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Isolation and molecular characterization of *oligopeptidase B* gene of *Trypanosoma evansi* from Indian dromedaries

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ABSTRACT

The aim of the study was isolation and molecular characterization of *oligopeptidase B* (*opdB*) gene of *Trypanosoma evansi* of camel. Genomic DNA of *T. evansi* was used to amplify the *opdB* gene by polymerase chain reaction then amplicon was cloned in a suitable plasmid vector and finally custom sequenced. The desired amplicon of *opdB* gene of *T. evansi* was amplified by PCR using gene specific primers. The amplicon of expected size was purified and then ligated to the pGEM- T Easy vector and ligated mixture was transformed into *Escherichia coli* JM109 strains for cloning. Screening of recombinants was done by restriction enzyme digestion of plasmid DNA. After confirmation of clone of *opdB* genes the plasmid DNA was sequenced and coding sequences of *opdB* gene according to the result obtained was of 2092 bp. Tree topology of *opdB* gene is based on the Neighbor-Joining method and and maximum parsimony method with 100% bootstrap values and identified *opdB* gene sequence showed a close homology with other *Trypanosoma* and *Leishmania spp*. gene sequences.

Key words: Indian dromedaries, Molecular characterization, *Oligopeptidase B* gene, *Trypanosoma evansi*

Trypanosoma evansi, cause of typanosomosis or surra, is extensively distributed geographically resulting in great economic loss. Horses and camels are mainly affected, followed by cattle, buffaloes, dogs, goats, sheep and pigs (Gill 1991), and outbreaks frequently occur during and after rainy season though sporadic cases are met with throughout the year (Pathak and Khanna 1995). Efforts towards the development of a vaccine against *Trypanosoma evansi* revealed several promising candidate vaccine antigens, including non-variant proteins of this parasite. Proteases, a ubiquitous group of enzymes, play key roles in the life cycle of parasites (McKerrow *et al.* 2006). Proteases are important for parasite survival; they are involved in the digestion of exogenous proteins for nutritive purposes (Rosenthal 1999), invasion of host cells and tissues (Roggwiller *et al.* 1996), and modification of host proteins (Caler *et al*. 1998). OpdB, a protease, being implicated as an important virulence factor in trypanosomosis (Burleigh and Woolsey 2002) has become the therapeutic target. Characterization of OpdB in kinetoplastid protozoan parasites should lead to the development of chemotherapeutic drugs for diseases that affect millions of people and livestock world-wide (Coetzer *et al*. 2008). In *T. evansi* infections OpdB has a major role to play in the manifestation of disease. It is shown to proteolytically cleave many of the host derived peptides and proteins like kinogen, atrial Natriuretic factor in the bloodstream of infected host etc. (Morty *et al*. 2005). Inability to inhibit this cysteine peptidase by host derived protease inhibitors makes OpdB important during pathogenesis, thus making it an attractive drug target and basis for vaccine development. The identification and research into new drug targets for trypanosomosis is necessary, and the development of novel, more effective, and less toxic drugs is an urgent priority. OpdB is emerging as an important virulence factor and therapeutic target in *Trypanosoma evansi* infection. Keeping in views of all these points, the present study was undertaken to characterize the *opdB* gene of *Trypanosoma evansi* of camel from Indian sub-continent at molecular level.

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MATERIALS AND METHODS

Preparation of trypanosome strains, DNA isolation and PCR amplification: After confirmation of *T. evansi* isolates by blood smear examination, blood from infected camel was inoculated intraperitoneally into Swiss albino mice (maintained at Small Animal Laboratory, NRC on Camel,

Bikaner) for propagation of trypanosomes. Mice with high parasitaemia were euthanized with chloroform and blood was collected into 5 ml disposable syringe containing 0.1 ml heparin solution by heart puncture. DEAE (diethyl amino ethyl) cellulose column chromatography method was used for purification of trypanosomes (Lanham and Godfrey 1970). The collected trypanosomes were pelleted by centrifugation at 1,000 rpm for 10 min and kept at –20°C for further processing. Genomic DNA isolation from collected pellet of *T. evansi* was done by illustra blood genomic prep mini spin kit using the manufacturer's protocol. The *opdB* gene of *T. evansi* was amplified from genomic DNA by the use of already designed forward 5'GGACACATATGATGC AAACTGAACGTGGTCC3' and reverse 5'TACGCTCATA TGCTACTTCCGCAGCAGCGGCC3' primer sequences by Morty *et al*. (2005). PCR amplification of the *opdB* gene was performed by cycling conditions as initial denaturation at 94°C for 4 min, 35 cycles of denaturation at 94°C for 30 sec, annealing at 57°C for 60 sec, extension at 72°C for 1 min and 30 sec, and final extension for 9 min at 72°C. The PCR amplified products were checked with 1.5 kb DNA molecular weight marker in 1.2% agorose gel.

Cloning and sequencing of opdB *gene:* The PCR products from low melting point agarose slices were purified by a commercial kit using the manufacturer's protocol. The DNA fragment of *opdB* gene and the pGEM- T Easy vector in which it is to be cloned were digested with T4 DNA ligase enzyme to generate compatible ends for ligation. The ligation was done (as per the Promega protocol with slight modification) in the reaction volume of 20 ml containing 10 ml of 2X Rapid ligation T4 DNA Ligase buffer [400mM Tris-HCl, 100mM $MgCl_2$, 100mM DTT, 5mM ATP (pH 7.8 at 25°C)], 6 ml PCR product, 2 ml pGEM- T Easy vector and 2 ml of T4 DNA ligase. The contents were vortexed, spun down in a micro centrifuge for 3–5 sec and incubated for overnight at 4°C. The ligation mix was then used directly for transformation in JM109 competent cells. After incubation of transformation culture 100 ml of transformation culture was plated onto antibiotic agar plates and incubated at 37°C for overnight (16–20 h). Colonies harbouring the recombinant plasmid were inoculated into LB broth and incubated at 37°C overnight with horizontal shaking. The plasmids DNA were extracted from culture using illustra plasmid prep mini spin kit. The positive clones were identified by restriction enzyme digestion of plasmid DNAs with *Eco*R1 and Colony PCR of plasmid colonies. Purified plasmid of *opdB* gene was sequenced in both directions at a commercial firm.

Sequence analysis: After confirmation of the *opdB* gene nucleotide sequence of *T. evansi* isolated from the host camel (*Camelus dromedarius*), the nucleotide sequence was submitted to GenBank, NCBI database. After getting the accession number of gene sequence Phylogenetic and sequence analysis of *opdB* gene of *T.evansi* was done. The phylogenetic and sequence analysis was done by use of Clustal X and MEGA5 softwares. Phylogenetic tree analysis of *opdB* gene was done by using Neighbor-Joining (NJ) method and maximum parsimony (MP) method and implemented with bootstrap test involving simple stepwise addition.

Building three dimensional structure model of opdB *protein using computational approach:* The 3D model of opdB protein was built by homology modeling based on high-resolution crystal structures of homologous proteins. A basic alignment search tool (BLAST, Altschul *et al*. 1990) search was performed for selecting the 3D models of the closest homologues available in the Brookhaven Protein Data Bank (PDB). The 2XE4 showed a high level of sequence identity with opdB. The coordinates of crystal structure of 2XE4 was used as template to build the initial model of opdB and the 3D model was generated by the automated homology modeling software MODELLER9v6 (http://salilab.org) on windows operating environment (Sali and Blundell 1993).

The protein model of opdB generated by comparative modeling and energy minimization was checked for stereochemical quality, regions of unusual geometry and overall structure assessment via the tool PROCHECKv3.4.4. The Ramachandran Plots generated was studied for the same and the G-factors was observed. RMSD (C-g) of the modeled protein, with respect to the best template (2XE4) was measured by pair wise structure alignment using the Combinatorial Extension algorithm (CE), available at the San Diego Supercomputer Center (http://cl.sdsc.edu).

RESULTS AND DISCUSSION

PCR- amplification of *opdB* gene of *T. evansi* revealed DNA fragment of *opdB* gene was 2092 bp (Fig. 1). There were several white colonies along with a few blue colonies were found after cloning of amplified PCR product. Two well separated DNA bands were seen in case of plasmid isolated from positive colonies upon digestion with *Eco*RI

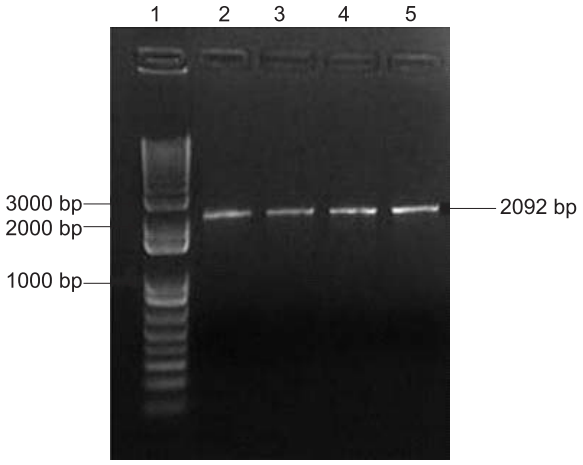

Fig.1. Amplification of *oligopeptidase B* gene of *T. evansi* by PCR.1. 1Kb plus DNA Ladder; 2 – 5. Amplicons.

(Fig. 2). Release of DNA fragments of around 2092 bp for *pfr1 opdB* was found after restriction enzyme digestion. Colony PCR was done for quick screening of plasmid inserts directly from *E. coli* colonies and amplification was found in wells of white colonies and also in one well of blue colony (Fig. 3).

After confirmation of *opdB* gene nucleotide sequence of *T. evansi* isolated from the host camel, the sequence was submitted to GenBank, NCBI database to which the assigned accession number is JQ909240. For phylogenetic analysis of *opdB* gene sequences of other *Trypanosomatidae* species already available in the genbank database were retrieved (Table 1). Tree topology based on the Neighbor-Joining method showed a close homology with other

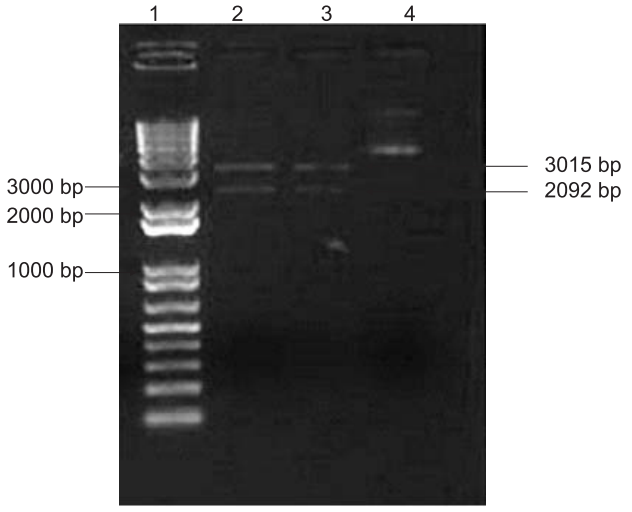

Fig. 2. *oligopeptidase B* gene fragments of *T. evansi* after restriction digestion of *opdB* gene plasmid.

1Kb plus DNA ladder; 2–3. *oligopeptidase B* gene clone; 4. uncut plasmid.

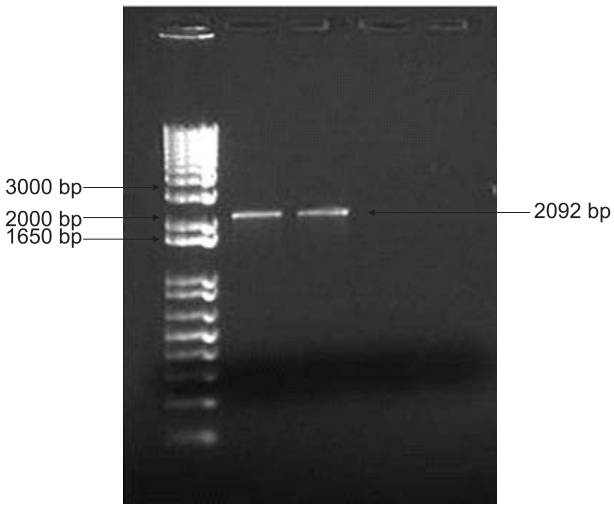

Fig. 3. Amplification of *oligopeptidase B* gene of *T. evansi* by colony-PCR. 1. 1Kb plus DNA ladder. 2–3. PCR reaction with white colony shows amplification. 4–5. PCR reaction with blue colony shows absence of amplification.

Table 1. Percent homology of *oligopeptidase B* gene sequence of *T. evansi* and other allied species

Identity of *opdB* genes and their accession numbers	*T.evansi* [India] JQ 909240	*T.brucei* [USA] AF 078916	*T.evansi* [Germany] AY 546084	*T.brucei* [UK] XM 824253	*T.cruzi* [USA] XM 804874	*T.cruzi* [USA] U69897	*L.amazonensis* [Brazil] EF 392367	*L.donovani* [USA] GQ 491028	*L.infantum* [UK] XM0014 63502	*L.major* [USA] AF 109875
T.evansi [India] JQ909240	**	99.6	100	99.0	75.7	68.4	77.6	76.2	75.9	76.2
T.brucei [USA] AF078916	99.6	**	99.6	99.2	75.8	75.1	77.9	82.7	76.2	76.4
T.evansi [Germany] AY546084	100	99.6	**	99.0	75.6	75.0	77.6	76.2	75.9	76.2
T.brucei [UK] XM824253	99.0	99.2	99.0	**	75.7	75.1	77.9	76.4	76.2	76.4
T.cruzi [USA] XM804874	75.7	75.8	75.6	75.7	**	98.3	72.2	72.2	72.1	75.0
T.cruzi [USA] U69897	68.4	75.1	75.0	75.1	98.3	**	74.6	74.5	74.7	73.6
L.amazonensis [Brazil] EF392367	77.6	77.9	77.6	77.9	72.2	74.6	**	93.9	93.8	92.9
L.donovani [USA] GQ491028	76.2	82.7	76.2	76.4	72.2	74.5	93.9	**	99.8	96.3
L.infantum [UK] XM001463502	75.9	76.2	75.9	76.2	72.1	74.7	93.8	99.8	**	96.3
L.major [USA] AF109875	76.2	76.4	76.2	76.4	75.0	73.6	92.9	96.3	96.3	**

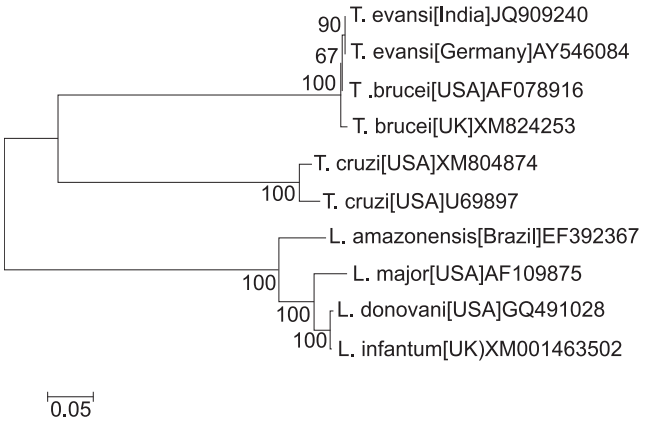

Fig. 4. Phylogenetic tree analysis of *oligopeptidase B* gene using the Neighbor-Joining method

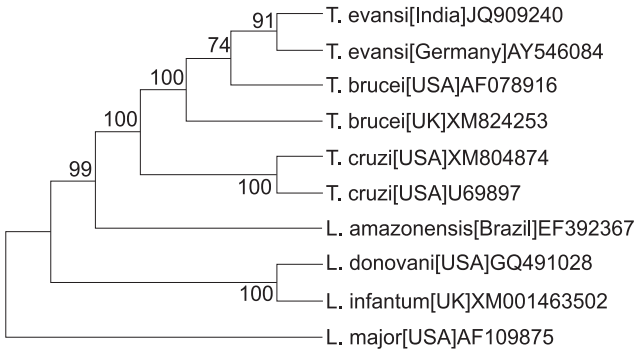

Fig. 5. Phylogenetic tree analysis of Oligopeptidase B gene using maximum parsimony method

Trypanosomatidae species sequences with100% bootstrap values (Fig. 4). The NJ, bootstrap consensus tree inferred from 1,000 replicates is taken to represent the evolutionary history of the taxa analyzed. Phylogenetic tree analysis of *opdB* gene using maximum parsimony (Fig. 5) also showed same topology as NJ method. The percentage of replicate tree in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches.

The final stable structure of *opdB* protein have 9 sheets, 4 beta alphabeta unit, 23 beta hairpins, 11 beta bulges, 39 strands, 22 helices, 20 helix-helix interfaces, 72 beta turns and 6 gamma turns. (Figs 6, 7).

In the present study, the Obtained *opdB* gene sequence showed 99.6% homology towards *T. brucei* (AF078916) and 99.0% similarities with *T.brucei* (XM824253). A 100% homology was found between obtained *opdB* sequence and *T. evansi* (AY546084). Lower homology was documented between the obtained *opdB* gene sequence and *T. cruzi* (XM804874) and *T. cruzi* (U69897). The comparison with *Leishmania amazonensis* (EF392367), *Leishmania donovani* (GQ491028), *Leishmania infantum* (XM001463502) and *Leishmania major* (AF109875) a member belonging to

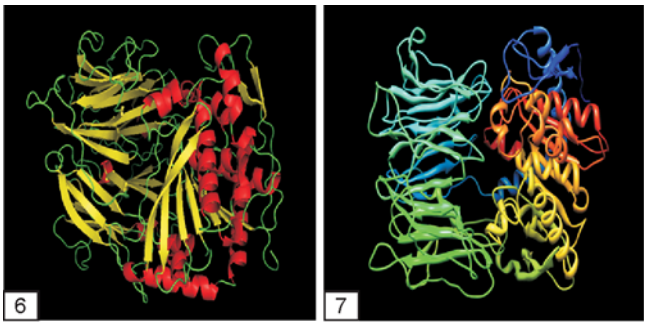

Fig. 6. A putative 3D model structure of *oligopeptidase B* protein with ribbon representation.

Fig. 7. Cartoon representation of 3D model of *oligopeptidase B* protein.

Trypanosomatidae family showed only 77.6%, 76.2%, 75.9% and 76.2% homology, respectively.

According to neighbor-joining phylogenetic tree analysis of *opdB* gene, *Trypanosoma cruzi* (XM804874) and *T. cruzi* (U69897) in the phylogenetic tree are placed as two sub cluster of one mega cluster. *Leishmania major* (AF109875), *Leishmania amazonensis* (EF392367), *Leishmania donovani* (GQ491028) and *Leishmania infantum* (XM001463502) as 4 sub cluster of 1 mega cluster; the other mega cluster comprising of rest of the species. The earlier reports of *T. evansi* by Morty *et al.* (2005) also showed 99.6% homology with *T. brucei* (AF078916) and 99.0% homology with *T. brucei* (XM824253). 75.0% to 77.6% sequence similarities was observed between *T. evansi opdB* gene and other species documented in this study.

Sequence analysis of the gene is the most appropriate method for the confirmation of specificity of the target region of any gene. In the present finding identified *opdB* gene sequence showed a close homology with other Trypanosome sequences like *T. brucei* (XM824253 and XM824253)), *T. evansi* (AY546084) and *T. cruzi* (XM804874 and. U69897). It could therefore be suggested that vaccine with *opdB* protein of trypanosomatidae parasite as the antigen could be effective against not only different strains within one Trypanosome species but also against other species of the same genus. In this study experiments are attempted to characterize the *opdB* gene which is gene of prime importance in *T. evansi* from Indian dromedaries. This gene could be the ideal vaccine and drug target in its own right for the control of trypanosomosis in India.

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