

# Immune Responses and Protective Immunity in *Pangasius Pangasius* (Hamilton, 1822) as Induced by Outer Membrane Proteins of *Edwardsiella Tarda* and Aluminium Hydroxide Adjuvant Complex

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## Research Article

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# Abstract

*Edwardsiella tarda* is considered one of the important bacterial fish pathogens. The outer membrane proteins (OMPs) of *E. tarda* are structurally and functionally conserved, and immunogenic. This study assessed the effects of the OMPs of *E. tarda* CGH9 as a vaccine without aluminium hydroxide [AH] (T1) and with AH adjuvant (T2) on the respiratory burst (ROB) activity, lymphocyte proliferation of head kidney (HK) leukocytes, and serum antibody production in pangas catfish *Pangasius pangasius*. The ROB activity and lymphocyte proliferation of HK leukocytes increased in both vaccinated groups compared to control. Nonetheless, the T2 group showed a gradual increase in ROB activity and lymphocyte proliferation of HK leukocytes up to 3-weeks post-vaccination (wpv). The serum antibody production in the T1 group decreased initially for up to 2-wpv and increased from 3-wpv; whereas, in the T2 group, the serum-specific antibody levels were significantly high from 1-wpv compared to control. Simultaneously, the protective efficacy in terms of relative percentage survival (RPS) in the T2 group after injecting with a lethal dose of *E. tarda* CGH9 was high ( $89.00 \pm 15.56$ ) compared to the T1 group ( $78.00 \pm 0.00$ ). Furthermore, the catfish administered with a booster dose of *E. tarda* OMPs with or without AH adjuvant showed no additional increase in immune response or protective immunity. These results suggested that *E. tarda* OMPs and AH adjuvant complex has a higher potential to induce protective immunity, which may be a good choice as a vaccine to combat *E. tarda* infection in catfish.

## Introduction

*Edwardsiella tarda*, a Gram-negative bacterial pathogen of the family Enterobacteriaceae, causes edwardsiellosis in many cultured fish species. It is considered as one of the important bacterial pathogens in global aquaculture notably in Nile tilapia *Oreochromis niloticus*, Japanese flounder *Paralichthys olivaceus*, turbot *Scophthalmus maximus*, carp *Labeo rohita*, channel catfish *Ictalurus punctatus*, striped bass *Morone saxatilis*, rainbow trout *Oncorhynchus mykiss* and pangas catfish *Pangasius pangasius* (Kodama et al. 1987; Park et al. 2012; Xu and Zhang 2014; Adikesavalu et al. 2016a). *Pangasius pangasius* is one of the important farmed freshwater catfish species with high economic values, especially in Asian countries including India (Mugaonkar et al. 2019). Control of *E. tarda* infection mostly relies on the use of antimicrobial compounds, mainly antibiotics. However, the selection and spread of antibiotic-resistant bacteria have raised concerns regarding antibiotic-based therapeutic procedures to control bacterial infection. The best way to combat bacterial diseases is through vaccination (Assefa and Abunna 2018). Currently, most aquaculture vaccines are aimed at different bacterial diseases, viz., vibriosis, furunculosis, and yersiniosis, although with varying degrees of success (Sommerset et al. 2005; Dadar et al. 2017; Assefa and Abunna 2018). However, the existence of antigenic diversity within the same bacterial species poses difficulty in developing an effective vaccine (Park et al. 2012). The past studies on vaccine development, as reviewed by Park et al. (2012), attempted numerous *E. tarda* antigen preparations, viz., formalin killed cells (FKC), lipopolysaccharides (LPS), extracellular proteins (ECP), live attenuated, avirulent bacterial strain, ghost cells, outer membrane proteins (OMPs), recombinant proteins and DNA vaccines in a different fish model by various research

groups. The two vaccine types, viz., killed bacterins and modified live (attenuated) bacteria are available against *Edwardsiella ictaluri*, which primarily affects catfish (Assefa and Abunna 2018). The live attenuated vaccine is presently sold commercially against edwardsiellosis (Dadar et al. 2017; Assefa and Abunna 2018). Recently, Wise et al. (2020) evaluated the efficacy of a live-attenuated *E. ictaluri* vaccine in *I. punctatus* fry administered through an oral vaccine delivery system. According to them, it improved the total harvest, survival and FCR significantly in experimental pond trials.

To date, several vaccine candidates have been reported for the edwardsiellosis disease (Cheng et al. 2010; Jiao et al. 2009, 2010; Sun et al. 2010, 2011; Tang et al. 2010). However, the immunogenic nature of the prepared vaccines was still debatable because inactivation procedures may alter the antigenicity of protective epitopes, attenuated vaccine candidate may revert to its virulent form and live avirulent vaccine candidate has the chance of acquiring virulent features through the transformation of genes that encode virulence factors (Dadar et al. 2017). Earlier reports by different research groups highlighted the fact that OMP homologues of bacterial species are both structurally and functionally conserved and therefore, could be immunogenic with broad protective coverage Sun et al. 2011; Dubey et al. 2019). Jalava et al. (2003) reported that the immunoprotective efficacy of a vaccine candidate against pathogens was improved by combining with adjuvants. Aluminium hydroxide (AH) adjuvant was considered safe for human vaccines and has been used now with great success. The aluminium adjuvant-antigen complex could effectively increase the antigen exposure time by acting as slow-release depots, which, in turn, leads to prolonged innate signalling that stimulates T helper-2-type (Th-2) lymphocyte response almost exclusively and thus, considered effective in promoting protective humoral immunity (Gupta et al. 1995; Jiao et al. 2010). The reports on *E. tarda* OMPs as a vaccine candidate in the catfish model were scanty. The present study, therefore, assessed the immunoprotective efficacy of *E. tarda* CGH9 OMPs with and without AH adjuvant in *P. pangasius* concerning respiratory burst (ROB) activity, in-vitro lymphocyte proliferation and serum antibody production.

## Materials And Methods

### Bacterial strain

The bacterial strain *Edwardsiella tarda* CGH9 (Accession no: KX159725) was originally isolated from African catfish *Clarias gariepinus* (Burchell 1822) with typical symptoms of dropsy (Adikesavalu et al. 2016a). The bacterial strain was grown in tryptone soya broth (TSB) at 30°C and stock cultures of 5 ml aliquots containing 20% glycerol were maintained frozen at -20°C until further use.

### Collection and maintenance of experimental fish

The experimental pangas catfish, *Pangasius pangasius* (61.71±2.36 g and 20.21±0.76 cm) were procured from Kantipota, South 24 Parganas district, West Bengal, India (Lat. 22°27'49" N; Long. 88°24'41" E), and disinfected by immersion in a 5 ppm potassium permanganate (KMnO<sub>4</sub>) solution for 15 min. The fish were stocked in 500 L capacity circular fibreglass reinforced plastic (FRP) tanks at 65 numbers/tank and acclimatized for 21 days before experimental vaccination. The fish were fed a

balanced basal dry pellet feed (CP9931, CP Pvt. Ltd., India) twice daily at 2% body weight. The wastes and faecal matter were siphoned out daily and 50% water was exchanged once in 3 days.

## Vaccine preparation

### Preparation of outer membrane proteins (OMPs) of *Edwardsiella tarda* CGH9

The suspension of *E. tarda* CGH9 OMPs was prepared by following Adikesavalu et al. (2016a). In brief, the strain was revived on tryptone soya agar (TSA) and cultured in TSB at 30°C for 24 h. The harvested bacterial cell pellet was washed thrice using sterile phosphate buffer saline (PBS) by centrifugation at 8000 rpm for 25 min at 25°C. The cell pellet was resuspended in 20 mL PBS, followed by treatment with 2% sodium dodecyl sulphate (SDS) and 2% mercaptoethanol for 20 min at 60°C for solubilisation. The supernatant was collected by centrifuging the solubilised extract at 8000 rpm for 30 min at 4°C. The supernatant was dialysed for 48 h against sterile PBS, followed by filter sterilization ( $\varphi$  0.22  $\mu$ ) and storage at -20°C until further use. The estimation of protein content was as per Lowry et al. (1951).

### Preparation of aluminium hydroxide (AH) adjuvant

The aluminium hydroxide (AH) adjuvant was prepared following the method of Jiao et al. (2010). Briefly, 5% sodium hydroxide (NaOH) and 5% aluminium sulfate ( $Al_2(SO_4)_3$ ) were prepared in distilled water, followed by filter ( $\varphi$  0.45  $\mu$ ) sterilization and incubation at 60°C for 30 min. Two volumes of 5% NaOH and five volumes of 5%  $Al_2(SO_4)_3$  were mixed with stirring, followed by centrifugation at 10,000 rpm for 5 min. After washing twice with sterile PBS, a diluted solution of aluminium hydroxide at 4 mg/mL concentration was prepared and stored at 4°C for further use.

## Vaccine formulation

The crude suspension containing *E. tarda* CGH9 OMPs at 500  $\mu$ g/mL was used directly as a vaccine. The other formulation contained a mixture of crude *E. tarda* CGH9 OMPs at 500  $\mu$ g/mL and AH adjuvant at 2 mg/mL concentration.

## Experimental vaccination

All vaccination experiments were performed for 4-weeks, in duplicate, with a group size of 65 fish. The catfish were divided randomly into five groups (65 fish/group) labelled as control (C), T1 (*E. tarda* CGH9 OMPs without AH adjuvant), T1B (booster *E. tarda* CGH9 OMPs without AH adjuvant), T2 (*E. tarda* CGH9 OMPs and AH adjuvant complex) and T2B (booster *E. tarda* CGH9 OMPs and AH adjuvant complex). Before vaccination, the fish were anaesthetized with clove oil (50  $\mu$ l/L). Each fish of the groups T1 and T1B was administered with 0.1 mL (50  $\mu$ g) of *E. tarda* CGH9 OMPs without AH adjuvant intramuscularly. Similarly, the T2 and T2B groups were injected intramuscularly with 0.1 mL of *E. tarda* CGH9 OMPs and AH adjuvant complex, i.e., 50  $\mu$ g OMPs and 0.2 mg AH. The C group received 0.1 mL of sterile PBS. The fish were maintained in their respective tanks and observed for mortality, external signs of infections and behavioural changes. One day after 2-weeks post-vaccination (wpv), fish from T1B and T2B groups were injected intramuscularly with a second dose of 0.1 mL each of the respective vaccine formulation and

maintained again in their respective tanks. Experimental protocols were approved by the Indian Council of Agricultural Research, Government of India, New Delhi under the Niche Area of Excellence programme and fulfilled the ethical guidelines including adherence to the legal requirements of India (CPCSEA, 2018).

## **Blood sampling and isolation of head kidney (HK) leukocytes**

On each sampling, viz., 1-, 2-, 3- and 4-wpv, four fish from each group were randomly sampled for blood collection and head kidney isolation to study the effect of the vaccine on the immune parameters such as ROB activity, in-vitro lymphocyte proliferation and serum antibody levels. Likewise, on 1- and 2-weeks post-booster vaccination (wpbv), the fish from T1B and T2B groups were sampled to assess the effect of booster dose on the immune parameters. This 1- and 2-wpbv corresponded with samples that were collected on 3- and 4-wpv from C, T1 and T2 groups. The assessment of serum antibody production alone was done up to 6-wpv in C, T1 and T2 groups. The fish serum collected up to 6-wpv corresponded with samples that were collected up to 4-wpbv in T1B and T2B groups.

The blood was collected from the anaesthetized fish using clove oil at 50  $\mu\text{L/L}$  water by caudal vein puncture and an aliquot of pooled blood was heparinized using 2.7% EDTA. The non-heparinized blood was allowed to clot at room temperature ( $\approx 30^\circ\text{C}$ ) and kept at  $4^\circ\text{C}$  overnight. The serum samples were collected by centrifugation at 2500 rpm for 15 min and pooled before storage at  $-20^\circ\text{C}$ . After blood collection, the fish were euthanized by increasing the clove oil dose to 100  $\mu\text{L/L}$  water. The leukocytes from the head kidney and spleen were collected as per Adikesavalu et al. (2016a). In brief, the head kidney and spleen were extirpated and prepared the cell suspension in PBS (pH 7.4) containing antibiotics, viz., penicillin 100 IU/mL (HiMedia, India) and streptomycin 100  $\mu\text{g/mL}$  (HiMedia, India) by squeezing the tissue pieces through sterile stainless steel screens. The cell suspension was layered over a density gradient, Histopaque (Sigma, USA) at 1:3 ratio and centrifuged at 1200 rpm for 30 min. The mononuclear cells present between plasma and density gradient were collected and washed with PBS. The cells were finally suspended in RPMI-1640 medium with 2.05 mM L-glutamine (Hyclone, USA) supplemented with 10% fetal calf serum (HiMedia, India) and antibiotics (penicillin 100 IU/mL and streptomycin 100  $\mu\text{g/mL}$ ). Trypan blue dye exclusion method using a haemocytometer was done for enumeration and determining the viability of the purified leukocytes.

## **Effect of vaccination on the immune parameters**

### **Respiratory burst (ROB) activity**

The ROB activity by neutrophils was determined by the reduction of nitroblue tetrazolium (NBT) to formazan following Mohanty and Sahoo (2010) with minor modification. Briefly, an equal volume of freshly collected heparinized blood and 0.2% NBT (Amresco, USA) in PBS was mixed and incubated at room temperature ( $30^\circ\text{C}$ ) for 30 min. After incubation, 50  $\mu\text{L}$  of this mixture was mixed with 1 mL of dimethylformamide (HiMedia, India) to solubilize the reduced formazan product and centrifuged at 2000

rpm for 5 min to collect the supernatant. The extent of NBT reduction was measured at an optical density of 540 nm using di-methyl formamide as blank.

## **In-vitro lymphocyte proliferation assay**

The proliferative response of the HK leukocytes was determined by the tetrazolium-based colourimetric assay as described by Adikesavalu et al. (2016a). Briefly, the HK leukocytes were distributed into the wells of a 96-well cell culture plate at  $1.50 \times 10^6$  cells/well. Mitogen concanavalin-A [Con-A] (Genei, Bangalore) stock solution was prepared at the concentration of 80  $\mu\text{g}/\text{mL}$  using a growth medium. To each well, 100  $\mu\text{L}$  with the final Con-A concentration of 40  $\mu\text{g}/\text{mL}$  was added. Similarly, 100  $\mu\text{L}$  of the suitably diluted stock solution of crude *E. tarda* CGH9 OMPs was added into the respective wells to achieve a final concentration of 40  $\mu\text{g}/\text{mL}$ . Meanwhile, a stock suspension of *E. tarda* CGH9 of approximately  $1.50 \times 10^8$  cells/mL was prepared using the growth medium. A multiplicity of infection of 1:10 (phagocytes: bacteria) ratio was used for infecting the HK leukocytes as described by Nakhro et al. (2013). This was achieved by adding 100  $\mu\text{L}$  of stock suspension of *E. tarda* CGH9 into appropriate test wells. The remaining wells were filled up to 200  $\mu\text{L}$  with the medium (negative control). The plate was incubated for 24 h at 30°C. After incubation, 20  $\mu\text{L}$  of filter-sterilized MTT [3-(4, 5-dimethyl thiazol- 2-yl)-2, 5-diphenyl tetrazolium bromide] (HiMedia, India) solution (10 mg MTT/mL) was added into all the wells and incubated again at 30°C for 6 h. After incubation, 150  $\mu\text{L}$  of the supernatant was carefully removed without disturbing the cells or the formazan precipitate. This was followed by the addition of 150  $\mu\text{L}$  of dimethyl sulphoxide (DMSO) and 20  $\mu\text{L}$  of glycine buffer (0.1 M glycine, 0.1M NaCl, pH 10.5) into all the wells. The contents of the wells were mixed thoroughly by pipetting and incubated for 10 min at 30°C. The cell culture plate was read on a Microtiter plate reader at 595 nm.

## **Serum antibody production**

Indirect ELISA was performed in a 96 well plate (Nune, Denmark) as per Mishra and Sekhar (1997). Briefly, the plate was coated in duplicate with a coating buffer (carbonate bicarbonate buffer, pH 9.6) containing antigen (2.5  $\mu\text{g}$  *E. tarda* CGH9 OMPs/well) and kept at 4°C overnight. After the antigen coating and blocking (5% skimmed milk powder in PBS) for 2 h at 37°C, the plate was washed thoroughly 4 times with PBS containing 0.05% Tween-20. Then 100  $\mu\text{L}$  of diluted (1:200 in PBS) test and control fish sera were added into the appropriate antigen-coated wells and incubated at 37°C for 2 h. The plate was washed again as above and 100  $\mu\text{L}$  each of diluted (1:100 in PBS) anti-pangas serum globulin enzyme (HRPO) immunoconjugate, developed as described by Adikesavalu et al. (2016b), was added into the wells and left for 2 h at 37°C. The plate was then thoroughly washed and 100  $\mu\text{L}$  of substrate solution (15  $\mu\text{L}$  of 6%  $\text{H}_2\text{O}_2$ , 0.025 g ortho-phenylenediamine dihydrochloride in 25 mL citrate buffer) was added into the wells in dark. The colour development was arrested after 20 min by adding 3N HCl (35% pure). The colour development was read at 492 nm using a Microplate reader.

## **Vaccine efficacy determination: Challenge with *Edwardsiella tarda* CGH9**

After 4-wpv, 40 each of catfish from all five groups, viz., C, T1, T2, T1B and T2B were transferred to 10 new tanks at 20 fish/tank and labelled properly. Each fish was challenged intramuscularly with 0.1 mL of  $\approx 10^8$  cells/mL (lethal dose) of *E. tarda* CGH9 and observed daily for mortality, external signs of infections and behavioural changes for 30 days. The effectiveness of the vaccine formulation was determined from the mortality data using the relative percentage survival (RPS) as described by Bader et al (2004). The RPS values above 60 were considered protective for this study.

$$\text{RPS} = \left( 1 - \left[ \frac{\text{Mortality in vaccinated group}}{\text{Mortality in unvaccinated control}} \right] \right) \times 100$$

## Statistical analyses

The data (mean  $\pm$  standard deviation) were analyzed by one-way repeated-measures analysis of variance (ANOVA) using OriginPro version 9.0.0. Pairwise comparisons of mean values were determined by the Tukey test, considering a probability level of  $P < 0.05$ .

## Results

The immune effects of *E. tarda* CGH9 OMPs were evaluated with or without AH adjuvant by quantifying the levels of ROB activity, in-vitro lymphocyte proliferation and serum antibody production. The vaccinated catfish exhibited inflammation at the site of injection, which lasted for a week when compared to control.

### Respiratory burst (ROB) activity

The ROB activity was significantly ( $P < 0.05$ ) high on 1-wpv in both *E. tarda* CGH9 OMPs vaccinated groups without AH adjuvant (T1) and with AH adjuvant (T2) compared to control (Fig. 1A). A significant ( $P < 0.05$ ) difference in ROB activity between the control and T1 groups was noted only up to 2-wpv. Whereas, the ROB activity increased gradually up to 3-wpv compared to the control in T2 group and reduced thereafter. In the T1 group, the ROB activity on 1-wpv (0.455 OD) was significantly ( $P < 0.05$ ) high compared to 3- and 4-wpv. The observed difference in ROB activity between 1-wpv and 2-wpv was insignificant ( $P > 0.05$ ). The ROB activity in the T2 group was significantly ( $P < 0.05$ ) high on 3-wpv (0.470 OD) when compared to 1-wpv and 4-wpv. However, the difference between 2-wpv and 3-wpv was insignificant ( $P > 0.05$ ; Fig. 1A). The differences in ROB activities between the T1 and T2 groups on 1-wpv as well as on 3-wpv were significant ( $P < 0.05$ ). The ROB activity in the T1 group was significantly ( $P < 0.05$ ) high on 1-wpv; while it was significantly ( $P < 0.05$ ) high in the T2 group on 3-wpv (Fig. 1A).

The ROB activities in booster groups, viz., T1B and T2B showed no further increase (Fig. 1B). In group T1B, the ROB activity on 1-wpbv was significantly ( $P < 0.05$ ) low compared to 2-wpv from the T1 group (Fig. 1B). The ROB activity in the T2B group showed an increase on 1-wpbv compared to 2-wpv from the T2 group (Fig. 1B). Nonetheless, the observed increase was insignificant ( $P > 0.05$ ). On 2-wpbv, the ROB

activities in T1B and T2B groups reached near normal, which corresponded to those observed in T1 and T2 groups on 4-wpv.

## In-vitro lymphocyte proliferation assay

The lymphocyte proliferation of HK leukocytes of the T1 group, when tested with a non-specific stimulator Con-A, showed a significant ( $P<0.05$ ) increase on 1-wpv when compared to control. It reduced thereafter to reach near control level on 4-wpv (Fig. 2A). The observed reduction in the levels of lymphocyte proliferation on 2-, 3- and 4-wpv was significant ( $P<0.05$ ) compared to 1-wpv; while the difference in the levels observed between 2-wpv and 3-wpv was insignificant ( $P>0.05$ ; Fig. 2A). In the T2 group, the lymphocyte proliferation of HK leukocytes, when tested with Con-A showed a significant ( $P<0.05$ ) increase up to 3-wpv, when compared to control and reduced thereafter (Fig. 2A). The differences in the levels of lymphocyte proliferation between 1-wpv and 2-wpv as well as 2-wpv and 3-wpv were insignificant ( $P>0.05$ ). The level of the proliferative response of leukocytes on 3-wpv (3.2310D) was significantly ( $P<0.05$ ) higher than those observed on 1-wpv and 4-wpv (Fig. 2A). The levels of proliferative responses in the T2 group were high on 2-, 3- and 4-wpv compared to the T1 group. However, the differences in the proliferative responses between the T1 and T2 groups were significant ( $P<0.05$ ) only on 3- and 4-wpv (Fig. 2A).

The lymphocyte proliferation of HK leukocytes of the T1B group, when tested with Con-A, no significant change was observed on 1-wpbv when compared to 2-wpv from the T1 group (Fig. 2B). The levels on 2-wpbv (0.887 OD) were significantly ( $P<0.05$ ) low when compared to 1-wpbv and also to 2-wpv from the T1 group. The lymphocyte proliferation of the T2B group, when tested with Con-A, showed no significant increase on 1-wpbv and 2-wpbv when compared to 2-wpv from the T2 group (Fig. 2B). However, the levels of proliferative responses in the T2B group were significantly ( $P<0.05$ ) high on 1- and 2-wpbv compared to the T1B group.

Further, the lymphocyte proliferation of HK leukocytes of the T1 group, when tested with *E. tarda* CGH9 OMPs, increased insignificantly ( $P>0.05$ ) up to 2-wpv and reduced on 3-wpv when compared to control (Fig. 3A). The levels of proliferative responses on 2-wpv (1.948 OD) were insignificantly higher than on 1-wpv ( $P>0.05$ ). The levels on 2-wpv were significantly ( $P<0.05$ ) higher than those recorded on 3-wpv and 4-wpv (Fig. 3A). In the T2 group, when tested with *E. tarda* CGH9 OMPs, the proliferative responses were significantly ( $P<0.05$ ) high on all wpv compared to control (Fig. 3A). The levels of proliferative responses increased up to 3-wpv (2.762 OD). It was observed to be significantly ( $P<0.05$ ) higher than on 1-wpv and 4-wpv; while the difference between 2-wpv and 3-wpv was insignificant ( $P>0.05$ ; Fig. 3A). The levels of proliferative responses in the T2 group were high on all wpv compared to the T1 group. However, as observed with Con-A, the differences in the levels of proliferative responses between the T1 and T2 groups were significant only on 3- and 4-wpv (Fig. 3A).

The proliferative responses of the T1B group, when tested with *E. tarda* CGH9 OMPs, displayed a significant ( $P<0.05$ ) decrease on 1-wpbv and 2-wpbv when compared to 2-wpv from the T1 group (Fig. 3B). The proliferative responses of the T2B group, when tested with *E. tarda* CGH9 OMPs, displayed

a significant ( $P<0.05$ ) increase on 1-wpbv (2.766 OD) when compared to 2-wpv from the T2 group (Fig. 3B). The responses decreased significantly on 2-wpbv and reached a level similar to that of 2-wpv from the T2 group. The differences in the levels of proliferative responses between the T1B and T2B groups were significant ( $P<0.05$ ) in both 1- and 2-wpbv (Fig. 3B).

Lymphocyte proliferation of HK leukocytes of T1 group, when tested with *E. tarda* CGH9 whole cells, showed an increase from control on 1-wpv, which later reduced to the level of control on 4-wpv (Fig. 4A). The differences in the levels of proliferation among 1-, 2- and 4-wpv were significant ( $P<0.05$ ); while the differences in the levels between 1-wpv and 3-wpv as well as 2-wpv and 3-wpv were insignificant ( $P>0.05$ ). The lymphocyte proliferation of the T2 group, when tested with *E. tarda* CGH9 whole cells, showed an increase from control on all wpv (Fig. 4A). However, significant ( $P<0.05$ ) differences in the levels of proliferation between control and different wpv were observed only from 2-wpv. Although the levels of lymphocyte proliferation on 3-wpv (3.682 OD), were higher than on 1-wpv and 2-wpv, the differences were insignificant ( $P>0.05$ ). Its level on 4-wpv was significantly ( $P<0.05$ ) lower than in other wpv (Fig. 4A). The levels of proliferative responses in the T2 group were high on all wpv compared to the T1 group. However, the differences in the levels of proliferative responses between T1 and T2 groups were significant ( $P<0.05$ ) only on 2- and 4-wpv (Fig. 4A).

The lymphocyte proliferation of the T1B group, when tested with *E. tarda* CGH9 whole cells, no significant changes were observed on 1-wpbv when compared to 2-wpv from the T1 group (Fig. 4B). The levels on 2-wpbv (2.121 OD) were low when compared to 1-wpbv and also to 2-wpv from the T1 group. Besides, the lymphocyte proliferation of the T2B group, when tested with *E. tarda* CGH9 whole cells, showed no significant changes on 1-wpbv and 2-wpbv when compared to 2-wpv from the T2 group. The differences in the levels of proliferative responses between the T1B and T2B groups were significant ( $P<0.05$ ) only on 1-wpbv (Fig. 4B).

## Serum antibody production

In T1 and T2 groups, indirect ELISA was performed to assess the antibody levels in the immune serum collected on different wpv. The results, expressed in OD at 492 nm, are shown in Fig. 5A. The antibody production in T1 group was reduced insignificantly up to 2-wpv ( $P>0.05$ ), which then increased from 3-wpv (0.283 OD) to 6-wpv (0.364 OD) compared to control. However, the increase was significant ( $P<0.05$ ) only from 4-wpv when compared to control. In the T2 group, the levels of antibody were significantly ( $P<0.05$ ) high on all wpv compared to control (Fig. 5A). Though the antibody production was increasing continuously, significant ( $P<0.05$ ) differences existed only on alternative weeks up to 4-wpv. In both T1 and T2 groups, the antibody production on 4- and 5-wpv were significantly ( $P<0.05$ ) high when compared individually with 1- and 2-wpv. The observed differences in the antibody production among 3-, 4- and 5-wpv were insignificant ( $P>0.05$ ). The levels of serum antibody on 6-wpv were significantly ( $P<0.05$ ) high when compared individually with 1-, 2- and 3-wpv in both groups. In contrast, the differences in the antibody levels among 4-, 5- and 6-wpv were insignificant ( $P>0.05$ ) in both groups. The antibody production observed in the T2 group was significantly ( $P<0.05$ ) high on all wpv compared to the T1 group.

In T1B and T2B groups, antibody production was observed on different wpbv. In the T1B group, the antibody production increased on all wpbv when compared to 2-wpv from the T1 group. However, the observed increase in different wpbv was significant ( $P < 0.05$ ) only on 3- and 4-wpbv. The antibody level on 3-wpbv was significantly ( $P < 0.05$ ) high compared to 1- and 2-wpbv. The difference in the antibody levels between 3-wpbv and 4-wpbv was, however, insignificant ( $P > 0.05$ ; Fig. 5B). In the T2B group, a slight but insignificant ( $P > 0.05$ ) reduction in antibody production was observed on 1-wpbv when compared to 2-wpv from the T2 group, followed by a significant ( $P < 0.05$ ) increase on 2-wpbv. The antibody production on 3-wpbv (0.580 OD) was reduced slightly but insignificantly ( $P > 0.05$ ) when compared to 2-wpbv (Fig. 5B). The serum antibody levels on 2- and 3-wpbv were significantly ( $P < 0.05$ ) high when compared individually with 1- and 4-wpbv. The serum antibody levels in the T2B group were significantly ( $P < 0.05$ ) high on all wpv compared to the T1B group (Fig. 5B).

### 3.4. Immunoprotective efficacy

After 4-wpv, the catfish from all five groups, viz., C, T1, T2, T1B and T2B were challenged with a lethal dose of *E. tarda* CGH9. The challenged control group exhibited hyperemic skin, anorexia, abnormal movement, haemorrhages with peeled skin and exposed muscle at the site of injection and petechial haemorrhages on the fin and skin. The challenged fish from the vaccinated groups showed comparatively less hyperemic skin and anorexic condition. The movement was normal when compared to control. Externally, inflammation with reddening at the site of injection alone was observed, which subsided and healed progressively. The immunoprotective efficacy in terms of RPS was markedly higher in T2 ( $89.00 \pm 15.56$ ) followed by T1 ( $78.00 \pm 0.00$ ) groups. In T1B and T2B groups, the RPS was  $55.50 \pm 31.82$  and  $78.00 \pm 31.11$ , respectively (Table 1).

Table 1  
Relative percentage survival (RPS) of catfish subjected to different vaccine treatments

Treatment group	RPS
T1 ( <i>Edwardsiella tarda</i> CGH9 OMPs without AH adjuvant) group	78.00
T2 ( <i>E. tarda</i> CGH9 OMPs with AH adjuvant) group	89.00
T1B (administered with a booster of <i>E. tarda</i> CGH9 OMPs without AH adjuvant) group	55.50
T2B (administered with a booster of <i>E. tarda</i> CGH9 OMPs and AH adjuvant) group	78.00
Values represent mean $\pm$ standard deviation of duplicate observations. The control group had $22.50 \pm 3.54\%$ mortalities. OMPs: Outer membrane proteins; Dose = <i>Edwardsiella tarda</i> CGH9 OMPs: 50 $\mu$ g/fish; Aluminium hydroxide (AH) adjuvant: 0.2 mg/fish.	

## Discussion

Although the immune responses after vaccination with several *E. tarda* vaccine candidates have been investigated in different fish species (Cheng et al. 2010; Jiao et al. 2009, 2010; Sun et al. 2010, 2011; Tang et al. 2010), information regarding OMPs of *E. tarda* as a vaccine candidate in pangas catfish is

limited. Previous reports suggested that OMPs of *E. tarda* are highly conserved, immunogenic and could confer broad protection (Kumar et al. 2007; Tang et al. 2010; Dubey et al. 2019; LiHua et al. 2019). In this study, we used crude OMPs of *E. tarda* CGH9 as a vaccine candidate. Usually, OMPs of bacterial species contain many immunogenic components that elicit protective immunity (Dubey et al. 2019). In the T1 group without AH adjuvant, the ROB activity was significantly high on 1-wpv (0.455 OD) compared to the control. Whereas, in the T2 group with AH adjuvant, a gradual increase in ROB activity was observed up to 3-wpv, indicating stimulation of immune responses for longer protection. Contrarily, in an earlier report, the stimulating effect of a recombinant subunit vaccine candidate along with AH adjuvant on the ROB activity of *P. olivaceus* was observed to be significantly high only on 1- and 7-day post-vaccination (pv) but not on 14- and 21-day pv (Sun et al. 2011). It has been demonstrated that the vaccine, when conjugated with AH adjuvant, helps in long-term antigen exposure (Gupta et al. 1995; Gupta 1998). Possibly, the crude *E. tarda* OMPs vaccine-AH adjuvant complex might have resulted in a gradual increase in the ROB activity of the T2 group. Surprisingly, no further increase in ROB activity of catfish was observed when administering a booster dose of *E. tarda* CGH9 OMPs with or without AH adjuvant at the same concentration.

The lymphocyte proliferation of HK leukocytes of the T1 group upon stimulation with Con-A showed a significant increase on 1-wpv compared to control and reduced thereafter to the level of control on 4-wpv. A similar trend was observed in the proliferative responses even with *E. tarda* CGH9 whole cells as the test antigen. On the other hand, when the *E. tarda* CGH9 OMPs were used as the antigen, the proliferative responses of HK leukocytes of the T1 group increased up to 2-wpv and were reduced thereafter. Contrastingly, the T2 group showed a significant increase in the proliferative responses up to 3-wpv in all the tested antigens, viz., Con-A, *E. tarda* CGH9 OMPs and *E. tarda* CGH9 whole cells. Our results corroborate the observations of LiHua et al. (2019), who reported an increased proliferation of whole blood cells on 21, 28 and 42 days post-immunization when *Anguilla japonica* was vaccinated with outer membrane protein A (OmpA) of *E. anguillarum* and Freund's incomplete adjuvant (FIA). In our study, the observed levels of proliferative responses in all the tested antigen groups were high on 2-, 3- and 4-wpv when compared to the T1 group. This suggested that the naïve lymphocytes of catfish administered with OMPs-adjuvant complexes are effectively stimulated for a longer period than the group-administered only the OMPs as a vaccine.

The T1B group showed no significant increase in lymphocyte proliferation with all tested antigens. Similarly, in the T2B group, the lymphocyte proliferation with all tested antigens showed no significant changes on 1-wpbv and 2-wpbv when compared to 2-wpv from the T2 group. Interestingly, the proliferative responses of the T2B group when tested with *E. tarda* CGH9 OMPs as the antigen displayed a significant increase on 1-wpbv (2.766 OD) compared to 2-wpv from the T2 group. However, the proliferative responses observed on 1-wpbv (2.766 OD) was almost similar to that of 3-wpv (2.762 OD) from the T2 group. Furthermore, the T1B and T2B groups showed no additional increase in lymphocyte proliferation when compared respectively to the T1 and T2 groups. Unfortunately, it was not possible to compare and discuss the observed results of the present study with the report of other authors, because

similar reports assessed the immunoprotective efficacy of OMPs of *E. tarda* with or without AH as an adjuvant by in-vitro lymphocyte proliferation were scanty.

The antibody-mediated humoral immunity, an important defence mechanism, was reported in *O. niloticus*, *O. mykiss*, *P. olivaceus* and *A. japonica* against bacterial infections caused by *Streptococcus iniae* (Shelby et al. 2002), *S. agalactiae* (Pasnik et al. 2006), *Flavobacterium psychrophilum* (LaFrentz et al. 2003), *Aeromonas hydrophila* (LaPatra et al. 2010) and *E. tarda* (Jiao et al. 2010; LiHua et al. 2019). In the present study, the serum antibody production in the T1 group reduced up to 2-wpv compared to the control. In contrast, its level was significantly high from 1-wpv when compared to control in the T2 group. The observed reduction up to 2-wpv in the serum antibody levels of the T1 group could be the result of false tolerance or false suppression as vaccination may lead to the substantial decrease of natural antibodies, which cross-reacts with most antigens (Sinyakov and Avtalion 2009). On the other hand, this false tolerance or suppression was not observed in the T2 group, which might probably be the effect of AH adjuvant. Additionally, Whyte (2007) described that the level of natural antibodies may vary among the fish species and it also depends on the environmental conditions in which they are present. The serum antibody production was observed to be the highest from 4-wpv to 6-wpv in both T1 and T2 groups. The observations of the present study confirmed many earlier reports performed in *P. olivaceus*, in which the antibody levels were observed to be highest at 4–8 weeks after vaccinating the fish with *E. tarda* antigen Eta21 (Jiao et al. 2009), *E. tarda* antigen Et49 (Jiao et al. 2010), attenuated *E. tarda* (Sun et al. 2010), recombinant *E. tarda* antigen rEta2 (Sun et al. 2011) and *E. tarda* OMPs (Tang et al. 2010). Furthermore, the T2 group recorded the highest level of serum antibody production on 6-wpv (0.581), which was >1.5-folds higher than those observed in the T1 group. This observation was in agreement with Jiao et al. (2010), which documented a significantly high level of specific antibody in *P. olivaceus* vaccinated with *E. tarda* major antigen Et49 and AH adjuvant.

The vaccinated groups with or without AH adjuvant that administered a booster dose of *E. tarda* CGH9 OMPs, i.e., T1B and T2B, recorded high serum antibody production on all wpbv when compared to 2-wpv. The time points 1-wpbv, 2-wpbv and 3-wpbv in T1B and T2B groups corresponded with the time point 3-wpv, 4-wpv and 5-wpv of the T1 and T2 groups without the booster. On comparing the groups vaccinated with *E. tarda* CGH9 OMPs and AH adjuvant complex, viz., T2 and T2B, the highest immune response in terms of antibody production was observed in the T2 group on 6-wpv. In the T2B group, the same level of antibody production was observed on 2-wpbv (0.587 OD), which was about two weeks earlier than the T2 group. Likewise, Cheng et al. (2010) reported that the group of *P. olivaceus* vaccinated with *E. tarda* ATCC 15947 via injection with boost produced the highest antibody titers. Contrarily, the levels of serum antibody in T1 and T1B groups vaccinated with *E. tarda* CGH9 OMPs without adjuvant were in the same range. These observations suggested that booster dose with AH adjuvant can able to increase the serum antibody levels quickly. Possibly, it effectively augmented the specific immunity induced by *E. tarda* CGH9 OMPs.

Many earlier reports by different research groups described the immunoprotective efficacy of different *E. tarda* vaccine candidates in *P. olivaceus* by examining RPS (Jiao et al. 2009, 2010; Sun et al. 2010, 2011;

Tang et al. 2010). As shown in Table 1, the high RPS in the T2 group ( $89.00 \pm 15.56$ ) could be attributed to the high levels of serum antibodies. Observations similar to the present work was made earlier in which a correlation between the serum antibody levels and protection against infection was observed in *P. olivaceus*, following intraperitoneal injection with OMPs of *E. tarda* in FIA (Tang et al. 2010) or with *E. tarda* antigen Et49 and AH adjuvant mixture (Jiao et al. 2010). The efficacy of *E. tarda* CGH9 OMPs and AH adjuvant mixture in terms of RPS of the present study was comparatively higher than the RPS (77.70) noted in *A. japonica* vaccinated with OmpA of *E. anguillarum* in FIA after the *E. anguillarum* challenge (LiHua et al. 2019). The present observations are also in close agreement with that of Jiao et al. (2010). The observed RPS in T1B ( $55.50 \pm 31.82$ ) and T2B ( $78.00 \pm 31.11$ ) groups were low when compared respectively to the T1 and T2 groups. A similar observation with low RPS in *I. punctatus* vaccinated with a booster dose of 12.5  $\mu\text{g}/100 \mu\text{L}$  of total *E. ictaluri* OMP was reported (Bader et al. 2004). Likewise, Tang et al. (2010) reported a low RPS in *P. olivaceus* vaccinated with a booster dose of 50  $\mu\text{g}$  OMPs of *E. tarda*. Interestingly, in the present study, serum antibody levels in the T1B and T2B groups were almost similar to the T1 and T2 groups but still showed a lower RPS. This suggested that the catfish that received a booster dose of 50  $\mu\text{g}$  *E. tarda* CGH9 OMPs with or without AH adjuvant might be too weak to resist the infection challenge. Because, this group was challenged immediately after 2-wpbv; while the vaccinated group without booster was challenged after 4-wpv.

## Conclusion

The vaccine candidate of the present study *E. tarda* CGH9 OMPs was observed to be a potent immunogen that elicited a strong protective immunity in catfish by inducing different immune responses as assessed by ROB activity, lymphocyte proliferation of HK leukocytes and serum antibody production. The *E. tarda* CGH9 OMPs, when administered with a cost-efficient AH adjuvant enhanced the protective function of the tested immune factors in catfish. Although the serum antibody levels increased in the vaccinated group administered with a booster dose of *E. tarda* CGH9 OMPs and AH adjuvant complex, it yielded no additional increase in RPS, ROB activity and lymphocyte proliferation of HK leukocytes at the test conditions. Our results suggested that *E. tarda* CGH9 OMPs and AH adjuvant complex could be applied as a vaccine either with or without a booster to combat *E. tarda* infection in commercial aquaculture involving pangas catfish and related species.

## Declarations

**Authors contributions** HA: Laboratory investigation, data generation, statistical analyses, and draft manuscript preparation; TJA: Conceptualization, project administration, supervision, resource mobilization, writing-reviewing and editing; SNJ: Conceptualization, methodology and interpretation of data. All authors agreed with the results and conclusions.

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## Compliance with ethical standards

**Conflict of Interest** The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

**Consent to participate** All authors consent to participate

**Consent for publication** All authors consent for publication

**Code availability** Not applicable.

**Ethics Approval** The current study was performed in compliance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India. All efforts were made to minimize the suffering of the fish. The experimental protocols under the Niche Area of Excellence program were approved by the Indian Council of Agricultural Research, New Delhi vide F.10(12)/2012–EPD.

**Availability of data and material** The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy restrictions.

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## Figures

### Figure 1

Respiratory burst activity by neutrophils of pangas catfish vaccinated with *Edwardsiella tarda* CGH9 outer membrane proteins (OMPs) with and/or without aluminium hydroxide (AH) adjuvant. A: Levels at different weeks post-vaccination (wpv) in T1 (*E. tarda* CGH9 OMPs without AH adjuvant) and T2 (*E. tarda* CGH9 OMPs and AH adjuvant) groups; B: Levels at different weeks post-booster vaccination (wpbv) in T1B (administered with a booster of *E. tarda* CGH9 OMPs without AH adjuvant) and T2B (administered with a booster of *E. tarda* OMPs and AH adjuvant) groups. \*: Groups differed significantly ( $P < 0.05$ ); A-F: Bars sharing common alphabets differed significantly ( $P < 0.05$ ).

### Figure 2

In-vitro lymphocyte proliferation of head kidney leukocytes of pangas catfish vaccinated with *Edwardsiella tarda* CGH9 outer membrane proteins (OMPs) with and/or without aluminium hydroxide (AH) adjuvant when tested with Con-A as the antigen. A: Levels at different weeks post-vaccination (wpv) in T1 (*E. tarda* CGH9 OMPs without AH adjuvant) and T2 (*E. tarda* CGH9 OMPs and AH adjuvant) groups; B: Levels at different weeks post-booster vaccination (wpbv) in T1B (administered with a booster of *E. tarda* CGH9 OMPs without AH adjuvant) and T2B (administered with a booster of *E. tarda* OMPs and AH adjuvant) groups. \*: Groups differed significantly ( $P < 0.05$ ); A-F: Bars sharing common alphabets differed significantly ( $P < 0.05$ ).

### Figure 3

In-vitro lymphocyte proliferation of head kidney leukocytes of pangas catfish vaccinated with *Edwardsiella tarda* CGH9 outer membrane proteins (OMPs) with and/or without aluminium hydroxide (AH) adjuvant when tested with *E. tarda* CGH9 OMPs as the antigen. A: Levels at different weeks post-vaccination (wpv) in T1 (*E. tarda* CGH9 OMPs without AH adjuvant) and T2 (*E. tarda* CGH9 OMPs and AH adjuvant) groups; B: Levels at different weeks post-booster vaccination (wpbv) in T1B (administered with a booster of *E. tarda* CGH9 OMPs without AH adjuvant) and T2B (administered with a booster of *E. tarda* OMPs and AH adjuvant) groups. \*: Groups differed significantly ( $P<0.05$ ); A-E: Bars sharing common alphabets differed significantly ( $P<0.05$ ).

#### Figure 4

In-vitro lymphocyte proliferation of head kidney leukocytes of pangas catfish vaccinated with *Edwardsiella tarda* CGH9 outer membrane proteins (OMPs) with and/or without aluminium hydroxide (AH) adjuvant when tested with *E. tarda* CGH9 whole cells as the antigen. A: Levels at different weeks post-vaccination (wpv) in T1 (*E. tarda* CGH9 OMPs without AH adjuvant) and T2 (*E. tarda* CGH9 OMPs and AH adjuvant) groups; B: Levels at different weeks post-booster vaccination (wpbv) in T1B (administered with a booster of *E. tarda* CGH9 OMPs without AH adjuvant) and T2B (administered with a booster of *E. tarda* OMPs and AH adjuvant) groups. \*: Groups differed significantly ( $P<0.05$ ); A-E: Bars sharing common alphabets differed significantly ( $P<0.05$ ).

#### Figure 5

Serum antibody production in pangas catfish vaccinated with *Edwardsiella tarda* CGH9 outer membrane proteins (OMPs) with and/or without aluminium hydroxide (AH) adjuvant. A: Levels at different weeks post-vaccination (wpv) in T1 (*E. tarda* CGH9 OMPs without AH adjuvant) and T2 (*E. tarda* CGH9 OMPs and AH adjuvant) groups; B: Levels at different weeks post-booster vaccination (wpbv) in T1B (administered with a booster of *E. tarda* CGH9 OMPs without AH adjuvant) and T2B (administered with a booster of *E. tarda* OMPs and AH adjuvant) groups. \*: Groups differed significantly ( $P<0.05$ ); A-M: Bars sharing common alphabets differed significantly ( $P<0.05$ ).