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The Effects of Inactivated Recombinant Cells Vaccine Encoding Outer Membrane Proteins (OMPs) of Aeromonas Hydrophila in African Catfish, Clarias Gariepinus (Burchell, 1822)

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Abstract

This study was carried out to determine the protective efficacy of inactivated recombinant cell vaccine expressing OmpTs (37 kDa) and OmpW (22 kDa) of *Aeromonas hydrophila* against the virulent strain of *A. hydrophila* strain Ah1sa5 in African catfish and to observe the presence of GALT in fish after vaccinated with the recombinant protein vaccine compared to control and placebo vaccine groups. Two hundred and forty African catfish were divided into five major groups. Three groups were vaccinated intraperitoneally (IP) with inactivated recombinant cells concentration of 1 x 10^7 CFU/ml and one group that was vaccinated with recombinant cell alone without insert and the last group was an unvaccinated group. Vaccines were injected on day 0 and booster dose given on day 14. On day 28 challenge test were done where all fish from all groups were IP injected with virulence bacteria, *A. hydrophila* strain Ah1sa5 inoculums with concentration of 4.0 x 10^8 CFU/ml. During the course of study, fish were collected randomly from all groups and guts were collected for histological examination to observe the aggregation of GALTs. All of the vaccinated groups had a significantly higher protection ($P < 0.05$) than placebo vaccine and control groups. This level of protection may be due to high antibody responses as demonstrated by aggregation of GALTs following vaccination. These results suggest that the recombinant cell vaccines OmpTs and OmpW could effectively stimulate both specific and non-specific immune responses and protect against *A. hydrophila* infection. Therefore, OmpTs and OmpW may be developed as potential vaccine candidates against *A. hydrophila* infection.

Keywords: Inactivated Recombinant Cell Vaccine; *Aeromonas Hydrophila;* Omps

Introduction

Aeromonas species is an important pathogen in aquaculture systems, and millions of dollars are estimated to be lost per annum due to diseases caused by this bacterium [1] and it has been reported to cause mass mortalities in several species of cultured and wild fish living in fresh, brackish and marine water environments including African catfish [2], Nile tilapia [3], gilthead seabream [4] and ornamental fish such as Dwarf Gourami,

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Discus Cichlids, and Tiger Barb [5]. Motile Aeromonas Septicemia (MAS) infection has been identified as a problem in *Pangasius* sp. culture in Malaysia causing up to 40% mortality which occurring mostly during dry season where study from 2008 till 2014 revealed *Aeromonas hydrophila* as the main culprit causing the outbreak [6]. While, surveillance studies reported the *A. hydrophila* infection occurred in cage culture red tilapia farm in Pahang and Terengganu [7] and in some of ornamental fish in retail pet shop in Terengganu [5]. It also caused infection in the African catfish farms in West Java, Indonesia [8,9] and aquaculture pond along Cross River, Nigeria [10] that caused high losses to the farmers. A study in Mekong Delta, Vietnam revealed *Aeromonas* spp. infection in 15 Vietnamese intensive catfish aquafarms and they also investigated and proved that *Aeromonas* spp. been highly resistance to antibiotic that were applied in multiple drugs in order to cure diseases [11].

Application of antibiotics to control disease outbreaks is no longer effective where only several types of antibiotics are allowed to be used and furthermore the pathogens fast development by emergence of drug resistance strains making the application of antibiotics ineffective towards diseases management plus its negative effect of immunosuppressive in fish [12]. Alternatively, vaccination widely applied in aquaculture to prevent disease outbreak [13,14]. In aquaculture sector, vaccines mainly work as an enhancer towards fish immune system by activating the immune system combating the specific pathogen based on the mode of action [15]. Anuradha, et al. (2010) [16] used two modes of vaccination which are intraperitoneally and orally fed with live recombinant aerolysin genes of *Lactocoocus lactis* in tilapia [16]. Hence, both modes of vaccination gave highest level of protection against *Aeromonas hydrophila* by eliciting the antibody production.

Research on recombinant subunit DNA recently become trending among scientists in developing the most effective way to combat pathogenic bacteria by building immune defense in host [17]. A number of virulence factors may contribute to the overall virulence of this bacterium. Outer membrane protein (OMP) that characterized by β-barrel structures maintain the connectivity and selective permeability of bacterial cell surface [18]. Besides, OMP also develop resistance on antimicrobial peptide, multidrug, bile salt and serum, it also involved in bacterial adaptive responses such as iron uptake [19]. A study by Mao, et al. (2007) revealed that OmpW, OmpV, OmpK, and OmpU are immunogenic and might be used for protection against *Vibrio parahaemolyticus* because these antigens can stimulate cell mediated immunity of large yellow croaker [20].

The fish immune system comprised of numerous distinct and interdependent immune components [21]. The gastro-intestinal tract serves as the route of infection of most bacterial pathogens in fish plus, the immune response genes in the gut produce substances that provide an initial defense during pathogen invasion [22]. Mucosal immunity or mucosa-associated lymphoid tissue (MALT) of fish constitute of the first line defense from infection [23]. Gut-associated lymphoid tissue (GALT) is the most important part in MALT due to its multifunctionality, antigen load and the endogenous microflora besides, the study of the GALT has intensified in recent years [24]. This study was carried out to determine the effects of inactivated recombinant cell vaccine expressing OmpTs and OmpW of *Aeromonas hydrophila* against the virulent strain of *A. hydrophila* Ah1sa5 in African catfish on survival rate and to observe the presence of GALT in fish after vaccinated with the recombinant cell vaccine.

Materials and Methods

Bacterial Strain, Plasmid and Culture Condition

Aeromonas hydrophila strain Ah1sa5 was cultured on Tryptic Soy Agar (TSA) plates incubated at 37˚C and maintained in Tryptic Soy Broth (TSB) with 20% glycerol at -80˚C until used. A non-expression host, One Shot TOP10 Chemically Competent Cells *E. coli* (Invitrogen, California, USA) and expression host, BL21 Star (DE3) One Shot Chemically Competent *E. coli* cells (Invitrogen, California, USA) were used as host for cloning and expression. The *E. coli* cells were grown on Luria Bertani (LB) agar at 37˚C incubation while when grown on LB broth agitation of 250 rpm was needed and after transformation process both were supplemented with 50 μg/ml ampicillin for growth. Competent cells *E. coli* stocked were stored at -80˚C until used. The expression vector, pET102/D-TOPO was obtained from Invitrogen, California, USA and stored at -20˚C until used.

PCR Amplification of the Outer Membrane Proteins (OMPs) Genes

DNA template was extracted from *Aeromonas hydrophila* strain Ah1sa5. In order to detect and isolate the genes of interest which are OmpTs and OmpW genes, two set of primers were designed based on the published sequences AF276639 [25] and HM063438 [1] respectively. The designated forward and reverse primer sequences were as in Table 1. PCR amplification was run using proofreading polymerase, *Pfu* DNA Polymerase (Thermo Scientific, Massachusetts, USA) was used to produce blunt-end PCR products. PCR was carried out in 50 μl volumes containing 5 μl 10x Buffer with 25 mM $MgSO_4$

solution, 0.5 µl 20 mM dNTPs, 1 µl 20 µM forward primer, 1 µl 20 µM reverse primer, 1 µl 50 ng template DNA, 41 µl sterile deionized water and 0.5 µl *Pfu* DNA Polymerase. PCR amplification was run in a programmable thermocycler (Eppendorf, Hamburg, Germany) having an initial denaturation at 95°C for three min and a final extension at 72°C for 10 min followed by 35 cycles of denaturation at 95°C for 30 sec, annealing at 53°C for 30 sec followed by extension at 72°C for two min and hold at 4°C. PCR amplification for both OmpTs and OmpW genes used the same setting. After amplification, 7 µl samples were mixed with 3 µl of loading dye (Thermo Scientific, Massachusetts, USA) and subjected to electrophoresis in a 1% agarose gel at 90 V for 60 min and visualized using Alpha Imager HP (Alpha Innotech, California, USA).

Primer	Oligonucleotide Sequence (5'- 3')*	PCR size (bp)	Protein size (kDa)
OmpTsF	CACC GCA GTG GTT TAT GAC AAA GAC G	1000	37
OmpTsR	TTA GAA GTT GTA TTG CAG GGC		
OmpWF	CACC ATG AAA AAG ATC CTT CCT CT	600	22
OmpWR	TCA GAA GCG ATA GCC GAC AC		

Table 1: List of primer pairs and the oligonucleotide sequences of OmpTs and OmpW genes.
*Underlined nucleotides are necessary for directional cloning in order the gene of interest were optimally expressed and fused in frame with any epitope tags following requirement by Champion™ pET Expression System (Invitrogen, California, USA).

Plasmid Construction

The Champion™ pET Directional TOPO® Expression Kits (Invitrogen, California, USA) was used to directionally cloned a blunt-end PCR product into selected vector which is pET102/D-TOPO® (Invitrogen, California, USA). The insert was ligated into the vector according to the manufacturer protocol. One Shot® TOP10 Chemically Competent *E. coli* (Invitrogen, California, USA) was used to transform the recombinant vectors and plated overnight at 37°C on LB agar supplemented with 50 µg/ml ampicillin. The selected colonies were directly screened using PCR amplification where the same designated forward and reverse primers were used accordingly for both OmpTs and OmpW recombinants. Purified plasmids were isolated according to the protocol by EZ-10 Spin Column Plasmid DNA Kit (Bio Basic Inc., Markham ON, Canada) for further DNA sequencing and the positive plasmids containing the insert were than transformed into expression host, Shot® *E. coli* BL21 Star™ (DE3) (Invitrogen, California, USA). The positive clones known as pET102/D-OmpTs and pET102/D-OmpW were stored as glycerol stock in LB broth supplemented with 50 µg/ml ampicillin at – 80°C.

Bioinformatics Analysis

The BioEdit software was used to analyze the arrangement of the OMPs gene in vector sequence and compared with other published sequences of *A. hydrophila* available in GenBank.

Preparation of Rabbit Polyclonal Antibody of Anti Whole Cells of Aeromonas Hydrophila

Rabbit antiserum against *A. hydrophila* was used to detect the OMP proteins in *A. hydrophila*. A white rabbit was immunized by one ml of the formalin-killed whole cells of 4.0 x 10^8 CFU/ml *A. hydrophila* strain Ah1sa5, emulsified with Freud's complete adjuvant (Sigma, Missouri, USA) at ratio 7:3. Booster doses of one ml FKC in Freud's incomplete adjuvant (Sigma, Missouri, USA) at ratio 7:3 respectively were given on day 14 and day 21. On day 28, the rabbit's blood was collected, clotted at room temperature for one hour, and stored at 4°C overnight. The blood was then centrifuged to separate and obtain the rabbit antiserum against *A. hydrophila*, and stored at -20°C.

Pilot Expression

Overnight cultures of recombinant *E. coli* cells were used for pilot expression where four ml culture was inoculated with 500 ml LB with 50 µg/ml ampicillin and incubated at 37 ˚C for three hours 15 min with 200 rpm until OD_{600} 0.5 - 0.8 was achieved. Next, to induce the protein expression, 1 mM IPTG was added to the cultures and incubation was continued with the same setting for another four hours. Cultures were then centrifuged at 10 000 rpm for 10 min and stored at -20°C until further used. Pellet cells were subjected to Bugbuster lysis buffer (Novagen Inc., WI, USA) to determine the expressed protein.

Western Immunoblotting

The supernatant, which contained soluble cytoplasmic fractions, was preceded with protein analysis by SDS PAGE and detection of the expressed protein by using rabbit polyclonal antibody of anti-whole cells of *A. hydrophila* as primary antibody.

Preparation of Inactivated Recombinant Cells Vaccine

Following induction with IPTG as described previously, cultures of the recombinant *E. coli* BL21 Star™ (DE3) expressing pET102/D-OmpTs, pET102/D-OmpW and pET102/D-TOPO without insert were harvested and killed in 4% formalin in phosphate buffered saline (PBS) overnight at 4°C. This was followed by washing three times in sterile PBS by centrifugation (5000 rpm) at 4°C for 10 min to ensure that formalin was completely removed. Finally, the inactivated recombinant cells were resuspended in sterile PBS as stock vaccine seed. For preparation of vaccine, the stock vaccine seed was added into adequate amount of sterile PBS to give a final concentration of 1 x 10^7 CFU/ml using McFarland method. The sterility of the inactivated recombinant vaccines was tested by inoculating 0.1 ml of the vaccine onto the TSA followed by incubation at 37°C for 24 hours. The vaccines were considered sterile when no growth appeared on TSA [26].

Preparation of Live Bacterial Inoculums for Challenge

Aeromonas hydrophila strain Ah1sa5 was used in preparation of live bacteria inoculums for challenge. The bacteria were subcultured onto TSA and incubated at 37°C for 24 hours before five colonies were selected and inoculated into 100 ml of TSB for 24 hours at 37°C with shaking at 250 rpm. Following incubation, one ml of the broth was injected intraperitoneal (IP) into a 100 g African catfish to enhance bacterial virulence. The infected fish was killed at 24 hours post challenged for reisolation of *A. hydrophila* before it was subcultured onto TSA and incubated at 37°C for 24 hours. Following the incubation, five colonies were selected and inoculated into 100 ml of TSB and incubated for 24 hours at 37˚C with shaking at 250 rpm. A serial dilution and standard plate count techniques were used in order to determine the bacteria concentration. Ten folds of dilutions prepared from highest dilution (10^1) to lowest dilution (10^9) where one ml of cultured broth *A. hydrophila* was serially added into nine ml of PBS respectively. Then one ml from the highest dilution was continuously diluted into another dilution till the lowest dilution. About 0.1 ml of each dilution was poured and spread onto the TSA and incubated in normal incubator at 37°C for 24 hours. Growth with 25 – 250 colonies were counted before the concentration was expressed as colony forming unit per milliliter (CFU/ml). The last concentration of live *A. hydrophila* was recorded. The challenge dose was 4.0 x 10^8 CFU/ml of live *A. hydrophila* and used immediately.

Animals

A total of 240 healthy African catfish (*Clarias gariepinus*) with mean weight 30±10 g was used. The fish were bought from a local commercial farm in Puchong, Selangor with no history from any infections. Fish were fed *ad libitum* daily with local commercial pellet diet during the acclimatization and the experimental period. Prior to experiment commence, fish were randomly killed and screened to ensure they were free from any bacterial infection.

Experimental Design

Acclimatized fish were divided into five groups with triplicate (Table 2). All groups were IP injected on day 0 (week 0) and booster dose given on day 14 in the amount of 1 ml/ 100 g per fish containing concentration of 1 x 10^7 CFU/ml. On day 28 challenge test were done where all fish from all groups were IP injected with virulence bacteria, *Aeromonas hydrophila* strain Ah1sa5 inoculums with the amount of 1 ml/ 100 g per fish with concentration of 4.0 x 10^8 CFU/ml.

Sampling was done prior vaccination every week where as three fish from each group was sacrificed for their gut. Following the post infection on day 28, all fish were monitored daily for 14 days and observation was done in-term of clinical signs, abnormal behavior, and mortality.

Group	Vaccine
OmpTs	Inactivated recombinant cells pET102/D-OmpTs
OmpW	Inactivated recombinant cells pET102/D-OmpW
OmpTs+ OmpW	Combination of inactivated recombinant pET102/D-OmpTs and pET102/D-OmpW cells with ration of 1:1
Placebo vaccine	Inactivated recombinant pET102/D-TOPO vector cells without insert protein
Control	Unvaccinated

Table 2: Five groups of African catfish designed for in-vivo test.

Bacterial Isolation

All dead fish were post mortem by isolating the bacteria from the internal organs (kidney, liver and spleen) and were inoculated on TSA and selective *Aeromonas* Agar Base (RYAN) (CONDA, Madrid, Spain) and incubated at

37°C for 24 hours. The plates were then examined for bacterial growth. The suspected *Aeromonas* sp. colony obtained were confirmed with colony PCR screening using *A. hydrophila* genes specific primers to ensure fish death were died due to infection by *Aeromonas* sp. Mortalities were considered to be due to *A. hydrophila* if the challenged strains were isolated as pure culture from internal organs and grow on selective agar with similar morphology as described by the manufacturer.

Relative Percentage Survival *(RPS)*

The mortality of the fish, clinical signs and the abnormal behavior were observed and recorded over 14 days post infection. The presence of *A. hydrophila* in the tissues was determined by bacterial culture in selective *Aeromonas* agar Base (RYAN) (CONDA, Madrid, Spain). The mortality data from all treatment groups and control group with *Aeromonas hydrophila* strain Ah1sa5 were used to calculate and determine the vaccine efficacy by relative percentage survival (RPS) as:

$$RPS = 1 - \frac{(\text{Mortality of vaccinated fish})}{\text{Mortality of unvaccinated control fish}} \times 100$$

The mean ± standard deviation for survival and mortality rate were calculated. The mean values were compared by one way ANOVA followed by Duncan's multiple range tests to determine significant difference at 5 % ($P < 0.05$) level.

Histopathology

Histological analysis was carried out to investigate for the presence of Gut-Associate Lymphoid Tissues (GALTs) in fish gut after vaccination. Fish were randomly sacrificed from each group for their guts from week 0 until week 5. The guts were fixed in 4% buffered formalin and histological process and examination was carried out at Histopathology Laboratory at Faculty of Veterinary, Universiti Putra Malaysia. Aggregations of GALTs were then observed under microscope (Leica Microsystem, Wetzlar, Germany).

Results and Discussion

Construction of Recombinant Vaccine Plasmids Expressing *OmpTs* and *OmpW*

The PCR products were purified and successfully cloned into pET102/D-TOPO® expression vector. The positive clones that grew on LB agar supplemented with ampicillin were screened by colony PCR using specific gene primers followed by sequencing analysis which revealed 795 bp and 612 bp full length ORF of OmpTs and OmpW genes were obtained respectively (Figure 1a). The restriction enzyme analysis of purified plasmids with SacI (Figure 1b) successfully cleaved the recombinants plasmids to produce a single band for each recombinant plasmid consist of combination vector (6315 bp) with insert (OmpTs; ~1000 bp or OmpW; ~600 bp) showed products of 7315 bp and 6915 bp respectively which are the correct size of successful cloning according to the manufacturer's protocol (Champion™ pET Directional TOPO® Expression Kits). From sequencing analysis, purified plasmids of pET102/D-OmpTs and pET102/D-OmpW proved that the genes in correct orientation in the plasmid.

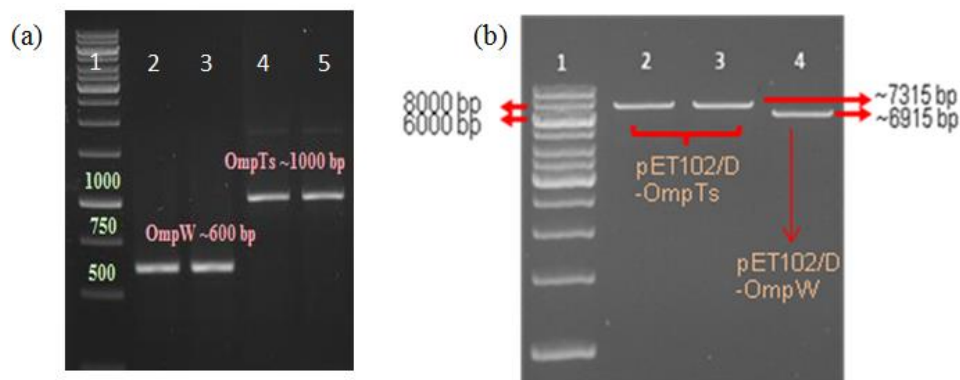

Figure 1: (a) Polymerase chain reaction (PCR) amplification of OmpW and OmpTs genes in *Aeromonas hydrophila* strain Ah1sa5. Lane 1, molecular weight marker (1 kb ladder); lanes 2-3, OmpW genes; lanes 4-5, OmpTs genes. (b) Restriction enzyme (Sac I) analysis of purified recombinant plasmids of OmpTs and OmpW. Lane 1, molecular weight marker (1 kb ladder); lanes 2-3, purified plasmids of pET102/D-OmpTs; lane 4, purified plasmids of pET102/D-OmpW.

Bioinformatics Analysis

Figure 2 and Figure 3 shows the full length of the OMP sequence, nBLAST analysis and antigenic site.

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>pET102/D-OmpTs

1     GCA GTG GTT TAT GAC AAA GAC GGT ACC TCT TTT GAT ATC TAT GGC   45
1      A   V   V   Y   D   K   D   G   T   S   F   D   I   Y   G   15

46    CGT GTT CAA GCC AAC TAC TAC GGT GAA CAC GAC GAT ACC GAC GCT   90
16     R   V   Q   A   N   Y   Y   G   E   H   D   D   T   D   A   30

91    GAA CTG GTT GGT TCC TCC CGT CTG GGC TGG TCC GGC AAA GTC GGC   135
31     E   L   V   G   S   S   R   L   G   W   S   G   K   V   G   45

136   CTG AAC AAC ACC TGG TCC GGT ATC GCC AAG ACC GAG TGG CAA GTA   180
46     L   N   N   T   W   S   G   I   A   K   T   E   W   Q   V   60

181   TCC GCC GAA AAC TCT GAC AAC AAG TTC AAC TCC CGT CAC GTA TAC   225
61     S   A   E   N   S   D   N   K   F   N   S   R   H   V   Y   75

226   GTC GGC CTG GAC GGT ACC CAG TAC GGC AAG ATC ATC TTC GGC CAG   270
76     V   G   L   D   G   T   Q   Y   G   K   I   I   F   G   Q   90

271   ACC GAC ACC GCG TTC TAC GAC GTG CTG GAG CCG ACC GAT ATC TTC   315
91     T   D   T   A   F   Y   D   V   L   E   P   T   D   I   F   105

316   AAC GAA TGG GGT GAC GTA GGT AAC TTC TAC GAC GGT CGT CAA GAA   360
106    N   E   W   G   D   V   G   N   F   Y   D   G   R   Q   E   120

361   GGC CAG ATC ATC TAC TCC AAC ACC TTC GGT GGC TTC AAG GGC AAA   405
121    G   Q   I   I   Y   S   N   T   F   G   G   F   K   G   K   135

406   GTG TCC TAC CAG ACC AAC GAT GAC GTA GCC GTC AAG GTT ACT GAC   450
136    V   S   Y   Q   T   N   D   D   V   A   V   K   V   T   D   150

451   ATC GGT CAA GGC ATC AAG GAA ACC GAC GTC TAC GGT CCG AAC GTC   495
151    I   G   Q   G   I   K   E   T   D   V   Y   G   P   N   V   165

496   AAG CGC AAC TAC GGC TAC GCC GCT GCT GCT GGC TAC GAC TTC GAC   540
166    K   R   N   Y   G   Y   A   A   A   A   G   Y   D   F   D   180

541   TTC GGT CTG GGC CTG AAC GCC GGT TAC GCT TAC TCC GAC CTG GAA   585
181    F   G   L   G   L   N   A   G   Y   A   Y   S   D   L   E   195

586   AGC ACC GTC AGC ACC GCT TCT GGC GAC AAG AAA AAT TGG GCC CTG   630
196    S   T   V   S   T   A   S   G   D   K   K   N   W   A   L   210

631   GGC GCA CAC TAC GCC ATC AAC GGT TTC TAC TTC GCC GGT ATG TAC   675
211    G   A   H   Y   A   I   N   G   F   Y   F   A   G   M   Y   225

676   ACC CAG GGG GGA CCT GAG CTA CCG ACA CCC CAC CCA AGG GAA CAA   720
226    T   Q   G   G   P   E   L   P   T   P   H   P   R   E   Q   240

721   CAA GGA CAA GGG CTG TGG CTA CAA ACT TGG TTG CTT CCT ACA ACG   765
241    Q   G   Q   G   L   W   L   Q   T   W   L   L   P   T   T   255

766   TTG AAC GCC TGG AAC CTT CCT TGG CGG GTT TAA   795
256    L   N   A   W   N   L   P   W   R   V   *
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Figure 2: Nucleotides and predicted amino acid sequences of OmpTs protein of *A. hydrophila* strain Ah1sa5. Underlined amino acids are antigenic sites and 11 antigenic sites were recognized. The stop codon is represented by an asterisk.

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>pET102/D-OmpW

1     ATG AAA AAG ATC CTT CCT CTG TTA ATC GCA GCC GCT TTT GCT TCC    45
1      M   K   K   I   L   P   L   L   I   A   A   A   F   A   S     15

46    CCC GTC GCC ATG GCA CAT CAG GCC GGT GAC ATC CTG GTC CGT GGT    90
16     P   V   A   M   A   H   Q   A   G   D   I   L   V   R   G     30

91    GGC CTG GCG TTT GTC TCT CCC CAG ACC AGC AGT GAC AAC GTG CTG    135
31     G   L   A   F   V   S   P   Q   T   S   S   D   N   V   L     45

136   GGG ACG GGC GAG CTG GAG ATC GAC AGC AAC ATG CAG CTT GGC CTG    180
46     G   T   G   E   L   E   I   D   S   N   M   Q   L   G   L     60

181   ACC CTC TCC TAC ATG TTG ACG GAC AAC TGG GGT GTC GAG CTG CTG    225
61     T   L   S   Y   M   L   T   D   N   W   G   V   E   L   L     75

226   GCC GCG ACC CCC TTC TCC CAC TCG GTG TCG ACT GCC GGC CTG GGT    270
76     A   A   T   P   F   S   H   S   V   S   T   A   G   L   G     90

271   GAG GTC GCC AAG GTC AAA CAC CTG CCG CCG ACC CTG ATG GCG CAG    315
91     E   V   A   K   V   K   H   L   P   P   T   L   M   A   Q     105

316   TAC TAC TTC GGT GAT GCC AAC AGC AAG GTG CGC CCC TAT GTG GGG    360
106    Y   Y   F   G   D   A   N   S   K   V   R   P   Y   V   G     120

361   GCC GGT ATC AAC TAC ACC ACC TTC TTC GAC GAG GAA GGG CGC GGT    405
121    A   G   I   N   Y   T   T   F   F   D   E   E   G   R   G     135

406   GCC CTG GCC GGG ACG GAT GTG AGC GTG GAC TCC TCC TGG GGG ATG    450
136    A   L   A   G   T   D   V   S   V   D   S   S   W   G   M     150

451   GCG GGT CAG GTG GGC CTG GAC ATG GCG ATC AAT GAC CGC TGG TTC    495
151    A   G   Q   V   G   L   D   M   A   I   N   D   R   W   F     165

496   GTC AAC GCC TCC GCC TGG CTC ATC GAC ATC GAT ACC GAT GTG CAT    540
166    V   N   A   S   A   W   L   I   D   I   D   T   D   V   H     180

541   ACC GCC GTG GGC ACC ATC AAC ACC TCC ATC GAT CCG GTG GCC TTC    585
181    T   A   V   G   T   I   N   T   S   I   D   P   V   A   F     195

586   ATG TTC GGT GTC GGC TAT CGC TTC AAG TAA    612
196    M   F   G   V   G   Y   R   F   K   *
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Figure 3: Nucleotides and predicted amino acid sequences of OmpW protein of *A. hydrophila* strain Ah1sa5. Underlined amino acids are antigenic sites and 9 antigenic sites were recognized. The stop codon is represented by an asterisk.

Expression and Western Blots Analysis of Recombinant Protein

The pET102/D-OmpTs and pET102/D-OmpW recombinant plasmids were successfully transformed into expression host, *E. coli* BL21 Star™ (DE3) cells which was verified by colony PCR. The recombinant proteins pET102/D-OmpTs and pET102/D-OmpW were expressed after four hours post induction with 1 mM IPTG. The expression of pET102/D-OmpTs and pET102/D-OmpW recombinant proteins were visualized by SDS PAGE analysis that showed prominent bands of 60 kDa and 45 kDa compared to control empty host *E. coli* alone. The Western blot analysis using rabbit polyclonal antibody of anti whole cells of *A. hydrophila* detected the presence of 60 kDa and 45 kDa molecular mass of recombinant

proteins bands respectively, corresponded to the recombinant fusion tag, of 23 kDa protein containing 37 kDa predicted size corresponded to recombinant proteins of OmpTs and 22 kDa predicted size corresponded to recombinant proteins of OmpW (Figure 4).

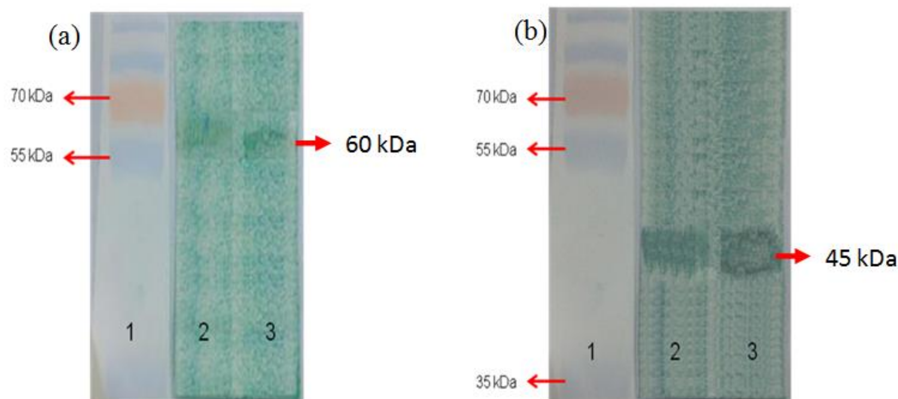

Figure 4: Western immunoblot analysis of the soluble fusion protein of the recombinant OmpTs and OmpW after expression in *E. coli* BL21 Star™ (DE3) using rabbit polyclonal antibody of anti whole cells of *A. hydrophila* as primary antibody, (a) fusion protein of the recombinant OmpTs. Lane 1, standard molecular weight marker; lane 2-3, the soluble cell protein of *E. coli* BL21 Star™ (DE3) expressing pET102/D-OmpTs. (b) fusion protein of the recombinant OmpW. Lane 1, standard molecular weight weight marker; lane 2-3, the soluble cell protein of *E. coli* BL21 Star™ (DE3) expressing pET102/D-OmpW.

Relative Percentage Survival

To confirm the effectiveness of the recombinant OMPs as vaccine candidates, naive African catfish were immunized with three different recombinant cells of vaccine as mentioned earlier. On day 28, they were challenged with *A. hydrophila* bacterial strain Ah1sa5. The post challenged results were summarized in Table 3. Results obtained showed RPS values of 100% in all African catfish groups vaccinated with the three recombinant cell vaccines series, as compared with the placebo vaccine group with 29.42% RPS (Figure 5). The vaccinated groups had a significantly higher protection ($P < 0.05$) following challenged with 4.0 x 10^8 CFU/ml live *Aeromonas hydrophila* than placebo vaccine and control groups. These data suggested that the recombinant cell vaccines expressing OmpTs and OmpW induced protection in African catfish against *A. hydrophila* infection.

Group	Total fish	Mortality (%)	Survival (%)^a	RPS^b
ompTs	10	0.00±0.00	100.00±0.00^x	100.00^x
ompW	10	0.00±0.00	100.00±0.00^x	100.00^x
ompTs+ompW	10	0.00±0.00	100.00±0.00^x	100.00^x
placebo vaccine	10	40.00±10.00	60.00±10.00^y	29.42^y
control	10	56.67±11.54	43.33±11.54^z	N/A

Table 3: Relative Percentage Survival of African catfish after 2 weeks post challenged with virulent strain *Aeromonas hydrophila* Ah1sa5.

^{a}Significant differences ($P < 0.05$) among vaccination groups were determined according to Duncan and are indicated in the table by different letters.
^{b}Relative percent survival = 1 – (% mortality vaccinated fish / % mortality control fish) × 100.

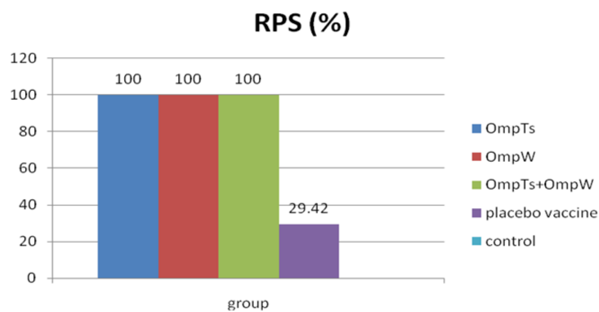

Figure 5: Relative percentage survival (%) in five African catfish groups post challenged by IP injection with 4.0 x 10^8 CFU/ml live *Aeromonas hydrophila*. Five groups which were OmpTs (vaccinated with inactivated recombinant pET102/D-OmpTs), OmpW (vaccinated with inactivated recombinant pET102/D-OmpW), OmpTs+ompW (vaccinated with the combination of both inactivated recombinant pET102/D-OmpTs + pET102/D-OmpW), placebo vaccine (vaccinated with inactivated pET102/D-TOPO vector without insert protein) and control group (unvaccinated).

Clinical Observation

Following infection by IP injection with 4.0 x 10^8 CFU/ml live *A. hydrophila*, the clinical signs became apparent between the control and placebo groups as early as 16 hours post infection. The infected African catfish showed symptoms of lethargy, skin lesions, fin rot, hemorrhagic ulcer, red eyes and hemorrhages on the base of the fins and vent (Figures 6a-6c). In the other hand, none of the clinical findings and mortality was observed in all of the vaccinated groups and fish were in normal healthy condition.

Post mortem examination of the dead African catfish was performed and the lesion correlated with the clinical findings. The lesion observed were pale liver, enlargement of liver, spleen and kidney (Figure 6d). Suspected *Aeromonas* sp. was successfully isolated from the liver, kidney and spleen from all dead fish of control and placebo vaccine groups on specific *Aeromonas* agar (Figure 6e). Moreover, colony PCR screening confirmed the isolates as *Aeromonas hydrophila* based on specific band of 600 bp by using gene specific primer, OmpW.

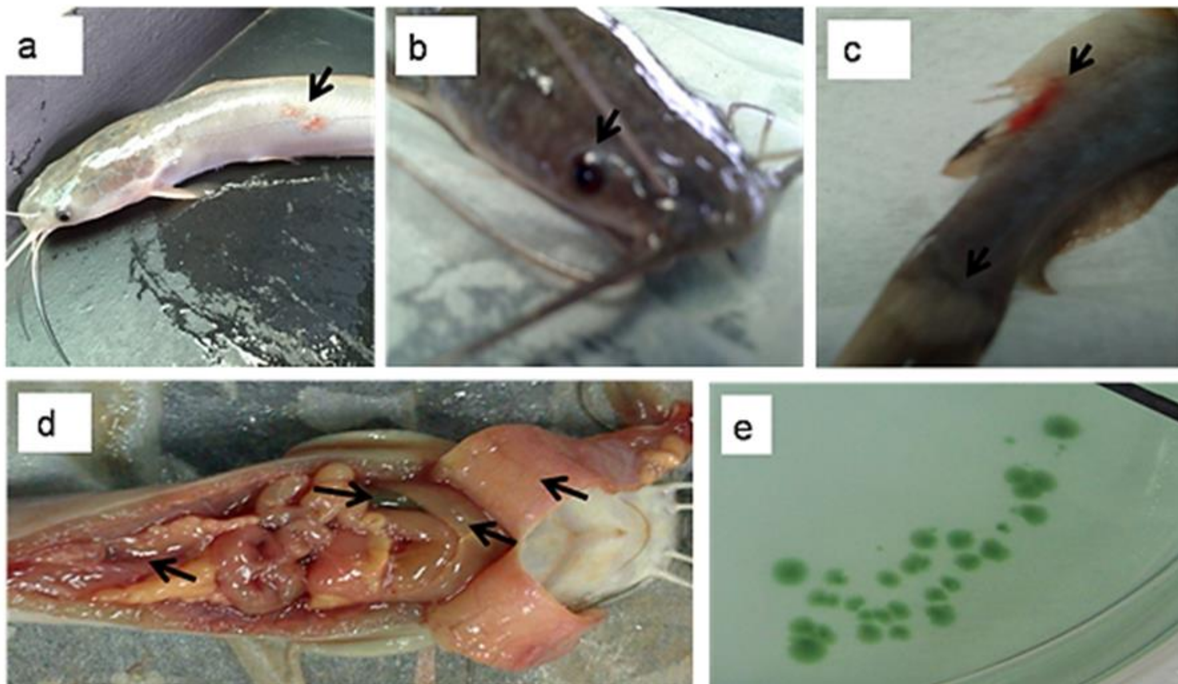

Figure 6: Clinical findings and post mortem analysis from control group 24 hours post challenged with IP injection with 4.0 x 10^8 CFU/ml live *Aeromonas hydrophila.* Black arrow showed (a) ulceration; (b) exophthalmia; (c) hemorrhage on fin and (d) enlargement of gall bladder fill with emerald green secretion, patchy liver, rotten muscle and friable kidney. (e) Suspected *Aeromonas* sp. was successfully isolated from the liver of dead fish on specific *Aeromonas* agar.

Histological Analysis

Histological analysis on the gut of African catfish revealed the existence of GALTs in all vaccinated groups. Vaccination by recombinant cells vaccines pET102/D-OmpTs, pET102/D-OmpW and combination of both pET102/D-OmpTs + pET102/D-OmpW successfully stimulated the aggregations of lymphoid cells within the lamina propria (Figures 7a-7c) and scattered lymphoid cells in the epithelium as early as week 2 post vaccination. There was no lymphoid cells aggregation found in the gut of African catfish of the placebo vaccine and control (unvaccinated) groups (Figures 7d & 7e).

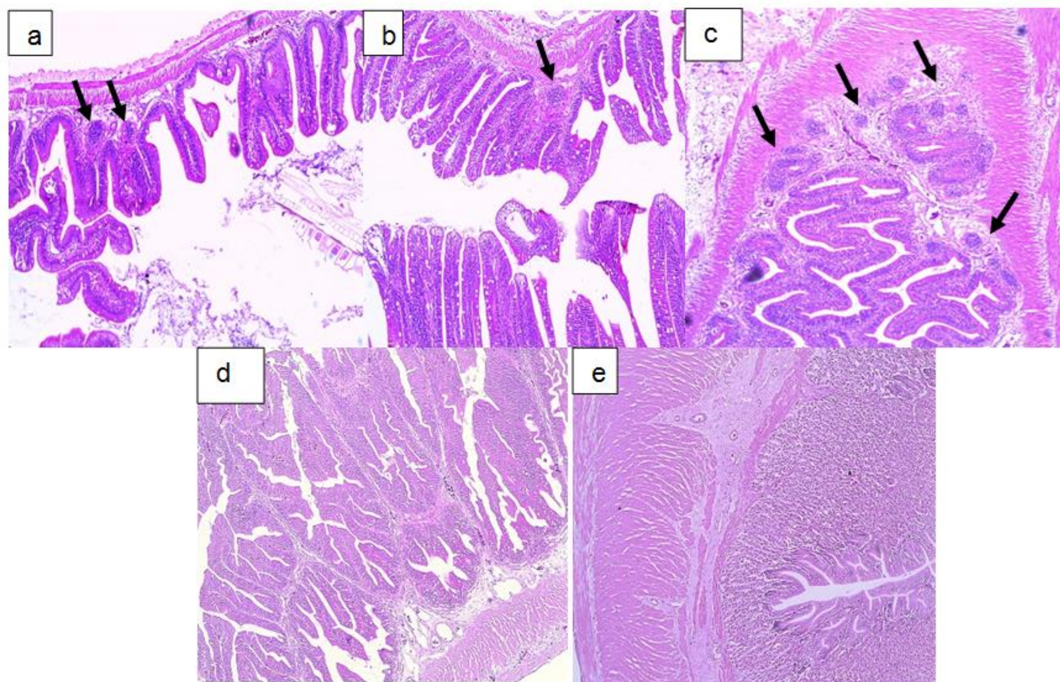

Figure 7: Cross sections of African catfish gut 2 weeks after booster dose given. Arrows show aggregation of lymphoid tissues (GALTs) in lamina propria of vaccinated groups with recombinant OmpTs (a); recombinant OmpW (b); recombinant OmpTs+OmpW (c); no GALTs were present in the lamina propria of African catfish in placebo vaccine group (d); and control group (e) under microscope (40x).

Discussion

Since OMPs are conserved in nature and highly immunogenic due to their exposed epitopes on the cell surface, the OMPs from *Aeromonas* spp. have been identified as suitable candidates for vaccine development in fish [27-29]. In present studies, two antigenic genes from the same species, OmpTs and OmpW were chosen to represent different pathogenic epitopes of *Aeromonas hydrophila* that served as antigens. In this study, the OmpTs gene exhibit homology with other known OMP genes detected in *Aeromonas* sp. isolates such as OmpTs and major adhesion protein (MAH) from *A. hydrophila* and *A. sobria*. While OmpW gene exhibit the homology with other published OmpW gene detected in *A. hydrophila* strains with 88 % - 84 % similarity. In Gram-negative pathogenic bacteria, the OMPs is very crucial acting as the bridge to the hosts which make their components communicate with the host immune system [30]. Conserved regions are important in structural roles, side-chain chemistry, solvent accessibility, and location in the molecule that act as conserved functions such as binding site [31]. Antigenic sites are the protein antigen regions that function in the antibodies binding sites which also known as epitopes [32]. In present study, the antigenic sites were determined. A total of 11 and 9 antigenic sites were identified in OmpTs and OmpW genes, respectively (Figures 2 & 3). The presence of OmpTs and OmpW genes in the outer membrane region of the bacterial cells may be the reason of the large number of exposed epitopes determined [33]. It is proved by study by Maiti, et al. (2009), OmpW was highly recommended in vaccine development due to it is very immunogenic [1]. Signal peptides exist at N-terminal of the amino acid chain, consisting of short amino acids where it directs the

protein to the secretory pathway and cleaved at the cleavage site after completing the translocation [34-36].

The construction of recombinant expression system was achieved by inserting the OmpTs and OmpW genes coding sequences into pET102/D-TOPO®. Results demonstrated that the recombinant fusion genes were properly constructed which were proved and verified by DNA sequencing where the correct orientation showed that the genes were well constructed and kept in frame in the plasmid. In this study, the recombinant proteins were expressed in *E. coli* BL21 cells under the control of the elements from bacteriophage T7 promoter where induction by IPTG induced basal transcription of the gene of interest in *E. coli* BL21 cells [37]. During the investigation, the expression level of OmpTs and OmpW fusion proteins could not be visualized by SDS-PAGE alone therefore, Western immunoblotting using rabbit polyclonal antibody of anti whole cells of *A. hydrophila* to detect and confirm the desired proteins. Immunoblotting of recombinant proteins pET102/D-OmpTs and pET102/D-OmpW revealed the presence of 60 kDa and 45 kDa protein bands respectively after four hours post induction with IPTG that corresponding to the inserted protein. This concluded that both recombinant proteins were successfully expressed with similar to the hypothetical calculation of the recombinant proteins together with fusion tags (Figure 4). Zhu and Wu (2008) used Anti-His monoclonal antibody as primary antibody to detect the expressed recombinant protein expressing OmpR gene revealed 17 kDa of protein band [38].

Some other successful cloning and expression studies involving OMPs were OmpR of rickettsia-like organism with molecular weight of 19.76 kDa applied as vaccine in oysters [38], Esa1 (87.1 kDa) of *Edwardsiella tarda* as vaccine candidate for Japanese flounder [39]. In the other report, a 40 kDa OmpF from *A. hydrophila* was successfully cloned and expressed and showed good immune protections in murine model [40]. Besides, the conserved OmpW works as effective vaccine candidate against *V. alginolyticus* infections in yellow croakers [41]. The presence of iron and osmotic stress influences the expression for OmpW gene and these findings lead to successful cloning and expression of OmpW (22 kDa) isolated from *A. hydrophila* by using rabbit hyper immune sera (1). Therefore, OmpW gene from this study could be an effective vaccine candidate as well as OmpTs (37 kDa) which is known to be highly immunogenic proteins that can produce and induce immunogenicity in Indian major carp [27,35] when challenged with virulence *A. hydrophila*.

To date, many researchers focus on development of recombinant vaccine as new vaccine strategy harmless effect to induce immune response against pathogen with [42]. Outer membrane protein (OMP) is one of the subunit parts that is highly immunogenic in bacteria [43]. Guo, et al. (2013) developed recombinant bivalent vaccine for American eels expressing OMP of porin II of *A. hydrophila* and OmpS2 of *Edwardsiella tarda* that positively affect specific and non-specific immune parameters that give protection against those two pathogens [44]. The intention in this study was to justify and determine the protective efficacy of inactivated recombinant cell vaccines expressing OmpTs and OmpW in African catfish when challenged with virulent strain *A. hydrophila* Ah1sa5. In these experiment trials, five groups were designed as listed in Table 2. The vaccinated groups consisted of two monovalent vaccines (OmpTs and OmpW) and one bivalent vaccine (OmpTs+OmpW). During the trials, vaccine was given intraperitoneally (IP) injection in the amount of 1 ml/ 100 g containing 1 x 10^7 CFU/ml of the recombinant cells. Vaccine was given twice during the study period, first immunization on day 0 followed by second dose as booster on day 14. Booster dose of vaccine is necessary as a strategy to induce adequate and efficient immune response against infection.

Clinical findings on infected African catfish was observed for two weeks post challenged and observation on day 28 revealed there were different clinical signs among each of the fish. In control and placebo groups, from 16 hours post challenged, some of the fish display one clinical sign while some others showed more than one clinical signs including ulcers, inflammation, with focal hemorrhage, dermal lesions, and hyperemia of the fin bases. A bleeding wound appear on skin, exophthalmia in both or only one eye with eventual bristle of the orbit, swollen with friable kidney and spleen, gall bladder containing emerald green bile, abdominal distention as a result of an edema with dark green pustules on the liver with yellowish foci on the surface, also an accumulation of fluid mix with blood in the scale pockets where blood drained from organs such as liver, kidney and spleen. This post mortem results are in concordance with Janda and Abbott (2010) where they reported that *A. hydrophila* causes hemorrhages sepsis, distinguished by small superficial cuts and localized bleeding which transform to epidermal cuts in fish such as perch, catfish, carp and salmon [45]. The most apparent clinical signs included opaqueness in both or one side of the eyes, coexist with exophthalmia and may end up with bristle of the orbit [16-48].

Skin lesions with inflammation and focal hemorrhage may be imparted to *Aeromonas hydrophila* infections associated with ulcerative skin and may be on the surface of organ or deep within tissue [49]. The present study discloses hemorrhages of the dorsal, pectoral, anal and caudal fins had been seen in the diseased fish. These

results were relatively equivalent to the previous study revealed that the hemorrhage of the fins is protuberant [50]. Findings gathered were also similar by Cipriano, et al. (2001) who stated that the chronic infections of *A. hydrophila* led to dermal ulceration lesions with focal haemorrhages and inflammation [47]. The present study shows that infected African catfish having damaged and showing deteriorated of kidney which is the same to the report by Suprapto, et al. (2005) who proclaimed that kidney attacked by bacterial toxins resulting kidney cells to lose their structural integrity [50]. This is confirmed with the presence of *A. hydrophila* in the tissues of lesion, liver and kidney of control and placebo groups after 16 hours post infection. However, the bacteria were not present in all vaccinated groups after 11 days post infection and the disappearance of the pathogenic bacteria could be due to the development of effective immunity [8]. In this experiment, inflammation can be observed within 24 hours post infection in control and placebo groups and fish began to die at 16 hours post infection. The study also showed that at the level of macroscopic and microscopic examinations, no parasites were observed.

The present study showed that the RPS in the African catfish vaccinated with recombinant cell vaccines (100 %) were significantly higher ($P<0.05$) than the placebo vaccine group (29.42 %). The recombinant cell vaccines could produce 100 % protective effect in African catfish against *A. hydrophila* challenge as compared to African catfish without vaccine immunization. Both the recombinant cells of bivalent and monovalent vaccines give same level protection in term of survival rate therefore, further study in immune response level is needed to further explain the vaccine efficacy in inducing immunity. The study was explained by Shoemaker, et al. (2012) [51] where antibody response of tilapia vaccinated with bivalent vaccine of *Streptococcus iniae* and *Vibrio vulnificus* showed significance differences to monovalent vaccine [51]. This indicated that the developed recombinant cell vaccines could at least provide the same magnitude of protection as the other native major adhesion. All these findings suggest that these recombinant cell vaccines have the potential to be developed into an effective vaccine in fish against *A. hydrophila*.

Early review by Hart and friends (1988) showed that presence of GALTs as indicator of immunity level increase as positive result and significance of vaccination in protection of fish against infectious disease [52]. Study by Firdaus-Nawi, et al. (2011) previously showed aggregations of GALTs were observed in lamina propria of the tilapia gut when orally vaccinated with formalin killed *Streptococcus agalactiae* [53]. Moreover, significant different of GALTs numbers observed between fish vaccinated once per week and five times per week and there were no GALTs observed in control fish, fed with only commercial feed. This is in agreement with our study where the presence of GALTs appeared in all vaccinated groups but not in control and placebo groups. This level of protection may be due to high antibody responses which increase the number of GALTs in vaccinated groups [54]. In future, study on evaluating these inactivated recombinant vaccines effectiveness and antibody responses produced after vaccination are necessary in order to develop and produce a good potential vaccine. In addition, dosage determination, application of adjuvant and administration method is also necessary to produce less cost and affordable vaccine for farmers.

Conclusion

We successfully cloned and expressed recombinant fusion protein pET102/D-OmpTs and pET102/D-OmpW. These potential genes were used as vaccine antigen to prevent bacterial infection in African catfish in the form of recombinant cell vaccines and as bivalent vaccine candidate. The high survival of fish and the presence of GALTs showed that our recombinant cell vaccines could be used in future aquaculture practice for freshwater fish.

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