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Phylogenetic studies on RoTat 1.2 VSG of *Trypanosoma evansi* isolate from semi arid India

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ABSTRACT

RoTat 1.2 variant surface glycoprotein (VSG) is considered to be one of the most important VSGs expressed in many of the isolates of *Trypanosoma evansi*. This accounts for its utility as a potent molecule for both molecular and serological based detection of Surra. Of late, there are reports of its absence and variation from a fewer isolates of *T. evansi*. The study of these variations by means of molecular phylogenetic studies is of immense significance in determining the evolutionary phylogeny of these *T. evansi* isolates. With this information in the background, the present study was designed to clone and characterize RoTat 1.2 VSG from horse isolate of *T. evansi* from semi arid regions of India. Thereafter, the gene was compared with various other isolates across the world. Interestingly, the isolate was found to be closer to camel isolates from Egypt than the other known isolates from India and Kenya. The finding is important from evolutionary point of view.

Key words: India, Molecular characterization, Phylogenetic studies, Rotat 1.2 VSG, *Trypanosoma evansi*

The trypanosome variant surface glycoprotein (VSG) is considered to be an integral part of parasite's surface coat. These VSGs have unique ability to undergo class switching in expression on regular basis which accounts for its immune evasion strategy against the host's immune response (Barry and McCullogh 2001). In comparison with the cyclically developing trypanosomes, *Trypanosoma evansi* is having limited VSG antigenic repertoire (Zhang and Baltz 1994). However, amongst all those VSG repertoires, RoTat 1.2 VSG is a predominant variant antigen type (VAT) expressed in early, middle and late stages of infection (Verloo *et al.* 2001) in many of the *T. evansi* isolates (Verloo *et al.* 2000, Devi *et al.* 2017). Of late, RoTat 1.2 VSG gene was found to be either having some structural variations (Jia *et al.* 2011, Sudan *et al.* 2017) or is altogether absent in some *T. evansi* isolates (Ngaira *et al.* 2004, Salim *et al.* 2011). The present study was designed with the aim to clone and characterize RoTat 1.2 VSG from horse isolate of *T. evansi* from semi arid regions of India and then to phylogenitically analyze it with various other isolates across India and the globe.

MATERIALS AND METHODS

Sample collection and DNA isolation: Blood samples (1 ml aliquot) were collected in clean sterile vacutainer, coated with EDTA anticoagulant, from the jugular vein of earlier confirmed Surra infected horse (through microscopic observation of blood smears). 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: Oligonucleotide primers targeting the RoTat 1.2 VSG of *T. evansi* (VSG F/R) were custom synthesized using the sequences available in the pubmed (Assession No. KU589274, KY457409). The PCR reactions were set up into 25 µl volume containing 12.5 µl of Green 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. The PCR conditions included initial denaturation at 95°C for 2 min followed by 35 cycles of denaturation at 95°C for 45 sec, primer annealing at 60°C for 1 min and extension at 72°C for 2 min. A final extension was given at 72°C for 10 min. The amplified amplicon was analyzed by agarose gel electrophoresis in 1.25% agarose gel.

Molecular cloning and characterization of RoTat 1.2

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VSG: The amplified RoTat 1.2 VSG gene was purified using GeneJet Gel 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 2001). Ligation reaction for cloning of amplified RoTat 1.2 VSG into CloneJet cloning vector (Fermentas) as well as transformation of DH5α cells was carried out as per manufacturer's protocol. The positive clones were identified by their suicidal vector technology. Further confirmation was done by colony PCR following standard protocol (Sambrook and Russel 2001). The colony PCR amplified products were again visualized in the ethidium bromide stained agarose gel following agarose electrophoresis. A subculture of positive clone harbouring the desired RoTat 1.2 VSG gene was subcultured into 100 ml of LB broth (Himedia) for overnight growth and thereafter plasmid was 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 softwares.

RESULTS AND DISCUSSION

PCR amplification, molecular cloning and molecular characterization of the RoTat 1.2 VSG gene of horse isolate of T. evansi*:* RoTat 1.2 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 862 bp. It was further purified for ligation into cloning vector. The selection of positive colonies was performed by colony PCR using the specific primers.

Data analysis: The sequence hence generated was submitted to NCBI and an accession number KY457408

(a) Percent identity (rows: Divergence)

	1	2	3	4	5	6		
1		99.8	99.4	100.0	100.0	99.6	1	KY457408.1 Mathura
2	0.2		99.8	96.4	99.8	95.9	2	JX134605.1 Bikaner
3	0.4	0.2		100.0	100.0	99.6	3	AF317914.1 Kenya
4	0.0	0.2	0.0		100.0	99.6	4	JX888091.1 Egypt
5	0.0	0.2	0.0	0.0		99.6	5	KF726106.1 Egypt
6	0.4	0.7	0.4	0.4	0.4		6	EE495337.1 Karnataka
	1	2	3	4	5	6		

(b) Percent identity (rows: Divergence)

	1	2	3	4	5	6		
1		99.3	99.3	100.0	98.7	100.0	1	KY457408.1 Mathura
2	0.7		99.3	95.9	94.6	99.3	2	JX134605.1 Bikaner
3	0.7	0.7		100.0	98.7	100.0	3	EE495337.1 Karnataka
4	0.0	0.7	0.0		98.7	100.0	4	JX888091.1 Egypt
5	1.3	2.1	1.3	1.3		98.7	5	AF317914.1 Kenya
6	0.0	0.7	0.0	0.0	1.3		6	KF726106.1 Egypt
	1	2	3	4	5	6		

Fig. 1. Divergence table of RoTat VSG 1.2 Mathura equine isolate with other known sequences. 1(a): Nucleotide homologies; and 1 (b): Protein homologies.

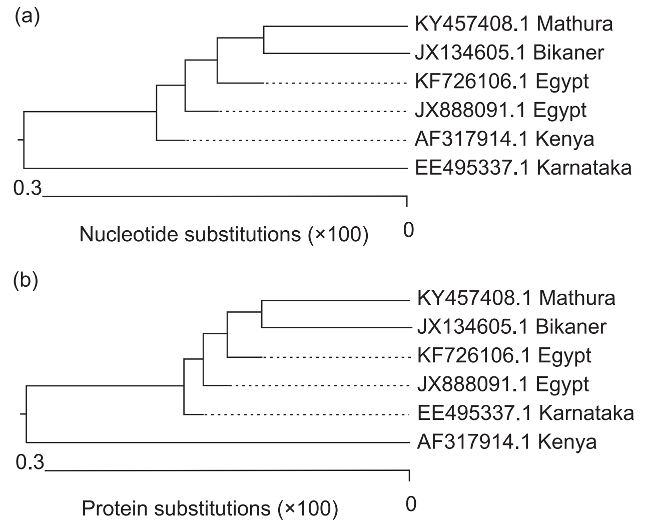

Fig. 2. Phylogenetic tree of RoTat VSG 1.2 Mathura equine isolate with other known sequences. 2(a): Nucleotide substitution; and 2(b): Protein substitution.

was obtained. The nucleotide sequence revealed 100.0% (Fig.1a) sequence homologies with that of isolates from Egypt (Accession numbers JX888091 and KF726106). However, the nucleotide similarity with camel and buffalo sequences from India revealed 99.8 and 99.6% homologies, (Accession numbers JX134605 and EF495337), respectively. Alongside, it also showed 99.4% homology with Kenyan isolate (Accession number AF317914). Likewise, it also showed 99.3, 100 and 98.7% homologies in protein patterns with various isolates from India, Egypt and Kenya, respectively (Fig. 1b). 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. 2 a, b).

The recent developments in the molecular techniques have imposed a considerable impact upon the trypanosome identification, characterization, accuracy and reliability at various taxonomic levels (Desquesnes and Davila 2002). There are ample reports of characterization studies on trypanosomes in general and *T. evansi* in particular. For instance, oligopeptidase B from *T. b. brucei*, variable surface glycoprotein (VSG) gene of *T. evansi* (Sengupta *et al.* 2012), ISG-75 gene of *T. b. gambiense* (Tran *et al.* 2008), hypoxanthine guanine phosphoribosyl transferase gene of *T.b. brucei* (Allen and Ullman 1993), beta-tubulin gene of *T. evansi* (Li *et al.* 2007) and actin gene of *T. evansi* (Li *et al.* 2009). All these molecules are used for phylogenetic analysis between the various isolates.

T. evansi possess marked importance in subtropics owing to its 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). The surface epitopes in live trypanosomes are confirmationally labile (Freymann *et al.* 1990) and anti-VSG antibodies are known to recognizes glycosylated and deglycosylated VSGs equally well (Reinwald 1985). These points make VSG a potent antigen in the diagnosis of *T. evansi* infection. Amongst all the studied VSGs, 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) like cattle (Sengupta *et al.* 2012), buffalo (Sengupta *et al.* 2010), horse (Sudan *et al.* 2017) and camel (Roge *et al.* 2013) across many parts of the globe. Of late, RoTat 1.2 VSG gene was found to be absent in some *T. evansi* trypanosomes (Ngaira *et al.* 2004, Salim *et al.* 2011).

The *T. evansi* horse isolated used in the present study showed cent percent homology with that from camel isolate from Egypt and yielded comparatively lesser homologies with that of isolates of camel and buffalo origin from India. Hence, it can be very much concluded that the present stock was more phylogenetically closer to Egyptian isolates than the Indian and the Kenyan isolates. The finding is significant from molecular evolutionary point of view.

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