

Molecular Characterization of Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) Gene of Field Isolate of *Edwardsiella tarda*

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Abstract

The *E. tarda* bacterial culture isolated from infected fish, was confirmed by morphological and biochemical characterization. GAPDH gene of 996 bp was amplified from bacterial genomic DNA. GAPDH gene was cloned in pTZ57R/T cloning vector. The positive clone was sequenced. The sequencing result showed very homology with published sequence from pathogenic *E. tarda*. The sequence (nucleotide and amino acid) divergence values were very low between *E. tarda* isolates from India (KF142190) and China (HQ697337-38) (Figure 4 and Figure 5). However, the divergence value was high when compared with *E. tarda* isolates from Japan (AB198939) and this value was higher than the inter-specific divergence value (*E. tarda*-*E. ictaluri*, *E. tarda*-*E. coli* & *E. tarda*-*Klebsiella* species).

Keywords

E. tarda, GAPDH Gene, Vaccine, Cloning

1. Introduction

Aquaculture is the fastest growing sector of agriculture in most part of the world. However, aquaculture growth has been hampered by economic loss due to fish diseases [1]. Ten percent of cultured aquatic animals are lost because of various infectious diseases [2]. Therefore, controlling disease problems in aquaculture industry are a major concern.

Out of various infectious bacterial diseases, Edwardsiellosis caused by *Edwardsiella tarda* (*E. tarda*) is a

dreadful problem. Lack of effective means of control and prevention, especially the lack of effective vaccines, causes heavy economic losses in aquaculture industries worldwide due to *E. tarda* infection. *E. tarda* is a member of family *Enterobacteriaceae*. It is a Gram-negative, motile and rod shaped bacterium which can affect warm and coldwater species of fish. The bacterium is a pathogen with a wide range of hosts, such as fishes, reptiles, birds and mammals including human. *E. tarda* is a problem in eel ponds, tilapia culture and channel catfish culture systems. It causes rapidly progressive necrotizing soft tissue infection in humans. Geographic range of *E. tarda* is worldwide and it is a versatile pathogen with a wide ecological niche.

The DNA based vaccination or genetic immunization is a very promising research field since long years ago. DNA vaccines represent a major advance in fight against infectious diseases. DNA vaccines have several practical and immunological advantages than traditional antigen vaccines. Many virulence genes of *E. tarda* and genes encoding antigenic proteins have been explored for development of a vaccine and immunodiagnosics.

GAPDH protein of *Edwardsiella* is reported to be highly antigenic, and is considered to be a strong vaccine candidate to counter both Gram-negative and Gram-positive bacterial infections [3] [4]. The aim of the present study was to clone and sequence the GAPDH gene of *E. tarda* bacteria isolated from infected fish, and to explore the potential of it as a vaccine candidate against Edwardsiellosis in future.

2. Materials and Methods

2.1. Morphological and Biochemical Characterization of Bacteria

The bacterium *E. tarda* used in the study was obtained from a field isolate, collected from central India. The bacterium was inoculated in Brain Heart Infusion Broth (BHI broth) (Himedia, India). The broth was incubated for 18 - 24 h at 37°C. One loop of bacteria from the incubated broth was streaked on Nutrient Agar and Salmonella Shigella Agar (SS agar) media.

Based on the principle of pH change and substrate utilisation biochemical tests were carried out. For biochemical analysis of bacteria Hi 25 *Enterobacteriaceae* identification kit (Himedia, India) was used. Briefly, a single isolated colony of *E. tarda* was picked and inoculated in 5 mL of BHI broth and incubated at 37°C for 4 - 6 hours. Kit was opened aseptically and each well was inoculated with 50 µL of inoculum by surface inoculation method. The kit was incubated at 37°C for 18 h. Results were interpreted using the chart supplied along with kit.

2.2. DNA Isolation from *E. tarda*

The genomic DNA from *E. tarda* was isolated by using GenElute bacterial genomic DNA kit (Sigma USA), according to manufacturer's protocol.

2.3. PCR Amplification of GAPDH Gene of *E. tarda*

Specific primer set containing forward primer "CTC GAG ATG ACT ATC AAA GTA GGT ATC A" and reverse primer ACG CGT TTA CTT AGA GAT GTG TGC GA was used to amplify the gene GAPDH. PCR was performed using 25 µL reaction volume, each containing 2 µL (100 µg) of template DNA, 2.5 µL of 10× buffer, 2 µL of 25 mM MgCl₂, 0.5 µL of 10 mM dNTP mix, 0.25 µL of *Taq* DNA polymerase (5U), 0.5 µL each of forward and reverse primers (25 pmol) and rest nuclease-free water. Amplification was carried out at the following cyclic conditions in the thermocycler (Quanta Biotech, England) 95°C for 5 min, 30 cycles of 95°C for 1 min, 56°C for 1 min and 72°C for 1 min, then a single step of final extension at 72°C for 10 min. Size of the amplified product was then determined on 1.2% agarose gel electrophoresis.

The 996 bp DNA fragment of GAPDH was eluted from gel using Fermentas GeneJET Gel Extraction Kit. The PCR product was eluted in 25 µL elution buffer. Concentration of elute was measured using Nano Drop (Thermo Scientific, USA).

2.4. Cloning of GAPDH Gene in pTZ57R/T Cloning Vector

InsTA cloning vector pTZ57R/T was supplied by Thermo Scientific, India. The host strain DH5α of *E. coli* was available in the Aquatic Animal Health Management Laboratory, CIFE.

Four µL of the gene eluted PCR product and 3 µL of the pTZ57R/T vector were ligated in 30 µL ligation reaction, with 5× ligation buffer, T₄ DNA ligase and nuclease-free water. This mixture was incubated at room temperature for 1 h.

2.5. Preparation of Competent *E. coli* Cells and Transformation

One loop from fresh colony of DH5 α cells was inoculated in 2 mL of C medium which was supplied with Transform Aid bacterial transformation kit (Fermentas, India) and incubated at 37°C overnight. One fifty μ L of overnight bacterial culture was added to 1.5 mL of prewarmed C medium and incubated for 20 min at 37°C in a shaker. Pelleted cells were resuspended in 300 μ L of T-solution and incubated for 5 min and centrifuged. The pellet was resuspended in 120 μ L of T-solution and to this 5 μ L of ligation mixture was added. This mix was incubated on ice for 5 min. The transformed cells were spread immediately on LB agar plates containing ampicillin (100 μ g/mL), IPTG (10 mM) and X GAL (2% W/V), under sterile conditions. Appropriate positive and negative controls were processed simultaneously. Plate was incubated at 37°C for 18 h.

2.6. Screening of Recombinant InsT/A Clones

Screening of the colonies was done by sub culturing white colonies in LB broth containing Ampicillin (100 μ g/mL) and incubated at 37°C overnight in a shaker incubator. The plasmid DNA was extracted by GeneJET Plasmid Miniprep Kit (Fermentas, USA). For plasmid extraction single colony from freshly streaked selective plate was picked and inoculated in 3 mL LB-ampicillin broth and incubated at 37°C in shaking incubator for 16 h. Bacterial cells were harvested by centrifugation at 8000 \times for 2 min. Pelleted cells were resuspended in 250 μ L of re-suspension solution lysed by adding 250 μ L of lysis solution. Then 350 μ L of neutralisation solution was added. The supernatant was transferred to the supplied GeneJET spin column. Plasmid was eluted in 25 μ L of nuclease free water, after washing with washing buffer.

2.7. Restriction Enzyme Analysis of Recombinant Clones

To confirm the cloning of GAPDH gene in T/A vector double enzyme digestion reaction was carried out. Plasmid DNA (1 μ L), 10 \times FD buffer (2 μ L), *Xho* I (1 μ L), *Mlu* I (1 μ L) and nuclease-free water (15 μ L) were mixed thoroughly to make a 20 μ L reaction volume. The mixture was incubated at 37°C for 5 min and 80°C for 10 min. The digested samples were analysed on 1.2% agarose gel in 0.5 \times TAE buffer.

2.8. Sequence Analysis of GAPDH Gene

The pTZ/GPD plasmid, 15 μ L was sent for sequencing to Bioinnovations, India. The nucleotide sequence was screened using VecScreen programme on the internet (<http://www.ncbi.nlm.nih.gov/VecScreen/>). The sequence was designated as E tardaGAPDH-MP-INDIA. This sequence was aligned with few available GAPDH sequences from E tarda and other bacteria (Table 1). To find homologous sequence single nucleotide-nucleotide alignment was done using the programme Basic Local Alignment Search Tool (BLAST) on internet using web site <http://www.ncbi.nlm.nih.gov/blast>. Nucleotide and amino acid based Neighbour-Joining (NJ) trees were constructed by implying Kimura 2 Parameter and Jones-Taylor-Thornton (JTT) substitution model, respectively using MEGA software v5.0 [5].

3. Result & Discussion

In the present study, GAPDH gene of an *Edwardsiella tarda* isolated from infected fish collected from central India was cloned and sequenced.

3.1. Characterization of *E. tarda*

The *E. tarda* was isolated from infected fish, from central India. Colonies of bacteria were identified as *E. tarda* on the basis of colony morphology, staining characteristics and biochemical properties. The colonies appeared transparent with black centre, after overnight incubation at 37°C in SS agar medium. On staining with Gram's stain it appeared Gram-negative small rods to coccobacilli. The length was about 2 - 4.5 times more than the width and was non-sporing and non-capsulated bacteria. Biochemical tests showed positive result, especially Indole, Methyl Red and H₂S production which are the clear distinguishing factors of *E. tarda* from *E. ictaluri* [6]. Also the bacteria showed positive catalase reaction with H₂O₂ (Figure 1(a) and Figure 1(b)).

3.2. Cloning and Sequencing of GAPDH Gene of *E. tarda*

After bacterial genomic DNA extraction, PCR amplification of GAPDH gene was achieved at annealing tem-

perature 56°C. A 996 base pair PCR-amplified GAPDH gene was obtained. The size of the gene was similar to the earlier reports [3].

Successful ligation of PCR amplified GAPDH gene was achieved at 22°C incubation. On transformation, in DH5 α cells, white and blue colonies on LB-ampicillin plates containing X-GAL and IPTG were produced. Plasmid from the white colonies were confirmed as positive clones by release of GAPDH fragment from the plasmid by double digestion with *Mlu* I and *Xho* I enzymes (Figure 2).

3.3. Sequence Analysis of GAPDH Gene

The nucleotide sequences obtained from GAPDH, is submitted to NCBI, Gene Bank (accession no. KF142190). The sequence showed a complete functional open reading frame of 996 bp containing initiation codon of ATG and termination codon TAA (Figure 3).

Table 1. List of GAPDH sequences from bacterial origin used for sequence similarity analysis.

Name of the culture	Bacteria name	Accession No.	Country
<i>E. tarda</i> -GAPDH-FJ605131.1	<i>Edwardsiella tarda</i>	F605131.1	China
<i>E. coli</i> -GAPDH-X02662	<i>Escherichia coli</i>	X02662	Pennsylvania
<i>E. ictaluri</i> -HQ697334	<i>Edwardsiella ictaluri</i>	HQ697334	China
<i>E. tarda</i> -GAP-AB198939	<i>Edwardsiella tarda</i>	AB198939	Japan
<i>E. tarda-ga</i> -HQ697338	<i>Edwardsiella tarda</i>	HQ697338	China
<i>E. tarda</i> -GapA-HQ697337	<i>Edwardsiella tarda</i>	HQ697337	Shanghai
<i>E. tarda</i> -GAPDH-MP-INDIA	<i>Edwardsiella tarda</i>	KF142190	India
<i>Klebsiella</i> -GAPDH-AJ875018	<i>Klebsiella pneumoniae</i>	AJ875018	France



Figure 1. (a) *Edwardsiella tarda* growth on Salmonella Shigella Agar, after 18 h growth at 37°C; (b) *Edwardsiella tarda* culture showing catalase positive reaction.

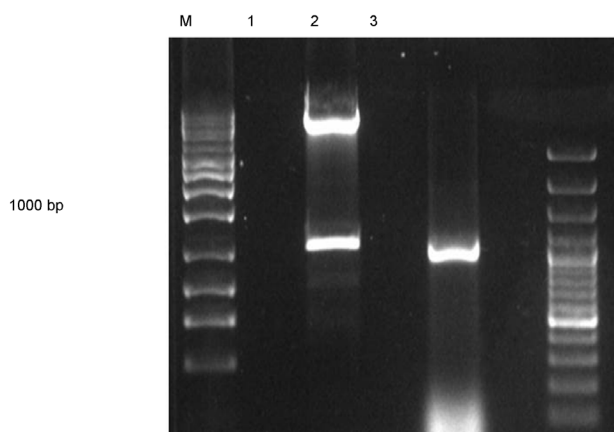


Figure 2. Agarose gel electrophoresis (1%) showing release of 996 bp GAPDH fragment from pTZ vector after restriction digestion with *Mlu* I and *Xho* I enzymes. Lane 1: release of 996 bp GAPDH fragment from pTZ vector, confirming the cloning of the gene in vector; Lane 2: PCR amplified 996 bp GAPDH gene fragment; Lane M: 1 Kb plus marker; and Lane 3: 100 bp DNA Molecular weight marker.

The sequence (nucleotide and amino acid) divergence values were very low between *E. tarda* isolates from India (KF142190) and China (HQ697337-38) (Figure 4 and Figure 5). However, the divergence value was high when compared with *E. tarda* isolates from Japan (AB198939) and this value was more than the Inter-specific divergence value (*E. tarda-E. ictaluri*, *E. tarda-E. coli* & *E. tarda-Klebsiella* species). This could be due to accumulation of more synonymous mutations in *E. tarda* isolates from Japan. As per the NJ tree of GAPDH sequences, Indian isolate of the *E. tarda* showed close clustering with other Asian countries isolates (Japan and China) (Figure 6 and Figure 7). Although GAPDH sequence of *E. ictaluri* clustered with *E. tarda*, this clustering does not have strong bootstrap value.

E. tarda is one of the most important pathogen in the context of dreadful disease in aquaculture. Because of the incapability of antibiotics and other subunit vaccines, researchers are focussing towards DNA vaccine candidates. A 37 kDa outer membrane protein was detected in several *E. tarda* serotype strains and was designated as an effective vaccine candidate against experimental *E. tarda* infection in Japanese flounder [3]. A recombinant GAPDH of *E. tarda* was reported to be an effective vaccine antigen against *E. tarda* infection in Japanese flounder [7]. The gene GAPDH is conserved in a series of bacteria including Gram-positive and Gram-negative.

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ATGACTATCAAAGTAGGTATCAACGGTTTTGGCCGTATCGGCCGTATTGTTTTCCGTGCTGCTCA
GGAACGTTGTGACATCGAAATCGTTGGCATCAACGATCTGCTGGATGCCAACTACATGGCATAAC
ATGCTGAAGTACGACTCTACTACGGTCTTTCAACGGCACTGTTGAAGTGAAAGATGGCCAC
CTGATCGTTAACGGTAAAAAATCCGTGTTACCGCTGAAAGAGATCCGGCTAACCTGAAAGTGG
GACGAAATCGGTGTGACGTAGTTGCCGAAGCAACGGTCTGTTCTGACCGACGAAACCGC
ACGTAAGCACATCGCTGCCGGCCGAAGAAAGTCGTCATGACTGGCCCGTCCAAAGATGCTAC
CCCGATGTTGTTATGGCGTAAACCAAGAAGTACGCTGGCCAGGAGATCGTTTCTAACGC
TTCTGCACCACTGCTGGCACCCTGGCTAAAGTCTGAAACGCAACTTCGGCATCGTT
GAAGCACTGATGACCACCGTTCACGCTACCACCGCTACCCAGAAAACCGTTGATGGCCCGTCC
ATGAAAGATTGGCGCGCGCCGTGGCGCTAGCCAGAACATCATCCCGTCTCTACCGGTGCT
GCCAAGGCTGTTGGCAAGGTTATCCCGGAACTGAACGGCAAACCTGACCGGTATGGCTTTCCG
CGTTCCGACCCCGAACGTTTCCGTTGTTGACCTGACTGCACGCTGGCCAAAGCCGGCTACCTAT
CAGCAGATCTGTGACGTGATGAAGGCCGCTTCTGAAGGCGAAATGAAAGGCGTTCTGGGCTA
CACCGACGAAGCCGTCGTTTCTACCGACTCAACGGCGAAGTTGACCTCTGTGTTGATGCC
GACGCCGTATCTCCTGAACGCAACTTCGTGAAACTGGTTTCTGGTATGACAAAGAACT
GGTACTCCAACAAGTTCTGGATCTGATCGCGCACATCTCCAATAA
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Figure 3. Nucleotide sequence of 996 bp GAPDH gene of *Edwardsiella tarda* isolate collected from infected fish from central India, submitted to NCBI GENBANK (Accession No. KF142190).

Species	1	2	3	4	5	6	7
1 <i>Edwardsiella tarda</i> _KF142190		0.019	0.002	0.028	0.000	0.010	0.019
2 <i>Escherichia coli</i> _X02662	0.167		0.019	0.024	0.019	0.020	0.012
3 <i>Edwardsiella ictaluri</i> _HQ697334	0.002	0.170		0.028	0.002	0.010	0.019
4 <i>Edwardsiella tarda</i> _AB198939	0.304	0.251	0.306		0.028	0.030	0.025
5 <i>Edwardsiella tarda</i> _HQ697338	0.000	0.167	0.002	0.304		0.010	0.019
6 <i>Edwardsiella tarda</i> _HQ679337	0.049	0.188	0.051	0.338	0.049		0.020
7 <i>Klebsiella sps</i> _AJ875018	0.169	0.078	0.172	0.262	0.169	0.189	

Figure 4. Percentage nucleotide similarity and divergence based on the 996 bp nucleotide sequences of GAPDH gene from different *Edwardsiella tarda* isolates. Sequence in the box is the one which is analysed. The analysis is done by Clustal W algorithm. Table showing sequence distance on the basis of pairwise sequence homology.

Species	1	2	3	4	5	6	7
1 <i>Edwardsiella tarda</i> _KF142190 (India)		0.000	0.028	0.004	0.022	0.025	0.005
2 <i>Edwardsiella tarda</i> _HQ697338 (China)	0.000		0.028	0.004	0.022	0.025	0.005
3 <i>Edwardsiella tarda</i> _AB198939 (Japan)	0.172	0.172		0.029	0.031	0.033	0.029
4 <i>Edwardsiella tarda</i> _HQ679337 (Shanghai)	0.005	0.005	0.178		0.022	0.025	0.007
5 <i>Escherichia coli</i> _X02662 (Pennsylvania)	0.092	0.092	0.153	0.097		0.012	0.023
6 <i>Klebsiella sps</i> _AJ875018	0.110	0.110	0.165	0.116	0.036		0.027
7 <i>Edwardsiella ictaluri</i> _HQ697334 (China)	0.005	0.005	0.178	0.010	0.097	0.116	

Figure 5. Percentage nucleotide similarity and divergence based on the deduced amino acid sequences of GAPDH gene from different *Edwardsiella tarda* isolates. Table showing sequence distance on the basis of pairwise sequence homology of aminoacid sequence.

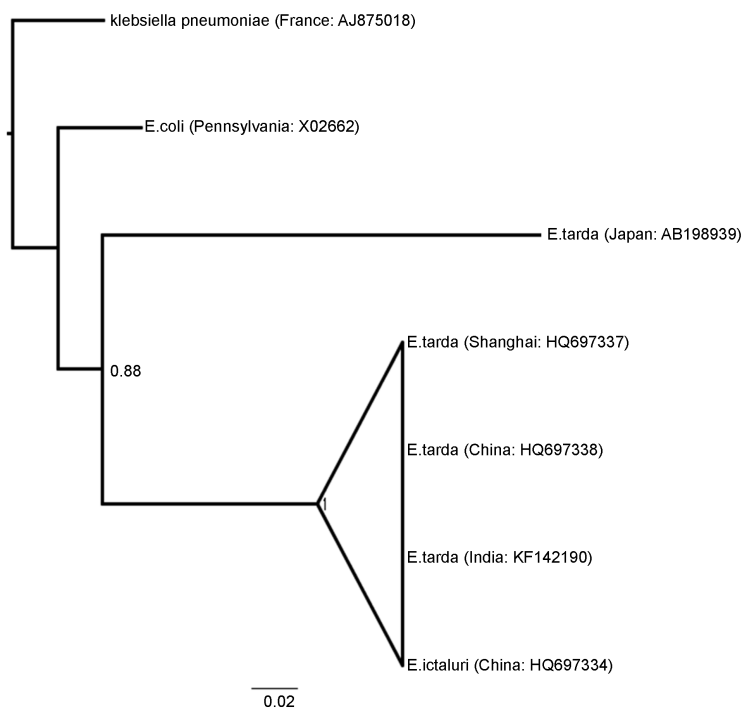


Figure 6. Neighbour-Joining tree (cladogram) based on deduced 996 bp nucleotide similarity of GAPDH gene from different *Edwardsiella tarda* isolates showing close cluster of *E. tarda* sequences with *E. ictaluri*.

So the vaccine construct is believed to work against a number of bacteria. In our study, as per the secondary structure prediction, GAPDH sequence showed high antigenic index and surface probability, which make it an ideal candidate for generating immunogenicity. This result is in agreement to the experimental work of Kawai *et al.* [3].

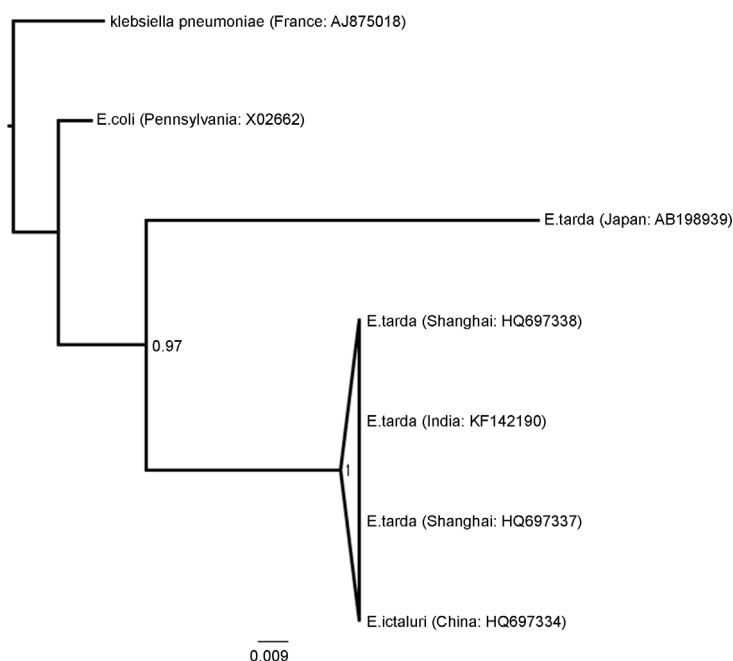


Figure 7. Neighbour-Joining tree (cladogram) based on deduced amino acid sequence similarity of GAPDH gene of different *Edwardsiella tarda* isolates showing close cluster of *E. tarda* aminoacid sequences with *E. ictaluri*.

4. Conclusion

Objective of our study was to characterize the GAPDH gene of an Indian isolate of *E. tarda*. The study confirmed that the bacterial GAPDH gene was highly conserved and could be exploited as the potential candidate as vaccine gene against *E. tarda* and other Gram-negative bacteria.

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