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Analysis of agglutinants elicited by antiserum of channel catfish immunized with extracellular proteins of virulent *Aeromonas hydrophila*

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ABSTRACT

Motile *Aeromonas* septicemia (MAS), caused by new virulent *Aeromonas hydrophila* (vAh) strains, has been one of the major diseases in channel catfish in recent years. Previous studies showed that channel catfish developed immunity against vAh infection after immunization with the pathogen's extracellular proteins (ECP). To understand the mechanisms associated with the immunity, anti-ECP fish serum (antiserum) was analyzed in this study. Our results revealed that the antiserum elicited agglutination of both ECP and cells of vAh. Five fish proteins were identified in ECP agglutinants, including two innate immunity associated proteins (serotransferrin and rhamnose-binding lectin), two immunoglobulin M (IgM) molecules (IgM heavy chain and light chain) and a constitutively-produced protein (warm temperature acclimation protein). More than 68 vAh proteins in ECP were recognized and caused to aggregate by IgM in the antiserum. IgM was isolated from vAh cell agglutinants and the native IgM was shown to form a tetramer that was responsible for bacterial agglutination. Immunoblotting analysis indicated that the isolated native IgM was able to recognize some proteins in ECP, such as aerolysin and hemolysin (in the form of a high molecular weight heterologous polymer). Gene expression analysis by quantitative PCR showed that fish immunized with vAh ECP had more transcripts of genes coding for IgM, serotransferrin and rhamnose binding lectin than mock-immunized fish. Both innate and antibody-mediated immune responses in serum and expressed genes contributed to fish immunity upon immunization with ECP. Results of this study shed light on the versatility of vAh antigens and catfish IgM, which would help identify specific antigens for vaccine development and antigen specific antibodies in catfish.

1. Introduction

The emergence of new virulent *Aeromonas hydrophila* (vAh) strains is of concern in aquaculture industries since the bacteria are capable of causing high mortality of warm-water fishes worldwide [1,2]. In the Southeastern United States, a severe outbreak of motile *Aeromonas* septicemia (MAS) caused by vAh was reported in 2009 in catfish farms [3]; the disease has since resulted in the loss of millions of pounds of market-size catfish annually [4,5]. The mechanism that leads to the development of MAS, however, is still largely unknown, although wounding on fish body surface was determined to be one of the important factors that initiated pathogenesis [6–8]. Prevention and control of MAS remain to be a challenging issue in aquaculture.

Prophylactic vaccination against vAh is under investigation as this strategy has been shown to be efficacious for many infectious fish diseases [9]. In laboratory trials, mutants of *A. hydrophila* attenuated by antibiotics (rifampicin and/or novobiocin) were used to immunize channel catfish and Nile tilapia [10] and common carp [11], resulting in greater than 80% protection (in terms of relative percentage of survival, RPS). Feeding of fish with *A. hydrophila* ghosts (empty bacterial cell envelopes) was shown to induce immune responses against *A. hydrophila* infection [12]. Immune protection against vAh infection was also evaluated by vaccinating fish with extracellular proteins (ECP) [13], recombinant DNA [14], recombinant virulence factors (aerolysin and hemolysin) [15], recombinant extracellular proteases [16], recombinant outer membrane proteins (OMP) [17–20] and recombinant fimbrial proteins [21]; 75–100% RPS were reported in these studies. Humoral immune responses were shown to play a significant role in these immunized fish for fighting against vAh infection. Previously, we demonstrated that serum from ECP-immunized channel catfish was able to recognize specific proteins in the vAh ECP (via immunoblotting assay) and aggregated vAh cells (causing agglutination), which mainly contributed to immune protection of fish against the establishment of pathogenesis [13]. Proteins in the anti-ECP serum merit further investigation to understand their immune properties.

In this study, we aimed to analyze and characterize agglutinants of

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ECP and cells of vAh, elicited by anti-ECP serum, to reveal the versatility of vAh antigens and catfish immunoglobulin M (IgM). Relative expression of genes coding for proteins, identified in anti-ECP serum, were also examined.

2. Materials and methods

2.1. ECP preparation

Virulent strains of *A. hydrophila* used in this study included ML10-51K, ML09-119, S14-452, S14-296, S04-690, and ZC1 [1], in which the first four were isolated from diseased channel catfish and the last one (ZC1) was isolated from grass carp. To prepare ECP, bacteria were cultured in tryptic soy broth (TSB; Bacto™, USA) at 28°C-shaking incubator for about 16 h when cell density reached approximately 3×10^9 cells mL^{-1} by optical density (OD_{600}). The bacterial cell suspension was centrifuged at 5000 × *g* for 30 min and the supernatant recovered was filtered through a 0.22 μm PES unit (33 mm Millex GP, Merck Millipore Ltd, Ireland). The filtrate was then concentrated to approximately 1000 times using a 9K-MWCO centrifugal concentrator (Pierce/Thermo Scientific, USA). The concentrated supernatant was hereafter referred to ECP. The total amount of proteins in ECP was estimated with the Coomassie Protein Assay Reagent (Thermo Scientific) using bovine serum albumin as the standard protein.

2.2. Fish immunization and vAh challenge

For fish immunization, ECP was diluted in sterile phosphate-buffered saline (PBS; Sigma) to obtain protein concentration of 20 μg mL^{-1}; equal volumes of the ECP solution and Freund's complete adjuvant (Sigma) were emulsified using previous published procedure [13]. Channel catfish, weighing 35 ± 5 g, were transferred to 50-L aquarium tanks, which were supplied with constant aeration and dechlorinated municipal water (27 ± 1 °C) at flow rate of 0.5 L min^{-1}. Fish were fed daily with Aquamax 300 (PMI Nutrition International, Brentwood, MO, USA) at approximately 3% of body weight. After two weeks' acclimation under these conditions, fish were anesthetized with tricaine methane sulfonate (100 mg L^{-1} of water) and immunized by intraperitoneal (IP) injection of 100 μl emulsified ECP. There were 60 fish immunized and equally distributed in five tanks. Another 60 fish (12 fish × 5 tanks) were subjected to mock immunization by IP injection of emulsion made with PBS and adjuvant. Three weeks after immunization, individual fish in three tanks of ECP-immunized group (36) and mock-immunized group (36) were challenged by IP injection of 100 μL TSB-cultured vAh cell suspension (approximately 2×10^8 colony-forming units per mL). Fish mortality was recorded daily for two weeks.

2.3. Serum and tissue sampling

Individual fish in the remaining two tanks of ECP-immunized (24) and mock-immunized (24) groups, were subjected to blood and head kidney collection at three weeks post immunization. Blood samples from each group were pooled and allowed to clot at 4 °C for 2 h. Sera were collected after centrifugation at 4 °C at 4500 × *g* for 20 min and stored at −80 °C until use. Sera obtained from ECP-immunized fish and mock-immunized fish were referred to anti-ECP serum and mock serum, respectively. The head kidney tissues from each group were sampled, pooled and stored in RNA*later*® Solution (Ambion, USA) until use.

2.4. RNA isolation and cDNA synthesis

Total RNA in head kidney tissues was isolated using RNeasy® Midi Kit (Qiagen, USA), including on-column DAase digestion. Purified RNA was quantified with NanoDrop ND-1000 (Wilmington, DE, USA) and stored at −80 °C until use. Synthesis of cDNA was performed using PrimeScript™ RT Reagent Kit (Clontech/Takara, USA) and the product was stored at −20 °C until use.

2.5. Bacterial cell agglutination and titration

Anti-ECP serum and mock serum samples were two-fold diluted in PBS in wells of a 96-well microtiter plate. Cells of ML10-51K cultured in tryptic soy agar (Bacto™) for approximately 24 h were suspended in PBS and centrifuged at 5000 × *g* for 10 min. The cell pellet was re-suspended in PBS and precipitated by centrifugation for two times. After re-suspension in PBS, bacterial cell density was adjusted to approximately $7.5–8.0 \times 10^8$ cells mL^{-1} by OD_{600} reading. Aliquots of 50 μl of this cell suspension were mixed into individual wells containing 50 μl of undiluted or diluted serum. The microtiter plate was then sealed by a film and kept at RT (room temperature, 23 ± 1 °C) for 3–4 h. The agglutination titer of the serum was determined by the reciprocal of the highest dilution factor that resulted in visible clumping (precipitated film) of bacterial cells. There were three replicates for each dilution.

2.6. Analysis of ECP agglutinants elicited by anti-ECP serum

An aliquot of 50 μl ECP (2 μg mL^{-1}) was mixed with 100 μl fish serum (either anti-ECP serum or mock-serum, described above) in 1 mL PBS. The mixture was kept at RT for 10–15 min (or until the solution became cloudy) and centrifuged at 10,000 × *g* for 10 min. The resulting pellet was washed twice (by re-suspension and centrifugation) with 500 μl PBS. After the final wash, the pellet was dissolved in 50 μl 1 × NuPAGE LDS sample buffer (Invitrogen/Thermo Scientific) and heated at 80 °C for 10 min. Proteins dissolved from the pellet were analyzed with SDS-PAGE using NuPAGE 4–12% Bis-Tris gel and MES-SDS running buffer (Invitrogen/Thermo Scientific). Protein bands revealed after staining with SimplyBlue™ solution (Invitrogen/Thermo Scientific) were excised from the gel for mass spectrometry analysis.

2.7. Analysis of anti-ECP serum proteins that agglutinated bacterial cells

A cell suspension of ML10-51K in PBS was made as described above and the cell density was adjusted to approximately 1.5×10^9 cells mL^{-1}. Aliquots of 5.5 mL of this suspension were mixed with 0.5 mL anti-ECP serum or mock serum in 10-mL test tubes. The tubes were kept at RT for approximately 10 min or until visible cloudiness (cell clumping) was formed in the tube containing anti-ECP serum. Low speed centrifugation (200 × *g*) was performed for 10 min to precipitate clumping cells and the pellet was washed three time with 6 mL PBS by re-suspension and centrifugation. After the last wash, the pellet was resuspended in 250 μl 0.5 M glycine (pH 4.0). The supernatant was recovered after the suspension was incubated at RT for 3 min and centrifuged at 8000 × *g* for 5 min. The recovered supernatant was neutralized to pH ≥ 7.0 with 1 M Tris solution (pH 8.5) and protein concentration was measured with the Coomassie reagent described above. The identity of proteins in the recovered supernatant was analyzed with SDS-PAGE (as described above) and native PAGE, the latter of which was performed using Novex™ 4–12% Tris-glycine gel and Tris-glycine native running buffer (Invitrogen/Thermo Scientific). Protein bands revealed after staining with SimplyBlue™ were excised from the gel for mass spectrometry analysis. Bio-reactivity of proteins in the recovered supernatant was determined by western blot analysis. An aliquot of ECP (approximately 15 μg) was separated by SDS-PAGE as described above. After electrophoresis, the gel was subjected to blotting using a 0.45 μm PVDF membrane and Novex minicell apparatus (Invitrogen). The blot was first probed with proteins in the recovered supernatant and, then, incubated with peroxidase-conjugated goat anti-catfish-IgM IgG (produced by Rockland, Gilbertsville, PA, USA), followed by colorimetric detection using 4-chloro-1-naphthol/H_2O_2 (Bio-RAD, USA). Visible bands were cut from the membrane and treated with a stripping buffer (0.1 M glycine, 20 mM magnesium acetate, 50 mM KCl, pH 2.2 [22]) to

Table 1
Real-time PCR primer pairs for measuring quantitative expression of following genes in the head kidney tissue of channel catfish: IgM heavy chain (IgMH), IgM light chain (IgML), Serotransferrin (TRS), Warm temperature acclimation protein (WTA), Rhamnose binding Lectin (RBL) and 18S rRNA (18S).

Gene	Position	Sequence (5' – 3')	Amplicon size (bp)	Accession#
IgMH	Forward	GAAATCCCTGTTTCCCGTGTGGCA	125	U67437
	Reverse	TCCCGCTCGCATCCTTCCATA		
IgML	Forward	ACACGTTTCACTGGGGTTCCAGATC	150	U25705
	Reverse	GGTTCCTCCACCGAAAGTCAACG		
TRS	Forward	CTGGAACATCCCCATCGGCAC	122	NM_001200320
	Reverse	TCGCTCCTGGTACACAACTGGCTG		
WTA	Forward	CATGGCCATGATTTCTCCAAGTTCG	146	NM_001200248
	Reverse	TGGCTGCACTGCTCTCTCTCGA		
RBL	Forward	GATTCGGTAACCTGCTCCAA	118	KF725630
	Reverse	CAGTGCATCTCCTCTGTCCA		
18S	Forward	TTGATAACCTCGGGCCGATCG	115	AF021880
	Reverse	CGTTACCCGTGGTCACCATG		

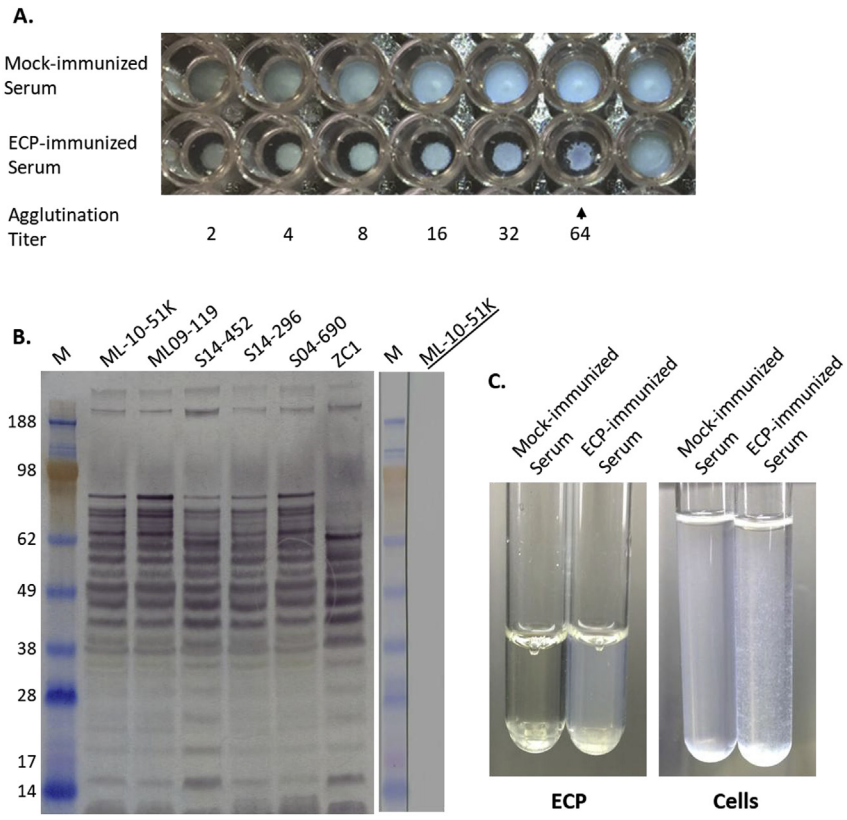

Fig. 1. Antiserum activities. Panel A-Agglutination of cells of ML 10–51K by antiserum of channel catfish immunized with ML 10–51K. The ECP-immunized serum had agglutination titer of 64 but the mock-immunized serum was unable to agglutinate cells of ML 10–51K (the titer < 2). Panel B – Western blot & immune stain of ECP prepared from ML 10–51K and other vAh strains. The blot was probed with antiserum of channel catfish immunized with ML-10-51K ECP. No antigen recognition was observed with the mock-immunized serum (Lane ML 10–51K with underline). All vAh strains were isolated from diseased channel catfish except for ZC1, which was isolated from diseased grass carp. M – SeeBlue Plus2 pre-Stained Stained standards. Panel C-Precipitation of ECP and cells of ML 10–51K after mixing with antiserum of channel catfish immunized with ML 10–51K ECP (Pictures were taken 5 min after addition of antiserum).

remove antibodies for mass spectrometry analysis.

2.8. Quantitative PCR

Gene transcripts coding for channel catfish proteins that were revealed in ECP agglutinants (subheading *2.6.* above) were analyzed by quantitative PCR (qPCR) using cDNA prepared from head kidney tissues as templates (subheading *2.4.* above). Primer pairs and associated references are shown in Table 1 (with channel catfish 18S rRNA gene as an internal calibrator for normalization). PCR was performed in triplicate for all samples in a volume of 20 μL mixture, containing 2 μL primer pair (5 μM each), 8 μL diluted cDNA (equivalent to 15 ng RNA) and 10 μL 2× iQ-SYBR (Bio-RAD). Amplification was conducted using Chromo 4 real-time PCR detection system (Bio-RAD), with following cycling parameters: one cycle of 95 °C for 3 min and 40 cycles of 95 °C for 15 S and 60 °C for 30 S. The threshold cycle (C_T) values of target genes were compared to the C_T of 18S RNA gene. Amplicons at the end of PCR cycling were analyzed for specificity with 2% agarose gel.

2.9. Data analysis

The relative expression levels of target genes in qPCR were determined by subtracting the mean C_T of the calibrator gene by that of the target gene (ΔC_T = C_T target gene – C_T calibrator). The relative changes of gene expression levels between samples of immunized (Imm) and mock-immunized (mock) tissues were calculated by $2^{-\Delta\Delta CT}$, where $\Delta\Delta C_T = \Delta C_{T\ Imm} - \Delta C_{T\ mock}$ [23]. Differences of gene expression levels were analyzed with one-way ANOVA using Graph Prism 6 software. Probabilities of 0.05 or less were considered statistically different.

3. Results

3.1. Fish immunity against ML10-51K infection

All 36 fish immunized with ECP survived the lethal challenge with vAh while all 36 mock-immunized fish died within 5 h post the

Table 2
Agglutinated proteins in ECP of virulent *A. hydrophila* induced by Channel catfish anti-ECP Serum.

Band ID	*A. hydrophila*			Catfish (*Ictalurus punctatus*)		
	Identity	Unique #	Accession #	Identity	Unique #	Accession #
AgP1	Aerolysin	8	WP_043159156			
	Hemolysin	12	WP_017410045			
AgP2	Polysaccharide export protein	40	WP_016351349			
	β-N-acetylhexosaminidase	14	WP_043162245			
AgP3	Extracellular lipase	31	WP_016348961	Serotransferrin	4	NP_001187249
	DeCa-heme cytochrome C	12	WP_075384576			
AgP4	Peptidylprolyl isomerase	39	WP_075384268	IgM μ (heavy) chain C region	18	A45804
	Carboxy-terminal protease	15	WP_016350681			
	S9 family peptidase	13	WP_016349495			
	M13 family peptidase	12	WP_075384375			
	TonB siderophore receptor	12	WP_016349237			
	3'-Nucleotidase	12	WP_075384417			
	Outer Membrane protein (OMP)	8	WP_016351217			
AgP5	Vitamin B12 transporter BtuB	17	WP_016348941	Warm temp acclimation protein	5	ABW07853
	Signal peptide peptidase SppA	17	WP_016351758			
	OMP assembly factor	8	WP_024943793			
	Alkaline phosphatase	8	WP_016350314			
AgP6	Peptide ABC transporter	22	WP_011706430			
	Membrane protein insertase	13	WP_011707925			
	Preprotein translocase subunit	12	WP_016350391			
	Paraquat-inducible protein B	11	WP_016352115			
	Peptidoglycan synthetase FtsI	10	WP_016352182			
	Hydrogenase 2 large subunit	8	WP_016351001			
	Peptide ABC transporter	8	WP_016351967			
AgP7	Serine peptidase DegQ	23	WP_011707600			
	NADH-quinone reductase subA	21	WP_011705061			
	Agglutination protein	19	WP_016351164			
	OMP channel protein	13	WP_026080288			
	Maltoporin	11	WP_075384379			
	D-alanyl-carboxypeptidase	10	WP_016351095			
	Succinyl-CoA synthetase sub-α	7	WP_080697644			
	Pilus assembly protein FimV	6	WP_043119142			
	Peptidase M48	6	WP_080755660			
AgP8	Peptidylprolyl isomerase SurA	16	WP_011704881			
	Cytochrome d ubiquinol oxidase	14	WP_011707370			
	Flagellar hook protein FlgE	12	WP_016351321			
	Translocation protein TolB	11	WP_011707363			
	Lipoprotein	10	WP_016349961			
	RND efflux transporter	10	WP_011704066			
	Elongation factor Tu	6	WP_005306325			
	Zinc metallopeptidase RseP	5	WP_016349871			
AgP9	OMP_Porin	28	WP_016349176			
	HflK protein	16	WP_016349609			
	D-alanine carboxypeptidase	13	WP_011707032			
	OMP assembly factor BamC	13	WP_029301731			
	OMP assember factor BamB	12	WP_016350408			
	Maltose ABC transporter	12	WP_011705558			
	Murein transglycosylase A	10	WP_011706178			
	Murein hydrolase B	8	WP_016350221			
	Phosphoglycerate kinase	7	WP_016349507			
AgP10	Uroporphyrin methyltransferase	17	WP_016349243	Rhamnose binding lectin	4	NP_001316192
	Putrescine ABC transporter	12	WP_016352333			
	Flagellin-like protein	12	WP_016350346			
	O-antigen determinant protein	11	WP_016351350			
	L-asparaginase II	11	WP_016352139			
	Fimbrial protein	10	WP_043118352			
AgP11	ATP-dependent Zn protease	13	WP_080630501			
	Glutamate ABC transporter	12	WP_016349490			
	Membrane protein	11	WP_011705056			
	NADH-quinone reductase sub C	10	WP_011705063			
AgP12	Major OMP OmpAI	26	WP_016349944	IgM kappa (light) chain	7	AAA82596
	Long-chain fatty acid transporter	13	WP_016350827			
	Succinate dehydrogenase	9	WP_011706985			
AgP13	Autotransporter	19	WP_016349813			
	OMP_W	6	WP_011707635			
AgP14	Membrane protein	17	WP_011705101			
	PTS glucose transporter	7	WP_016351511			

Unique#: Number of exclusive unique peptides (threshold ≥ 99.0%) identified by mass spectrometry.

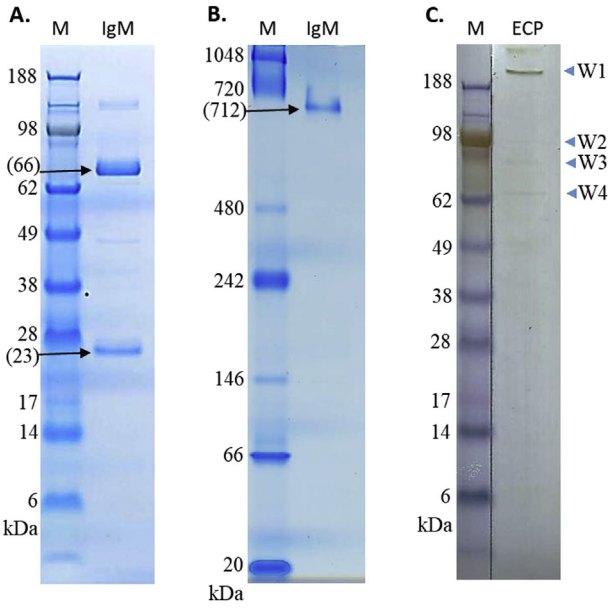

Fig. 2. Analyses of channel catfish antiserum proteins (IgM) co-precipitated with cells of *Aeromonas hydrophila* ML 10–51K. Panel A: Two prominent bands with estimated molecular weights of 66 kDa (upper) and 23 kDa (lower) were visualized after SDS-PAGE and Coomassie staining; these two proteins were identified as IgM heavy chain and light chain, respectively, by mass spectrometry. Panel B: Native PAGE of the same protein preparation shown inPanel A:a single band was shown after Coomassie staining with estimated molecular weight of 712 kDa. Panel C: Western blotting using the same protein preparation shown in Panel A as first antibody against extracellular proteins of ML 10–51K (visible bands, w1 -w4, were indicated by arrows). M: SeeBlue pluse2 Pre-stained standards (Panel A & C) and NativeMark Protein Standards (Panel B).

Table 3
Expression of anti-ECP serum-associated protein genes in the head kidney of channel catfish.

Protein encoding gene	Immunized vs Mock-Immunized genes	C_T ± SD	Relative to Calibrator (ΔC_T)	Fold Change ($2^{-\Delta\Delta CT}$)
IgMH	Imm	18.17 ± 0.06	8.32	1.77*
	Mock	19.15 ± 0.04	9.14	–
IgML	Imm	20.44 ± 0.02	10.59	1.75*
	Mock	21.41 ± 0.04	11.40	
TRS	Imm	10.68 ± 0.11	0.83	1.27*
	Mock	11.19 ± 0.13	1.82	–
WTA	Imm	7.14 ± 0.05	−2.71	−1.01
	Mock	7.31 ± 0.07	−2.70	–
RBL	Imm	15.42 ± 0.12	5.57	1.62*
	Mock	16.28 ± 0.17	6.27	–

Calibrator: 18S rRNA; $\Delta C_T = C_{T\ target\ gene} - C_{T\ calibrator}$; $\Delta\Delta C_T = \Delta C_{T\ Imm} - \Delta C_{T\ Mock}$. Significant difference ($p < 0.05$) was marked by an asterisk.

challenge. Bacterial cell agglutination assay confirmed that sera collected from ECP-immunized fish (antiserum) had an agglutination titer of 64 against vAh cells and sera of mock-immunized fish were unable to produce agglutination (titer < 2) (Fig. 1A). The antiserum contained specific antibodies that bound antigens in ECP of ML10-51 and other vAh strains in western blotting assay (Fig. 1B) while no antigen recognition was observed with the mock-immunized serum. By antigen-recognition patterns, all channel catfish vAh strains were similar but different from the grass carp strain (ZC1).

3.2. Agglutinants from ECP of ML10-51K

ECP-immunized sera caused proteins in ECP of ML10-51K to precipitate within 5 min at RT; but no precipitation occurred for mock-immunized sera under the same experimental conditions (Fig. 1C).

SDS-PAGE analysis revealed that the banding pattern of ECP agglutinants was different from the pattern of ECP that was used for immunization (Supplemental Figure 1). Both ML10-51K and fish proteins were identified in ECP agglutinants. The identities of major proteins in individual bands (AgP1 – AgP16) were resolved by mass spectrometry. There were more than 100 ML10-51K proteins identified (data not shown) and the most prominent 68 proteins (threshold ≥ 99.0%; probability = 100%) were shown in Table 2. Individual bands contained more than two proteins; their migrating distances in the gel were closely matched with their molecular weights (MW), such as the MWs of seven proteins in band AgP4 ranging from 70 to 79 kDa. An exception is band AgP1. The apparent MW of band AgP1 is estimated to be over 220 kDa; however, the calculated MWs of the two proteins identified, aerolysin and hemolysin, are approximately 52 kDa and 66 kDa, respectively. Additionally, there were 5 channel catfish proteins identified, including IgM heavy chain (IgM-H), IgM light chain (IgM-L), serotransferrin, warm temperature acclimation protein and rhamnose binding lectin.

3.3. Agglutinants that co-precipitated with cells of ML10-51K

Proteins that co-precipitated with cells of ML10-51K (Fig. 1C) were released by glycine (0.5 M, pH 4.0). Approximately 35 μg of proteins were recovered from the assay. Upon SDS-PAGE, the recovered proteins were separated to two prominent bands (Fig. 2A). They were identified as IgM-H and IgM-L of channel catfish by mass spectrometry. Their molecular weights were estimated to be 66 kDa (IgM-H) and 23 kDa (IgM-L), respectively. By native PAGE, the recovered protein was shown as a single band with molecular weight being approximately 712 kDa (likely a tetrameric form of IgM: (66 + 23) x 2 × 4 [24]) (Fig. 2B). Using the recovered protein as a primary antibody against ECP of ML10-51K, four high-molecular weight proteins (W1 –W4) were detected in western blotting (Fig. 2C). Protein in band W1 were identified as a mixture of aerolysin and hemolysin by mass spectrometry but the identities of other three bands were unresolved.

3.4. Expression of antiserum-associated genes in the head kidney tissue of channel catfish

All individual qPCR samples showed a single peak following melt curves analysis and each gene had one amplicon with a prospective size (Supplemental Figure 2). Significantly-higher levels of gene transcripts were observed for IgM-H, IgM-L, serotransferrin and rhamnose binding lectin in the head kidney of ECP-immunized fish, compared with the same genes in the same tissue of mock-immunized fish (Table 3). Based on comparative quantification ($2^{-\Delta\Delta CT}$), the fold changes were 1.77 (or 77% increase), 1.75 (or 75%), 1.27 (or 27%) and 1.62 (62%) for gene transcripts of IgM-H, IgM-L, serotransferrin and rhamnose binding lectin, respectively. The levels of gene transcripts for warm water acclimation protein were approximately the same between ECP-immunized and mock-immunized fish (no significant difference, $P < 0.05$).

4. Discussion

Fish developed immunity against infection by vAh after immunization with the pathogen's extracellular proteins (ECP) and anti-ECP fish serum exhibited a high agglutination titer against cells of the bacterium. These claims were supported by this and previous studies [13]. ECP-immunized fish evidently produced antiserum with specific antibodies that were able to bind specific antigens and cause

aggregation (precipitation), an important humoral immune response that neutralizes pathogen's infectivity [25]. Although three classes of immunoglobulins, IgM, IgD and IgT, have been described in teleost fish, IgM is a main systemic immunoglobulin [26] and rich in the serum [27].

Catfish IgM was identified in antiserum-induced agglutinants of ECP and cells of vAh in this study. Two protein bands were revealed in SDS-PAGE gel and recognized as IgM-H and IgM-L, with apparent molecular weights of approximately 66 and 23 kDa, respectively. The estimated MW of IgM-H was higher than the calculated MW, which was approximately 60.6 kDa based on the amino acid residues of the mature peptide (GenBank Accession# AAA79003 [28]). The discrepancy could be due to the heavy glycosylation of IgM-H as it was observed that the carbohydrate moiety of Atlantic cod (*Gadus morhua*) serum IgM could account for as high as 10% of the MW of the immunoglobulin [29]. Analysis of native IgM molecules indicated that a tetrameric assembly was formed when they bound and agglutinated cells of vAh. The MW of the tetramer, approximately 712 kDa shown in native gel, was in agreement with the above estimation. The native IgM tetramer appeared to be functionally active in western blotting assay but only a few faint protein bands were revealed. This is likely because: 1) the IgM tetramers cross-linking cells of vAh were specific for cell surface antigens and 2) tetrameric IgM, though having higher avidity, has lower affinity than monomeric antibodies [30]. As indicated in western blot analysis (Fig. 1B) that the antiserum generated from one vAh strain (i.e. ML10-51K) could recognize ECP from vAh strains isolated from multiple locations and different fish species [1], it implies that ECP-immunized fish would have immunity against other vAh strains and the antiserum could be applied for diagnosis of field isolates.

Proteins in the ECP agglutinants were most likely antigens that were bound by specific IgM present in antiserum. Colloidal instability (aggregation into masses) led to precipitation of the antigen-antibody complexes. The amount and variety of proteins with MW varied from 9 to 95 kDa shown in Table 1 may imply equal diversities of IgM molecules produced in the antiserum. Some of these vAh proteins have been investigated and proved to be effective immunogens that elicited humoral immune responses against *A. hydrophila* infection in fish, such as aerolysin and hemolysin [15], OMP- maltoporin [17], OMP-A1 [18], OMP-porin [19] and OMP-W [20]. One particular finding in this study was that a large polymer (AgP1 in Supplemental Figure 1 and W1 in Fig. 2C) was formed by two smaller proteins, aerolysin and hemolysin. Based on the apparent MW ($\geq$220 kDa), the polymer may be constituted by two copies of aerolysin and hemolysin each. This polymer also appeared to be resistant to heat and detergent (SDS) treatments. Both aerolysin and hemolysin are hemolytic proteins [15] and have been recognized as important virulence factors of *A. hydrophila* [31,32]. Although earlier studies indicated that aerolysin could oligomerize to a channel (hole)-forming structure [33,34] and disrupt eukaryotic cell membrane, it was unknown until this study that aerolysin and hemolysin can form a heterologous polymer. Functionality of this heterologous polymer merits further investigation. Other proteins identified in the ECP agglutinant are potential immunogens for vaccine development although individual roles have yet to be determined using purified or recombinant proteins.

Additionally, there were three catfish-originated proteins found in ECP agglutinants. Among the three serum proteins, the serotransferrin was reported to play an important role in iron metabolism and be involved in fish defense against diseases [35]; the rhamnose-binding lectin was also associated with innate immunity and could participate in agglutination of bacterial lipopolysaccharides [36,37]. The warm temperature acclimation protein, matched with Wap65-1 [38], was however a constitutively-produced protein in fish in response to temperature changes.

Analysis of genes coding for the five fish proteins found in anti-ECP serum showed that all but the warm temperature acclimation protein gene were expressed more in ECP-immunized fish than in mock-immunized fish, indicating the enhanced immune competency of fish following ECP immunization. Particularly, an array of specific antibodies (IgM) in antiserum were inventoried for cell surface and secreted proteins of vAh, as evidenced by ECP and cell agglutination assays and immunoblotting. Insight into the repertoire of the versatile IgM genes (the recombination of variable, diverse and joining regions from germline genes) will help understand fish immune responses to specific antigens. This laboratory is currently undertaking cloning and sequence analysis of differentially expressed IgM genes.

In conclusion, channel catfish developed both innate and antibody-mediated immune responses in serum and expressed genes upon immunization with ECP of virulent *A. hydrophila*, which accounted for the fish immunity against infection.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2018.11.033.

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