Indian Journal of Animal Sciences **87** (8): 974–976, August 2017/Short communication

Molecular characterization and phylogenetic sequence analysis of unique conserved portion of VSG of *Trypanosoma evansi*

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Received: 25 February 2017; Accepted: 18 March 2017

Key words: Molecular characterization, *Trypanosoma evansi,* VSG

The trypanosome variant surface glycoprotein (VSG) is an integral part of parasite's surface coat and is known to be expressed in early, middle and late stages of surra (Verloo *et al.* 2001). *Trypanosoma evansi* is known to have limited VSG antigenic repertoire in contrast to the cyclically developing trypanosomes (Zhang and Baltz 1994). Of late, specific VSG was found to be absent in some *T. evansi* trypanosome isolates (Ngaira *et al.* 2004, Salim *et al.* 2011); alongside, there are a fewer reports of its structural variations within the stocks (Jia *et al.* 2011).The present communication deals with molecular cloning and characterization of unique truncated portion of VSG which was later found to be conserved in all known stocks of the parasite across India as well as the globe through phylogenetic analysis.

Sample collection and DNA isolation from blood: Blood sample (1 ml aliquot) was collected in clean sterile vacutainer, coated with EDTA anticoagulant, from the jugular vein of earlier surra confirmed horse. DNA was isolated from blood using standard phenol chloroform method with minor modifications. Briefly, 200 µl of blood was added into equal volume in TE lysis solution consisting of 10 mM of Tris acid and 1 mM of EDTA. Then 5 µl of 25 mg/ml of Proteinase K and 50 µl of 10% SDS were added to it and the sample was kept at 65°C for 4 h. Thereafter, the DNA was extracted as per standard phenol chloroform protocol (Sambrook and Russel 2001).

Primer selection and PCR amplification: Primers were derived from the VSG sequence (AF317914). Using DNA sequence homology search programs to interrogate databases GenBank, primer sequences were identified within the region (608–812 bp). Oligonucleotide primers targeting the VSG of *T. evansi* (VSG F/R) were custom synthesized using the primer sequence published elsewhere (Claes *et al*. 2004). The PCR reactions were set up into 25 µl volume containing 12.5 µl Green Taq PCR Master Mix (0.05/µl Taq DNA polymerase in reaction buffer, 4 mM $MgCl_2$, 0.4 mM dATP, 0.4 mM dCTP, 0.4 mM dGTP, and 0.4 mM dTTP), 1.5 µl of each primer (VSG F/R, 20 pmol of each primer) and 2 µl of the extracted DNA template. The total volume of the PCR mix was made up to 25 µl using nuclease-free water. Details of primers alongside expected PCR products and thermal cycling profile are shown in Table 1. The amplified amplicon was analyzed by agarose gel electrophoresis in 1.5% agarose gel.

Molecular cloning and characterization of VSG: The amplified product of VSG gene was purified using GeneJet DNA purification kit (Fermentas) following manufacturer's protocol. Thereafter, competent *Escherichia coli* DH5α cells (Puregene) were prepared following the standard calcium chloride treatment method (Sambrook and Russel

Table 1. Thermocyclic profile along with primer sequence of truncated VSG

Primer	Primer sequence				Amplicon size
VSG F	5'GCG GGG TGT TTA AAG CAA TA 3'				205 bp
VSG R	5'ATT AGT GCT GCG TGT GTT CG 3'				
	Thermal cycling profile				
	Initial denaturation	Denaturation	Hybridization	Extension	Termination
PCR with VSG gene	94°C, 240 sec	94°C, 60 sec	59°C, 60 sec	72°C, 60 sec	72°C, 300 sec
			× 35 cycles		

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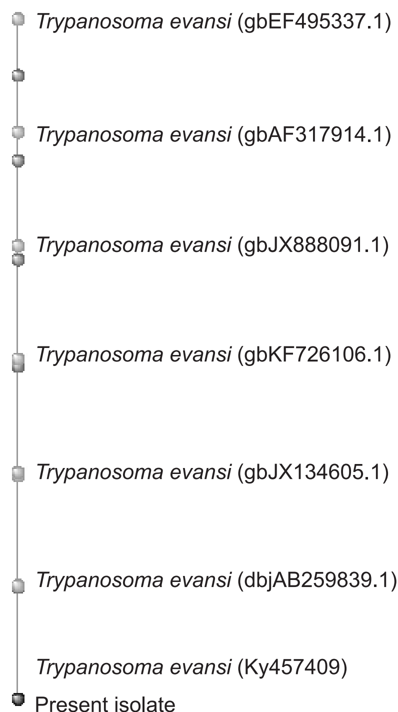

Fig. 1. Phylogenetic tree of VSG Mathura equine isolate with other known sequences.

2001). Ligation reaction for cloning of amplified VSG into CloneJet Vector (Fermentas) as well as transformation of DH5α cells was carried out as per manufacturer's protocol. The positive clones were identified by suicidal vector technology of the vector. Further confirmation was done by colony PCR. A subculture of positive clone harbouring the desired VSG gene was subcultured into 100 ml of LB broth (Himedia) for overnight growth and plasmids were isolated using Plasmid Purification Kit (Fermentas) following manufacturer's protocol. Thereafter the plasmids were sent for custom sequencing using M13 universal primers. The sequence information received was analyzed using DNASTAR and GeneTool software.

PCR amplification, molecular cloning and molecular characterization of the VSG gene: VSG gene was amplified from the genomic DNA of horse isolate of *T. evansi* using the specific forward and reverse primers. The amplicons were resolved as a single band of 205 bp product. It was further purified for ligation in CloneJet cloning vector. The selection of positive colonies was performed by colony PCR using the specific primers. The result of colony PCR was again checked by agarose gel electrophoresis.

Data analysis: The sequence, hence generated, was submitted to NCBI and an accession number KY457409 was obtained. A phylogenetic association, for analyzing the identity between strains and testing the robustness of the association, was done using the online bootstrap method (http://blast.ncbi.nlm.nih.gov/) to delineate its relationship with other referral stains (Fig. 1). The nucleotide sequence revealed 100.0% sequence homologies with all the other known *T. evansi* sequences, viz. buffalo, camel and horse isolates from Karnataka and Bikaner, India (EF495337, JX134605, AB259839); Kenyan isolate (AF317914); camel and cattle isolates from Egypt (JX888091, KF726106), respectively.

T. evansi possess marked importance in subtropics owing to its vast economic impact. The parasite is covered by a thick uniform coat of VSG which shields the invariant surface proteins from host immune system effectors and prevents complement activation (Turner *et al.* 1985). These VSGs are expressed at early, middle and later stages of infection (Verloo *et al.* 2001). Inspite of being the major determinant of immune evasion process, the host immune system still elicits sufficient level of antibody production against the parasite VSG (Gadelha *et al.* 2011). RoTat 1.2 VSG is the predominant variant antigen type and is known to be expressed in many *T. evansi* stocks (Verloo *et al.* 2000) across many parts of the globe. Of late, this gene was found to be absent in some *T. evansi* trypanosomes (Ngaira *et al.* 2004, Salim *et al.* 2011) and it also showed marked variation in fewer isolates (Jia *et al.* 2011). Hence, there is need for identification of that molecule that is conserved in all the isolates. The present truncated fragment of VSG is a good alternative in this regard. It was found to show cent percent homology with other isolates, thereby suggesting, that it could be used as a good alternative both in molecular detection using PCR as well as in serology using recombinant protein. Further research in this regard is thereby, warrented to use this molecule for molecular and serological studies.

SUMMARY

VSG is considered to be an important molecule for both molecular and serological based detection of surra. Of late, there are reports of variation and/or absence of RoTat 1.2 *T. evansi* VSG from a fewer isolates of murine and wild life origin. Hence, in order to use VSG for routine diagnosis of trypanosomiosis, there is need for selection of that portion of gene, which is conserved in all trypanosomes irrespective of species and geographical distribution. Hence, we identified a 205 bp portion of VSG, which is conserved in Trypanosomes.The present communication deals with molecular cloning and characterization of this *Trypanozoan* specific VSG gene from *T. evansi* of horse origin from semi arid region of India and checked its uniqueness by comparing it with various other isolates across the world. The selected truncated showed cent percent homology with all other isolates, thereby, justifying its uniqueness.

ACKNOWLEDGEMENTS

The authors are thankful to Dean and Vice Chancellor DUVASU for the facilities provided. Authors also acknowledge the help of Dr Harkirat Singh, GADVASU, during sequence analysis.

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