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IDENTIFICATION AND MOLECULAR CLONING OF CYSTEINE PROTEASE GENE OF *Trypanosoma evansi* ISOLATED FROM CAMEL

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ABSTRACT

A molecular study was carried out to isolate cysteine protease gene of *Trypanosoma evansi* using PCR. The desired amplicons of cysteine protease gene from the genomic DNA of *T. evansi* were successfully amplified by PCR using gene specific primers at annealing temperature of 55°C. Amplified PCR product was identified on the basis of its size in agarose gel electrophoresis as 1533 bp. For cloning the purified DNA fragment was ligated to the pGEM- T Easy vector and the ligated mixture was transformed into *Escherichia coli* JM109 strains. The cells containing recombinant plasmid were identified on the basis of white/blue colony selection on LB agar containing X-Gal, IPTG and ampicillin. Screening of recombinants was done by restriction enzyme digestion of plasmid DNA using EcoRI and confirmed on the basis of gene size, i. e. 1533 bp for cysteine protease 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, cloning, cysteine protease, *Trypanosoma evansi*

Trypanosoma evansi is widely distributed amongst the domesticated and wild animals and it is the causative agent of trypanosomosis in camels, horses, mules, the Indian elephant, ruminants, etc (Gill, 1977). Trypanosomosis is the most pathogenic parasitic disease of camelids in all camel rising countries causing high morbidity and mortality (Luckins, 1992).

Proteases, also called peptidases are peptide-hydrolysing enzymes. Proteases are classified according to the initial position at which they cleave the peptide substrate. Based on reactive residues found in the active site, these enzymes are further categorised into 7 main groups: serine, cysteine, aspartic, metallo, threonine, glutamate and asparagine proteases (Rawlings *et al,* 2004). Cysteine proteases are good targets for comparative studies of trypanosomes because they serve vital role in development of trypanosomatids in their life cycles in vertebrate hosts and vectors contributing not only to the infectivity and pathogenesis but also to protective immune response (Atkinson *et al,* 2009; Caffrey and Steverding, 2009). Cysteine proteases have been characterised extensively in *T. cruzi*, *T. congolense* and *T. b. brucei* at the biochemical, molecular and immunological levels (Sajid and McKerrow, 2009; Lalmanach *et al,* 2002). Characterisation of the genes encoding cysteine proteases is helpful as first step in understanding cysteine protease enzymes in *T. evansi*. Thus, present study was carried out to isolate the cysteine protease gene of *Trypanosoma evansi* using PCR and cloning of the gene.

Materials and Methods

After confirmation of *T. evansi* infection by blood smear examination, blood from infected host was collected and inoculated intra-peritoneally in Swiss albino mice (maintained at Small Animal Laboratory, NRC on Camel, Bikaner). DNA isolation from collected pellet of *Trypanosoma evansi* was done as per the protocols given by ready to use kit from Illustra blood genomic prep. mini kit. The cysteine protease gene of *T. evansi* was amplified from genomic DNA using specific forward 5′ ACACCCACGCAAGCAGTAA 3′ and reverse 5′ ACGTCGATCGCGGACACATA 3′ primer sequences designed from published sequence of *T. cruzi* (Accession No. U41454.1). Cycling conditions for PCR were initial denaturation at 94°C for 4 minute, 35 cycles of 30 seconds at 94°C, 45 seconds at annealing temperatures of 55°C and 1 min. and 30 seconds at 72°C, followed by a final extension for 10 minute at

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72°C. The PCR amplified products were checked with 1.5 kb DNA molecular weight marker in 1% agarose gel.

The PCR product from low melting point agarose slices were purified using Illustra GFX PCR DNA and Gel Band Purification Kit. The DNA fragments of cysteine protease gene and the pGEM-T Easy vector in which it was to be cloned were digested with T4 DNA ligase enzyme to generate compatible ends for ligation. The ligation was done in the reaction volume of 20 µl containing 10µl of 2X Rapid ligation T4 DNA Ligase buffer [400mM Tris-HCl, 100mM $MgCl_2$, 100mM DTT, 5mM ATP (pH 7.8 at 25°C)], 6 µl PCR product, 2 µl pGEM- T Easy vector and 2 µl of T4 DNA ligase. The contents were mixed well by tapping and it was spun down in a micro centrifuge for 3-5 seconds and incubated for overnight at 4°C. The ligation mix was used directly for transformation in JM109 high efficiency competent cells. After incubation 100 µl transformation culture was plated onto antibiotic agar plates in duplicate and incubated at 37°C for overnight (16-20 hr). Colonies harbouring recombinant plasmids were inoculated into LB broth and incubated at 37°C overnight with horizontal shaking. The plasmid DNA was extracted from culture using illustra plasmid prep mini spin kit. The positive clone was confirmed by Restriction Enzyme digestion of plasmid DNAs with EcoR1 and colony PCR of plasmid colonies.

Results and Discussion

The genomic DNA was analysed in 0.8% analytical agarose gel and was found to be intact without much shearing. Gene specific forward and reverse primers were used for amplification of cysteine protease gene and the amplicons were analysed by agarose gel electrophoresis. An intensely amplified DNA was seen in lanes B and C using genomic DNA (Fig 1). The size of the intense band was deduced from the standard curve drawn between the log molecular sizes of the marker bands against their respective mobility and found to be 1533 bp.

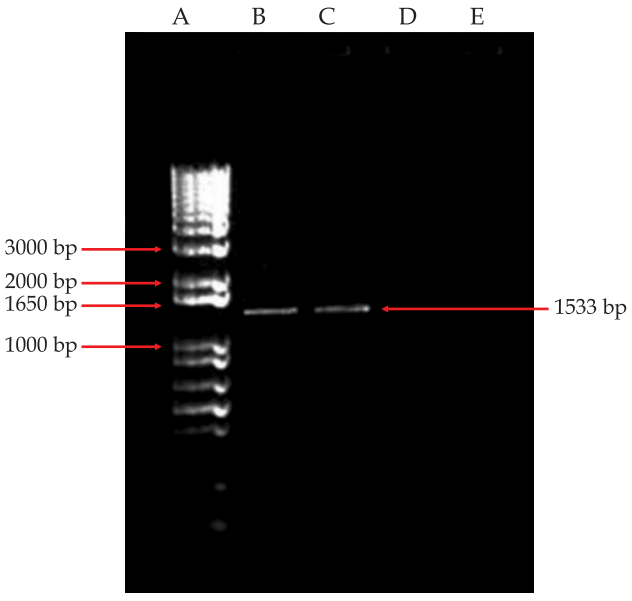

Fig 1. Amplification of cysteine protease gene of *T. evansi* by PCR. A. 1Kb plus DNA Ladder B-C. Amplicons.

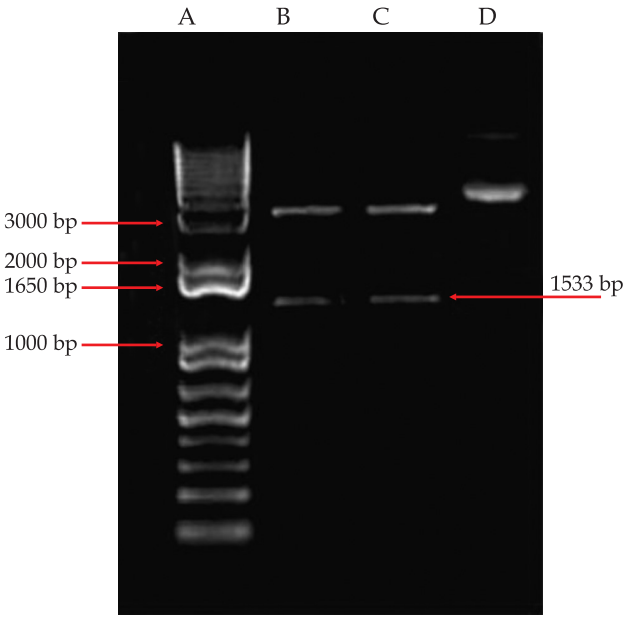

Fig 2. Cysteine Protease gene fragments of *T. evansi* after restriction digestion of *cysteine protease* plasmid Legends. A. 1Kb plus DNA Ladder B–C. *Cysteine protease* gene clone D. Uncut plasmid.

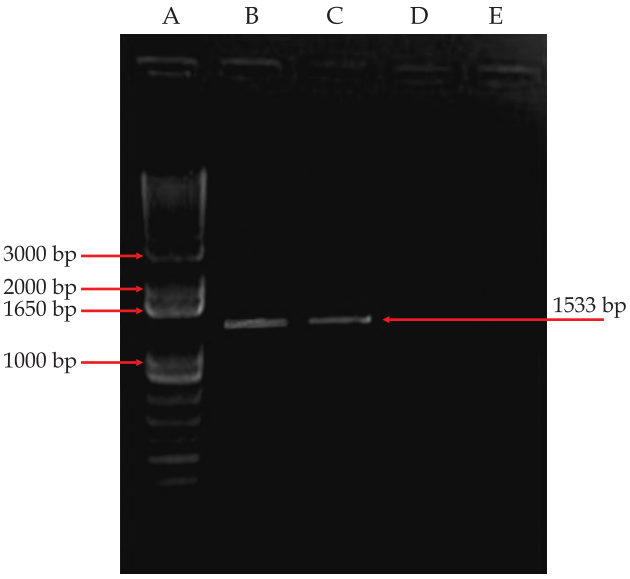

Fig 3. Amplification of Cysteine Protease gene of *T. evansi* by Colony- PCR. A. 1Kb plus DNA Ladder B–C. PCR reaction with white colony shows Amplification, D. PCR reaction with Blue colony shows no Amplification.

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 and several white colonies along with a few blue colonies were obtained. The blue colonies represented the presence of vector alone but few of them contained vector with insert. The white colonies 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 Eco RI, the less intense lower band may correspond to the insert (Fig 2). Release of DNA fragments of around 1533 bp for cysteine protease 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 blue colony (Fig 3).

The cysteine protease gene of *Trypanosoma evansi* is a nonvariant gene. Due to stability against the host immune response, this gene may be explored to make possible immune prophylaxis to control the disease effectively and inexpensively. It may also be investigated for identification of diagnostic markers and drug targets of this parasite to develop improved methods of prevention, diagnosis and treatment. In the present study, the amplicon size obtained was of 1533 bp. Gonzatti *et al* (1999) identified 453 bp partial cds of *Trypanosoma evansi* cysteine protease evansain gene (Accession no. AF165115). Tomas and Kelly (1996) reported *Trypanosoma cruzi* cysteine protease cruzipain gene of a tandem array complete cds of 1466 bp (Gen Bank Accession no. U41444) and Omara-Opyene and Gedamu (1998) observed that complete cds of the gene was 1677 bp (GenBank Accession no. AF004594). *Trypanosoma cruzi* cysteine protease gene, partial cds with 495 bp was identified by Eakin *et al* (1993; GenBank Accession no. M27305). However, in this study, the DNA fragment amplified in the PCR reaction was of expected size (1533bp) and highly target specific region of cysteine protease gene of *T. evansi*. With the cloning and sequencing of cysteine protease gene of *T. evansi* and expression of this protein it can make a great impact on the discovery of new protective antigen.

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