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Isolation and characterization of outer membrane proteins of *Edwardsiella tarda* and its application in immunoassays

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Abstract

Edwardsiella tarda is an important cause for hemorrhagic septicemia in fish and also for gastro intestinal infections in humans. Sodium lauryl sarcosinate extraction method was used for the isolation of outer membrane proteins (OMPs) from 16 isolates of *E. tarda*, recovered from snakeheads (*Ophiocephalus punctatus*). Sodium dodecyl sulphate polyacrylamide gel electrophoresis profiles of OMPs from *E. tarda* isolates had one major and common protein band of 44 kilodalton (kDa) and 9 other variable protein bands. Rabbit polyclonal antibodies (RPAbs) against OMPs of *E. tarda* ET-7 detected mainly one common 44 kDa protein in all isolates by western blotting. RPAbs strongly reacted with all isolates of *E. tarda* by indirect enzyme linked immunosorbent assay (ELISA). These polyclonal antibodies showed no cross reactivity with OMPs or whole cells of other selected aquatic bacteria, i.e., *Escherichia coli*, *Pseudomonas fluorescens*, *Aeromonas hydrophila, Vibrio cholerae* and *Flavobacterium ferrugineum*. This study indicated that RPAbs against OMPs is useful for detection of *E. tarda* by ELISA.

Keywords: *Edwardsiella tarda*; Outer membrane proteins; Western blotting; ELISA

1. Introduction

Edwardsiella tarda is the causative agent for edwardsiellosis in freshwater and marine fish species throughout the world and it also causes gastro intestinal infections in humans. Moreover, *E. tarda* is known to infect reptiles, amphibians, marine mammals and other warm blooded animals. *E. tarda* infections with economically important losses have been reported in Japanese eel, *Anguilla japonica*, Channel catfish, *Ictalurus punctatus*, Chinook salmon, *Oncorhynchus tshawytscha*, Rainbow trout, *Oncorhynchus mykiss*, Nile tilapia, *Oreochromis niloticus* and Olive flounder, *Paralichthys olivaceus* in different geographical areas (Plumb, 1993; Austin and Austin, 1999).

The outer membrane proteins (OMPs) of Gram-negative bacteria have an important role as virulence factors (Zierler and Galan, 1995) and these proteins have been used as epidemiological markers for human and animal pathogens (Barenkamp et al., 1981). OMPs of bacteria are associated with pathogenicity and protective antigenicity (Suzuki et al., 1994; Lutwyche et al., 1995). These proteins are located in the outer most surface of the bacteria and play a major role in suppressing the immune

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defense factors of the host (Ling et al., 2000; Srinivasa Rao et al., 2001). Two major OMPs of 36 and 46 kDa were reported in *E. tarda* (Aoki and Holland, 1985), whereas, Tu and Kawai (1998) extracted three major OMPs at 37, 40 and 43 kDa from *E. tarda* EF-1. Further, these authors reported the presence of 37 kDa OMP as a heat resistant antigen in various *E. tarda* strains (Tu and Kawai, 1999). The 37 kDa OMP has been detected in different *E. tarda* serotypes and has been evaluated as an effective vaccine candidate against *E. tarda* infection (Kawai et al., 2004).

Researchers from India have also done work on various aspects of *E. tarda*. Infectivity pattern and pathology of *E. tarda* has been studied in *Anabas testudineus* (Sahoo et al., 2000). Further, endotoxin of *E. tarda* has been found to elicit protection against *E. tarda* infection in *Heteropneustes fossilis* fingerlings (Das et al., 2001). Swain et al. (2002) investigated vaccination in *Labeo rohita* and *Catla catla* using formalin killed *E. tarda* bacterial suspension. Swain et al. (2001) developed dot-ELISA for detection of *E. tarda* in fish using adsorbed hyperimmune rabbit sera against whole cells of *E. tarda*. Further, they have developed different serological tests to detect antibodies against *E. tarda* in naturally infected fish (Swain and Nayak, 2003).

The objectives of this study were to isolate and characterize *E. tarda* from snakeheads (*Ophiocephalus punctatus*), based on phenotypic biochemical tests and their OMP profiles. These OMPs were further used as antigens to raise polyclonal antibodies in rabbits. The specificity and sensitivity of polyclonal antibodies were also examined to develop a specific diagnostic assay for detection of *E. tarda*.

2. Materials and methods

2.1. Isolation of E. tarda and culture conditions

Sixty samples of diseased and apparently healthy snakeheads (*O. punctatus*) were collected from freshwater ponds and fish markets of Uttar Pradesh, India during the period 2002–2004 (Table 1), and processed for *E. tarda* isolation on Salmonella–Shigella agar (SS). The presumptive isolates of *E. tarda* appeared as small, transparent colonies with minute black center on SS agar. These isolates were purified and subjected to following biochemical tests: Gram's stain, motility, oxidase, catalase, glucose with gas production, oxidation-fermentation reaction, indole, methyl red (M.R), Voges-Proskauer (V.P), Simmon's citrate, Christensen's citrate, hydrogen sulfide production on triple sugar iron (TSI) agar slant, urease, lipase, hemolysin, decarboxylation of arginine, lysine, ornithine and fermentation of mannitol, sucrose, arabinose and trehalose. The biochemical identification of *E. tarda* was done according to Barrow and Feltham (1992). For long term storage, the isolates were stored at −80 °C in brain heart infusion (BHI, Hi-Media, India) broth with 15% glycerol.

One reference strain of *E. tarda* MTCC 2400 was obtained from IMTECH, Chandigarh, India for comparative studies. Sixteen isolates of *E. tarda* and MTCC 2400 were cultured in BHI broth at 30 °C. Other selected aquatic bacteria, i.e., *E. coli*, *Salmonella arizonae*, *P. fluorescens, A. hydrophila*, *V. cholerae* were also cultured in BHI broth at 37 °C for 24 h and *F. ferrugineum* (MTCC 2955) was cultured in Shieh broth at 28 °C.

2.2. Isolation of OMPs

OMPs from 16 isolates of *E. tarda*, MTCC 2400 and other selected aquatic bacteria were obtained as per the method described by Darwish et al. (2001). Protein concentrations of OMPs were determined by the method of Lowry et al. (1951).

2.3. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis

The OMP profiles of 16 *E. tarda* isolates and MTCC 2400 were analyzed by SDS-PAGE as described by Laemmli (1970). Samples were mixed in 2 × SDS gel loading buffer and heated at 100 °C for 5 min. Electrophoresis was carried out on in 5% stacking and 15% separating gel at 60 V for 4 h. The gel

Table 1
Isolation details of *E. tarda* from snakeheads (*Ophiocephalus punctatus*)

Isolates no.	Clinical status	Tissues	Duration
ET1	Diseased-ulcers on the lateral body	Kidney	July 2002
ET2	Diseased-hemorrhages on fins	Muscle	July 2002
ET3	Apparently healthy	Muscle	August 2002
ET4	Diseased-hemorrhages on fins	Muscle	June 2003
ET5	Diseased-hemorrhages on bases of caudal fins	Muscle	July 2003
ET6	Diseased-hemorrhages on fins, lesions on lateral parts	Muscle	September 2003
ET7	Diseased-ulcers on the body, hemorrhages on fins and lesions on postero-lateral parts	Kidney	June 2004
ET8	Diseased-hemorrhages on caudal fins	Muscle	June 2004
ET9	Diseased-hemorrhages on fins, lesions on lateral parts	Muscle	July 2004
ET10	Diseased-hemorrhages on fins	Muscle	July 2004
ET11	Diseased-hemorrhages on ventral fins, skin erosions in ventral parts	Muscle	July 2004
ET12	Diseased-hemorrhages on fins, lesions on lateral parts	Kidney	August 2004
ET13	Apparently healthy	Muscle	August 2004
ET14	Diseased-hemorrhages on fins, skin erosions on lateral parts	Muscle	August 2004
ET15	Diseased-hemorrhages on fins, skin erosions on lateral parts	Kidney	September 2004
ET16	Apparently healthy	Muscle	September 2004

Table 2
Variation in the biochemical tests of 16 isolates of *E. tarda*

Tests	Reaction of isolates															
	ET1	ET2	ET3	ET4	ET5	ET6	ET7	ET8	ET9	ET10	ET11	ET12	ET13	ET14	ET15	ET16
Simmon's citrate	+	–	+	–	–	+	–	–	–	–	–	+	+	–	–	–
Christensen's citrate	+	+	+	+	+	–	+	–	+	+	+	+	+	+	+	+
Hemolysin	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–
Mannitol	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Sucrose	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Arabinose	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Trehalose	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–

+=positive reaction; −=negative reaction.

was stained with 0.25% (w/v) Coomassie brilliant blue R-250 in 50% (v/v) methanol, 10% (v/v) acetic acid and then destained. Estimations of the apparent molecular masses of OMPs were made by LabWorks Image Acquisition and Analysis Software version 4.5 (UVP, Inc., UK).

2.4. Production of polyclonal antibodies in rabbits (RPAbs)

Hemolysin positive *E. tarda* ET-7 isolate was selected to raise polyclonal antibodies in two adult New Zealand white rabbits. One fifty microgram of purified OMPs of *E. tarda* isolate ET-7 (E-OMPs) was emulsified with Freund's incomplete adjuvant (Pierce, USA) and injected subcutaneously at multiple sites over the back region of rabbits. This was followed by three injections of similar emulsion at 2, 4 and 6 weeks intervals. Ten days after the 3rd and 4th injections, the rabbits were bled and antisera titers were determined by ELISA using E-OMPs (1 μg/ml) antigen coated ELISA plates (Nunc ImmunoTM plate, MaxiSorp, Denmark). The blood samples from rabbits showing high titers were collected and separated sera were stored in sterile vials at −20 °C for further use.

2.5. Western blotting of OMPs

Western blotting was performed to determine the reactivity of the rabbit antisera to OMPs. The OMPs from 16 isolates of *E. tarda* were electrophoresed by SDS-PAGE using the standard protocol mentioned above. After electrophoresis, the proteins were transferred on to a nitrocellulose membrane (pore size 0.45 μm; Millipore, USA) in transfer buffer, as described by Towbin et al. (1979). The transfer was performed at 300 mA constant current for 2 h on a semi dry apparatus (Amersham Pharmacia Biotech, USA). After the transfer, the membrane was washed in deionized water and blocked with 5% skimmed milk powder solution in phosphate buffer saline containing 0.05% Tween-20 (PBS-T) at 37 °C for 1 h. Membrane was then incubated with RPAbs (1:20,000 dilution) at 37 °C for 1 h, washed three times in PBS-T and again incubated with 1:10,000 dilution of goat anti-rabbit IgG-HRP conjugate (Genei, India) for 1 h at 37 °C. Following further washes, bound antibodies were detected by addition of 3,3′,5,5′-tetrametylbenzidine membrane peroxidase substrate (KPL, USA) and the colour reaction was stopped by rinsing the membrane extensively with deionized water.

2.6. Immunoassays

Indirect ELISA and competitive ELISA were performed to assess the reactivity and specificity of RPAbs.

2.6.1. Reactivity of RPAbs to E-OMPs by indirect ELISA

Optimal concentration of purified E-OMPs and RPAbs were determined by a checkerboard titration as per the method described by Crowther (2001). ELISA plates were coated individually with OMPs (1 μg/ml) of *E. tarda* including reference strain or other selected aquatic bacteria in carbonate-bicarbonate buffer (pH 9.6) and incubated overnight at 4 °C. The wells were washed three times with PBS-T followed by blocking with 50 μl of blocking buffer (PBS-T containing 3% BSA) for 1 h at 37 °C. After incubation, plates were washed three times with PBS-T followed by addition of 50 μl of RPAbs (1:20,000 dilution) to each well. The plates were further incubated for 1 h at 37 °C and washed. Thereafter, 50 μl of a 1:10,000 dilution of goat anti-rabbit IgG-HRP conjugate was added to each well and the plates were incubated for 1 h at 37 °C and washed. Finally, 50 μl of substrate solution [1 mg/ml of O-phenylene diamine

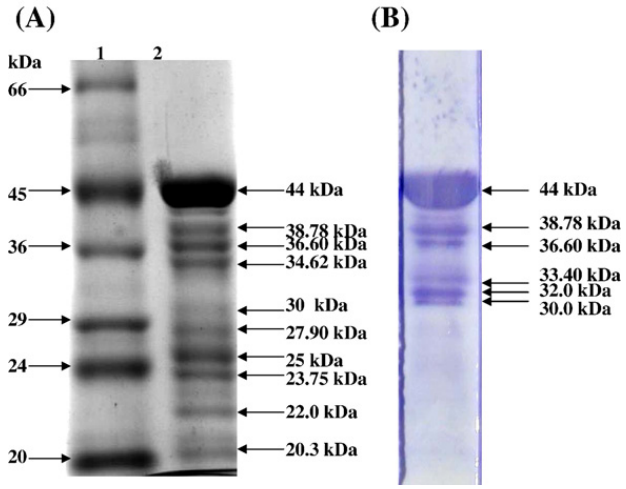

Fig. 1. SDS-PAGE and western blot analysis of outer membrane proteins (OMPs) of *E. tarda* ET-7. (A) Lane 1, Molecular weight proteins marker (Sigma); Lane 2, OMPs of *E. tarda* stained with Coomassie blue. (B) Western blotting profile of OMPs of *E. tarda* ET-7.

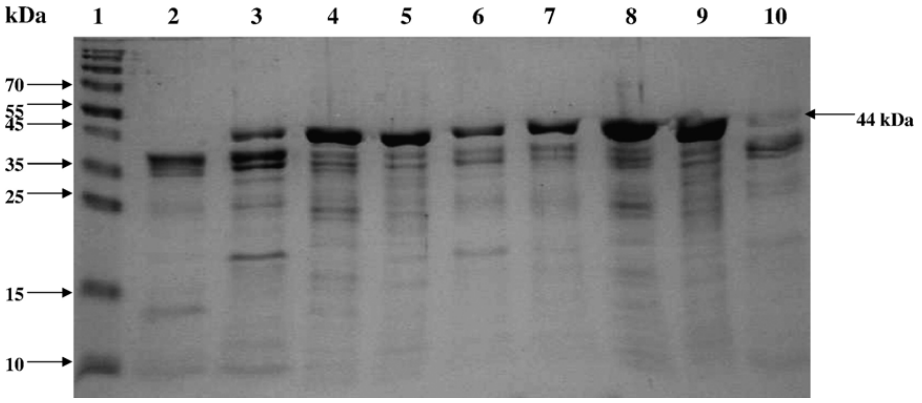

Fig. 2. SDS-PAGE profiles of outer membrane proteins of *E. tarda* isolates. Lane 1, Molecular weight proteins marker (Fermentas); Lane 2, reference strain of *E. tarda* MTCC 2400, Lane 3, ET2, Lane 4, ET4, Lane 5, ET5, Lane 6, ET6, Lane 7, ET7, Lane 8, ET8, Lane 10, ET9.

dihydrochloride and 1 µl of H_2O_2/ml in citrate buffer, pH 5.0] was added to each well. The reaction was stopped after 15 min by adding 50 µl of 2N sulphuric acid to all wells. The optical density (OD) of wells was measured at 492 nm in an ELISA plate reader (TECAN, Austria).

The sensitivity of the RPAbs to detect formalin killed whole cells of *E. tarda* and other selected aquatic bacteria were also tested by indirect ELISA. Briefly, ELISA plates were coated and dried overnight with 50 µl of ten fold dilutions solution [10^8 to 10^1 colony forming unit (CFU)/ml] of whole bacterial cells diluted in carbonate-bicarbonate buffer. The subsequent steps were similar to those described as above.

2.6.2. Competitive ELISA for determination of specificity of RPAbs

The competitive ELISA was performed as per the method described by Crowther (2001). ELISA plates were coated with E-OMPs (0.5 µg/ml) of *E. tarda* ET-7. The plates were incubated at 37 °C for 1 h. After blocking and washing the plates with PBS-T, competition was carried out for binding, by incubating the plates with 50 µl/well of serially diluted competitor (E-OMPs or other selected bacterial OMPs from *E. coli*, *S. arizonae*, *P. fluorescens*, *A. hydrophila*, *V. cholerae* and *F. ferrugineum*) with 50 µl of optimal dilution of RPAbs (1:80,000) in dilution buffer, at 4 °C for overnight. After washing the wells with PBS-T, goat anti-rabbit IgG-HRP conjugate was added and incubated for 1 h at 37 °C. The colour was developed, stopped and the OD recorded as described above. Percentage competition was calculated using following formula: % Competition = 100 − {(OD of test OMPs ÷ mean OD of 0% competition) × 100}.

3. Results

3.1. Biochemical tests of E. tarda isolates

All isolates of *E. tarda* were Gram-negative short rods, fermentative, positive for motility, catalase, acid and gas production from glucose, indole, M.R, hydrogen sulfide production on TSI agar slant, decarboxylation of lysine and ornithine, but negative for oxidase, urease, lipase, V.P and decarboxylation of arginine. Some variations in the biochemical tests were also found within isolates of *E. tarda* (Table 2).

3.2. SDS-PAGE profiles of OMPs

The SDS-PAGE profiles of OMPs from *E. tarda* ET-7 had one major protein band of 44 kDa and 9 other protein bands of 38.8, 36.5, 34.5, 30, 28, 25, 23.75, 22 and 20.3 kDa (Fig. 1A). The OMPs profiles of 16 *E. tarda* isolates also showed a similar major OMP band at 44 kDa. MTCC 2400 showed a major protein band at 38.8 kDa. SDS-PAGE profiles of 8 *E. tarda* isolates are shown in Fig. 2. Lower intensity of 44 kDa was noted in ET-9 (Fig. 2). In addition, ET-2 showed predominant bands at 38.8, 36.6 and 20.3 kDa.

3.3. Western blotting profiles of OMPs

In western blotting, the RPAbs detected 4 protein bands of E-OMPs similar to the SDS-PAGE at 44, 38.8, 36.5 and 30 kDa along with extra two additional protein bands of 33.40 and 32 kDa (Fig. 1B). This indicated that six of the twelve proteins

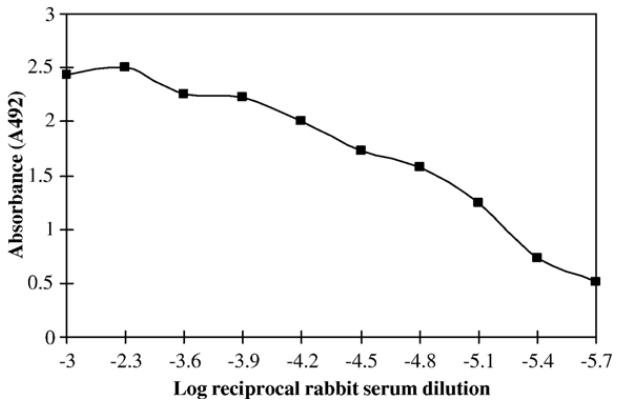

Fig. 3. Titration of rabbit polyclonal antibodies (RPAbs) to *E. tarda* ET-7 outer membrane proteins (E-OMPs) in indirect ELISA. Fixed quantity of E-OMPs (1 ug/ml) were coated to the plate. RPAbs 2 fold dilutions from 1 to 10 (1:1000 to 5,12000).

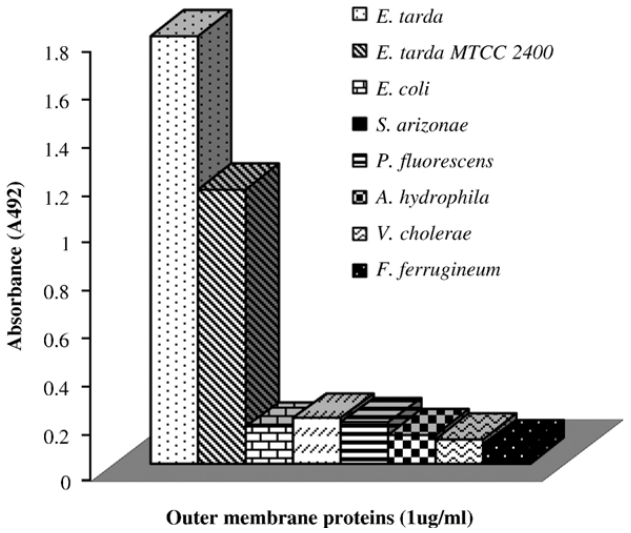

Fig. 4. Reactivity of rabbit polyclonal antibodies (RPAbs) to OMPs of *E. tarda*, reference strain of *E. tarda* MTCC 2400 and other important aquatic bacteria in indirect ELISA. A fixed quantity of OMPs (1 ug/ml) were coated to the plate and optimal dilution of RPAbs (1:20,000) was used as primary antibody.

are antigenic. A regular feature seen in western blotting was that the 44 kDa protein band was main antigenic protein present in OMPs of all our *E. tarda* isolates (data not shown).

3.3.1. Immunoassays

By indirect ELISA, the titer of RPAbs to E-OMPs is shown in Fig. 3. It is noted that the antibody titer of RPAbs was of a very high level and even at a dilution of 1:64, 000; it showed an absorbance (A_{492}) of 1.57. These antibodies showed high degree of reactivity with all *E. tarda* isolates, including MTCC 2400. No cross reactivity was observed with OMPs from *E. coli*, *S. arizonae*, *P. fluorescens*, *A. hydrophila*, *V. cholerae* and *F. ferrugineum* (Fig. 4). However, RPAbs reacted slightly with whole cells of *S. arizonae* at a cell number above 10^6 CFU/ml (Fig. 5). It is further noted from

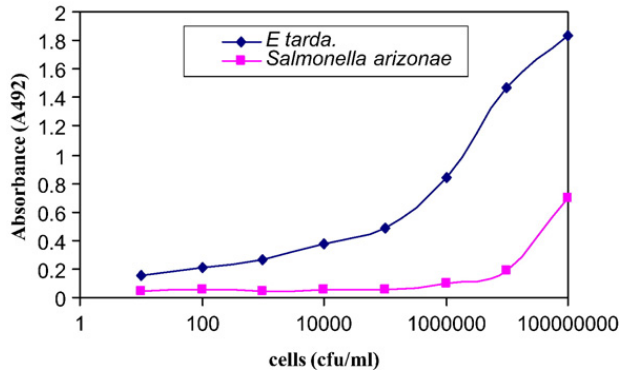

Fig. 5. Reactivity of rabbit polyclonal antibodies (RPAbs) to formalin killed whole bacterial cells of *E. tarda* and *Salmonella arizonae* in indirect ELISA. A fixed quantity of RPAbs (1/20,000) was used as primary antibody for detection of whole bacterial cells.

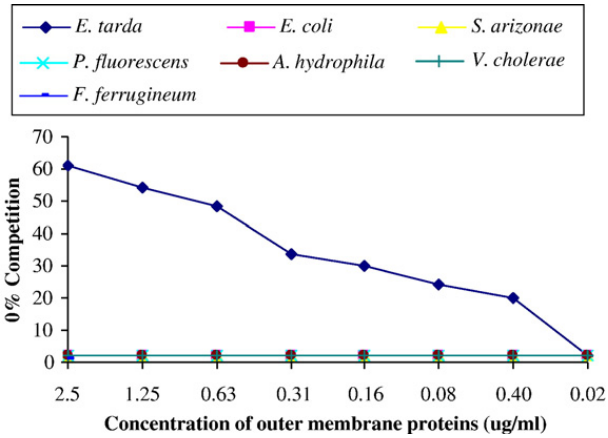

Fig. 6. Percentage competition of rabbit polyclonal antibodies (RPAbs) to outer membrane proteins of *E. tarda* (E-OMPs) or OMPs of other test bacteria. Competition was carried out by incubating serially diluted competitor (OMPs of *E. tarda* or test OMPs of *E. coli*, *S. arizonae*, *P. fluorescens*, *A. hydrophila*, *V. cholerae* and *F. ferrugineum*) along with optimal dilution of RPAbs (1:20,000) on E-OMPs (0.5 ug/ml) coated plates.

Fig. 5 that the sensitivity of RPAbs to detect whole cells of *E. tarda* was up to a level of 1×10^3 CFU/ml.

The reactivity of RPAbs to E-OMPs of *E. tarda* or OMPs from other selected aquatic bacteria has been also demonstrated by a competitive ELISA. Results are shown in Fig. 6, where serially diluted E-OMPs and optimal dilution of RPAbs were added simultaneously to wells coated with fixed quantity of E-OMPs. A clear competition in the binding of RPAbs to the coated antigen was observed, with a maximum competition of 61.2% at 2.5 μg/ml of E-OMPs. No cross reactivity was observed between E-OMPs and OMPs from other selected aquatic bacteria using RPAbs in competitive ELISA.

4. Discussion

The OMPs have been shown to play not only a role in bacterial defence against host factors but also in virulence related functions such as adhesion, invasion and host cell modulation (Ling et al., 2000; Srinivasa Rao et al., 2001). The OMPs profile of present *E. tarda* isolates showed one predominant and common protein band of 44 kDa and 9 other variable bands ranging from 20.3 kDa to 38.8 kDa. Elsewhere, three major OMPs of 37, 40 and 43 kDa from the *E. tarda* (EF-1) were isolated from Japanese eel (*A. japonica*). Among these 37 kDa band was common in all the Japanese strains, whereas, this protein band was not found in ATCC 15469 and also in F-1 strains isolated from Taiwan (Tu and Kawai, 1998). Our results indicate that the 44 kDa protein band which was common in all *E. tarda* isolates recovered from *O. punctatus*, was not noted in reference strain of *E. tarda* MTCC 2400. MTCC 2400 strain showed a major protein band at 38.8 kDa. Acharya et al. (2007) have also observed genotypic variation

between fish isolates of *E. tarda* and MTCC 2400 by PCR-RFLP. The other OMP bands of 25, 28, 34.5 and 36.5 kDa of *E. tarda* in this study were found to be similar to the ATCC 15947, as reported by Darwish et al. (2001), except a 40 kDa protein band which was not present in any of our isolates. The 44 kDa protein band in this study appears to be similar to a major protein band in the range of 45.2–46.5 kDa of *E. tarda* (Aoki and Holland, 1985) and to 44 kDa protein from field isolates of *E. tarda* (Darwish et al., 2001). This 44 kDa band was also major immunogenic protein found in OMPs of our *E. tarda* isolates. This is in contrast to Japanese strains of *E. tarda*, where 37 kDa protein was the most predominant antigen reacting to polyclonal antibodies against formalin killed cells of *E. tarda* EF-1 (Tu and Kawai, 1998). These differences in the OMP profile of our isolates prove that *E. tarda* is a heterogeneous species as also reported by Darwish et al. (2001).

Heterogeneity among isolates were found in few biochemical tests i.e. citrate utilization tests and fermentation of sugars, which showed variation among the isolates of *E. tarda* from snakehead. On the basis of fermentation of sugars, only one ET2 isolate was found to as *E. tarda* biogroup 1. Most of the other isolates belonged to *E. tarda* wild type. However, among wild type isolates differences were observed for utilization of Simmon's citrate. These phenotypic variations in our study too suggest that *E. tarda* is a heterogeneous group. In support of phenotypic variations, genetic variations between *E. tarda* strains have been reported by Yamada and Wakabayashi (1998) using PCR-RFLP analysis of 16S rDNA. Similarly, variations in the plasmid profile (Janda et al., 1991) and lipopolysaccaride pattern (Nomura and Aoki, 1985) of *E. tarda* have also been reported. Recently, genotypic variations have been also found between fish, water and sediment isolates of *E. tarda* using PCR-RFLP of 16S rDNA (Acharya et al., 2007). From these studies, it can be safely assumed that *E. tarda* exhibits a lot of phenotypic and genotypic diversity in different geographic locations as well as habitats.

The diagnosis of edwardsiellosis based on clinical signs, gross and histopathological lesions followed by elaborate laboratory procedures of bacterial isolation and characterization are not only time consuming but also expensive (Sahoo et al., 2000). In recent times, the ELISA is widely used in fish disease diagnosis (Klesius et al., 1991; Rodak et al., 1993; Swain and Nayak, 2003). Swain et al. (2001) have used dot-ELISA for rapid and confirmatory identification of *E. tarda* in infected and dead fish through detection of its antigen in the tissues like liver, spleen and kidney using adsorbed hyperimmune rabbit sera against whole cells of *E. tarda*. This test has been used specifically for the detection of *E. tarda* antigen in decayed tissues within 48–72 h after death of fish. In their study, *E. tarda* antigen concentration was found higher in kidney tissue followed by the spleen and liver. Swain and Nayak (2003) have also standardized a competitive ELISA for seromonitoring of *E. tarda* infection in sera of *L. rohita* and *C. catla*. However, rabbit polyclonal sera against whole cells and whole cell products of *E. tarda* were noted to cross react with *A. hydrophila* and *P. fluorescens* (Swain et al., 2003). Our study shows that indirect ELISA was capable of detecting approximately 1000 bacterial cells of *E. tarda*, thereby making it a highly useful diagnostic test. Moreover, the RPAbs showed no cross reactivity with either OMPs or whole cells of other selected aquatic bacteria except to *S. arizonae* whole cells at 10^6 CFU/ml. This may indicate that the RPAbs recognizes epitopes, which are mostly specific to *E. tarda* isolates in our study.

The present study has thus shown that the E-OMPs are very good immunogens for production of antisera with high titers in rabbits. These antibodies are useful in specific detection of *E. tarda* isolates by employing ELISA. It is also suggested that the purified OMPs of *E. tarda* can be used as an antigen in indirect ELISA for sero-monitoring and surveillance of edwardsiellosis of fishes in enzootic areas.

In conclusion, RPAbs against E-OMPs can be used for development of a diagnostic assay for sensitive and specific detection of *E. tarda*. Further study is in progress for making a more specific assay by raising monoclonal antibodies against 44 kDa OMP.

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