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AMPLIFICATION, CLONING AND SEQUENCE ANALYSIS OF ALTERNATIVE OXIDASE GENE OF *Trypanosoma evansi* ISOLATED FROM INDIAN DROMEDARIES

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ABSTRACT

The present study was carried out to isolate the Alternative oxidase (*aox*) gene of *Trypanosoma evansi* by polymerase chain reaction, clone the amplicons in a suitable bacterial plasmid vector and characterisation of the gene through sequencing. The desired amplicon of *aox* gene of *T. evansi* was amplified by PCR using gene specific primers and identified on the basis of size of the gene. The amplicon of expected size was purified from the 1% low melting agarose gel. The DNA fragment of interest was then ligated to the pGEM- T Easy vector and ligated mixture was transformed into *Escherichia coli* JM109 strains for cloning. After cloning, screening of recombinants was done by Restriction Enzyme digestion of plasmid DNA and by colony PCR. After confirmation of clone, the plasmid DNA was sequenced and coding sequence of *aox* gene according to the results obtained was of 990 bp. Tree topology of *aox* gene is based on the Neighbor-Joining method with 100% bootstrap values and identified *aox* gene sequence showed a close homology with other *Trypanosoma* spp. gene sequences.

Key words: Alternative oxidase gene, camel, cloning, sequence analysis, *Trypanosoma evansi*

Alternative oxidase (*aox*) is a membrane bound di-iron mitochondrial protein encoded in the nucleus and translated as a precursor protein with a mitochondrial targeting sequence that is removed during import into the mitochondrion (Chaudhuri *et al,* 2005).

An *aox* gene has important role in general metabolism, cellular metabolism, virulence, oxidative stress, respiration, stress response, protection against oxidative stress, programmed cell death, maintenance of the cellular redox balance, oxidative defense mechanism and inhibition of the main respiratory chain (Atteia *et al,* 2004). Trypanosomes mitochondrial cyanide sensitive respiration possesses an alternative oxidase which is inhibited specifically by salicyl hydroxamic acid (SHAM). The mammalian respiratory chain does not contain alternative oxidase, which therefore represents a potential drug target against trypanosomes, which may rely on alternative oxidase activity to survive the environmental stresses during host infection.

An *aox* gene of *T. evansi* is a nonvarient gene and emerging as an important virulence factor and therapeutic target in *Trypanosoma evansi* infection. Due to stability against the host immune response, this gene may be explored to make possible immune prophylaxis to control the disease effectively and inexpensively. Alternative oxidase have been found in a wide variety of organisms but not in mammals so considered to be a promising drug target for the treatment of trypanosomiasis (Kido *et al,* 2010; Saimoto *et al,* 2012). Thus, the present study was undertaken to isolate Alternative oxidase gene of *Trypanosoma evansi* from camel by polymerase chain reaction, clone the amplicon and characterisation of *aox* gene through sequencing.

Materials and Methods

After confirmation of *T. evansi* infection by blood smear examination, blood from infected host was collected and inoculated intra-peritoneal into Swiss albino mice (maintained at Small Animal Laboratory, NRC on Camel, Bikaner). DNA isolation from collected pellet of *Trypanosoma evansi* was done as per the protocols given by ready to use kit from Illustra blood genomic prep. mini kit with slight modifications. The *aox* gene of *T. evansi* was amplified from genomic DNA using specific forward 5′AGGTAATATCTTGACACCAAGAGC3′ and

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reverse 5′ACGTGTTTGTTTACATTACTCGCA3′ primer sequences designed from published sequence (Accession No. AB188573). Cycling condition for PCR were initial denaturation at 94°C for 4 minute, 35 cycles of 30 seconds at 94°C, 60 second at annealing temperatures of 49°C and 60 second at 72°C, followed by a final extension for 10 minute at 72°C. The PCR amplified products were checked with 1.5 kb DNA molecular weight marker in 1.2% agarose gel.

Cloning and sequencing of aox gene

The PCR product from low melting point agarose slices were purified using Illustra GFX PCR DNA and Gel Band Purification Kit. The DNA fragments of *aox* gene and the pGEM- T Easy vector in which it was to be cloned were digested with T4 DNA ligase enzyme to generate compatible ends for ligation. The ligation was done in the reaction volume of 20 µl containing 10µl 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 µl PCR product, 2 µl pGEM- T Easy vector and 2 µl of T4 DNA ligase. The contents were mixed well by tapping and it was spun down in a micro centrifuge for 3-5 seconds and incubated for overnight at 4°C. The ligation mix was used directly for transformation in JM109 high efficiency competent cells. After incubation of 100 µl of each transformation culture were plated onto antibiotic agar plates in duplicate and incubated at 37°C for overnight (16-20 hr). Colonies harbouring recombinant plasmids 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 clone was confirmed by Restriction Enzyme digestion of plasmid DNAs with *EcoR*1 and colony PCR of plasmid colonies. Purified plasmid of *aox* gene was sequenced in both directions at Eurofins Genomics India Pvt Ltd., Whitefield, Bengaluru.

Sequence analysis

After getting the accession number of gene sequence Phylogenetic and sequence analysis of the *aox* gene of *T. evansi* was done. The phylogenetic and sequence analysis was done by use of Clustal X, MEGA5 and Praline softwares. Phylogenetic tree analysis of *aox* gene was done by using Neighbour-Joining (NJ) method and Maximum Parsimony (MP) method and implemented with bootstrap test involving simple stepwise addition.

Results and Discussion

The amplification band of *aox* gene was obtained in between 1000 bp and 500 bp and the size

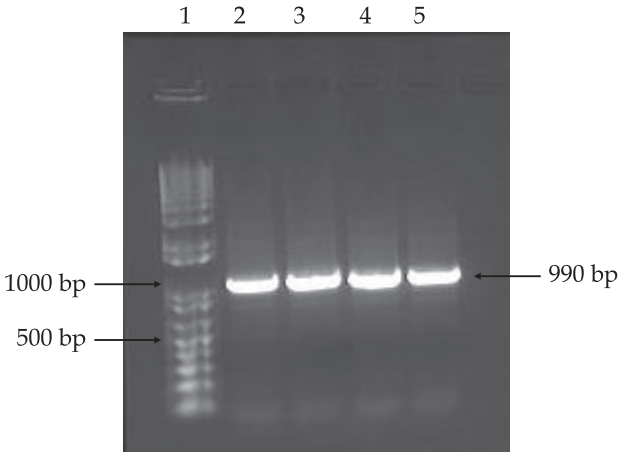

Fig 1. Amplification of Alternative oxidase gene of *T. evansi* by PCR 1.1Kb plus DNA Ladder. 2-5. Amplicons.

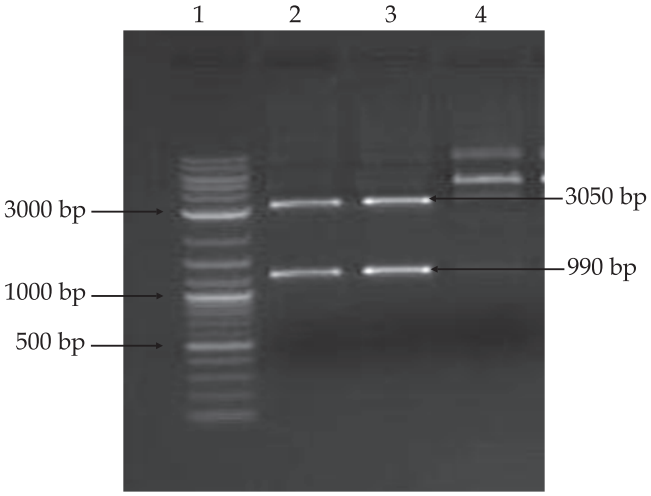

Fig 2. Alternative oxidase gene fragments of *T. evansi* after restriction digestion of *aox* gene plasmid. 1.1Kb plus DNA Ladder (2 Log DNA). 2-3. Alternative oxidase gene clone. 4. Uncut plasmid.

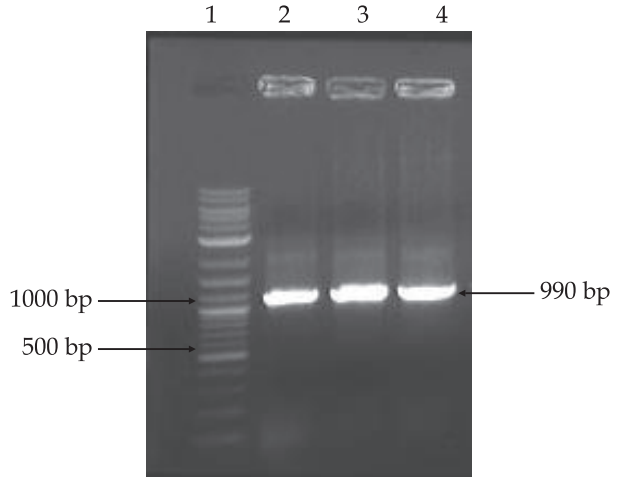

Fig 3. Amplification of Alternative oxidase gene of *T. evansi* by Colony-PCR 1.1Kb plus DNA Ladder 2-3. PCR reaction with white colony shows amplification 4. PCR reaction with blue colony shows absence of amplification.

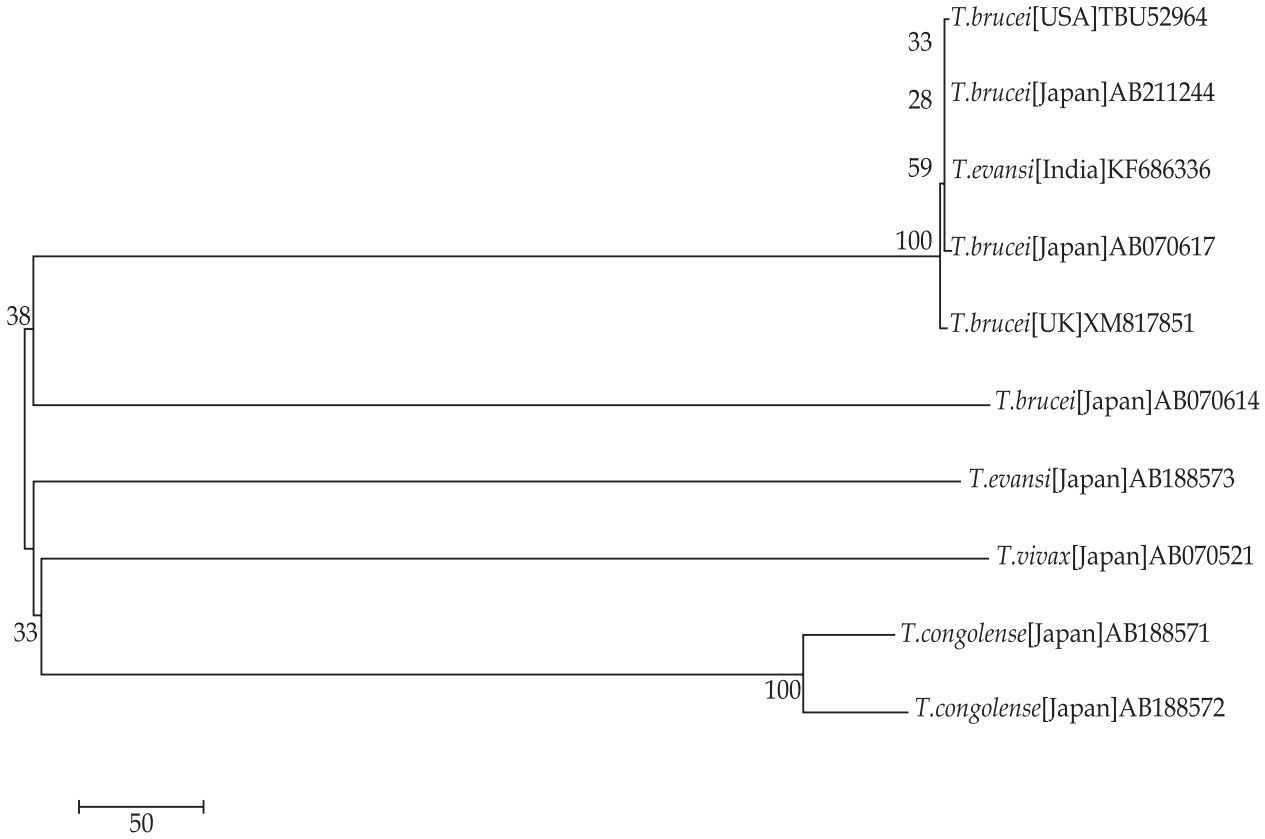

Fig 4. Phylogenetic tree analysis of Alternative oxidase gene using the Neighbor-Joining method.

of the amplicons was deduced from the standard log molecular sizes of the marker bands against their respective mobility. It was found to be 990 bp (Fig 1). There were several white colonies along with a few blue colonies. The blue colonies represented the presence of vector alone but few blue colonies may contain vector with insert. The white colonies usually represent recombinant clones carrying insert in the plasmid. The white colonies were screened for the presence of vector with insert. Two well separated DNA bands were seen in case of plasmids isolated from positive colonies upon Restriction Enzyme digestion with *Eco*R1, the less intense lower band corresponded to the insert (Fig 2). Release of DNA fragments of around 990 bp for *aox* gene 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 (Fig 3)

The coding sequence of *aox* gene according to the result obtained was of 990 bp. After confirmation of the *aox* gene nucleotide sequence of *T. evansi* isolated from camel, the sequence was submitted to GenBank, NCBI database to which the assigned accession number is KF686336. For phylogenetic analysis of *aox* gene sequences of other Trypanosomatidae species already available in the genebank database were retrieved. Tree topology is based on the Neighbour-Joining (NJ) method showed a close homology with other Trypanosomatidae species sequences with100% bootstrap values (Fig 4). The NJ, bootstrap consensus tree inferred from 1000 replicates was taken to represent the evolutionary history of the taxa analysed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) was shown next to the branches.

Sequence of the Alternative oxidase gene obtained in the present study (KF686336) showed 99.9% homology towards *T. brucei* (AB070614, AB211244) and 99% similarities with *T. brucei* (TBU52964, AB070617 and XM817851). A 100 per cent homology was found between obtained *aox* sequence and *T. evansi* (AB188573). Lower homology was documented between the obtained *aox* gene sequence and *T. congolense* (AB188571 and AB188572) and *T. vivax* (AB070521) a member belonging to Trypanosomatidae family showed only 76% and 68% homology, respectively.

Based on the above homology, *Trypanosoma congolense* (AB188571) and *T. congolense* (AB188572) in the phylogenetic tree are placed as two sub cluster of one mega cluster. *Trypanosoma brucei* (XM817851), *T. brucei* (TBU52964), *T. brucei* (AB070617) and *T. brucei* (AB211244) as four sub cluster of one mega cluster; the other mega cluster comprising of rest of the species. The earlier reports of *T. evansi* by Suzuki *et al* (2005) also showed 99% homology with *T. congolense* (AB188571 and AB188572). 68 to 76% sequence similarities was observed between *T. evansi aox* gene and other species documented in this study. The *aox* gene isolated from *T. evansi* by Suzuki *et al* (2005) contained an open reading frame of 1128 bp encoding a polypeptide of 329 amino acids. In the present study, it revealed a sequence homology of 99.9% with *T. brucei*, suggesting a close relationship between *T. evansi* and *T. brucei*. This is also consistent with previous study of ribosomal RNA genes of *T. brucei* (Urakawa *et al,* 1998) and it was considered as *T. evansi* as a variant of *T. brucei* or the basis of sequence homology.

The *aox* molecule is thought to have enabled some organisms to adapt to situations of decreased oxygen pressure, because it requires fewer oxygen molecules for a glucose degradation compared with cytochrome pathway (Kita *et al,* 2003). Moreover, because *Trypanosoma brucei* alternative oxidase functions as a terminal oxidase in *T. brucei* at the mitochondrial membrane inside mammalian hosts where oxygen pressure is lower than it is in the atmosphere, it may have contributed to the ability of African trypanosomes to survive in the blood stream of the mammalian hosts (Suzuki *et al,* 2005).

The sequence identity of obtained *aox* nucleotide sequence of *T. evansi* with other Trypanosomes species indicating that *aox* gene is highly conserved in the kinetoplastid species. It could therefore be suggested that vaccine with *aox* protein of trypanosomatidae parasite as the antigen could be effective against not only different strains within one trypanosomes species but also against other species of the same genus.

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