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Regular Article

Sequencing and analysis of invariant surface glycoprotein (ISG) gene from *Trypanosoma evansi* dog isolate

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***Trypanosoma (T) evansi* causes a chronic wasting disease called "surra" in cattle, buffaloes, horses, canines, felines etc. In the present study *T.evansi* isolated from dog from Karnataka state in India was used to sequence invariant surface glycoprotein (ISG) gene. The sequence obtained was analyzed to elucidate its relationship with other isolates/ species. The ISG gene sequences obtained from four recombinant clones revealed the open reading frame (ORF) of 1521 nucleotide (nt) encoding a polypeptide of 506 amino acids (aa) and belongs ISG-75 gene family. Sequence analysis revealed respectively, 92-99% and 65-99% similarity at nucleotide and amino acid levels, with other isolates/ species. Hence, the present studied *T.evansi* isolate belongs to the RoTat 1.2 strain.**

Keywords: Trypanosomosis; Dog isolate; *T.evansi*; ISG-75; Phylogenetic analysis.

"Trypanosomosis" or "surra", a chronic wasting disease in cattle, buffaloes, horses, mules, donkeys, camels, canines, felines including domestic and wild animals is caused by *Trypanosoma evansi.* The disease is characterized by recurrent fever, chronic weakness, less feed intake, poor body weight gain, poor drauhgtability etc. The reproductive problems such as stillbirth and abortions in buffaloes and other infected animals have also been reported due to "surra". Reduced calving rate/reduced fertility in infected bulls has also been reported in high risk areas of infection (Dargantes *et al.,* 2009). The animals serve as carriers of the disease by exhibiting low level of fluctuating parasitaemia for years even after recovery. Buffaloes and cattle are probably the main parasite reservoir hosts for *T.evansi* (Dobson *et al.,* 2009). The sub clinical stage of the disease can even cause a significant decrease in milk production in lactating cows (Pholpark *et al.,* 1999). The etiological agent is transmitted mechanically by the biting of tabanid flies or carnivorism. The disease transmission has also been observed in the carnivores after feeding on infected meat (Raina *et al.,* 1985). Mortality and morbidity of the disease can be up to 70%, a high percentage (12.74%) of *T.evansi* infection in horses has been reported from India (Laha and Sasmal, 2008). The detection

of carrier status and subsequent treatment of animals helps in the effective control of disease.

T.evansi is evolved from *T.brucei* due to the loss of kinetoplastid DNA and is considered as petite mutant of *Trypanosoma brucei* (Lai *et al.,* 2008; Field and Carrington, 2009). The blood stream stage specific ISGs are uniformly distributed over the entire surface of the trypanosomes (Nolan *et al.,* 1997) and do not exhibit antigenic variation, unlike variable surface glycoprotein (VSG) (Tran *et al.,* 2008). Many blood stream forms of ISGs have been identified and characterized such as ISG 64 (Jackson *et al.,* 1993), ISG 65 or ISG 70 (Zeigelbauer and Overath, 1992), ISG 75 (Zeigelbauer *et al.,* 1992) and ISG 100 (Nolan *et al.,* 1997). ISG-75 is conserved among all the species and subspecies of the *trypanozoon* subgenus including *T. evansi, T. b. gambiense, T.b.rhodesiense and T. equiperdum* (Tran *et al.,* 2008) and found as multiple copies in trypanosomes (Pays and Nolan, 1997). ISGs are composed of four domains such as, small signal peptide at the amino terminal region, large extra cellular domain, single pass transmembrane α helical domain and small cytoplasmic domain at the carboxy terminal region (Tran *et al.,* 2006).

Several parasitological/serological diagnostic techniques are available for detection of trypanosomosis such as thick or wet blood smear for parasitological tests, indirect fluorescent antibody test (IFAT) or enzyme linked immunosorbent assay (ELISA) or card agglutination test for trypanosomiasis, for serological tests. The conventional blood smear examination technique which is widely practiced at the field level, can diagnose only clinical stages of infections satisfactorily but not latent or chronic infection (Fernandez *et al.,* 2009). The detection of carrier status of trypanosomosis has been improved by the development and application of DNA based techniques such as PCR, which is very sensitive and effective method for the detection of chronic stage or prepatent period of disease (Davila *et al.,* 2003). Killed trypanosomal DNA does not remain in the blood for more than 24-48 hours, thus PCR based assay helps in the detection of only active infections after the drug therapy (OIE, 2010). Several genes have been investigated and exploited as potential tools for the molecular diagnosis of trypanosomosis which includes VSG gene (Sengupta *et al.,* 2010), ISG gene (Rudramurthy *et al.,* 2013), repetitive nuclear DNA sequences (Masiga *et al.,* 1992), ribosomal DNA (Ijaz *et al.,* 1998), a region from r-RNA internal transcribed spacer 1 (ITS-1) (Taylor *et al.,* 2008), loop mediated isothermal amplification (Thekisoe *et al.,* 2005) etc. The present study is aimed at sequencing and analysis of ISG gene of *Trypanosoma evansi* (dog isolate).

Materials and methods

T.evansi isolate

T.evansi dog isolate maintained in the Parasitology laboratory of NIVEDI, Bangalore was used in the present study. The isolate was maintained *in vitro* by cryopreservation in liquid nitrogen. The presence of parasite in the blood was detected by wet blood film and thin blood smear. In wet blood film a drop of blood was diluted (1:1 volume in Alsever's solution), spread on the clean slide as a thin layer using a glass cover slip and was examined using light microscopy for the presence of parasite. However in thin blood smear, a thin smear of blood was fixed with methanol and stained with Giemsa's stain. After staining the slide was examined under the microscope.

Experimental animal infection and purification of trypanosomes

Two Wistar albino male rats, used as experimental animals in the present study were screened for blood parasites and found negative. The rats of were injected intra-peritoneally with 1x 10^5 trypanosomes. The blood was collected from the tail vein of rats (10-20µl) in 10% ethylenediamine tetra acetic acid (EDTA) solution daily to screen the

presence of trypanosomes through wet film and thin blood smear preparation. The rats were sacrificed at peak parasitaemia (6th day post infection) and the blood was collected as per the methodologies described by Sengupta *et al.* (2010). The trypanosomes from the plasma were purified by diethylaminoethyl (DEAE) cellulose column chromatography (Lanham and Godfrey, 1970). The purified trypanosomes were preserved at -80^{0}C in aliquots till further use.

Primers, reverse transcription PCR (RT-PCR) and PCR

A set of primer – Tef-ISGF (forward) (5′ CTG ACA AGC TGA GGA AGT GGT GAT C 3′) and Tef-ISGR (reverse) (5′ GTG GTT AAA TAT CAC TGT CAA GA 3′) were used in the present study (Rudramurthy *et al.,* 2013). Total RNA from the purified trypanosomes was extracted by Trizol LS reagent (Gibco BRL), following the standard protocol and preserved at -80^{0}C till further use. RT-PCR was carried out from the total RNA to synthesize ISG specific cDNA using Tef-ISGF and Tef-ISGR by following the standard protocol (Sengupta *et al.,* 2010). The cDNA obtained was subjected to PCR for the amplification of ISG-75 gene.

The 50µl PCR reaction mixture was prepared by 5µl of cDNA, 1µl of each primer (20 pmol), 5µl of 10x PCR buffer, 4µl of 25mM $MgCl_2$, 1µl of each of dNTPs (10mM) and 3.0 unit (U) of *Taq* DNA polymerase (Fermentas, USA). The step up PCR was carried out with an initial denaturation at 95^{o}C for 2 minutes (min) followed by 9 cycles of 45 seconds (sec) denaturation at 94^{0}C, 1 min primer annealing at 50^{0}C (+1^{0}C/cycle) and 2 min primer extension at 72^{0}C which is followed by 25 cycles of 45 sec denaturation at 94^{0}C, 1 min primer annealing at 60^{0}C and 2 min primer extension at 72^{0}C. The final primer extension was carried out for 10 min at 72^{0}C. The amplified DNA product was run through 1% agarose gel stained with ethidium bromide and visualized under UV transillumination.

Sequencing and analysis

The ISG amplicon (1.6 kb) generated by PCR was purified from the agarose gel (wizard plus®, SV miniprep gel extraction kit, Promega) and cloned into PGEM®-T-Easy vector (Promega). The recombinant plasmid DNA (PGEM®-T-Easy+ISG-75) was transformed in to *Escherichia coli* (Top 10) competent cells by heat shock method, following the standard protocol. The transformed cells were plated on LB agar medium containing ampicillin (50µg/ml) and IPTG (100mM/ml) and X-gal (20µg/ml). The recombinant clones (white) were selected by blue - white colony screening, colony lysis, and colony touch PCR. Furthermore, the recombinant clones were confirmed by restriction enzyme digestion (*Eco*RI single digestion) using the recombinant plasmid DNA isolated from recombinant clones (mini prep plasmid DNA isolation kit, Qiagen).

The recombinant plasmid DNA extracted from the four positive recombinant clones was sequenced in both orientation using T7 (forward) and Sp6 (reverse) primers to generate the contiguous sequence. The ORF was determined by sequence alignment and ORF nt sequence homology was searched using NCBI BLAST server. The sequence identity was determined by assembling ORF nt sequences and the deduced aa sequences into multiple sequence alignment using Clustal W program of Lasergene 6.0 (DNASTAR Inc., USA). The sequence from the present study (KF734091) was compared with published sequences by multiple sequence alignment.

Phylogenetic (bootstrap consensus) tree was constructed using molecular evolutionary genetics analysis (MEGA) version 4 based on the deduced aa sequences. The alignment gaps were excluded by pair wise distance estimations, while interior branch test of neighbor joining method was used to evaluate tree topologies. After 500 replications the bootstrap P-values were obtained and the robustness of the

predicted tree was evaluated using the bootstrap method.

Results and Discussion

Sequence homology and phylogenetic analysis

The primer set amplified 1.6 kb ISG gene sequence by PCR (Fig.1A), further, the colony PCR and restriction enzyme (*Eco*RI) digestion confirmed 1.6 kb DNA fragment of ISG (Fig. 1B and 1C). The ORF of ISG from all the four recombinant clones were of 1521nt (GenBank, accession number KF734091), encoding a polypeptide of 506 aa. The sequence homology search at the nucleotide level using NCBI BLAST server showed that the sequences recorded in present study had 94% to 99% homology among *T.evansi* isolates, 96% to 99% similarity with *T. brucei rhodesiense, T. brucei gambiense,* and *T. equiperdum,* and 92% to 99% similarity with *T. brucei brucei*. While, the sequence homology at the amino acid level showed, 68% to 99% similarity among *T.evansi* isolates, 66% to 99% similarity with *T. brucei gambiense,* 65% to 99% similarity with *T. brucei rhodesiense,* 66% to 98% similarity with *T. equiperdum* and 66% to 99% similarity with *T. brucei brucei*. The multiple sequence alignment with ORF nt sequences revealed that the sequence (KF734091) is highly conserved (Fig. not shown). Similarly, comparison of the deduced amino acid sequences by multiple alignment (Fig. 2) also showed that the protein is highly conserved, except 42nd position which is substituted by Arginine (R).

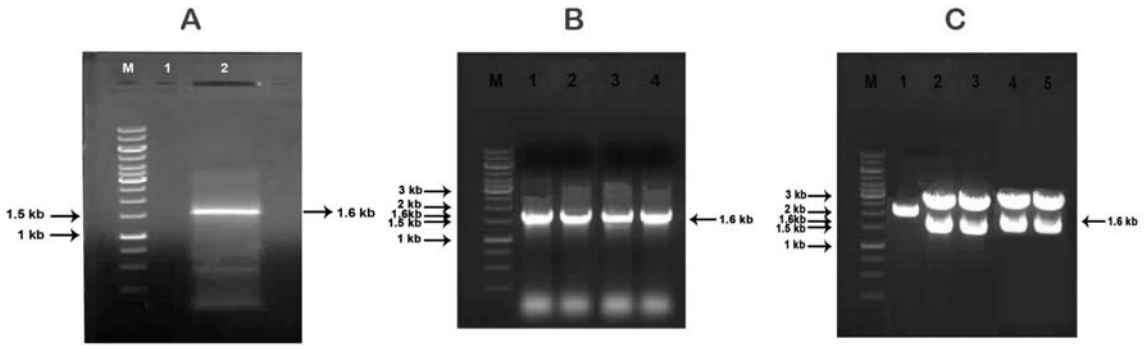

Fig. 1: RT PCR and PCR, colony PCR and restriction enzyme digestion (RED) assay.
Lane M = 1 kb DNA ladder. **A: PCR assay**, lane 1: PCR control, lane 2: PCR product. **B: Colony PCR,** lanes 1, 2, 3 and 4 = colony PCR from clones 1, 2, 3 and 4 respectively. **C: RED,** lane 1: plasmid pGEMT@easy + ISG-75 (undigested), lanes 2, 3, 4 and 5: plasmid pGEMT@easy + ISG-75, RED (*Eco*RI) samples from clones 1, 2, 3 and 4 respectively.

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Majority                          M S T M P V T L R T T A T V F L L C G I C A L D V T W A E E L S V A Q K Q Y V T A H G R Q L V G Q G
                                  ----------+---------+---------+---------+---------+---------+---------+---------+---------+
                                            10        20        30        40        50
                                  ----------+---------+---------+---------+---------+---------+---------+---------+---------+
T.evansi (RoTat 1.2)(ABB22149)    . . . . . .                                                                               50
T. b. gambiense(LiTat 1.3)(ABB22219) . . . . . .                                                                            50
T. brucei (MITat)(AAC41568)       . . . . . S K . S H . P . . Y . . . . . M N . . K . D . E A                                50
T. equiperdum(Alfort)(ABB22206)   . . . . . S K . S N . P . . Y . . . . . M N . . K . D . E A                                50
T.b. rhodesiense(STIB850)(ABB22172) . . . . . .                                                                             50
T.evansi (RoTat 1.2)(ABB22139)    . X . . . . . X X . . . . . X X . . . . . X . . . . . X X . . . . . . . . . X . X . . . .  50
T.evansi (RoTat 1.2)(ABB22140)    . . . . . S K . S N . P . . Y . . . . . M N . . K . D . E A                                50
T.evansi (RoTat 1.2)(ABB22141)    . . . . . .                                                                               50
T.evansi (RoTat 1.2)(ABB22142)    . . . . . .                                                                               50
T.evansi (RoTat 1.2)(ABB22143)    . . . . . S K . S N . P . . Y . . . . . M N . . K . . . . .                                50
T.evansi (RoTat 1.2)(ABB22145)    . . . . . .                                                                               50
T.evansi (RoTat 1.2)(ABB22146)    . . . . . G A . . . . . .                                                                 50
T.evansi (RoTat 1.2)(ABB22147)    . . . . . .                                                                               50
T.evansi (RoTat 1.2)(ABB22148)    . . . . . G A . . . . . .                                                                 50
T.evansi (RoTat 1.2)(ABB22150)    . . . . . .                                                                               50
T.evansi (RoTat 1.2)(AER62858)    . . . . . G A . . . D . . .                                                               50
T.evansi (RoTat 1.2) (AHC31981)   . . . . . G A . . . . . .                                                                 50
T.evansi(RoTat 1.2)(AHC31980)     . . . . . R . . . . . .                                                                   50
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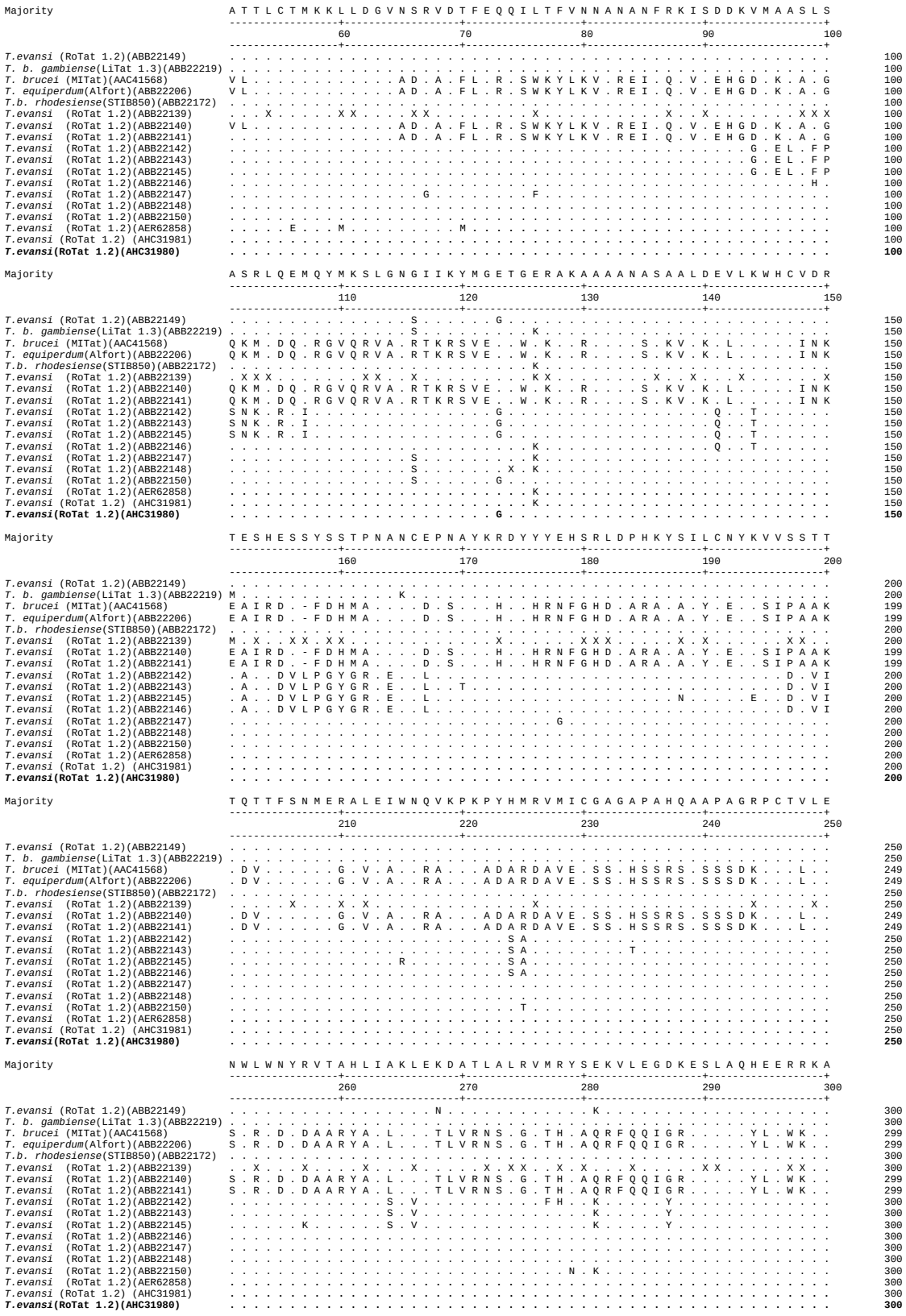
Majority A T T L C T M K K L L D G V N S R V D T F E Q Q I L T F V N N A N A N F R K I S D D K V M A A S L S
60 70 80 90 100
T.evansi (RoTat 1.2)(ABB22149) 100
T. b. gambiense(LiTat 1.3)(ABB22219) 100
T. brucei (MITat)(AAC41568) 100
T. equiperdum(Alfort)(ABB22206) 100
T.b. rhodesiense(STIB850)(ABB22172) 100
T.evansi (RoTat 1.2)(ABB22139) 100
T.evansi (RoTat 1.2)(ABB22140) 100
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T.evansi (RoTat 1.2)(ABB22142) 100
T.evansi (RoTat 1.2)(ABB22143) 100
T.evansi (RoTat 1.2)(ABB22145) 100
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T.evansi (RoTat 1.2)(ABB22147) 100
T.evansi (RoTat 1.2)(ABB22148) 100
T.evansi (RoTat 1.2)(ABB22150) 100
T.evansi (RoTat 1.2)(AER62858) 100
T.evansi (RoTat 1.2) (AHC31981) 100
T.evansi(RoTat 1.2)(AHC31980) 100
Majority A S R L Q E M Q Y M K S L G N G I I K Y M G E T G E R A K A A A A N A S A A L D E V L K W H C V D R
110 120 130 140 150
T.evansi (RoTat 1.2)(ABB22149) 150
T. b. gambiense(LiTat 1.3)(ABB22219) 150
T. brucei (MITat)(AAC41568) 150
T. equiperdum(Alfort)(ABB22206) 150
T.b. rhodesiense(STIB850)(ABB22172) 150
T.evansi (RoTat 1.2)(ABB22139) 150
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T.evansi (RoTat 1.2)(ABB22146) 150
T.evansi (RoTat 1.2)(ABB22147) 150
T.evansi (RoTat 1.2)(ABB22148) 150
T.evansi (RoTat 1.2)(ABB22150) 150
T.evansi (RoTat 1.2)(AER62858) 150
T.evansi (RoTat 1.2) (AHC31981) 150
T.evansi(RoTat 1.2)(AHC31980) 150
Majority T E S H E S S Y S S T P N A N C E P N A Y K R D Y Y Y E H S R L D P H K Y S I L C N Y K V V S S T T
160 170 180 190 200
T.evansi (RoTat 1.2)(ABB22149) 200
T. b. gambiense(LiTat 1.3)(ABB22219) 200
T. brucei (MITat)(AAC41568) 199
T. equiperdum(Alfort)(ABB22206) 199
T.b. rhodesiense(STIB850)(ABB22172) 200
T.evansi (RoTat 1.2)(ABB22139) 200
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T.evansi (RoTat 1.2)(ABB22150) 200
T.evansi (RoTat 1.2)(AER62858) 200
T.evansi (RoTat 1.2) (AHC31981) 200
T.evansi(RoTat 1.2)(AHC31980) 200
Majority T Q T T F S N M E R A L E I W N Q V K P K P Y H M R V M I C G A G A P A H Q A A P A G R P C T V L E
210 220 230 240 250
T.evansi (RoTat 1.2)(ABB22149) 250
T. b. gambiense(LiTat 1.3)(ABB22219) 250
T. brucei (MITat)(AAC41568) 249
T. equiperdum(Alfort)(ABB22206) 249
T.b. rhodesiense(STIB850)(ABB22172) 250
T.evansi (RoTat 1.2)(ABB22139) 250
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T.evansi (RoTat 1.2)(ABB22142) 250
T.evansi (RoTat 1.2)(ABB22143) 250
T.evansi (RoTat 1.2)(ABB22145) 250
T.evansi (RoTat 1.2)(ABB22146) 250
T.evansi (RoTat 1.2)(ABB22147) 250
T.evansi (RoTat 1.2)(ABB22148) 250
T.evansi (RoTat 1.2)(ABB22150) 250
T.evansi (RoTat 1.2)(AER62858) 250
T.evansi (RoTat 1.2) (AHC31981) 250
T.evansi(RoTat 1.2)(AHC31980) 250
Majority N W L W N Y R V T A H L I A K L E K D A T L A L R V M R Y S E K V L E G D K E S L A Q H E E R R K A
260 270 280 290 300
T.evansi (RoTat 1.2)(ABB22149) 300
T. b. gambiense(LiTat 1.3)(ABB22219) 300
T. brucei (MITat)(AAC41568) 299
T. equiperdum(Alfort)(ABB22206) 299
T.b. rhodesiense(STIB850)(ABB22172) 300
T.evansi (RoTat 1.2)(ABB22139) 300
T.evansi (RoTat 1.2)(ABB22140) 299
T.evansi (RoTat 1.2)(ABB22141) 299
T.evansi (RoTat 1.2)(ABB22142) 300
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T.evansi (RoTat 1.2)(ABB22145) 300
T.evansi (RoTat 1.2)(ABB22146) 300
T.evansi (RoTat 1.2)(ABB22147) 300
T.evansi (RoTat 1.2)(ABB22148) 300
T.evansi (RoTat 1.2)(ABB22150) 300
T.evansi (RoTat 1.2)(AER62858) 300
T.evansi (RoTat 1.2) (AHC31981) 300
T.evansi(RoTat 1.2)(AHC31980) 300

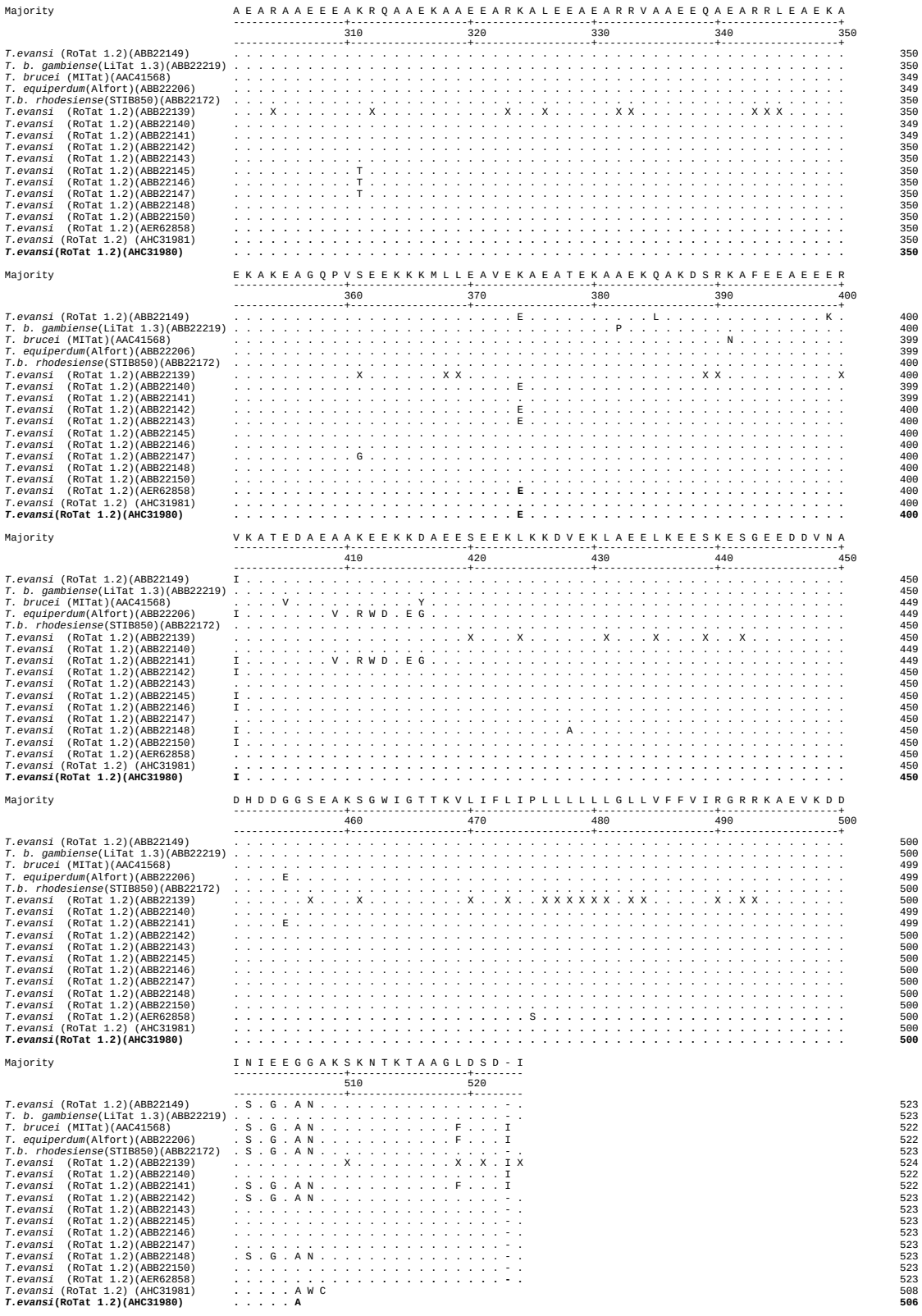

Fig. 2: Multiple sequence alignment of *T.evansi* ISG-75 deduced amino acid sequences: The difference in amino acid sequence is represented by a single letter amino acid code and the present study is shaded. Dot (.); sequence identity, spaces or dash (–); gap generated during alignment.

Phylogenetic analysis of ISG-75 from different isolates / species at aa level indicated two major lineages. The present studied *T.evansi* isolate (AHC31980) belongs to separate sub cluster with 64% bootstrap confidence value and is closely related to *T.evansi* RoTat 1.2 (ABB22149) with a bootstrap confidence value of 31%. (Fig. 3).

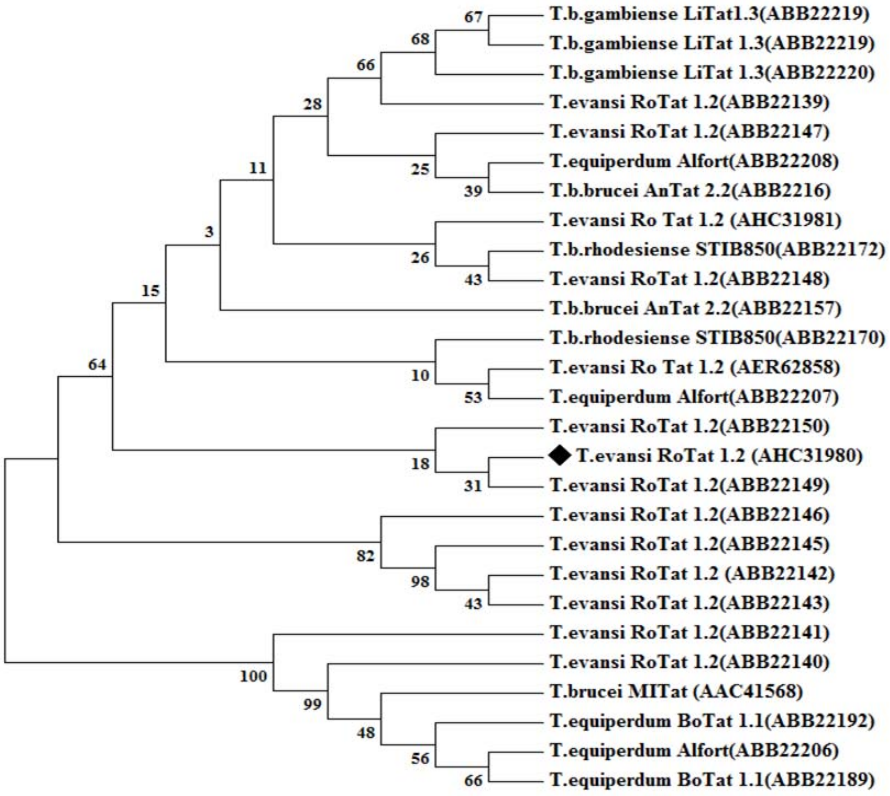

Fig. 3: Phylogenetic tree: Bar represents the genetic distance, branch values represent the bootstrap confidence values,◆; present study.

The sequence analysis of the ISG gene of *T.evansi* carried out in the present study revealed that the sequence (KF734091) is highly conserved among *T.evansi* isolates/strains and different species of trypanosomes. Multiple sequence alignment with deduced amino acid sequences demonstrated that the ISG sequence is highly conserved among different isolates and species of trypanosomes. The NCBI- BLAST analysis at the nucleotide and amino acid level revealed that the sequence of the present study belongs to ISG-75 gene family and recognized RoTat 1.2 strain of *T.evansi.* Moreover, phylogenetic analysis also showed that the *T.evansi* isolate of the present study is closely related to *T.evansi* RoTat 1.2, thus it can be concluded that our isolate (dog) belongs to RoTat 1.2 strain of *T.evansi.* The multicopy gene family encoding ISG-75 consist of two main groups, the cDNA and gDNA sequences of these two groups share 75% and 77% similarities (Tran *et al.,* 2006). Group I (EPNAYKR-DYYYEHSRLD) and group II (DPSAYKH-DYHRNFGHDD) differ in their putative cleavage sites. The sequence obtained in this study contains EPNAYKR-DYYYEHSRLD sequence corresponding to 167-183 aa, which is highly similar to group I putative cleavage

site, hence the ISG-75 gene of the present study belongs to group I gene family. Highly conserved region of a particular gene(s) can be of immense value in the development of diagnostic PCR for differential diagnosis and molecular epidemiological studies. Earlier study by Rudramurthy *et al.* (2013) reported that, the diagnostic primers (TeDISG-F/R) targeting ISG gene, can diagnose carrier status of trypanosomosis effectively.

Conclusion

The present finding can be used in the design of diagnostic primers for the molecular diagnosis of trypanosomosis in carrier animals caused by *T.evansi*, *T.equiperdum* and *T.brucei*. The designed primers can be further evaluated for sensitivity, specificity and potentiality to diagnose trypanosomosis in different host systems. As per literature search, this is the first report of sequencing ISG-75 gene of *T. evansi*, dog isolate from India.

References

Dargantes AP, Mercado RT, Dobson RJ, Reid SA. (2009). Estimating the impact of *Trypanosoma evansi* infection (surra) on buffalo population dynamics in southern Philippines using data from cross-sectional surveys, *Int J Parasitol* 39, 1109–1114.

Davila AM, Herrera HM, Schlesinger T, Souza SS, Traub-Cseko YM. (2003). Using PCR for unraveling the cryptic epizootiology of livestock trypanosomosis in the Pantanal, Brazil, *Vet Parasitol* 117, 1-13.

Dobson RJ, Dargantes AP, Mercado RT, Reid SA. (2009). Models for *Trypanosoma evansi* (surra), its control and economic impact on small –hold livestock owners in the Philippines, *Int J Parasitol* 39, 1115-1123.

Fernandez D, Gonzalez-Baradat B, Eleizalde M, Gonzalez–Marcano E, Perrone T, Mendoza M. (2009). *Trypanosoma evansi*: a comparison of PCR and parasitological diagnostic tests in experimentally infected mice, *Exp Parasitol* 121, 1-7.

Field MC, Carrington M. (2009). The trypanosome flagellar pocket, *Nature rev Microbiol* 7, 775-786.

Ijaz MK, Nur-E-Kamal MSA, Mohamed AIA, Dar FK. (1998). Comparative studies on the sensitivity of polymerase chain reaction and microscopic examination for the detection of *Trypanosoma evansi* in experimentally infected mice, Comp Immunol *Microbiol Infect Dis* 21, 215-223.

Jackson DG, Windle HJ, Voorheis HP. (1993). The identification, purification, and characterization of two invariant surface glycoproteins located beneath the surface coat barrier of bloodstream forms of *Trypanosoma brucei*, *J Biol Chem* 268, 8085-8095.

Laha R, Sasmal N K. (2008). Endemic status of *Trypanosoma evansi* infection in a horse stable of eastern region of India- a field investigation, *Trop Anim Health Prod* 40, 357-361.

Lai DH, Hashimi H, Lun ZR, Ayalag FJ, Lukes J. (2008). Adaptations of *Trypanosoma brucei* to gradual loss of kinetoplast DNA: *Trypanosoma equiperdum* and *Trypanosoma evansi* are petite mutants of *T. brucei, Proc Natl Acad Sci* 105, 1999-2004.

Lanham SM, Godfrey DG. (1970). Isolation of salivarian trypanosomes from man and other mammals using DEAE-cellulose, *Exp Parasitol* 28**,** 521-534.

Masiga DK, Smyth A J, Hayes P, Bromidge TJ, Gibson WC. (1992). Sensitive detection of trypanosomes in tsetse flies by DNA amplification. *Int J Parasitol* 22**,** 909-918.

Nolan DP, Jackson DG, Windle HJ, Pays A, Geuskens M, Micheli A, Voorheis HP, Pays E. (1997). Characterization of a novel, stage-specific, invariant surface protein in *Trypanosoma brucei* containing an internal, serine-rich, repetitive motif, *J Biol Chem* 272, 29212–29221.

OIE. (2010). *Trypanosoma evansi* (surra). In: Manual of diagnostic tests and vaccines for terrestrial animals, World organization for animal health, Paris, cited at: http://www.oie.int.

Pays E, Nolan DP. (1997). Expression and functions of surface proteins in *Trypanosoma brucei*. The trypanosome surface, Proceedings of the third francqui colloquium, May 26-27, Brussels, pp.28-32.

Pholpark S, Pholpark M, Polsar C, Charoenchai A, Paengpassa Y, Kashiwazaki Y. (1999). Influence of *Trypanosoma evansi* infection on milk yield of dairy cattle in northeast Thailand, *Prev Vet Med* 42, 39-44.

Raina AK, Kumar R, Rajora VS, Sridhar, Singh RP. (1985). Oral transmission of *Trypanosoma evansi* infection in dogs and mice. *Vet Parasitol* 18, 67-69.

Rudramurthy G R, Sengupta PP, Balamurugan V, Prabhudas K, Rahman H. (2013). PCR based diagnosis of trypanosomiasis exploring invariant surface glycoprotein (ISG) 75 gene, *Vet Parasitol* 193, 47-58.

Sengupta PP, Balumahendiran M, Suryanarayana VVS, Raghavendra AG, Shome BR, Ganjendragad MR, Prabhudas K. (2010). PCR-based diagnosis of surra-targeting VSG gene: Experimental studies in small laboratory rodents and buffalo, *Vet Parasitol* 171, 22-31.

Taylor TK, Boyle DB, Bingham J. (2008). Development of TaqMan PCR assay for the detection of *Trypanosoma evansi* the agent of surra, *Vet Parasitol* 153, 255-264.

Thekisoe OMM, Inoue N, Kuboki, Tuntasuvan D, Bunnoy W, Borisut-suwan S, Igarashi I, Sugimoto C. (2005). Evaluation of loop mediated isothermal amplification (LAMP), PCR and parasitological tests for detection of *Trypanosoma evansi* in experimentally infected pigs, *Vet Parasitol* 130, 327-330.

Tran T, Claes F, Dujardin JC, Buscher P. (2006). The invariant surface glycol-protein ISG 75 gene family consists of two main groups in the *Trypanozoon* subgenus, *Parasitol* 133, 613-621.

Tran T, Buscher P, Vandenbussche G, Wyns L, Messens J, Greve HD. (2008). Hetero-logous expression, purification and characterisation of the extracellular domain of trypanosome invariant surface glycoprotein ISG75, *J Biotech* 135, 247–254.

Ziegelbauer K, Overath P. (1992). Identification of invariant surface glycoproteins in the bloodstream stage of *Trypanosoma brucei*, *J Biol Chem* 267, 10791-10796.

Ziegelbauer K, Multhaup G, Overath P. (1992). Molecular Characterization of two invariant surface glycoproteins specific for the bloodstream stage of *Typanosoma brucei, J Biol Chem* 267, 10797-10803.