Profile and protective role of 61kDa and 47kDa antigens of *Edwardsiella tarda* in rohu (*Labeo rohita*)

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Abstract

Edwardsiella tarda is a versatile pathogen that could survive in different environmental conditions and infect economically important fish species. The whole cell antigenic proteins were extracted from *E. tarda* and assessed via Western blot using anti *E. trada* rohu and anti *E. trada* rabbit serum. Two strong reacted proteins with antibodies were recovered *viz* 61 kDa and 47 kDa from SDS PAGE gels to evaluate their vaccine potential in rohu fish. Fish were vaccinated 10 µg of antigenic proteins intraperitoneally as immunogens 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 47 kDaproteins had significantly higher RPS, white blood cell count, specific and nonspecific parameters over control group. 61 kDa protein gave significantly high NBT activity, lysozyme activity and specific antibodies for *E. tarda*. 47 kDa protein hadsignificantly high phagocytic index and myeloperoxidase activity. The phagocytic capacity was significantly high at 3rd week of 47 kDaprotein. 61 kDaand 47kDa proteins had RPS of 59% and 52% respectively after challenge studies. Non specific immune parameters were more active at the first four weeks of immunization and specific antibodies for *E. tarda* were significantly higher since second week and peaked at 4th week in this study. Therefore 61 kDa could be considered as best vaccine candidateover 47 kDa for *E. tarda* infection in rohu fish.

Keywords: *Edwardsiella tarda*, Protective antigens, *Labeo rohita*, 61kDa protein

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Introduction

Edwardsiella septicaemia is an acute to chronic disease of primarily warm water fish (Farmer and McWhorter 1984). *E. tarda* has been reported all over the world in tropical, temperate and marine fish species Eels (*Anguilla spp*.), channel catfish, *Ictalurus punctatus* (Alcaide, Herraiz & Esteve 2006); chinook salmon (*Oncorhynchus tshawytscha),* brook trout, *Salvelinus fontinalis* (Amandi,Hiu, Rohovec & Fryer 1982; Uhland, Helie & Higgins 2000); turbots, *Scophthalmus maximus* (Xiao, Wang ,Liu, Wang, Liu & Zhang 2009), Japanese flounder, *Paralichthys olivaceus* (Tang, Zhan, Sheng & Chi 2010); African sharp tooth catfish (*Clarias gariepinus*), Nile tilapia,*Oreochromis niloticus* (Ibrahem,Iman, Shaheed, Yazeed & Korani 2011), catla (*Catla catla*) androhu, *Labeo rohita* (Swain &Nayak, 2003). *E. tarda* has been isolated from bird, land mammals and even reptiles (Owens, Nelson &Addison1974; Goldstein, Agyare, Vagvolgyi & Halpern1981). *E. tarda* is a zoonotic disease causing gastrointestinal and extra-intestinal infection in humans (Janda & Abbott 1993; Lowry & Smith 2007).

There are numerous efforts were made to control *Edwardsiella septicaemia* in a variety of fish species through vaccination Japanese flounder, *Paralichthys olivaceus* (Takano,Matsuyama, Oseko, Sakai,Kamaishi, Nakayasu, Sano & Iida 2010), eel, *Anguilla japonica* (Salati,Ono & Kusuda 1991), common carp (Maiti, Shetty, Shekar, Karunasager & Karunasager 2011) & tilapia, *Oreochromis spp* (Kwon,Nam, Kim &Kim2006) due to heavy economic losses. The vaccination attempts were oral administration of anti-*E. tarda* chicken egg yolk immunoglobulin (Ig) Y (Gutierrez, Miyazaki, Hatta & Kim 1993), formalin-killed cells (Gutierrez & Miyazaki 1994), *E. tarda* ghost cells (Kwon *et al.,* 2006), lipopolysaccharides (Gutierrez & Miyazaki 1994; Salati, Kawai & Kusuda1994), outer membrane proteins (OMPs) (Tu & Kawai 1998; Kawai, Liu, Ohnishi & Oshima 2004; Tang *et al.,* 2010) and DNA vaccines (Sun, Liu & Sun 2011). A commercial vaccine for the prevention of *E. tarda* infection is not widely available (Hou, Zhang & Sun 2009).

The use of immunogenic proteins as sub-unit vaccine is a novel area of fish vaccination. Some of the immunogenic proteins have a capable of protective antigenicity over a pathogen invasion. Several major antigenic proteins of *E.tarda* have been used to evaluate their immuno- protectively in Japanese flounder fish i.e., *Esa1* (Sun, Liu & Sun 2010), *Et18* and *EseB* (Hou *et al.,* 2009), *Eta 21* (Jiao, Dang , Hua & Sun 2009a), *FliC* and *Eta6* (Jiao, Zhang, Hu & Sun 2009b). The immuno- protectively of *E. tarda* protein as protective antigen(S) in Indian major carp Rohu is hardly available at present.

Materials and Methods

Bacterial Cultures

Edwardsiella tarda ATCC 15947 strain was obtained from Microbiologics ®, USA and Indian field isolates (*ET-1*, *ET-2* and *ET-3*) were gratis from Dr. Gaurav Rathore, Principal Scientist, Central Institute of Fisheries Education (CIFE), Mumbai, India.

Experimental animals

Fish

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

Rabbits

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

Characterization of *E. tarda*isolates

E. tarda isolates were streaked on Salmonella Shigella agar (SS agar) (Himedia, India) to get characteristic colonies. The bacterial isolates were identified from VITEK® 2 System (bio Mérieux, 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 *sodB* was amplified from PCR (Han,Kim, Lee, Kim & Park 2006).

Purification of Rohu IgM

Twenty-five numbers of rohu fish were immunized with bovine serum albumin (BSA). The blood was drawn by puncturing *vena caudalis* and serum was separated (Swain ,Mohanty & Sahu 2004). Total immunoglobulins were collected by ammonium sulfate precipitation and salt fractionation (Hay & Westwood 2002).The anion exchange chromatography was conducted for purification of rohu immunoglobulin(Ig)(Hudson & Hay 1991) using DEAE cellulose (Merck) column.

Production of Anti-Rohu Immunoglobulin Antibody in Rabbit

Purified rohu Ig was mixed with Freund's complete adjuvant (FCA) (Merck) at 1:1 ratio to make an emulsion. The rabbit was injected subcutaneously 200 µg of purified rohuIg present in 1 ml emulsion. Boosters were administered at 7^{th}, 14^{th}, 21^{st} and 28^{th} days with Freund's incomplete adjuvant (FIA). The rabbit was bled on 35^{th} day from marginal ear vein. The blood was clotted at room temperature, kept

at 4 ^{o}C overnight and centrifuged at 3000 g for 5 min for collection of serum. Rabbit IgG was purified from IgG purification kit (Bangalore Genei, India). Purified anti rohuIgrabbit was stored at -20^{0}C.

SDS-PAGE analysis of purified rohu IgM and rabbit IgG

SDS PAGE (Sambrook,Russel, Janssen &Irwuin2001) was conducted from vertical electrophoresis Maxi: 20 x 10 cms(MERCK MILLIPORE®) unit. The separating gel was 15% and stacking gel was 5%. 20 µl of rohuIgMwas diluted with equal volume of 2X sample loading buffer and boiled for 5 min. Samples were kept in ice for 10 min and load the adjacent wells of SDS PAGE along with molecular weight marker (Puregene, pre-stained protein marker, Genetic Biotech). The SDS PAGE was stained using Coomassie blue and destained until the background became clear afterthe electrophoresis. The gel was analyzed by Gel-Quant software (DNr Bio imaging systems, Israel) to determine the molecular weight of heavy chain and light chain of rohu immunoglobulin.The same procedure was conducted for SDS-PAGE analysis of purified rabbit IgG.

Agarose gel precipitation test (AGPT) and Western blot

Specificity and cross-reactivity of anti-rohu rabbit IgG was carried out using agarose gel precipitation test (AGPT) and Western blot (Sambrook*et al.,* 2001).The purified rohuIgM was separated in SDS-PAGE gel. Protein bands present in SDS-PAGE were transferred to PVDF membrane (Amresco, Total blot +™) using semi dry blotting apparatus (BioRad, USA).The nonspecific sites were blocked using 3% skim milk powder in PBS. The phosphate buffer saline (PBS, pH 7.2) containing Tween-20 (PBS-T) was used for washing in end of each steps (4 × 5 min). Anti-rohu rabbit IgG in PBS was the primary anti body (1: 5000).The conjugate was goat anti-rabbit HRP conjugate (1:1000 in PBS, Genei™, India) and the substrate wasdiaminobenzidine(10 mg of diaminobenzidine in 10 ml of PBS) (Sigma) containing 10 µl of Hydrogen peroxide (MERCK). Finally the reaction was stopped using tap water. The image of stained gel and membranes were analyzed by Gel-Quant software (DNI Bio imaging systems, Israel), once membrane air dried at room temperature.

Development of anti *E. tarda* polyclonal rabbit serum

Anti *E. tarda* immunoglobulin was raised in rabbit using formalin killed *E. tarda* with slight modification of Dresser (1986).The immunization was carried out formalin killed *E. tarda*1 × 10^{9}cfu/ ml suspended in PBS. The rabbit was bled through marginal ear vein prior to immunization. The serum was confirmed by agglutination test for absence of anti *E. tarda* antibodies. *E. tarda* suspended in PBS was injected to marginal ear vein on 1st, 4th, 6th, 8th,

10^{th} and 14^{th} days. Initial injected volume was 0.1ml 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 as Swain,Behura , Dash & Nayak (2007). Pre-immunization serum was used as the control.

Development of anti *E. tarda*antibodies in rohu fish

Apparently healthy 10 number of Rohu fish of size 40 - 50 gm were used for development of anti *E. tarda* rohu antibodies. Fish were injected 100 µl of formalin killed *E. tarda*in PBS (pH 7.4) containing 1.0X 10^5cfuintraperitoneally. Fish were injected 3 doses at 10 day intervals and bled one week after last injection for serumcollection. The antibody titer was measured fromagglutination test (Swain *et al*., 2007a).

Identification of immunogenic proteins of *E. tarda* inrohu fish using Western blot

The concept of Sakai, Matsuyama, Nishioka, Nakayasu, Kamaishi, Yamaguchi &Iida (2009) was followed for identification of immunogenic proteins from *E. tarda*. Whole cell antigens of *E. tarda* was separated by two 15% SDS PAGE gels along with 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) as mentioned.

The separated proteins of one SDS PAGE was transferred to PVDF (Amresco, Total blot + ™) membranes by electro blotting. The nonspecific sites were blocked using 3% skim milk powder in PBS. PBS-T was used for washing at theend 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™, India) was used as a conjugate and diaminobanzidine (Sigma, USA) was the substrate for enzymatic reaction (10 mgof diaminobanzidine10 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

Extraction of immunogenic proteins from *E.tarda* was conducted as Hardy,Santana, Sosa, Herna´ndez, Patron &Serra (1996) with slight modification. Two mmtickness 15% preparative SDS PAGE was prepared in vertical electrophoresis Maxi: 20 x 10 cms (MERCK MILLIPORE®) apparatus. SDS PAGE was stained by reverse stain technique using imidazole (Merck) and zinc sulfate (Merck). Briefly, SDS PAGE was rinsed with distilled

water for 30 to 60 seconds and incubated with 0.2 M imidazole solution containing 0.1% SDS for 25 minutes. The gel was immersed in 0.2 M zinc sulfate solution until the gel background become deep white. The reaction was stopped by rinsing the gel with abundant distilled water. Protein bands appeared, transparent and colourless in white a background. The protein bands of 61 kDa and 47 kDa was excised which rinsed with PBS containing 100 mM EDTA (2 × 10 min). Gel slices were washedtwice 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 gelpieces. The gel pieces were frozen at - 20ºC. The protein fluid present in gel pieces leaves the gel and freeze. PBS contained proteins were collected while melting of the gel pieces. The concentrations of proteins were measured using Nanodrop (Thermo Scientific, USA). The extracted proteins were concentrated using 10K Nanosep® Centrifugal Devices. The extracted proteins were stored at -20ºC. A number of SDS PAGES gels were used for extraction of whole cell *E.tarda* antigens.

Experimental design and vaccination

The fish were transferred to plastic tanks filled with 70 liters of water for experiment. There were 10 numbers of fishes in tank each and experimental period was conducted for 30 days. Two immunogenic proteins were extracted from SDS PAGE gels *viz*61kDa and 47 kDa. The fish were vaccinated 10 µg of immunogenic protein in PBS intraperitoneally emulsified with Freund's incomplete adjuvant (Jiao, Cheng, Hu & Sun 2010) (FIA) (Merck). The injected volume was 100 µl per 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 of vaccination. 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 Studies

Fish were challenged with *ET-1* 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 Amend (1981).

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 count of blood samples were determined by Neubauer's counting chamber of haemocytometer. The diluting fluid was Shaw's solutions, as described by Hesser (1960). Blood

smears of each fish were stained with Wright's and Giemsa for the determination of abundance of different leukocytesand percentages of each cell types. The identification of neutrophils, monocytes, and lymphocytes were according to Hibiya (1982) and Chinabut, Limsuwan & Kitswat (1991).

Non-specific immunity

Nitrobluetetrazolium (NBT) Test

Nitrobluetetrazolium assay was done by the method of Secombes (1990) as modified by Stasiack and Baumana (1996). 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 the loaded wells were washed using PBS for three times. 100µl of 0.2% NBT was added to each well and incubated for 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 dimethyl sulphoxide (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 activity of neutrophils and monocytes in blood was determined as Anderson and Siwicki (1995) using *Staphylococcus aureus* (Microbiologics® USA). A sample (0.1 mL) of blood was placed in a microtiter plate well. 0.1 ml of *S. aureus*1×10^7cfu / ml cells suspended in PBS (pH 7.2) was added and mixed well. The bacteria–blood solution was incubated for 25 minutes at room temperature. A smear was made in clean glass slide from 5 µL of bacteria–blood solution. 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 is the number of bacteria engulfed cells divided by the total number of neutrophils and monocytes (phagocytes) examined. Phagocytic index is 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) with slight modification by Sahoo,Kumari&Mishra (2005). About 10 µl of serum was 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 level was measured using the turbidimetric assay following Sankaran and Gurnani (1972) with slight modification by Sahoo *et al.,* (2005) using chicken egg white lysozyme (Sigma) as a standard. A solution was prepared dissolving 20 mg of *Micrococcus lysodeikticus* (Sigma,USA) in 100 ml acetate buffer (0.02 M, pH 5.5). Microtiter plate wells were lorded 15 µl of serum and 150 µl of the above suspension. The OD was taken at 450 nm at the beginning and after 1 h incubation at 25^0C. The difference of 0.001 in ΔOD at 450 nm observed at 1 h is taken as the measure of enzyme activity.

Specific immunity

Quantification of Serum Immunoglobulin by Enzyme Linked ImmunoSorbent Assay (ELISA)

The serum antibody specific to *E. tarda* was detected by indirect ELISA. *E. tarda* bacterin was prepared according to Swain and Nayak (2003). The optimum concentration of antigen, secondary antibody and conjugate was estimated by checker board titration (Crowther 1995). The wells of microtitre plates were coated each with 5 µg of ELISA antigen diluted in 50 µl of carbonate–bicarbonate buffer (pH 9.3) and kept overnight at 4^0C (Swain&Nayak2003). The plates were washed 4 times in phosphate buffer saline (PBS, pH 7.2) containing Tween-20 (PBS-T). The nonspecific sites were blocked using 50 µl of 3% skim milk powder for 2 h at 37 ^{0}C and washed 4 times in PBS-T to remove of excess skim milk. 50 µl of rohu serum was serially diluted across the plate and incubated at 37 ^{0}C for 1 hr. 50 µl of anti-rohu rabbit IgG (1:500) was added to all wells and incubated at room temperature for 1 hr. The plate was washed again 4 times with PBS-T. 50 µl of goat anti-rabbit HRP conjugate (1:10,000) was added and incubated at 37 ^{0}C for 1 hr and washed with PBS-T. 50 µl of tetramethylbenzidine(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 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.

Result

Authenticity of *E.trada* isolates

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

Pathogenicity of *E.tarda* isolates

Experimentally *E.trada*injected fish were shownabdominal dropsy and hemorrhages, especially around the vent, base of fins and ventral body surface. The friable liver covered with fibrinous exudates. The congested spleen and edematous heart was present in abdominal cavity, accumulated with bloody ascitic 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 analysis of rohu IgM had two bands of 85 kDa and 23 kDa corresponding to heavy chain and light chain respectively. There was a 52 kDa band of heavy chain and 24 kDa light chain was present in SDS PAGE analysis anti-rohuIgM rabbit IgG. Specificity of anti-rohu IgM rabbit to purified rohu IgM 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-rohu IgM rabbit to rohu IgM. The antibody titer of anti *E.tarda* rabbit serum and anti *E.tarda* rohu serum was 256 and 8 respectively from 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 band number was present in *E T-3*(Plate 1).There were six major protein bands located at *75*kDa, 61 kDa, 50 kDa, 47 kDa, 33 kDa and 30 kDa, was reacted with anti *E. tarda* rohu serum in Western blot (Plate 2).The anti *E. tarda* rabbit serum was reacted with protein band located at 84 kDa, 61 kDa, 47 kDa and 20 kDa levels (Plate 3). 61 kDa and 47 kDa were present in both Western blots as prominent bands. Therefore 61 kDa and 47 kDa protein bands were extracted from preparative SDS PADE gels and assessed its purity from SDS PAGE for hematological and immunological studies.

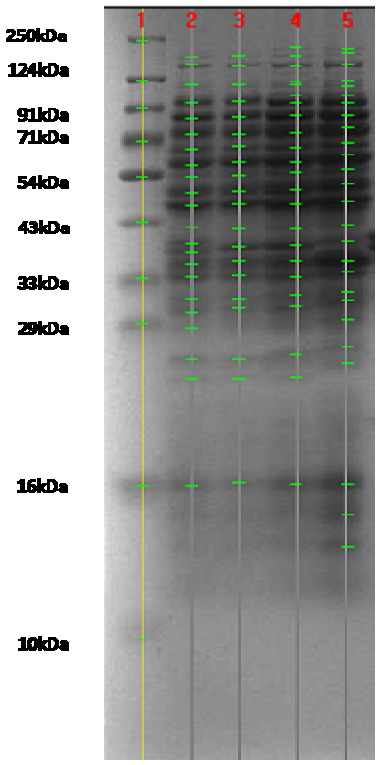

Plate 1 Protein profile of*E.tarda*antigens (SDS PAGE 5%Resolving gel)
Lane 1: Protein marker, Lane 2: Whole Cell antigen of *E.tardaATCC15947* strain, Lane 3: Whole Cell antigens of *E.tarda* isolate *ET-1*, Lane 4: Whole Cell antigens of *E.tarda* isolate *ET-2*, Lane 5: Whole Cell antigens of *E.tarda* isolate *ET-3*

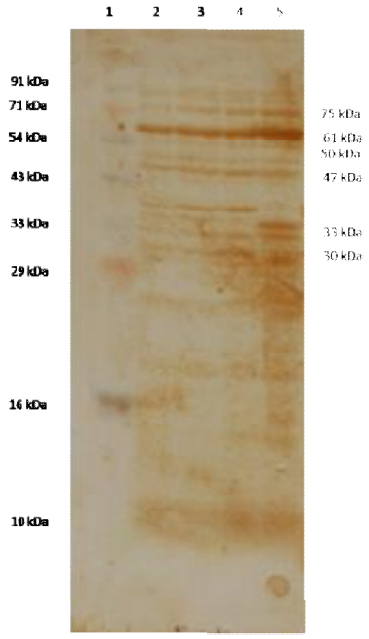

Plate 2 Western blot profile of*E.tarda*antigens (SDS PAGE 15% Resolving gel) using anti *E.tarda*rohu serum as primary antibody

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*

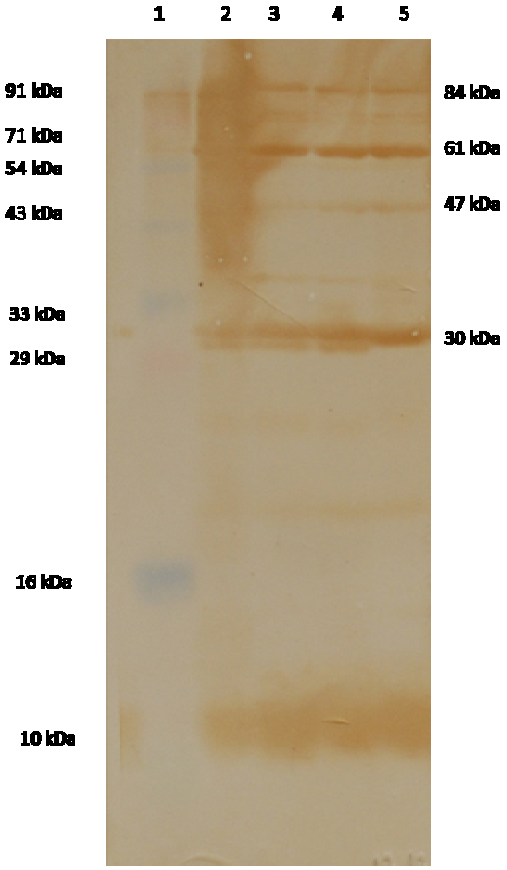

Plate 3 Western blot profile of*E.tarda*antigens (SDS PAGE 15% Resolving gel) using anti *E. tarda* rabbit serum as primary antibody

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*

Nonspecific and specific immune parameters

The treatment groups of 61 kDa and 47 kDahad significantly higher($p \leq 0.05$) leukocyte count (Fig. 1), NBT activity (Fig. 2),phagocytic index (PI)(Fig.3), phagocytic capacity (PC)(Fig. 4), lysozyme activity (Fig. 5), myeloperoxidase activity(MPO) (Fig. 6) and specific antibody production(Fig. 7)over control group. The NBT activity and lysozyme activity were significantly high ($p \leq 0.05$) in 47 kDa at 1^{st} week of immunization. But NBT activity (2^{nd} and 3^{rd} weeks) and lysozyme activity (2^{nd}, 3^{rd} and 4^{th} weeks) were significantly high ($p \leq 0.05$) in 61 kDa at subsequent weeks of experiment. 47 kDa protein had significantly high ($p \leq 0.05$) myeloperoxidase (MPO) activity over 61 kDa at 2^{nd}, 3^{rd} and 4^{th} weeks. 47 kDa protein had significantly high ($p \leq 0.05$) phagocytic index (PI) over 61 kDa at 1^{st}, 2^{nd} and 3^{rd} weeks. 47 kDaprotein had significantly high ($p \leq 0.05$) phagocytic capacity at 3^{rd} and 4^{th} weeks of immunization. The specific antibody production (mean ELISA reading) was significantly high ($p \leq 0.05$) in 61 kDa protein over 47 kDa at 2^{nd}, 3^{rd} and 4^{th} weeks.

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 36.66 % and RPS was 59.25 % (Fig. 8 & 9). The CM of47 kDawas 43.33% and RPS was 51.84 % (Fig. 8 & 9).

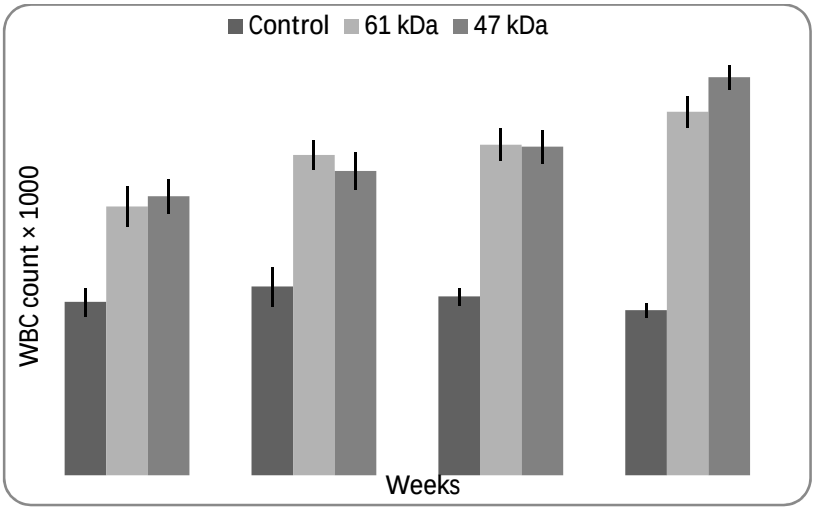

Figure 1 Effect of 61 kDa and 47 kDa*E. tarda* proteins as immunogen on white blood cell count in rohu blood

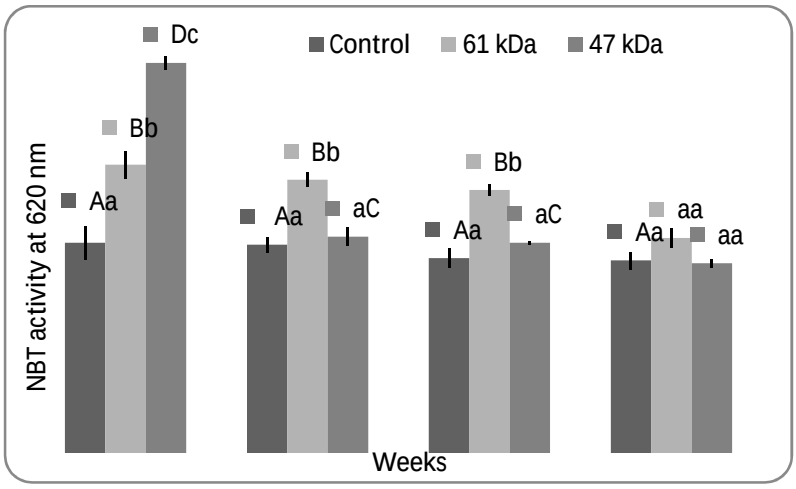

Figure 2 Effect of 61 kDa and 47 kDa*E. tarda* proteins as immunogenon NBT activity in rohu blood

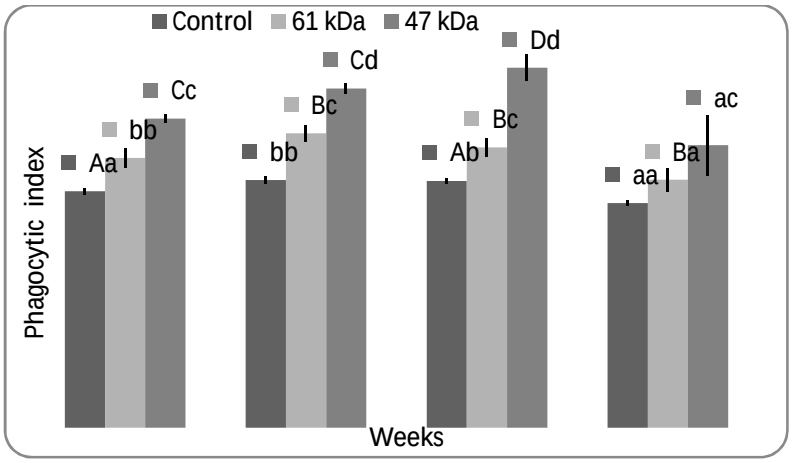

Figure 3 Effect of 61 kDa and 47 kDa *E. tarda* proteins as immunogen on phagocytic index in rohu blood

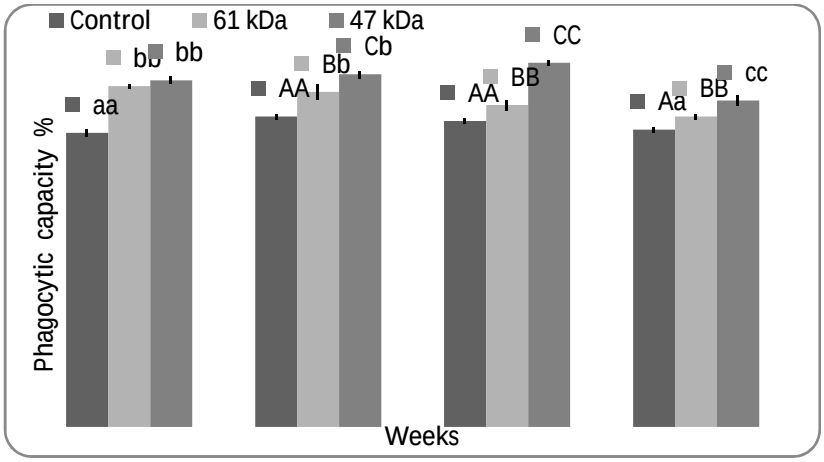

Figure 4 Effect of 61 kDa and 47 kDa*E. tarda* proteins as immunogenon phagocytic capacity in rohu blood

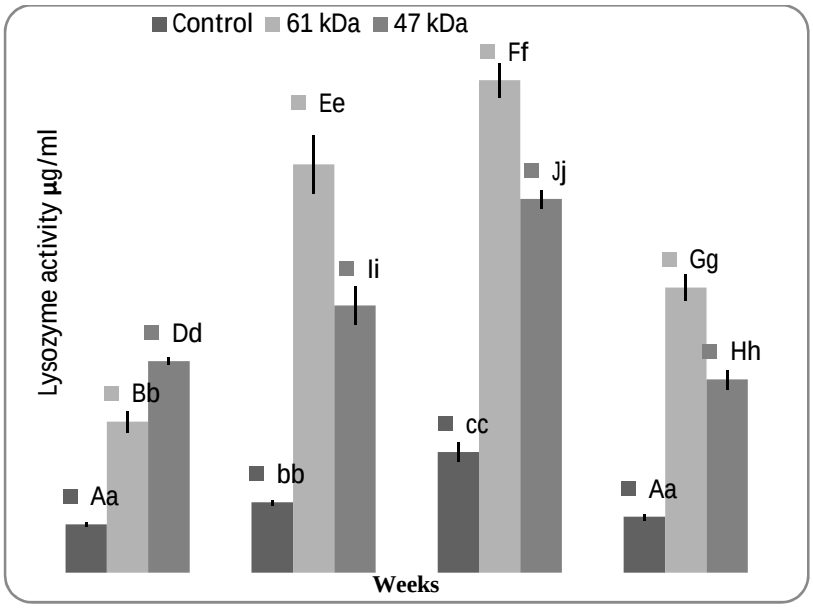

Figure 5 Effect of 61 kDa and 47 kDa *E. tarda* proteins as immunogen on lysozyme activity in rohu serum

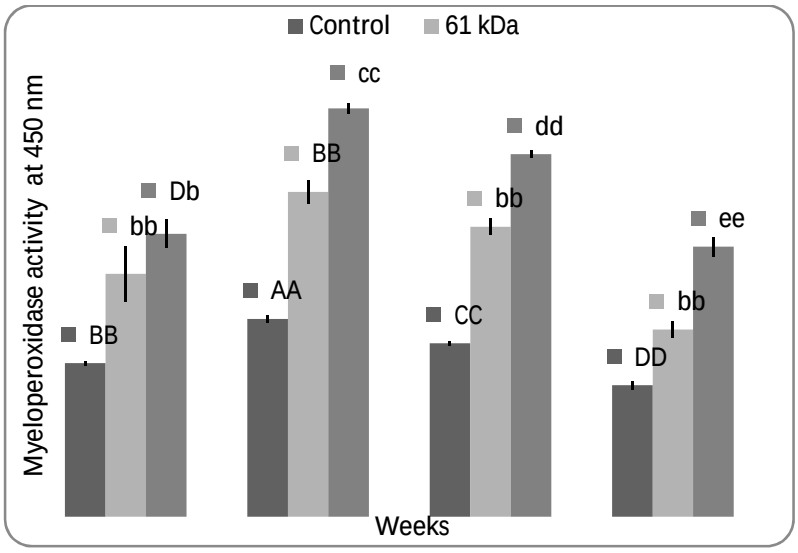

Figure 6 Effect of 61 kDa and 47 kDa *E. tarda* proteins as immunogen on myeloperoxidase activity in rohu serum

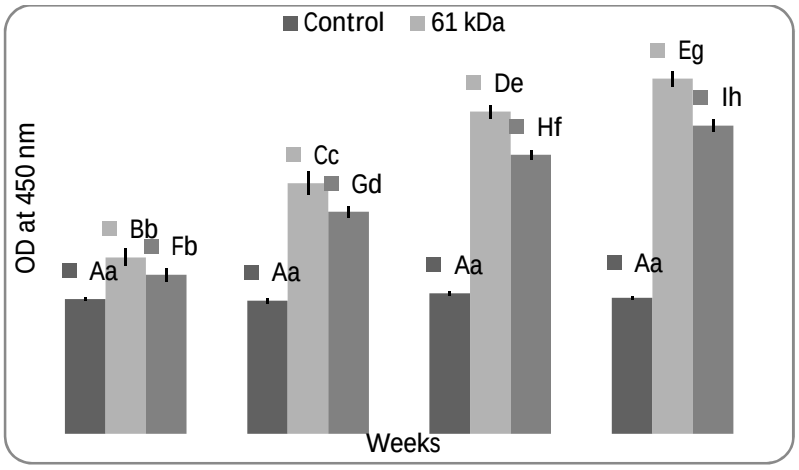

Figure 7 Effect of 61 kDa and 47 kDa*E. tarda* proteins as immunogen on on specific antibody production against *E.tarda* (ELISA reading at OD 450nm) in rohu serum

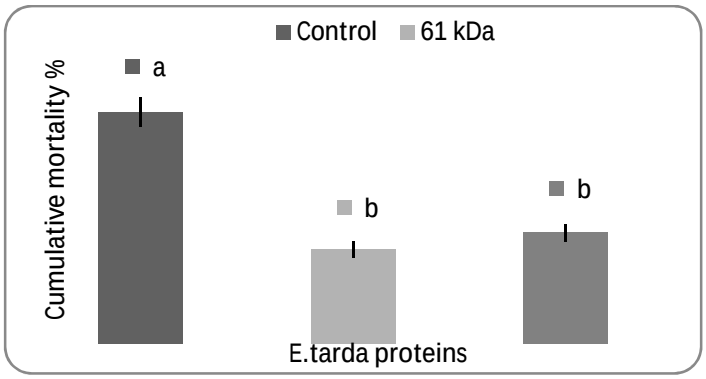

Figure 8 Effect of 61kDa and 47 kDa*E. tarda* proteins as immunogen on mean cumulative mortality in rohu after challenge with *E. tarda*

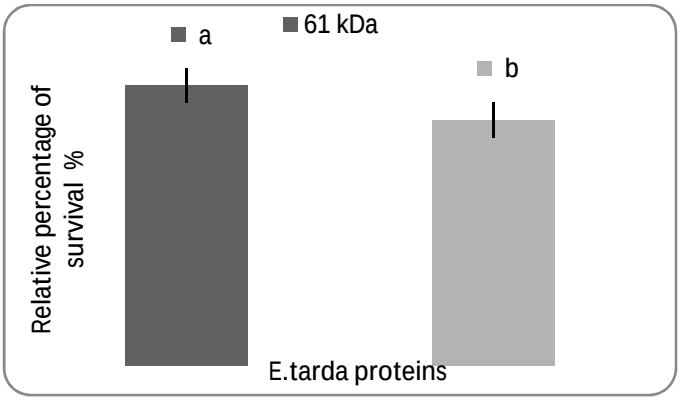

Figure 9 Effect of 61 kDa and 47 kDa*E. tarda* proteins as immunogen on mean RPS in rohu after challenge with *E. tarda*

Discussion

The molecular weights of heavy and light chains of rohu IgM were estimated as 85kDa and 23 kDa (Suresh ,Shankar, Honnananda, Vijaya kumara Swamy, Prasannashama, Suryanarayana & Dechamma 2008). The molecular weight of rabbit IgG heavy chain and light chain were 52 kDa and 24 kDa respectively (Carayannopoulos & Capra 1993). Specificity of rabbit anti-rohuIgG with rohuIgM was proved by the Western blot and AGPT.

E. tarda is heterogeneous bacteria among different isolates (Panangala, Shoemaker, McNulty, Arias & Klesius 2006) even isolated within a limited geographic location (Darwish, Newton & Plumb 2001). It was indicated by presence of different number of major protein bands in SDS PAGE of different isolates. The strongly recognized proteins *via* anti *E.trada* rohu and rabbit serum ,were participated in cell antigenicity and carried a common epitope specific to *E. tarda* species (Kawai *et al.,* 2004). 61 kDa and 47 kDa were strongly reacted with anti *E.trada* rabbit and rohu serum. NBT was significantly increased on 1st and 7th day in Japanese flounder injected with *rEta2* (17.4 kDa), outer membrane protein of *E. tarda* (Sun *et al.*, 2011). Phagocytic activity was significantly high in fish vaccinated with formalin killed whole cells of *A. bestiarum* in the 1st week of immunization (Kozinska & Guz 2004). The peak phagocytosis was recorded two weeks after immunization of red sea bream with formalin killed *E.tarda*cells (Salati,Hamaguchi & Kusuda 1987). Serum lysozyme activity, phagocytic activity and bactericidal activity of head kidney leukocytes was correlated with resistance of fish against bacterial infections (Robertsen *et al.,* 1994). Lysozyme activity and PI of Chinese breams wereincreased by OMP 38 recombinant protein of *A.s hydrophila* in the 14 day of vaccination (Wang,Yang, Zang, Liu & Lu 2013). The recombinant *E. tarda* proteins of *Eta6* (Jiao *et al.,* 2009b), *Eta* 21 (Jiao *et al.,* 2009a), *rEta2* (Sun *et al.,* 2011) and *Esa1* (Sun *et al.,* 2010) were developed specific antibodies after 4 weeks of immunization. The peak antibody titer for OMP 38 was at 21st day of immunization (Wang *et al.,* 2013). 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* (Tang *et al.,* 2010). The non specific response was significantly active within first few weeks of present study. Non-specific immune response may extend up to 4-8 weeks with miner effect of non-specific protection (Ellis 1999).

In conclusion specific antibody development is more important in protective antigencity than non specific immune response. 61 kDa was the best in specific immune response and strongly reacted with anti *E.tarda* rabbit and rohu serum in Western Blot. Therefore 61 kDa proteins of *E.tarda* could be

considered as best vaccine candidate that would contribute to the development of an effective vaccine against different serotype of *E.tarda.*

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