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Production of monoclonal antibodies specific to major outer membrane protein of *Edwardsiella tarda*

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Abstract

Edwardsiella tarda is an important cause for hemorrhagic septicemia in fish and gastro and extra-intestinal infections in humans. Monoclonal antibodies (MAbs) were produced against outer membrane proteins (OMPs) of *E. tarda* ET-7, isolated from diseased snakehead (*Ophiocephalus punctatus*). Two stable hybridoma clones, designated as 3F10 and 2C3 MAbs were found to be potentially specific for *E. tarda* by indirect enzyme linked immunosorbent assay (ELISA). These MAbs recognized major immunogenic OMP band at 44 kDa in Western blotting. Both MAbs belonged to the IgG1 isotype and recognized different epitopes of OMP as seen by competitive ELISA. These MAbs strongly reacted with all 17 isolates of *E. tarda* used in our study by indirect ELISA and Western blotting. Interestingly, no reaction was observed with the reference strain of *E. tarda* (MTCC 2400). The sensitivity of 3F10 MAb to detect whole cells of *E. tarda* was up to a level of 1×10^4 CFU/ml in indirect ELISA. No cross-reactivity of MAbs were seen with *Escherichia coli*, *Salmonella arizonae*, *Pseudomonas fluorescens*, *Aeromonas hydrophila*, *Vibrio cholerae*, *Flavobacterium ferrugineum* and *Mycobacterium tuberculosis*. These MAbs could be used for specific detection of *E. tarda* infection in fish by immunoassays.
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Résumé

Edwardsiella tarda est une cause importante de la septicémie hémorragique chez les poissons et pour les infections gastro-intestinales et extra-intestinales de l'homme. Des anticorps monoclonaux (MAbs) ont été produits contre les protéines externes de membrane (OMPs) de *E. tarda* ET-7, isolée à partir de *Ophiocephalus punctatus*. Deux clones d'hybridome désignés comme 3F10 et 2C3 MAbs se sont avérés spécifiques de la détection du *E. tarda* par ELISA. Ces MAbs ont permis de reconnaître les OMPS de 44 kDa et ont réagi fortement avec tous les isolats de *E. tarda* utilisés à partir de poissons et d'eau d'étang (17). Il n'y a pas eu d'activité hétérospécifique avec des anticorps monoclonaux ou des cellules bactériennes entières de *Escherichia coli*, *Salmonella arizonae*, *Pseudomonas fluorescens*, *Aeromonas hydrophila*, *Vibrio cholerae*, *Flavobacterium ferrugineum* et *Mycobacterium tuberculosis*. Ces Mabs pourraient être utilisés pour la détection de *E. tarda* chez les poisons.
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Mots clés : *Edwardsiella tarda* ; protéines externes de membrane ; anticorps monoclonaux ; ELISA*Keywords:* *Edwardsiella tarda*; Outer membrane proteins; Monoclonal antibodies; ELISA

1. Introduction

Edwardsiella tarda is the causative agent for edwardsiellosis in freshwater and marine fish species throughout the world. *E. tarda* infections with economically important losses have been reported in a variety of fish species from different geographical areas. In fish, this bacterium is associated with acute to chronic diseases of fry, fingerlings and adults resulting in severe economic loss. *E. tarda* infection has been reported from many cultured fish species in India [1,2]. It has a broad host range and causes diseases in reptiles, amphibians, marine mammals and other warm blooded animals including humans [3]. In humans, it causes gastro and extra-intestinal infections like skin, soft tissue and biliary tract infection, bacteremia, peritonitis, intra-abdominal abscess, tubo-ovarian abscess, liver abscess, meningitis, cholecystits, osteomyelitis, salpingitis, endocarditis, septic arthritis, myonecrosis, maternal colonization and neonatal sepsis and empyema [4,5]. Liver abscess caused by *E. tarda* has been also reported first time in India from human [6].

The outer membrane proteins (OMPs) of Gram negative pathogenic bacteria has an important role in the interaction with hosts and in the bacterial pathogenicity during adherence and uptake of nutrients from the host. It also helps in eliminating host defence mechanisms and has protective antigenicity. This is because the components of the OMPs are easily recognized as foreign substances by immunological defence systems of hosts [7]. OMP-based enzyme linked immunosorbent assay (ELISA) have gathered increased attention of researchers for the development of rapid detection methods for pathogens such as *Listeria monocytogenes* [8] and *Brucella melitensis* [9].

Immunological techniques have been developed for the diagnosis of edwardsiellosis in fish using rabbit polyclonal antibodies. Swain et al. [10] developed dot-ELISA for the detection of *E. tarda* by using rabbit polyclonal anti-sera. Further, Swain and Nayak [11] have developed different serological tests viz. indirect ELISA, indirect blocking ELISA, competitive ELISA and serum agglutination tests to detect antibodies against *E. tarda* in

naturally infected *Labeo rohita* and *Catla catla*. Rabbit polyclonal sera against whole cells and whole cell products of *E. tarda* were noted to cross-react with *A. hydrophila* and *P. fluorescens* [12]. Our previous study also showed cross-reactivity of rabbit polyclonal antibodies against E-OMPs (RPAbs) with *Salmonella arizonae* [13]. The use of polyclonal antibodies may produce a high number of false positive results due to cross-reactions with antigens from other bacteria.

Despite the availability of such diagnostic tools, specificity and sensitivity problems associated with the polyclonal antibodies indicate that specific tests are required for early diagnosis of edwardsiellosis. Therefore, the production of monoclonal antibodies (MAbs) against unique epitopes of *E. tarda* would be a logical first step to develop specific immunodiagnostic tests. MAbs have the advantage of better reproducibility of results after their application, provides the ability to enhance immunological or serological analysis by providing a uniform reagent and can be produced reliably in unlimited amounts. MAbs are powerful reagents for testing the presence of a desired epitope, so they are highly useful as a diagnostic reagent. Several reports have been available for the production of MAbs against *E. ictaluri* causing enteric septicemia in fish [14–16]. However, very few reports on production of MAbs against *E. tarda* are available from isolates of Chinese origin only [17,18]. *E. tarda* isolate of different regions are known to vary in their antigenic and genetic characteristics [19–22,13]. Hence, the present study on the production and characterization of MAbs against OMPs of *E. tarda* will be a step forward for the development of specific immunoassays for this important fish pathogen in India. Further, the potential of these MAbs in detection of *E. tarda* has been discussed.

2. Materials and methods

2.1. Bacterial isolates

A total of 18 isolates of *E. tarda* including the reference strain of *E. tarda* (MTCC 2400) used in this study have been described previously [13]. Briefly, 16 isolates were recovered from diseased and apparently healthy snakeheads (*Ophiocephalus punctatus*) and 1 isolate from freshwater pond of Uttar Pradesh, India. Other selected bacterial strains, i.e., *Escherichia coli*, *Salmonella arizonae*, *Pseudomonas fluorescens*, *Aeromonas hydrophila*, *Vibrio cholerae* and *Flavobacterium ferrugineum* were also used for cross-reactivity of MAbs in this study. *Mycobacterium tuberculosis* (H37RV) antigen was obtained from the mycobacterial repository of the National JALMA Institute for Leprosy & Other Mycobacterial Diseases (ICMR), Agra, India.

2.2. Preparation of OMPs

The OMPs from all isolates of *E. tarda* including reference strain and other selected bacterial strains except *M. tuberculosis* were purified as per the method described by Darwish et al. [19]. Briefly, all isolates of *E. tarda* and reference strain were cultured in brain heart infusion (BHI, HiMedia, India) broth at 30 °C. Other selected bacterial strains, i.e., *E. coli*, *S. arizonae*, *P. fluorescens*, *A. hydrophila*, *V. cholerae* were also cultured in BHI

broth at 37 °C and *F. ferrugineum* was cultured in shieh broth at 28 °C. The bacteria were collected by centrifugation at 10,000 × *g* for 15 min at 4 °C and then washed three times with chilled phosphate buffer saline (PBS). The pelleted bacteria were suspended in 7 ml of 10 mM HEPES buffer, pH 7.4 and sonicated. Intact cells and large debris were removed by centrifugation at 10,000 × *g* at 4 °C for 15 min. Supernatants were ultra-centrifuged at 100,000 × *g* at 4 °C for 1 h. The pellet was resuspended in 1 ml 10 mM HEPES buffer and inner membrane proteins solubilized by the addition of 10 ml of 1.5% sodium lauroyl sarcosine in 10 mM HEPES buffer for 30 min at room temperature and ultra-centrifuged at 100,000 × *g* at 4 °C for 1 h. The pellet was suspended in 1 ml PBS. Protein concentrations of OMPs were determined by the method of Lowry et al. [23]. Purified OMPs of haemolysin positive isolate of *E. tarda* ET-7 was designated as E-OMPs.

2.3. Preparation of formalin killed whole bacterial cells

Formalin killed whole bacterial cells were prepared as per the method described by Swain and Nayak [11]. All isolates of *E. tarda*, reference strain and other selected bacterial strains were separately cultured in appropriate broth for 18–24 h. After incubation the total bacterial count was enumerated by plate count method. The bacteria were inactivated with 1% formalin overnight at 4 °C. Bacteria were pelleted by centrifugation and washed twice with PBS. Finally, individual bacterial suspension containing 10^8 to 10^1 CFU/ml was made in carbonate–bicarbonate buffer, pH 9.6.

2.4. Production of MAbs

Standard procedures were used for producing MAbs [24] with some modifications, i.e., use of purified E-OMPs antigen for immunization of mice and feeder cells (peritoneal macrophages) for hybridoma production. Briefly, five female BALB/c mice were immunized intraperitoneally with 0.2 ml of purified E-OMPs (50 μg per mouse) emulsified in Freund's incomplete adjuvant (Pierce, USA) on 0 day and boosted with the same dose after 2 and 5 weeks. After 1 week of second booster, antibodies titre of immunized mice were checked by indirect ELISA on E-OMPs coated plates. The mouse producing highest ELISA titre was selected for hybridoma production. A final injection (25 μg) was given to the mouse intravenously without adjuvant 3 days before fusion.

Myeloma cells (SP2/0-Ag-14) obtained from National Centre for Cell Line, Pune, India and spleen lymphocytes from immunized mouse were prepared for fusion. The spleen lymphocytes (10^8) were fused to myeloma cells (10^7) with 50% polyethylene glycol (MW 1300–1600). Selection of hybridomas was carried out using Dulbecco's Modified Eagle's Medium with hypoxanthine, aminopterin and thymidine. After 12–15 days, supernatants were screened using indirect ELISA to detect hybridomas secreting antibodies for E-OMPs. The hybridoma culture supernatants that gave at least five times the A_{492} values of 0 day mouse serum (1:1000 dilution) were considered positive for E-OMPs. Selected positive hybridomas were cloned twice by limiting dilution method [24]. The isotypes of MAbs were determined by testing the hybridoma cell culture fluid using mouse monoclonal antibody isotyping kit (Sigma, USA). Two of the strongly reacting MAbs

(designated as 3F10 and 2C3) were used for further characterizations and applications in immunoassays.

2.5. Western blotting for antigenic characterization of MAbs

OMPs from all isolates of *E. tarda* were separated on 15% SDS-PAGE gel as described Kumar et al. [13]. For Western blot analysis, the OMPs samples resolved by SDS-PAGE were transferred on to a nitrocellulose membrane in transfer buffer, as described by Towbin et al. [25]. 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 3% BSA in PBS-T at 37 °C for 1 h. Membrane was then incubated with hybridoma supernatant of selected MAbs (3F10 and 2C3) at 37 °C for 1 h, washed three times in PBS-T and again incubated with 1:5000 of goat anti-mouse IgG peroxidase conjugate (Sigma, USA) for 1 h at 37 °C. Following further washes, bound antibodies were detected by addition of 3,3′, 5,5′-tetramethylbenzidine membrane peroxidase substrate (KPL, USA) and the colour reaction was stopped by rinsing the membrane extensively with deionized water.

2.6. Immunoassays

2.6.1. Indirect ELISA for reactivity of MAbs

Indirect ELISA was performed to assess the reactivity of selected MAbs (3F10 and 2C3) to OMPs or formalin whole cells of all *E. tarda* isolates including reference strain or other selected bacterial strains. Optimal concentration of purified OMPs, purified MAbs and conjugate were determined by a checkerboard titration as per Crowther [26]. ELISA plates (Nunc ImmunoTM plate, MaxiSorp, Denmark) were coated with 50 μl per well of OMPs (1 μg/ml) or formalin killed whole bacterial cells (10^8 CFU/ml) in carbonate–bicarbonate buffer (pH 9.6) and incubated overnight at 4 °C. All tests were performed in duplicate. The wells were washed three times with PBS containing 0.05% Tween-20 (PBS-T) and blocked 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 1:400 dilution of purified MAbs (3F10 and 2C3) to each well. The plates were further incubated for 1 h at 37 °C and washed. Thereafter, 50 μl of a 1:5000 dilution of goat anti-mouse IgG peroxidase conjugate (Sigma, USA) 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 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 of wells was measured at 492 nm in an ELISA plate reader (TECAN, Austria).

Indirect ELISA was also used to test the sensitivity of the MAbs to detect formalin killed whole cells or OMPs of *E. tarda*. Briefly, ELISA plates were coated and dried overnight with 50 μl of 10-fold diluted solutions (10^8 to 10^1 CFU/ml) of whole bacterial cells or different concentrations of OMPs (2 μg/ml to 4 ng/ml) diluted in carbonate–bicarbonate buffer. The subsequent steps were similar as mentioned above.

2.6.2. Competitive ELISA for epitope analysis

For competitive ELISA, 3F10 MAb was labelled with horse radish peroxidase (HRP) using HRP labelling kit and the optimum dilution of HRP labelled 3F10 MAb was determined by indirect ELISA. This dilution was used in the assay for determining the competition with the unlabelled 2C3 MAb. For the test, ELISA plate coated with E-OMPs (1 μg/ml), was incubated with 1:200 dilution of labelled 3F10 MAb and simultaneously with decreasing concentrations of an unlabelled 2C3 MAb. The subsequent steps were similar as mentioned above.

3. Results

3.1. Production and characterization of MAbs to E-OMPs

The OMPs profile of all 17 isolates of *E. tarda* including E-OMPs used in our study has been demonstrated previously through SDS-PAGE [13]. Briefly, E-OMPs consisted of a strong band with a molecular mass of approximately 44 kDa and nine other protein bands of 38.8, 36.5, 34.5, 30, 28, 25, 23.75, 22 and 20.3 kDa. This preparation was used as an antigen in the immunization protocol and also in indirect ELISA for coating for the selection of positive clones.

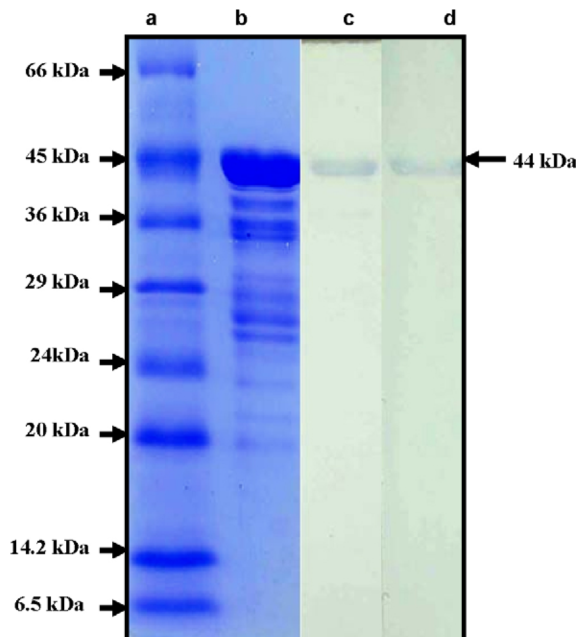

Fig. 1. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis of outer membrane proteins (OMPs) of *Edwardsiella tarda* ET-7. lane a, molecular weight marker proteins; lane b, SDS-PAGE profiles of OMPs stained with Coomassie blue; lanes c and d, Western blot of E-OMPs treated with 3F10 MAb and 2C3 MAb, respectively.

There was a significant increase in indirect ELISA signal after immunization of BALB/c mice, when sera samples were tested on the 35th day of the immunization schedule (data not shown) as compared to 0 day mice serum, thereby suggesting immunogenic nature of E-OMPs. A total of 15 positive clones consistently specific to E-OMPs were obtained after screening. Out of which, two strong positive hybridoma clones (3F10 and 2C3 MAbs) were selected on the basis of their reactivity in indirect ELISA and stability in culture. The clones exhibited especially good growth characteristics and antibody production. These clones were subjected to single cell cloning and subcloning. The limiting dilution method of hybridoma clones was adopted for this purpose. Monoclonality of a clone was accepted only when all the wells of a microtitre plate with growing cells gave a positive reaction in indirect ELISA after repeated subcloning. Antibodies secreted by both hybridoma cell lines (3F10 and 2C3) were found to be IgG1 isotype. In Western blotting, both the MAbs recognized predominant 44 kDa OMP band of E-OMPs (Fig. 1). In all other *E. tarda* isolates also, 44 kDa OMP band was detected using 2C3 MAb (Fig. 2). Both the MAbs were not able to detect reference isolate of *E. tarda* (MTCC 2400) by Western blotting.

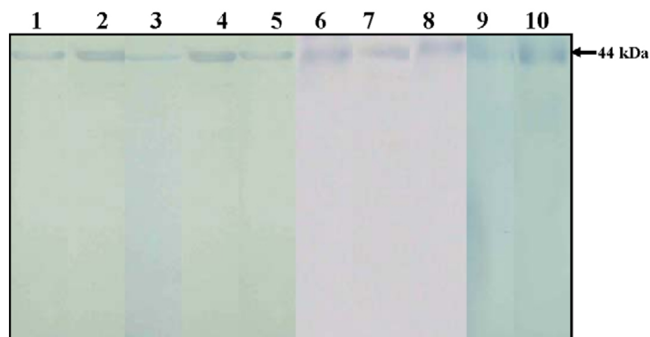

Fig. 2. Western blotting analysis of OMPs of *E. tarda* isolates by 2C3 MAb. lane 1, ET8; lane 2, ET9; lane 3, ET10; lane 4, ET11; lane 5, ET12; lane 6, ET13; lane 7, ET14; lane 8, ET15; lane 9, ET16; lane 10, ET17.

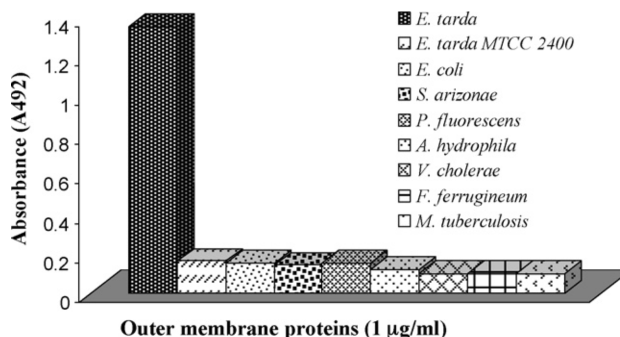

Fig. 3. Reactivity of 3F10 monoclonal antibody to OMPs of *Edwardsiella tarda*, reference strain of *Edwardsiella tarda* MTCC 2400 and test bacterial strains in indirect ELISA. A fixed quantity of OMPs (1 µg/ml) were coated to the plate and 1:400 dilution of 3F10 MAb was used as primary antibody.

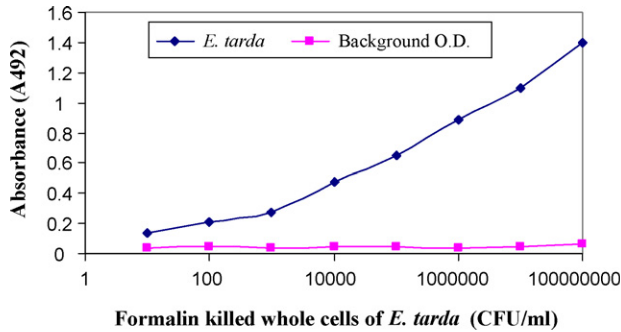

Fig. 4. Sensitivity of 3F10 monoclonal antibody to detect formalin killed whole bacterial cells of *Edwardsiella tarda* by indirect ELISA. ELISA plate was coated with 10-fold diluted solutions (10^8 to 10^1 CFU/ml) of formalin killed whole cells of *E. tarda* and 1:400 dilution of 3F10 MAb was used as primary antibody.

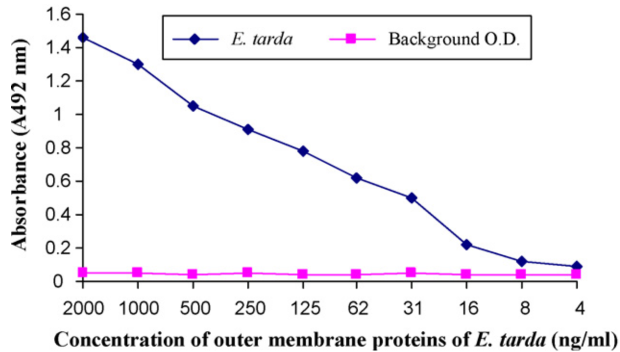

Fig. 5. Sensitivity of 3F10 monoclonal antibody to detect outer membrane proteins of *Edwardsiella tarda* by indirect ELISA. ELISA plate was coated with different concentrations of OMPs of *E. tarda* (2000–4 ng/ml) and 1:400 dilution of 3F10 MAb was used as primary antibody.

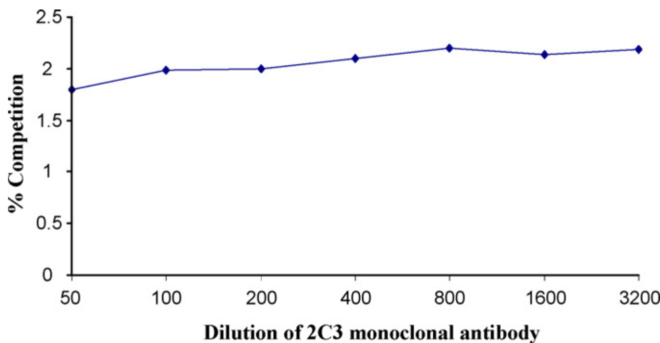

Fig. 6. Competition of HRP-labelled 3F10 MAb to unlabelled 2C3 MAb of *Edwardsiella tarda*. Competition was carried out by incubating optimal dilution of HRP-labelled 3F10 MAb (1:200) along with twofold serially diluted (1:50–1:3200) unlabelled 2C3 MAb on E-OMPs (1 μg/ml) coated plate.

3.2. Immunoassays

3F10 and 2C3 MAbs reacted strongly to formalin killed whole bacterial cells and also to OMPs from all isolates of *E. tarda* but not with reference strain of *E. tarda* (MTCC 2400). These MAbs also did not react with either the OMPs or whole bacterial cells of *E. coli*, *S. arizonae*, *P. fluorescens*, *A. hydrophila*, *V. cholerae*, *F. ferrugineum* and *M. tuberculosis* (Fig. 3). The sensitivity of MAbs to detect whole cells of *E. tarda* was up to a level of 1×10^4 CFU/ml (Fig. 4). The purified OMPs of *E. tarda* were detected up to a level of 31 ng/ml (Fig. 5). In a competitive ELISA, 3F10 MAb did not prevent the binding of 2C3 MAb, indicating that the antibodies recognized different epitopes (Fig. 6).

4. Discussion

Edwardsiellosis is one of the important diseases of freshwater and marine fish, which is caused by *E. tarda*. It has a significant economic impact on the aquaculture industry in country like India. In humans, gastroenteritis caused by *E. tarda* appears to be more common in tropical and subtropical countries, where dietary habits include the eating of raw fish [4].

The specific diagnosis of edwardsiellosis based on gross lesions and elaborate laboratory procedures of bacterial isolation are time consuming and expensive. Quick and precise laboratory diagnosis of disease therefore, requires specific assays. Application of MAbs are important tools for antigenic characterization of various bacterial pathogens and for specific detection. Previously, MAbs have been utilized for specific diagnosis against variety of fish bacterial pathogens, i.e., *A. hydrophila* [27], *A. salmonicida* [28], *Piscirickettsia salmonis* [29], *Photobacterium damselae* ssp. *Piscicida* [30] and *Vibrio harveyi* [31]. However, MAbs to *E. ictaluri* have been used extensively for diagnosis and characterization of OMPs [14,16].

In the present investigation, using E-OMPs as immunogen, two MAbs (3F10 and 2C3) were obtained, characterized and their specificity were tested. Both of MAbs reacted with all isolates of *E. tarda*, as determined by indirect ELISA and Western blot analysis. Both of the MAbs recognized only major 44 kDa OMP band in all isolates of *E. tarda*. However, these MAbs did not detect reference strain of *E. tarda* (MTCC 2400), because 44 kDa OMP band was absent in MTCC 2400 strain. This variation in the OMP profile could be due to differences in the origin of the reference strain. Such antigenic and genetic variations exists between *E. tarda* strains isolated from different fish species as seen by immunoblotting and random amplified polymorphic DNA (RAPD) analysis [21]. In a similar study, variation have been reported in *E. tarda* strains isolated from fish and human from different countries by using RAPD method [20].

Studies have also shown that *E. tarda* isolates of a particular origin in a geographical location are the most oftenly similar in antigenic profile. A 37 kDa OMP was commonly detected among Japanese strains of *E. tarda* and was strongly recognized by rabbit anti-serum raised against whole cells of *E. tarda* [32]. In USA, six common antigenic proteins bands at 15, 18, 37, 46, 66 and 71 kDa have identified in nine reference isolates of *E. tarda* by using a goat polyclonal anti-serum [33]. All these studies suggest the prevalence of antigenic heterogeneity among the *E. tarda* isolates from different geographical locations.

Recently production of MAbs to *E. tarda* isolate of Chinese origin have been reported. However, considering the differences in antigenic characteristics of *E. tarda* from various regions, use of MAbs in diagnostic assays may be limited to a particular expanse only. Therefore, the present study carried out by us in raising MAbs against *E. tarda* of Indian origin is of great relevance.

Specificity of diagnostic reagents is one of the important criterion in evaluating the efficacy of a assay. Studies have shown that polyclonal antibodies have their own limitations of specificity in immunoassays for detection of *E. tarda*. Swain et al. [10] have used dot ELISA for rapid and confirmatory identification of *E. tarda* in fish tissues. However, rabbit polyclonal anti-sera against bacterial whole cells, whole cells lysates, somatic 'O' antigens, lipopolysaccharides and extracellular products of *E. tarda* were noted to cross-react with *A. hydrophila* and *P. fluorescens* [12]. Similarly, in our earlier study also RPAbs cross-reacted to whole cells of *Salmonella arizonae* above 10^6 CFU/ml [13]. However, in our present study, no cross-reactivity of the MAbs were observed with other test bacterial strains, thereby improving the specificity of detection. This also indicates that the 44 kDa OMP may contain at least two antigenic epitopes, which are specific to our *E. tarda* isolates. A conserved 37 kDa OMP of *E. tarda* has been evaluated as an effective vaccine candidate in Japanese flounder, *Paralichthys olivaceus* for protection against *E. tarda* infection. *N*-terminal amino acid sequence analysis showed that the 37 kDa OMP is homologous to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) of *E. tarda* [34]. In another study, Liu et al. [35] concluded that recombinant GAPDH protein offered protection to Japanese flounder against *E. tarda* infection and evaluated its use as an effective and practical vaccine antigen against edwardsiellosis. Going further, this recombinant GAPDH of *E. tarda* showed protective antigenicity even against *Vibrio anguillarum* in Japanese flounder [36]. The presence of a conserved epitope on the 44 kDa OMP band of our *E. tarda* isolates suggests that this OMP band could be further evaluated as candidate antigen for the development of a new vaccine.

Our previous studies and other research findings have proven that *E. tarda* is a heterogeneous species. Therefore, ideally there is need to develop an immuno-diagnostic kit based on panel of MAbs including 3F10 and 2C3 MAbs for comprehensive detection of *E. tarda* from different geographic locations. Rapid and specific diagnosis of the disease will be essential for control and prevention of edwardsiellosis.

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