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IDENTIFICATION AND MOLECULAR CLONING OF *RoTat* VSG GENE OF *Trypanosoma evansi* OF CAMEL IN INDIA

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ABSTRACT

The present study was carried out to isolate the Rode Trypanozoon Antigen Type VSG (*RoTat* VSG) gene of *Trypanosoma evansi* using PCR and cloning of the gene. The desired amplicons of *RoTat* VSG gene from genome 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 *RoTat* VSG gene using 25mM $Mgcl_2$ and at annealing temperature of 51°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 1450 bp for *RoTat* VSG 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, *RoTat* VSG, *Trypanosoma evansi*

Rode Trypanozoon antigen type 1.2 (*RoTat* 1.2) is a variable antigen type 1.2 which was cloned from *T. evansi* isolated from an Indonesian buffalo in 1982 (Bajyana Songa and Hamers, 1988). It is one of the predominant VATs and has been found early in the infection in the majority of *T. evansi* strains. Test using *RoTat* 1.2 do not cross react with antibodies to *T. theileri* or other pathogenic trypanosomes, although they cannot distinguish between infections with trypanosomes currently classified as *T. evansi* and *T. equiperdum* (Claes *et al,* 2002). The *RoTat* 1.2 gene is a fairy specific marker for *T. evansi* type A strains, but is not present in *T. evansi* type B strains isolated from Kenya, which may limit its diagnostic utility (Claes *et al,* 2004; Ngaira *et al,* 2005).

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 lack of accurate diagnostic tests and tools for characterisation of *T. evansi* forms a major constraint to treatment and deployment of rational control strategies for surra. The present study was carried out to isolate the Rode Trypanozoon Antigen Type VSG (*RoTat* VSG) gene of *Trypanosoma evansi* using PCR and cloning of the gene.

Materials and Methods

Initially, camels suspected of trypanosomosis were identified in the National Research Centre on Camel, Bikaner (Rajasthan). Geimsa stain after proper fixation with methanol. The Geimsa stained blood films were examined under compound microscope and after confirmation of *T. evansi* isolates, 5 ml blood from infected host was collected from the jugular vein using 9 ml vaccutainer tube containing EDTA (ethyl diamine tetra acetic acid). Intraperitoneal inoculation with 0.5 ml blood was done into the each experimental animal which were Swiss albino mice. The blood of mice was collected from heart region 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 RoTat 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 plane blood. The total genomic DNA was

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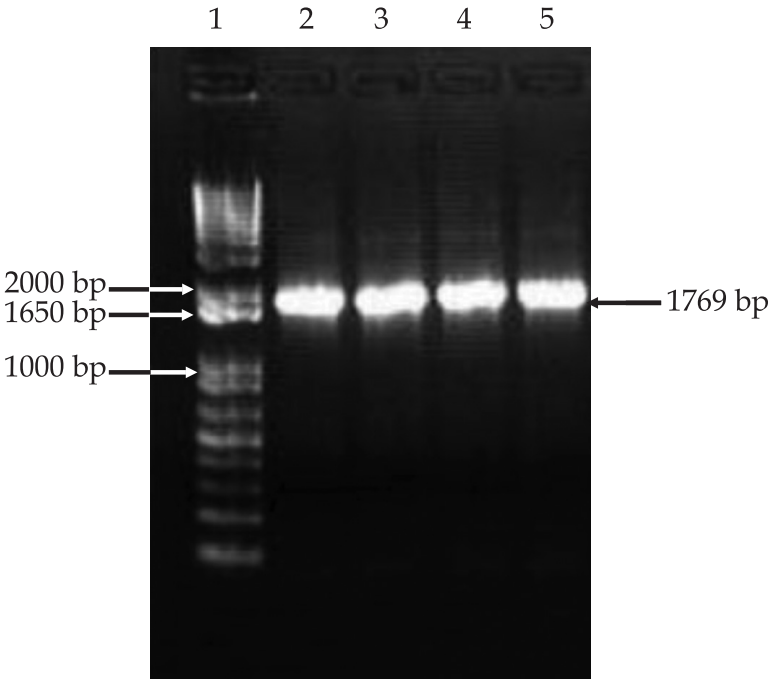

Fig 1. Amplification of *RoTat* VSG gene of *T. evansi* by PCR

Lane1. 10 Kb plus DNA Ladder, Lane2 – 5. Amplicons, Lane 6. Control.

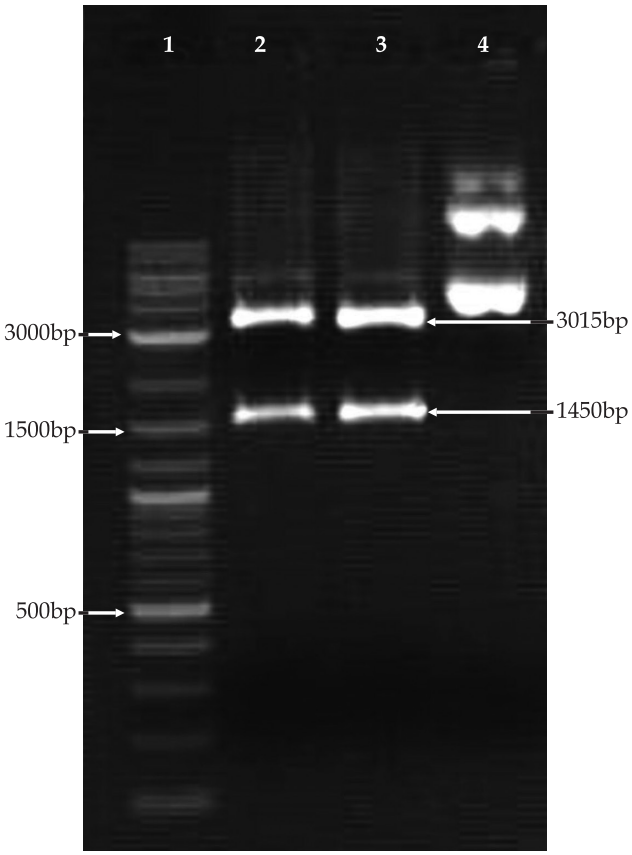

Fig 2. *RoTat* VSG gene fragments of *T. evansi* after restriction digestion of *RoTat* VSG gene plasmid Legends

Lane 1. 10 Kb plus DNA Ladder, Lane 2 – 3. *RoTat* VSG gene clone, Lane 4. Uncut plasmid.

isolated from the pellets of *T. evansi* using Proteinase K digestion and subsequent phenol-chloroform extraction method. Gene specific Forward 5′ CACTGCTTTACGCCATCACTC 3′ and Reverse 5′ GCATTCTTTTCCATCCCATTTGC 3′ primer sequences for *RoTat* VSG gene were designed from published sequences Accession No. HQ286335 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 0.25 µl of Taq DNA polymerase. After initial denaturation of the template at 94°C for 4 minutes the mix 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 51°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 RoTat VSG gene

After electrophoresis of amplified product in low meltig 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 EcoR1 enzyme. In 10 µl digestion mixture 5 µl plasmid DNA, 1 µl EcoR1 (Promega), 1 µl EcoR1 buffer (Promega) and 3 µl ultrapure water was added. After 4 hrs. digestion at 37°C in water bath 2 µl 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

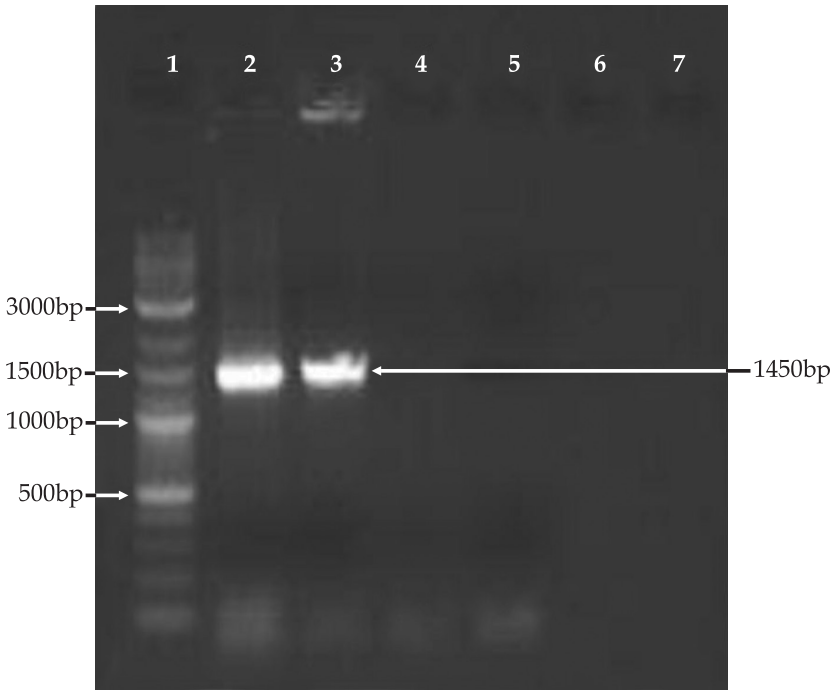

Fig 3. Amplification of *RoTat* VSG gene of *T. evansi* by Colony-PCR.

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 absence of Amplification, Lane 5. Control.

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 RoTat VSG 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 *RoTat* VSG gene was obtained in between 1000 bp and 3000 bp (Fig 1).

Cloning of RoTat VSG 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 *Eco*RI 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 *Eco*RI (lanes 2 & 3), the less intense lower band may correspond to the insert. Release of DNA fragments of around 1450 bp for *RoTat* VSG 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 *RoTat* VSG gene was found in wells of white colonies but was not found in blue colony (Fig 3).

Discussion

Trypanosomes undergo antigenic variation that enables them to evade the host's immune system (Barry and Turner, 1991; Baron, 1996; Donelson *et al,* 1998). VSG covers the entire parasite including the flagellum presumably as a protective shield against host antibodies (Englund *et al,* 1982; Pays *et al,* 2001; Machado *et al,* 2006). This protective coat which determines the antigenic features of the parasite, is easily recognised by the host and is highly immunogenic.

RoTat 1.2 gene based PCR test (Urakawa *et al,* 2001) has been considered reliable as it detects both dyskinetoplastic and kinetoplastic isolates (Claes *et al,* 2004). However, recent reports of the occurrence of *RoTat* 1.2 negative isolates in Kenya (Ngaira *et al,* 2004) may deny this marker the expected universality.

The present study revealed that *RoTat* VSG gene was appropriated marker for detection of *T. evansi*

strains in camels except the Kenyan *T. evansi* type B. This finding agrees with Claes *et al* (2004) in their study in Variable Surface Glycoprotein (VSG) *RoTat* 1.2 PCR as diagnostic tool for the detection of *T. evansi* infection. A total of 39 different trypanosome stocks were tested using the *RoTat* 1.2 based Polymerase Chain Reaction (PCR). The expression of VSG in the early, middle and late stages of infection favours its use in early diagnosis (in prepatency) as well as in carrier status where the conventional microscopy fails to diagnose (Robinson *et al*, 1999). This would make the use of VSG-based diagnosis more feasible in *T. evansi* infection than in cyclically transmitted trypanosome species. The primers designed for amplification of *RoTat* VSG gene in this study could be used in the diagnosis of surra caused by *T. evansi* type A.

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