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Molecular Isolation and Characterization of *Trypanosoma evansi* in Dromedary Camels from Different Regions of Sudan

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Abstract
Trypanosoma evansi is classified under the subgenus Trypanozoon along with the *T. brucei spp*. and *T. equiperdumi*, and they are main causative agents of Animal trypanosomoses throughout the world. *T. evansi* causes Surra via mechanical transmission in Camels. Whole blood samples were collected from 102 suspected camels from 18 different geographic regions in Sudan. *T. evansi*-affected camels were detected by Wet smear method, and the positive blood samples (10 out of 102) were subjected to DNA extraction and TBR-PCR examination and phylogenetic analysis. Basic Local Alignment Search Tool data of the obtained TBR sequences revealed that partial sequence of most of them corresponded to those of *T. evansi* (VSG), Malaysian isolate selanger 2 (AM497934.1) with the homology of 100%. The phylogenetic tree inferred from the TBR nucleotide sequences (137bp) clearly showed the genetic diversity of the parasites. Phylogenetic and molecular analyses of this region of variable surface glycoprotein (VSG) showed that three distinct genotypes of *T. evansi* in Sudanese dromedary camels are present. This study suggests that *T. evansi* could be a polyphyltic group with 4 clades or a monophlytic group and all isolates have a common ancestor. More informative genetic marker is required in order to have ultimate conclusion.

1. Introduction

Trypanosomes are haemoflagellates protozoan parasites, found in blood and sometimes tissue of mammals (involving human). They belong to the Phylum Sarcomastigophora, order Kinetoplastida, family Trypanosomatidae and genus *Trypanosoma*. African Trypanosomes are pathogenic to both animals and humans. The two subspecies *T. brucei gambiense* and *T. brucei rhodesiense* cause sleeping sickness, a major disease in human, while *T. brucei brucei*, *T. vivax, T. conglense, T. equiperdum*

and *T. evansi* are pathogenic to animals, cause a very serious disease to livestock called African Animal Trypanosomiosis (AAT) that transmitted cyclically by tsetse flies (genus *Glossina*) and mechanically by horse flies (*Tabanus* spp) and Stable flies (*Stomoxys* spp) [21]. This disease and its vectors affects areas of Sub Saharan Africa which threats around 48 million cattles in 37 African countries (FAO -Vingt-deuxi`eme conf´erence r´egionale pourl' Afrique) and also threats other domestic animals. It causes anemia, fever, nervous symptoms and weakness leading to huge losses in the production (milk, meat, fertility, draught power and manure). Thus Animal trypanosomiosis represents very important obstacle to economic and agricultural advancement in the impacted regions [14] resulting in losses of over 1.5 billion dollars annually [3]. Trypanosomosis in Sudan was first reported in 1904 in cattle arriving from upper Nile to Khartoum [9].

Biologically *T. evansi* is very similar to *T. equiperdum*, the causative agent of dourine [4] and [5], and morphologically resembles the slender forms of the tsetse-transmitted species, *T. brucei brucei*, *T. b. gambiense* and *T. b. rhodesiense*. Most of the molecular characterizations indicate that various strains of *T. evansi* isolated from Asia, Africa and South America are very homogeneous and may have a single origin [24], but other workers suggest that *T. evansi* could have emerged from *T. brucei* in several instances [8, 12]. *T. evansi* is a monomorphic thin trypomastigote with long slender form and occasionally appears pleomorphic in some strains with intermediate and stumpy forms. Strains from different host sources and various geographical areas are indistinguishable [7]. The three Trypanozoon (*T. brucei*, *T. evansi* and *T. equiperdum*) subgenus members are morphologically indistinguishable.

Many scientists in the different regions of the world had been employed multitechniques for detection of camel's trypanosomiosis. The most sensitive test is that of satellite DNA using TBR primers [13]. However, in areas where host species potentially infected with otherT rypanozoon such *T. brucei brucei*, species confirmation can be obtained with more specific primers such as TEPAN [18], or TE2249/2250 [2]. Other primers specific for RoTat [6, 25] or none–RoTat strain [16] and other techniques such as the loop–mediated isothermal amplification (LAMP) [22], Taqman [23] can also be used.

The objectives of this study were to use TBR-PCR to characterize field isolates of *T. evansi* from East, North-East, North, and Central and Western regions of Sudan during the period February 2011 – July 2012.

2. Materials & Methods

2.1. Study Area

This study was carried out in different regions of Sudan in the period February 2011 to June 2012. The collection sites included five States namely; The Red Sea State (Arbaat, Bulaib, Oshairy, Saloom, Sulaim and Swakin Port); Kassala State (Kassala, NewHalfa, Sidoon and Butana); Gedaref State (Showak, Gedaref and Galabat); River Nile State (Berber, Abidiya, Atbara and AdDamir). Samples collected from North Kordofan State (Khawai) during the period May 2005–June 2006 is also included in this study. Figure 1 shows the samples collection sites from different regions of the Sudan.

2.2. Blood Samples Collection from Camels

A small drop of blood (2–3µl) from suspected camel was examined parasitological in a wet smear method in order to detect any motile trypanosomes. Samples from infected blood from each camel that were confirmed positive, were collected by placing a drop of blood on 3MM filter paper, these were kept in sterile plastic bag and stored at –20°C. In addition, 3 ml of infected blood were collected from the same camel and mixed in cryiovial with equal volume of phosphate saline glucose buffer (PSG) (50 mM $Na_2HPO_4.H_2O$, 2 mM $NaH_2PO_4.2H_2O$, 36 mM NaCl and 1.5% glucose; pH 8.0) and were stored in the liquid nitrogen container (-196°C) until to be used.

2.3. Isolation of Trypanosome DNA by Chelex-100 from Camel Blood

DNA isolation by chelex-100 was performed with a modification of the methods described previously [26, 1]. A volume of 10 µl aliquot of the frozen blood was added to 1 ml of 0.15% Saponin (Stigma, USA) in phosphat-buffer saline (PBS pH 7.2) in a 1.5 microcenrifuge tube for removal of red blood cells, and was incubated 10 min. on ice. The parasites were collected by centrifugation at 10000 rpm and room temperature for 1 min. and were then washed once with cold PBS. The result cells pellet was added to 200 µl of 5% Chelex-100 (Bio-rad, USA) in distilled water, incubated at 56°C for 15 min, and subjected to boiling water for 8 min. The Chelex was removed by centrifugation at 10000 rpm at room temperature for 1 min, and the supernatant was saved in a fresh tube. A volume of 2 µl aliquot of supernatant was used for PCR amplification. For dried blood samples from Camels, a small piece (about 5 mm^2) of filter paper with a 10 µl blood spot was initially soaked in 0.15% Saponin in PBS and processed as described above. The DNA was quantified by NanoDrop before PCR procedure was performed.

2.4. PCRA Mplification and Sequencing

The PCR amplification was performed in a total volume of 25 µl containing the following reagents: 2.5 µl of 10x buffer (Bioline), 10 mM dNTPs, 10 µM of each TBR-1 forward [CGAATGAATATTAAACAATGCGCAG] and TBR-2 reverse [AGAACCATTTATTAGCTTTGTTGC] primers, 50 mM $MgCl_2$ (Bioline), 0.0025 U Taq polymerase and 1.0 µl DNA. The following thermal cycling program was used: The reactions were cycled in a programmable heating block (Robocycler® machine), incubated at 94°C for 3min. in an

initial denaturing step and were subjected to 30 cycles. The cycles involved denaturation at 94°C for 1 min., annealing at 60°C for 1 min, and extension at 72°C for 1 min., the final extension reaction was increased to 7 min. to ensure maximum full-length product. The amplified product was separated on 1.5% agarose gel in TBE buffer. The Gel documentation system was used to visualize the ethidium bromide-stained gel. TBR-sequencing total reaction was 50 µl volume that contained Master mix (10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 5% Glycerol, 0.08% IGEPAL®CA-630, 0.05% Tween® 20 and 25 U/ml Taq DNA Polymerase, pH 8.6). The positive samples are sequenced at Bioneer (Republic of South Korea). The sequences were manually edited before searching the BLAST database of the National Center for Biotechnology Information (NCBI) Genebank for sequence matches. Sequences alignment was performed using the program CLUSTAL2.1. The phylogenetic analysis is carried out using the MegAlign program of the Software suite for Sequence Analysis (DNASTAR, Lasergene).

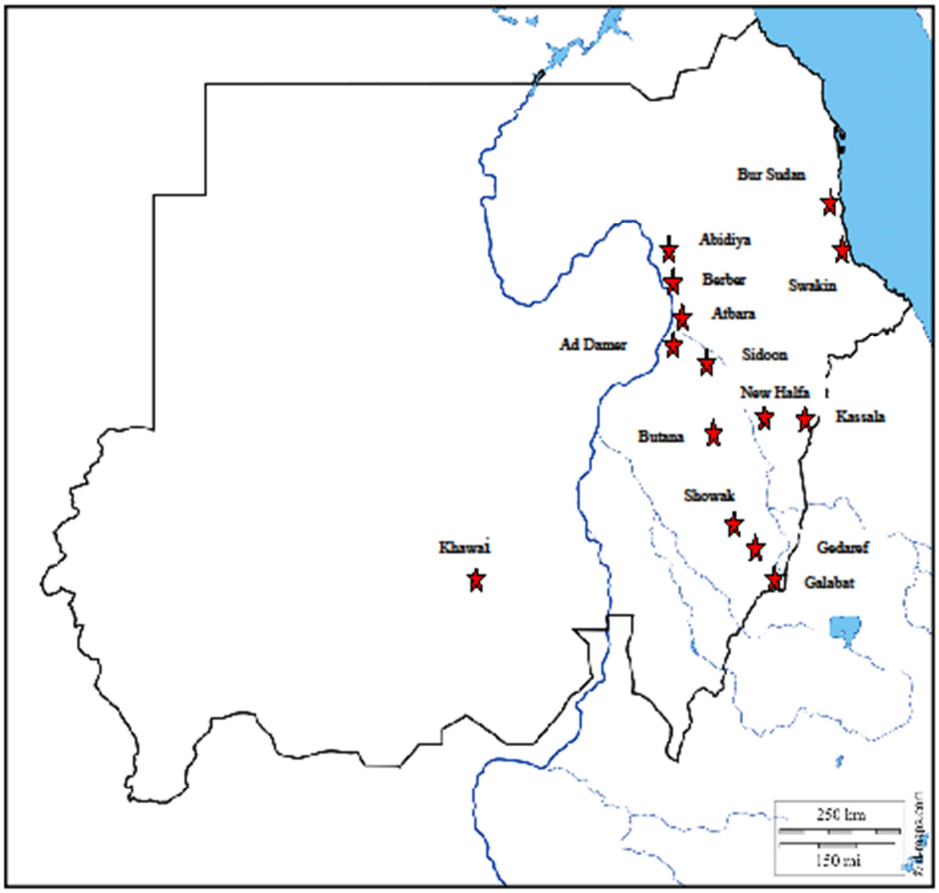

Figure 1. *Map of Sudan showing the location of the sampling areas.*

3. Results

In the molecular characterization of *T. evansi* from camels using TBR-PCR, as shown in Figure 1, a band size of 177 bp as expected was obtained by all the analyzed field collected samples from different regions of the Sudan. Very interesting, same samples were also positive in the parasitological tests.

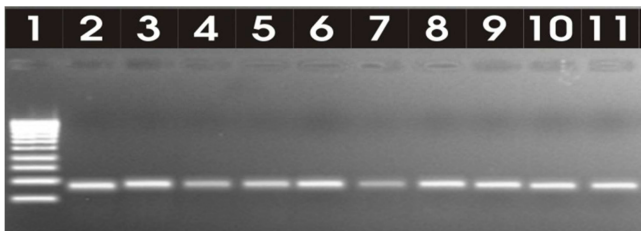

Figure 2. *PCR amplification of field collected samples using TBR primers.*

A volume of 6 µl of each PCR reaction were loaded on 1.5% TBE agarose gel stained with ethidium bromide and electrophoretically separated at 110 voltage. Lane 1 is 1 kb DNA ladder. Lanes 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 are *T. evansi* samples from different geographical regions in Sudan; BurSudan (PS-A-1), Sidoon (Si-2), Ad Damer (DA-2), AdDamer (DA-10), New Halfa (H-5), New Halfa (H-6-2), Gedaref (Ged-7), Gedaref (Ged-9), Kassala (KS-10) and Gedaref (Ged-2); respectively.

In this study, sequences comparison has been performed to look for similarities and differences in order to deduce relationships. There are several different computer programs that can generate an alignment. All of them try to maximize the number of matches among all sequences being aligned by changing the positions of the sequences relative to one another and/or adding gaps.

All the *T. evansi* TBR sequences have been deposited in the GenBank (Accession numbers: MF142290 – MF142299). As shown in Figure 3, all the ten TBR1 sequences have large number of identical nucleotides particularly in the middle part of the sequence alignment. While, the 5-primed end of the alignment is the most variable part followed by the 3-primed end. Sequence SU6-TBR1 (H-6) is very different in the middle part in comparison to the other ones. A SNP in an alignment is a position where the letters in a column do not match. Because an indel is an addition or removal of a pair of DNA bases, an indel in an alignment will be represented by a gap (sometimes a dash) in the alignment. In Figure 3, this shown by both sequences SU6-TBR1 (H-6) and SU7-TBR1 (Ged-7). These two sequences have 7 differences in common in the 3-primed end of the alignment.

The analysis of the 133 bp of the 10 obtained TBR1 sequences in terms of differences is shown in Table 1. Sample SU10-TBR1 (Ged-2) is the only one that has insertion type of mutation with a percent of 0.75 based on the compared sequence. However, samples SU3-TBR1, SU6-TBR1 and SU7-TBR1 have deletion type of mutation with percent of 0.75, 0.75 and 1.50, respectively. Seventy percent (7 samples) have all SNP type of mutation. Sample SU6-TBR1 has the highest level of SNPs (21) with a percent of 15.78, followed by sample SU7-TBR1 (9) with a percent of 6.67.

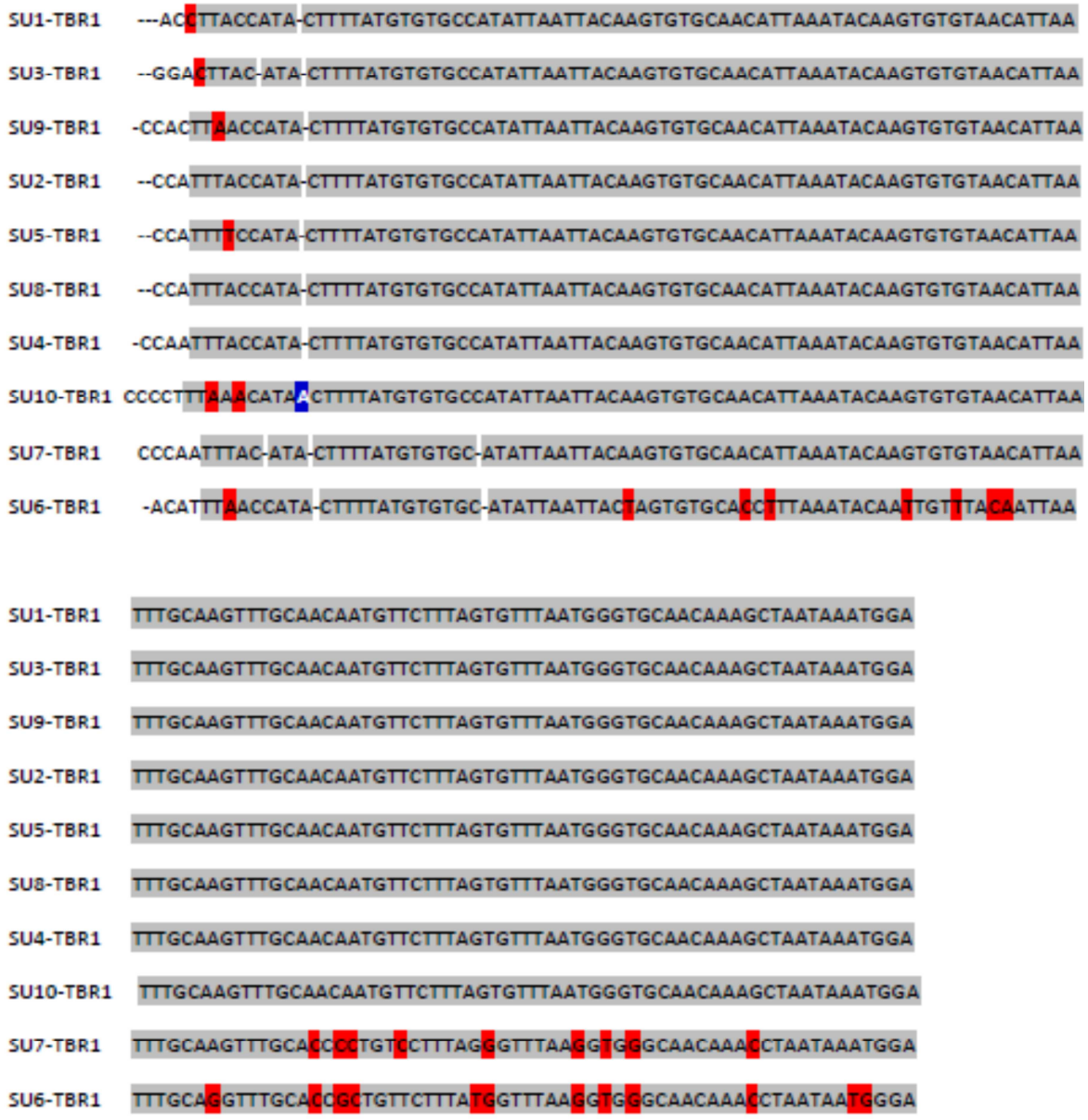

Figure 3. *CLUSTAL multiple sequence alignment of T. evansi TBR1 sequences.*

The gap is inserted to maximize the similarity between the aligned sequences. The grey shaded nucleotides represent the identical ones or the conserved nucleotides. The red shaded nucleotides in the alignment represent the different ones. The blue shaded nucleotide represents the insert one. The un-shaded nucleotides in the aligned sequences represent the un-identical or variable ones in all the ten sequences.

Table 1. *Analysis of the mutations (SNPs and Indel) of the T. evansi TBR1 sequences.*

Sequence	SNPs		Insertions		Deletions	
	Number	%	Number	%	Number	%
SU1-TBR1	2	1.50	0	0	0	0.00
SU2-TBR1	0	0.00	0	0	0	0.00
SU3-TBR1	1	0.75	0	0	1	0.75
SU4-TBR1	0	0.00	0	0	0	0.00
SU5-TBR1	1	0.75	0	0	0	0.00
SU6-TBR1	21	15.78	0	0	1	0.75
SU7-TBR1	9	6.76	0	0	2	1.50
SU8-TBR1	0	0.00	0	0	0	0.00
SU9-TBR1	2	1.50	0	0	0	0.00
SU10-TBR1	3	2.25	1	0.75	0	0.00

When the ten TBR1 obtained sequences were compared using the program MegAlign, they were aligned by Clustal W method. Table 2 summarized the findings, where samples SU2 and SU8 have shown 100% identity. Sample SU6 has the lowest identity level that lies between 80.60 and 85.07%, followed by sample SU7 which has identity level lies between 85.07 and 91.73%. Overall, sample SU6 is the most divergent one. The slight difference in the results may be due to the fact that the whole sequence for each sample was analyzed by the program Clustal 2.1, irrespective to the quality of the start of each one.

Table 2. *Sequences distances for T. evansi TBR–created by Clustal W. Percent Identity Divergence.*

	1	2	3	4	5	6	7	8	9	10		
1		97.7	98.5	98.5	97.0	83.3	91.6	97.7	98.5	96.2	1	SU1-TBR1.ab1
2	2.3		97.7	99.3	99.3	83.5	92.4	100.0	97.8	97.8	2	SU2-TBR1.ab1
3	1.5	2.3		97.7	97.0	81.8	90.9	97.7	96.2	95.5	3	SU3-TBR1.ab1
4	1.5	0.8	2.3		98.5	83.6	93.2	99.3	98.5	97.0	4	SU4-TBR1.ab1
5	3.1	0.8	3.1	1.5		82.7	91.7	99.3	97.0	97.0	5	SU5-TBR1.ab1
6	18.9	18.9	20.9	18.7	19.9		88.0	83.5	84.3	83.6	6	SU6-TBR1.ab1
7	8.9	8.0	9.7	7.1	8.9	13.2		92.4	91.7	90.3	7	SU7-TBR1.ab1
8	2.3	0.0	2.3	0.8	0.8	18.9	8.0		97.8	97.8	8	SU8-TBR1.ab1
9	1.5	2.3	3.9	1.5	3.1	17.7	8.8	2.3		97.8	9	SU9-TBR1.ab1
10	3.9	2.3	4.7	3.0	3.1	18.7	10.5	2.3	2.3		10	SU10-TBR1.ab1
	1	2	3	4	5	6	7	8	9	10		

Table 2 displays the divergence and percent identity values of each sequence pair in the TBR1 sequences alignment. Divergence is calculated by comparing sequence pairs in relation to the phylogeny reconstructed by MegAlign. Percent Identity compares sequences directly, without accounting for phylogenetic relationships. SU2-TBR1 and SU8-TBR1 are 100% identical. SU6-TBR1 has the lowest identity (80.3%) with SU3-TBR1 and consequently the highest divergence level (23.0). Overall, sample SU6-TBR1 is the most divergent one and has less identity to all the sequences under investigation (80.3-85.1%) and the highest divergence (16.8–23.0).

The phylogenetic Tree is shown in Figure 4, Residues substitutions are shown below. The tree is drawn using 1000 Bootstrap.

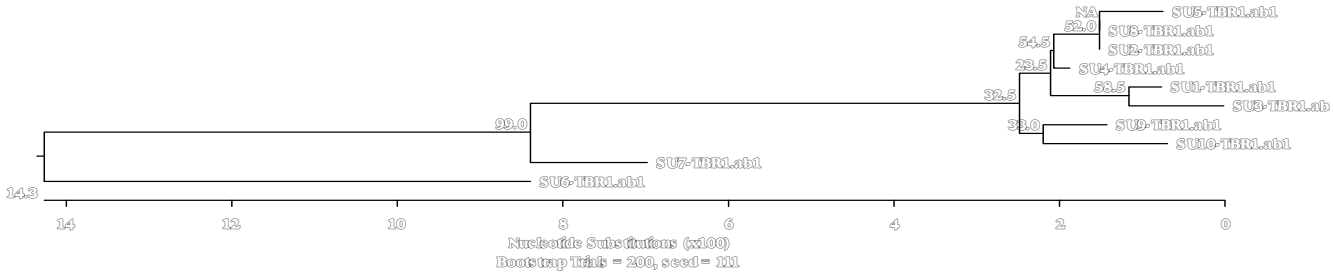

Figure 4. *Phylogenetic Tree of T. evansi TBR sequences of Clustal W alignment.*

4. Discussion

For most African trypanosome species, repetitive nuclear DNA sequences have been used as targets for PCR amplification since the minicircle kinetoplast DNA sequences are highly heterogeneous [10]. Therefore, in this study the TBR primers were chosen in a purpose to confirm the presence of the trypanosome species in the field collected sample.

Good quality forward (TBR1) and reverse (TBR2) sequences were searched in database for similarities. Most of

them showed 100% identity for *T. evansi* (VSG), Malaysian isolate selanger 2 (AM497934.1) with the homology of 100%. Only sequence SU6-TBR1 showed very different result that is 100% identical to *T. equiperdum* DNA. A very interesting observation on this sequence analysis that almost all of the four clones of the *T. evansi* from the database are identical, which may suggest some sort of evidence that *T. evansi* is monophyletic organism. However, alignment of the nucleotide sequences of SU1-SU10 TBR1-PCR showed considerable variation (80.60 – 99.25%) among isolates of *T. evansi* in Sudan. However, samples SU2 (Si-2), SU4-TBR1 (DA-10) and SU8-TBR1 (Ged-9) are 100% identical. This indicates that all the three samples belong to the same trypanosome species. One can predict, though the TBR is not considered as informative genetic marker, that there is no variation in the genetic pool of *T. evansi* isolates from the three different geographical regions namely; North (Ad Damer and Sidoon), and East (Gedaref).

It is well known that as organisms evolve and diverge, their DNA sequences accumulate differences, also known as mutations. There are two types of mutations namely; Single nucleotide polymorphisms (SNPs) and Indels (insertions/deletions). A SNP is a change of one DNA base pair in to another. More distantly related species have had more time elapse since they shared a common ancestor; therefore, they have had more time for mutations to accumulate in their DNA. Closely related species have had little time to accumulate mutations since their last common ancestor, so their DNA sequences are much more similar. As shown in Table 2, the very interesting finding of this analysis, that samples SU2-TBR1, SU4-TBR1 and SU8-TBR1 have no change in the sequence or any type of mutation. This suggests that they are very close to each other and less divergent from the ancestor species. It has been observed that samples SU1-TBR1 and SU9-TBR1 have the same level of mutation, which may suggest their very close genetic relationship and also may be included in the same subspecies of trypanosome.

There are several lines of evidence on the close resemblance between *T. evansi* and *T. equiperdum*, for example in a previous study [4], the two parasites *T. evansi* and *T. equiperdum* were compared regarding their ultrastructure, their mammalian hosts, way of transmission, pathogenicity, diagnosis and treatment, and biochemical and molecular characteristics. Electron microscopic investigation revealed no ultrastructural differences between the two species except that there were more coated vesicles in the flagellar pocket of *T. equiperdum*. Biological, biochemical and molecular studies showed many similarities between *T. evansi* and *T. equiperdum*. The most prominent differences between the two species are the presence of maxicircles in *T. equiperdum*, which are missing in *T. evansi*, and the route of transmission. While, *T. evansi* is transmitted by biting flies, *T. equiperdum* is transmitted from one equine host to another during copulation when mucous membranes come in to contact. Otherwise the two species are remarkably similar. The phylogenetic analysis between the two species and *T. b. brucei* was investigated, and they proposed that *T. evansi* arose from a clone of *T. equiperdum* which lost its maxicircles [4]. Result obtained by this study suggests that the trypanosome species (H-6) isolated from camel may be the most divergent from all others isolated ones from the different geographical regions. This assumption can support the fact that *T. evansi* is polyphyletic organism. On the other hand, since all the others analyzed trypanosome species are belonging to the same strain of *T. evansi*, this may also suggest that they are less diverged and can support the fact that *T. evansi* is monophyletic organism. Moreover, since all the obtained sequences have shown 100 identities to four clones of the *T. evansi* from the database this give the strong evidence that *T. evansi* has monophyletic origin. This speculation is in disagreement with a previous study that assumes *T. evansi* and *T. equiperdum* trypanosomes are not monophyletic clades and do not qualify for species status and should be considered as two subspecies [12].

The cladogram in Figure 4, revealed that *T. evansi* isolate from New Halfa (SU6-TBR1) is the most divergent one, followed by Gedaref isolate (SU7-TBR1). Since the branch lengths are proportional to the amount of inferred evolutionary change, the phylogenetic tree inferred from the TBR nucleotide sequences (137 bp out of 177 bp expected) clearly showed the genetic diversity of the parasites. Phylogenetic and molecular analyses of this region showed that three distinct genotypes of *T. evansi* in Sudanese dromedary camels do exist. Moreover, New Halfa isolate H-5 (SU6-TBR1) represents the outgroup species in this analysis, since it diverges early from the common ancestor. Gedaref isolate Ged-7 (SU7-TBR1) represents separate clade and also diverged early but later than New Halfa isolate H-5 (SU6-TBR1) and this node is well supported as the confidence level is 99% at a bootstrap value of 200. Sidoon isolate Si-2 (SU2-TBR1) and Gedaref isolate Ged-9 (SU8-TBR1) are identical *T. evansi* species, although they belong to two different geographical regions.

The phylogentic analysis obtained by MegAlign program has revealed low bootstrap values and this could be supportive to our datasets as to be more realistic in contrast to the level of reliability of node construction in the obtained tree. However, 6 branches of the Outgroup rooted tree are less significant based on the low bootstrap values, which would suggest the divergence of these *T. evansi* isolates is recently and low genetic diversity exists. Therefore, this study suggests that the Sudanese *T. evansi* is polyphlytic trypanosome.

The obtained results of *T. evansi* TBR sequences, using *T. brucei* primers is expected as the previous assumption that both *T. evansi* and *T. equiperdum* are petite mutants of *T. brucei* [12]. The repetitive nuclear DNA sequence was chosen for comparative analysis of *T. evansi* Sudanese isolates instead of minicircle kinetoplast DNA as the former is tends to be semi-conservative and the latter known to have two major sequence variants types A and B [17]. Based on the type of TBR primers used in this study which target specifically repetitive nuclear DNA of *T. brucei* s.l and as

stated before [20, 15], the sequence heterogeneity in these repeats is limited and restricted to certain positions.

Several studies have confirmed that *T. evansi* and *T. equiperdum* are morphologically indistinguishable parasites and although they are evolved from a common ancestor but they acquired biological differences, including host range, mode of transmission, distribution, clinical symptoms and pathogenicity [19]. However, in this study the detection of *T. evansi* and *T. equiperdum* in camels from different geographical regions of Sudan was conducted by PCR from blood collected on a filter paper and one genetic marker was used to amplify and sequence the repetitive nuclear DNA (TBR). The molecular identification of *T. equiperdum* based on the repetitive DNA sequence from camel during the current study may possibly be due to the fact that Tabanids are the causative agents for "Surra" in both camels and horses. Since *T. equiperdum* is known to have transmission through coitus only, and *T. evansi* is known to have a mechanical mode of transmission, therefore, tabanid vectors might be first fed on horses and become contaminated with *T. equiperdum* in their mouthparts, then secondly fed on camel where it transmitted *T. equiperdum* to this camel. Most interesting observation that the *T. equiperdum* can survive in the camel host; one suggestion is that may bet aking the advantage of changing their VSG coat.

5. Conclusions

The results reported here indicate that dried blood spots on filter papers are a useful source of DNA for detection of trypanosomes by PCR, which in agreement with previous report [11]. This study suggests that *T. evansi* could be a polyphyltic group with 4 clades or a monophlytic group and all isolates have a common ancestor. More informative genetic marker such as internal transcribed spacer-2 (ITS-2), mobile genetic element PCR (MGE-PCR) and microsatellite have to be carried out in both *Tabanus* flies and domestic animals (camels, horses, dogs) in order to have ultimate conclusion. More geographic locations should be included in the study; particularly the western regions.

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