



Monoclonal antibodies against recombinant GAPDH of *Edwardsiella tarda* reveal the conserved nature of the protein

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ABSTRACT

The outer membrane protein, encoded by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene, of *Edwardsiella tarda* is a highly conserved immunogenic protein. The GAPDH was cloned and expressed in *Escherichia coli*. The purified protein was used to produce mouse monoclonal antibodies (MAbs). Four stable hybridomas producing MAbs (3G12, 4E9, 5A11 and 9G1) against rGAPDH were obtained. The heavy chains of antibodies produced by the hybridomas were of the isotypes IgG1 and IgM. Cross reactivity of MAbs (3G12 and 9G1) was observed with GAPDH of *Aeromonas hydrophila* and *Micrococcus luteus*. MAbs 3G12 and 4E9 reacted with *Vibrio cholerae*, *Salmonella enterica* and *Penaeus monodon* tissues but not with vertebrate GAPDH. None of the MAbs reacted with *Staphylococcus aureus*. The results indicate that the level of conservation of GAPDH is high among evolutionarily close species. The MAbs developed will be a useful tool to study the evolutionarily conserved and functionally diverse GAPDH.

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Introduction

Bacterial diseases cause considerable loss to fresh water aquaculture industry. Among the bacterial pathogens, motile *Aeromonas* and *Edwardsiella* sp. are the most important affecting a wide range of hosts ranging from lower vertebrates such as fish to reptiles, amphibians and mammals, including human. They cause serious infection in fishes, especially warm water fishes. *Edwardsiella tarda* causes edwardsiellosis, a subacute to chronic disease in carp, tilapia, eel, catfish, mullet, salmon, trout and flounder. The disease is reported in North and Central America, Europe, Africa, Australia and Asia. In India the disease has been reported in Indian major carps, Asian catfish, snakehead (Kumar, Rathore, Sengupta, Kapoor, & Lakra, 2010) and striped catfish (Shetty, Maiti, Venugopal, Karunasagar, & Karunasagar, 2014).

E. tarda belongs to the Enterobacteriaceae family and is Gram negative, motile with peritrichous flagella, facultative anaerobic measuring 1 µm in diameter and 2–3 µm in length. They ferment glucose, maltose, mannose, mannitol and arabinose and produce H₂S. They are positive for lysine decarboxylase and Ornithine decarboxylase and negative for urease (Shetty et al., 2014).

The outer membrane proteins (OMP) of bacteria constitute about 50% of the outer membrane mass. Out of all the major OMPs of *E. tarda*, the 37 kDa OMP is the most conserved protein and immunodominant antigen (Kasagala, Prasad, Makesh, & Gireesh Babu, 2016; Maiti, Shetty, Shekar, Karunasagar, & Karunasagar, 2011). This OMP has a glycoprotein profile and is proved to be a good vaccine candidate for *E. tarda*. The 20 N-terminal amino acid sequences of the 37 kDa OMP have a high homology to glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which catalyzes glycolysis in eukaryotes. GAPDH is also found on the bacterial cell surface and is used as a house keeping gene and protein in quantitative polymerase chain reaction (PCR) and western blot analysis respectively (Jin, Wei, Niu, Li, & Yang, 2014; Ueno, Nishiguchi, Takeshita, Yamaguchi, & Oda, 2017; Wu et al., 2012; Yu et al., 2011; Zainuddin, Chua, Rahim, & Makpol, 2010).

Currently, there are numerous molecular as well as serological techniques reported to detect *E. tarda* infection in fish. The molecular techniques reported include fluorescence in situ hybridization (FISH), PCR (Kumar, Swaminathan, Rathore, Sood, & Kapoor, 2008), real-time PCR (Reichley, Ware, Greenway, Wise, & Griffin, 2015; Xie, Huang, et al., 2012) and LAMP (Xie, Zhang, et al., 2012). The serological techniques reported are agglutination test, Indirect ELISA, dot-ELISA, Indirect blocking ELISA and competitive ELISA. Among these methods, serological tests are relatively inexpensive, provide rapid results and can be easily performed under field conditions.

All the serological tests reported for *E. tarda* detection use polyclonal antibodies raised in laboratory animals. However, polyclonal antibodies have the disadvantage that they can cross react with closely related species. For serodiagnosis monoclonal antibodies (MAbs) are important reagents, which are highly specific for an epitope in comparison to polyclonal antibodies. Although polyclonal antibodies and MAbs against GAPDH are commercially available, they are produced against eukaryotic GAPDH or synthetic peptides within eukaryotic GAPDH. There is no report of monoclonal antibody specific to GAPDH membrane protein of *E. tarda*. Hence the present study was undertaken for the production and characterization of MAb against *E. tarda* GAPDH outer membrane protein, which can be used to develop specific immunoassays for the identification of *E. tarda*.

Materials and methods

Bacterial strain and growth conditions

The bacterial isolates and *E. tarda* ATCC strain No. 15947 maintained in the fish processing and aquatic animal health laboratories of Central Institute of Fisheries Education (CIFE), Mumbai were used in this study. The *E. tarda* strain was revived and grown on Salmonella Shigella Agar (SS agar) media (Himedia, India) at 30°C for 24 h. Bacterial colonies with black centers in SS agar were used for further studies. *Escherichia coli* strains DH5 α and BL21 (DE3) pLys S were used for cloning and protein expression, respectively. The recombinant-DH5 α was grown at 37°C on ampicillin supplemented Luria-Bertani agar whereas both ampicillin and chloramphenicol were supplemented for BL21 (DE3) pLys S. Apart from these *Aeromonas hydrophila*, *Micrococcus luteus*, *Staphylococcus aureus*, *Vibrio cholerae* and *Salmonella enterica* were used for cross reactivity studies. For bacterial enumeration, cells were washed and harvested with phosphate-buffered saline (PBS), serially diluted, spread on plates and expressed as colony forming units

(CFU) per ml. For inactivation of *E. tarda* the bacteria were grown overnight and incubated at 70°C for 10 min with citric acid (0.9%) and formalin (0.1%).

Biochemical tests

The biochemical tests were performed using biochemical test kit for Enterobacteriaceae (Himedia, India). The tests carried out were citrate, lysine and ornithine utilization, urease, phenylalanine deamination, nitrate reduction, H₂S production and carbohydrate utilization *viz.*, glucose, adonitol, lactose, arabinose and sorbitol. Each well in the test strip was inoculated with 50 µl of fresh overnight bacterial culture and incubated at 30°C for 24 h. The results were read following the protocol prescribed by the manufacturer of the kit.

Cloning and GAPDH protein expression

Genomic DNA extraction and PCR for GAPDH gene

Genomic DNA of *E. tarda* was isolated by phenol-chloroform method. Briefly overnight grown bacterial culture was centrifuged at 12,000 rpm for 3 min and the pellet was resuspended in 1× TE buffer. For cell lysis 10% SDS, 20 mg/ml proteinase K and 5 M NaCl were added to the suspension. The DNA was separated by adding phenol-chloroform followed by 0.6 volume of isopropanol.

For PCR reaction, primers were designed to amplify the gene encoding GAPDH of *E. tarda* (GAPDH F2 5'-CGGGATCCCAACGGTTTTGGCCGTATCG-3' and GAPDH R2 5'-CGAAGCTTGGAGTAGCCAGTTTCGTTGTC-3') with restriction sites for *Bam* HI and *Hind* III at the 5' end of the forward and reverse primers, respectively, taking care to align the gene in correct reading frame when cloned into pRSET B expression vector. The annealing temperature for the primers was standardized by performing gradient PCR. Initial denaturation was carried out at 94°C for 3 min. followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 63–67°C for 30 s and extension at 72°C for 90 s. A final extension was carried out at 72°C for 5 min at the end of 30 cycles and then held at 4°C. The PCR products were purified from agarose gels using GeneJET Gel Extraction Kit (Thermo scientific, USA) following the manufacturer's protocol.

Bacterial transformation of GAPDH gene

GAPDH gene was cloned into pTZ57R/T using InsTAclone PCR Cloning Kit (Thermo scientific, USA) following the manufacturer's protocol. The ligation mix was transformed into DH5α cells and plated on LB agar plate containing IPTG (isopropyl-β-D-thiogalactopyranoside) and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). Positive colonies were selected by blue white selection method. The plasmid containing the insert was purified using GeneJET Plasmid Miniprep Kit (Thermo scientific, USA).

The purified recombinant pTZ57R/T containing GAPDH insert and the expression vector pRSET B (Invitrogen, USA) were double digested with FastDigest *Bam* HI and *Hind* III (Thermo scientific, USA). The products were electrophoresed on a 1% agarose gel and the GAPDH insert and linearized pRSET B were purified from gel slices using

GeneJET Gel Extraction Kit (Thermo scientific, USA). The GAPDH insert was ligated to the linearized pRSET B and transferred into DH5 α competent cells using TransformAid bacterial transformation kit (Thermo scientific, USA). For protein expression the recombinant plasmid was purified and transformed into the expression host BL21 (DE3) pLys S (Invitrogen, USA). Positive colonies were identified by colony PCR.

Gene sequencing and phylogenetic study

The GAPDH gene was sequenced using the commercial services of Eurofins (India). The nucleotide sequences were screened using the NCBI VecScreen programme ([http:// www.ncbi.nlm.nih.gov/VecScreen/](http://www.ncbi.nlm.nih.gov/VecScreen/)). To find homologous sequences, single nucleotide–nucleotide alignment was performed using the program Basic Local Alignment Search Tool (BLAST) of NCBI (<http://www.ncbi.nlm.nih.gov/blast>).

For phylogenetic analysis except *E. tarda* GAPDH, which was obtained by sequencing, all GAPDH sequence used were retrieved from NCBI GenBank. All sequences were aligned in MEGA6 (Tamura, Stecher, Peterson, Filipski, & Kumar, 2013) by using ClustalW algorithm. Phylogenetic analysis of GAPDH gene sequences were performed through Kimura 2-parameter distance model and neighbor-joining method available in MEGA6. The reliability of phylogenetic groupings was evaluated by using the bootstrap test with 1000 bootstrap replications.

Protein expression and purification

A pilot expression study was performed to standardize the expression conditions. The culture was induced with IPTG to a final concentration of 1 mM and samples were collected after every hour up to 6 h post induction. The cells were lysed by repeated freeze thawing and the pellet was resuspended in 100 μ l of phosphate buffer at neutral pH. The expression of the recombinant GAPDH (rGAPDH) protein was detected by SDS-PAGE of the induced as well as the uninduced bacterial cells on a 12% polyacrylamide gel.

After confirmation, bulk protein was produced by harvesting the induced cells at appropriate period according to pilot study result by centrifuging at 5000g for 5 min. Cells were lysed with guanidium lysis buffer and sonicated on ice with three 5-s pulses at high intensity. The histidine tagged recombinant protein was purified from the bacterial lysate using Ni-NTA agarose (Invitrogen, USA) under denaturing conditions following manufacturer's instructions. The protein was eluted as 1 ml fractions and fractions that contain the peak absorbance at 280 nm were pooled and dialyzed against 10 mM Tris, pH 8.0, 0.1% Triton X-100 overnight at 4°C to remove the urea. The protein was concentrated by sprinkling polyethylene glycol (PEG) over the dialysis bag.

Monoclonal antibody production

The animal experiments were carried out after the due approval of the institutional animal ethics committee. Two eight-weeks-old female balb/c mice were immunized each with 50 μ g of recombinant protein emulsified with Freund's complete adjuvant (FCA) and boosted intraperitoneally on 14th, and 28th day with the same dose of purified protein

emulsified with Freund's incomplete adjuvant (FIA). The animals were test bled 10 days after the second booster dose and serum antibody titer was determined by ELISA. On 36th day the mouse having high antibody titer was injected with 50 µg of purified protein intravenously and after three days of the final booster, the mouse was killed and spleen was removed for fusion.

The cryopreserved SP2/0 myeloma cells were thawed rapidly at 37°C and washed with Iscove's Modified Dulbecco's Medium (IMDM) (Thermofisher, USA). The cells were finally suspended in IMDM containing 15% fetal bovine serum (FBS) (Thermofisher, USA), transferred to a 25 cm² tissue culture flask and incubated at 37°C with 5% CO₂. Feeder cells were prepared two days prior to fusion. The peritoneal macrophage and spleen cells of unimmunized mice were used as feeder cells. For fusion the spleen cells and myeloma cells were mixed in the ratio of 10:1 and fused using 1 ml of sterile PEG (Hybrimax, Sigma, USA). Finally the cells were resuspended in IMDM with 20% FBS with 2× HAT (hypoxanthine aminopterin thymidine) supplement (Thermofisher, USA). The cells were added to the plates containing feeder cells at the rate of 100 µl per well. After two weeks, supernatants from the wells were screened for the presence of antibodies by indirect ELISA using ELISA plates coated with rGAPDH and killed *E. tarda* and positive clones were expanded by sub-culturing. Cells in the logarithmic phase of growth were harvested and cryopreserved in liquid nitrogen.

Characterization of monoclonal antibody

Western blotting

For western blotting, purified rGAPDH protein, *E. tarda* cell lysate and molecular weight marker were resolved on 12% polyacrylamide gel and blotted on to a PVDF membrane using a semi dry electro blotting apparatus at a constant voltage of 60 V for 15 min. At the end of the transfer, the membrane was removed, soaked in methanol, air dried and blocked with 3% BSA in PBS at 4°C overnight. The membrane was washed three times in PBST and cut into number of strips, each pertaining to one sample lane in SDS-PAGE gel. Each strip was incubated with the hybridoma supernatant from the culture of each clone for 2 h at room temperature (RT) followed by three times washing and incubation with rabbit anti-mouse HRP conjugate at 1:2000 dilution in PBS for 1 h at RT. The strips were washed again four times in PBS and freshly prepared substrate solution consisting of 0.05% diaminobenzidine (Sigma, USA) in PBS containing 0.1% H₂O₂ was added and allowed to react for 5 min. The membrane was washed in tap water and air dried.

Cross reaction

The cross reactivity of MAbs against GAPDH of *E. tarda* with that of *A. hydrophila*, *V. cholerae*, *S. enterica*, *M. luteus*, *S. aureus*, fish (*Pangasius pangasius*), shrimp tissue (*Penaeus monodon*), chicken blood and human blood was checked by western blotting. The PVDF membrane was incubated with a hybridoma culture supernatant for 90 min at RT. After washing and subsequent incubation with anti-mouse IgG-HRP conjugate and substrate, the membrane was washed with tap water and air dried.

Mouse antibody isotyping

Determination of monoclonal antibody subclass is helpful for characterization of the antibody, for choosing detection reagents and for deciding on the purification scheme. The isotyping was done using IsoQuick™ mouse monoclonal isotyping kit (Sigma) following the manufacturer's protocol.

Results

Colony morphology and biochemical tests

The *E. tarda* colonies appeared transparent with black center after overnight incubation at 30°C in Salmonella Shigella agar plate. In other solid media such as Nutrient agar, the colonies appeared as smooth, circular (0.5–1 mm), whitish translucent and raised about 0.2 mm from base. The results of the biochemical tests (Table 1) were suggestive of *E. tarda*.

Cloning and expression of GAPDH gene of *E. tarda*

Extraction of genomic DNA from *E. tarda* and PCR amplification

The concentration of the genomic DNA extracted from *E. tarda* was 87.4 ng/μl. The gene encoding the GAPDH of *E. tarda* was amplified from purified genomic DNA of *E. tarda* by PCR. The optimal annealing temperature of the primers was found to be 65°C based on gradient PCR (Figure 1(A)). Upon PCR a product of about 940 bp was obtained as expected.

Bacterial transformation of GAPDH gene

Colony PCR for the bacterial colonies transformed with recombinant pTZ57R/T and pRSETB resulted in a 940 bp product. The presence of the insert in the recombinant pRSETB was verified by restriction digestion of the recombinant pRSETB and upon digestion a 940 bp insert was released from the plasmid (Figure 1(B)), thus confirming the cloning of GAPDH into the expression vector pRSETB.

Table 1. Results of biochemical tests for *Edwardsiella tarda* ATCC strain 15947.

Biochemical tests	Results
1. Citrate utilization	+
2. Lysine utilization	+
3. Ornithine utilization	+
4. Urease	–
5. TDA (Phenylalanine deamination)	–
6. Nitrate reduction	+
7. H ₂ S production	+
8. Glucose	+
9. Adonitol	–
10. Lactose	–
11. Arabinose	+
12. Sorbitol	–

Notes: +, positive; –, negative.

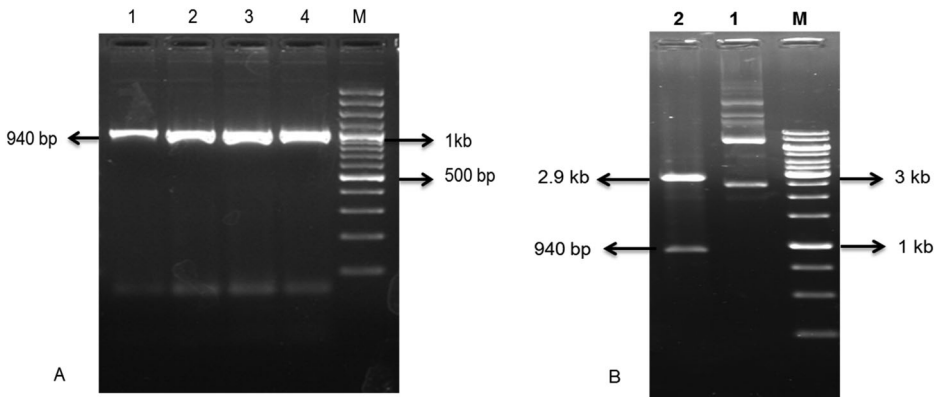


Figure 1. (A): Gradient PCR for GAPDH gene of *E. tarda* (Lanes 1–4: PCR samples with annealing temperature 60–67°C, Lane M: 100 bp Marker). (B) Restriction enzyme digestion of recombinant pRSETB (Lane M: 1 kb plus DNA marker, Lane 1: undigested pRSETB and Lane 2: *Bam*HI and *Hind*III digested recombinant pRSETB with GAPDH insert).

Analysis of gene sequence and phylogenetic tree

Upon sequencing both the strands of the GAPDH gene, a 939 bp sequence specific for GAPDH was obtained (Figure 2). The GAPDH gene sequence of *E. tarda* was compared with the GenBank database of the National Center for Biotechnology Information (NCBI) by using the BLAST program (<http://www.ncbi.nlm.nih.gov/>). The nucleotide blast results of the GAPDH gene of *E. tarda* revealed high homology of 99% with published gene sequences of *E. tarda* ATCC 15947 glyceraldehyde-3-phosphate dehydrogenase (*gapA*) gene (GenBank ACCESSION No. HQ697338), *Edwardsiella ictaluri* ATCC 33202 GAPDH gene (GenBank ACCESSION No. HQ697334) and 97% with *Edwardsiella piscicida* C07–087 (GenBank ACCESSION No. CP004141) complete genome. The CLUSTAL analysis of 939 bp GAPDH gene sequence of *E. tarda* with 996 bp GAPDH gene of *A. hydrophila*, 881 bp GAPDH gene of *S. aureus*, 856 bp GAPDH gene of *V. cholerae* and 924 bp gene of *S. enterica* has shown 82%, 56%, 77.91% and 83.51% of aligned score, respectively.

The phylogenetic analysis revealed that the sequenced GAPDH is more related to *Edwardsiella* spp. sequences than to other sequence (Figure 3). Among the other species *A. hydrophila* and *V. cholerae* seems to be more closely related with high bootstrap

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CAACGGTTTTGGCCGATCGGGCGTATTGTTTTCCGTGCTGCTCAGGAACGTTCTGACA
TCGAAATCGTTGGCATCAACGATCTGCTGGATGCCAACTACATGGCATACATGCTGAAGT
ACGACTCTACTCACGGTTCGTTTCAACGGCACTGTTGAAGTGAAGATGGCCACCTGATC
GTTAACGGTAAAAAATCCGTGTTACCGCTGAAAGAGATCCGGCTAACCTGAAAGTGGGA
CGAAATCGGTGTTGACGTAGTTGCCGAAGCAACCGGTCTGTTCTGACCGACGAAACC
GCACGTAAGCACATCGCTGCCGGCGCCAAGAAAAGTCGTATGACTGGCCCGTCCAAA
GATGCTACCCCGATGTTTCGTTATGGGCGTAAACCACAAGAAGTACGCTGGCCAGGAGAT
CGTTTTAAACGCTTCTGCAACCACTGCCTGGCACCCGCTGGCTAAAGTCTGAAAC
GACAACTTCGGCATCGTTGAAGCACTGATGACCACCGTTTACGCTACCACCGCTACCC
AGAAAACCGTTGATGGCCCGTCCATGAAAGATTGGCGCGGCGCCGTTGGCGCTAGCC
AGAACATCATCCCGTCTCTACCGGTGCTGCCAAGGCTGTTGGCAAGGTTATCCCGGA
ACTGAACGGCAAACCTGACCGGTATGGCTTTCGCGCTTCCGACCCGAAACGTTCCGTT
GTTGACCTGACTGCACGCTGGCCAAGCCGGCTACCTATCAGCAGATCTGTGACGTGA
TGAAGGCCGCTTCTGAAGGCGGAAATGAAAGGCGTTCTGGGCTACACCGACGAAGCCG
TCGTTTTACCGACTTCAACGGCGAAGTTGCACCTCTGTGTTTGTGACCGACGCGGT
ATCTCCCTGAACGCAACTTCGTGAAACTGGTTTCTGGTATGACAACGAACTGGCTA
CTCCAA

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Figure 2. Partial sequence of *Edwardsiella tarda* ATCC 15947 GAPDH gene (939 bp).

support. The eukaryotic GAPDH of human, primates, birds and *Staphylococcus* spp. were distantly related to *E. tarda*.

Expression and purification of rGAPDH

The pilot expression resulted in the over expression of the 37 kDa rGAPDH protein in the IPTG-induced cultures, which was not observed in the uninduced culture. The maximum protein expression was observed 6 h after IPTG induction (Figure 4).

The recombinant protein was purified under denaturing conditions using Ni-NTA columns. The recombinant protein was eluted in the first two fractions collected during the elution steps. The rest of the fractions also contained less quantity of protein comparatively (Figure 5(A)). The first two fractions were pooled, dialyzed and quantified. The concentration of the purified protein was 5.91 mg/ml. The purified protein upon SDS-PAGE analysis produced a distinct intense band at 37 kDa (Figure 5(B)).

Generation and characterization of MAb to purified GAPDH protein

A successful fusion was achieved using spleen cells from mouse immunized with purified GAPDH protein and myeloma cells. Out of 10 seeded plates, 83 hybridoma clones were obtained, of which 36 hybridomas produced antibodies against GAPDH as detected by indirect ELISA. Among the 36 wells containing positive hybridoma clones, 4 wells having single clone and having high OD values as tested by indirect ELISA were selected. The heavy chain isotypes of the MAbs were of IgM and IgG1 while that of light chain was kappa (Table 2).

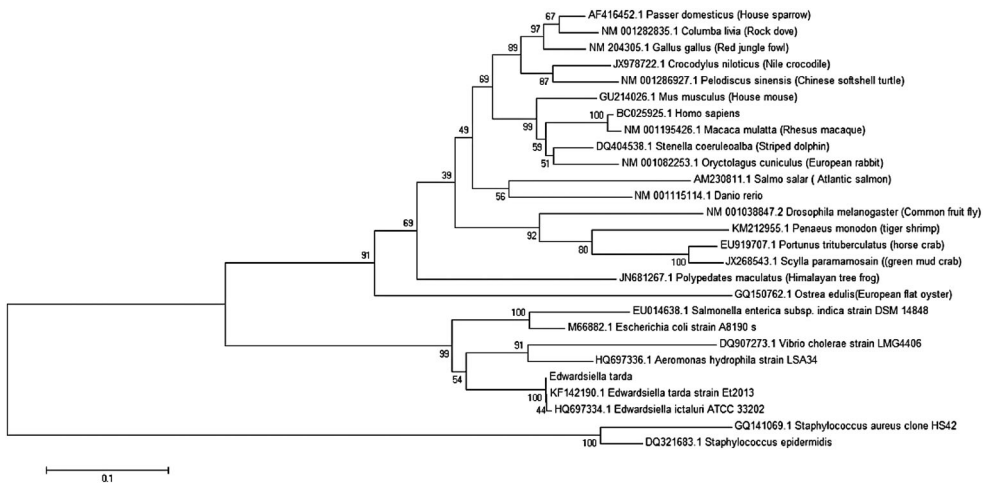


Figure 3. Phylogenetic analysis of GAPDH gene sequences using neighbor-joining method available in Mega 6. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated. The sequence of GAPDH obtained in this study is encircled.

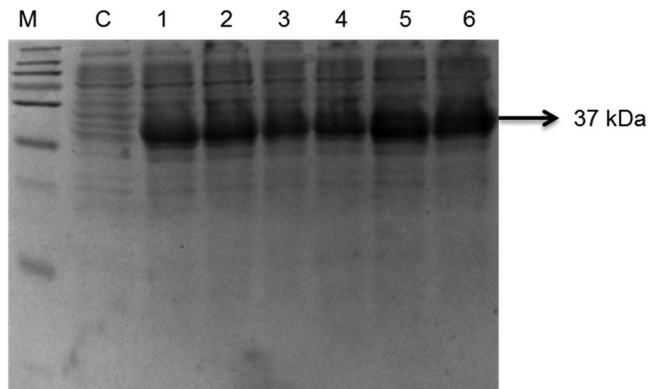


Figure 4. SDS-PAGE for pilot protein expression of rGAPDH (Lane M: protein ladder, Lane C: uninduced bacterial lysate, Lanes 1– 6: bacterial lysate of samples collected every hour from 1 h to 6 h after IPTG induction).

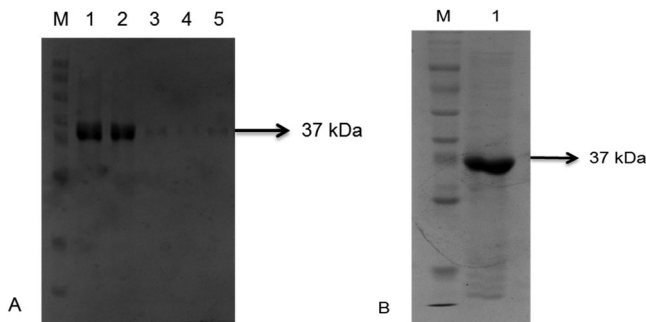


Figure 5. (A): SDS-PAGE of purified GAPDH protein fractions. (Lane M: protein marker Lanes 1–5: Elutes 1–5 of the Ni-NTA column respectively). (B) SDS-PAGE of purified rGAPDH protein (Lane M: Marker, Lane 1: purified protein).

Western blotting

Western blot was carried out to check the specificity of MABs to the purified GAPDH and *E. tarda*. Specific reactions were detected with all the four MABs. The MABs designated as 3G12, 9G1, 5A11 and 4E9 showed a clear specific reaction with the purified recombinant protein and inactivated *E. tarda* lysate at molecular weight of 37 kDa (Figure 6).

Cross reactivity and isotype analysis

The cross reactivity of MABs against GAPDH of *E. tarda* with that of *A. hydrophila*, *V. cholerae*, *S. enterica*, *M. luteus*, *S. aureus*, pangasius fish (*P. pangasius*) and tiger shrimp tissues

Table 2. Isotyping of MABs.

MABs Nos.	Heavy chain specificity	Light chain specificity
3G12	IgG1	kappa
4E9	IgM	kappa
5A11	IgG1	kappa
9G1	IgM	kappa

(*P. monodon*) was checked by western blotting. MAb 9G1 and 3G12 reacted with GAPDH of *A. hydrophila*, *M. luteus* and *E. tarda* (Figure 7(A)). MAb 3G12 also cross reacted with *V. cholerae*, *S. enterica* (Figure 7(B)) and shrimp tissues (*P. monodon*) (Figure 7(C)). However MAb 3G12 did not react with any of the fish tissues (*P. pangasius*) (Figure 7(C)) tested. MAb 4E9 also cross reacted with *V. cholerae*, *S. enterica* and shrimp tissues (*P. monodon*) (Figure 8(B)) MAbs 4E9 and 5A11 did not react with GAPDH of *A. hydrophila*, *M. luteus* and *S. aureus* (Figure 8(A)). MAb 5A11 also showed some non-specific reactions with *A. hydrophila* and *M. luteus*. Interestingly, none of the MAbs reacted with *S. aureus* and vertebrate tissues although some reacted with shrimp GAPDH. The results of western blotting are summarized in Table 3.

Discussion

GAPDH is a ubiquitous enzyme present in all living organisms involved in the breakdown of glucose resulting in energy production. The enzyme catalyzes the sixth step in glycolysis, converting glyceraldehyde 3-phosphate to D-glycerate 1,3-bisphosphate (1,3-BPG). The GAPDH gene and the protein are used as reference gene and protein in PCR (Zainuddin et al., 2010) and western blot analysis (Wu et al., 2012), respectively. Studies have revealed that this protein is multifunctional and perform diverse activities (Sirover, 2011). GAPDH is also found on the bacterial cell surface and is a major outer membrane protein of *E. tarda* (Kasagala et al., 2016; Maiti et al., 2011).

Kawai, Liu, Ohnishi, and Oshima (2004) made the first analysis of the 37 kDa OMP of *E. tarda* and reported that 20 N-terminal amino acid sequences have high homology with GAPDH. In another study, Liu, Oshima, and Kawai (2007) reported that 37 kDa OMP cross reacted with GAPDH antiserum. Similar size OMP has also been described for *E. coli*, *Klebsiella pneumoniae*, *Enterobacter aerogenes*, *Salmonella* spp. and *V. harveyi*. Further, high homology of the GAPDH gene of *E. tarda* with all other *E. tarda* strain as well as with other Edwardsiella species in GenBank database shows that GAPDH gene is a highly conserved OMP gene within and across the species. Sequencing and western blot in the present study also confirms the highly conserved nature of this gene and that the antibodies developed against one species can react with many other species.

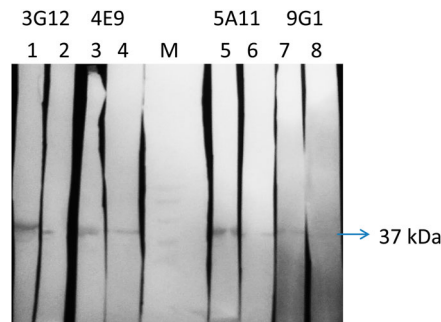


Figure 6. Western blot for anti-GAPDH MAbs against rGAPDH and *E. tarda* (Lane M: protein marker, Lanes 1, 3, 5 and 7: Western blot of MAbs 3G12, 4E9, 5A11 and 9G1 against rGAPDH, Lanes 2, 4, 6 and 8: Western blot of MAbs 3G12, 4E9, 5A11 and 9G1 against *E. tarda* lysate).

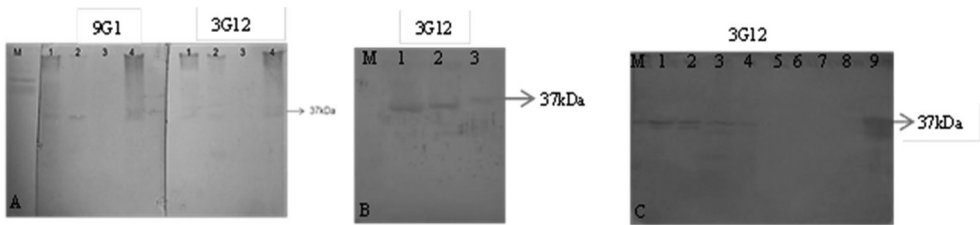


Figure 7. (A) Western blot for MAb (9G1 and 3G12) cross reactivity (Lane M: Molecular weight Marker; Lane 1: *A. hydrophila*; Lane 2: *M. luteus*; Lane 3: *S. aureus*; Lane 4: *E. tarda*). (B) Western blot for MAb 3G12 (Lane M: Molecular weight Marker; Lane 1: *V. cholerae*; Lane 2: *S. enterica*; Lane 3: rGAPDH) (C) western blot for MAb 3G12 with shrimp (*P. monodon*) and fish (*P. pangasius*) tissues (Lane 1:shrimp muscle, Lane 2: shrimp gill, Lane 3: hepatopancreas, Lane 4: hemolymph, Lane 5: Fish Muscle, Lane 6: fish gill, Lane 7: fish kidney, Lane 8: fish heart, Lane 9:rGAPDH).

E. tarda is highly pathogenic having a wide host range, including freshwater and marine fishes, reptiles, amphibians, birds and mammals throughout the world. It causes edwardsiellosis in fish and intestinal and extra intestinal infections in humans. Till date no effective vaccine has been developed owing to its large number of serotypes. The GAPDH protein of *E. tarda* is highly immunogenic and offers protection to the fish against *E. tarda* when injected into them (Maiti et al., 2011). Similarly tilapia immunized with *E. ictaluri* GAPDH produced higher specific antibody titer compared to control fish (Cao et al., 2014). Since the GAPDH is highly conserved with in *E. tarda* serotypes, it can be used as an immunogen to protect fishes against edwardsiellosis. Further, Liu et al. (2007) has reported that rGAPDH of *E. tarda* showed protective antigenicity against *V. anguillarum* in Japanese flounder. Hence the rGAPDH produced in the present study might have potential as a vaccine against multiple bacterial infections in freshwater and brackishwater fishes.

Identification and treatment of edwardsiellosis is a challenge due to its large number of serotypes. Monoclonal antibody has made significant contribution to the diagnosis and treatment of many clinical and veterinary diseases. Over the last decade they have been used as important tools for antigenic characterization of various bacterial pathogens and for specific detection of fish pathogens. Previously MABs have been utilized for

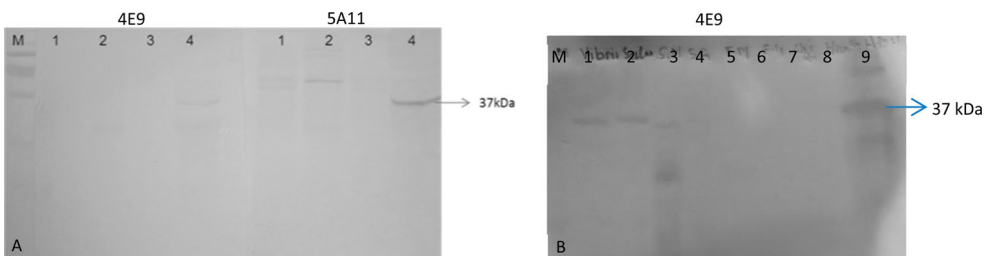


Figure 8. (A) Western blot for MAb (4E9 and 5A11) cross reactivity (Lane M: Molecular weight Marker; Lane 1: *A. hydrophila*; Lane 2: *M. luteus*; Lane 3: *S. aureus*; and Lane 4: *E. tarda*). (B) Western blot for MAb 4E9 (Lane M: Molecular weight marker; Lane 1:*V. cholerae*; Lane 2: *S. enterica*; Lane 3: *P. monodon* muscle; Lane 4: *P. monodon* gill; Lane 5: *P. pangasius* muscle; Lane 6: *P. pangasius* gill; Lane 7: chicken blood; Lane 8: Human blood; and Lane 9: rGAPDH).

Table 3. Summary of western blot for MAbs against various samples.

Sample	Monoclonal antibody			
	3G12	4E9	5A11	9G1
<i>E. tarda</i>	+	+	+	+
rGAPDH	+	+	+	+
<i>A. hydrophila</i>	+	–		+
<i>M. luteus</i>	+	–	–	+
<i>S. aureus</i>	–	–	–	–
<i>V. cholerae</i>	+	+	NT	NT
<i>S. enterica</i>	+	+	NT	NT
<i>P. monodon</i>	+	+	NT	NT
<i>P. pangasius</i>	–	–	NT	NT
Chicken blood	NT	–	NT	NT
Human blood	NT	–	NT	NT

Notes: +, positive; –, negative; NT, not tested.

specific diagnosis of a variety of fish bacterial pathogens, viz., *A. hydrophila*, *A. salmonicida*, *Piscirickettsia salmonis*, *Photobacterium damsela* ssp. *Piscicida* and *Vibrio alginolyticus* (Sithigorngul et al., 2006). MAbs to *E. ictaluri* and *E. tarda* have been used extensively for diagnosis and characterization of OMPs (Kumar et al., 2010). However the present study is probably the first report of MAbs produced against GAPDH of *E. tarda*.

All the four stable hybridomas (3G12, 4E9, 5A11 and 9G1) secreting MAbs against rGAPDH reacted with whole cell bacteria also confirm that the GAPDH is also found on the outer membrane of *E. tarda*. All the four MAbs reacted with the denatured protein in western blot and hence they are all directed against linear epitopes of GAPDH.

The MAbs generated against the rGAPDH cross reacted with GAPDH of a wide range of bacterial species and shrimp tissues. Although MAb 5A11 did not react with *A. hydrophila*, *M. luteus* and *S. aureus*, it was not tested with other bacteria and animal tissues. From the western blot experiment, it can be construed that the level of conservation is high among evolutionarily close species. Vertebrates being evolutionarily far from prokaryotes did not react with any of the MAbs. However GAPDH of *S. aureus* too did not react with any of the MAbs probably because *S. aureus* has been shown to be well separated from other bacterial species and there is a wide evolutionary gap between *S. aureus* and other bacterial species (Jacklyn et al., 2009). Further, the percentage similarity of *S. aureus* GAPDH gene sequence with that of *E. tarda* is low (56%) compared to other species. Hence the MAbs generated may not be useful to identify *E. tarda*, but it may prove to be a useful tool to study one of the most conserved and functionally diverse protein, the GAPDH.

Conclusion

The present study has yielded rGAPDH and four hybridoma clones secreting MAbs to GAPDH of *E. tarda*. The recombinant protein has the potential to be used as a vaccine in fish species as this protein is reported to be highly immunogenic and protective in nature. The MAbs cross reacted with many other bacterial species and shrimp tissues but did not react with evolutionarily distant vertebrates. The MAbs will be a useful tool to study GAPDH, one of the most conserved and functionally diverse protein.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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