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Comparative Protective Antigenicity of 37 Kda Major Outer Membrane Protein (Omp) and 61 Kda whole Cell Extracted Protein of *Edwardsiella tarda* in Rohu (*Labeo rohita*)

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Research Article

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Abstract

Edwardsiella septicemia is an economically important fish disease, caused by a bacterium *Edwardsiella tarda* having zoonotic potential. It affects many fish species and various animal groups including reptiles, birds and mammals. The whole cell antigenic proteins and outer membrane proteins (OMP) of *E.tarda* were extracted from different isolates and assessed their reactivity with anti *E. trada* rohu and anti *E. trada* rabbit serum via Western blot. The strongly reacted proteins were considered as immunogenic proteins. The 61 kDa whole cell proteins and 37 kDa OMP were extracted from SDS PAGE gelsto evaluate their vaccine potential in rohu fish. Fish were vaccinated 10µg of individual proteins intraperitoneally as immunogen with Freund's incomplete adjuvant (FIA). The boosters were given on the 10th day of immunization with PBS. The blood was drawn from *vena caudalis* at every 7th day for hematological and immunological studies. Fish were challenged on 35th day with 1× 10^{6} cfu *E. tarda* field isolate *ET-1*. The cumulative mortality (CM) was recorded over one week and relative percentage survival (RPS) was calculated. The 61 kDa and 37 kDa proteins had significantly higher ($p \leq 0.05$) nonspecific and specific parameters and relative percentage survival (RPS) over control group. The non specific immune parameters were more active in first four weeks of vaccination. Specific antibody production for *E. tarda* in both proteins was not significantly different at the end of experiment. However, the significantly higher RPS of 61 kDa (63%) over 37 kDa (53%) reflect the antibodies for 61 kDa could more effective against *E.tarda* and a better vaccine can did ate over 37 kDa to control *E. tarda* in rohu. The presence of 61 kDa in all isolates indicated it could use as an antigen in indirect ELISA for the sero-monitoring and surveillance of edwardsiellosis.

Keywords: *Edwardsiella tarda*; Protective antigens; Outer membrane proteins; 61kDa protein; 37 kDa protein; Rohu

Introduction

Edwardsiella tarda is a zoonotic pathogen of *Enterobacteriaceae* family [1-3]. It has been isolated from bird, land mammals, reptiles [4-6] and economically important fish species in many parts of the world. *E.tarda* causes *Edwardsiella* septicemia in tropical, temperate and

marine fish species including eels (*Anguilla spp*.), channel catfish (*Ictalurus punctatus)* [7], chinook salmon (*Oncorhyn chustshawytscha),* brook trout (*Salvelinus fontinalis)* [8,9], turbots (*Scophthalmus maximus*) [10], Japanese flounder (*Paralichthys olivaceus*) [11], African sharp tooth catfish (*Clarias gariepinus*), Nile tilapia (*Oreochromis niloticus*) [12], catla (*Catla catla*) and rohu (*Labeo rohita*) [13].

Several attempts were made to control *E.tarda* in fish including vaccination. Oral administration of anti-*E.tarda* chicken egg yolk immunoglobulin (Ig) Y [14], formalin-killed cells [15], *E.tarda* ghost cells [16], lipopolysaccharides [15,16], a virulent *Edwardsiella tarda* strains as a live vaccine [17] and DNA vaccines [18]. But still commercial vaccine for the prevention of *E. tarda* infection is not widely available [19].

The use of immunogenic proteins and outer membrane protein (OMP) of pathogenic bacteria as protective antigens is a novel vaccine strategy. Several major antigenic proteins of *E. tarda* have been used to evaluate their immuno- protectively in Japanese flounder fish i.e*., Esa1* [20], *Et18* and *EseB* [19], *Eta 21* [21], *FliC* and *Eta6* [22]. The 37-kDa OMP is conserved in different serotype strains of *E.tarda* and N-terminal amino acid sequence of 37 kDa and it components have shown the same amino acid residues; 20 residues of 37 kDa have high similarity to GAPDH [23] and effective multi-purpose vaccine candidate against the different kinds of pathogenic bacteria [24]. The 61 kDa protein of present study was a major protein band of Western bloat analysis and present in different isolates of *E.tarda.* This study was to comparison of protective antigenicity of 37 kDa and 61 kDa proteins of *Edwardsiella tarda* in Indian major carp Rohu (*Labeo rohita*).

Materials and Methods

Experimental animals

Fish: Clinically healthy, 40-50 gm rohu fish were procured from commercial fish farm in Nallasopara, Maharashtra state, India. Fish were acclimatized in 300 liter tanks for one month and fed @ 5% of the fish body mass using a commercial fish feed. The temperature, pH, dissolved oxygen, unionized ammonia and nitrite were maintained at favorable range and measured as per APHA (1998).

New Zealand white Rabbits: Clinically healthy, New Zealand white rabbits of 8-10 months old and weighing 2.5 kg were procured from National Institute of Nutrition, Hyderabad, India. Rabbits were fed with commercial rabbit feed and the immunization was carried out after due approval from the Animal Care and Ethics Committee of the Central Institute of Fisheries Education (CIFE).

***E. tarda* cultures and characterization:** Indian field isolates (*ET-1, ET-2* and *ET-3*) were gratis from Dr. Gaurav Rathore, Principal Scientist, CIFE, Mumbai, India and *E.tarda* reference strain (*ATCC 15947*) was obtained from Microbiologics®, USA. The bacterial isolates were identified from VITEK® 2 System (bioMérieux, France) and 16s rRNA gene sequencing (Bio Innovation, India).

Pathogenicity of *E.tarda* isolates: Fish were injected intraperitonelly, $1x10^6$ cfu *E. tarda* isolates suspended in 100 µl PBS to test their pathogenicity. The bacteria re-isolated from moribund fish were confirmed as *E. tarda* by VITEK® 2 System (bioMérieux, France). The virulent gene *sod B* was amplified by PCR [25].

Purification of Rohu IgM: Rohu fish were immunized using bovine serum albumin (BSA), 25 in numbers. Fish blood was drawn from *vena caudalis* and serum was separated [26]. The ammonium sulfate precipitation and salt fractionation [27] was conducted to collect rohu immunoglobulin (Ig). The Ig was purified [28] by DEAE cellulose (Merck) column.

SDS-PAGE analysis of purified rohu IgM: The vertical electrophoresis Maxi: 20 x 10 cms (MERCK MILLIPORE®) unit was used to conduct SDS PAGE [29]. The separating gel was 15% and stacking gel was 5%. 20 µl of rohu IgM was diluted in20 µl of 2 × sample loading buffer and boiled for 5 min. The rohu IgM was kept in ice for 10 min and load the SDS PAGE along with molecular weight marker (Puregene, pre-stained protein marker, Genetic Biotech) for electrophoresis. SDS PAGE was stained by Coomassie blue and destained until the background became clear. The SDS PAGE was analyzed by Gel-Quant software (DNr Bio imaging systems, Israel) to determine the molecular weight of heavy chain and light chain of rohuIgM.

Production of Anti-Rohu Immunoglobulin Antibody in Rabbit: Freund's complete adjuvant (FCA) (Merck) and purified rohu Ig were made an emulsion at 1:1 ratio. The rabbit was injected subcutaneously, 1 ml of emulsion containing 200 µg of purified rohu Ig. The boosters were administered at 7th, 14th, 21st and 28th days with Freund's incomplete adjuvant (FIA). Rabbit was bled on 35th day

from marginal ear vein for serum separation. Rabbit IgG was purified by commercial kit (Bangalore Genei, India). SDS-PAGE was conducted to determine molecular weight of heavy and light chains.

Agarose gel precipitation test (AGPT) and Western blot: Specificity and cross-reactivity of anti-rohu rabbit IgG with rohu IgM was conducted by Agarose gel precipitation test (AGPT) and Western blot [29]. The separated protein bands of purified rohu IgM in SDS-PAGE were transferred to PVDF membrane (Amresco, Total blot +™) using semi dry blotting apparatus (BioRad, USA). The nonspecific sites were blocked by 3% skim milk powder in phosphate buffer saline (PBS). The PBS (pH 7.2) containing Tween-20 (PBS-T) was used for washing in end of each step (4 × 5 min). Anti-rohu rabbit IgG in PBS was the primary antibody (1: 5000). The conjugate was goat anti-rabbit HRP conjugate (1:1000 in PBS, Genei™, India) and diaminobenzidine (10 mg of diaminobenzidine in 10 ml of PBS) (Sigma) containing 10 µl of Hydrogen peroxide (MERCK) was the substrate. Finally, the reaction was stopped by distilled water. The image of stained gel and membranes were analyzed by Gel-Quant software (DNI Bio imaging systems, Israel), once membrane was air dried at room temperature.

Development of anti *E. tarda* polyclonal rabbit serum: Anti *E. tarda* immunoglobulin was raised in rabbit with slight modification of Dresser (1986). Formalin killed *E. tarda* 1 × 10^9cfu/ml suspended in PBS was used for immunization. The rabbit was bled from marginal ear vein prior to immunization to confirm absence of anti *E. tarda* antibodies by agglutination test. The antigen was injected to marginal ear vein on 1st, 4th, 6th, 8th, 10th and 14th days. The injected volume was 0.1ml initially and subsequent volumes were 0.25 ml, 0.5 ml, 1 ml, 2 ml and 3 ml respectively. The rabbits were bled from marginal ear vein one week after the last immunization and the serum was separated. The titration of antiserum was conducted [30]. The control was pre-immunization serum.

Development of anti *E. tarda* antibodies in rohu fish: The 10 number of Fish were injected intraperitoneally 100 µl of formalin killed *E. tarda* (1.0X 10^5cfu) in PBS (pH 7.4). Two boosters were given at 10 day intervals and bled one week after last injection for serum collection. The antibody titer was measured by agglutination test [30].

Extraction of whole cell antigens and outer membrane proteins of *E. tarda*: *E. tarda* antigens were extracted from *ATCC 15947* strains and field isolates using SDS- PAGE sample buffer as described by [31]. Briefly, overnight grown *E. tarda* cultures in BHI broth was centrifuged (5000g for 5 min). The recovered bacterial pellets were washed 3 times in PBS. The bacterial pellets were lysed with SDS- PAGE sample buffer by boiling at 100°C for 3 minutes. Finally, samples were kept in ice for 5-10 minutes.

Sarkosyl method was used to extract outer membrane protein from *ATCC 15947* strain and field isolates as described by Kawai (1998). Briefly, overnight grown *E. tarda* cultures in BHI broths were centrifuged (5000g for 5 min) and the supernatant was discarded. The recovered bacterial pellets were washed three times with 20 mMTris/HCl (pH 8.0) and disrupted by sonicator three times each for 30 seconds. The suspensions were centrifuged at 4000× g for 10 min to remove intact cells. The cell outer membranes samples were recovered by centrifugation at 43 000 × g for 30 min. The recovered outer membrane samples were treated with Sarkosyl (Himedia, India) at protein /detergent ratio of 1:6 (mg/ml) for 30 min at 32°C. The mixtures were centrifuged again at 43 000 × g for 30 min. The recovered outer membrane protein pellets were washed 3 times 20 mMTris/ HCl (pH 7.4) and stored at -20°C.

Identification of immunogenic proteins of *E. tarda* in rohu fish using Western blot: The concept of [31] was followed to identify immunogenic proteins from *E. tarda*. Whole cell antigens of *E. tarda* isolates and outer membrane proteins of each isolates were separated by two 15% SDS PAGE gels with a pre-stained protein marker (Puregene, pre-stained protein marker, Genetic Biotech, India). The molecular weights of whole cell antigenic proteins were measured by Gel-Quant software (DNr Bio imaging systems, Israel).

The separated proteins of one SDS PAGE was transferred to PVDF (Amresco, Total blot + ™) membranes by electro blotting. The nonspecific sites were blocked by 3% skim milk powder in PBS. PBS-T was used for washing at the end of each step (4 × 5 min). The anti *E. tarda* rohu serum in PBS (1: 200) and anti-rohu rabbit IgG in PBS (1: 500) were used as primary and secondary antibody respectively. Goat anti-rabbit HRP conjugate (1:1000 in PBS, Genei™, and India) was used as a conjugate and diaminobanzidine (Sigma, USA) was the substrate for enzyme reaction (10 mg of diaminobanzidine 10 µl of hydrogen peroxide (Merck) in 10 ml of PBS). The color reaction was stopped using distilled water.

Western blot was conducted using anti *E. tarda* rabbit serum (1:1000) as primary antibody to detect immunogenic proteins reacted with rabbit serum.

Extraction of immunogenic proteins from SDS PAGE gel: The procedure of Hardly et al. (1996) [32] was conducted with a modification to extract immunogenic protein from *E.tarda*. Preparative SDS PAGE (2 mm thickness, 15%) was prepared in vertical electrophoresis Maxi: 20 x 10 cms (MERCK MILLIPORE®) apparatus to extract whole cell *E. tarda* proteins. Separate SDS PAGEs were used for extraction of OMPs. A number of SDS PAGEs were used to harvest required amount of immunogenic proteins. SDS PAGE was stained by reverse stain technique. Briefly, SDS PAGE was rinsed with distilled water for 30 to 60 seconds and incubated with 0.2 M imidazole (Merck) solution containing 0.1% SDS for 25 minutes. The gel was immersed in 0.2 M zinc sulfate (Merck) solution until the gel background become deep white. The reaction was stopped by rinsing the gel with abundant distilled water. Protein bands appeared as transparent and colorless lines in white a background. The protein bands of 61 kDa from whole cell extraction and 37 kDa of OMP were excised which rinsed with PBS containing 100 mM EDTA (2 × 10 min). Gel slices were washed twice in PBS to remove excess EDTA. Gel renaturation was done by stocking the gel slices in PBS containing 0.1% Triton X-100 (3× 10 min). The gel was washed twice in PBS to remove excess detergent. The protein recovery was done by passive elution from crushed gel pieces by incubation in PBS (2× 10 min) under vigorous shaking till PBS absorb to gel pieces. The gel pieces were frozen at - 20°C. The protein fluid present in gel pieces leaves the gel and freeze. PBS contained proteins were collected while melting of the gel pieces. Proteins concentrations were measured by Nanodrop (Thermo Scientific, USA). The extracted proteins were concentrated using 10K Nanosep® Centrifugal Devices and stored at -20°C.

Experimental design and vaccination: The fish were transferred to plastic tanks filled with 70 liters of water. Ten numbers of fishes in tank each and the experimental period conducted for 30 days. The fish were vaccinated 10 µg of protein in PBS intraperitoneally emulsified with Freund's incomplete adjuvant [20] (FIA) (Merck). The injected volume was 100 µl per fish. FIA emulsified with PBS was injected to control fish. A single booster of 10 µg protein in PBS was injected after 10 days of primary vaccination. The experiment was conducted with triplicates for each protein and controls. The blood was drawn end of every week (7,14,21,28 days) from 5 fish of each tank for hematological and immunological studies.

Challenge study for relative percentage survival: Fish were challenged with *ET-1* field isolate of *E. tarda,* 1 x10^6 cfu in 100µl of PBS at 35th day intraperitoneally. The disease incidence was observed over one week. The cumulative mortality (CM) and relative percentage of survival (RPS) were calculated as [33].

CM % = (Number of death at end of experiment / Total number fish) x 100

RPS % = (1-[% Mortality of vaccinated fish / %mortality of unvaccinated control fish]) ×100

Haematology: White blood cell counts were determined by Neubauer's counting chamber of haemocytometer with Shaw's solutions, as described by Hesser (1960) [34]. Blood smears of each fish were stained with Wright's and Giemsa for differential count. The identification of neutrophils, monocytes, and lymphocytes were conducted by Hibiya (1982) [35] and Chinabut (1991) [36].

Non-specific immunity

Nitrobluetetrazolium (NBT) Test: NBT activity was made by the method of [37] as modified by [38]. 100 µl of blood was placed in wells of flat bottom microtiter plates and incubated at 37°C in water bath for 1 hr to facilitate cell adhesion. The supernatant was removed and wells were washed by PBS for three times. 100µl of 0.2% NBT was added to each well and incubated further 1 hr at 37°C. The phagocytic cells were fixed with 100% methanol for 2-3 min and washed once with 70% methanol. The plates were air dried. 120 µl of freshly prepared 2N KOH and 140 µl dimethylsulphoxide (DMSO) were added into each well to dissolve the formazone blue precipitate formed. The OD of blue colored solution was read in ELISA reader at 620 nm.

Phagocytic index and activity: Phagocytic index and activity of neutrophils and monocytes were determined as [39]. 100 µl of blood was placed in a microtiter plate well. 100 µl of *Staphylococcus aureus* 1×10^7cfu / ml cells (Microbiologics ® USA) suspended in PBS (pH 7.2) was added to plate wells and mixed. The bacteria–blood solution was incubated for 25 minutes at room temperature. A 5 µL of bacteria–blood solution was used to prepare a smear. The smear was air dried, fixed with ethanol (95%) for 5 min and stained with 50% Giemsa for 15 min. A total of 100 neutrophils and monocytes from each smear were observed under oil objective of light

microscope. The number of phagocytic cells and the number of bacteria engulfed by the phagocytes were counted. Phagocytic capacity was the number of bacteria engulfed cells divided by the total number of neutrophils and monocytes (phagocytes) examined. Phagocytic index was expressed as the total number of bacteria engulfed by the phagocytes, divided by the total number of phagocytes containing engulfed bacteria.

Myeloperoxidase (MPO) activity: Total MPO content present in serum was measured according to Quade and Roth (1997) [40] with slight modification by Sahoo [41]. 10 µl of serum diluted with 90 µl of Hank's balanced salt solution (HBSS) without Ca^{2+} or Mg^{2+} in 96-well plates. Then 35 µl of 20 mM 3, 3'-5, 5'-tetramethylbenzidine hydrochloride (TMB) (Sigma, USA) and 5 mM H_2O_2(Merck) (freshly prepared) were added. The colour change reaction was stopped after 2 min by adding 35 µl of 4 M sulphuric acid (H_2SO_4). The OD was read at 450 nm from a micro plate reader (µ Quant, Universal micro plate spectrophotometer).

Serum lysozyme activity: The lysozyme activity was measured by a turbidimetric assay of Sankaran and Gurnani [42] with slight modification by Sahoo [41] with chicken egg white lysozyme (Sigma) as a standard. 20 mg of *Micrococcuslysodeiktic us* (Sigma, USA) was dissolved in 100 ml acetate buffer (0.02 M, pH 5.5). Microtiter plate wells were lorded 15 µl of serum and 150 µl of bacterial suspension. The OD was taken at 450 nm at the beginning and 1 hr after incubation at 25°C. The difference of 0.001 in ΔOD at 450 nm observed at 1h is taken as the measure of lysozyme activity.

Specific immunity

Quantification of Serum Immunoglobulin by Enzyme Linked Immuno Sorbent Assay (ELISA): The specific anti *E. tarda* antibodies were detected by antigen capture ELISA. *E. tarda* bacterin was prepared to coat ELISA plates [13]. The optimum concentration of *E. tarda* bacterin, primary anti body, secondary antibody and conjugate were estimated by checker board titration. The each well of microtitre plates were coated with 5 µg of *E. tarda* bacteria diluted in 50 µl of carbonate–bicarbonate buffer (pH 9.3) and kept overnight at 4ºC [13]. The plates were washed 4 times in PBS-T at the end of below steps each. The nonspecific sites were blocked by 50 µl of 3% skim milk powder for 2 h at 37°C. 50 µl of rohu serum was serially diluted across the plate and incubated at 37°C for 1 hr. 50 µl of anti-rohu rabbit Ig G (1: 500) was added to all wells and incubated at room temperature for 1 hr. 50 µl of goat anti-rabbit HRP conjugate (1:10,000) was added and incubated at 37°C for 1 hr. 50 µl of *Tetramethyl Benzidine* (TMB) Hydrogen Peroxide solution was added per well and kept in dark for 10 min. 4 M Sulphuric acid (H_2SO_4) was used to stop the reaction. The absorbance was measured at 450 nm in ELISA reader (BioRad, USA). Antigen control (without rabbit serum) and negative serum control (healthy rabbit serum) were used for test validation.

Statistical analysis

The data were statistically analyzed by statistical package SPSS version 16 (SPSS Inc., Chicago, Illinois, USA). Data were subjected to one way ANOVA and Duncan's multiple range tests was used to determine the significant differences between the variables. The t-test was used in parameters of CM and RPS. Comparisons were made at the 5% probability level.

Results

Authenticity of *E. tarda* isolates

E. tarda field isolates were confirmed by automated VITEK® 2 System (bioMérieux, France) and 16s rRNA sequencing. The virulent *so dB* gene was present in all *E. tarda* isolates including *ATCC 15947* reference strain.

Pathogenicity of *E. tarda* isolates

The fish injected experimentally *E.trada* were having abdominal dropsy and hemorrhages around the vent, base of fins and ventral body surface. The friable liver covered with fibrinous exudates. The spleen was congested and heart was edematous. *Peritoneal* cavity was filled with bloody ascetic fluid. The bacteria isolated from moribund fishes was reconfirmed as *E. tarda* by VITEK® 2 System (bioMérieux, France).

Specificity of rabbit anti-rohuIgG with rohuIgM and serum agglutination test

SDS PAGE of rohuIgM had two bands of 85 kDa and 23 kDa corresponding to heavy chain and light chain respectively. The 52 kDa band of heavy chain and 24 kDa light chain were present in SDS PAGE of anti-rohuIgM rabbit IgG. Specificity of anti-rohuIgM rabbit to purified rohuIgM was indicated by sharp distinct precipitation in contact zones in AGPT. Specific reaction at 85 kDa level in Western blot indicated the specificity of anti-rohuIgM rabbit to heavy chain of rohu IgM. The antibody titer of

anti *E.tarda* rabbit serum and anti *E.tarda* rohu serum was 256 and 8 respectively by agglutination test.

Protein profile of *E.tarda* antigens

The protein bands number was varied in each *E. tarda* isolate from SDS PAGE analysis. The highest number of whole cell bands was present in *E T-3* and highest number of OMP bands were in *ATCC 15947* (Plate 1).There were six major protein bands in whole cell antigens located at *75*kDa, 61 kDa, 50 kDa, 47kDa, 33 kDa and 30 kDa and two major OMP bands of 37 kDa and 40 kDa were reacted with anti *E. tarda* rohu serum in Western blot (Plate 2).The anti *E. tarda* rabbit serum was reacted with whole cell antigens of 84 kDa, 61 kDa, 47 kDa and 20 kDa l and OMPs of 37 kDa and 40 kDa (Plate 3). 61 kDa was the prominent whole cell antigen and 37 kDa was potential vaccine candidate described by many authors. 61 kDa and 37 kDa protein bands were extracted from preparative SDS PADE gels and assessed its purity from SDS PAGE for hematological and immunological studies.

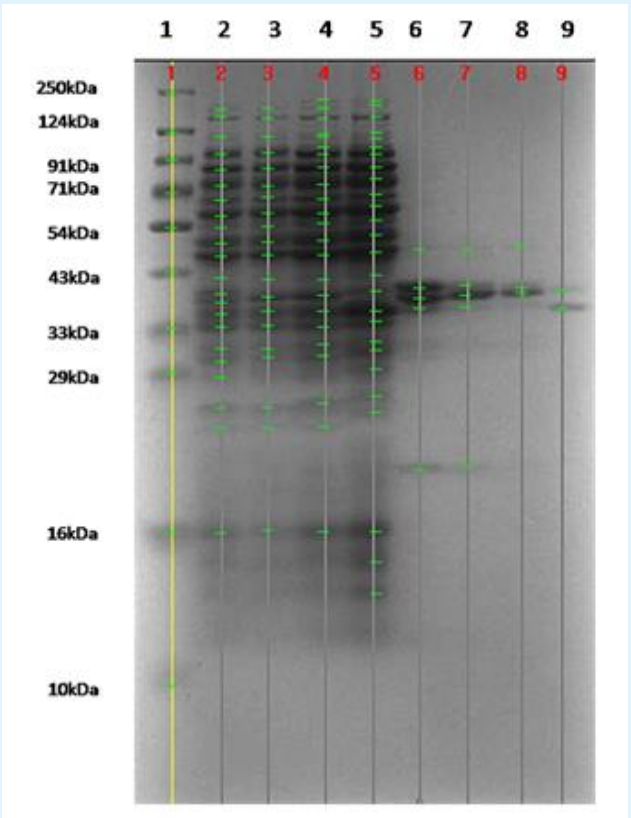

Plate 1: Protein profile of *E.tarda* antigens and Omps (SDS PAGE 15% Resolving gel).

Lane 1: Protein marker
Lane 2: Whole Cell antigen of *E.tarda ATCC15947* strain
Lane 3: Whole Cell antigens of *E.tarda* isolate *ET-1*
Lane 4: Whole Cell antigens of *E.tarda* isolate *ET-2*
Lane 5: Whole Cell antigens of *E.tarda* isolate *ET-3*
Lane 6: Outer membrane of *E.tarda ATCC15947* strain
Lane 7: Outer membranes of *E.tarda* isolate *ET-1*
Lane 8: Outer membrane of *E.tarda* isolates *ET-2*
Lane 9: Outer membrane of *E.tarda* isolates *ET-3*

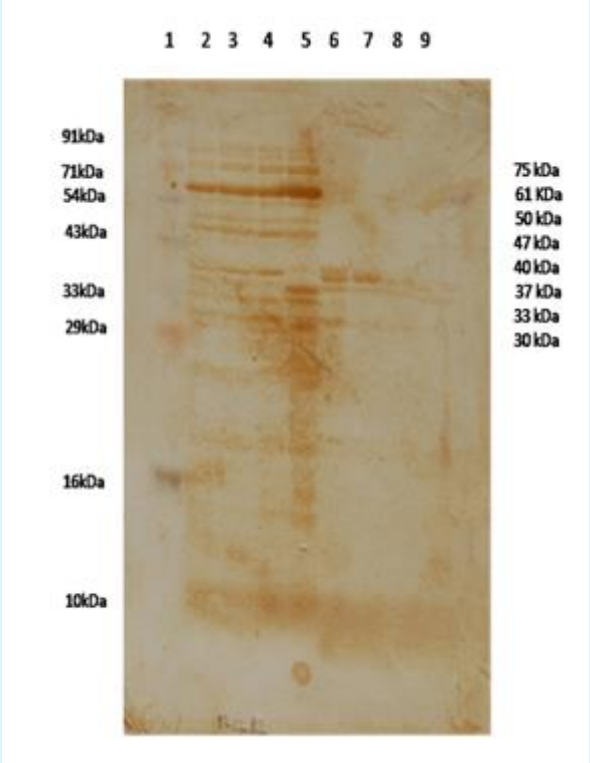

Plate 2: Western blot profile of *E.tarda* antigens and Omps (SDS PAGE 15% Resolving gel) using anti *E.tarda* rohu serum as primary antibody.
Lane 1: Protein marker
Lane 2: Whole Cell antigen of *E.tardaATCC15947* strain
Lane 3: Whole Cell antigens of *E.tarda* isolate *ET-1*
Lane 4: Whole Cell antigens of *E.tarda* isolate *ET-2*
Lane 5: Whole Cell antigens of *E.tarda* isolate *ET-3*
Lane 6: Outer membrane of *E.tardaATCC15947* strain
Lane 7: Outer membranes of E.tarda isolate *ET-1*
Lane 8: Outer membrane of *E.tarda* isolates *ET-2*
Lane 9: Outer membrane of *E.tarda* isolates *ET-3*

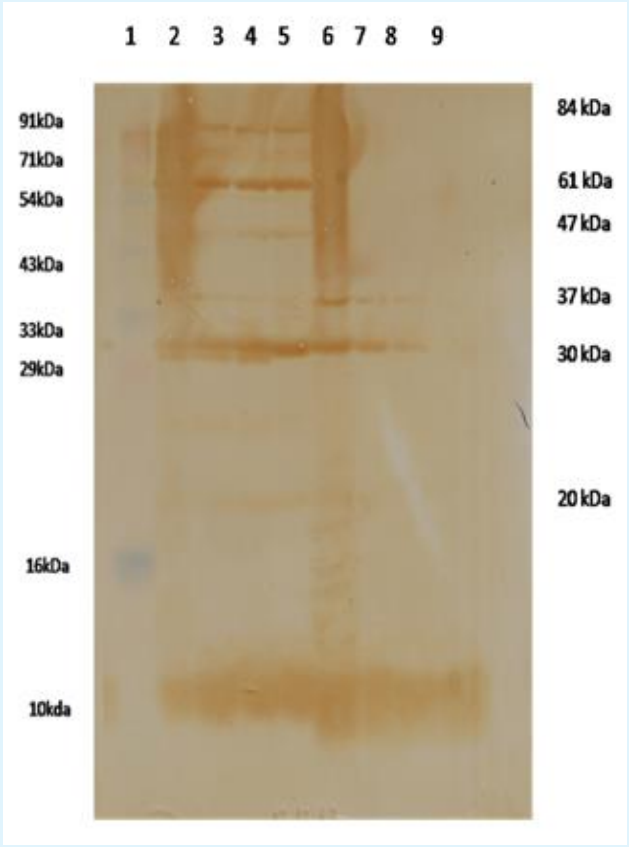

Plate 3: Western blot profile of *E.tarda* antigens and Omps (SDS PAGE 15% Resolving gel) using anti *E.tarda* rabbit serum as primary antibody.

Lane 1: Protein marker
Lane 2: Whole Cell antigen of *E.tardaATCC15947* strain
Lane 3: Whole Cell antigens of *E.tarda* isolate *ET-1*
Lane 4: Whole Cell antigens of *E.tarda* isolate *ET-2*
Lane 5: Whole Cell antigens of *E.tarda* isolate *ET-3*
Lane 6: Outer membrane of *E.tardaATCC15947* strain
Lane 7: Outer membranes of *E.tarda* isolate *ET-1*
Lane 8: Outer membrane of *E.tarda* isolates *ET-2*
Lane 9: Outer membrane of *E.tarda* isolates *ET-3*

Nonspecific and specific immune parameters

The treatment groups of 61 kDa and 37 kDa had significantly higher ($p \leq 0.05$) leukocyte count (Figure1), NBT activity (Figure 2), phagocytic index (PI) (Figure 3), phagocytic capacity (PC) (Figure 4), lysozyme activity (Figure 5), myeloperoxidase activity(MPO) (Figure 6) and specific antibody production (Figure 7) over control group.

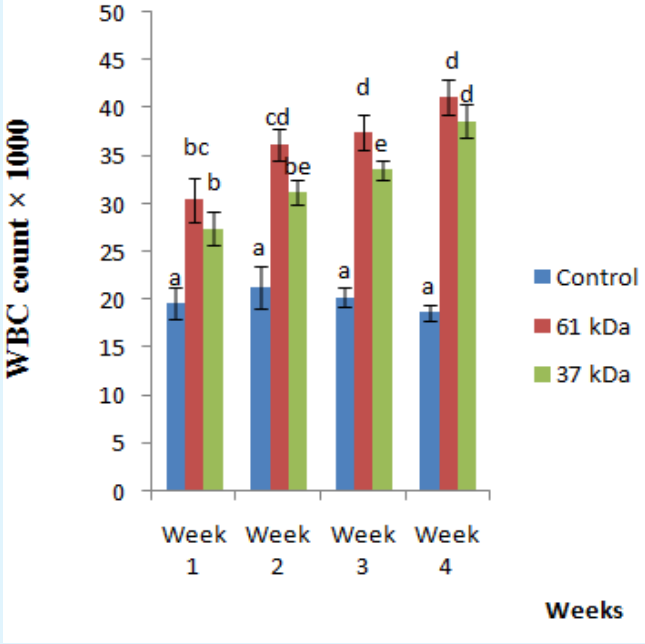

Figure 1: Effect of 61 kDa and 37 kDa *E. tarda* proteins as immunogen on white blood cell count in rohu blood.

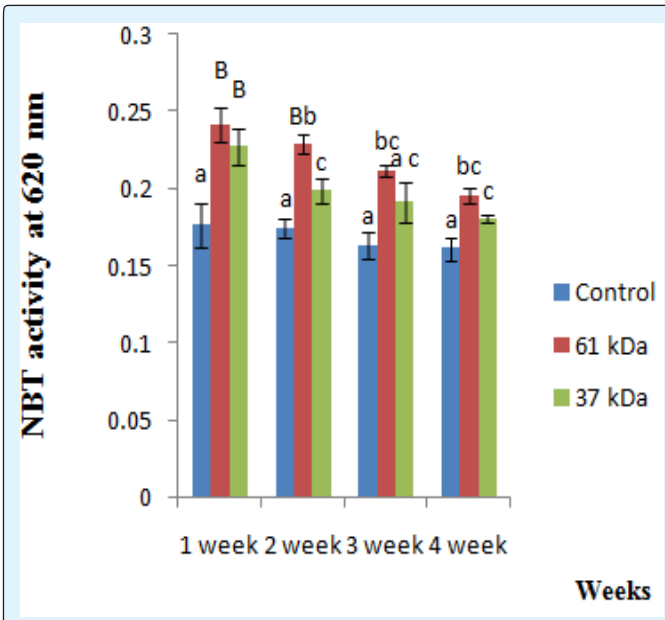

Figure 2: Effect of 61 kDa and 37 kDa *E. tarda* proteins as immunogen on NBT activity in rohu blood.

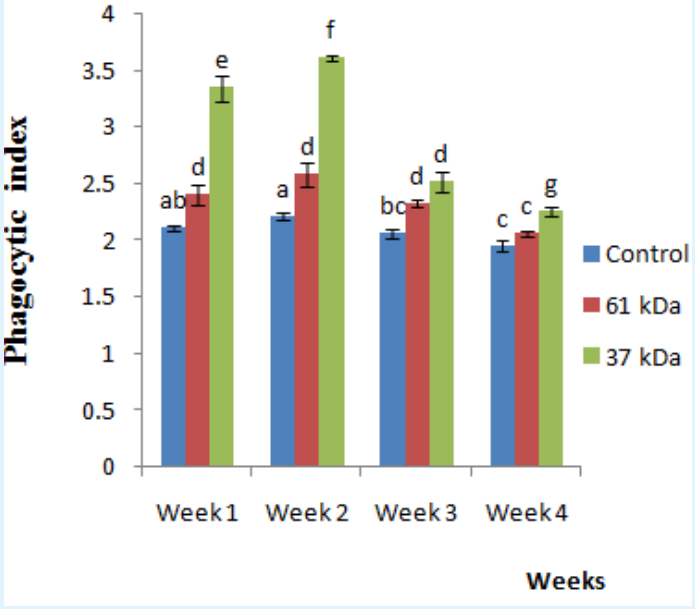

Figure 3: Effect of 61 kDa and 37 kDa *E. tarda* proteins as immunogen on phagocytic index in rohu blood.

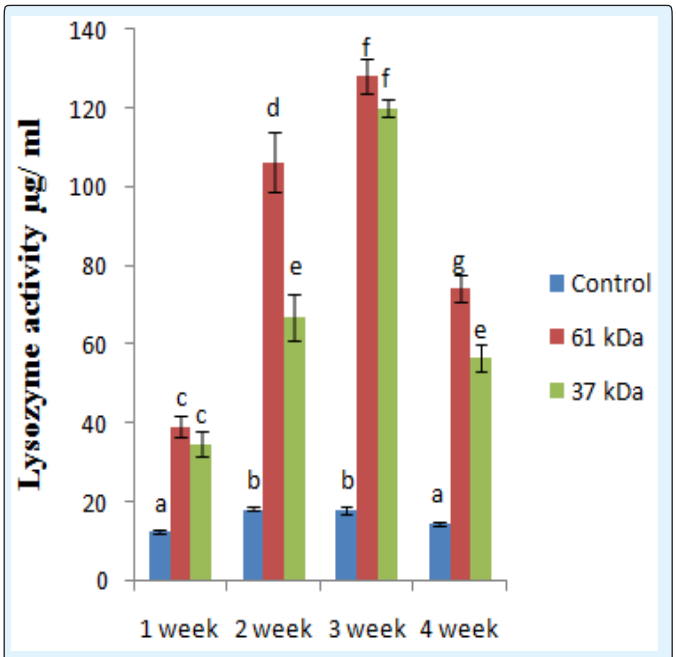

Figure 5: Effect of 61 kDa and 37 kDa *E. tarda* proteins as immunogen on lysozyme activity in rohu serum.

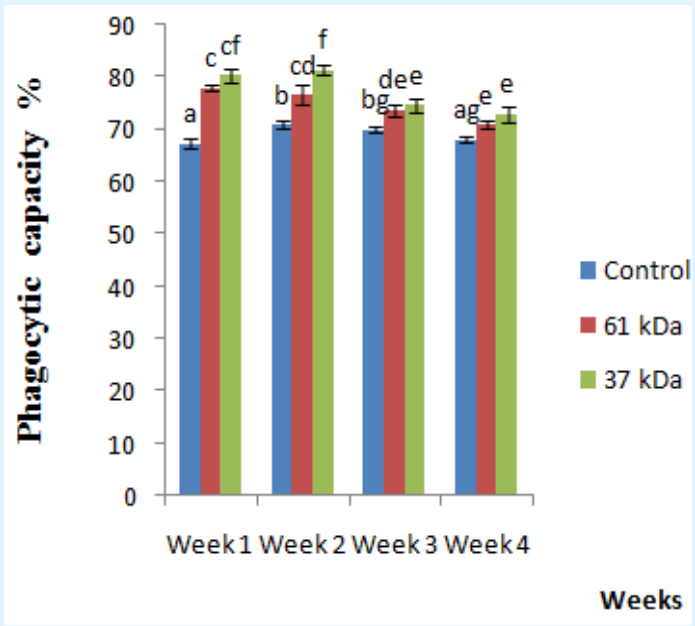

Figure 4: Effect of 61 kDa and 37 kDa *E. tarda* proteins as immunogen on phagocytic capacity in rohu blood.

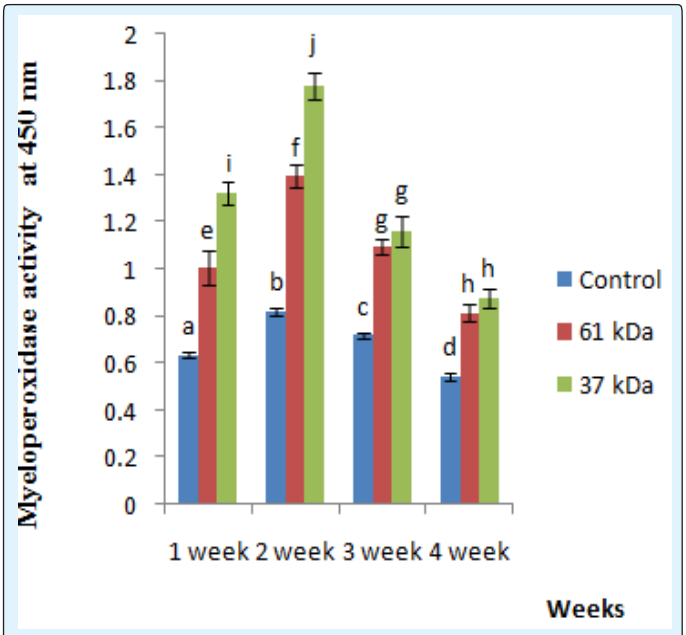

Figure 6: Effect of 61 kDa and 37 kDa *E. tarda* proteins as immunogen on myeloperoxidase activity in rohu serum.

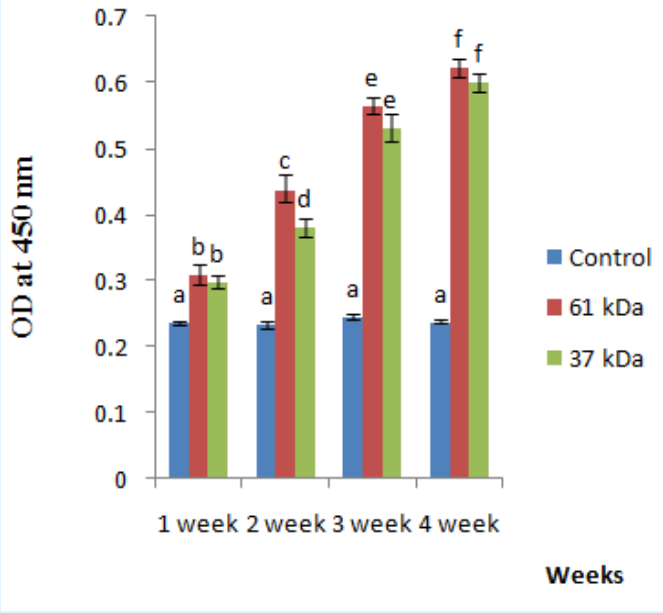

Figure 7: Effect of 61 kDa and 37 kDa *E. tarda* proteins as immunogen on specific antibody production against *E.tarda* (ELISA reading at OD 450nm) in rohu serum.

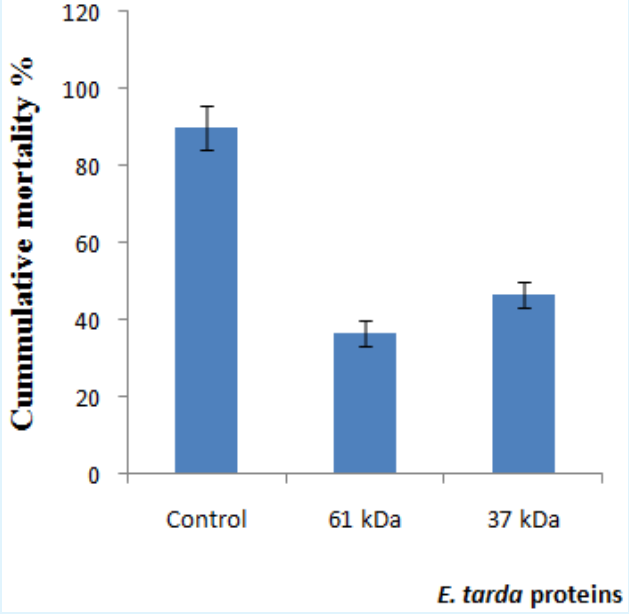

Figure 8: Effect of 61kDa and 37 kDa *E. tarda* proteins as immunogen on mean cumulative mortality in rohu after challenge with *E. tarda.*

The 61 kDa protein as immunogen was increased NBT activity at 2nd week and the lysozyme activity at 2nd and 4th week of immunization significantly ($p \leq 0.05$). The 37 kDa OMP was significantly increased phagocytic index (PI) at 2nd, 3rd and 4th weeks of immunization and phagocytic capacity (PC) at 2nd week. Myeloperoxidase activity (MPO) was significantly high in 37 kDa protein in 1st and 2nd weeks of immunization. The specific antibody production (mean ELISA reading) was significantly high ($p \leq 0.05$) in 61 kDa protein over 37 kDa at 2nd week of immunization. But there was not any significant difference at subsequent weeks of experiment.

Cumulative mortality (CM) and relative percentage survival (RPS)

The CM was significantly low and RPS was significantly high in treatment groups over control fish. The CM of 61 kDa was 36 % and RPS was 63 % (Figure 8 & 9). The CM of37 kDa was 46% and RPS was 53 % (Figure 8 & 9).

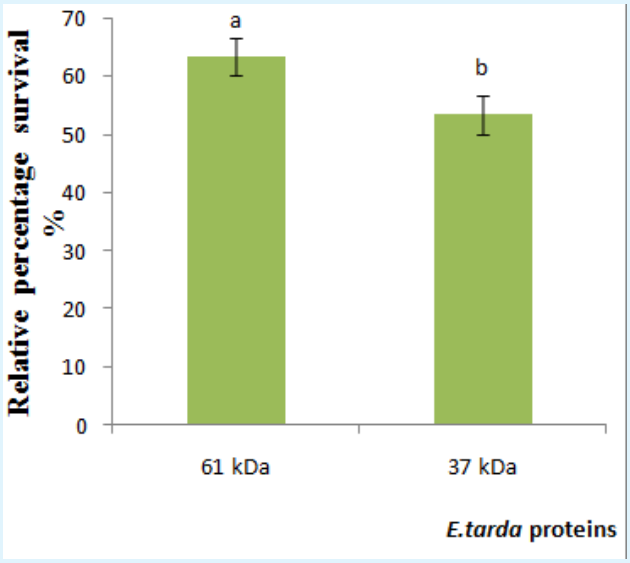

Figure 9: Effect of 61 kDa and 37 kDa *E. tarda* proteins as immunogen on mean RPS in rohu after challenge with *E. tarda.*

Discussion

Suresh [43] estimated molecular weights of heavy and light chains of rohu IgM as 85kDa and 23 kDa. The rabbit IgG heavy chain and light chain were 52 kDa and 24 kDa [44]. Western blot and AGPT confirmed the specificity of rabbit anti-rohu IgG with rohu IgM. Several *E.tarda* isolates and reference strain were collected for this study due to heterogeneity of *E.tarda* isolate seven within a limited geographic location [45,46]. There were different numbers of major protein bands and OMPs in SDS PAGE among isolates. The protein bands strongly reacted with anti *E.trada* rohu and rabbit serum, were participated in cell antigenicity and carried a common epitope specific to *E.tarda* species [23]. 61 kDa whole cell protein and 37 kDa OMP were strongly reacted with anti *E.trada* rabbit and rohu serum. 37 kDa OMP was reported by several authors as a vaccine candidate for *E.trada* [23,24,47]. 61 kDa was a new protein best of our knowledge.

The non specific response was significantly active within first few weeks of present study. *rEta2* (17.4 kDa), an OMP of *E. tarda* was significantly increased NBT activity on 1st and 7th day in Japanese flounder [18]. The formalin killed whole cells of *Areomonas* significantly increased phagocytic activity at the 1st week of immunization [48]. The peak phagocytosis was recorded at the 2nd week of red sea bream immunized with formalin killed *E.tarda* cells [49]. Lysozyme activity and phagocytic index of Chinese breams were increased by OMP 38 recombinant protein of *Aeromonashydrophila*in the 14th day of immunization [50]. Non-specific immune response may extend up to 4-8 weeks with minor effect of non-specific protection [51]. However, serum lysozyme activity, phagocytic activity and bactericidal activity of head kidney leukocytes was correlated with resistance of fish against bacterial infections [52].

The recombinant *E.tarda* proteins of *Eta6* [22], *Eta* 21 [22], *rEta2* [18] and *Esa1* [20] were developed specific antibodies after 4 weeks of immunization. The highest peaks of serum IgM and surface Ig-positive cell number were reported at the 4th week in Japanese flounder immunized by outer membrane proteins of *E.trada* [11]. The peak antibody titer for OMP 38 was at 21st day [50].

Recombinant *Esa1* had 84% RPS [20]. *Et18* and *EseB* proteins gave 61% and 51.3% of RPS respectively the hybrid of *Et18* and *EseB* proteins had 71% of better RPS. The Filched 70% CM and 26% RPS [22]. The putative protein of *Eta6* gave 45 % CM and 53 % RPS [22]. The common carp vaccinated with recombinant 36 kDa (OmpA) had 54.3 % of RPS [53]. The recombinant Eta2 of has given RPS of 80% in Japanese Flounder 4 - 8 weeks of post vaccination.

Specific antibody production was not significantly different among two proteins after the 2nd week of immunization in present study. The higher RPS of 61 kDa indicated that antibodies for 61 kDa were more effective against *E.tarda* rather than antibodies for 37 kDa.

Conclusion

In conclusion 61 kDa proteins of *E.tarda* could be considered as best vaccine candidate for development of an effective vaccine against different serotype of *E.tarda.* 61 kDa was the prominent whole cell antigen and it could be used as coating antigen of antigen capture ELISA for sero-monitoring and surveillance of edwardsiellosis.

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