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MOLECULAR CHARACTERISATION OF PARAFLAGELLAR ROD 1 GENE OF *Trypanosoma evansi* ISOLATED FROM INDIAN DROMEDARIES

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ABSTRACT

The study was conducted for characterisation of Paraflagellar Rod 1 (*pfr1*) gene of *Trypanosoma evansi* from camel at molecular level. Genomic DNA of *T. evansi* from camel was used to amplify the *pfr1* gene by polymerase chain reaction. Cloning of the amplicon was done in a suitable bacterial plasmid vector and characterisation of *pfr1* gene was carried out through sequencing. The desired amplicon of *pfr1* gene of *T. evansi* was amplified by PCR using gene specific primers and identified on the basis of size of the *pfr1* gene. The amplicon of expected size was purified from the 1% low melting agarose gel. DNA fragment of interest was then ligated to the pGEM- T Easy vector and ligated mixture was transformed into *Escherichia coli* JM109 strains for cloning. Screening of recombinants was done by restriction enzyme digestion of plasmid DNA and by colony PCR for quick screening of plasmid insert directly from *E. coli* colonies in the presence of insert specific primers. After confirmation of clone of *pfr1* genes the plasmid DNA was sequenced and coding sequences of *pfr1* gene according to the result obtained was of 1769 bp. Tree topology of *pfr1* gene is based on the Neighbor-Joining method and maximum parsimony method with 100% bootstrap values and identified *pfr1* gene sequence showed a close homology with other *Trypanosoma* and *Leishmania* spp. gene sequences.

Key words: Camel, cloning, India, paraflagellar Rod 1 gene, sequencing, *Trypanosoma evansi*

Trypanosomosis caused by protozoan parasite of the genus *Trypanosoma*, 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. Camel trypanosomosis commonly known as 'surra' is caused by blood protozoan parasite *Trypanosoma evansi*. Trypanosomosis is the most pathogenic parasitic disease of camelids in all camel raising countries causing high morbidity and mortality (Luckins, 1992). In India, the disease in camels is very common and outbreaks frequently occur during and after rainy season though sporadic cases are met with throughout the year (Pathak & Khanna, 1995). Control of trypanosomosis is based principally on insecticide spraying to control the vector population and on regular treatment of livestock at risk in endemic area through chemoprophylaxis and chemotherapy. The high cost of regular drug and insecticidal treatment, the limited effectiveness of insecticides application in high rainfall areas, the possibility of environmental pollution by insecticides, the increasing incidence of parasite resistance to available drugs and absence of new drugs to replace them, are the major problems that make vector and trypanosomosis control difficult and expensive. Vaccine development against *T. evansi* is also hampered due to variation of surface proteins as antigen. Variant surface glycoprotein coat of *T. evansi* is changeable when host forms antibodies against it.

The major researches today are being focused on identifying various invariant protein components of trypanosome as potential drug target and basis for vaccines. The paraflagellar rod (PFR) is a functionally important structure that is present in pathogenic trypanosomatids but absent in their mammalian hosts. The *T. evansi* gene Paraflagellar Rod 1 (*pfr1*), which encodes the invariant protein *Pfr1* major structural component of the PFR is highly pathogenic gene and restricted to the flagella of kinetoplastids. The *pfr1* gene of *T. evansi* from Indian dromedaries have not yet been characterised at molecular level. Sequencing of *pfr1* gene of *T. evansi* may be useful to identify the function of this gene and encoding protein. Therefore, a study was carried out to amplify the *pfr1* gene of *T. evansi* from camel by polymerase chain reaction, clone the amplicon and characterisation of *pfr1* gene through sequencing.

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Materials and Methods

Preparation of trypanosome strains, DNA isolation and PCR amplification

After confirmation of *T. evansi* isolates by blood smear examination, blood from infected camel was inoculated intraperitoneally into Swiss albino mice (maintained at Small Animal Laboratory, NRC on Camel, Bikaner) for propagation of trypanosomes. Mice with high parasitaemia were euthanised with ethyl-ether and blood was collected into 5 ml disposable syringe containing 0.1 ml heparin solution by heart puncture. Diethyl amino ethyl cellulose column chromatography method was used for purification of trypanosomes (Lanham and Godfrey, 1970). The collected trypanosomes were pelleted by centrifugation at 1000 rpm for 10 min and kept at -20°C for further processing. DNA isolation from collected pellet of *T. evansi* was done according to the method utilized by Desquesnes and Davila (2002) for the preparation of animal trypanosomes DNA from plane blood. Nucleotide primers for the amplification of *pfr1* gene of *T. evansi* were designed using the published sequence of *pfr1* gene (Accession No. EU366960): forward primer 5′-ATGGCCGCAGTTGACGATGCCAC-3′ and reverse primer 5′-CTATTCGAGGCGTGCCGGTG-3′. PCR amplification of the *pfr1* gene was performed by cycling conditions as initial denaturation at 94°C for 4 min, 35 cycles of denaturation at 94°C for 30 sec, annealing at 63°C for 60 sec, extension at 72°C for 1 min and 30 sec, and final extension for 9 min at 72°C. The PCR amplified products were checked with 1.5 kb DNA molecular weight marker in 1.2% agorose gel.

Table 1. PCR reaction mixture for *pfr1* 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 (Promega)	0.25 µl	1.5 Units
Distilled Water	34.75 µl	–
Total volume	**50 µl**	**–**

Cloning and sequencing of pfr1 gene

The PCR products from low melting point agarose slices were purified by illustra GFX PCR DNA and Gel Band Purification Kit (GE healthcare, USA) using the manufacturer's protocol. The DNA fragment of *pfr1* gene and the pGEM- T Easy vector (Promega, USA) in which it is to be cloned were digested with T4 DNA ligase enzyme to generate compatible ends for ligation. The ligation was done (as per the Promega protocol with slight modification) in the reaction volume of 20 µl containing 10µl of 2X Rapid ligation T4 DNA Ligase buffer [400mM Tris-HCl, 100mM $MgCl_2$, 100mM DTT, 5mM ATP (pH 7.8 at 25°C)], 6 µl PCR product, 2 µl pGEM- T Easy vector and 2 µl of T4 DNA ligase. The contents were vortexed, spun down in a microcentrifuge for 3-5 seconds and incubated for overnight at 4°C. The ligation mix was then used directly for transformation in JM109 competent cells (Promega, USA). After incubation of transformation culture 100 µl of transformation culture was plated onto antibiotic agar plates and incubated at 37°C for overnight (16-20 hr). Colonies harbouring the recombinant plasmid were inoculated into LB broth

Table 2. Detail of *pfr1* gene nucleotide and amino acid sequence of encoding protein of trypanosomatidae species from various animal species.

Sl. No.	Identity of *pfr1* gene	NCBI Accession No.	Collection Country and year of isolation	Per cent identity with *T. evansi* from India, 2012 (JQ909241)	
				Nucleotide	Amino acid
1.	*T. evansi*	EU366960	China, 2008	99.9	99.0
2.	*T. evansi*	FJ968743	India, 2009	100.0	100.0
3.	*T. brucei*	XM838928	USA, 2012	99.8	100.0
4.	*T. brucei*	Z25827	Switzerland, 2004	99.4	93.0
5.	*T. cruzi*	XM804737	USA, 2008	84.4	93.0
6.	*T. cruzi*	AF005195	USA, 1998	84.2	93.0
7.	*L. infantum*	AY702344	Spain, 2004	83.6	84.0
8.	*L.major*	XM003722211	UK, 2012	83.6	85.0
9.	*L. infantum*	XM003392645	UK, 2011	83.9	85.0

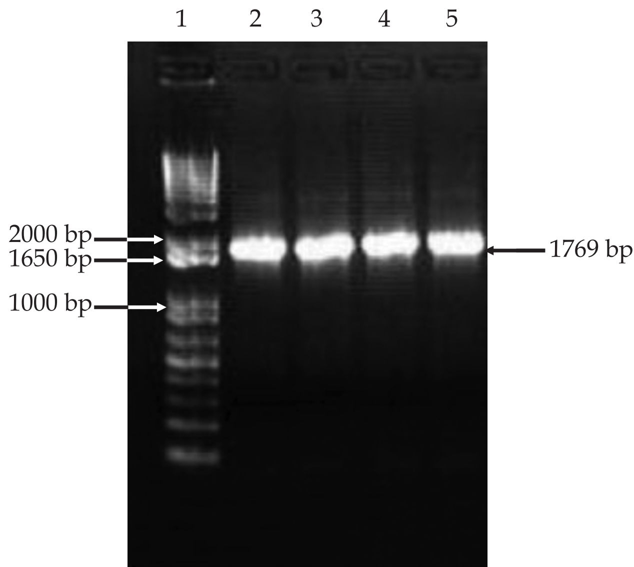

Fig 1. Amplification of Paraflagellar rod 1 gene of *T. evansi* by PCR
1. 1Kb plus DNA Ladder, 2-5. Amplicons

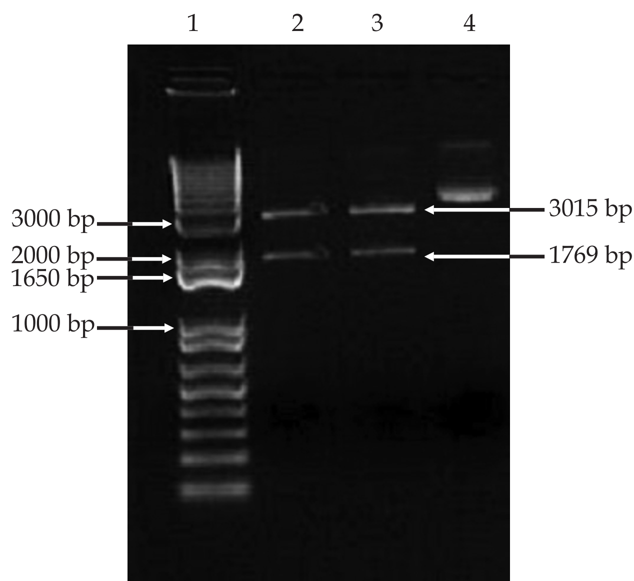

Fig 2. Paraflagellar rod 1 gene fragments of *T. evansi* after restriction digestion of pfr1 gene plasmid
1. 1Kb plus DNA Ladder, 2-3. Paraflagellar Rod 2 gene clone, 4. Uncut plasmid

and incubated at 37°C overnight with horizontal shaking. The plasmids DNA were extracted from culture using illustra plasmid prep mini spin kit (GE healthcare, USA) according to the manufacturer's instructions. The positive clones were identified by Restriction Enzyme digestion of plasmid DNAs with EcoR1 and colony PCR of plasmid colonies. Purified plasmid of *pfr1* gene was sequenced in both directions at Eurofins Genomics India Pvt Ltd., Whitefield, Bangalore.

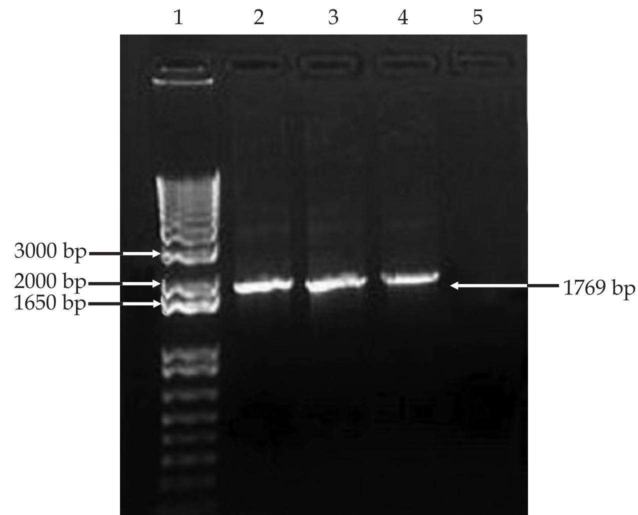

Fig 3. Amplification of Paraflagellar rod 1 gene of *T. evansi* by Colony-PCR.
1. 1Kb plus DNA Ladder, 2-3. PCR reaction with white colony shows amplification, 4. PCR reaction with blue colony shows amplification, 5. PCR reaction with blue colony shows absence of amplification.

Sequence analysis

After confirmation of the *pfr1* gene nucleotide sequence of *T. evansi* isolated from the host camel (*Camelus dromedarius*), the nucleotide sequence was submitted to GenBank, NCBI database. After getting the accession number of gene sequence Phylogenetic and sequence analysis of *pfr1* gene of *T.evansi* was done. The phylogenetic and sequence analysis was done by use of Clustal X and MEGA5 softwares. Phylogenetic tree analysis of *pfr1* gene was done by using Neighbor-Joining (NJ) method and maximum parsimony (MP) method and implemented with bootstrap test involving simple stepwise addition.

Building three dimensional structure model of pfr1 *protein using computational approach*

The 3D model of *pfr1* protein was built by homology modeling based on high-resolution crystal structures of homologous proteins. A basic alignment search tool (BLAST, Altschul *et al*, 1990) search was performed for selecting the 3D models of the closest homologues available in the Brookhaven Protein Data Bank (PDB). The 3LP5 showed a high level of sequence identity with *Pfr1*. The coordinates of crystal structure of 3LP5 was used as template to build the initial model of *pfr1* and the 3D model was generated by the automated homology modeling software MODELLER9v6 (http://salilab.org) on windows operating environment (Sali & Blundell, 1993).

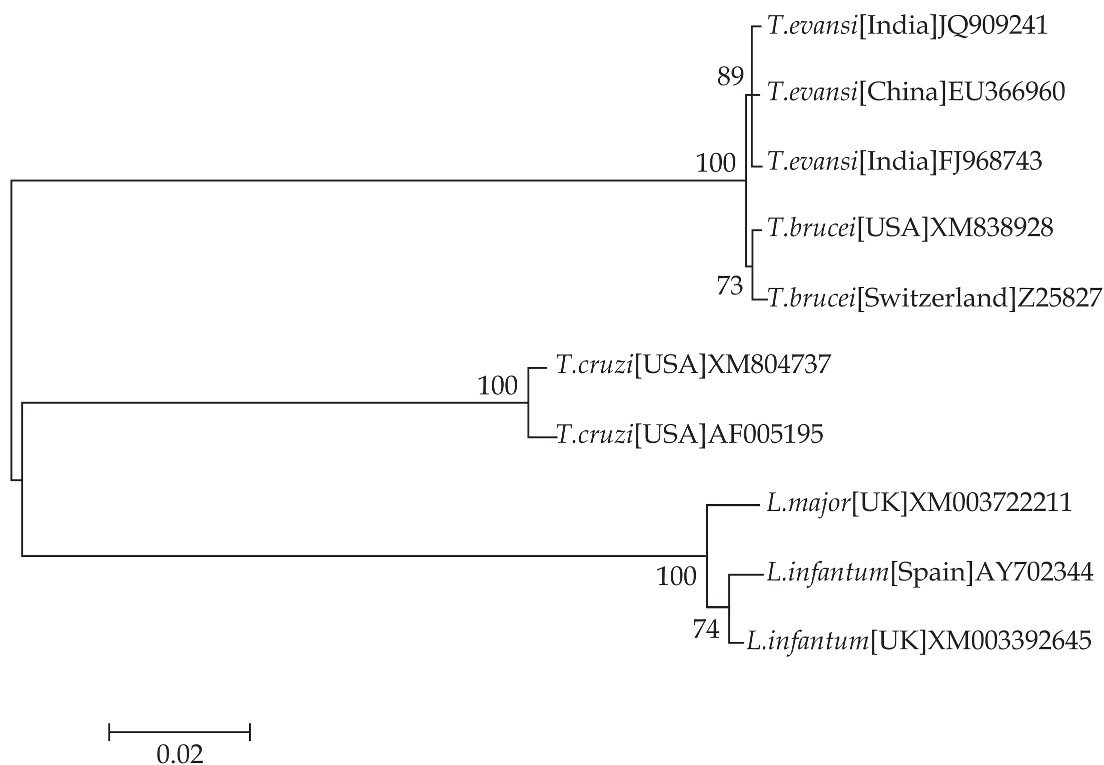

Fig 4. Phylogenetic tree analysis of Paraflagellar rod 1 gene using the Neighbor- Joining method.

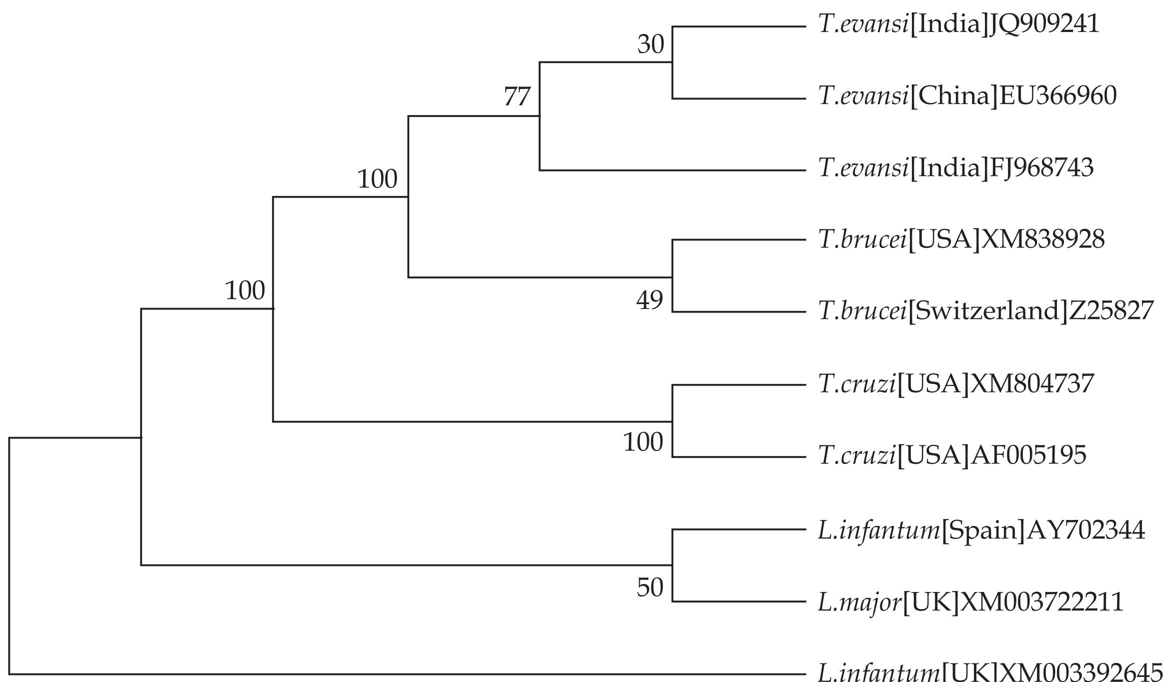

Fig 5. Phylogenetic tree analysis of Paraflagellar rod 1 gene using maximum parsimony method.

Results and Discussion

PCR- amplification of *pfr1* gene of *T. evansi* revealed that the amplification band of obtained DNA fragment of *pfr1* gene was in between 2000 bp and 1650 bp (Fig 1). There were several white colonies along with a few blue colonies were found after cloning of amplified PCR product. The blue colonies represent the presence of vector alone but few blue

colonies may contain vector with insert. The white colonies may represent recombinant clones carrying insert in the plasmid. Two well separated DNA bands were seen in case of plasmid isolated from positive colonies upon digestion with EcoRI, the less intense lower band may correspond to the insert (Fig 2). Release of DNA fragments of around 1769 bp for *pfr1* gene was found after restriction enzyme digestion. Colony PCR was done for quick screening of plasmid inserts directly from *E. coli* colonies and amplification was found in wells of white colonies and also in one well of blue colony (Fig 3).

The coding sequence of *pfr1* gene according to the result obtained was of 1769 bp encoding a protein of 589 amino acids with a predicted molecular weight of 73 kDa. After confirmation of *pfr1* gene nucleotide sequence of *T. evansi* isolated from the host camel, the sequence was submitted to GenBank, NCBI database to which the assigned accession number is JQ909241. For phylogenetic analysis of *pfr1* gene sequences of other Trypanosomatidae species already available in the genbank database were retrieved (Table 2). Tree topology based on the Neighbor-Joining method showed a close homology with other Trypanosomatidae species sequences with100% bootstrap values (Fig 4). The NJ, bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analysed. Phylogenetic tree analysis of *pfr1* gene using maximum parsimony also showed same topology as NJ method. The percentage of replicate tree in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches.

The final stable structure of *pfr1* protein have 4 sheets, 1 beta-alpha-beta unit, 2 beta hairpins, 2 beta bulges, 9 strands, 35 helices, 57 helix-helix interfaces, 105 beta turns and 16 gamma turns (Fig 6 & 7).

In the present study, the paraflagellar rod 1 gene sequence showed 99.9% homology with *T. evansi*, GenBank Accession No. EU366960, 99.8% with *T. brucei*, GenBank Accession No. XM838928 and 99.4% with *T. brucei*, GenBank Accession No. Z25827. 100% sequence similarity was found between obtained *pfr1* gene sequence and *T. evansi*, GenBank Accession No. FJ968743. Slightly lower homology was documented between the obtained *pfr1* gene sequence and T. cruzi, GenBank Accession No. XM804737 and T. cruzi, GenBank Accession No. AF005195. The comparison with *Leishmania infantum*, GenBank Accession No. AY702344, *Leishmania major*, GenBank Accession No. XM003722211 and *Leishmania infantum*, GenBank Accession No. XM003392645 a member belonging to Trypanosomatidae family showed only 83.6%, 83.6%, and 83.9% homology, respectively.

According to neighbor-joining phylogenetic tree analysis of *pfr1* gene, *T. cruzi*, GenBank Accession No. XM804737 and *T. cruzi*, GenBank Accession No. AF005195 are placed as two sub cluster of one mega cluster. *Leishmania major*, GenBank Accession No. XM003722211, *Leishmania infantum*, GenBank Accession No. AY702344 and *Leishmania infantum*, GenBank Accession No. XM003392645 as three sub

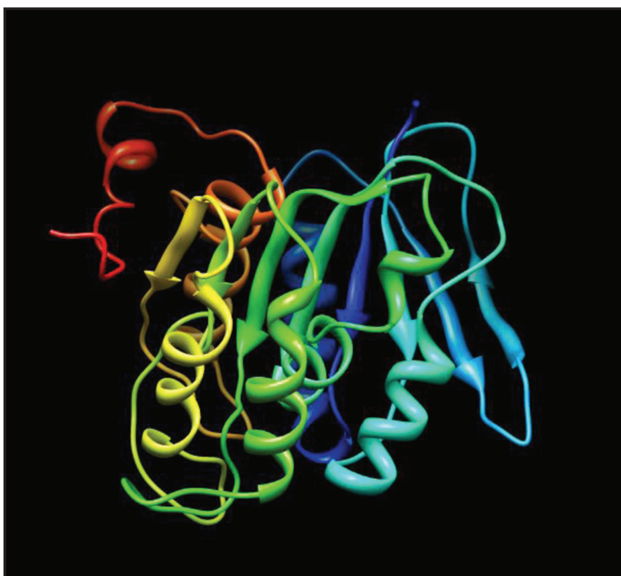

Fig 6. Cartoon representation of 3D model of Paraflagellar rod 1 protein.

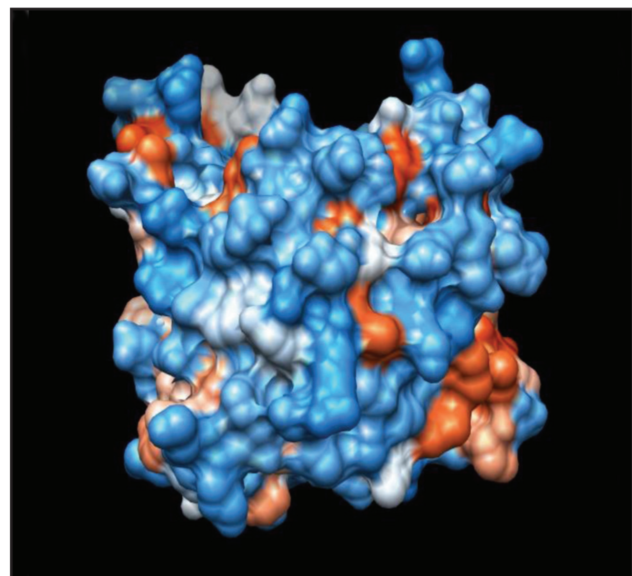

Fig 7. Crystallographic 3D structure of Paraflagellar rod 1 protein.

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*, GenBank Accession No. FJ968743 and other documented species. The earlier reports of *T. evansi* by Abdille *et al* (2008) showed 99.9% homology with obtained *pfr1* sequence and sequence of *T. evansi*, GenBank Accession No. FJ968743 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 *pfr1* gene in *Trypanosoma evansi* of Izatnagar isolate from horse of India (Maharana *et al*, 2011) revealed 99.8% homology with the China isolates and only one nucleotide change at 867 bp was detected. The nucleotide sequence of Izatnagar isolate also showed 99.8, 82.1, 79.9, 72.9 per cent homology with *Trypanosoma brucei*, *Trypanosoma cruzi, Leishmania infantum* and *Crithidia daenei*, respectively. The deduced amino acid sequence of *T. evansi pfr1* revealed 99.7% homology between Izatnagar and China isolate.

This Paraflagellar rod protein present in the kinetoplastid flagellum is a unique structure of trypanosoma flagellum due to presence of paracrystaline structure. PFR is vital for trypanosome motility (Bastin *et al,* 1998) and is unique among the kinetoplastids as their heteropolymers provide the building block of flagellum (Abdille *et al,* 2008). Identified *pfr1* gene also found in close homology with other trypanosome sequences like *T. brucei* (GenBank Accession No. XM838928 and GenBank Accession No. Z25827), *T. evansi* (GenBank Accession No. FJ968743 and GenBank Accession No. EU366960) and T. cruzi (GenBank Accession No. XM804737 and Gene bank Accession No. AF005195). The sequence identity of obtained *pfr1* nucleotide sequence of *T. evansi* with other trypanosomes species indicating that *pfr1* gene is highly conserved in the kinetoplastid species. It could therefore be suggested that vaccine with *pfr1* protein of trypanosomatidae parasite as the antigen could be effective against not only different strains within one trypanosome species but also against other species of the same genus.

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