

Molecular Characterization and Phylogenetic Analysis of *Trypanosoma evansi* from Local and Imported Camels in Egypt

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Abstract

Trypanosoma evansi, the agent of trypanosomiasis commonly known as Surra or Guffar, is regarded as one of the most economically important animal parasitic pathogen affecting livestock in Egypt. The current study aims to discuss genetic characterization and phylogenetic analysis of *Trypanosoma* isolates from local and imported naturally infected camels in Egypt. The study was initially started with parasitological and molecular surveillance on 411 native and 117 imported camels by using PCR-RoTat 1.2 VSG gene targeting 205 bp. Further, the molecular characterization and sequencing were achieved on four positive samples using PCR-TR3/TR4 primers that derived from a trypanosome-specific repetitive nucleotide sequence fragment. Product sequences were aligned against the corresponding GenBank sequences of known isolates of *T. evansi* and subjected to phylogenetic analysis. Results revealed that *T. evansi* was present in 66.67% and 74.36% of the local and imported camels respectively, regardless of age and sex factors. Basic Local Alignment Search Tool (BLAST) data of the obtained PCR TR3/TR4 gene sequences revealed that they corresponded to those of *T. evansi*, with the homology of 93% to 99%. Phylogenetic and molecular analyses of this gene showed that three genotypes of *T. evansi* in Egypt are present showed two common SNPs (G136A and G189T) in all samples, two SNPs in ISM and ISD (C3T and A207G) and six SNPs in HSA (T12C, C14T, T15C, G19C, C21G, and G22C). We conclude that *T. evansi* is described as presenting genotype variability among its isolates according to geographical distribution in Egypt.

Keywords: *Trypanosoma evansi*; DNA sequencing; Phylogenetic analysis

Introduction

Trypanosomiasis is the most important and serious pathogenic protozoal disease of camel caused in Egypt [1]. There are 11 different pathogenic trypanosomes known to exist in Africa [2]. The subgenus of Trypanozoon comprises three socio-economically important and highly pathogenic African trypanosomes; *Trypanosoma brucei*, *T. evansi* and *T. equiperdum* [3]. Neither parasitological nor serological tests are sensitive and specific enough to differentiate between them, thus various kinds of genetic and molecular methods have been continually updated in order to enhance greater precision in the diagnosis of Trypanozoon species and differentiation of these pathogens [4]. In addition, characterization and comparative analysis of genomes of such closely-related *T. evansi* allow their identification at the strain and may generate a testable hypothesis of genes that might be responsible for differences in pathologies between parasite strains or subspecies [5]. A series of techniques based on PCR have been used for phylogenetic analysis and/or characterization of polymorphisms in *T. evansi* populations around the world. Minisatellite markers [6,7], and random amplified polymorphic DNA (RAPD) [8,9] evidenced this connection. Similar results have been found by using amplified restriction fragment length polymorphism analysis (AFLP) [10,11], and inter-simple sequence repeat PCR (ISSR) [12]. Few studies have been carried out to investigate whether *T. evansi* populations are genetically sub-structured using the internal transcribed spacer (ITS) region including (ITS1 and ITS2), that are located between the repeating array of nuclear 18S, 5.8S, and 28S ribosomal RNA genes [13-17]. In addition, diversity of the transferrin receptor-encoding gene located at expression-site-associated (ESAGs 6 and 7) genes [18-21] are utilized in molecular classification of trypanosomes. Those studies demonstrated that the first and second internal transcribed spacers (ITS-1 and ITS-2, respectively) of the rDNA are useful targets for species delineation and for inferring phylogenetic relationships of *Trypanosoma* spp. with some limitations [1,16,22,23]. On the other hand, RoTat 1.2 PCR VSG gene amplified 205 bp is more specific [22], but failed to differentiate some Egyptian strains that have been previously identified as *T.*

evansi [24]. Furthermore, targeting the highly repeated sequence of minichromosome satellite DNA (TBR1/2 primer set) was more often seen in the PCR method compared to targeting ITS1 or RoTat 1.2 VSG sequences [16].

In Egypt, thousands of camels are regularly imported from neighboring countries (Libya, Sudan, and Somalia), for the purpose of breeding and slaughter. During their journey in Egypt, they mix with local animals at borders leading to mixed genotyping and strain difference might occur between Egyptian sub-populations and imported strains of *T. evansi* [10,24]. A few studies on genetic variability and characterization of *T. evansi* isolate in Sudanese originating camels and in other domestic animals were conducted [10,18,22], but the genetic information is lacking in local dromedary camels. The objective of this study was to ascertain the usefulness of sequence analysis of the region targeted by TR3/TR4 in assessing the genetic variability and characterization of *Trypanosoma* circulating in imported and local camels in Egypt, and as a tool to study phylogenetic and potential relationships of various *T. evansi* isolates.

Materials and Methods

Study areas

The present study requires a comparative analysis of several isolates. Therefore, it was initially started with a spot survey to study the prevalence of *T. evansi* in both native camels, mostly from nomads at the border

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provinces, and the imported camels from the main abattoirs in Egypt. Six different sites of Egypt located in the Red Sea and Matrouh governorates were selected, where rearing camels are the main activity and *T. evansi* infections were expected. Regions are the major Egyptian southern and western entrances (from The Sudan and Libya, respectively), where camels are imported legally (Figure 1). Due to migratory communities which usually move from one place to another, regions were grouped as geographic origin of the stocks used in this study.

Animals and blood sample collection

A total of 528 randomly selected camels (411 native, 117 imported), were examined for trypanosomiasis regardless of ages and sexes during the period from May 2011 to September 2014. Blood samples were collected in Vacutainer tubes containing ethylene di-amine tetra-acetic acid (EDTA), and then divided into two parts. One was for microscopical examination and the remaining sample was preserved at -20°C for DNA extraction of trypanosomes for PCR. For parasitological examination, thin blood smears were air-dried, fixed in methanol, stained with a 10% solution of Giemsa in phosphate-buffered saline (pH 7.2), and then subjected to microscopic examination with light microscopy (x40 and oil immersion objectives) according to Njiru [3].

Extraction of genomic DNA and PCR amplification using RoTat1.2 VSG gene

Genomic DNA from whole blood samples was extracted using a commercially available DNeasy Blood & Tissue Kit (Qiagen Inc. Germany) according to the manufacturer's instructions. All samples were analyzed with RoTat1.2 PCR depends on detection of a gene for the predominant surface glycoprotein of the *T. evansi*, and to exclude other trypanosomes [25,26]. Primer sequences were as follows: F5'-GCGGGGTGTTTAAAGCAATA-3', R5'-ATTAGTGCTGCGTGTGTTTCG-3' (Invitrogen, USA). PCR amplification reaction was performed in a total reaction volume of 25 µl containing 50 ng of template DNA and 12.5 µl of commercially

available PCR master mix (Qiagen, Germany). The primers (RoTat1.2) were used at a concentration of 10 pmol/µl. The PCR amplifications were carried out in a Biometra thermocycler. Cycling conditions were as follows: initial denaturation 94°C/3 min (1 cycle), followed by 94°C/1 min. (denaturation), 57°C/1 min. (annealing), and polymerization 72°C/1 min. (40 cycles), final extension at 72°C/5 min. Then the product of each sample was electrophoresed through 1.5% agarose containing ethidium bromide (1 µg/mL), and the voltage was set at 60 V for the electrophoretic mobility to visualize the amplified DNA and compared to a standard DNA 100 bp (Qiagen, USA).

Statistical analysis

All the collected data were entered into MS excel spreadsheet program to create a database and filtered before analyzed by using SPSS version 20. Descriptive statistics was used to determine the prevalence of the disease. All statistics were considered significant at $P \leq 0.05$.

Molecular characterization of *T. evansi* isolates

Samples, primers and PCR cycling: Twenty-four positive samples with RoTat1.2-PCR were selected representing imported and local camels. They were divided into four groups containing 6 samples/group (one group produced only a specific band at 205 bp and the other three groups produced from 4-8 multiple bands). The molecular characterization was achieved by *T. evansi* DNA species-specific primers TR3 (5'-GCGCGGATTCTTTGCAGACGA-3') and TR4 (5'-TGCAGACACTGGAATGTTACT-3') (Invitrogen, USA). They were derived from a trypanosome-specific repetitive nucleotide sequence fragment that amplified 257 bp [27]. PCR amplifications were carried out in 50 µl reaction mixture containing 100 ng of template DNA and 25 µl of commercially available PCR master mix (Promega, UK). The reaction mixtures were cycled in a programmed PCR machine at the same conditions with RoTat1.2. Further, four of them were submitted to sequencing and phylogenetic analysis.

DNA sequencing and phylogenetic analyses: The four PCR products of amplified specific repetitive nucleotide sequence gene were purified and sequenced in Macrogen, Inc. (Seoul, South Korea). The nucleotide sequences were aligned with existing sequences of *T. evansi* in the GenBank databases using BLAST programs and databases of the NCBI (National Center for Biotechnology Information, Bethesda, MD, USA) (www.blast.ncbi.nlm.nih.gov/Blast.cgi). Four sets of nucleotide sequence HSA, ISM, ISD and NWC (220 bp) from Egypt were separately aligned against homologous sequences reference in GenBank using ClustalW method in the Bioedit software version 7.2.5. A phylogenetic tree was constructed using maximum parsimony (MP) version 3.3 and neighbor-joining (NJ) algorithms version 3.6a2.1 of Bioedit software [28].

ID of query sequence: DNA sequences generated from this study were blasted to the databases of the GenBank to find the homologous recorded sequences in ID 8W26EU701R, 8W2CZTCD01R, 8W96AJEX01R, and 8YKBM92G013.

Results

Microscopic examination and PCR detection of *T. evansi*

The present study indicated that *T. evansi* infection was prevalent in the study areas. Regardless of age and sex factors, *T. evansi* infections in thin blood smears were observed microscopically in samples from the Red Sea governorate 35 (21.6%), Matrouh governorate 52 (20.9%) and from abattoirs 28 (22.22%) with low parasitemia levels in most cases. Whereas, the PCR-positive rates for *T. evansi* in blood samples from

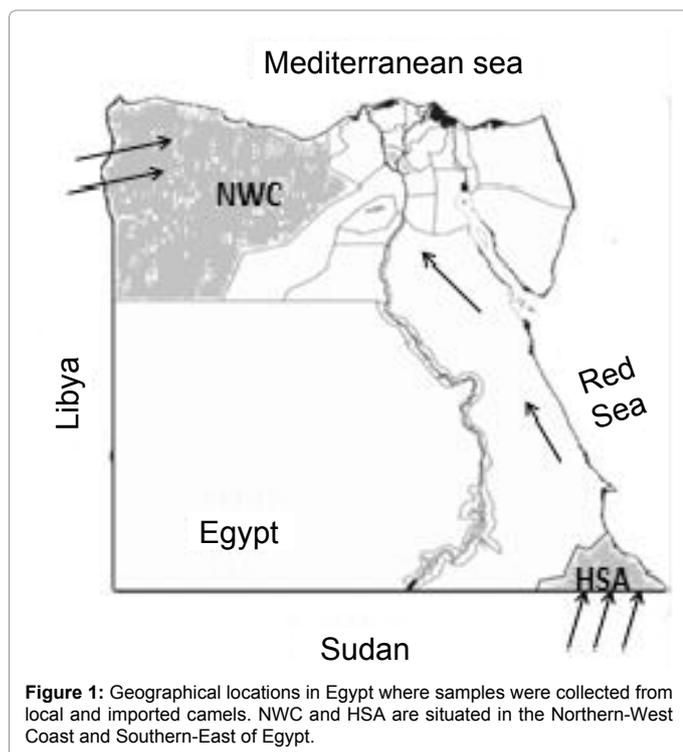


Figure 1: Geographical locations in Egypt where samples were collected from local and imported camels. NWC and HSA are situated in the Northern-West Coast and Southern-East of Egypt.

the Red Sea and Matrouh governorates and from abattoirs were 67.9%, 65.9%, and 74.36%, respectively. All blood smear-positive samples were also PCR-positive for *T. evansi* parasites with RoTat1.2 even though that having different morphological shapes in camels and in mice inoculated from infected camels (Figure 2). PCR results with RoTat 1.2 primers revealed that many not all local camel strains in HSA produced between four and eight specific bands with molecular weights of 94 bp, 205 bp, 355 bp, 429 bp, 553 bp, 861 bp, 1110 bp, and 1526 bp sharing with Sudanese and Somali imported strains in five of them (94 bp, 205 bp, 355 bp, 429 bp, 1526 bp). While, the majority of local camels in Matrouh Governorate of Egypt did not demonstrate these bands, but produced mostly one specific for its genotype with molecular weights of 205 bp (Figure 3). Compared with microscopy of blood smears, the PCR method had higher sensitivity and specificity. Epidemiology and risk factors affecting trypanosomiasis in these regions were discussed in detailed [25,29]. Results of this survey are shown in Table 1.

Genetic characterization

Genomic DNAs from imported and local camels were amplified producing a single fragment of expected size (257 bp) instead of multiple fragments as in RoTat1.2 amplified 205 bp. Sequence Alignments of VSG gene for infected camels from the four different regions yielded 220 base pairs with four stop codon and no gap. The results of sequencing reported existence SNPs between the four different samples where that HSA has 6 SNPs, ISD and ISM have 2 SNPs compare with NWC which mean presence three genotypes as shown in Figure 3. The nucleotide sequences derived from the four PCR products amplifying of four infected camels (ISD, NWC, HSA, and ISM) were released in GenBank with ID 8W26EU701R, 8W2CZTCD01R, 8W96AJEX01R, and 8YKBM92G013, respectively. BLAST data showed different identical from 99% to 93% between the sequence of Egypt *T. evansi* and eight sequences of *T. evansi* selected globally from GenBank as summarized in Table 2

Phylogenetic analysis

Phylogenetic analysis exhibited sequence divergence between *T. evansi* from Egypt and eight selected globally from GenBank ranging

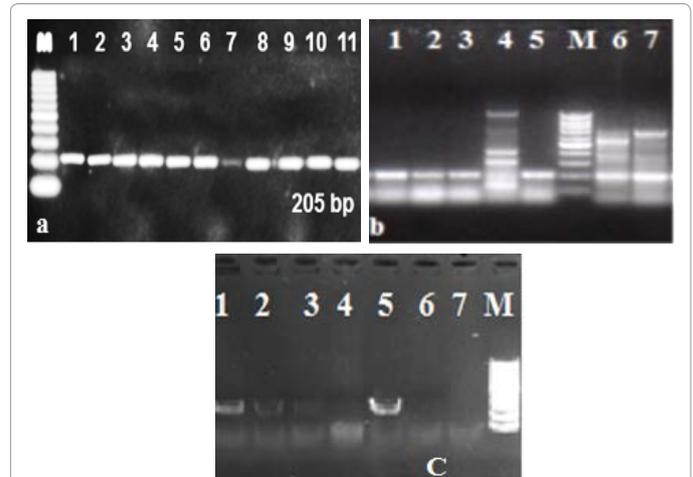


Figure 3: Agarose gel electrophoresis of RoTat 1.2 PCR-based assay products of only 205 bp (a) and/or represent other fragments of 205 bp, 355 bp, 429 bp, 861 bp, 1110 bp, 1526 bp and 1626 bp were amplified from *T. evansi* (b). Wherever, TR3/TR4 primers amplified only 257 bp in all positive samples. M, 100 bp molecular size marker (Qiagen®, Germany).

from 0.9% to 3.6%. The four isolates gave two common substitutions at G136A and G189T. Whereas the two imported isolates of ISD and ISM gave other two substitutions at C3T and A207G, the local isolate of HSA showed six others of substitutions of T12C, C14T, T15C, G19C, C21G, and G22C as represented in Table 3 and demonstrated in Figure 4. The phylogenetic tree shows also the evolutionary relationship of the sequences in which the length of the branch was proportional to the estimated genetic distance between the sequences (Figure 5). It showed that ISD and ISM identical, also they are close to NWC. The length of the branch is very small between *T. evansi* strain H4 from Israel and *T. evansi* from Palestine, strain C6 from Israel, Philippine, Ludhiana, India and Punjab, India. And they are very close to *T. evansi* from NWC and close to *T. evansi* from ISM or ISD. Evolutionary distance to each of *T. evansi* from HSA, American and India strain CIV are large.

Discussion

In Egypt, *T. evansi* is the most widespread blood parasite causing significant morbidity and mortality in camels and is given the first priority in its order of importance among camel diseases; in particular, it was presently associated with bacterial and parasitological secondary infections [29,30]. In the present study, during the blood collection process, cases of suspected trypanosomiasis were investigated. Despite the survey lasted three years and was conducted at different times of the year in Southeastern and Northwestern Egypt, the prevalence of trypanosomiasis seems to be similar and no significant difference was reported. This may due to lack of health care and veterinary services in the two mentioned regions as previously reported in Egypt [10,26,31]. A similar explanation was reported in Sudan [16]. Compared with microscopy of blood smears, the PCR method had higher sensitivity and was recommended to be utilized in the diagnosis of trypanosomiasis. To study of molecular characterization and phylogenetic analysis of *T. evansi* isolates from local and imported naturally infected camels in Egypt, we decided to use PCR-TR3/TR4 that amplified 257 bp instead of RoTat 1.2, followed by sequence analysis of this fragment. Whereas, PCR-RoTat1.2 produced mostly several bands other than the specific one (205 bp) in the majority of imported and local camels. This finding is in agreement with the results of [10,24], in addition to the absence of RoTat 1.2 VSG gene in some positive samples with TBR1.2 primer targeting the highly repeated sequence of minichromosome

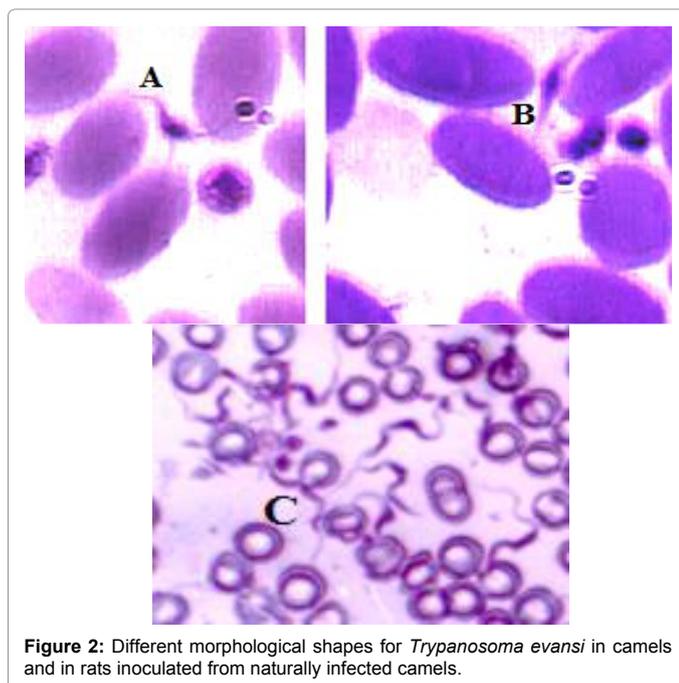


Figure 2: Different morphological shapes for *Trypanosoma evansi* in camels and in rats inoculated from naturally infected camels.

Origin (Identity)	Collection sites	Governorate (Region*)	Prevalence of <i>T. evansi</i> in camels		
			No.	Microscopic Examination	PCR (RoTat1.2)
local	Halaib El Shalateen Abu-Ramad	The Red Sea (Southern East Area)	162	35 (21.6%)	110 (67.9%)
	Mersa-Matrouh El-Negeila Sidi-Barrani	Matrouh (Northern West Area)	249	52 (20.9%)	164 (65.9%)
	Total		411	87 (21.17%)	274 (66.67%)
Imported	Al-Moneib	Giza	26	5 (19.23%)	17 (65.38%)
	Al-Bassatein	Cairo	91	23 (25.27%)	70 (76.92%)
	Total		117	28 (22.22%)	87 (74.36%)

*Collection sites were grouped as geographic regions of the stocks used in this study. No significant differences in *T. evansi* infection have been found between imported and local camels. However, there was a significant difference ($P < 0.001$) between PCR and parasitological tests in detection of *T. evansi*.

Table 1: Prevalence of *Trypanosoma evansi* in both imported and local camels in Egypt.

Selected Sequences	Current Egyptian <i>T. evansi</i> isolates				Reference
	(ISD) 8W26EU701R	(NWC) 8W2CZTCD01R	(HSA) 8W96AJEX01R	(ISM) 8YKBM92G013	
HM209055.1 (H4 Israel)	98%	99%	96%	98%	[36]
HM209054.1 (C6 Israel)	97%	98%	95%	97%	[37]
EU931247.1 (Palestine)	97%	98%	95%	97%	Unpublished
JQ653273.1 (Philippine)	98%	99%	96%	98%	unpublished
JF894242.1 (American)	99%	95%	93%	99%	Unpublished
LC008133.1 (CIV India)	99%	99%	96%	99%	[38]
JQ0303878.1 (Punjab, India)	96%	97%	94%	96%	Unpublished
AB979445.1 (Ludhiana, India)	99%	99%	96%	99%	[39]

*Coded isolates: ISD (ID. 8W26EU701R) and ISM (ID. 8YKBM92G013) were from imported camels originated from Sudan and Somali, respectively. Whereas, NWC (ID. 8W2CZTCD01R) and HSA (ID. 8W96AJEX01R) were from local camels originated from northern-west and southern-east Egypt, respectively.

Table 2: The divergence and identity between the current four *Trypanosoma evansi* isolates from Egypt and eight selected sequences circulating globally from GenBank.

Sample	NWC	ISM and ISD	HSA
Substitutions	G136A, G189T	G136A, G189T C3T A207G	G136A, G189T T12C C14T T15C G19C C21G G22C

Table 3: Substitutions between four isolates of *Trypanosoma evansi* from Egypt.

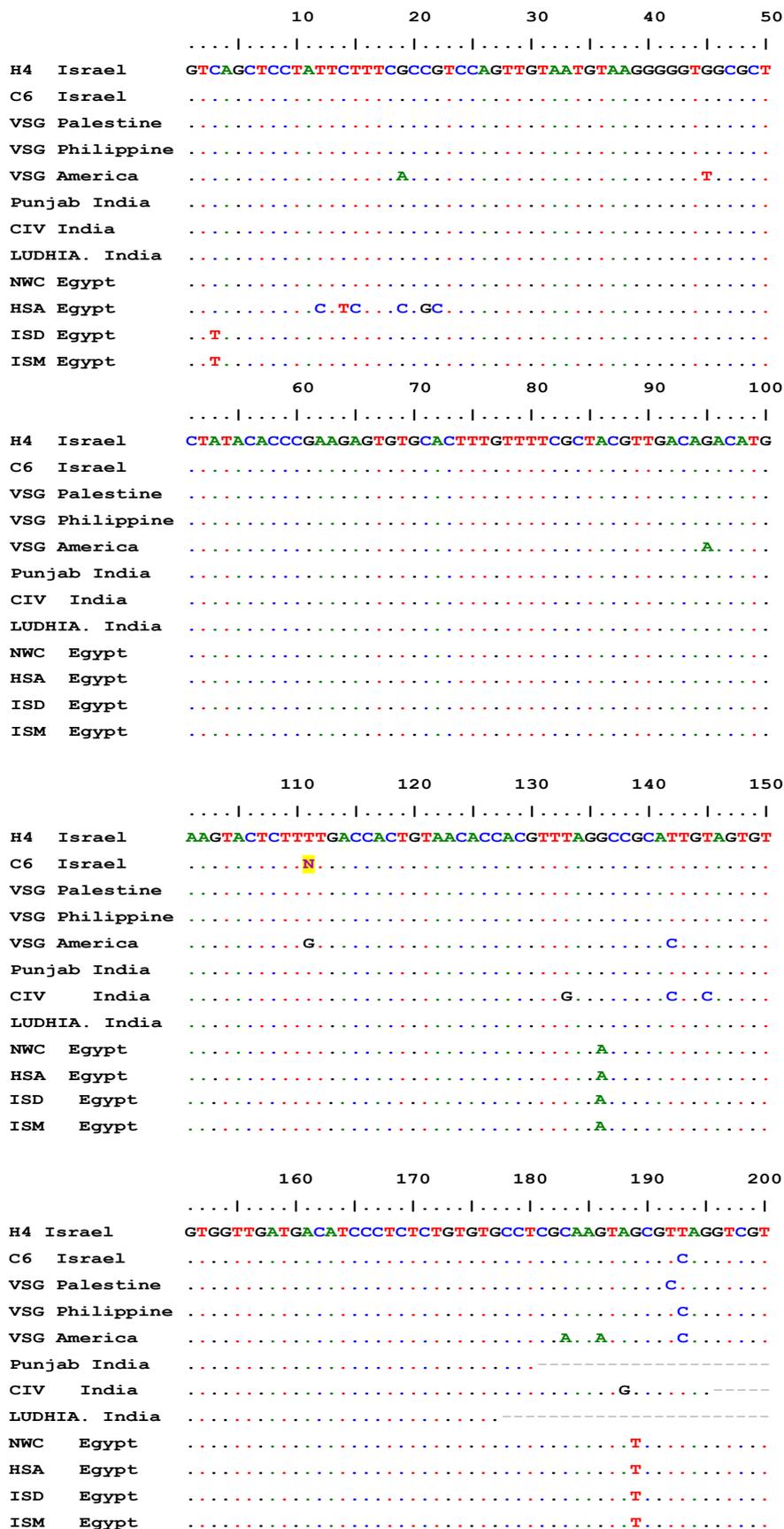
satellite DNA [25,32]. These results were explained as (a) occurrence of mutants due to mixed genotyping occurred between some *T. evansi* isolates with other Trypanozoon (b) presence of *T. brucei* and/or *T. equiperdum* infection in the Northern West border area of Egypt (c) the present of *T. evansi* lacking the RoTat 1.2 VSG gene in the study area as reported in Kenya [33].

Whereas, low genotype variability would be present in endemic trypanosomiasis areas as a result of the elimination of virulent trypanosome strains [34]. It is described as presenting genetic variability among its isolates according to geographical distribution [10]. However, those isolates were identified with degrees of heterogeneity, describing that natural populations of *T. evansi* in Egypt revealed the presence of higher levels of intra-specific genetic variability of *T. evansi* [24,35]. Thus, this study intended to apply TR3/TR4 as a molecular tool to characterize trypanosomes in Egypt. The current search was the third study on *T. evansi* genotypes in imported dromedary camels in Egypt after [18,22], but it was the first studied *T. evansi* genotypes in both imported and local camels. A partial sequence of RoTat 1.2 VSG gene was identical to the *T. evansi* sequences reported from India and Kenya, but the varied similarity was seen when aligned with other Egyptian *T. evansi* sequences [22]. Whilst, analysis of transferrin receptor-encoding

gene (ESAG6) showed variable repertoire in the studied isolates, which may indicate to a novel structure of *T. evansi* population from Egypt and/or a difference in host range [18]. In the present study, the results of the phylogenies obtained from the Maximum Parsimony (MP) method suggest that *T. evansi* isolates from HSA (Egypt), America and CIV (India) are more diverse than other isolates. Phylogenetic analysis showed a robust tree clustering all those from known strains of *T. evansi* circulating globally and retrieved from GenBank with values at relevant nodes. The phylogenetic tree showed the evolutionary relationship of the sequences in which the length of the horizontal line was proportional to the estimated genetic distance between the sequences. This indicated that the evolutionary distance between groups is very short suggesting that the genetic divergence is recent (Figure 4).

Conclusion

In conclusion, the present study indicated that surra is highly prevalent in local and imported camels. Though RoTat 1.2 PCR should have been better than any other diagnostic tests for *T. evansi*, it amplified multiple fragments in some cases than the case of TR3/TR4-PCR that could detect also those trypanosomes amplifying one fragment. This data could be applicable for the survey of parasite dynamics, epidemiological studies as well as prevention and control of the disease. Moreover, we cloned and sequenced the genes from *T. evansi* isolates from imported and local camels in Egypt and found that the local HSA gene sequences showed obvious differences to those from NWC and other *T. evansi* sequences from imported camels that were closely related. Consistent with the BLAST results, the phylogenetic tree constructed in this study showed that one newly sequenced *T. evansi* isolate appeared in the same clade as HSA, while the others appeared in the same clade as closely related isolates.



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      . . . . | . .
H4  Israel      CTGCAA
C6  Israel      . . . . .
VSG Palestine . . . . .
VSG Philippine . . . . .
VSG America    . . . . .
Punjab India   - - - - -
CIV  India     - - - - -
LUDHIA. India  - - - - -
NWC  Egypt     . . . . .
HSA  Egypt     . . . . .
ISD  Egypt     . . . . . G
ISM  Egypt     . . . . . G
    
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Figure 4: Nucleotide sequence alignment of *T. evansi* specific repetitive nucleotide sequence fragment from imported and local Egyptian camels with other VSG *T. evansi* isolates from India, Palestine, Israel, Philippines, and America. Identical bases are shown as dots and sites of variation are shown.

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                                     .01 + ISD (Egypt)
                                     +-10
                                     0 +--9 + ISM (Egypt)
                                     ! !
      .01                               .03
+-----8-----+-----HSA (Egypt)
0 !                               0!
+--7                               + NWC (Egypt)
! ! .005
! ! + VSG Palestine
! +--6
! + LUDHIANA. India
---1
! 0 + Punjab India
! +--4 .035
! .005! +-----VSG America
! +--2
!0 ! !0 + C6 Israel
+--5 +--3
! ! + VSG Philippine
! ! .02
! +-----CIV India
!
+ H4 Israel
    
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Figure 5: Phylogenetic relationships of *T. evansi* isolates from Egypt compared to reference sequences of different *T. evansi* in database were based on trypanosome specific repetitive nucleotide sequence fragment that amplified 257 bp.

Author Contributions

SB conceived and designed the experiment, contributed reagents/materials/analysis tools, carried out the molecular analyses, participated in the data analysis, did the field collection, statistical analysis and drafted the manuscript. TA participated in field collection and assisted in obtaining funding. AD helped to conceive the study, carried out phylogenetic analysis, PCR cycling, and helped to review the manuscript. All authors read and approved the final manuscript.

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Conflict of Interests

The authors declare that they have no conflict of interests regarding the publication of this paper.

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