DOI : 10.5958/2277-8934.2014.00006.X Vol 21 No 1, p 31-34

MOLECULAR CLONING OF ADENOSINE TRANSPORTER 1 GENE OF *Trypanosoma evansi* OF INDIAN CAMEL

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ABSTRACT

The present study was carried out to isolate the adenosine transporter 1(*TevAT1*) gene of *Trypanosoma evansi* using PCR and cloning of the gene. The desired amplicon of *TevAT1* gene from genomic DNA of *T. evansi* was successfully amplified by PCR using gene specific primers. Amplified PCR product was identified on the basis of size of the *TevAT1* gene using 25mM $MgCl_2$ and at annealing temperature of 49°C. For cloning the purified DNA fragment was ligated to the pGEM-T Easy vector and ligated mixture was transformed into *Escherichia coli* JM109 strains. The cells containing recombinant plasmid was identified on the basis of white/blue colony selection on LB agar containing X-Gal, IPTG and ampicillin. Screening of recombinant was done by Restriction Enzyme digestion of plasmid DNAs using *Eco*RI and confirmed on the basis of gene size, i. e. 1413 bp for *TevAT1* gene. Colony PCR was done for quick screening of plasmid inserts directly from *E. coli* colonies in the presence of insert specific primers.

Key words: Camel, Molecular Cloning, *TevAT1* gene, *Trypanosoma evansi*

Camel trypanosomosis is the most important parasitic disease that causes high morbidity and mortality among camel population. It has a wide distribution in Africa, Asia, South America and Middle East (Hoare, 1972). Recently human infections with *T. evansi* have been reported in India making it a potential human pathogen (Joshi *et al,* 2005).

Drug resistance to trypanosomes is now a problem, but its underlying mechanisms are not fully understood. Cellular uptake of the major trypanocidal drugs is thought to occur through an adenosine transporter. The adenosine transporter-1 gene, *TbAT1*, encoding a P2-like nucleoside transporter has previously been cloned from *Trypanosoma brucei brucei*, and when expressed in yeast, it showed very similar substrate specificity to the P2-nucleoside transporter, but could not transport diamidines (pentamidine and diminazene). Witola *et al* (2004) had cloned and sequenced a similar gene (*TevAT1*) from *Trypanosoma evansi* and found it to have 99.7% identity to the *TbAT1* gene. It was noticed that camel trypanosomosis incidence has increased and there was a wide increase of trypanocidals resistance for *T. evansi* reported from different part of Sudan (El Rayah *et al,* 1999). The number of anti-trypanosomal preparations available is limited and their value in disease control and eradication is reduced by the development of drug resistance in trypanosome populations (Leach and Roberts, 1981). So far, there is no suitable method for assessing drug resistance of trypanosomes.

Cloning of the purified PCR product of a gene is essentially required for the replication of DNA molecules to generate a large population of cells containing identical DNA molecules. The problem of drug resistance in *T. evansi* appears to be increasing in the field. Therefore, there is an urgent need to characterise resistant genes of *T. evansi,* so as to formulate a proper disease control policy.

Materials and Methods

Initially, camels suspected of trypanosomosis were identified in the National Research Centre on Camel, Bikaner (Rajasthan). Blood smear from suspected animals were prepared and stained with Geimsa stain after proper fixation with methanol. Properly stained blood films were examined under compound microscope to confirm the infection of *T. evansi* in the camels. After confirmation of *T. evansi* isolates, blood from infected host was

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collected. For this 5 ml blood was collected from jugular vein using 9 ml vaccutainer tube containing EDTA (Ethyl diamine tetra acetic acid). The 0.5 ml blood was inoculated intraperitoneally into the each experimental Swiss albino mice. The blood of mice was collected from heart in 5 ml disposable syringe containing 0.1 ml heparin solution after dissecting the mice which had massive infection. DEAE (Diethyl amino ethyl) cellulose column chromatography method was used for purification of trypanosomes (Lanham and Godfrey, 1970).

Amplification of TevAT1 gene by PCR

DNA isolation from collected pellet of *Trypanosoma evansi* was done as per the method utilised by Desquesnes and Davila (2002) for the preparation of animal trypanosomes DNA from plain blood. The total genomic DNA was isolated from the pellets of *T. evansi* using Proteinase K digestion and subsequent phenol-chloroform extraction method. Gene specific Forward 5′ CGGGTTTGACTCAGCCAATGA 3′ and Reverse 5′ CGTTTTACGTTTATGTCGTGACC 3′ primer sequences for *TevAT1* gene were designed from published sequences Accession No. AB124588 for the present study. Polymerase chain reaction was conducted in 50µl reaction mixture which contained 10 µl of 5x buffer, 25mM $MgCl_2$, 1 µl of 10 mM of dNTPs, 1µl of each nucleotide primer, 0.5 µl of DNA sample and 0.25µl of Taq DNA polymerase. After initial denaturation of the template at 94°C for 4 minutes the mixture was subjected to 36 cycles of amplification in thermo cycler. Each cycle consisted of a denaturation phase of 30 secs at 94°C; followed by annealing at 49°C for 45 secs and primer extension at 72°C for 1.30 min. The PCR product was analysed by electrophoresis in 1.2% agarose gel.

Cloning of PCR product of TevAT1 gene

After electrophoresis of amplified product in low melting agarose (LMP) DNA of interest from LMP agarose slices was purified using illustra GFX PCR DNA and Gel Band Purification Kit involving the steps as manufacturer protocol. The purified DNA was cloned in pGEM-T Easy vector (Promega, USA). The recombinant plasmid DNA was transferred into *Escherichia coli* JM109 competent cells and plated on Luria–Bertani (LB) agar medium. The recombinant clones were selected initially by blue–white screening followed by colony lysis. The recombinant plasmid DNA was isolated using mini-prep plasmid DNA isolation kit (Qiagen).

Confirmation of clones was done by Restriction Enzyme digestion of plasmid DNAs and Colony PCR of plasmid colonies. Restriction enzyme digestion was done with the use of *Eco*R1 enzyme. In 10 µl digestion mixture 5 µl plasmid DNA, 1 µl *Eco*R1 (Promega), 1 µl *Eco*R1 buffer (Promega) and 3 µl ultrapure water was added. After 4 hrs digestion at 37°C in water bath 2µl 6X loading dye was added to the mixture and analysed by running 1.2% agarose gel electrophoresis. Release of the expected size fragment confirmed the recombinants. For 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 colony was added in one tube.

Results

Amplification of TevAT1 gene of T. evansi by PCR

Total genomic DNA was isolated from the pellets of *T. evansi* and used as template for amplification by PCR. Gene specific forward and reverse primers were used for amplification and the amplicons analysed by agarose gel electrophoresis. To confirm the size of amplicons, the sample (amplicon) was run on gel electrophoresis using molecular weight marker (10 kbs plus DNA ladder). The amplification band of *TevAT1* gene was obtained in between 1000 bp and 3000 bp (Fig 1).

Cloning of TevAT1 gene of T. evansi into pGEM-T Easy vector

The amplified product was 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. The blue colonies represent the presence of vector alone but few blue colonies may contain vector with insert. The white colonies represented recombinant clones of carrying insert in the plasmid. The white colonies were screened for the presence of vector with insert.

Confirmation of clones by restriction digestion

Plasmid DNAs were extracted from positive colonies grown in LB medium containing ampicillin, digested with EcoRI and analysed by 1.2% analytical agarose gel electrophoresis using 10 kb plus molecular weight marker. Two well separated DNA bands were seen in case of plasmids isolated from positive colonies upon digestion with EcoRI (lanes 2 & 3),

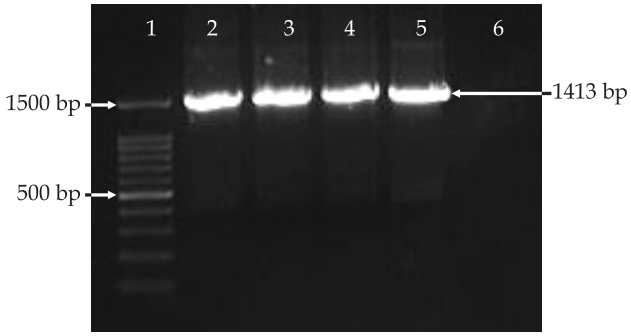

Fig 1. Amplification of *TevAT1* gene of *T. evansi* by PCR.
Lane 1. 10 Kb plus DNA Ladder, Lane 2-5. Amplicons.

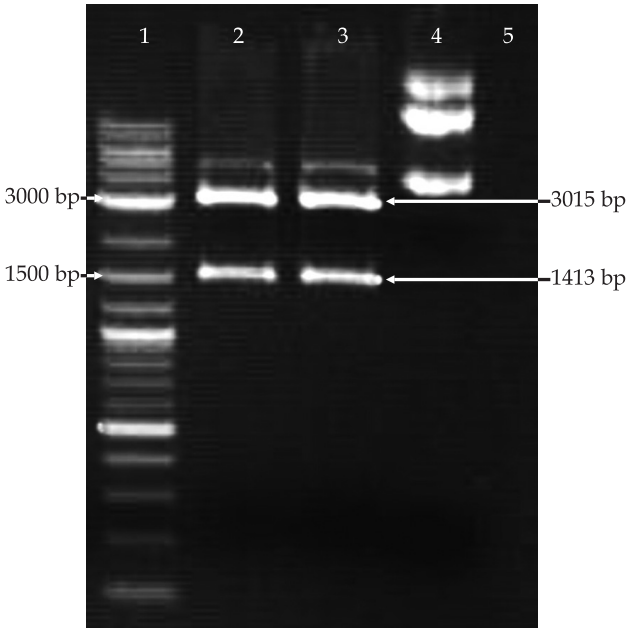

Fig 2. *TevAT1* gene fragments of *T. evansi* after restriction digestion of *TevAT1* gene plasmid Legends.
Lane 1. 10 Kb plus DNA Ladder, Lane 2-3. *TevAT1* gene clone, Lane 4. Uncut plasmid.

the less intense lower band may correspond to the insert. Release of DNA fragments of around 1413 bp for *TevAT1* gene was found after restriction enzyme digestion (Fig 2).

Confirmation of clones by Colony PCR

Colony PCR results were analysed by agarose gel electrophoresis using 10 kb plus molecular weight marker. Amplifications of *TevAT1* gene was found in both wells of white and blue colonies (Fig 3).

Disscusion

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. Control

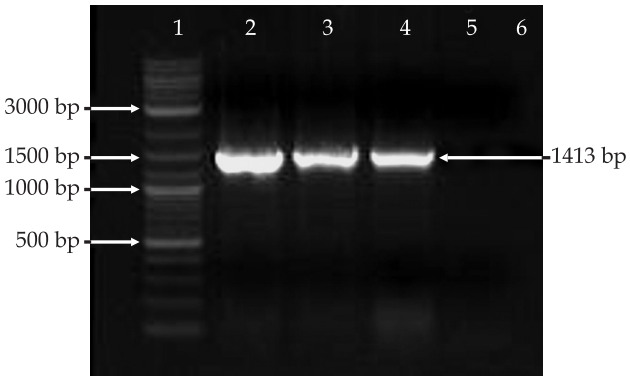

Fig 3. Amplification of *TevAT1* gene of *T. evansi* by Colony-PCR.
Lane 1. 10 Kb plus DNA Ladder, Lane 2-3. PCR reaction with white colony shows Amplification, Lane 4. PCR reaction with blue colony shows Amplification, Lane 5. Control.

of the disease remains a challenge, as chemotherapy has only modest success in disease treatment. The resistance of parasite to current drugs is increasingly becoming a major problem while there are no prospects for the development of new anti-trypanosome drugs. Drug tolerance develops in the field is derived basically from under-dosing due to incorrect estimation of body weight. A high incidence of trypanosomosis in conjunction with the irregular use of prophylactic and therapeutic drugs also favours the emergence of drug resistant trypanosome. The number of anti-trypanosomal preparations available is limited and their value in disease control and eradication is reduced by the development of drug resistance in trypanosome populations (Leach and Roberts, 1981).

In the present study, the desired amplicons of *TevAT1* gene from genome DNA of *T. evansi* was successfully amplified by PCR using gene specific primers. For cloning the purified DNA fragment was ligated to the pGEM- T Easy vector and ligated mixture was transformed into *Escherichia coli* JM109 strains. The cells containing recombinant plasmid was identified on the basis of white/blue colony selection on LB agar containing X-Gal, IPTG and ampicillin. Similar work was conducted by Witola *et al* (2004) in Japan, who had cloned and sequenced *TevAT1* gene from *Trypanosoma evansi* and found it to have 99.7% identity to the *TbAT1* gene. Liao and Shen (2010) studied the antrycide-resistance of *Trypanosoma brucei evansi* in China in some aspects in the last recent several years, the analysis of quinapyramine-sensitive situation of *T. b. evansi* in China, biological characteristics of *T. b. evansi* population in quinapyramine-resistance and biological materials of quinapyramine-resistance in *T. b. evansi*

population. They collected 12 *T.b. evansi* isolates from buffaloes, horses, mules and camels across 9 provinces of China. At genetic level, the gene, *TbAT1*, was amplified from the *T. b. evansi* isolates sensitive to quinapyramine-sensitivity but the *T. b. evansi* isolates with quinapyramine-resistance using not only the RT-PCR technique, but also PCR technique.

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FORM IV

(See Rule 8)

1.	Place of Publication	:	Camel Publishing House, 67, Gandhi Nagar West, Near Lalgarh Palace, Bikaner 334001, India.
2.	Periodicity of its publication	:	Biannual
3.	Printer's Name	:	Smt. Leela Devi
	(Whether citizen of India)	:	Yes
	Address	:	Gahlot Kutir, Nagani Road, Bikaner (Raj.)
4.	Publisher's Name	:	Smt. Leela Devi
	(Whether citizen of India)	:	Yes
	Address	:	Gahlot Kutir, Nagani Road, Bikaner (Raj.)
5.	Editor's Name	:	Dr. T.K. Gahlot
	(Whether citizen of India)	:	Yes
	Address	:	Department of Veterinary Surgery and Radiology, College of Veterinary and Animal Sciences, Bikaner 334001 (India).
6.	Names and address of individual who own the newspaper and partners or share holders holding more than one per cent of total capital.	:	Dr. T.K. Gahlot 67, Gandhi Nagar West, Near Lalgarh Palace, Bikaner 334001 (India)

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Dated : 01.06.2014

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