



# Utilizing outer membrane proteins (OMPs) as a vaccine to manage motile *Aeromonas* septicemia (MAS) in Nile tilapia (*Oreochromis niloticus*) with evaluating the vaccine immunoprotective effectiveness

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## Abstract

Motile aeromonads are an opportunistic and heterogenic bacteria that cause motile *Aeromonas* septicemia (MAS) which is accompanied with variety of clinical signs and lesions in fish. It has been found that their outer membrane proteins (OMPs) are highly immunogenic and may be used as a vaccine candidate. The present study was undertaken to evaluate OMP protection against *Aeromonas hydrophila* (*A. hydrophila*) strain ATCC 7966 (A5 and A8) and *A. hydrophila* strain DSM 30,187 (A6). Pathogenicity tests revealed that out of 12 isolated strains, seven were highly pathogenic, three were moderate, and two were avirulent. SDS-PAGE analysis of OMPs showed several protein bands (7–22) of different molecular weights ranging from 14 to 346 kDa. The common protein band was 39 kDa. It seems that pathogenicity and the number of protein bands are related in the highly pathogenic strains. Challenged *A. hydrophila* for Nile tilapia (*O. niloticus*) previously actively immunized with OMPs (A4, A5, A6, A8, and A11) showed a significant increase in relative percent survival (RPS) ranging from 50 to 100%. Immunological parameters such as immunoglobulin M (IgM), antioxidants (SOD, GSH, and CAT), and nitric oxide (NO) and expression of pro-inflammatory cytokines, interleukin 1 $\beta$  (IL-1 $\beta$ ), and tumor necrosis factor- $\alpha$  (TNF $\alpha$ ) showed a significant increase in OMP-immunized fish. Challenged *A. hydrophila* for *O. niloticus* previously passively immunized with OMPs (A4, A5, A6, A8, and A11) in fish-raised sera showed a significant increase in RPS (20 to 100%) in the vaccinated groups. It is concluded that OMPs from *Aeromonas* spp. have been identified as suitable target for protection development, especially in the case of heterogenic bacteria.

**Keywords** *Aeromonas hydrophila* · OMPs · *Oreochromis niloticus* · Immune response · Protection

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## Introduction

Egypt is first in Africa and sixth globally in aquaculture production, specifically in tilapia production, which is the main freshwater cultured fish (USDA 2022). The aquaculture industry has been subjected to some emerging and re-emerging outbreaks, coupled with insufficient diagnostic labs and diagnostic tools may be the main cause of high losses (Dadar et al. 2017; Faisal et al. 1989). Motile *Aeromonas* septicemia (MAS) is a common serious disease caused by *Aeromonas* spp. These are the most important bacteria for the aquaculture industry, mainly *Aeromonas hydrophila* (*A. hydrophila*) (Faisal et al. 1989). An emerging pathotype of *Aeromonads* have been responsible for widespread severe economic losses in the aquaculture industry (Faisal et al. 1989). Type III secretion system (T3SS), lipopolysaccharide (LPS), outer membrane proteins (OMPs), pili and flagella, and extracellular factors like exo-toxin, aerolysins, hemolysins, enterotoxin, and siderophore are some of the virulence factors that contribute to the complex and multifaceted pathogenicity of *Aeromonads* infection (Chopra et al. 2000; Peatman et al. 2018; Pablos et al. 2009). Fish manifested signs of MAS such as severe hemorrhagic dermatitis, orbital cellulitis, and ocular rupture (Janda and Abbott 2010). Because *A. hydrophila* isolates are heterogenic and the bacteria are becoming more resistant to a variety of antibiotics, it is challenging to stop disease outbreaks caused by this bacterium (Baumgartner et al. 2017). Thus, immunoprophylaxis is a risk-free way to stop outbreaks in aquaculture. But there is no commercial vaccination for *A. hydrophila* at this time (Daskalov 2006). This may be because of the insufficient capacity to meet the efficacy, safety, or cost-effectiveness necessary for the vaccine formulations that have been tried to date (Fang et al. 2004). Bacterial OMPs have exposed epitopes on their cell surface that enable them to readily engage with the host immune system, making them highly immunogenic and promising candidates for vaccine development (Clark and Cassidy-Hanley 2005; Lin et al. 2002). Fang et al. (2004) showed significant protection against two isolates of *A. hydrophila* in blue gourami, *Trichogaster trichopterus*, immunized with a recombinant 43 kDa OMP. More recently, a recombinant OMP (37 kDa) of *A. hydrophila* was produced and proved to be immunogenic in rohu carp (Thirumalaikumar et al. 2022).

Understanding the fish immune system is crucial for the creation of complementary therapies and preventative measures against disease outbreaks (Khushiramani et al. 2007). Immunoglobulin M and cytokine genes are crucial for controlling fish immunological function (Aoki and Ikuo 2006). After other antioxidant molecules run out, antioxidant enzymes become essential in the fight against oxidative stress brought on by toxicants (Lindenstrom et al. 2004). These enzymes are essential in oxidative stress to deal with free radicals, causing multiple disruptions. They include superoxide dismutase (SOD), which converts superoxide anion radicals to hydrogen peroxide, and catalase (CAT), which reduces hydrogen peroxide to water (Martinez-Alvarez et al. 2005; Pinto et al. 2003; Tripathi et al. 2006). To test the immunoprotective effectiveness of *Aeromonad* isolates of outer membrane proteins (OMPs) on *Oreochromis niloticus*, a potential vaccine was being developed. It is concluded that OMPs from *Aeromonas* spp. have been identified as suitable targets for protection development, especially in the case of heterogenic bacteria.

## Materials and methods

### Fish and experimental conditions

Batch of apparently healthy *O. niloticus* ( $30 \pm 5$  g body weight) used in this study were obtained from certified private fish farm in Kafr El Sheikh and transferred to the laboratory of Aquatic Animal Medicine Department, Faculty of Veterinary Medicine, Benha University, Moshtohor, Egypt, in large tanks supplied with oxygen with no previous history of disease underwent external inspection before being assigned to experimental groups then also examined for health status immediately upon arrival, including abnormal movement, faded or darkened pigmentation, skin lesions, fin and tail erosion, and external fungi or parasites (Amlacher 1970).

For 2 weeks, fish were housed in 750-L fiber glass tanks for acclimation to the lab conditions. Dechlorinated tap water and an air pump were provided for every tank. A static system was used where the water was renewed at a rate of 20% every 24 h and uneaten foods were removed every day.

The water temperature was adjusted to  $26 \pm 2$  °C, dissolved oxygen content to  $6 \pm 0.5$  mg/L, ammonia concentration  $0.53 \pm 0.07$  mg/L, and pH to  $7 \pm 0.2$ . Fish were fed a basal diet at 4% of their body weight twice a day. Fish samples were sacrificed at random, and their organs were tested for parasites, mycoses, and bacteria to ensure they were free from any pathogens as described by Austin and Austin (2007).

### Aeromonad strains

Twelve well-identified aeromonads bacterial spp. named (A1, A2, A3, A4, A5, A6, A7, A8, A9, A10, A11, and A12) isolated from clinically diseased freshwater fishes (*Oreochromis niloticus*, *Mugil cephalus*, and *Clarias gariepinus*) (Matter et al. 2018) were used in this study. The preserved bacterial isolates at  $-80$  °C (Hollander and Nell 1954) were also identified again using API 20E and molecular identification to ensure that the preserve culture was pure and were not contaminated then were inoculated into 20 ml Tryptic Soya Broth (TSB) media and grown at 28 °C for 24-h incubation and then different doses were adjusted for challenging fish. The bacterial growth was harvested in sterile physiological saline NaCl (0.9%) and bacterial cell concentrations (1.5, 3, 6,  $9 \times 10^8$  cells/ml) was adjusted using spectrophotometer with absorbance 0.1, 0.257, 0.451, and 0.582, respectively, at wavelength 620 nm (El- Asely et al. 2014).

### Phylogenetic analyses for identification of challenged *A. hydrophila* strains

Seven selected PCR products as representative strains were subjected to sequencing to identify *A. hydrophila* strains. Using the QIAquick PCR Purification Kit (QIAGEN, USA), the PCR products were purified. Purified PCR products were sequenced on an AB 3500 Genetic Analyzer using a Big Dye Terminator Kit, in accordance with the manufacturer's instructions. The sequences that were produced were examined using Applied Biosystem software (version 6). The resulting sequence was examined against the database of strain types and published, accepted prokaryotic nomenclature using the

neighbor joining blast tree and the Basic Local Alignment Search Tool (BLAST) tool from the National Center for Biotechnology Information (NCBI).

### Assessment of *Aeromonad* isolate virulence

The intensity of *Aeromonad* isolate virulence was evaluated as follows; a total of 500 fish were divided into 13 groups. Each group from the first 12 groups was divided into 4 subgroups. Ten fish in each subgroup were injected i.p. (0.2 mL) with different bacterial cell concentration (0.3, 0.6, 1.2,  $1.8 \times 10^8$  cells/fish) for the 12 *Aeromonad* isolates. Group No. 13 was kept as control ( $n=20$  fish) which was inoculated with sterile phosphate buffer saline (PBS, pH 7.5). On the day of the challenge, the experimental fish received no feed. After the challenge the following day, food was provided twice a day for the duration of the trial at a rate of 4% of body weight. For 7 days following the injection, the clinical signs and mortality rates in both the infected and control groups were documented every day. After being re-isolated from several organs (liver, kidneys, and spleen), the bacterial strains were fully identified as previously described (Matter et al. 2018). The restored bacteria from  $-80^\circ\text{C}$  freezing should be passage several times into fish (Nile tilapia) to restore their virulence due to long freezing led to loss the virulence of previously isolated bacteria.

### Extraction of OMPs

The bacterial isolates were cultured on brain-heart infusion broth at  $28^\circ\text{C}$  for 18 h in order to extract outer membrane proteins. The cells were then recovered by centrifugation at  $2550 \times g$  for 20 min at  $4^\circ\text{C}$ . Once the cells reached an OD620 (1.5), they were again suspended in 5 mL of 0.5 TRIS HCl (pH 7.4) and sonicated five times for a duration of 1 min on ice. Centrifugation was used to remove cell debris for 10 min at  $4^\circ\text{C}$  at  $2550 \times g$ . A 20% N-lauroyl sarcosine sodium salt (Sigma, USA) was then added in 0.5 mL and allowed to sit at room temperature for 30 min. After being centrifuged at  $17,650 \times g$  for an hour at  $4^\circ\text{C}$ , the OMPs were twice cleaned with PBS, suspended in fresh PBS and frozen at  $-20^\circ\text{C}$  (Abbass et al. 2010).

### SDS-PAGE

Electrophoresis for 12 *Aeromonad* OMPs was performed using pre-stained high molecular weight standard markers (Sigma, USA). OMPs were run on a 12% (w/v) acrylamide separating gel after being placed onto a 4% stacking gel. Gels were de-stained with 10% (v/v) acetic acid and 40% (v/v) methanol after being stained with Coomassie brilliant blue (R-250), Sigma, USA. Dice similarity index was calculated for every isolate as described by Laemmli (1970) and dendrogram was then constructed using (Advanced American biotechnology, UPG MA, USA).

### Immunoprotective efficiency of *Aeromonad* OMPs antigen in *O. niloticus*

#### Safety test

Safety of crude OMPs antigen on *O. niloticus* ( $30 \pm 5$  g body weight) was performed according to Midtlyng (2016) with minor modification briefly; three fish were IP

inoculated with 0.1 ml of OMPs antigen suspension (20 µg protein) of the highly virulent *A. hydrophila* (A6) in triplicate, keeping three fish in separate aquarium as control which were injected IP with sterile saline. All fish were placed under investigation for 7 days, with recording of clinical signs, developed lesions, and mortality rates.

## Active immunization of *O. niloticus* with OMPs antigens

**Preparation of the OMPs** Adequate amount of separated crude OMPs from the five representative strains of Aeromonads (four were separated from *O. niloticus* isolates: A4, A5, A6, and A11). While the fifth was separated from *C. gariepinus*, (A8) was used. Protein concentration in OMPs suspension was carried out by the method of Lowry et al. (1951), and preserved at -20 till use.

**Preparation of challenge bacteria** The bacteria virulent strains (A5, A6, and A8) were grown on brain-heart infusion broth at 28 °C for 18 h. The bacterial cells were collected by centrifugation at 2550 × g for 20 min at 4 °C and adjusted for 9 × 10<sup>8</sup> cells/mL (El-Asely et al. 2014).

**Experimental design** A total of 288 *O. niloticus* (30 ± 5 g body weight) from the acclimated stock were divided into six groups (16 fish each in triplicate) and placed in well-prepared aquaria. One group was kept as control (control positive) and the remaining five groups (A, B, C, D, and E) were injected 0.1 mL of emulsified OMPs of A4, A5, A6, A8, and A11, respectively, with Freund's complete adjuvant (FCA) (first dose). Thirty-four days post injection (Poobalane 2007), fish received booster dose at the same dose (0.1 mL) of emulsified OMPs with Freund's incomplete adjuvant (FIA) for 17 days (Poobalane 2007). The control groups (negative and positive) were injected with PBS. Fifty-one days post injection (at the end of the experiment), serum and tissue samples (liver and spleen) were collected from 18 fish in each group that were properly preserved till use. For evaluation of OMPs protection, the remaining 30 fish from each group were challenged IP with 0.2 mL of 1.8 × 10<sup>8</sup> cells/fish for the three *A. hydrophila* isolates (A5, A6, and A8) at the opposite side where they had been injected with the booster injection. Briefly, the challenged bacteria were injected as the following: group (B) was challenged with its corresponding *A. hydrophila* (A5), group (C) was challenged with its corresponding *A. hydrophila* (A6), and group (D) was challenged with its corresponding *A. hydrophila* (A8). Groups (A), (E), and (cont. positive) were challenged with A6.

**Calculation of relative percentage survival (RPS)** After 28 days post challenge, RPS was calculated using the formula of Amend (1981).

## Effect of active immunization on immune parameters

### Evaluation of serum immunoglobulin M (IgM)

Serum IgM levels in immunized fish was measured spectrophotometrically following the protocol of ELISA kits (Cusabio Biotech Co. Ltd, USA).

## Determination of antioxidant enzymes activities and nitric oxide

Superoxide dismutase enzyme activity (SOD) and catalase activity (CAT) were measured spectrophotometrically at 510 nm and calculated according to Fossati et al. (1980). Glutathione reductase enzyme activity (GSH) was determined colorimetrically at 405 nm and calculated according to Satoh (1978). Nitric oxide enzyme activity (NO) was estimated according to Rajaraman et al. (1998). All enzyme activities were measured by using Bio-diagnostic kits (Egypt).

## Gene expression estimation

After being immunized with *Aeromonad* OMPs, *O. niloticus* immune response was evaluated by assessing the expression of genes that encode the pro-inflammatory cytokines TNF- $\alpha$  and IL-1 $\beta$ . To investigate variations in IL-1 $\beta$  and TNF- $\alpha$  expression, quantitative real-time PCR was performed on specific genes extracted from the spleens of all treated groups as well as the control group. Using the RNeasy Mini Kit (Qiagen, USA) and the manufacturer's instructions, total RNA was extracted. Gene-specific primers were employed in qPCR to measure variations in the expression levels of the genes and listed in Table 1. The cycling conditions of SYBR green real time PCR were performed according to Quantitect SYBR green PCR kit.

The amplification profile was carried out as follows: 40 cycles of primary denaturation at 94°C for 15 s, optimal primer annealing temperature at 62°C for 30 s, and extension at 72°C for 30 s were performed after the initial denaturation at 94°C for 5 min. One cycle of the dissociation curve consists of 1 min of secondary denaturation at 94°C, 1 min of primer annealing at 62°C, and 1 min of final denaturation at 94°C. The software strata gene MX3005P was used to determine CT values and amplification curves. The CT of each sample was compared with that of the control group in order to quantify the variance of gene expression on the RNA of the various samples according to the “ $\Delta\Delta Ct$ ” method stated by Yuan et al. (2006), using the following ratio: ( $2^{-\Delta\Delta Ct}$ ), whereas  $\Delta\Delta Ct = \Delta Ct_{reference} - \Delta Ct_{target}$ .

$$\Delta Ct_{target} = Ct_{control} - Ct_{treatment} \text{ and } \Delta Ct_{reference} = Ct_{control} - Ct_{treatment}$$

**Table 1** The list of oligonucleotide primers and SYBR Green real time PCR made from *O. niloticus* spleen tissue for relative gene expression analysis. “F” and “R” indicate the forward and reverse primers, respectively

Gene	Primer sequence (5'-3')	Reference No.
EF-1 $\alpha$	CCTTCAACGCTCAGGTCATC (F) TGTGGGCAGTGTGGCAATC (R)	65
IL-1 $\beta$	GCTGGAGAGTGTGTGGAAGAACATATAG (F) CCTGGAGCATCATGGCGTG (R)	66
TNF $\alpha$	CCAGAAGCACTAAAGGCGAAGA (F) CCTTGGCTTGTGCTGCTGATC (R)	67

## Passive immunization of *O. niloticus* with anti-OMPs serum

### Experimental design

A total of 90 *O. niloticus* ( $30 \pm 5$  g body weight) from the acclimated stock were divided into six groups: five fish each in triplicate and placed in well-prepared aquaria. One group is the control (cont.) and the remaining 5 groups (1, 2, 3, 4, and 5) were injected 0.1 mL of *O. niloticus* sera raised against the OMPs (A4, A5, A6, A8, and A11, respectively). Cont. group was injected with serum collected from non-vaccinated *O. niloticus*. For the evaluation of OMPs antisera protection, after 24 h, all fish were challenged with i.p. injection of 0.2 mL of  $1.8 \times 10^8$  cells/fish for the three *A. hydrophila* isolates (A5, A6, and A8) at the opposite side where they had been injected with the antiserum (Lafrentz et al. 2003). Briefly, the challenged bacteria were injected as the following: group (2) was challenged with its corresponding *A. hydrophila* (A5) and group (4) was challenged with *A. hydrophila* (A8). Group (3) was challenged with its corresponding *A. hydrophila* (A6), while groups (1), (5), and (cont.) were challenged with (A6).

### Calculation of relative percentage survival (RPS)

After 28 days, the relative percentage survival (RPS) was calculated as in active immunization.

### Statistical analysis

Statistical significance across groups was analyzed using one-way analysis of variance with post hoc Duncan tests, using SPSS 16.0 software to determine the significant changes in different parameters. A  $p$ -value  $< 0.05$  was the threshold for significance (Yuan et al. 2006).

## Results

### Virulence of Aeromonad isolates

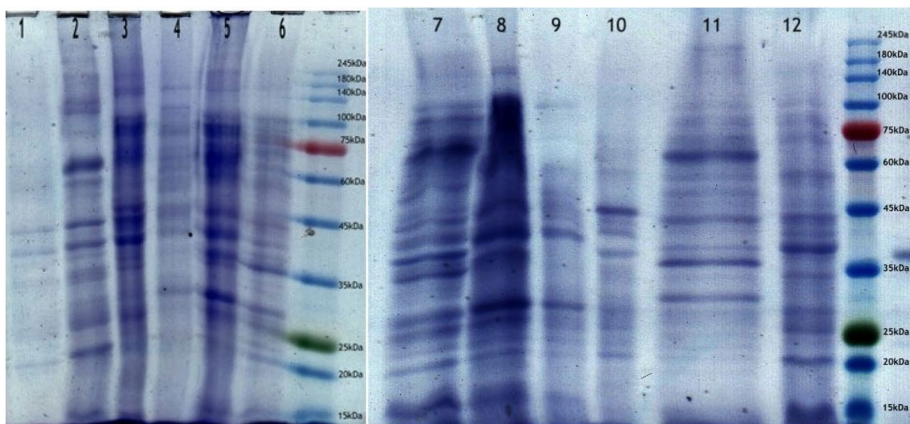
Starting from the 1st day until the 3rd day, the mortality rates in infected fish showed a high rate, and then decreased gradually or stopped till the 7th day post-injection. Out of the 12 tested Aeromonad isolates, 7 isolates (A2, A3, A4, A5, A6, A11, and A12) were found to be virulent strains, resulting in 80–100% mortality when injected with a dose  $1.8 \times 10^8$  cells/fish. The remainder of the three strains (A7, A8, and A9) showed moderate virulence, reaching 40, 60, and 60%, respectively, while A1 and A10 recorded no mortalities (avirulent strains) (Table 2).

### SDS-PAGE analysis of OMPs

Considerable differences were found in the number of OMP bands from the tested Aeromonads examined by SDS-PAGE, which ranged from 7 to 22 with molecular weights ranging from 14 to 346 kDa (Fig. 1). The most common bands were 22, 37, and 39 kDa in most of the strains. The dominant band (39 kDa) was seen in 8 out of the 12 isolates.

**Table 2** Mortality rate in intra-peritoneally infected *O. niloticus* with 0.2 ml *Aeromonas* isolates at doses 0.3, 0.6, 1.2,  $1.8 \times 10^8$  cells/fish

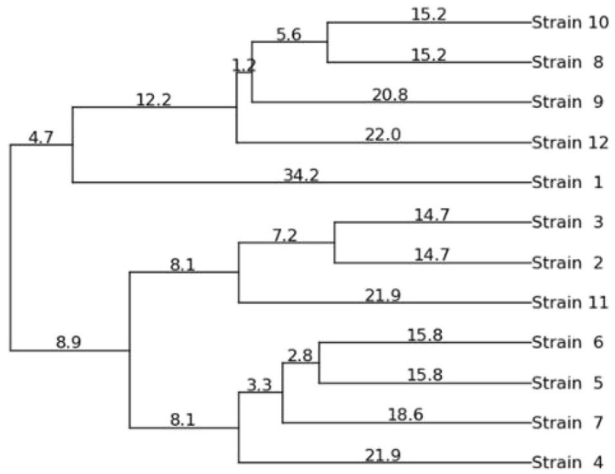
Aeromonad isolates	No. of fish/ group	Mortality %/bacterial cell concentrations (cells/fish)			
		$0.3 \times 10^8$	$0.6 \times 10^8$	$1.2 \times 10^8$	$1.8 \times 10^8$
A1	5	0	0	0	0
A2	5	0	20	60	100
A3	5	0	0	40	80
A4	5	0	20	60	100
A5	5	0	20	60	100
A6	5	20	40	80	100
A7	5	0	0	20	40
A8	5	0	0	20	60
A9	5	0	0	20	60
A10	5	0	0	0	0
A11	5	20	20	60	80
A12	5	0	20	60	80
Sterile saline as cont.	10	0	0	0	0

**Fig. 1** Coomassie blue stained SDS-PAGE analysis of OMPs extracted from *Aeromonas* spp. Lanes (1–12); M, marker; lane (1, 2): *Aeromonas* sp. OMPs protein bands isolated from *Mugil cephalus*; lane (3, 4, 5, 6, 7, 11, and 12): *Aeromonas* sp. OMPs protein bands isolated from *Oreochromis niloticus*; lane (8, 9, and 10): *Aeromonas* spp. OMPs protein bands isolated from *Clarias gariepinus*. M indicates the migration of the protein molecular weight (kDa) marker

Dendrogram analysis revealed that 12 aeromonad isolates were divided into 2 clusters. The first cluster includes A1, A8, A9, A10, and A12, which are then divided into two sub-clusters. The first sub-cluster includes A8, A9, A10, and A12, and the second sub-cluster includes only A1. The first sub-cluster was divided into two groups. The first group includes A8, A9, and A10, with no difference between A8 and A10, while the second group includes only A12 (Fig. 2). The second cluster includes A2, A3, A4, A5, A6, A7, and A11, and then is divided into two sub-clusters. The first sub-cluster includes A2, A3, and A11,



**Fig. 2** Similarity dendrograms of *Aeromonas* species isolated from different fish species. 1–12 represent the tested strains



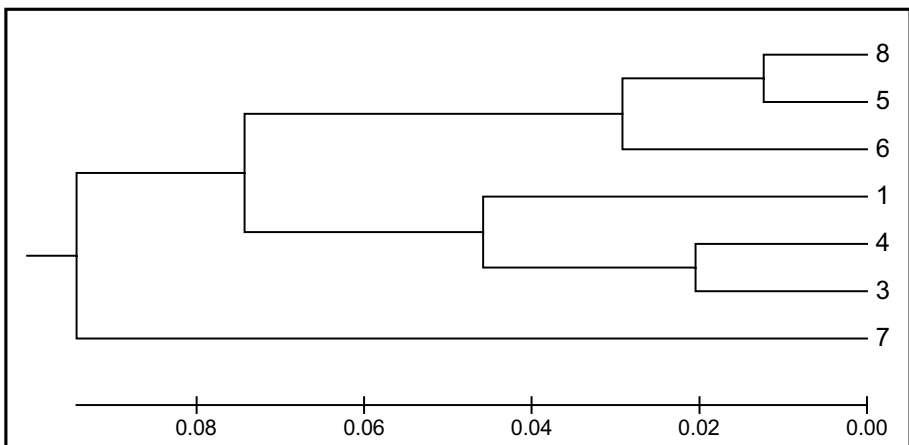
with no difference between A2 and A3, and the second sub-cluster includes A4, A5, A6, A7, then divided into two groups. The first group includes A5, A6, and A7, with no difference between A5 and A6, while the second group includes only A4 (Fig. 2).

**Relationship between pathogenicity and OMP profile**

Highly pathogenic strains (A2, A3, A4, A5, A6, A11, and A12) contained OMP bands (18, 17, 15, 17, 22, 14, and 13), respectively, in corresponding strains.

**Phylogenetic analyses for identification of challenged *A. hydrophila* strains**

Based upon the sequence analysis of the 7 *Aeromonad* isolates using housekeeping genes *gyrB*, A5, A6, and A8 were identified as *A. hydrophila*, showing high phylogenetic



**Fig. 3** Similarity dendrogram of *Aeromonas* species isolates constructed on the basis of UPGMA analysis

relatedness (Fig. 3), and A3 and A4 are *A. finlandiensis* with 100% similarity, while A7 was identified as *A. bestiarum* (Fig. 3) and was assembled in a separate tree, with only 50% similarity with other isolates, and A1 was recognized as *A. rivipollensis*, which showed 50% similarity with A3 and A4 (Fig. 3).

## Active immunization of *O. niloticus* with OMP antigens

### Safety test

The safety test revealed that the injected crude OMPs were safe; no clinical signs or lesions were reported.

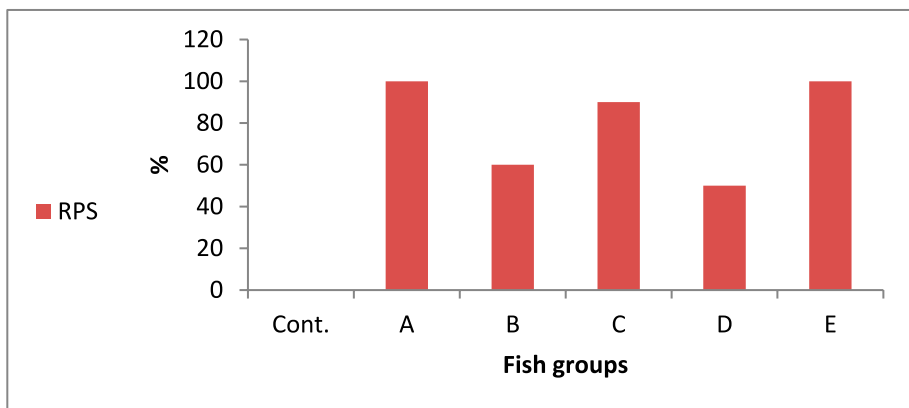
### Evaluation of the protective effect of OMP antigen on *O. niloticus* (RPS)

The relative percentage survival (RPS) 4 weeks post-challenge with pathogenic *A. hydrophila* isolates showed that group (B) was 60% when challenged with *A. hydrophila* (A5) while group (C) was 90% RPS when challenged with *A. hydrophila* (A6). Group (D) was 50% RPS when challenged with *A. hydrophila* (A8). In addition, groups (A), (E), and (cont.) had 100, 100, and 0% RPS, respectively, when challenged with the highly virulent strain *A. hydrophila* (A6) (Fig. 4).

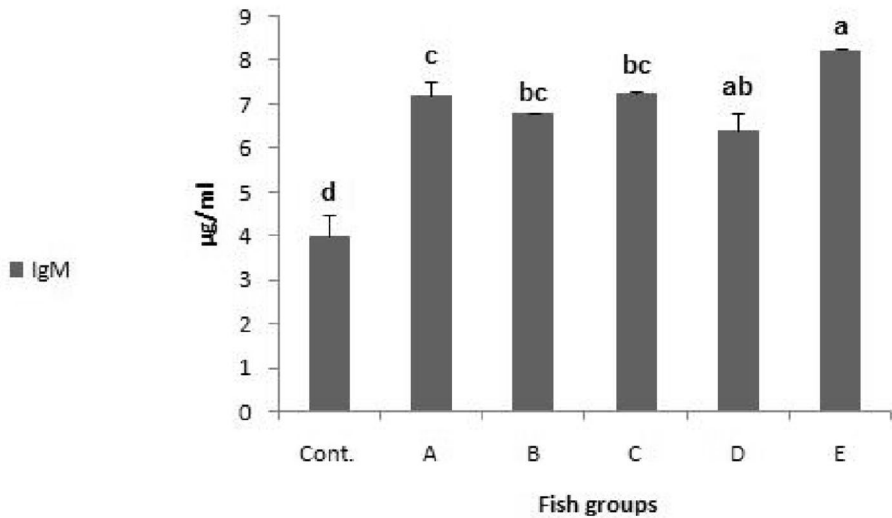
## Effect of active immunization on immune parameters

### Effect on IgM levels

*O. niloticus*-immunized groups (A, B, C, D, and E) with crude OMPs showed a significant increase ( $p < 0.05$ ) in serum IgM levels compared to the control group, with the highest levels recorded in groups (A), (C), and (E) and the lowest detected in group (D) (Fig. 5).



**Fig. 4** Relative percent survival (RPS) of the OMPs-immunized and PBS-immunized *O. niloticus* (cont.) after challenged with *A. hydrophila*. Cont. indicates control group and A to E indicate treated groups



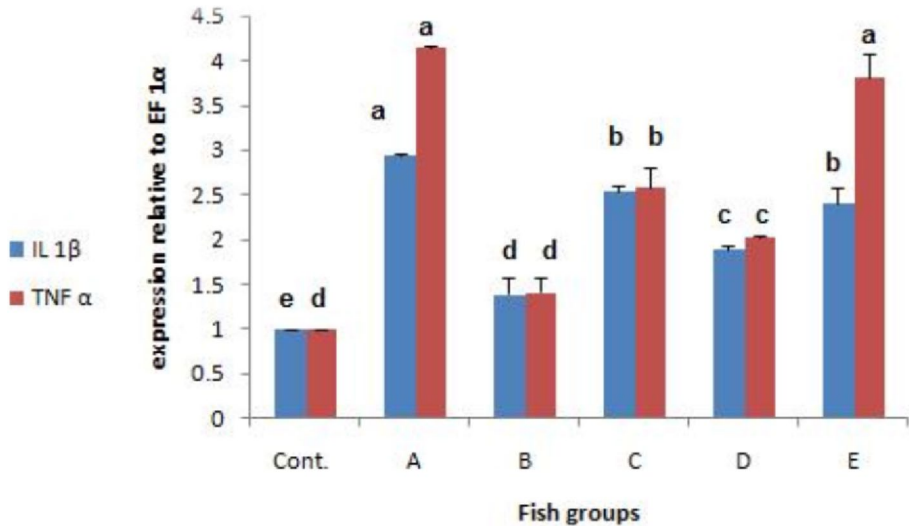
**Fig. 5** Effect of OMPs immunization in immune parameters (IgM level). Cont. indicates unimmunized fish and A to E indicate immunized groups. The data represent mean  $\pm$  SD for at least six fish in each group

**Table 3** The effect of outer membrane proteins (OMPs) antigen with adjuvant on antioxidant enzymes and NO activities (data are represented by Average  $\pm$  SD), value with different letters in the same column are significantly different ( $p < 0.05$ ,  $n = 3$ )

Groups	SOD(U/g. tissues)	CAT (U/g. tissues)	GSH (mg/g. tissue)	NO ( $\mu\text{mol/L}$ )
Cont.	1120.70 <sup>d</sup> $\pm$ 19.91	352.74 <sup>d</sup> $\pm$ 26.39357	17.55 <sup>a</sup> $\pm$ 0.53	25.2800 <sup>d</sup> $\pm$ 0.99304
A	1236.10 <sup>a</sup> $\pm$ 10.98	631.72 <sup>a</sup> $\pm$ 5.40689	18.07 <sup>a</sup> $\pm$ 1.05	63.6767 <sup>c</sup> $\pm$ 1.15759
B	1189.70 <sup>b</sup> $\pm$ 19.94	300.80 <sup>d</sup> $\pm$ 2.04382	16.01 <sup>a</sup> $\pm$ 0.77	13.0033 <sup>d</sup> $\pm$ 0.88335
C	1258.60 <sup>a</sup> $\pm$ 19.91	501.84 <sup>b</sup> $\pm$ 10.90326	19.06 <sup>a</sup> $\pm$ 0.04	72.2367 <sup>a</sup> $\pm$ 1.39430
D	1189.70 <sup>b</sup> $\pm$ 19.91	317.22 <sup>d</sup> $\pm$ 3.54204	16.57 <sup>a</sup> $\pm$ 1.92	12.1767 <sup>d</sup> $\pm$ 1.44049
E	1262.70 <sup>a</sup> $\pm$ 22.28	440.21 <sup>c</sup> $\pm$ 11.25833	19.19 <sup>a</sup> $\pm$ 0.60	57.6933 <sup>b</sup> $\pm$ 1.39430

### Effect of active immunization on antioxidant enzymes and nitric oxide (NO)

It was recorded that the SOD activity of all OMP-immunized groups (A, B, C, D, and E) was significantly increased ( $p < 0.05$ ) in comparison with the control group (Table 3). The catalase activity of *O. niloticus*-immunized groups (A, C, and E) showed a significant ( $p < 0.05$ ) increase compared to the control group, but no significant changes were recorded in the catalase activity in groups (B) and (D) (Table 3). Glutathione reductase enzyme activity (GSH) did not show significant changes in all immunized groups compared to control (Table 3). NO levels showed a significant increase ( $p < 0.05$ ) in immunized groups (A, C, and E), while no significant changes were observed in the values of NO in groups (B) and (D) (Table 3).



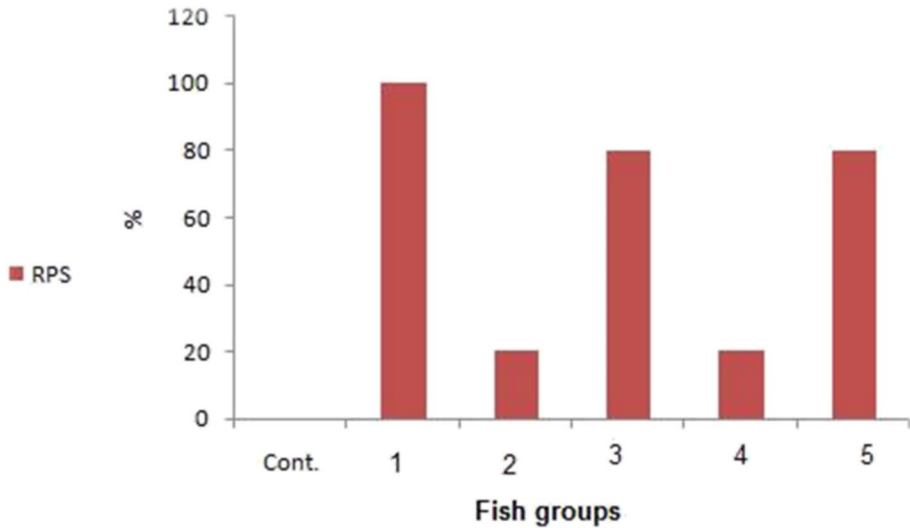
**Fig. 6** Expression of IL- 1 $\beta$  and TNF $\alpha$  relative to EF-1 $\alpha$  in the spleen of *O. niloticus* in the immunized groups at the end of experiment (51 days). “cont.” indicates unimmunized fish and A to E indicate immunized groups. The data represent mean  $\pm$  SD

### Effect of active immunization on expression of IL-1 $\beta$ and TNF- $\alpha$ in the spleen of *O. niloticus*

IL-1 $\beta$  expression was significantly upregulated in treated groups (A), (C), and (E) than in the control group. But groups (B) and (D) exhibited a slight upregulation of IL-1 $\beta$  (Fig. 6). The same results were observed in TNF $\alpha$  expression, which showed significant upregulation in groups immunized (A), (C), and (E) compared to the control group. No significant changes were recorded in the expression level of TNF $\alpha$  in the spleen tissue of the *O. niloticus* immunized group (B) (Fig. 6).

### Passive immunization

Sera rose against crude OMPs used to passively immunize fish. Groups that received sera against A4, A6, and A11 and were challenged with *A. hydrophila* (A6) showed the highest degree of protection with RPS 100, 80, and 80%, respectively, while fish immunized with sera against A5 and A8 showed the same lowest protection with RPS 20% when challenged with each corresponding strain (Fig. 7).



**Fig. 7** Relative percent survival (RPS) of the OMPs and cont. groups raised sera in *O. niloticus* after challenged with *A. hydrophila*. Cont. indicates control group and 1 to 5 indicate immunized groups

## Discussion

Aeromonads are thought to be a significant bacterial issue for the advancement of aquaculture. According to certain theories, aquatic environments are significant aeromonad reservoirs, and these bacteria can proliferate and flourish in a range of limnetic conditions (Lafrentz et al. 2003).

Mortality in the current investigation started on the first day following the challenge. After injecting each of the 12 tested isolates with  $1.8 \times 10^8$  cells/mL, seven of them proved to be virulent, and *A. hydrophila*(A6) was the most virulent strain with a 100% mortality rate. In the same respect, Rasmia Hanafy (2005), El Deen et al. (2014), and Basma et al. (2016) observed that mortality rates among the experimentally infected *O. niloticus* varied according to the strains of *A. hydrophila*, with mortalities ranging from 50 to 100% by i.p. injection of each with 0.2 mL of  $9 \times 10^8$  cells/mL suspension. A variety of virulence factors are produced by virulent strains and *Aeromonas* species that have varying mortalities. These virulence factors can be found in two forms: extracellular products such as cytotoxic, hemolytic, and enterotoxic proteins, and cell-associated structures such as pili, flagella, outer membrane proteins, lipopolysaccharide, and capsules (Handfield et al. 1996). Because of their virulence, the bacteria are able to infiltrate, enter, multiply, and harm the tissues of their hosts. They can also evade the host's defensive mechanism and spread, ultimately leading to the host's death (Abdullah et al. 2003; Pang et al. 2015).

The OMP profiles of the Aeromonads isolated from three freshwater fishes revealed three bands in several isolates and weighed around 22, 37, and 39 kDa, with the major band being 39 kDa. Thangaviji et al. (2012) found a band around 37 kDa in OMP profiles in three *Aeromonas* spp. strains isolated from carp and goldfish. Meanwhile, Khushiramani et al. (2008) isolated 3–4 high-intensity OMP bands from 40 strains of *Aeromonas* spp. with a range of 25–45 kDa.

The relatedness between pathogenicity and the number of protein bands showed that highly pathogenic strains (A2, A3, A4, A5, A6, A11, and A12) contained several OMP bands (18, 17, 15, 17, 22, 14, and 13 bands, respectively) corresponding to strains. Using SDS gel electrophoresis, Peng et al. (2014) evaluated isolates of *A. hydrophila* against eight antimicrobial drugs and categorized the samples according to the outer membrane protein (OMP) pattern. The discovery by the scientists that the strains exhibiting comparable OMP profiles also exhibited comparable resistance suggests the possibility of other variables contributing to *A. hydrophila* antimicrobial resistance. The gram-negative bacteria's outer membrane serves as a molecular filter for substances that are hydrophilic (Hancock 1997). Permeability changes in the outer membrane are one of the reasons bacteria have resistance to antibiotics (Chevalier et al. 2000). The development of pores by OMPs is one of their most crucial roles in maintaining the permeability of the outer membrane. This membrane's permeability is a major factor in how susceptible it is to antibiotics. Antibiotic resistance may be influenced by modifications to OMP profiles (Delcour 2009).

Seven strains of *Aeromonads* that were isolated from different fish species and collected from different locations were evaluated for genetic variability and relatedness using UPGMA analysis, which revealed heterogeneity and distance within the phylogenetic tree. Similarly, six isolates of *Aeromonads* were identified by Sarkar et al. (2012) as being *A. hydrophila*; these isolates came from various sources where genetic variability and distance within the phylogenetic tree were seen.

It has been demonstrated that fish that have been actively vaccinated with different *Aeromonad* isolate OMPs exhibit varying degrees of protection when challenged with the relevant pathogens, with RPS reaching 87.5–100%. Even though only a small number of fish were used in the research, OMPs could provide a good vaccine candidate to combat this pathogen. Nearly similar results were obtained by Poobalane (2007), who found that 50 kDa protein induced protection in goldfish against *A. hydrophila* in the active immunization trial. Additionally, Thangaviji et al. (2012) discovered that *C. auratus* vaccinated with recombinant OMP demonstrated a higher percentage of survival (more than 70%) following the virulent *A. hydrophila* challenge. Additionally, they discovered that *C. auratus* vaccinated with *A. hydrophila* OMP and the OMP gene demonstrated protection and that the OMP vaccine was particularly influential in improving the survival rate (80%) following the virulent *A. hydrophila* challenge.

Estimation of immunoglobulin M (IgM) revealed that groups were immunized with A4, A6, and A11 OMPs antigens, which have immunogenic effects in *O. niloticus*. These findings are supported by the results of Thangaviji et al. (2011), who found *C. auratus* vaccinated with *A. hydrophila* OMP and the OMP gene showed protection, and the OMP vaccine was highly influenced to improve immunological parameters. The authors added that bacterial OMP reduced the specific bacteria after the *A. hydrophila* challenge by increasing the production of a higher antibody, and the higher antibody production level may suppress the growth of the *A. hydrophila* load. Similarly, Yin et al. (2008) recorded that protection of *A. hydrophila* with OMP + herbal (*Lonicera japonica* and *Ganoderma lucidum*) immunoadjuvant helped to increase the immunoglobulin M level in *O. niloticus* against the *A. hydrophila* challenge.

In OMPs inoculated with *O. niloticus*, there was a considerable increase in SOD, CAT activity, and NO levels. Halyna et al. (2014) reported nearly similar findings, demonstrating that the OMPs vaccine against furunculosis exposed trout to increased levels of oxidative stress and antioxidant enzyme responses (catalase, glutathione reductase, glutathione peroxidase, and nitric oxide) when compared to the control group. These responses were more

severe in the liver than in other tissues. Similar findings of elevated SOD activity in carp tissues after exposure to the OMP vaccination have been reported (Ural 2013; Oruc 2010).

The immune response of *O. niloticus* after immunization with *Aeromonas* OMPs was assessed by measuring the expression of genes encoding IL-1 $\beta$  and TNF $\alpha$ . IL-1 $\beta$  and TNF $\alpha$  expression were significantly upregulated in all immunized groups compared with control. The higher upregulation is found in the group immunized with A4, A6, and A11, with the maximum level in the group receiving A11. Zhang et al. (2018) reported nearly identical results: the group immunized with recombinant *Lactobacillus casei* expressing OmpAI had significantly ( $p < 0.05$ ) upregulated expression of IL-10, IL-1 $\beta$ , IFN- $\gamma$ , and TNF $\alpha$  genes as compared to control groups, suggesting that inflammatory response and cell immune response were triggered. These cytokines are observable upon stimulation with OMPs, LPs, or microorganisms. TNF- $\alpha$  and IL-8 are two of the inflammatory cytokines that are induced when IL-1 $\beta$  is expressed. Furthermore, Jiang et al. (2016) reported that the expression profiles of several immune-related genes at various points after immunization demonstrated variable upregulations of immune-related genes, such as the pro-inflammatory cytokine IL-1 $\beta$ , the chemokine IL-10, and the liver and spleen of common carp injected in the vaccines with the live attenuated *A. hydrophila* strain XX1LA and the formalin-killed *A. hydrophila* (FKA). The protection seen in passively immunized *O. niloticus* raised sera against *A. hydrophila* with RPS reaches 80–100%. These findings were supported by the results of Poobalane (Poobalane 2007), who found that sera raised against OMP protein bands of 50 kDa induce protection in goldfish against *A. hydrophila*. Also, Zhang et al. (2016) found that the anti-OMP serum could provide the fish with immediate protection against *A. hydrophila* with RPS 100%, as evidenced by passive immunization.

## Conclusion

*Aeromonas hydrophila* OMPs are promising candidates for vaccine development because they are highly immunogenic, protect against motile *Aeromonas* septicemia (MAS) in *Oreochromis niloticus*, and promote protection against the disease.

**Author contribution** AM, AE, AS and AA designed the study, AM performed the experimental part, and AM, AE, AS and AA drafted the manuscript. All authors reviewed the manuscript.

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**Data availability** No datasets were generated or analysed during the current study.

## Declarations

**Ethical acceptance** The Committee of Animals Welfare and Research Ethics of Benha University's Faculty of Veterinary Medicine, Egypt, provided recommendations for doing this research.

**Competing interests** The authors declare no competing interests.

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