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Adoption of backyard poultry practices in Bhandara district of Maharashtra

Ph.D. (Veterinary Parasitology) THESIS 2013 SHINDE, N. G.

To,

The Dean,

Post graduate studies

RAJUVAS

Bikaner (Raj.)

Through: Dean, CVAS, Bikaner

Sub:- Submission of thesis for evaluation

Sir,

Please enclosed here with one copy of thesis "**Molecular characterization of Alternative oxidase and Trans-sialidase genes of *Trypanosoma evansi* of camel**" submitted by **Shinde Nitinkumar Gajanan**, **Ph.D.** student of this department. The thesis seminar was held on 28.10.2013. It is requested that necessary action for evaluation of thesis be taken please.

A panel for examiners in sealed envelope is also submitted herewith for needful, please.

Enclosed:-

1. Two copy of Thesis
2. One copy of Panel of Examiners

Head

Department of
Veterinary Parasitology

College of Veterinary & Animal Science, Bikaner

No. VCB/
Date

Forwarded in original to Dean, PGS, RAJUVAS, Bikaner, thesis copy for evaluation.

DEAN

DEPARTMENT OF VETERINARY PARASITOLOGY

COLLEGE OF VETERINARY AND ANIMAL SCIENCE, RAJUVAS, BIKANER

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LIST OF ABBREVIATIONS

Abbreviation	Meaning
DW	Distilled water
DEPC	Di ethyl pyro carbonate
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotide triphosphate
EDTA	Ethylene diamine tetra acetic acid
EtBr	Ethidium bromide
IPTG	Isopropyl – β- D- thiogalactoside
LB	Luria- Bretani
LMP	Low Melting Point
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate buffer saline
PCR	Polymerase chain Reaction
PK Buffer	Proteinase K Buffer
PK Enzyme	Proteinase K Enzyme
R.T.	Room temperature
RE	Restriction enzyme
RPM	Revolutions per minute
SDS	Sodium dodecyl sulphate
SOC	Super Optimal Broath with Catabolite Repression
TAE	Tris- Acetate EDTA
TE	Tris EDTA
UV	Ultra Violet
X- gal	5- bromo-4-chloro- 3 – indolylβDgalatase

Units of Measurement

Abbreviation	Meaning
%	Percentage
µg	Microgram
µl	Microlitre
^{0}C	Degree Celcius
A	Absorbance
bp	Base pair
cm	Centimeter
Da	Dalton
g	Gram (s)
h	Hour (s)
IU	International unit
Kbp	Kilo base pair
kDa	Kilo Dalton
Kg	Kilogram
M	Molar
ma	Milli ampere
mg	Milli gram
min	Minute (s)
ml	Milli litre
mm	Mili meter
mM	Milli molar
ng	Nano gram
OD	Optical density
pmol	Picomole
sec	Second (s)
U	Unit
V	Volt

V / cm	Volt per centimeter
V / V	Volume / volume
W / V	Weight / volume

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ABSTRACT

The present study was carried out to isolate the Alternative oxidase and Trans-sialidase genes of *Trypanosoma evansi* using PCR, clone the amplicons in a suitable plasmid vector and then characterization of above genes through sequencing. For this investigation, morphologically suspected *T. evansi* infected camel was confirmed by examination of Giemsa stained blood smear of camel blood.

After confirming infection, 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 desired amplicons of *aox* and *ts* genes were then amplified by PCR using gene specific primers. Amplified PCR products were analyzed on 1.2% agarose gel stained with ethidium bromide and identified on the basis of size of the *aox* and *ts* genes. The amplicons of expected size were purified from the 1% low melting agarose gel employing Illustra GFX PCR DNA and Gel Band Purification Kit. The DNA fragment of interest was then ligated to the pGEM- T Easy vector and ligated mixture was transformed into *Escherichia coli* JM109 strains. The cells containing recombinant plasmid could be identified on the basis of blue/white colony selection on LB agar containing X-Gal, IPTG and ampicillin. Screening of recombinants was done by Restriction Enzyme digestion of plasmid DNAs using *Eco*RI and found

that the release of DNA fragments around 990 bp for *aox* and 2241 bp for *ts* gene. Colony PCR was done for quick screening of plasmid inserts directly from *E. coli* colonies in the presence of insert specific primers. After confirmation of clones of *aox and ts* genes, the plasmid DNAs were sequenced and coding sequences of *aox and ts* genes according to the results obtained were of 990 and 2241 bp respectively. The phylogenetic and sequence analysis was done by use of Praline, Clustal X and MEGA5 softwares. Tree topology of *aox and ts* gene is based on the Neighbor-Joining method and maximum parsimony with 100% bootstrap values. Multiple sequence alignment of obtained protein sequences of *aox* and *ts* genes was performed with Praline sequence software. Identified *aox* and *ts* gene sequences showed a close homology with other *Trypanosoma spp*. gene sequences.

9. APPENDICES

APPENDIX – I

1. Phosphate Buffered Saline buffer (1X), pH 7.4

KCl	0.20 gm
KH_2PO_4	0.24 gm
NaCl	8.0 gm
$Na_2HPO_4.2H_2O$	1.44 gm
Distilled water to make	1000 ml

2. Agarose Gel Electrophoresis buffer

2.1. TAE buffer (50x)

Stock solution:

0.5 M EDTA acid (pH 8.0)	50 ml
Glacial acetic acid	28.55 gm
Tris base	121 gm
Water upto	500 ml

Working concentration of TAE buffer (1x)

TAE buffer	10 ml

Water	490 ml

2.2. TE Buffer (Tris/EDTA Buffer)

EDTA	1 mM
Tris-HCl (pH 7.5)	10 mM

Make from 1M stock of Tris-HCl (pH 7.5) and 500 mM stock of EDTA (pH 8.0).

Working solution:

500mM EDTA (pH 8.0)	0.2 ml
1M Tris-HCl (pH 7.5)	1.0 ml
Water upto	100 ml

3. Proteinase K buffer

EDTA	10mM
NaCl	50mM
SDS	2%
Tris base (ph 8)	100mM
β Mercapto ethanol	20mM

4. Trypanosome separation buffer (PSG buffer, pH 8.0)

4.1. Solution A

Na_2HPO_4 (anhydrous)	8.000 gm
$NaH_2PO_4.2H_2O$	0.780 gm
NaCl	4.250 gm
Distilled water to make	1000 ml

4.2. Solution B

Glucose solution dextrose	10 gm
Distilled water to make	400 ml

Just before use 6 parts of solution A was mixed with 4 part of solution B

APPENDIX – II

1. Agarose (0.8%)

Agarose	800 mg
TAE buffer	100 ml

2. Agarose (1.2%)

Agarose	1.2 gm
TAE buffer	100 ml

3. Ethidium bromide solution (10 mg/ ml)

Ethidium bromide	0.2gm
Sterile Water	20 ml

4. Giemsa stain

T.evansi pellet fixed for five minute with methnol and air-dried for five minute. Slides were stained for 45 minute.

Giemsa stain	1 ml
Distilled water	9 ml

5. 6X loading dye

Bromophenol blue	0.25%
Xylene cyanol	0.25%
Glycerol	50%
EDTA	2 mM

APPENDIX – III

1. Alcohol (70%)

Alcohol	70 ml
Water	30 ml

2. Ampicillin solution

Ampicillin	50µg/ml

Dissolved in distilled water.

To make 25ml of LB broth medium containing 50µg/ml Ampicillin, 125 µl of 10mg/ml ampicillin stock was used.

For 500 ml LB agar 2.5 ml of 10 mg/ml ampicillin stock was added, ensure that LB agar cooled to 50°C before adding ampicillin.

3. IPTG solution

IPTG	100mM

Dissolved in distilled water.

100µl IPTG was used for 25-30ml LB agar medium in LB plate.

4. Luria Bertani (LB) Medium (500 ml)

NaCl	0.5% (2.5 gm)

Trypton	1.0% (5 gm)
Yeast extract	0.5% (2.5 gm)

Adjust the pH to 7.0 with NaOH

For LB plates, add 1.5% (7.5 gm) agar to the LB broth and autoclave.

5. Luria Bertani (LB) Agar (500 ml)

Agar	1.5% (7.5 gm)
NaCl	0.5% (2.5 gm)
Trypton	1.0% (5 gm)
Yeast extract	0.5% (2.5 gm)

Adjust the pH to 7.0 with NaOH

6. LB plates with ampicillin/ IPTG/ X- Gal

LB plates with ampicillin were made by adding ampicillin to a final concentration of 50µg/ml after cooling of LB agar to 50^0C. Then 100 µl of 100mM IPTG and 20 µl of 20mg/ml X-Gal was spreaded over the surface of the LB- ampicillin plate and allowed to absorb for 30 minutes at 37ºC prior to use.

7. Phenol: Chloroform: Iso amyl alcohol; 25:24:1

Phenol	250 ml
Chloroform	240 ml
Iso amyl alcohol	100 ml

8. SOC medium (100ml)

Glucose (2M)	1ml
KCl (1M)	0.25 ml
Mg+2 stock (2M)	1ml
NaCl (1M)	1 ml
Trypton	2 gm
Yeast extract	0.5 gm

2M Mg^{+2} stock (100ml)

$MgCl_2 . 6H_2O$	20.33 gm
$MgSO_4 . 7H_2O$	24.65 gm

9. X-GAL solution

X-GAL	20mg/ml

Dissolved in 100% N,N dimethyl formamide.

20 µl X-GAL was used for 25-30ml LB agar medium in LB plate.

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5. DISCUSSION

Trypanosomes have the capacity for antigenic variation, which is the basis of their ability to escape the host immune response and because of this; prospects for the development of a vaccine against trypanosomosis have been considered poor. Trypanosomes change their variant surface glycoprotein coat very quickly which is the main hurdle in vaccine development. Target of alternative invariant proteins like protozoan oxidases and trans-sialidase proteins may be helpful in vaccine evolution against trypanosomosis. An option for developing vaccines and chemotherapeutic agents against trypanosomosis is to target pathogenic factors released by the parasite during infection, namely an “anti-disease” approach (Authie *et al*., 2001).

During the present study initially, *T. evansi* was isolated from the naturally infected camel. Following this, the desired genes, *aox* and *ts*

were amplified employing PCR. The purified PCR products were cloned into pGEM-T Easy vector. The genes were sequenced from purified plasmid of the obtained clones. The sequences of desired genes were compared with other related species gene sequences. After analysis, the gene sequences showed a wide range of homology within the organisms of Trypanosomatidae family.

In the present study, the amplicons size of *aox* gene was obtained as 990 bp. However, *Trypanosoma evansi aox* gene mRNA for alternative oxidase, complete cds was identified 1128 bp (Suzuki *et al.,* 2005a; AB188573). Chaudhuri and Hill (2004) reported *Trypanosoma brucei brucei* alternative oxidase (aox) mRNA, complete cds of 990 bp (TBU52964) and Berriman *et al.,* (2012) observed that partial cds of the gene was 990 bp (XM817851). Likewise Fukai *et al.,* (2011) reported that complete cds of the gene was 990 bp (AB070617). Also Kita (2001) observed that complete cds of the gene was 1147 bp (AB070614). Further, Nakamura *et al.,* (2006) reported that complete cds of the gene was 990 bp (AB211244). Complete gene sequence of *aox* gene from mRNA of *Trypanosoma congolense* was done by Suzuki *et al.,* (2005b) and identified sequence gene size was 1050 bp (AB188571). Suzuki *et al.,* (2005c) did the complete gene sequence of *aox* gene of *Trypanosoma congolense* and identified sequence size of gene was 1016 bp (AB188572). Similarly, complete gene sequence of *aox* gene form mRNA of *Trypanosoma vivax* was done by Suzuki *et al.,* (2004a) and identified gene sequence was of 1193 bp (AB070521).

Obtained Alternative oxidase gene sequence showed 99.9% homology towards *T. brucei* (AB070614 and (AB211244), and 99.0% similarities with *T. brucei* (TBU52964, AB070617 and XM817851). A 100 percent homology was found between obtained *aox* sequence and *T. evansi* (AB188573). 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.

Molecular mass of the obtained protein of alternative oxidase gene ranged from 33-38 kDa (Li, *et al.,* 2007). Open reading frame from deduced amino acids predicted a molecular mass of 38 kDa, in the present study. The significant amount of similarities of *aox* gene of *T. evansi* from blood stream, revealed the conserved nature of the gene of Alternative oxidase. This result may also indicate that the gene may be able to produce a functional alternative oxidase protein.

Alternative oxidase, an important virulence factor and therapeutic target has been characterized molecularly. Alternative oxidase has been found in a wide variety of organisms but not in mammals, so considered to be a promising drug target for the treatment of trypanosomosis (Chaudhuri *et al.,* 2006; Kido *et al.,* 2010a; Nihei *et al.,* 2003; Ott *et al.,* 2006; Saimoto *et al.,* 2012). Studies of Chaudhuri and Hill (1996) showed that a 33 kDa mitochondrial protein of *T. brucei* was identified as an essential component associated with trypanosome alternative oxidase activity. Kornblatt *et al.* (1992) also purified a protein from the membranes of blood stream forms of *T. b. brucei* contained a single polypeptide chain of molecular mass 67 kDa as judged by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Won-ki *et al.* (1999) stated that the *aox*1 gene, which encodes an alternative oxidase, was isolated from the genomic DNA library of *Candida albicans*. The gene encodes a polypeptide consisting of 379 amino acids with a calculated molecular mass of 43,975 Da. Also Henriquez *et al.* (2009) showed that open reading frames of 1113 and 1125 bp, respectively with a predicted molecular weight of 42 kDa of Acaox protein has a predicted cleavable

mitochondrial targeting sequence. Besides, all *aox* genes, so far, analyzed having a greater extent of sequence identity, possessed an open reading frame of 328 amino acid residues. It is thus opined that the *aox* are likely to be transcribed in the blood stream stage of the mammalian host (Suzuki *et al.,* 2005a). This is consistent with the present study where in the *aox* gene is amplified and identical amino acid sequences were derived.

Lower homology was documented between the obtained *aox* gene sequence and *T. congolense* (AB188571 and AB188572) and *T. vivax* (AB070521), other members 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 clusters of one mega cluster. Similarly, *Trypanosoma brucei* (XM817851), *T. brucei* (TBU52964), *T. brucei* (AB070617) and *T. brucei* (AB211244) as four sub clusters of one mega cluster; the other mega cluster comprising of rest of the species. The earlier reports of *T. evansi* by Suzuki *et al.,* (2005b&c) 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.,* (2005b&c) contained an open reading frame of 1128 bp encoding a polypeptide of 329 amino acids.

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.,* 2005a).

In the present study, the amplicons size of *ts* gene was obtained as 2241 bp. However, *Trypanosoma evansi* trans-sialidase gene, partial cds was identified as 2248 bp (Yakubu *et al.,* 2009; FJ597949). Berriman *et al.,* (2012a) reported *Trypanosoma brucei* trans-sialidase gene partial cds of 1284 bp (XM841210). Further, Berriman *et al.* (2012b) observed that partial cds of the gene was 2316 bp (XM841212). Likewise, the complete cds of the gene has been reported as 3313 bp (AF181287) by Engstler (2000) and 2316 bp (AF310232) by Montagna *et al.* (2002a). The partial cds of the gene have been reported as 2247 bp (XM842471), 2247 bp (XM842470), 2451 bp (XM839645) and 2109 bp (XM946607) by Berriman *et al.* (2012c), (2012d), (2012e) and (2012f), respectively.

The Trans-sialidase gene sequence showed 99% homology with *T. evansi* (KF686337), 99% with *T. brucei* (XM841212) and 99% with *T. evansi* (FJ597949). This emphasizes further that the gene amplified in a *ts* gene and was duly expressed in the blood stream stage of *T. evansi*. Slightly lower homology was documented between the obtained *ts* gene sequence and *T. brucei* (AF310232, AF181287 and XM841210). However, the comparison with *T. brucei* (XM842470), *T. brucei,* (XM842471) and *T. brucei* **(**XM946607) genes only 49%, 48%, and 28% homology, respectively, was observed.

Montagna *et al.* (2002a) showed that *ts* genes are present at a small copy number, at variance with American trypanosomes where a large gene family is present. Its N-terminus contains a region of 372 amino acids that is 45% identical to the catalytic domain of *Trypanosoma cruzi* trans-sialidase and contains the relevant residues

required for catalysis. Yakubu *et al.* (2011) studied a PCR based approach of a trans-sialidase gene from the bloodstream form of *T. evansi* obtained from the blood of infected camels. Sequence analysis of the cloned *Trypanosoma evansi* trans-sialidase gene indicated 99% identity to some African trypanosomes trans-sialidase genes.

According to neighbor-joining phylogenetic tree analysis of *ts* gene, *T. brucei* (XM842470 and XM842471) are placed as two sub cluster of one mega cluster. *T. brucei* (XM841212, AF310232, XM841210 and XM839645) as four sub cluster of one mega cluster and the other mega cluster comprising of rest of the species. Exactly same type of sequence similarities was observed between *T. evansi* (KF686337) and other documented species. The earlier reports of *T. evansi* by Yakubu *et al.* (2009) showed 99% homology with obtained *ts* sequence and sequence of *T. evansi* (FJ597949) and very few differences in sequence similarities were observed between these sequences when compared with other documented species. In open reading frame of nucleotide sequence of *ts* gene in *Trypanosoma brucei* by Montagna *et al*.; (2002) revealed 99% homology with the 2316 bp encoding a polypeptide of 372 amino acids. The detection of *ts* gene in *T. evansi* suggests that this gene along with sialidase gene may play specific role in adaptation and/or pathogenesis in mammalian host. This specific role in adaptation in host followed by pathogenesis has also been reported in other protozoan parasites, viz, *T. cruzi, Entamoeba histolytica* and *Trichomonas vaginalis* (Taylor *et al.,* 1992).

The principal finding of the present study was the identification of the *aox* and *ts* gene of *T. evansi* from camel by sequencing the recombinant plasmid pGEM-T Easy –*aox*/*ts* in both directions with forward and reverse primers. The present findings therefore suggest that the identified *aox* gene showed a close homology with *T. brucei*

(AB070614 and AB211244), *T. evansi* (AB188573) and *T. congolense* (AB188571 and AB188572). Identified *ts* gene was also found in close homology with other trypanosome sequences like *T. brucei* (XM841212 and AF310232), *T. evansi* (FJ597949) and *T. brucei* (AF181287 and XM841210). The sequence identity of obtained *ts* nucleotide sequences of *T. evansi* with other trypanosome species indicated that *ts* genes are highly conserved in the kinetoplastid species. As mentioned in results, amino acid sequences of obtained Aox and Ts proteins of *T. evansi* showed high level of homology with amino acid sequence of respective proteins of other trypanosome species. It could therefore be hypothesized and suggested that vaccine with Aox and Ts proteins 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.

Sequence analysis of the gene is the most appropriate method for the confirmation of specificity of the target region of any gene. Therefore aforementioned experiments were attempted to characterize the genes of prime importance in *T. evansi* from Indian dromedarius. These genes would be the ideal vaccine and drug target in its own right for the control of trypanosomosis in India.

1. INTRODUCTION

The animal pathogenic protozoan, *Trypanosoma evansi*, causes a wasting disease commonly known as “Surra” in equines, camels, cattle and buffaloes. It is transmitted mechanically by biting flies like *Tabanus*, *Haematobia, Stomoxys*, *Chrysops, Lyperosia* and *Pangonia* and is prevalent in the Northern Africa, Middle East, India and the Far East Asian countries (Lukins, 1992). Trypanosomosis is a very serious and often fatal blood protozoan disease of domestic animals severely limiting their productivity in the subtropical and tropical regions of the world. *Trypanosoma evansi* was discovered by Griffith Evans in 1880 in infected camels and equids in the Dera Ismail Khan (presently in Pakistan) district of Punjab (Indrakamhang, 1998). Chronic infection of *T. evansi* may be present for three or more years in camels and this form of the disease is commonly known as “Tibersa”. In South Africa, another form of Trypanosomosis is prevalent which is known as "Nagana".

Trypanosoma belongs to the Family *Trypanosomatidae* and is parasitic in the blood of invertebrates and vertebrates, including humans. Most species live a part of their life cycle in the intestine of insects and other invertebrates, the flagellate stage being found only in

the vertebrate host. The difference in species depends on size and shape of the body, position of nucleus, degree of development of the undulating membrane and flagellum (Smyth, 1996). Acute infection causes a severe febrile illness characterized by coughing, dyspnea, pulmonary edema, idiopathic cardiomyopathy, hepato-splenomegaly, immunosuppression and death due to respiratory distress or cardiac failure (Hoare, 1972; Brun *et al*., 1998). Chronic trypanosomosis has significant impact resulting in infertility, reduced milk yield and weight gain, contributing to the lower provision of animal proteins and agricultural products due to lack of working livestock (Boid *et al*., 1996).

Trypanosomosis caused by *Trypanosoma evansi*, is the most pathogenic parasitic disease of camelids in all camel raising countries causing high morbidity and mortality (Luckins, 1992). This parasite has a wide range of distribution throughout tropical and sub-tropical regions of the world. Traditionally *T. evansi* infection has been observed in domestic and wild animals; recent reports however, suggest their ability to infect humans (Desquesnes *et al.,* 2013). Diagnostic tests have been prescribed by OIE but not the vaccines against this disease. *T. evansi* consists of a large number of morphologically identical populations that differ significantly in various biological characteristics such as host range, virulence, pathogenicity and drug sensitivity.

Camel trypanosomosis has been recognized as the most important single cause of economic losses in camel rearing areas. According to one estimate, *T. evansi* causes about 30% morbidity and 3% mortality in camels (Njiru *et al.*, 2002). Besides causing disease, the trypanosomes are also responsible for producing a state of severe immuno-suppression, which renders the infected host more susceptible to secondary infections and produce poor immune response to bacterial and viral vaccines (Holmes, 1980).

Chemotherapy is the most efficient control methods, which depends on very effective drugs. Unfortunately, with the development of resistance to drugs, their effective use is threatened, emphasizing a need to find new drugs (Gillingwater *et al.* 2009). Unavailability of immunoprophylaxis is due to its variant surface glycoprotein coat which is changeable when host forms antibody against it. *Trypanosoma evansi* possesses about 1000 VSG genes and only one gene being active at a time (Janz and Clayton, 1994).

Vaccine development against animal trypanosomiasis based on variant surface glycoprotein was totally abandoned (Donelson *et al*., 1998). So there is an urgent need of advance research based on nonvariant protein from trypanosomes for its detection and effective control. Because of the wide geographic range of surra, its control has attracted international attention with a focus on formulating and implementing effective strategies aimed at increasing productivity and achieving a decrease in mortality and morbidity.

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 (Atteia *et al.,* 2004; Chaudhuri *et al.* 2005). Alternative oxidase has been found in a wide variety of organisms but not in mammals and thus is considered to be a promising drug target for the treatment of trypanosomiasis (Chaudhuri *et al.,* 2006; Kido *et al.,* 2010a; Nihei *et al.,* 2003; Ott *et al.,* 2006; Saimoto *et al.,* 2012).

Alternative oxidase has important roles 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 (Akhter *et al.,* 2003; Atteia *et al.,* 2004). Trypanosomes mitochondrial cyanide sensitive respiration possesses an alternative oxidase which is inhibited specifically by salicyl hydroxamic acid (SHAM). *T. brucei* lacks the cytochrome pathway activity and completely relies on alternative oxidase activity for respiration. 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.

Trans-sialidase (*ts*) is a GPI-anchored (Glycosylphosphatidylino -sitol) cell surface enzyme that was first described in *T. cruzi* and is used to acquire sialic acid from host glycol-conjugates for sialylation of plasma membrane glycoproteins of parasite. The sialic acid is transferred to mucin-like glycoproteins that are the most abundant molecules on the surface of trypomastigotes (Costa *et al.,* 1998; Koliwer-Brandl *et al.,* 2011). *T. cruzi* trans-sialidase *(Tcts)* is a member of the sialidase superfamily encoding both active and inactive proteins and functions as a sialidase in the absence of a carbohydrate acceptor. The inactive proteins physically interact with sialic acid containing molecules on host cells and may play a role in immune evasion, adhesion and invasion or interaction of host cell (Todeschini *et al.,* 2000 and 2002).

Trans-sialidase is located on the external surface of the parasite and sheds to the medium, the relatively broad specificity shown by the enzyme with respect to protein and lipid-linked oligosaccharides which may alter the sialic acid distribution in glycoproteins and glycolipids of the mammalian host (Ferrero-Garcia *et al.,* 1993). Besides, *ts* plays a key role in the recognition of protein and induces the expression of

adhesion molecules in the host cells, stimulates parasite entry and blocks apoptosis generated by growth factor depletion (Ming *et al.,* 1993; Dias *et al*., 2008). It may be involved in parasite survival from the complement-mediated host immune response, host cell invasion and pathogenesis due to its capacity of sialylating the mucin molecules that cover the parasite surface with a dense protective layer (Paris *et al.,* 2005).

Trans-sialidase strengthens the innate immunity of the parasites by sialylation, masking antigens, protecting trypanosomes from proteases, disturbing the host's cytokine network, impairing its immune response, manipulating multiple host cell-signaling pathways, inhibiting apoptosis, and facilitating recognition and uptake of trypanosomes by host cells (Santos *et al.,* 1997). Since *T. cruzi* is unable to synthesize sialic acids, *ts* specifically transfers alpha (2-3)-linked sialic acid from extrinsic host derived macromolecules to parasite surface molecules, leading to the assembly of Ssp-3, a trypomastigote-specific epitope, is required for target cell recognition and invasion of host cells and enables the parasite to escape immune response (Carvalho *et al.,* 2010; Schenkman *et al.,* 1991).

Trans-sialidase belongs to a highly heterogeneous family of surface molecules which contain a long stretch of 12 amino acid repeats at the C terminus which are necessary for the catalytic activity (Briones *et al.,* 1995a; Engstler *et al.,* 1993; Buschiazzo *et al.,* 2002). SAPA (shed acute phase antigen) contains a unique trans-sialidase activity that is essential for penetration of the parasite into mammalian cells. Ts protein consists of two different domains, enzymatic domains

which contain four copies of an amino acid motif conserved in bacterial neuraminidase and other domain which is highly antigenic consisting of the repeats essential to attract the immune response by protecting the enzyme activity (Cazzulo and Frasch, 1992).

Trans-sialidase strongly contributes to the pathogenicity of the trypanosomes by scavenging sialic acids from the host or blood meal to coat the parasite surface; this aids their survival strategy in the insects intestine, host cells and blood circulation which serves to compromise the immune system of human or animal host (Schauer and Kamerling, 2011). Due to its absence in the mammalian host, *ts* acts as a potential biological target that may be useful for drug design and gene product (Buschiazzo *et al.* 2012).

Recent research progress has clarified that mammalian sialidases indeed contribute to the regulation of various cellular functions as well as lysosomal catabolism, unlike the sialidases of microbial origin that probably play roles limited to nutrition and pathogenesis (Monti and Miyagi 2012). Interest in *ts* has increased rapidly in recent years owing to its great relevance to the pathogenicity of trypanosomes and its possible application in the region specific synthesis of sialylated carbohydrates and glycol-conjugates (Yakubu *et al.,* 2011).

Alternative oxidase and Trans-sialidase gene of *T. evansi* are nonvarient genes. Due to stability against the host immune response, these genes may be explored to make possible immune prophylaxis to control the disease effectively and inexpensively. Due to lack of understanding of the pathogenesis of trypanosomiasis, development of effective drugs and vaccines against this protozoan parasite is hampered and this becomes worse by the complexity of host-parasite interactions. Alternative oxidase is emerging as an important virulence factor and therapeutic target in *T. evansi* infection. Alternative oxidase

has been found in a wide variety of organisms but not in mammals so it is considered to be a promising drug target for the treatment of trypanosomiasis (Chaudhuri *et al.,* 2006; Kido *et al.,* 2010a; Nihei *et al.,* 2003; Ott *et al.,* 2006; Saimoto *et al.,* 2012). Trans-sialidase is a functionally important structure that is presents in pathogenic trypanosomatids but absent from their mammalian hosts. As such, Trans-sialidase is a logical target for therapeutic intervention in the infection process (Santos *et al.,* 1997; Buschiazzo *et al.,* 2012).

The identification of peptidases/proteases in protozoan parasites that are able to process regulatory host peptides under physiological conditions gave clues to possible mechanisms of parasite virulence that can be targeted to prevent these debilitating diseases.

Keeping in view of above facts, the present study of Alternative oxidase and Trans-sialidase genes of *Trypanosoma evansi* was undertaken with the following objectives:

1. **To isolate Alternative oxidase and Trans-sialidase genes of *Trypanosoma evansi* from camel by polymerase chain reaction and clone the amplicons,**

2. **Characterization of above genes through sequencing**

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3. MATERIALS AND METHODS

3.1. Material

3.1.1. Experimental organism

For isolation of Alternative oxidase and Trans-sialidase genes, the experimental organism is *Trypanosoma evansi*, a blood protozoan parasite under Phylum *Sarcomastigophora*, Sub-phylum *Mastigophora*, Class *Zoomastigophorea*, Order *Kinetoplastida* and Family *Trypanosomatidae*. It is an important parasitic protozoan of camelids responsible for high morbidity and mortality.

3.1.2. Equipments

- Deep Freeze (-20°C) - Heto, Denmark
- Dry heating block - Thermocon, Genei, India
- Gel Documentation System – AlphaImager 2200, USA
- Horizontal Agarose Gel Electrophoresis apparatus with power supply - Genei, India
- Horizontal laminar air flow – Telstar, AV- 30/70, USA
- Incubator Shaker - Lab-Therm, Kuhner, Switzerland
- Incubator– Model BD-53, Binder, USA
- Microwave oven - Kenstar Electronic Ltd., India

- Micropipettes: 2.5, 10, 20, 100, 200 and 1000 µl capacity – Eppendorf, Germany and Nichipet, USA
- pH meter– µ pH system 361, Systronics, India
- Refrigerated high-speed centrifuge - Biofuge Primo R, Heraeus, Germany
- Refrigerated micro centrifuge - Hettich, model- Mikro 22 R, Germany
- Single Pan digital balance - Precisa, 125 A SCS, Switzerland
- Thermocycler – Mastercycler Gradient, Eppendorf, 5330, Germany
- Trans illuminator - UVP, USA
- Trinocular Compound microscope, Nikon, USA
- Ultra low freezer (-80°C) - Model U410, NBS, USA
- Water Bath cum shaker - Aqua shake, Kuhner, Switzerland
- Water Purification System – Elix-Milli-Q, Synthesis Type, Millipore, USA
- UV- vis Spectrophotometer– UV mini 1240, Shimadzu, USA

3.1.3. Chemicals

- Agarose – Analytical and Preparative grades
- Ampicillin, Bovine serum fraction-V (BSA), Bromophenol blue, RNAse-A, Sodium dodecyl sulphate (SDS), Sucrose and Xylene cyanol
- DNAse
- DNA purification kit and Plasmid isolation kit.
- DNA molecular size markers
- dNTPs
- Glycerol
- Proteinase K, Tris Base, Ethidium bromide
- Seakem Low Melting Point Agarose

3.1.4. Glass and plastic ware

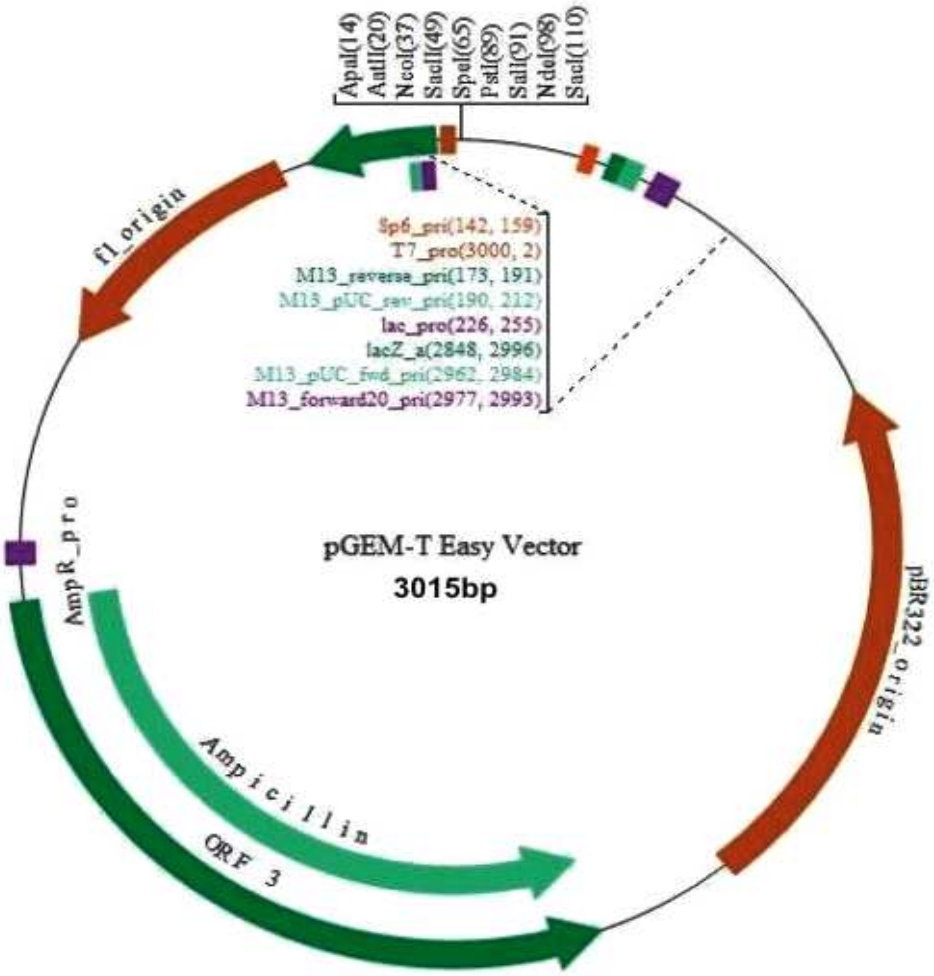
Glassware used in this study was procured from Borosil India Ltd., and Duran Schott, Germany. All the Glassware were thoroughly washed and sterilized as per standard protocols before their use. Micro centrifuge tubes and micropipette tips were from Axygen and Eppendorf, India Ltd.

3.1.5. Buffers and Media

The composition of buffers, media and solutions that were used in this study is given in the Appendix No. I to III

3.1.6. Vector

pGEM- T Easy vector (Promega) available in the laboratory was used. The vector was propagated in *Escherichia coli*, JM109 cells, extracted and used for cloning studies. The map of the pGEM- T Easy vector is shown in plate 1.



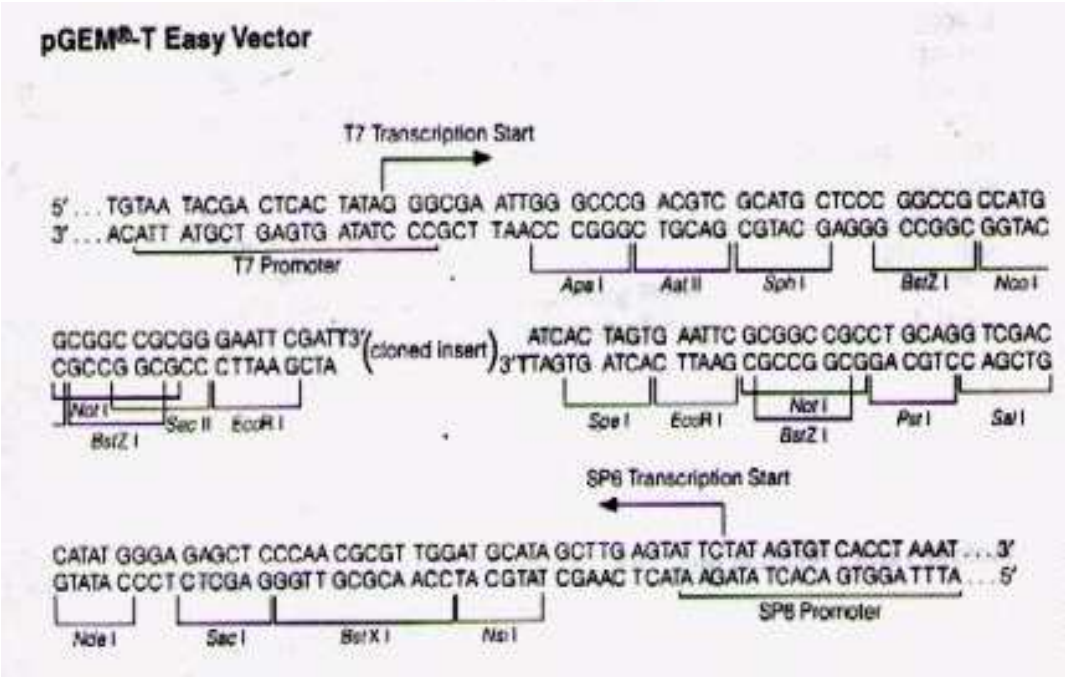


Plate1. Map of the pGEM- T Easy vector

3.1.7. Host Systems

Escherichia coli strain JM109 (Promega) stored at –70^{0}C was used for propagation of plasmids.

3.1.8. Primers

Gene specific primer for *aox* gene was from published sequences (Accession No. AB188573) using the primer designing tool at NCBI. For *ts* gene already designed primer sequences by Montagna *et al*. (2002) were used. All the primer sequences were stretched 5' to 3' end. The primers obtained in lyophilized form from Eurofins, India was reconstituted as 100 mmol. stock in sterile TE buffer. Primers at working concentrations of 10 pmol in sterile TE buffer were used for PCR amplification.

Table 3.1: Primer sequences used to amplify *aox* and *ts* genes

Genes of *T. evansi*	**Forward and reverse Primers (5' to 3')**
aox	**F** 5'AGGTAATATCTTGACACCAAGAGC3' **R** 5'ACGTGTTTGTTTACATTACTCGCA3'
ts	**F** 5'ATGGAGGAACTCCACCAACAAAT3' **R** 5'TATAGATCTTCAAATCGCCAACACATACAT3'

3.1.9. Enzymes

Restriction enzymes *Eco*RI and *Taq* DNA polymerase were used from Promega, USA.

3.2. Methods

During the present study attempts were made to identify the Alternative oxidase and Trans-sialidase genes of *T. evansi* from camel (*Camelus dromedarius*). The procedure followed has been described in details as under:

3.2.1. Identification of *Trypanosoma evansi* infected camel

Trypanosomosis suspected camels were identified in the National Research Centre on Camel, Bikaner (Rajasthan). Blood smears from these animals were prepared and stained with Giemsa stain after proper fixation with methanol (Appendix-II.4) and examined under compound microscope to confirm the infection of *T. evansi* in the camels. After confirmation, blood from infected host was collected. For this, 5 ml blood was collected from the jugular vein using 9 ml vaccutainer tube containing EDTA (ethyl diamine tetra acetic acid). 0.5 ml blood (with the help of insulin syringe) was inoculated intra-peritoneally into each experimental animal ie. Swiss albino mice (maintained at Small Animal Laboratory, NRC on Camel, Bikaner).

3.2.2. Propagation of trypanosomes

A convenient passage dose/interval for mice for each of the isolates of *T. evansi* was arrived at and routinely used throughout the investigation, based on the infectivity titration as given by Lumsden *et al*. (1973). The method consisted of examination of wet blood films from peripheral blood of the laboratory hosts (mice/rats) and scoring the degree of parasitaemia. The scheme described by Desowitz and Watson (1951) was followed for interpretation of degree of parasitaemia:

S **:** Scanty infection i.e. less than one trypanosome per microscopic field

+ **:** Average one to five trypanosomes per microscopic field

++ **:** Average six to ten trypanosomes per microscopic field

+++ **:** Average eleven to twenty trypanosomes per microscopic field

++++ **:** Average more than twenty trypanosomes per microscopic field

M **:** Massive infection i.e. trypanosomes equal or exceeding the number of erythrocytes in the field

The estimation of the working/infectivity dose for routine passage in mice was then arrived at by dilution of the suspension of trypanosomes collected through tail blood (++++ or M).

3.2.3. Isolation of genomic DNA of *Trypanosoma evansi* from camel blood:

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 and involving five major steps:

- Blood cell lysis
- Genomic DNA binding
- Washing of DNA
- Drying of DNA
- Elution

600 µl of parasitologically positive camel blood was taken in eppendorf tube, to which 300 µl lysis buffer was added, mixed well and incubated for 5 minute at room temperature. The mixture was centrifuged at 500 rpm for 2 minutes at room temperature. The upper phase supernatant was discarded and 150 µl PBS was added then vortexed to resuspend the pellet. Mixture was transferred to a fresh

1.5 ml microcentrifuge tube in which 20 µl Proteinase-K and 400 µl lysis buffer Type X was added. The mixture was incubated for 10 minutes at room temperature. It was loaded into assembled column and collection tube, centrifuged at 11,000 rpm for 1 minute and flow through was discarded. 500 µl lysis buffer Type X was again added to it and centrifuged at 11000 rpm and flow through was discarded. 500 µl wash buffer Type VI was added and centrifuged at 11,000 rpm and flow through was discarded. The column was inserted into a clean DNase free microcentrifuge tube. 200 µl elution buffer Type V was added in microcentrifuge tube and heated upto 70º C then incubated for 1 minute at room temperature and centrifuged at 11,000 rpm for 1 minute. Then elute was collected and purified genomic DNA was stored at -20º C. Then the concentration and purity of DNA sample was determined.

3.2.4. Quantitative and Qualitative assessment of DNA

3.2.4.1. Spectrophotometric Determination

For quantifying the amount of DNA, O.D. values were recorded at wavelengths of 260 and 280 nm (The reading at 260 nm allows calculation of the concentration of nucleic acid in the sample and an O.D. of 1 corresponds to approximately 50 µg/ml for the double stranded oligonucleotides).The DNA was diluted with TE Buffer at the ratio of 1: 50 and mixed accordingly. TE buffer was taken as a blank, and respective O.D. was recorded to calibrate at zero.

After setting spectrophotometer with blank, the O.D. values at 260 nm and 280 nm were recorded for DNA respectively. The concentration of unknown double stranded DNA sample was estimated, using the following formula:

DNA concentration (µg /ml) =Absorbance at 260nm x dilution factor x50

= A1 X 50 X 50

The ratio between the readings at 260 nm and 280 nm (O.D. 260/280) provides estimates of purity of nucleic acid. The ratio of O.D. values of high quality DNA ranged between 1.8 and 2.

A1/A2 = Absorbance at 260/280 = 1.85

3.2.4.2. Checking of quality of DNA

The genomic DNA isolated from *T. evansi* was checked for quality, purity and concentration. Only the DNA samples of good quality were used for further analysis.

Horizontal submarine agarose gel electrophoresis was carried out to check the quality of genomic DNA using 0.8 % w/v agarose. At the start, the gel casting tray was prepared by sealing it's both end with adhesive tape and then the comb was set over it in a way to keep a gap of atleast 0.5 mm between the tips of comb teeth and floor of the casting tray, so that the wells got completely sealed by agarose.

Subsequently, 0.8% agarose (w/v) suspension in 1 X TAE buffer was made and heated on an electric heater or in microwave until the agarose was completely melted and dissolved to give a clear transparent solution. After cooling it to about 50^0C, ethidium bromide (10 mg/ml) was added to a final concentration of 0.5 µg/ml and was mixed gently. The agarose solution was poured into the sealed casting tray. The gel was prepared to about 4 mm thickness. The agarose gel was allowed to set completely at 4^0C temperature before the comb was gently removed. The adhesive tape was also detached and gel casting platform was submerged in the electrophoresis tank containing 1 X TAE buffer.

For loading the samples, 10 µl of autoclaved triple distilled water was mixed with 5 µl DNA. 1 µl of 6 X gel loading dye (Appendix-II.5) was mixed with 5 µl diluted DNA for loading in each well of gel. Electrophoresis was performed at 4V/cm (60-70V) for ~1 hour after loading the DNA sample into the well. Once the electrophoresis was over, the gel was visualized under UV trans-illuminator and documented by photography. Only DNA sample showing intact bands were used for further analysis.

3.2.5. Amplification of Alternative oxidase and Trans-sialidase genes by PCR

Principle of PCR

The purpose of PCR (polymerase chain reaction) is to make a huge number of copies of a gene. This is necessary to have enough starting template for sequencing. There are three major steps in a PCR, which are repeated for 30 to 40 cycles. This is done on an automated thermo cycler, which can heat and cool the tubes with the reaction mixture in a very short time. During denaturation, the double strand melts and opens to single stranded DNA after all enzymatic reaction. The primers are annealing around, annealing being caused by the Brownian motion. Ionic bonds are constantly formed and broken between the single stranded primers and the single stranded template. The more stable bonds last a little bit longer (primers) that fit exactly on the little piece of double stranded DNA (primers and template), the polymerase can attach and start copying the template. Once there are a few bases built in, the ionic bond is so strong between the template and the primer that does not break any more. This is the ideal working temperature for the polymerase. The primers, where there are a few bases built in, already have to a stronger ionic attraction to the template than the forces breaking these attractions. Primers that are on positions with on exact match get loose again

(because of higher temperature) and don't give an extension of the fragment. The bases (complementary to the template) are coupled to the primer on the 3' side (the polymerase add dNTP's from 5' to 3', reading the template from 3' to 5'side, bases are added complementary to the template). Because both of strands are copied during PCR, there is exponential increase in the number of copies of the gene.

3.2.5.1. Optimization of PCR Parameters

Various combinations of reaction chemical were tried to optimize the concentration of each component. The PCR parameter, viz. annealing temperature [2(A+T) +4(G+C)] and cycling conditions were optimized to obtain a specific amplified product in sufficient quantity. The reaction volume was kept constant at 50 µl. After proper mixing, 50 µl reaction mixture was divided in four PCR tubes. The standardized concentrations of components used in the reaction mixture are given in Table 3.2 – 3.3.

Table 3.2: PCR reaction mixture for Alternative oxidase gene

Components for Master mix	Vol. / reaction	Final conc. / reaction
5X Flexi PCR Buffer	10 µl	1x
dNTP mix (10 mM each)	1 µl	200 µM of each dNTP
$MgCl_2$ (25 mM)	3 µl	2.5 mM of Mg^{2+}
Primer F	0.5 µl	10 pM
Primer R	0.5 µl	10 pM
Template DNA	0.5 µl	100ng
Taq DNA polymerase	0.25 µl	1.5 Units
Distilled Water	34.25 µl	-
Total volume	**50 µl**	-

Table 3.3: PCR reaction mixture for Trans-sialidase gene

Components for Master mix	Vol. / reaction	Final conc. / reaction
5X Flexi PCR Buffer	10 µl	1x
dNTP mix (10 mM each)	1 µl	200 µM of each dNTP
$MgCl_2$ (25 mM)	3 µl	2.5 mM of Mg^{2+}
Primer F	0.25 µl	10 pM
Primer R	0.25 µl	10 pM
Template DNA	0.5 µl	100ng
Taq DNA polymerase	0.25 µl	1.5 Units
Distilled Water	34.75 µl	-
Total volume	**50 µl**	-

PCR amplification was carried out in thermal cycler using the optimized condition as given in table 3.2 - 3.3.

3.2.5.2. Protocol for amplification of Alternative oxidase *(aox)* and Trans-sialidase *(ts)* genes of *Trypanosoma evansi* by PCR

At first, a PCR master mixture, containing all the reaction components except genomic DNA, was prepared in ice. Care was taken to add *Taq* DNA polymerase at the end of preparation. After addition of all the components, the master mix was mixed gently, followed by spinning by table top micro centrifuge. Then, 49.5µl of master mix was added to each pre labeled PCR tubes (eppendorf tube) of 0.2 ml capacity. Finally, 0.5µl of good quality genomic DNA was added to each tube. The contents of tube were mixed gently and spinned at 9000 rpm at 10 sec. Finally, the PCR tubes were arranged in a preprogrammed thermo cycler. PCR products obtained, after the completion of the programme, were kept at 4^0C in refrigerator for further analysis.

3.2.5.3. PCR programme

Several combinations of PCR programmes were tried before finalizing one programme giving the best amplification of the desired fragments. The standardized programmes for different genes are given into tables 3.4 and 3.5

Table 3.4: PCR conditions for Alternative oxidase gene

Step	Temperature	Time	No. of cycle
I. Initial Denaturation	94°C	4 min.	One
II. Cycle (i) Denaturation (ii) Annealing (iii) Synthesis	 94°C 49°C 72°C	 30 sec. 1 min. 1 min. and 30 sec.	Over all total 35 cycle
III. Final extension	72°C	9 min.	One
IV. Hold	4°C		
Thermal Cycler lid temperature = 105°C			

Table 3.5: PCR conditions for Trans-sialidase gene

Step	Temperature	Time	No. of cycle
I. Initial Denaturation	94°C	4 min.	One
II. Cycle (i) Denaturation (ii) Annealing (iii) Synthesis	 94°C 51°C 72°C	 30 sec. 1 min. 1 min. and 30 sec.	Over all total 35 cycle
III. Final extension	72°C	9 min.	One
IV. Hold	4°C		
Thermal Cycler lid temperature = 105°C			

3.2.5.4. Analytical Agarose Gel Electrophoresis

PCR amplified DNA was analyzed by analytical agarose gel electrophoresis as per the procedure described by Sambrook and Russel (2001). The procedure of electrophoresis was same as mentioned in qualitative confirmation of RNA. DNA to be analyzed was charged into wells of gel alongside DNA molecular weight marker for the confirmation of molecular size of the DNA bands in relation to molecular weight marker.

3.2.5.5. Elution of DNA from low melting point agarose gel (preparatory)

DNA required for various manipulations was purified after electrophoresing the DNA in preparatory agarose gel. The method of gel preparation and casting was essentially the same as was done with the analytical method except for the usage of 1% Low Melting Point (LMP) Agarose in place of the analytical grade used earlier. The DNA was electrophoresed at a constant low voltage of 4mV/cm to

facilitate clear separation of the DNA bands. The agarose slice containing the DNA band of interest was carefully excised from the gel under UV illumination with the help of a sterile scalpel and used for elution of the DNA within.

3.2.5.6. DNA purification (Illustra GFX PCR DNA and Gel Band Purification Kit) method

DNA from LMP agarose slices was purified using Illustra GFX PCR DNA and Gel Band Purification Kit according to manufacturer's instruction with the following steps

Capture buffer Type III was added to the weighed agarose gel slice according to the weight of agarose slice. It was mixed by inversion and incubated at 60^0C for 15-30 minutes until the agarose was completely dissolved. When the agarose was completely dissolved then color of capture buffer Type III sample mix was checked and that was yellow. Then it was centrifuged for collecting the liquid at the bottom of the tube. One GFX Micro spin column was placed into one collection tube. Then up to 800 µl of capture buffer type 3-sample mix was transferred on to the assembled GFX Micro spin column and collection tube. It was incubated at room temperature for 1 minute and centrifuged at 16000 x g for 30 seconds. The flow through was discarded by emptying the collection tube and placed the GFX Micro spin column back inside the collection tube. Simple binding steps were repeated until total sample volume was loaded. 500 µl Wash buffer type 1 was added to the GFX Microspin column and then spined the assembled column and collection tube at 16000 x g for 30 seconds. Collection tube was discarded and the GFX Microspin column was transferred to a DNase free 1.5 ml micro centrifuge tube. 50 µl elution buffer type 4 was added to the center of the membrane in the assembled GFX Microspin column and simple collection tube then incubated at room temperature for 1 minute. Then assembled column

and sample collection tube was spined at 16000 x g for 1 minute to recover the purified DNA. Purified DNA was stored at -20^{0}C for further use.

3.2.6. Cloning of DNA fragments

Cloning of DNA fragments into pGEM- T Easy vectors involved the following steps, which are described below.

3.2.6.1. Ligation of DNA fragment with pGEM- T Easy vector

The DNA fragments of *aox* and *ts* gene and the pGEM- T Easy vector 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 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^{0}C)], 6 µl PCR product, 2 µl pGEM- T Easy vector and 2 µl of T4 DNA ligase. The contents were vortexed, spun down in a micro centrifuge for 3-5 seconds and incubated for overnight at 4^{0}C. The ligation mix was used directly for transformation and unused ligation mixture was stored at -20^{0}C for future use.

3.2.6.2. Transformation

The frozen aliquot of JM109 high efficiency competent cells was removed from -70°C storage and placed in an ice bath for thawing (for 5-10 min.). Competent cells were mixed by gentle flicking the tube. 2 µl ligation mixture and 50 µl JM109 competent cells were mixed in 1.5 ml eppendorf tube and placed the tube in ice for 20 minutes. The cells were given heat shock at 42^{0}C for 45 sec before snap cooling on ice for 2 minutes. Immediately thereafter 950 µl of pre warmed SOC (Appendix–III.6) was added to the cells and the suspension was

incubated at 37^0C for 1 hr and 30 min. with shaking of 150 rpm in a shaking incubator. 100 µl IPTG and 20 µl X-GAL was spreaded over the surface of LB agar plates with ampicillin (50µg/ml). After incubation of transformation culture 100 µl of each transformation culture were plated onto antibiotic agar plates in duplicate and incubated at 37^0C overnight (16-20 hr). Colonies that appeared were screened for the presence of plasmids.

3.2.6.3. Screening for recombinants

Both white and blue colonies were grown in the plate. Larger single white colonies were picked up individually and inoculated into LB broth (Appendix–III.4) containing final concentration of ampicilin (50µg/ml) and kept in a water bath cum shaker (at 37^oC and 150 rpm) for 16 hrs. Subsequently, the plasmid DNA was isolated from all the bacterial cultures using the kit.

3.2.6.4. Protocol for plasmid preparation (by using illustra plasmid prep mini spin kit)

3.2.6.4.1. Harvesting of bacterial culture

1.5 ml bacterial culture was taken in eppendorf tube, centrifuged for 30 second at 16000 x g, poured off and supernatant was discarded.

3.2.6.4.2. Lysis

175 µl lysis buffer type 7 was added and pellet was resuspended. Then 175 µl lysis buffer type 8 was added and mixed by gentle inversion till solution became clear and viscous. After 5 min of lysis reaction 350 µl lysis buffer type 9 was added and mixed by gentle inversion till precipitate was evenly dispersed. Then it was centrifuged for 4 min at 16000 x g.

3.2.6.4.3. Plasmid binding

Supernatant was transferred into plasmid mini column inside collection tube. Then it was centrifuged at 30 sec at 16000 x g and flowthrough was discarded.

3.2.6.4.4. Wash and dry

400 µl Wash buffer type 1 was added to the plasmid mini column and then spined the assembled column and collection tube at 16000 x g for 30 seconds. Flow through and collection tube was discarded and the plasmid mini column was transfered to a new DNase free 1.5 ml micro centrifuge tube.

3.2.6.4.5. Elution

100 µl elution buffer type 4 was added to the center of the membrane in the assembled plasmid mini column and simple collection tube then incubated at room temperature for 30 sec. Then assembled column and sample collection tube was spined at 16000 x g for 30 sec to recover the purified plasmid DNA. Purified plasmid DNA was kept at -20^{0}C for storage.

3.2.6.5. Confirmation of clones

Confirmation of clones was done by Restriction Enzyme digestion of plasmid DNAs and Colony PCR of plasmid colonies.

3.2.6.5.1. Restriction Enzyme digestion (to check the insert size)

After checking the quality of the plasmid DNAs in agarose gel electrophoresis, they were subjected to restriction enzyme digestion using *Eco*RI. In 10 µl digestion mixture 5 µl plasmid DNA, 1 µl *Eco*RI (Promega), 1 µl *Eco*RI buffer (Promega) and 3 µl ultrapure water was

added. After 4 hours digestion at 37°C in water bath 2µl 6X loading dye was added to the mixture and analyzed by running 1.2% agarose gel electrophoresis alongside a DNA molecular weight marker. Release of the expected size fragment confirmed the recombinants.

3.2.6.5.2. Colony PCR

This protocol was designed to quickly screen the plasmid inserts directly from *E. coli* colonies. Colonies were screened for recombinants by colony PCR as per procedure described in Promega protocols. PCR was carried out in the presence of insert specific primers. The colony PCR reaction mixture was similar to PCR reaction mixture, only template DNA was not added. To each PCR tube containing the PCR reaction, a single colony was added. For each amplification reaction white colonies were added in two PCR tubes and blue colonies were added in two tubes. A fine yellow pipette tip attached to a pipetter was used to separate a colony from culture and pipetted up and down to mix the colony into PCR reaction mixture (The amount of cells were small, just a touch was done, the small amount required to fill the end of the opening was sufficient). Sufficient mixing of the colony in PCR tube was done for complete cell lysis and high yields. The conditions of amplification applied were similar to those applied for the amplification of the specific genes. The amplified products were analyzed by agarose gel electrophoresis using standard molecular size markers.

3.2.7. Sequencing

Purified plasmids of *aox* and *ts* genes were sequenced from Eurofins Genomics India Pvt. Ltd., Whitefield, Bengaluru. The sequence obtained was then matched using BLAST (Biological Local Alignment Search Tool) software. After confirmation of the *aox* and *ts* genes nucleotide sequences of *T. evansi* isolated from the host camel

(*Camelus dromedarius*), the nucleotide sequences were submitted to GenBank, NCBI database.

3.2.7.1. Sequence analysis

After getting the accession numbers of individual gene sequences phylogenetic and sequence analysis of the *aox* and *ts* genes 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* and *ts* genes was done by using Neighbor-Joining (NJ) method and maximum parsimony (MP) method and implemented with bootstrap test involving simple stepwise addition.

3.2.7.2. Multiple sequence alignments

The amino acid sequences of the *aox* and *ts* genes from *T. evansi* was BLASTed against similar sequences in the public database (http://www.ncbi.nlm.nih.gov/). The sequences of Aox proteins of *T. brucei* (GenBank Accession Nos. TBU52964, XM817851, AB070617 AB070614, and AB211244), *T. evansi* (GenBank Accession No. AB188573), *T. congolense* (GenBank Accession No. AB188571 and AB188572), *T. vivax* (GenBank Accession No. AB070521) were collected and sequence homology between species was determined. For sequence homology determination of Ts protein, the sequences of *T. brucei* (GenBank Accession Nos. XM841210, XM841212, AF181287, AF310232, XM842471, XM842470, XM839645, and XM946607), and *T. evansi* (GenBank Accession No. FJ597949) were collected. Multiple sequence alignments of obtained protein sequences of *aox* and *ts* genes were performed with Praline sequence alignment software with default parameters.

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4. Results

Trypanosomosis is a very serious and often fatal blood protozoan disease of camels and has been observed in domestic and wild animals and recent reports suggest its ability to infect humans with widest geographical range. Chemotherapeutic, chemoprophylactic and fly control strategies are not quite enough to control this disease. Recent effort towards the development of a vaccine against *Trypanosoma evansi* has identified several promising candidate vaccine antigens, including non-variant genes of this parasite. Keeping this in view, the present study was undertaken to amplify the

aox and *ts* genes of *T. evansi* from camels by Polymerase Chain Reaction (PCR), clone the amplicons in a suitable plasmid vector and sequence the genes as these candidate genes could be helpful in future for developing vaccines against *Trypanosoma evansi*. The results obtained are as follows:

4.1. Identification of Camel (*Camelus dromedarius*) infected with *Trypanosoma evansi* infection and preparation of stained blood film

Trypanosoma evansi is a highly pathogenic protozoan of camel which is having clinical signs depending on strain pathogenicity, host species, general stresses on the host and local epidemiological conditions.

In this regard, a male camel calf was reported to be off-feed and in lethargic condition (Plate 2). The camel was also having profuse lacrimation and nasal discharge, conjuctival mucosa found to be icteric and there was also rise of body temperature (104-105F^{o}). Wet smear examination of the blood collected from the camel revealed active lashing movements of the extracellular parasites.

The camel suffering from live, long, slender trypanosomes with well developed undulating membrane and free flagellum from the Giemsa stained blood smear has been presented in Plate 3. Further, *Trypanosoma evansi* was identified by its morphological characteristics.

4.2. Isolation and qualitative confirmation of genomic DNA

The total genomic DNA was isolated from the blood of camel and *Trypanosoma evansi* as per the protocols given by ready to use kit as mentioned in materials and methods. The genomic DNA was

analyzed in 0.8% analytical agarose gels and was found to be intact near the well without smearing and has been presented in Plate 4.

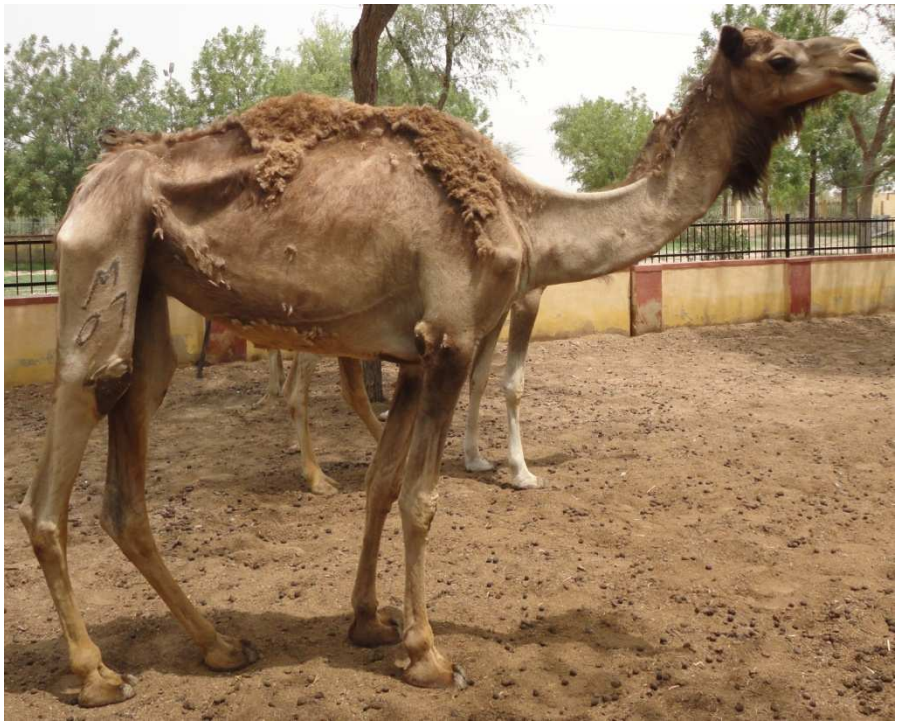

Plate 2. Camel infected with 'Surra' disease

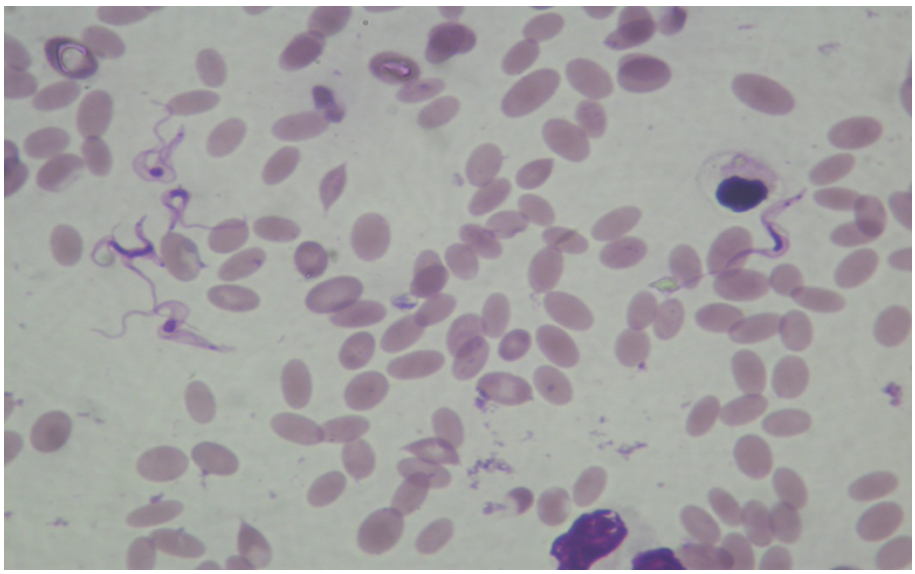

Plate 3. Giemsa stained blood smear of *Trypanosma evansi* from camel (Magnification-400X)

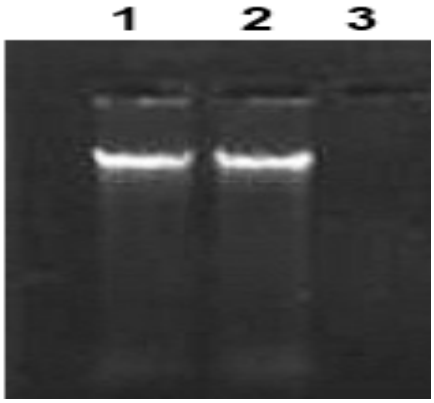

Plate 4. Genomic DNA of *Trypanosoma evansi* in lane 1 and 2

4.3. Amplification of Alternative oxidase and Trans-sialidase genes of *Trypanosoma evansi* by PCR

Total genomic DNA was isolated from the blood of camel and *T. evansi* used as template for amplification. The gene specific primers for a*ox* gene Forward 5'AGGTAATATCTTGACACCAAGAGC3' and Reverse 5'ACGTGTTTGTTTACATTACTCGCA3' and for *ts* gene

Forward 5'ATGGAGGAACTCCACCAACAAAT3' and Reverse 5'TATAGATCTTCAAATCGCCAACACATACAT3' were used.

PCR amplified DNA was charged into wells of gel alongside DNA molecular weight marker for the confirmation of molecular size of the amplicons. For both the genes, these were single and intensely amplified product was seen and this was absent in corresponding control amplifications where no target DNA was used. The results for both genes were reproducible when the amplifications were repeated. The amplification band of *aox* gene was obtained in between 1000 bp and 500 bp, which has been presented in Plate 5 and the size 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. The amplification band of *ts* gene was obtained in between 3000 bp and 2000 bp, which has been presented in Plate 6 and it, was found to be 2241 bp.

4.4. Cloning of Alternative oxidase and Trans-sialidase genes of *Trypanosoma evansi* into pGEM-T Easy vector

The amplicons (obtained from genomic DNA amplification) were purified from the LMP agarose gel and ligated with pGEM-T Easy vector (Promega). 100 µl of transformation culture was plated onto X-gal-IPTG-Ampicillin agar plate. There were several white colonies along with a few blue colonies (plate no. 7). The blue colonies represent 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.

4.4.1. Confirmation of clones by restriction digestion

Plasmid DNAs were extracted from positive colonies (white) grown in LB medium containing ampicillin, digested with *Eco*RI and analyzed by 1.2% analytical agarose gel electrophoresis using 1kb plus

molecular weight marker. Two well separated DNA bands were seen in case of plasmids isolated from positive colonies upon digestion with *Eco*RI (lanes 2 & 3), the less intense lower band may correspond to the insert. Release of DNA fragments of around 990 bp for *aox* and 2241 bp for *ts* gene were found after restriction enzyme digestion, the results of which has been presented in Plate 8 and 9.

4.4.2. Confirmation of clones by Colony PCR

Further, colony PCR was done for quick screening of plasmid inserts directly from *E. coli* colonies. The conditions of amplifications were similar to those applied for the amplification of the specific genes. The amplified products were analyzed by agarose gel electrophoresis using standard molecular size markers. For both the genes amplifications were found with white colonies and no amplification was found with blue colony (Plate 10 and 11) in the present study.

4.5. Sequencing

After confirmation of clones of *aox* and *ts* genes the plasmid DNAs along with their respective forward and reverse primers were sent to Eurofins Genomics India Pvt Ltd., Whitefield, Bengaluru, for getting the sequences. The coding sequences of *aox* and *ts* genes according to the results obtained were of 990 bp and 2241 bp respectively. These sequences were then matched using BLAST software. After confirmation of the *aox* and *ts* genes nucleotide sequences of *T. evansi* isolated from the host camel (*Camelus dromedarius*), the sequences were submitted to GenBank, NCBI database to which the assigned accession numbers are KF686336 for *aox* gene (Plate 12) and KF686337 for *ts* gene (Plate 16).

4.5.1. Sequence analysis

Sequence analysis revealed that the length of the coding sequences of *aox* and *ts* genes of *T. evansi* from Bikaner, India were 990 bp and 2241 bp respectively. The phylogenetic and sequence

analysis was done by use of Clustal X and MEGA5 softwares. Tree topology is based on the Neighbor-Joining (NJ) method with100% bootstrap values. The NJ, bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. 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. Phylogenetic tree analysis of *aox* and *ts* genes using maximum parsimony (MP) also showed same topology as NJ method. The sequences of desired genes were compared with other related species gene sequences.

After analysis, the gene sequences showed a wide range of homology within the organisms of Trypanosomatidae family. The obtained Alternative oxidase gene sequence showed 99.9% homology towards *T. brucei* (AB070614, AB211244) and 99.0% similarities with *T. brucei* (TBU52964, AB070617 and XM817851). A 100 percent 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 Trans-sialidase gene sequence showed 99% homology with *T. evansi* (KF686337), 99% with *T. brucei* (XM841212). 99% sequence

similarity was found between obtained *ts* gene sequence and *T. evansi* (FJ597949). Slightly lower homology was documented between the obtained *ts* gene sequence and *T. brucei* (AF310232 and AF181287) and *T. brucei* (XM841210). The comparison with *T. brucei* (XM842470), *T. brucei* (XM842471) and *T. brucei* (XM946607) a member belonging to Trypanosomatidae family showed only 49%, 48%, and 28% homology, respectively.

According to neighbor-joining phylogenetic tree analysis of *ts* gene, *T. brucei* (XM842470) and *T. brucei* (XM842471) are placed as two sub cluster of one mega cluster. *T. brucei* (XM841212), *T. brucei* (AF310232), *T. brucei* (XM841210) and *T. brucei* (XM839645) as four sub cluster of one mega cluster and the other mega cluster comprising of rest of the species.

The assigned accession numbers and the names of workers who submitted them are presented in Plate 13, 14, 17, 18 and Tables 4.1 to 4.4.

4.5.2. Multiple sequence alignment

Multiple sequence alignment of obtained protein sequences of *aox* and *ts* genes was performed with Praline sequence software. The amino acid alignments are presented in Plate 15 and 16. The asterisks below the alignments indicate the positions of highly conserved amino acid residues.

The obtained Aox protein sequence of *Trypanosoma evansi* was of 329 amino acids (KF686336) which showed 100% amino acid sequence identity of *Trypanosoma evansi* (AB188573). Similarly, 99% sequence identity of *Trypanosoma evansi* to *Trypanosoma brucei* (AB070614, AB211244, XM817851, TBU52964 and AB070617) was documented. Likewise 76% sequence identity of *Trypanosoma evansi* to *Trypanosoma congolense* (AB188571 and AB188572) and 68%

sequence identity of *Trypanosoma evansi* to *Trypanosoma vivax* (AB070521) was reported.

The obtained Ts protein sequence of *Trypanosoma evansi* was of 745 amino acids (KF686337) which showed 99% amino acid sequence identity of *Trypanosoma evansi* (FJ597949).

Similarly 99% sequence identity of *Trypanosoma evansi* to *Trypanosoma brucei* (XM841212) was reported. Likewise 98% sequence identity of *Trypanosoma evansi* to *Trypanosoma brucei* (AF310232 and AF181287) was documented.

The 93% of sequence identity of *Trypanosoma evansi* to *Trypanosoma brucei* (XM841210) was reported. The lower sequence identity, 49% of *Trypanosoma evansi to Trypanosoma brucei* (XM842470) was seen. The 48% sequence identity of *Trypanosoma evansi* to *Trypanosoma brucei* (XM842471 and XM839645) was documented.

At last, the lowest sequence identity, 28% of *Trypanosoma evansi* to *Trypanosoma brucei* (XM946607) was reported.

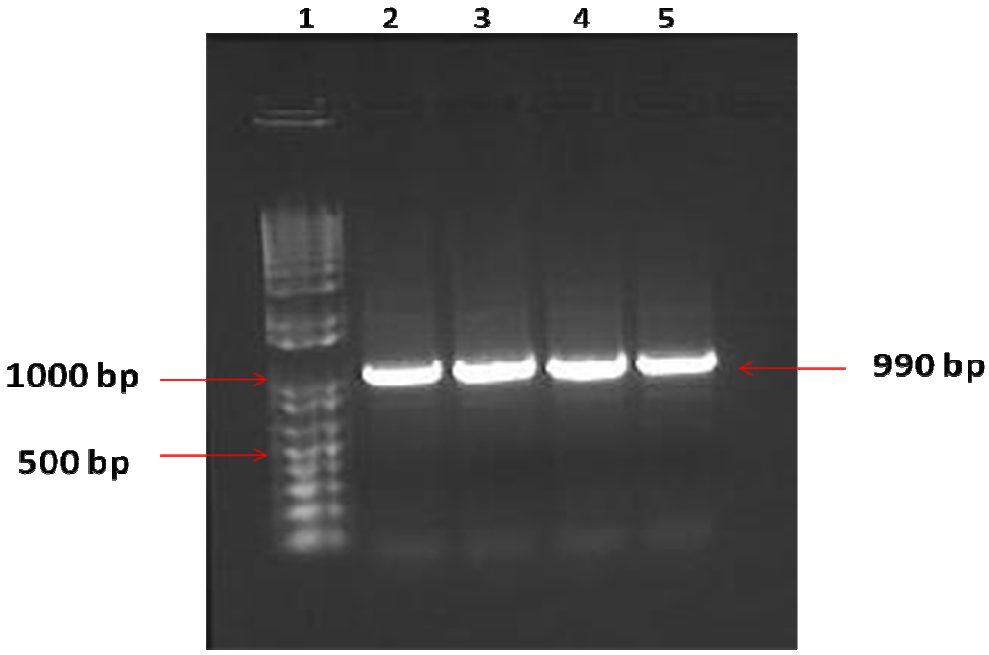

Plate 5. Amplification of Alternative oxidase gene of *Trypanosoma evansi* by PCR

1. 1Kb plus DNA Ladder

2 – 5. Amplicons

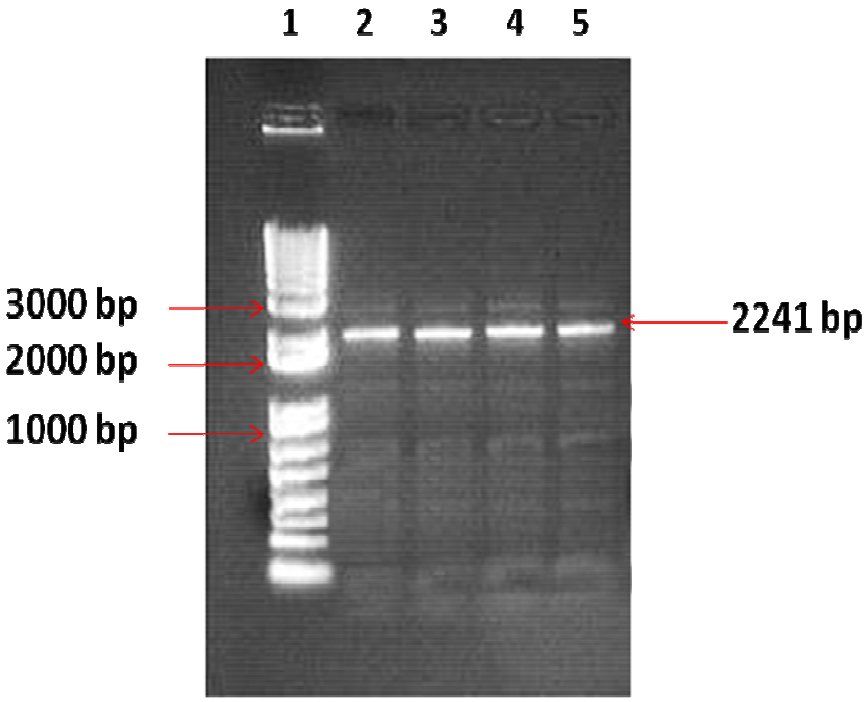

Plate 6. Amplification of Trans-sialidase gene of *Trypanosoma evansi* by PCR

1. 1Kb plus DNA Ladder

2 – 5. Amplicons

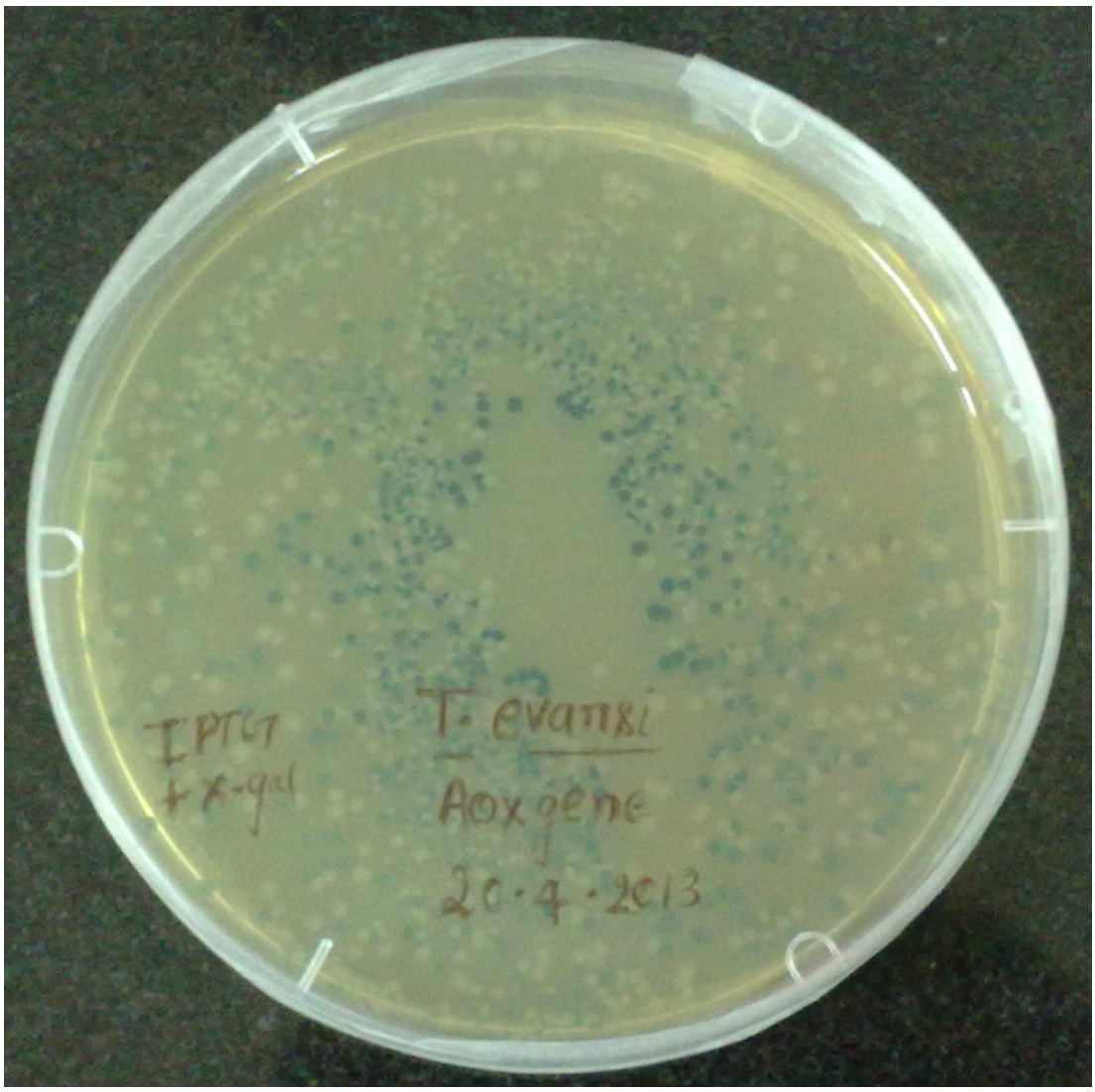

Plate 7. Appearance of blue and white colony in LB agar plate showing culture of Alternative oxidase gene of *Trypanosoma evansi*

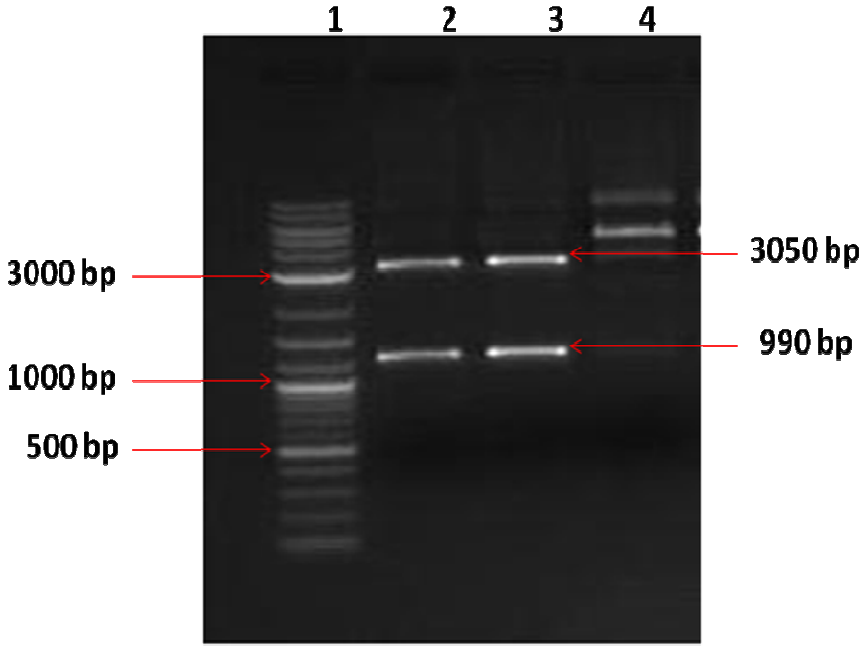

Plate 8. Alternative oxidase gene fragments of *Trypanosoma evansi* after restriction digestion of *aox* gene plasmid

1. 1Kb plus DNA Ladder (2 Log DNA)

2 – 3. Alternative oxidase gene clone

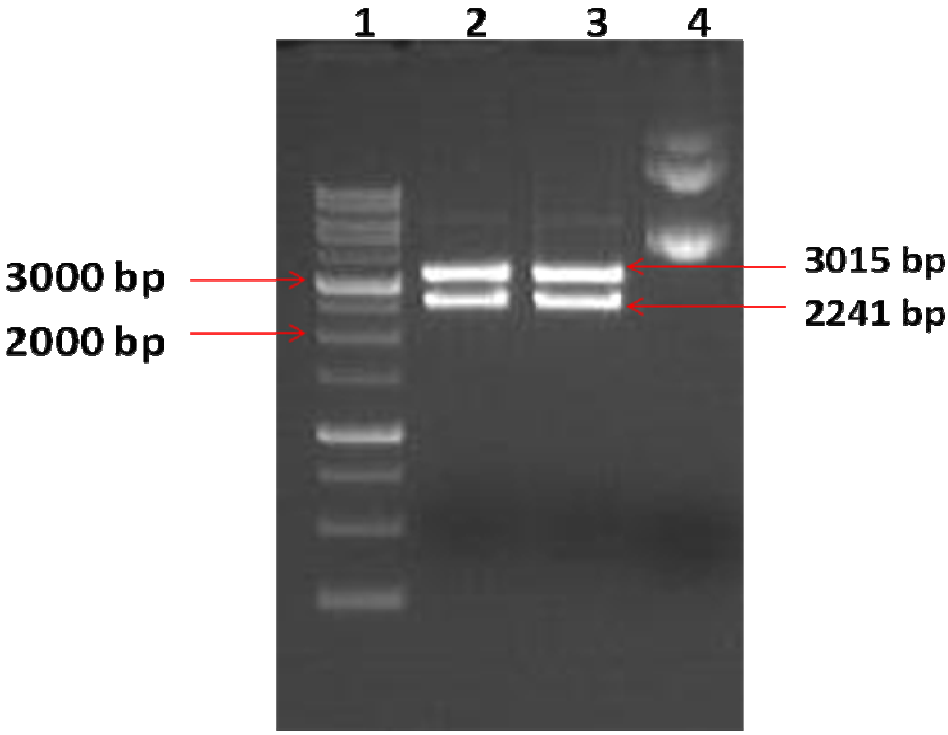

Plate 9. Trans-sialidase gene fragments of *Trypanosoma evansi* after restriction digestion of *ts* gene plasmid

1. 1Kb plus DNA Ladder

2 -3. Trans-sialidase gene clone

4. Uncut plasmid

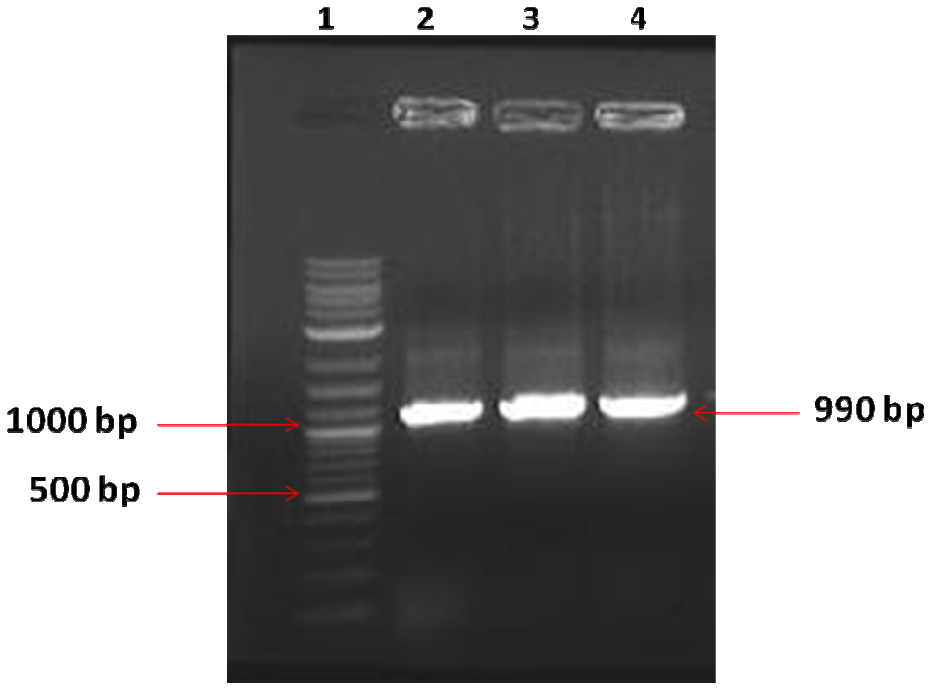

Plate 10. Amplification of Alternative oxidase gene of *Trypanosoma evansi* by Colony-PCR

1. 1Kb plus DNA Ladder

2 – 3. PCR reaction with white colony shows amplification

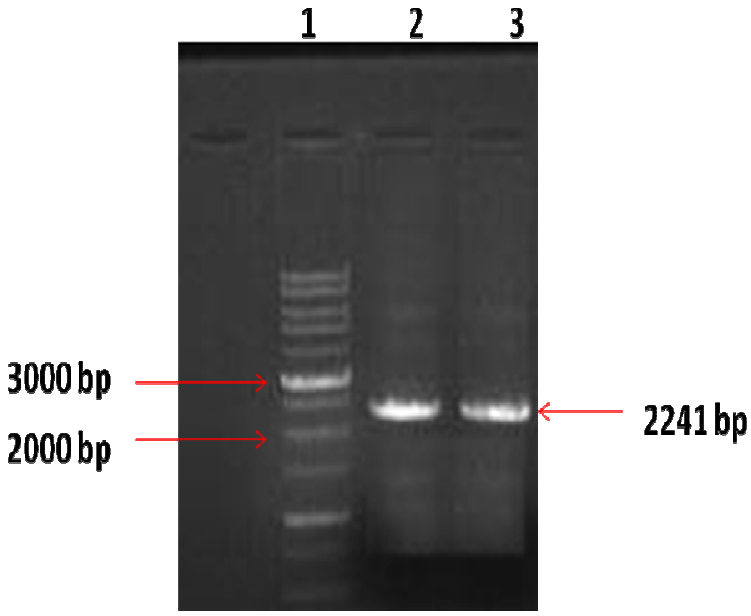

Plate 10. Amplification of Trans-sialidase gene of *Trypanosoma evansi* by Colony-PCR

1. 1Kb plus DNA Ladder

2 – 3. PCR reaction with white colony shows amplification

ORIGIN

```
        1  ATGTTTCGTA  ACCACGCATC  GAGGATCACT  GCCGCAGCTG
CGCCTTGGGT GCTCCGGACG
       61  GCTTGCCGCC  AGAAGTCTGA  CGCCAAAACA  CCTGTGTGGG
GACACACTCA ACTGAACCGT
      121  CTCAGTTTTT  TGGAAACCGT  GCCTGTCGTT  CCTTTGCGTG
TTTCCGATGA AAGCAGTGAG
      181  GACCGCCCCA  CCTGGAGCCT  TCCCGATATT  GAGAATGTGG
CCATAACGCA CAAGAAGCCA
      241  AACGGCCTCG  TTGATACACT  CGCCTACCGC  AGCGTCCGCA
CCTGCCGCTG GTTATTTGAC
      301  ACATTCTCTC  TCTACCGTTT  CGGTTCCATC  ACGGAGAGCA
AAGTCATCAG CCGCTGTCTT
      361  TTTCTTGAAA  CTGTTGCCGG  TGTCCCGGGG  ATGGTCGGTG
GAATGTTGCG CCACCTTTCA
      421  TCATTGCGGT  ACATGACGCG  CGACAAGGGT  TGGATTAACA
CTCTTCTTGT TGAAGCAGAG
      481  AATGAGCGCA  TGCACCTCAT  GACGTTCATT  GAACTTCGCC
AGCCAGGGCT CCCCCTACGC
      541  GTTTCCATCA  TTATTACGCA  AGCCATTATG  TACCTCTTCC
TCCTGGTCGC CTATGTGATT
      601  TCCCCCCGTT  TTGTACACCG  CTTTGTCGGT  TACCTTGAAG
AGGAAGCCGT CATTACATAC
      661  ACCGGCGTTA  TGAGAGCAAT  TGACGAAGGA  AGGCTGCGCC
CTACCAAAAA TGATGTTCCC
      721  GAAGTGGCTC  GCGTGTACTG  GAACCTCAGC  AAAAATGCCA
CATTCCGCGA CCTCATCAAC
      781  GTGATCCGAG  CTGACGAGGC  GGAGCACCGT  GTCGTTAACC
ACACATTTGC TGACATGCAC
      841  GAAAAACGCC  TGCAAAACAG  TGTCAACCCC  TTCGTTGTTC
TGAAGAAGAA CCCGGAGGAA
      901  ATGTACTCCA  ACCAACCAAG  TGGTAAGACA  AGGACGGATT
TTGGAAGCGA AGGCGCCAAA
      961  ACTGCGAGTA  ATGTAAACAA  ACACGTGTAA
```

Plate 12. The coding sequence of Alternative oxidase gene of *Trypanosoma evansi* isolated from *Camelus dromedarius*

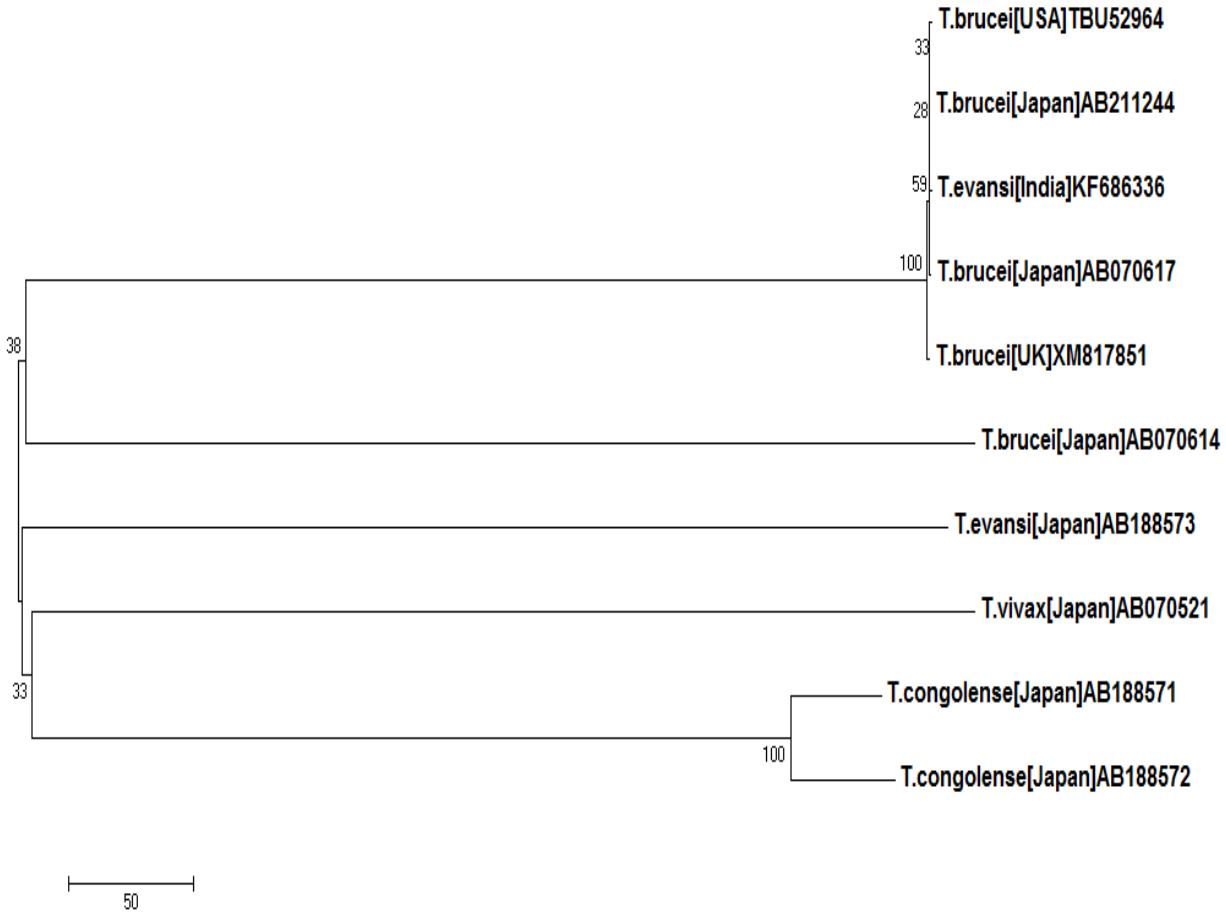

Plate 13a. Phylogenetic tree analysis of Alternative oxidase gene using the Neighbor-Joining method

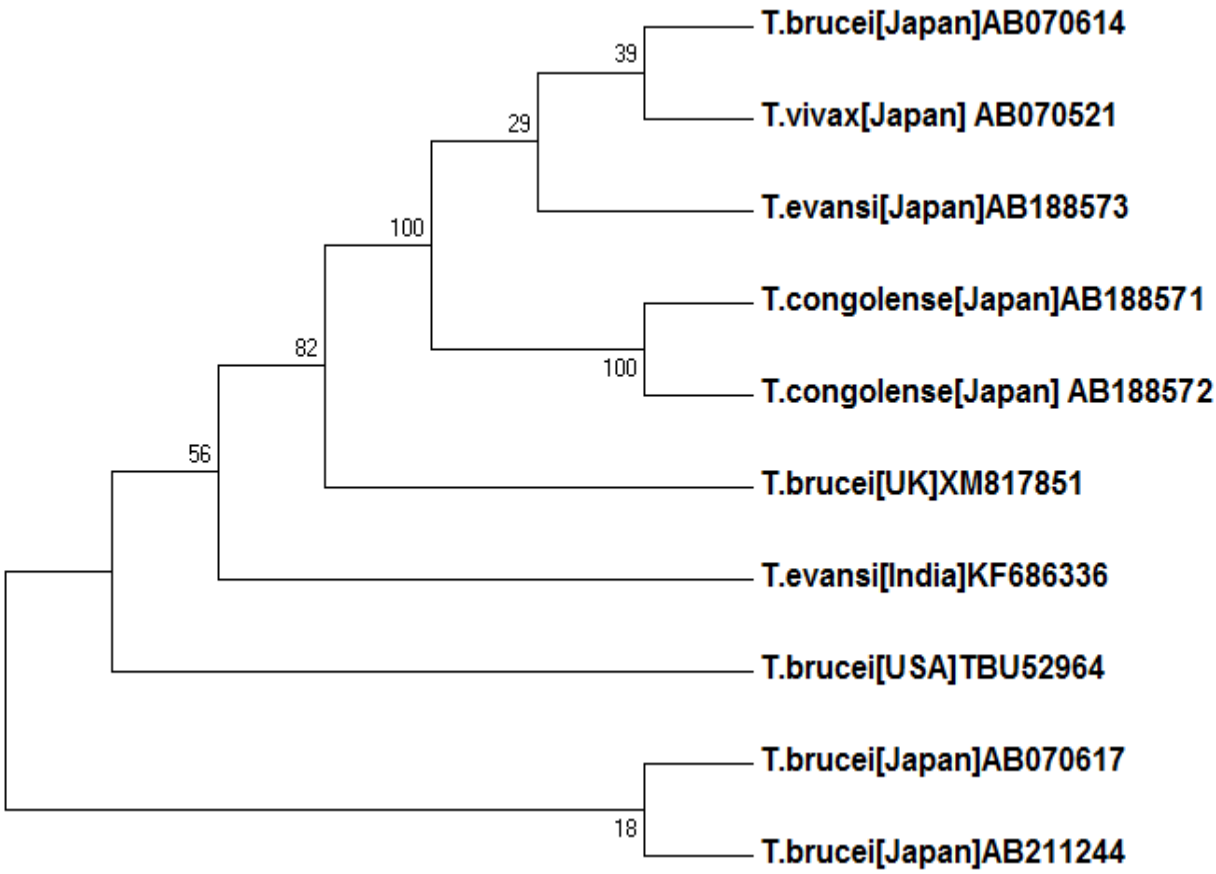

Plate 13b. Phylogenetic tree analysis of Alternative oxidase gene using maximum parsimony method

Table 4.1: Sequence identity of Alternative oxidase gene of *Trypanosoma evansi* with other allied species

Identity of *aox* gene for *Trypanosoma evansi*	*T.congolense* [Japan] AB188571	*T.congolense* [Japan] AB188572	*T.evansi* [India] KF686336	*T.evansi* [Japan] AB188573	*T.brucei* [Japan] AB070614	*T.brucei* [Japan] AB211244	*T.brucei* [UK] XM817851	*T.brucei* [USA] TBU52964	*T.brucei* [Japan] AB070617	*T.vivax* [Japan] AB070521
T.congolense[Japan] AB188571	**	91.6	76.0	75.2	75.3	76.1	76.0	76.0	76.0	69.3
T.congolense[Japan] AB188572	91.6	**	76.1	75.5	75.4	76.0	75.9	75.9	75.9	68.8
T.evansi [India] KF686336	76.0	76.1	**	100.0	99.9	99.9	99.7	99.8	99.8	68.5
T.evansi [Japan] AB188573	75.2	75.5	100.0	**	99.9	99.9	99.7	99.8	99.8	66.2
T.brucei [Japan] AB070614	76.3	75.4	99.9	99.9	**	100.0	99.8	99.9	99.9	66.3
T.brucei [Japan] AB211244	76.1	76.0	99.9	99.9	100.0	**	99.8	99.9	99.9	68.6
T.brucei [UK] XM817851	76.0	75.9	99.7	99.7	99.8	99.8	**	99.7	99.7	68.5
T.brucei [USA] TBU52964	76.0	75.9	99.8	99.8	99.9	99.9	99.7	**	99.8	68.7
T.brucei [Japan] AB070617	76.0	75.9	99.8	99.8	99.9	99.9	99.7	99.8	**	68.7
T.vivax [Japan] AB070521	69.3	68.8	68.5	66.2	66.3	68.6	68.5	68.7	68.7	**

Table 4.2: Alternative oxidase gene for different species of *Trypanosoma* as submitted by various workers

Sl. No.	Identity of *aox* gene for *Trypanosoma evansi*	Accession No.	Product Length	Collection Country	Reference
1.	*T. evansi*	KF686336	990 bp	India	Shinde *et al.* (2013)
2.	*T. brucei*	XM817851	990 bp	UK	Berriman *et al.* (2012)
3.	*T. brucei*	AB070617	990 bp	Japan	Fukai *et al.* (2011)
4.	*T. brucei*	AB211244	990 bp	Japan	Nakamura *et al.* (2006)
5.	*T. evansi*	AB188573	1128 bp	Japan	Suzuki *et al.* (2005)
6.	*T. congolense*	AB188571	1050 bp	Japan	Suzuki *et al.* (2005)
7.	*T. congolense*	AB188572	1016 bp	Japan	Suzuki *et al.* (2005)
8.	*T. brucei*	TBU52964	990 bp	USA	Chaudhuri and Hill (2004)
9.	*T. vivax*	AB070521	1193 bp	Japan	Suzuki *et al.* (2004)
10.	*T. brucei*	AB070614	1147 bp	Japan	Kita, K. (2001)

Unconserved 0 1 2 3 4 5 6 7 8 9 10 **Conserved**

```
                                  . . . . . . . . . 10 . . . . . . . . . 20 . . . . . . . . . 30 . . . . . . . . . 40 . . . . . . . . . 50
T.evansi(KF686336)                 F R N H A S R I  T A A A A P W V L R  T A - C R Q K S D A  K T P V W G H T Q L  N R L S F L E T V P  V
T.brucei(TBU52964)                M F R N H A S R I  T A A A A P W V L R  T A - C R Q K S D A  K T P V W G H T Q L  N R L S F L E T V P  V
T.brucei(XM817851)                M F R N H A S R I  T A A A A P W V L R  T A - C R Q K S D A  K T P V W G H T Q L  N R L S F L E T V P  V
T.brucei(AB070617)                M F R N H A S R I  T A A A A P W V L R  T A - C R Q K S D A  K T P V W G H T Q L  N R L S F L E T V P  V
T.brucei(AB070614)                M F R N H A S R I  T A A A A P W V L R  T A - C R Q K S D A  K T P V W G H T Q L  N R L S F L E T V P  V
T.evansi(AB188573)                M F R N H A S R I  T A A A A P W V L R  T A - C R Q K S D A  K T P V W G H T Q L  N R L S F L E T V P  V
T.brucei(AB211244)                M F R N H A S R I  T A A A A P W V L R  T A - C R Q K S D A  K T P V W G H T Q L  N R L S F L E T V P  V
T.vivax(AB070521)                 M L R Y R T P T L  A A A A A M K V Q Q  M F T H S S S S S N  K T P V W G Y T Q V  N R L S F V D L V P  R
T.congolense(AB188571)            M F R I N A S C I  N A A A A T T T L W  S A - M R L R S D G  K A P V W G H T Q L  N R L S F I D A V P  A
T.congolense(AB188572)            M L R I S A S C I  N F A A A T T T M W  S A - M R L R S D G  K A P V W G H T Q L  N R L S F I D V V P  V
Consistency                       * 8 * 5 6 8 8 5 9  7 8 * * * 6 5 8 7 6  7 8 0 5 8 6 7 * 8 6  * 8 * * * 9 * * 9  * * * * * 8 8 7 * *  7

                                  . . . . . . . . . 60 . . . . . . . . . 70 . . . . . . . . . 80 . . . . . . . . . 90 . . . . . . . . . 100
T.evansi(KF686336)                V P L R V S D E S  S E D R P T W S L P  D I E N V A I T H K  K P N G L V D T L A  Y R S V R T C R W L  F
T.brucei(TBU52964)                V P L R V S D E S  S E D R P T W S L P  D I E N V A I T H K  K P N G L V D T L A  Y R S V R T C R W L  F
T.brucei(XM817851)                V P L R V S D E S  S E D R P T W S L P  D I E N V A I T H K  K P N G L V D T L A  Y R S V R T C R W L  F
T.brucei(AB070617)                V P L R V S D E S  S E D R P T W S L P  D I E N V A I T H K  K P N G L V D T L A  Y R S V R T C R W L  F
T.brucei(AB070614)_               V P L R V S D E S  S E D R P T W S L P  D I E N V A I T H K  K P N G L V D T L A  Y R S V R T C R W L  F
T.evansi(AB188573)_               V P L R V S D E S  S E D R P T W S L P  D I E N V A I T H K  K P N G L V D T L A  Y R S V R T C R W L  F
T.brucei(AB211244)                V P L R V S D E S  S E D R P T W S L P  D I E N V A I T H K  K P N G L V D T L A  Y R S V R T C R W L  F
T.vivax(AB070521)                 V P A R E E D E S  S S E R P H W N L P  D I E K V A I T H K  P A E G I V D T L A  Y R L V R T C R W A  F
T.congolense(AB188571)            V P Q R V G D E S  S E D R P T W Q L A  D V E N V A I T H K  K P N G L V D T L A  Y R G V R T C R W A  F
T.congolense(AB188572)            V P H R V G D E S  S E E R P T W Q L A  D V E N V A I T H K  K P N G L V D T L A  Y R G V R T C R W A  F
Consistency                       * * 5 * 8 7 * * *  * 8 8 * * 8 * 7 * 7  * 9 * 8 * * * * * *  8 8 8 * 9 * * * * *  * * 6 * * * * * * 7  *

                                  . . . . . . . . . 110 . . . . . . . . . 120 . . . . . . . . . 130 . . . . . . . . . 140 . . . . . . . . . 150
T.evansi(KF686336)                D T F S L Y R F G  S I T E S K V I S R  C L F L E T V A G V  P G M V G G M L R H  L S S L R Y M T R D  K
T.brucei(TBU52964)                D T F S L Y R F G  S I T E S K V I S R  C L F L E T V A G V  P G M V G G M L R H  L S S L R Y M T R D  K
T.brucei(XM817851)                D T F S L Y R F G  S I T E S K V I S R  C L F L E T V A G V  P G M V G G M L R H  L S S L R Y M T R D  K
T.brucei(AB070617)                D T F S L Y R F G  S I T E S K V I S R  C L F L E T V A G V  P G M V G G M L R H  L S S L R Y M T R D  K
T.brucei(AB070614)                D T F S L Y R F G  S I T E S K V I S R  C L F L E T V A G V  P G M V G G M L R H  L S S L R Y M T R D  K
T.evansi(AB188573)_               D T F S L Y R F G  S I T E S K V I S R  C L F L E T V A G V  P G M V G G M L R H  L S S L R Y M T R D  K
T.brucei(AB211244)                D T F S L Y R F G  S I T E S K V I S R  C L F L E T V A G V  P G M V G G M L R H  L S S L R Y M T R D  K
T.vivax(AB070521)                 D T F S L Y R F G  S L T E Q K V I N R  C L F L E T V A G V  P G M V G G M L R H  L T S L R Q M R R D  K
T.congolense(AB188571)            D T F S L Y R F G  N L T E G K V I N R  C L F L E T V A G V  P G M V G G M L R H  L K S L R Y M T R D  K
T.congolense(AB188572)            D T F S L Y R F G  S L T E G K V I N R  C L F L E T V A G V  P G M V G G M L R H  L K S L R Y M T R D  K
Consistency                       * * * * * * * * *  9 8 * * 7 * * * 7 *  * * * * * * * * * *  * * * * * * * * *  * 7 * * * 8 * 8 * * *

                                  . . . . . . . . . 160 . . . . . . . . . 170 . . . . . . . . . 180 . . . . . . . . . 190 . . . . . . . . . 200
T.evansi(KF686336)                G W I N T L L V E  A E N E R M H L M T  F I E L R Q P G L P  L R V S I I I T Q A  I M Y L F L L V A Y  V
T.brucei(TBU52964)                G W I N T L L V E  A E N E R M H L M T  F I E L R Q P G L P  L R V S I I I T Q A  I M Y L F L L V A Y  V
T.brucei(XM817851)                G W I N T L L V E  A E N E R M H L M T  F I E L R Q P G L P  L R V S I I I T Q A  I M Y L F L L V A Y  V
T.brucei(AB070617)                G W I N T L L V E  A E N E R M H L M T  F I E L R Q P G L P  L R V S I I I T Q A  I M Y L F L L V A Y  V
T.brucei(AB070614)_               G W I N T L L V E  A E N E R M H L M T  F I E L R Q P G L P  L R V S I I I T Q A  I M Y L F L L V A Y  V
T.evansi(AB188573)                G W I N T L L V E  A E N E R M H L M T  F I E L R Q P G L P  L R V S I I I T Q A  I M Y L F L L V A Y  V
T.brucei(AB211244)                G W I N T L L V E  A E N E R M H L M T  F I E L R Q P G L P  L R V S I I I T Q A  I M Y L F L L V A Y  V
T.vivax(AB070521)                 G W I N T L L V E  A E N E R M H L M T  F I E L R Q P G V V  F R L S I K I T Q A  I M Y S F L L I A Y  I
T.congolense(AB188571)            G W I N T L L I E  A E N E R M H L M T  F I E L R Q P G F A  F R V S I I V T Q A  I M Y L F L L T A Y  I
T.congolense(AB188572)            G W I N T L L V E  A E N E R M H L M T  F I E L R Q P G F T  F R V S I I V T Q A  I M Y L F L L T A Y  I
Consistency                       * * * * * * * 9 *  * * * * * * * * * *  * * * * * * * 7 6  7 * 9 * * 8 9 * * *  * * * 8 * * * 7 * *  9

                                  . . . . . . . . . 210 . . . . . . . . . 220 . . . . . . . . . 230 . . . . . . . . . 240 . . . . . . . . . 250
T.evansi(KF686336)                I S P R F V H R F  V G Y L E E E A V I  T Y T G V M R A I D  E G R L R P T K N D  V P E V A R V Y W N  L
T.brucei(TBU52964)                I S P R F V H R F  V G Y L E E E A V I  T Y T G V M R A I D  E G R L R P T K N D  V P E V A R V Y W N  L
T.brucei(XM817851)                I S P R F V H R F  V G Y L E E E A V I  T Y T G V M R A I D  E G R L R P T K N D  V P E V A R V Y W N  L
T.brucei(AB070617)                I S P R F V H R F  V G Y L E E E A V I  T Y T G V M R A I D  E G R L R P T K N D  V P E V A R V Y W N  L
T.brucei(AB070614)_               I S P R F V H R F  V G Y L E E E A V I  T Y T G V M R A I D  E G R L R P T K N D  V P E V A R V Y W N  L
T.evansi(AB188573)_               I S P R F V H R F  V G Y L E E E A V I  T Y T G V M R A I D  E G R L R P T K N D  V P E V A R V Y W N  L
T.brucei(AB211244)                I S P R F V H R F  V G Y L E E E A V I  T Y T G V M R A I D  E G R L R P T K N D  V P E V A R V Y W N  L
T.vivax(AB070521)                 T S P R F V H R F  V G Y L E E E A V V  T Y T G I L R A I D  D G R L P P M K N A  V P D V A R V Y W G  L
T.congolense(AB188571)            I S P R F V H R F  V G Y L E E E A V I  T Y T S I L R A I D  E G R L R P T K S D  V P E V A R V Y W N  L
T.congolense(AB188572)            I S P R F V H R F  V G Y L E E E A V I  T Y T S I L R A I D  E G R L R P T K S D  V P E V A R V Y W N  L
Consistency                       8 * * * * * * * *  * * * * * * * * * 9  * * * 8 9 8 * * * *  9 * * * 8 * 8 * 8 8  * * 9 * * * * * * 8  *

                                  . . . . . . . . . 260 . . . . . . . . . 270 . . . . . . . . . 280 . . . . . . . . . 290 . . . . . . . . . 300
T.evansi(KF686336)                S K N A T F R D L  I N V I R A D E A E  H R V V N H T F A D  M H E K R L Q N S V  N P F V V L K K N P  E
T.brucei(TBU52964)                S K N A T F R D L  I N V I R A D E A E  H R V V N H T F A D  M H E K R L Q N S V  N P F V V L K K N P  E
T.brucei(XM817851)                S K N A T F R D L  I N V I R A D E A E  H R V V N H T F A D  M H E K R L Q N S V  N P F V V L K K N P  E
T.brucei(AB070617)                S K N A T F R D L  I N V I R A D E A E  H R V V N H T F A D  M H E K R L Q N S V  N P F V V L K K N P  E
T.brucei(AB070614)_               S K N A T F R D L  I N V I R A D E A E  H R V V N H T F A D  M H E K R L Q N S V  N P F V V L K K N P  E
T.evansi(AB188573)_               S K N A T F R D L  I N V I R A D E A E  H R V V N H T F A D  M H E K R L Q N S V  N P F V V L K K N P  E
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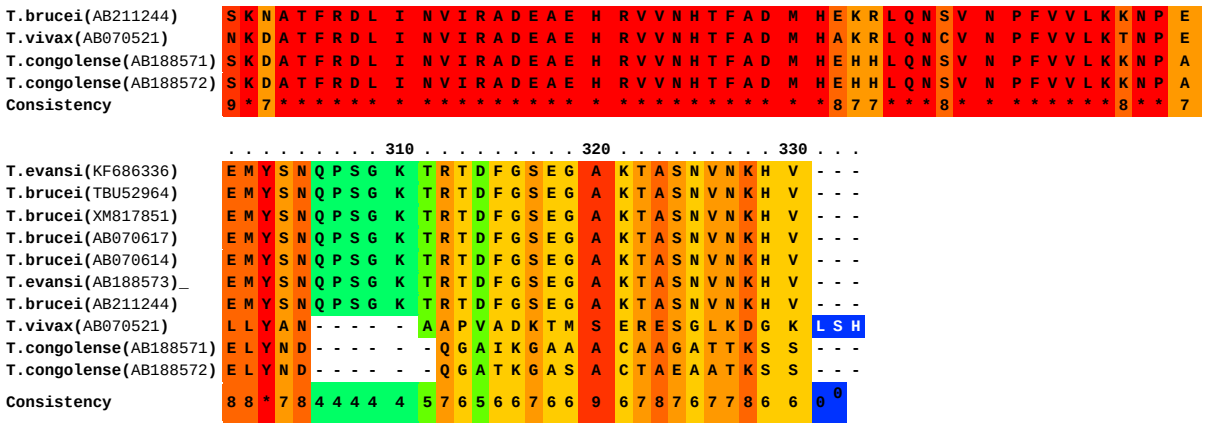

Plate 14. Multiple Sequence Alignment of Alternative oxidase gene with PRALINE sequence alignment software.

ORIGIN

```
        1  TGCGCTCTGA  CTTCCAAGGC  TGCGGGCAAG  AGGACGACGC  GTGAGGCATT
TCTGTCCGGC
       61  GGTTCGTGGG  CTTTGAGAAA  GAAGCTGAGC  GAGAAGGATG  GTGAAGTGTG
GTGGCGGCAG
      121  GATGGACCCA  ATTGGAAGGA  TAAGTATGAT  AAGGAATGGG  AGAGATGGTT
CAAAGAAGAG
      181  AAAGGTCCCT  GGGGAGGGTC  TGAGAAGCGT  AGCGAATGGT  TCGCTCGAAT
GACAGGTGGG
      241  TACATAACGC  TTGGCAAAAC  GAAGATACTT  TCATCTGCTA  TTGAGGGTAG
TGATAAGGTA
```

301 GAGCGCACTG TGCATTCCTT TCGTATTCCT TCGTTTGTTG AGGTTGATGG
GGTGCTGATG
361 GGTATTGGTG ATGCCCGGTA TCTTACCTCC ACGGATTACT TCTTCACCGA
CACCGTTGCT
421 AAATACAGTG CGGACGGGGG CAAAACATGG AAAACAGAGG TCATCATTGA
AAATGGTCGC
481 GTAGATCCCA CATACTCTCG TGTTGTGGAT CCTACTGTCG TTGCTAAGGC
GGACAGTGTC
541 TTTGTGCTTG TGGCAAGATA CAATGTCACG AAGGGGTATT GGCACAACGA
AAACAACGCA
601 GCGGGTATAG CGGATTGGGA ACCTTTCATG TACAAGGGTG TAGTGACTAA
GGGTGCCGAC
661 GGTAAAACCA GTGATGTGCG GATCTCTTGG ACAAAGACAC CACTGAAACC
CCTCTACGAC
721 TTCACTGTTG CAGGAAGCAA GGGCACGCAG TTCATTGGAG GAGCTGGTAA
CGGCGTTGTA
781 ACATTGAACG GTACAATATT GTTTCCGGTG CAGGCGAGGA ATGAAGACAA
TGCCGTTGTA
841 AGCATGGTTA TGTACTCTGT TGACGATGGT GTGAGTTGGC ATTTTGCCCG
TGGTGAAACG
901 GCGCTTCTCA CATCGGAAGC TTCTCTTACT GAGTGGAATG GGAAACTGCT
GATGAGCGCG
961 CGGACAGACA CTTCTGGCGT TAACGTAGAA GGTGGGTTCC GCAAGGTGTT
CGAATCTAAC
1021 AACCTTGGGG CAACGTGGGA GGAATCACTC GGAACGATTT CCCGCGTAAT
TGGGAACTCA
1081 CCCGACCGTA CGAAACCGTC TCCAACGGCC AACTATCCCG GTAGTTCGGG
GGCTCTTATT
1141 ACTGTGACGC TTGGGGATGT GCCTGTGATG TTGATTACCC ACCCGAAAAA
CACAAAGGGG
1201 GCATGGAGCC GGGACCGTCT ACAGCTGTGG ATGACAGATG GTAACCGTAT
GTGGCTTGTT
1261 GGCCAGATAT CGGAGGGCGA CGATAACAGC GCTTACAGTT CTTTGCTGTT
GGCCCGTGAT
1321 GGACTGCTTT ATTGTCTGCA CGAGCAGAAC ATCGACGAAG TGTATAGTCT
TCATCTTGTG
1381 CACCTTGTGG ATGAGTTGGA GAAGGTTAAC GCGACGGTGC GGAAATGGAA
GGCCCAGGAC
1441 GCCTTACTTG CTGGCCTTTG CTCTTCATCA CGGAAAAAGA ACGACCCCAC
GTGCTCCGGT
1501 GTCCCTACCG ATGGCCTTGT TGGTTTACTC GCCGGCCCTG TTGGTGCGAG
TGTGTGGGCT
1561 GATGTGTACG ACTGCGTGAA TGCCAGTATC TCTGATGGTG TGAAGGTTTC
GGAAGGCGTG
1621 CAGCTGGGAG GCAAAAGAAA CAGCCGTGTG CTGCGGCCTG TGAGCGAGCA
GGGACAGGAC
1681 CAGAGGTATT ACTTCGCCAA CACGCACTTT ACGCTGCTTG CCACCGTTCG
GTTCGCGGGG
1741 GAACCGAAGG CGGAGGCACC GCTGATGGGA TTTTCAAACG CAGAGGGGAA
AACGAGCGAA

```
1801  ACTTTGAGTC  TCACAGTTGG  CGGCAAGAAG  TGGGTCCTAA  CGTACGGCTC
CGTCCGTAAA
1861  GAGGGCCCAA  CCACGTCGAT  GGATTGGAAT  CAAACCCATC  AGATTGCGCT
CACACTGCGT
1921  GATGGTAAAG  TGGATGCTCA  TGCCAATGGA  GAGCTCATAA  TAAAAGAAGT
GAGTGTAGGC
1981  GCTTCTGAAT  CTTCTGCACA  TCTACATCTC  TCACACTTTT  TTATTGGAGC
GCCGGTAAAT
2041  GACAGTGGGG  AGGGAGGCAA  TAATGTGATC  GTCAGAAATG  TTCTTCTGTA
CAATCGAAAG
2101  CTCGATGAGG  ACGAACTGCA  AGTGCTATAT  AGCAACAGGG  AAAAGATACA
ACCGGTCGTC
2161  AGTGCAGTTG  GTATCCCCGA  AGGTATGAGC  GCACCTCGGT  TATGTTGTCT
GCTGATCTTA
2221 ATGTATGTGT TGGCGATTTG A
```

Plate 15. The coding sequence of Trans-sialidase gene of *Trypanosoma evansi* isolated from *Camelus dromedarius*

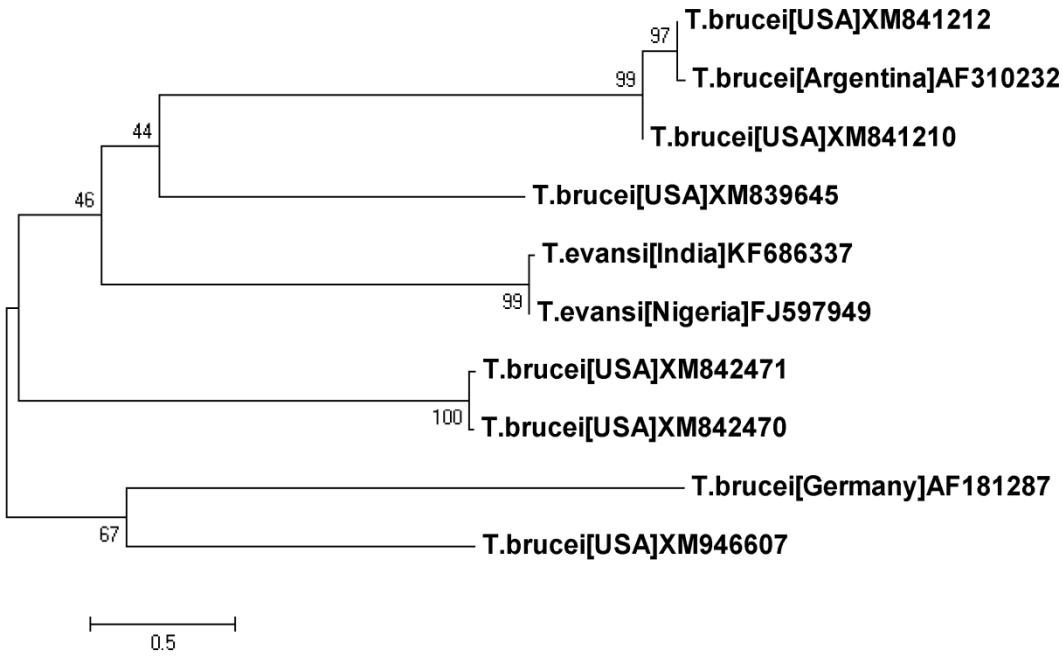

Plate 16a. Phylogenetic tree analysis of Trans-sialidase gene using the Neighbor-Joining method

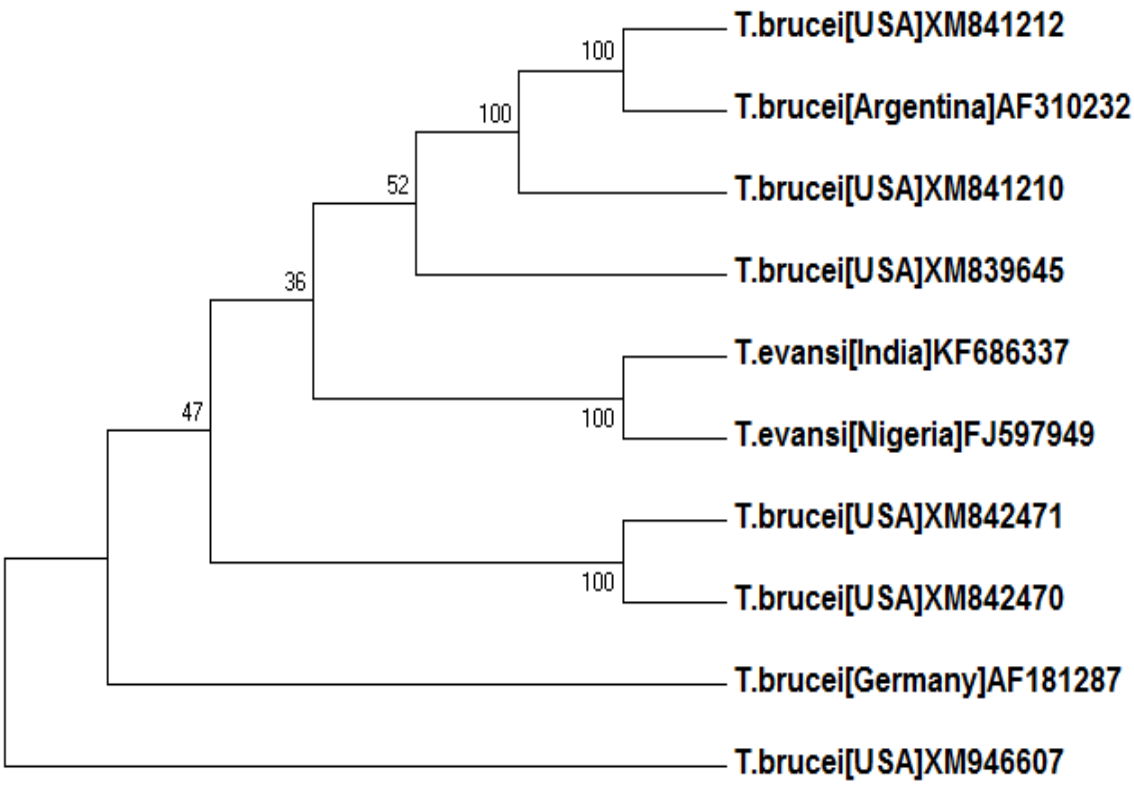

Plate 16b. Phylogenetic tree analysis of Trans-sialidase gene using maximum parsimony method

Table 4.3: Sequence identity of Trans-sialidase gene of *Trypanosoma evansi* with other allied species

Identity of *ts* gene for *Trypanosoma evansi*	*T.brucei*[USA] XM842471	*T.brucei*[USA] XM842470	*T.brucei*[USA] XM839645	*T.brucei*[USA] XM841210	*T.brucei*[USA] XM841212	*T.brucei* [Germany] AF181287	*T.evansi* [India] KF686337	*T.evansi* [Nigeria] FJ597949	*T.brucei* [Argentina] AF310232	*T.brucei*[USA] XM946607
T.brucei [USA] XM842471	**	96.5	50.0	46.9	49.0	48.5	48.8	49.3	48.7	30.6
T.brucei [USA] XM842470	96.5	**	50.0	46.6	49.4	49.4	49.4	49.9	49.3	30.2
T.brucei [USA] XM839645	50.7	50.0	**	42.3	47.5	47.3	48.1	48.6	46.7	28.4
T.brucei [USA] XM841210	46.9	46.6	42.3	**	94.6	94.3	93.5	94.2	93.6	25.2
T.brucei [USA] XM841212	49.0	49.4	47.5	94.6	**	99.4	99.0	99.4	98.5	28.6
T.brucei [Germany] AF181287	48.5	49.4	47.3	94.3	99.4	**	98.7	99.2	98.3	28.6
T.evansi [India] KF686337	48.8	49.4	48.1	93.5	99.0	98.7	**	99.0	98.7	28.8
T.evansi [Nigeria] FJ597949	49.3	49.9	48.6	94.2	99.4	99.2	99.0	**	98.9	28.9
T.brucei [Argentina] AF310232	48.7	49.3	46.7	93.6	98.5	98.3	98.7	98.9	**	28.1
T.brucei [USA] XM946607	30.6	30.2	28.4	25.2	28.6	28.6	28.8	28.9	28.1	**

Table 4.4: Trans-sialidase gene for different species of *Trypanosoma* as submitted by various workers

Sl. No.	Identity of *ts* gene for *Trypanosoma evansi*	Accession No.	Product length	Collection Country	Reference
1.	*T. evansi*	KF686337	2291 bp	India	Shinde *et al.* (2013)
2.	*T. brucei*	XM841210	1284 bp	USA	Berriman *et al.* (2012)
3.	*T. brucei*	XM841212	2316 bp	USA	Berriman *et al.* (2012)
4.	*T. brucei*	XM842471	2247 bp	USA	Berriman *et al.* (2012)
5.	*T. brucei*	XM842470	2247 bp	USA	Berriman *et al.* (2012)
6.	*T. brucei*	XM839645	2451 bp	USA	Berriman *et al.* (2012)
7.	*T. brucei*	XM946607	2109 bp	USA	Berriman *et al.* (2012)
8.	*T. evansi*	FJ597949	2248 bp	Nigeria	Yakubu *et al.* (2009)
9.	*T. brucei*	AF310232	2316 bp	Argentina	Montagna *et al.* (2002)
10.	*T. brucei*	AF181287	3313 bp	Germany	Engstler, M. (2000)

```
Unconserved   0 1 2 3 4 5 6 7 8 9 10 Conserved

                        .         10         .         20         .         30         .         40         .         50
T.evansi(KF686337)      - - - - - - - - - -  - - - - - - - - - -  - - - - - - - - - -  - - - - - - - - - A  L T S K A A G K R T
T.evansi(FJ597949)      - - - - - - - - - -  - - - - - - - - - -  - - - - - - - - - -  - - - - - - - - C A  L T S K A A G K G T
T.brucei(XM841212)      M E E L - - - - - -  - - - - - - H Q Q M  R M P - I S R L L L  I F T A V C H C C A  L T S K A A G K G T
T.brucei(AF310232)      M E E L - - - - - -  - - - - - - H Q Q M  H M P - I S R L L L  I F T A V C H C C A  L T S K A A G K G T
T.brucei(AF181287)      M E E L - - - - - -  - - - - - - H Q Q M  R M P - I S R L L L  I F T A V C H C C A  L T S K A A G K G T
T.brucei(XM842470)      - - - - - - - - - -  - - - - - - - - - -  - - - M A S Y M L L  L V A I V W H C C -  - - - - - - - - - -
T.brucei(XM842471)      - - - - - - - - - -  - - - - - - - - - -  - - - M A S Y M L L  L V A I V W H C C -  - - - - - - - - - -
T.brucei(XM839645)      M K R L P V R P C C  V T G E G S F S A L  T F S F S L Y A L L  V L F A L L Y C C D  L V S S K I Y E R T
T.brucei(XM946607)      - - - - - - - - - -  - - - - - - - - - -  - - - - - - - - - -  - - - - - - - - - -  - - - - - - - - - -
T.brucei(XM841210)      M E E L - - - - - -  - - - - - - H Q Q M  R M P - I S R L L L  I F T A V C H C C A  L T S K A A G K G T
Consistency             2 1 1 2 0 0 0 0 0 0  0 0 0 0 0 0 1 1 1 1  1 1 1 0 2 3 2 3 4 4  3 2 2 3 4 2 4 4 6 3  4 3 4 3 3 3 3 4 2 4

                        .         60         .         70         .         80         .         90         .         100
T.evansi(KF686337)      T R E A F L S G - G  S W A L R K K L S E  K D G E V W W R Q D  G P N W K D K Y D K  E W E R W F K E E K
T.evansi(FJ597949)      T R E A F L S G - G  A W A L R K K L S E  K D G E V W W W Q D  G P D W K D K Y D K  E W E R W F K E E K
T.brucei(XM841212)      T R E A F L S G - G  A W A L R K K L S E  K D G E V W W W Q D  G P N W K D K Y D K  E W E R W F K E E K
T.brucei(AF310232)      T R E A F L S G - G  S W A L R K K L S E  K D G E V W W W Q D  G P N W K D K Y D K  E W E R W F K E E K
T.brucei(AF181287)      T R E A F L S G - G  A W A L R K K L S E  K D G E V W W W Q D  G P N W K D K Y D K  E W E R W F K E E K
T.brucei(XM842470)      - - - - - - - S V L  V V D A K E G T T R  - - - E M F L - - G  G G L W V - - - - -  V N E G C - - - - -
T.brucei(XM842471)      - - - - - - - S V L  V V D A K E G T T R  - - - E M F L - - G  G G L W V - - - - -  V N E G C - - - - -
T.brucei(XM839645)      T R E V F L E G G R  W V R K S E W E K G  - - - S W K T - - S  P E W N A - - G Y E  W W A W C - - - - -
T.brucei(XM946607)      - - - - - - - - - -  - - - - - M R V V Y Q  - - - R F F P - - V  L L L L L - - - - -  S L E H L - - - - -
T.brucei(XM841210)      T R E A F L S G - G  A W A L R K K L S E  K D G E V W W W Q D  G P N W K D K Y D K  E W E R W F K E E K
Consistency             4 4 4 4 4 4 3 6 0 3  4 4 4 5 6 7 4 5 6 6  3 3 3 7 6 5 4 2 3 5  6 4 3 6 5 3 3 3 3 4  5 5 8 4 5 3 3 3 3 3

                        .         110        .         120        .         130        .         140        .         150
T.evansi(KF686337)      G P W G G S E K R S  E W F A R M T G G -  - - - Y I T L G K T  K I L S S A I E G S  - - D K V E R T V H
T.evansi(FJ597949)      G P W G G S E K R S  E W F A R M T G G -  - - - Y I T L G K T  K I L S S A I E G S  - - D K V E R T V H
T.brucei(XM841212)      G P W G G S E K R S  E W F A R M T G G -  - - - Y I T L G K T  K I L S S A I E G S  - - D K V E R T V H
T.brucei(AF310232)      G P W G G S E K R S  E W F A R M T G G -  - - - Y I T L G K T  K I L S S A I E G S  - - D K V E R T V H
T.brucei(AF181287)      G P W G G S E K R S  E W F A R M T G G -  - - - Y I T L G K T  K I L S S A I E G S  - - D K V E R T V H
T.brucei(XM842470)      - - L S V G K D P K  S T Y R G S R R - -  - - - - - - - - C I  L E P K D T S P N I  R - K E G M K D V H
T.brucei(XM842471)      - - L S V G K D P K  S T Y R G S R R - -  - - - - - - - - C I  L E P K D T S P N I  R - K E G M K D V H
T.brucei(XM839645)      - - M D S V A K E A  K G E V C R K E W L  S Q R K K G Y T L V  P R T K V P F R E K  N G T Q W M R N V H
T.brucei(XM946607)      - - C S R H A C A D  E G E E G - - - - -  - - - - - - - - - -  - - - G V N H T V G  - - - G N L T G T H
T.brucei(XM841210)      G P W G G S E K R S  E W F A R M T G G -  - - - Y I T L G K T  K I L S S A I E G S  - - D K V E R T V H
Consistency             3 3 5 6 4 5 6 6 5 6  7 4 6 5 5 5 5 4 3 0  0 0 0 3 3 3 3 3 4 5  4 4 4 6 5 5 5 5 5 5  0 0 4 6 4 5 7 5 8 *

                        .         160        .         170        .         180        .         190        .         200
T.evansi(KF686337)      S F R I P S F V E V  D G V L M G I G D A  R Y L T S T D Y F F  T D T V A K Y S A -  - - - - - - - D G G
T.evansi(FJ597949)      S F R I P S F V E V  D G V L M G I G D A  R Y L T S T D Y F F  T D T V A K Y S A -  - - - - - - - D G G
T.brucei(XM841212)      S F R I P S F V E V  D G V L M G I G D A  R Y L T S T D Y F F  T D T V A K Y S A -  - - - - - - - D G G
T.brucei(AF310232)      S F R I P S F V E V  D G V L M G I G D A  R Y L T S T D Y F F  T D T V A K Y S A -  - - - - - - - D G G
T.brucei(AF181287)      S F R I P S F V E V  D G V L M G I G D A  R Y L T S T D Y F F  T D T V A K Y S A -  - - - - - - - D G G
T.brucei(XM842470)      S F R I P S L I E V  D G V I I G I A D C  R Y T S S E D F A F  I D T V A R Y S A -  - - - - - - - D G G
T.brucei(XM842471)      S F R I P S L I E V  D G V I I G I A D C  R Y T S S E D F A F  I D T V A R Y S A -  - - - - - - - D G G
T.brucei(XM839645)      S F R V P S F V E I  S G V L V G I A D V  R Y I S S A D F T F  T E T V A K Y S A -  - - - - - - - D G G
T.brucei(XM946607)      S Y R S P S L L S I  G D S L V T V S E T  W D P S - D T E Q Y  V D V I T E Y S A H  Y G A P A V T Q V A
T.brucei(XM841210)      S F R I P S F V E V  D G V L M G I G D A  R Y L T S T D Y F F  T D T V A K Y S A -  - - - - - - - D G G
Consistency             * 9 * 8 * * 7 8 8 9  7 8 8 9 7 8 9 6 9 6  8 8 5 7 8 5 8 7 5 9  7 9 8 9 8 8 * * *  0 0 0 0 0 0 0 8 8 8

                        .         210        .         220        .         230        .         240        .         250
T.evansi(KF686337)      K T W K T E V I I E  N G R V D P T Y S R  V V D P T V V A K A  D S V F V L V A R Y  N V T K G Y W H N E
T.evansi(FJ597949)      K T W K T E V I I E  N G R V D P T Y S R  V V D P T V V A K A  D S V F V L V A R Y  N V T K G Y W H N E
T.brucei(XM841212)      K T W K T E V I I E  N G R V D P T Y S R  V V D P T V V A K A  D S V F V L V A R Y  N V T K G Y W H N E
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T.brucei(AF310232) KTWKTEAIIE NGRVDPTYSR VVDPTVVAKA DSVFVLVARY NVTKGYWHNE
T.brucei(AF181287) KTWKTEVIIE NGRVDPTYSR VVDPTVVAKA DSVFVLVARY NVTKGYWHNE
T.brucei(XM842470) RTWKTEIIFE NARVNKDHSR VVDPTVVVKN NTVFVLVGRY NNSK---TWW
T.brucei(XM842471) RTWKTEIIFE NARVNKDHSR VVDPTVVVKN NTVFVLVGRY NNSK---TWW
T.brucei(XM839645) DTWKTKVIIE NSRVNTNFSR VVDPTVAVKG NNIFVLVGRY NTSS---KYW
T.brucei(XM946607) VRNDTLDFHA VYKHKEDRES TMHPTAVAID GMIFVLIFCK NIG-----AS
T.brucei(XM841210) KTWKTEVIIE NGRVDPTYSR VVDPTVVAKA DSVFVLVARY NVTKGYWHNE
Consistency        6888*7686 8 85986566 88 898**99785 669**9688 *567333445

                   .........260 .........270 .........280 .........290 .........300
T.evansi(KF686337) NNAAGIADWE PFMYKGVVTK G-ADGKTSDV RISWTKTPLK PLYD------
T.evansi(FJ597949) NNAAGIADWE PFMYKGVVTK G-ADGKTSDV RISWTKTPLK PLYD------
T.brucei(XM841212) NNAAGIADWE PFMYKGVVTK G-ADGKTSDV RISWTKTPLK PLYD------
T.brucei(AF310232) NNAAGIADWE PFVYKGVVTK G-ADGKTSDV RISWTKTPLK PLYD------
T.brucei(AF181287) TNAAGIADWE PFMYKGVVTK G-ADGKTSDV RISWTKTPLK PLYD------
T.brucei(XM842470) PLMNDGDDWD ILMYKGTVQK TVDESGNASA TIVWKDPQYL KSLLG----T
T.brucei(XM842471) TGQANGDDWD ILMYKGTVQK TVDESGNASA TIVWKDPQYL KSLLG----T
T.brucei(XM839645) TWQHYGEDWD ILMYKGTVI- ----KEEKDG NVTASITFEA PQNLKFLLAT
T.brucei(XM946607) GNQTGDAVIM PHVAKGVVLP ---GGATGNV WVNWTQLMPI RSLLP-----
T.brucei(XM841210) NNAAGIADWE PFMYKGVVTK G-ADGKTSDV RISWTKTPLK PLYD------
Consistency        55666468 86 5688**7*56 40456566 76 596875644 5 6554000000

                   .........310 .........320 .........330 .........340 .........350
T.evansi(KF686337) -FTVAGSKGT QFIGGAGNGV VTLNGTILFP VQARNEDNAV VSMVMYSVDD
T.evansi(FJ597949) -FTVAGSKGT QFIGGAGNGV VTLNGTILFP VQARNEDNAV VSMVMYSVDD
T.brucei(XM841212) -FTVAGSKGT QFIGGAGNGV VTLNGTILFP VQARNEDNAV VSMVMYSVDD
T.brucei(AF310232) -FTVAGSKGT QFIGGAGNGV VTLNGTILFP VQARNEDNAV VSMVMYSVDD
T.brucei(AF181287) -FTVAGSKGT QFIGGAGNGV VTLNGTILFP VQARNEDNAV VSMVMYSVDD
T.brucei(XM842470) VGKINGRSLI QYLGGVGNGI VTPNGTVVFP VQILNTENYL AAMILYSDDD
T.brucei(XM842471) VGKINGRSPI QYLGGVGNGI VTPNGTVVFP VQILDAENYL TAMILYSDDD
T.brucei(XM839645) VPSPGGHPPS QFLGGVGNTA VTPDGAIVFS VQVKNTWNQV VGKLLYSTDD
T.brucei(XM946607) -KVIEGKRVS MFFGGGGNTI VMTKGTLVMP VQMVRTDDQR FATIIYSSNN
T.brucei(XM841210) -FTVAGSKGT QFIGGAGNGV VTLNGTILFP VQARNEDNAV VSMVMYSVDD
Consistency        04675*6646 887**6**78 *857*89888 **65766957 677888**599

                   .........360 .........370 .........380 .........390 .........400
T.evansi(KF686337) GVSWHFARGE TALLTSEASL TEWNGKLLMS ARTDTSGVNV EGGFRKVFES
T.evansi(FJ597949) GVSWHFARGE TALLTSEASL TEWNGKLLMS ARTDTSGVNV EGGFRKVFES
T.brucei(XM841212) GVSWHFARGE TALLTSEASL TEWNGKLLMS ARTDTSGVNV EGGFRKVFES
T.brucei(AF310232) GVSWHFARGE TALLTSEASL TEWNGKLLMS ARTDTSGVNV EGGFRKVLES
T.brucei(AF181287) GVSWHFARGE TALLTSEASL TEWNGKLLMS ARTDTSGVNV EGGFRKVFES
T.brucei(XM842470) GKTWEFSKGA TPIGTTESSI VWWGERLLLN GRTDRPQSNV DAGYRKVFES
T.brucei(XM842471) GKTWEFSKGA TPIGTTESSI VWWGERLLLN GRTDKVGRYV DAGYRKVFES
T.brucei(XM839645) GKTWHFSAGE TPVGSTESSA VWWKDKLLVN AR-----TDE HIGCRRVFET
T.brucei(XM946607) GTSWTVAKGF TDSGCRESSV LIWKRKMLLV ARSD------ -DGYTKVFES
T.brucei(XM841210) GVSWHFARGE TALLTSEASL TEWNGKLLMS ARTDTSGVNV EGGFRKVFES
Consistency        *58*6887*6 *574778*7 64*65898*86 8*78444456 55*7898*8*9

                   .........410 .........420 .........430 .........440 .........450
T.evansi(KF686337) NNLGATWEES LGTISRVIGN SPDRTKPSPT ANYPGSSGAL ITVTLGD-VP
T.evansi(FJ597949) SNLGATWEES LGTISRVIGN SPDRTKPSPT ANYPGSSGAL ITVTLGD-VP
T.brucei(XM841212) NNLGATWEES LGTISRVIGN SPDRTKPSPT ANYPGSSGAL ITVTLGD-VP
T.brucei(AF310232) SNLGATWEES LGTISRVIGN SPDRTKPSPT ANYPGSSGAL ITVTLGD-VP
T.brucei(AF181287) NNLGATWEES LGTISRVIGN SPDRTKPSPT ANYPGSSGAL ITVTLGD-VP
T.brucei(XM842470) SDMGATWVES LGTISRVIGN SPE------- RNQPGSSGSS IKVTFDD-VP
T.brucei(XM842471) SDMGATWVES LGTISRVIGN SPE------- RNQPGSSGSS IKVTVDD-VP
T.brucei(XM839645) SDLGNTLKES IRTLSRVIGN SPL------- RNQPGSSGSA ISITVEG-MD
T.brucei(XM946607) DYMGDVWTES LGTISRVLGN SPD------- RRGPGNQGSA ITIPLGN-ET
T.brucei(XM841210) SNLGATWEES LGTISRVIGN SPD------- RTLYLV--LL LCKCSAKRRR
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Consistency        7 6 8 * 7 8 8 5 * *   9 8 * 9 * * * 9 * *   * * 6 2 2 2 2 2 2   6 7 4 8 8 7 7 8 6 6   9 5 8 7 6 5 6 0 6 6

                   . . . . . . . . . 460 . . . . . . . . . 470 . . . . . . . . . 480 . . . . . . . . . 490 . . . . . . . . . 500
T.evansi(KF686337) V M L I T H P K N T   K G A - - - - - - W   S R D R L Q L W M T   D G N R M W L V G Q   I S E G D D N S A Y
T.evansi(FJ597949) V M L I T H P K N T   K G A - - - - - - W   S R D R L Q L W M T   D G N R M W L V G Q   I S E G D D N S A Y
T.brucei(XM841212) V M L I T H P K N T   K G A - - - - - - W   S R D R L Q L W M T   D G N R M W L V G Q   I S E G D D N S A Y
T.brucei(AF310232) V M L I T H P K N T   K G A - - - - - - W   S R D R L Q L W M T   D G N R M W L V G Q   I S E G D D N S A Y
T.brucei(AF181287) V M L I T H P K N T   K G A - - - - - - W   S R D R L Q L W M T   D G N R M W L V G Q   I S E G D D N S A Y
T.brucei(XM842470) V M L V T Q P K N I   H G K - - - - - - W   I R D R L Q L W L T   D G N R V F F V G Q   I S V G D D S S P Y
T.brucei(XM842471) V M L V T Q P K N I   H G K - - - - - - W   I R D R L Q L W L T   D G N R V F F V G Q   I S V G D D S S P Y
T.brucei(XM839645) V M L I S Q P K N T   K G R - - - - - - F   S R D R L Q L W L T   D G T R V F M I G Q   I S Q G D D N S P Y
T.brucei(XM946607) V M F F T Q T T V S   N S S D S N E G D I   N R I D Q R I W F S   D G S R M V K V G H   I Y W N D H L S S S
T.brucei(XM841210) K I V P A V P G L A   C R - - - - - - - K   S C W Q L V M V Y -   - - - - - - - - - -   - - - - - - - - - -
Consistency        8 9 8 6 8 5 8 7 6 6   5 7 4 0 0 0 0 0 0 5   6 8 6 7 8 7 8 8 6 7   8 8 6 8 6 4 4 7 8 6   8 6 3 6 8 6 5 8 4 6

                   . . . . . . . . . 510 . . . . . . . . . 520 . . . . . . . . . 530 . . . . . . . . . 540 . . . . . . . . . 550
T.evansi(KF686337) S S L L L A R D G L   L Y C L H E Q N I D   E V Y S L H L V H L   V D E L E K V N A T   V R K W K A Q D A L
T.evansi(FJ597949) S S L L L A R D G L   L Y C L H E Q N I D   E V Y S L H L V H L   V D E L E K V N A T   V R K W K A Q D A L
T.brucei(XM841212) S S L L L A R D G L   L Y C L H E Q N I D   E V Y G L H L V H L   V D E L E K V N A T   V R K W K A Q D A L
T.brucei(AF310232) S S L L L A R D G L   L Y C L H E Q N I D   E V Y S L H L V H L   V D E L E K V N A T   V R K W K A Q D A L
T.brucei(AF181287) S S L L L A R D G L   L Y C L H E R N I D   E V Y S L H L V H L   V D E L E K V N A T   V R K W K A Q D A L
T.brucei(XM842470) S S L L Y T K T G E   L H C L Y E Q I V D   S G V N I Y L T H L   V D E L E L I R S T   V R L W K A Q D R L
T.brucei(XM842471) S S L L Y T K T G E   L H C L H E E S I V   G V V S L H L V H L   V D E L E L I R S T   V R L W K A Q D R L
T.brucei(XM839645) S S L L Y T S D G K   L Y C L Y E Q N I E   E V F T I Y L A R L   V D E M K M I K W V   V L L W K A Q D T L
T.brucei(XM946607) R S Y L L Y S N N R   L L C A Y E M G A E   K A Y A I T V R S L   V G E L E N A R F A   R E T W A R Q D A Y
T.brucei(XM841210) - - - - - - - - - -   - - - - - - - - - -   - - - - - - - - - -   - - - - - - - - - -   - - - - - - - - - -
Consistency        6 8 6 8 5 4 5 5 6 3   8 5 8 6 6 8 5 4 6 5   5 5 5 5 7 5 7 5 5 8   8 6 8 7 7 4 6 5 4 6   6 5 4 8 6 6 8 8 5 6

                   . . . . . . . . . 560 . . . . . . . . . 570 . . . . . . . . . 580 . . . . . . . . . 590 . . . . . . . . . 600
T.evansi(KF686337) L A G L C S S S R K   K N D P T C S G V P   T D G L V G L L A G   P V G A S V W A D V   Y D C V N A S I S D
T.evansi(FJ597949) L A G L C S S S R K   K N D P T C S G V P   T D G L V G L L A G   P V G A S V W A D V   Y D C V N A S I S D
T.brucei(XM841212) L A G L C S S S R K   K N D P T C S G V P   T D G L V G L L A G   P V G A S V W A D V   Y D C V N A S I S D
T.brucei(AF310232) L A G L C S S S R K   K N D P T C S G V P   T D G L V G L L A G   P V G A S V W A D V   Y D C V N A S I S D
T.brucei(AF181287) L A G L C S S S R K   K N D P T C S G V P   T D G L V G L L A G   P V G A S V W A D V   Y D C V N A S I S D
T.brucei(XM842470) L A G T C S S D V T   D E T - T C T G I P   T A G L V G L L A G   P A V G T V W S D A   Y Q C V N A S V G G
T.brucei(XM842471) L A G T C S S D V T   D E T - T C T G I P   T A G L V G L L A G   P A V G T V W S D A   Y Q C V N A S V G G
T.brucei(XM839645) L V G D C L S S A G   G T G - P C R G I P   V G G L A G L L S G   P A V G H V W P D V   Y K C V Y A S V S G
T.brucei(XM946607) I S G I C S S A K E   S A P - C A S G V P   V D G L V G L L S S   M V N G T A W V D A   Y F S V N A N F V G
T.brucei(XM841210) - - - - - - - - - -   - - - - - - - - - -   - - - - - - - - - -   - - - - - - - - - -   - - - - - - - - - -
Consistency        7 6 8 4 8 6 8 5 4 4   4 4 4 2 5 6 5 8 7 8   6 4 8 8 7 8 8 8 6 7   6 6 3 5 5 7 8 5 8 6   8 4 6 8 6 8 7 6 5 4

                   . . . . . . . . . 610 . . . . . . . . . 620 . . . . . . . . . 630 . . . . . . . . . 640 . . . . . . . . . 650
T.evansi(KF686337) G V K V S E G V Q L   G G K R N S R V L R   P V S E Q G Q D Q R   Y Y F A N T H F T L   L A T V R F A G E P
T.evansi(FJ597949) G V K V S E G V Q L   G G K R N S R V L W   P V S E Q G Q D Q R   Y Y F A N T H F T L   L A T V R F A G E P
T.brucei(XM841212) G V K V S E G V Q L   G G K R N S R V L W   P V S E Q G Q D Q R   Y Y F A N T H F T L   L A T V R F A G E P
T.brucei(AF310232) G V K V S E G V Q L   G G K R N S P L L W   P V S E Q G Q D Q R   Y Y F A N T H F T L   L A T V R F A G E P
T.brucei(AF181287) G V K V S E G V Q L   G G K R N S R V L W   P V S E Q G Q D Q R   Y Y F A N T H F T L   L A T V R F A G E P
T.brucei(XM842470) A A V I D D G L Q L   S G K N D S S V S W   P V S E Q G Q D Q R   Y H F A N T H F T L   V V T V Q L A E I T
T.brucei(XM842471) A A V I D D G L Q L   S G K N D S S V S W   P V S E Q G Q D Q R   Y H F A N T H F T L   V V T V Q L A E I T
T.brucei(XM839645) A V A N K D G V V L   G G T G K D R V V W   P V G E Q G Q D Q R   Y Y F A N T H F T I   V A T V Q F G V V P
T.brucei(XM946607) G L A G P A G L T F   E G - T A R G G R W   P V G V Q G Q N Q R   F H F A N T H F T L   V L T L T I H E Q T
T.brucei(XM841210) - - - - - - - - - -   - - - - - - - - - -   - - - - - - - - - -   - - - - - - - - - -   - - - - - - - - - -
Consistency        5 5 4 4 4 5 8 6 5 7   5 8 5 4 4 5 4 5 4 6   8 8 6 6 8 8 8 7 8 8   7 6 8 8 8 8 8 8 8 7   6 5 8 7 5 5 5 3 4 5

                   . . . . . . . . . 660 . . . . . . . . . 670 . . . . . . . . . 680 . . . . . . . . . 690 . . . . . . . . . 700
T.evansi(KF686337) K A E A P L M G F S   N A E G K T S E T L   S L T V G G - K K W   V L T Y G S V R K E   G P T T S M D W N Q
T.evansi(FJ597949) K A E A P L M G F S   N A E G K T S E T L   S L T V G G - K K W   V L T Y G S V R K E   G P T T S M D W N Q
T.brucei(XM841212) K A E A P L M G F S   N A E G K T S E T L   S L T V G G - K K W   V L T Y G S V R K E   G P T T S M D W N Q
T.brucei(AF310232) K A E A P L M G F S   N A E G K T S E T L   S L T V G G - K K W   V L T Y G S V R K E   G P T T S M D W N Q
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T.brucei(AF181287) KAEAPLMGFS  NAEGKTSETL  SLTVGG-KKW  VLTYGSVRKE  GPTTSMDWNQ
T.brucei(XM842470) QNETSLVGFV  THDGQTSKYI  TLSLVKDVCR  HGAGQVSGAV  EDEQSPGTNG
T.brucei(XM842471) QNETSLVGFV  THDGQTSKYI  TLSLVKDVCR  HGAGQVSGAV  EDEQSPGTNG
T.brucei(XM839645) QRDTPLIGFV  NGEKNANKTL  MLSIK-NKKW  FLTYGRIRSE  GSPVPSNLEG
T.brucei(XM946607) APRAPVLVTR  LYENGSYIDL  EFSYTDDKRW  HIKYGAEY-G  STSGQWALNQ
T.brucei(XM841210) ----------  ----------  ----------  ----------  ----------
Consistency        5465576664  4365465447  5765331545  3455544444  4444453436 4

                   .........710 .........720 .........730 .........740 .........750
T.evansi(KF686337) THQIALTLRD  GKVDAHANGE  LIIKEVSVGA  SESSAHLHLS  HFFIGAPVND
T.evansi(FJ597949) THQIALTLRD  GKVDAHVNGE  LIIKEVSVGA  SESSAHLHLS  HFFIGAPVND
T.brucei(XM841212) THQIALTLRD  GKVDAHVNGE  LIIKEVSVGA  SESSAHLHLS  HFFIGAPVND
T.brucei(AF310232) THQIALTLRD  GKVDAHANGE  LIIKEVSVGA  SESSAHLHLS  HFFIGAPVND
T.brucei(AF181287) THQIALTLRD  GXVDAHVNGE  LIIKEVSVGA  SESSAHLHLS  HFFIGAPVND
T.brucei(XM842470) IHQVALTLSA  GKVFAHLDGK  HLPDMDT-IV  TGAGKLLNIS  RFFVGHPGVQ
T.brucei(XM842471) IHQVALTLSA  GKVFAHLDGK  HLPDMDT-IV  TGAGKLLNIS  RFFVGHPGVQ
T.brucei(XM839645) SHQIALTLQD  GLVTAYVDGK  LAVAAINVRK  FGRVGFLNIR  RFFVGTPVSI
T.brucei(XM946607) EHQLAFVLRG  GTIAVYLDGK  RMPTMAR-ML  AGNGNLLNIT  HFYIGGYGTV
T.brucei(XM841210) ----------  ----------  ----------  ----------  ----------
Consistency        4887877854  8-21474836  4873765586  4544445334  5454438566  5877846443

                   .........760 .........770 .........780 .........790 .........800
T.evansi(KF686337) SGEGGNNVIV  RNVLLYNRKL  DEDELQVLYS  NREKIQP---  ----------
T.evansi(FJ597949) SGEGGNNVIV  RNVLLYNRKL  DEDELQVLYS  NREKIQP---  ----------
T.brucei(XM841212) SGEGGNNVIV  RNVLLYNRKL  DEDELQLLYS  NREKIQP---  ----------
T.brucei(AF310232) SGEGGNNVIV  RNVLLYNRKL  DEDELQVLYS  NREKIQP---  ----------
T.brucei(AF181287) SGEGGNNVIV  RNVLLYNRKL  DEDELQLLYS  NREKIQP---  ----------
T.brucei(XM842470) DVAAGDGVVV  KNVLLYNRQL  SGSELRSLYL  NNNVIAVSQL  SPEGRSPSLL
T.brucei(XM842471) DVAAGDGVVV  KNVLLYNRQL  SGSELRSLYL  NNNVIAVSQL  SPEGRSPSLL
T.brucei(XM839645) RTSSHTTVTV  HNALLYNRRL  SEGELQLVFT  NREVIRAANP  TTPPPTPFES
T.brucei(XM946607) KQSPRDRLTI  RNAMLYNRPL  KKAEIDKLFA  AKSGITAATK  GIEPLTPKWN
T.brucei(XM841210) ----------  ----------  ----------  ----------  ----------
Consistency        4354554757  5867888858  5448753764  6553844100  0000001000

                   .........810 .........820 .........830 .........840 .........850
T.evansi(KF686337) ----------  ----------  ----------  -VVSAVGIPE  GMSAPRLCCL
T.evansi(FJ597949) ----------  ----------  ----------  -VVSAVGIPE  GMSAPRLCCL
T.brucei(XM841212) ----------  ----------  ----------  -VVSAVGIPE  GMSAPRLCCL
T.brucei(AF310232) ----------  ----------  ----------  -VVSAVGIPE  GMSAPRLCCL
T.brucei(AF181287) ----------  ----------  ----------  -VVSAVGIPE  GMSAPRLCCL
T.brucei(XM842470) -------KDI  AEGIGNEMNE  NCASLKLVVI  SDGGVRVHRL  LYLLMGLGLL
T.brucei(XM842471) -------KDI  AEGIGNEMNE  NCASLKLVVI  SDGGVRVHRL  LYLLMGLGLL
T.brucei(XM839645) SAGGDEQSHD  RAGSTFGVPF  LSGGSMYCEG  DALERVYILF  IITLCALALF
T.brucei(XM946607) ----------  ----------  ------HKTK  DLEPQSGDGR  ADHAFRTPSW
T.brucei(XM841210) ----------  ----------  ----------  ----------  ----------
Consistency        0000000000  0000000000  0000000000  0434444433  3445346345

                   .........860 .......
T.evansi(KF686337) LILMYVLAI-  -------
T.evansi(FJ597949) LILMYVLAI-  -------
T.brucei(XM841212) LILMYVLAI-  -------
T.brucei(AF310232) LILMYVLAI-  -------
T.brucei(AF181287) LILMYVLAI-  -------
T.brucei(XM842470) QLLSA-----  -------
T.brucei(XM842471) QLLSA-----  -------
T.brucei(XM839645) MLVLVFQKQW  DGDSTII
T.brucei(XM946607) ALPLFVLHLY  ---SLFM
T.brucei(XM841210) ----------  -------
Consistency
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Plate 17. Multiple sequence alignment of Trans-sialidase Amino acid sequences with PRALINE sequence alignment software.