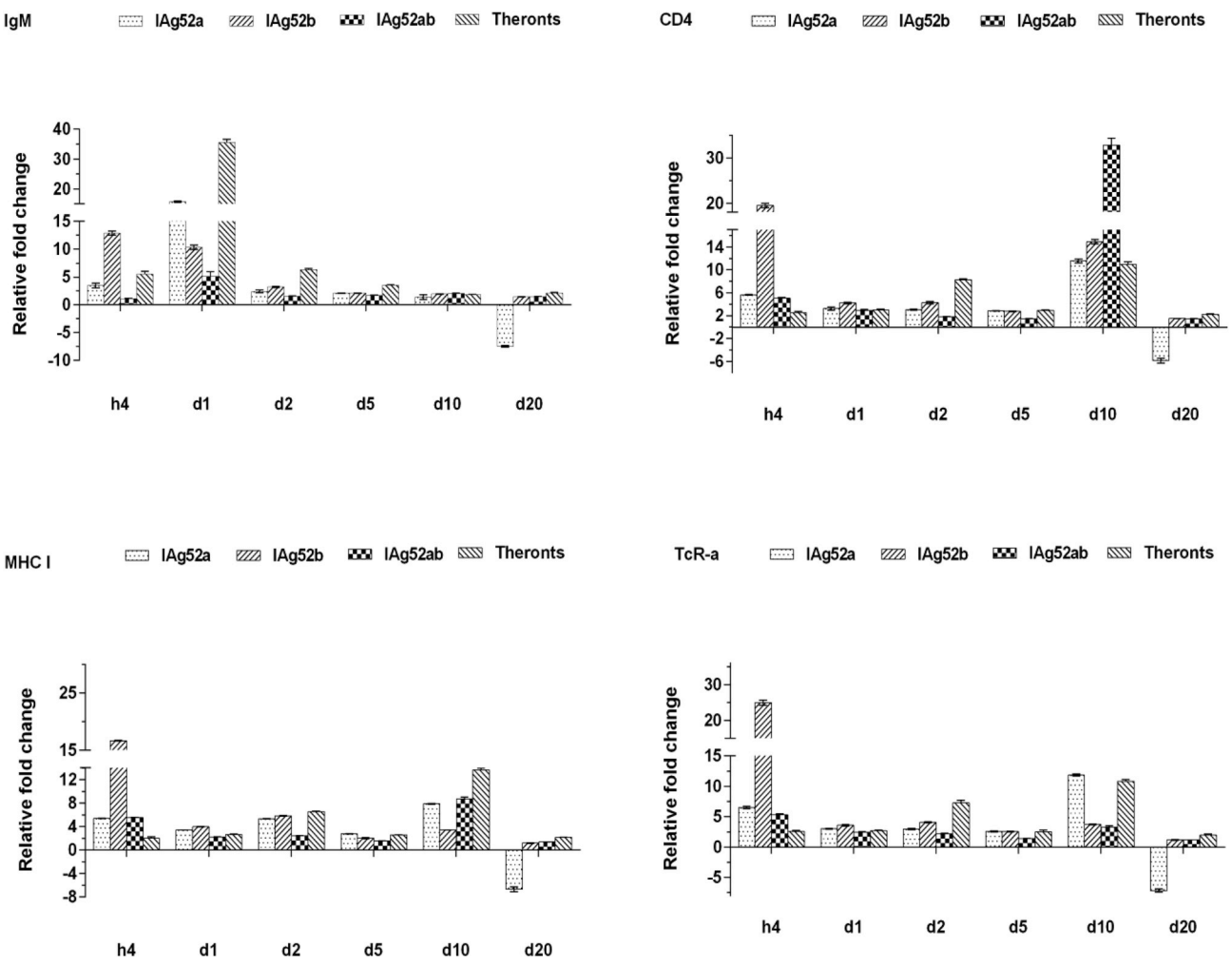

Fig. 6. Expression of immunoglobulin and immune cell receptor genes in the head kidney of channel catfish post vaccination with plasmid DNA vaccines and theronts of *Ichthyophthirius multifiliis*. Data are presented as relative fold change of genes from immunized fish at different time-points compared with fish prior vaccination (h0). IgM: immunoglobulin M, CD: cluster of differentiation, MHC: major histocompatibility complex, TcR: T cell receptor.

4.1. DNA vaccines apparently stimulated the expression of innate immune

Genes in the vaccinated catfish. In this study, genes of the cytokine IFN-γ, complement factor C3 and toll-like receptor TLR-1 were rapidly up-regulated in the head kidney from h4 and then maintained high expression levels until d10 post vaccination. Similar expression patterns of IFN-γ, C3 and TLR-1 were observed between fish vaccinated with DNA vaccines and those with live Ich theronts. However, there was a difference in interleukin-1 (IL-1) expression between fish vaccinated with DNA vaccines and fish with Ich theronts. Fish vaccinated with live Ich theronts exhibited up-regulation from h4 to d10 post vaccination while fish vaccinated with DNA vaccines mainly showed a down regulation of IL-1 expression. Interferons and interleukins are a group of small proteins that belong to cytokines produced by macrophages, lymphocytes, granulocytes, or epithelial cells [28]. Cytokines are secreted by activated immune-related cells upon induction by various antigens and modulate the balance between humoral and cellular immune responses [29]. It is not clear why channel catfish vaccinated with DNA vaccines showed low IL-1 gene expression. The difference in IL-1 expression may be attributed to antigenicity of DNA vaccines and live Ich theronts. In a study of response of rainbow trout fry to infection with *I. multifiliis*, Heinecke and Buchmann [17] also found that IL-1, IL-6, and IL-8 were up-regulated after fish were exposed to Ich theronts.

The complement system is a part of the innate immune system and helps phagocytic cells to clear pathogens from host. Complement-mediated killing occurs when complement is activated either directly by pathogen components and/or by antibody-antigen complexes [30]. Von Gersdorff Jørgensen et al. [31] found that complement factors C3, C5 and factor B were upregulated in head kidney of rainbow trout following immunization with live Ich theronts. Toll-like receptors (TLRs) play a crucial role in the activation of innate immunity by recognizing pathogen-associated molecules [32]. The expression of toll-like receptors TLR-1 and TLR-2 were observed to be significantly up-regulated in the head kidney of channel catfish after infection by Ich [33]. The results in this study were mostly in agreement with those findings in other studies [17,29–32].

Fish vaccinated with pcDNA3.1-IAg52b showed enhanced expression of adaptive and innate immune genes and higher anti-Ich antibodies compared to sham-vaccinated fish. When challenged with Ich theronts, the vaccinated fish showed partial survival with lower Ich infection levels and longer MDD than the sham-vaccinated control group. However, the vaccination with pcDNA3.1-IAg52b did not result in satisfactory protection against Ich. Further research is needed to enhance fish immune responses against Ich infection by improving transfection efficiency or using adjuvants. In this study, the DNA vaccines were mixed with lipofectamine 3000 and then the pDNA-lipid complexes were used for fish vaccination. Lipofectamine is a transfection reagent and based on the lipofection method, in which the negatively charged pDNA is trapped in a cationic lipid vacuole from the reagent to form liposome. The liposome fuses with the cell membrane

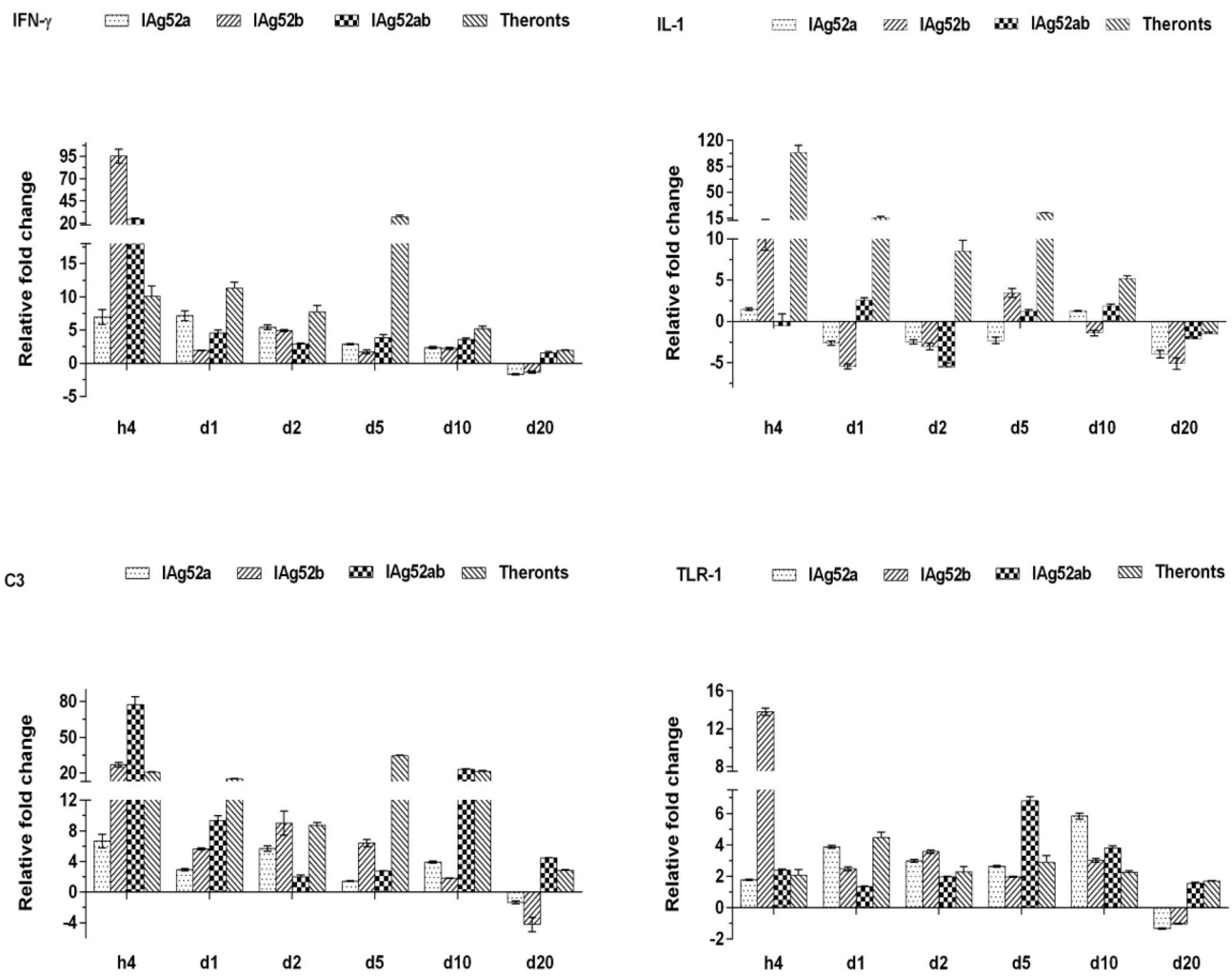

Fig. 7. Expression of cytokines (IFN-γ, IL-1), complement factor (C3) and toll-like receptor (TLR-1) genes in the head kidney of channel catfish post vaccination with plasmid DNA vaccines and theronts of *Ichthyophthirius multifiliis*. Data are presented as relative fold change of genes from immunized fish at different time-points compared with fish prior vaccination (h0). IL: interleukin, IFN: interferon, C3: complement component 3, TLR: toll-like receptor.

and thereby releases pDNA into the cell [34]. To our knowledge, most commercially available transfection reagents are optimized for mammalian cells and these reagents only lead to weak transfection efficiencies in fish cells [34]. In the present study, our transfection efficiency was only around 10% in several attempts when optimizing Lipofectamine 3000 in fish cells. Among factors influencing transfection and transgene expression, nucleic acids show poor intrinsic transfection efficiency due to their large size and negative charge [27]. Low transgene expression may fail to induce protective immune responses in fish [27] and may be partly responsible for the poor IL-1 response.

DNA vaccines were reported to possess their own adjuvant activity because of the presence of unmethylated CpG motifs in the plasmid backbone of DNA vaccines. The DNA vaccines containing CpG motifs can stimulate lymphocytes, NK cells, and antigen-presenting cells (APCs) to proliferate and/or secrete immunomodulatory cytokines, chemokines, and polyreactive IgM [35]. No attempts were made to evaluate the use of adjuvants with the DNA vaccines in this study. Improving transfection efficiency and the use of an appropriate adjuvant may help to increase the immunogenicity of the transgene antigen, thereby enhancing the immune response and increasing vaccine efficacy [35].

Fish vaccinated with live Ich theronts developed strong immune responses, such as a high level of anti-Ich antibodies, little to no parasite infection and high fish survival [8,11,32,36]. When catfish are vaccinated with live Ich theronts by IP injection, theronts can survive for several days and up to 3 weeks within the peritoneal cavity [4]. The continuation of stimulation by live theronts is one important factor for fish to develop a robust immune response. The GPI-anchored surface membrane proteins and secreted products from live theronts provide fish complete antigens and further stimulate strong immune responses. In many previous studies, fish immunized with dead theronts, trophonts, or subunit components of Ich, mainly resulted in no or partial protection [8,14,36]. Those Ich vaccines could not provide long stimulation like live theronts or may have lacked some antigens from live theronts. In contrast to live theronts, each DNA vaccine used in this study encoded only a portion of the i-antigen gene. The DNA vaccines in the current study do not contain complete antigens like live theronts so the use of DNA vaccines in the current forms may not provide long or strong immune stimulation. In a study on rainbow trout vaccinated with plasmid DNA vaccines encoding i-antigens and cysteine protease, the vaccination could not provide fish significant protection [15]. Those results suggest an insufficiency of DNA vaccination alone to trigger protective mechanisms against Ich and additional parasite antigens may be required for a successful DNA vaccine [15]. Further studies are needed to evaluate additional parasite antigens so that efficacy of DNA vaccines can be improved.

In summary, two plasmid DNA vaccines were constructed and evaluated for the immune protection of channel catfish against parasite Ich. The IAG52A and IAG52B antigens were detected in the transfected G1b cells using anti-Ich specific antibodies. The DNA vaccines were

distributed and expressed in different tissues of catfish. There was a positive correlation between pcDNA3.1-IAg52b concentration and expression of IAg52b gene, suggesting expression of IAg52b in catfish tissues. Administration of DNA vaccines by IM injection induced significant gene up-regulation in the head kidney, including IgM, CD4, MHC I, TcR-α, cytokine IFN-γ, complement component 3 (C3), and toll-like receptor (TLR) from h4 to d5 post immunization. Fish vaccinated with DNA vaccines produced anti-Ich antibodies and showed lower parasite infection than those mock-vaccinated with pcDNA3.1 after theront challenge. However, vaccination with DNA vaccines against Ich resulted in only 20% of pcDNA3.1-IAg52b vaccinated fish surviving theront challenge. Further research is needed to improve efficiencies of DNA vaccines for induction of immune protection, including improvement of transfection efficiency, employment of adjuvants and/or the addition of novel parasite antigen genes.

Acknowledgments

The authors would like to thank Dr. Harry W. Dickerson at the University of Georgia for kindly providing monoclonal antibody MAb G3–61. This research was supported by USDA/ARS CRISProject #6010-32000-024-00D. Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the US Department of Agriculture.

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