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Original papers

Molecular changes in *Trypanosoma evansi* after treatment against trypanosomosis

Safaa Mohamed Barghash

Parasitology Unit, Animal Health Department, Desert Research Center (DRC), Mathaf El Mataria St, El- Naam, Cairo, Egypt; e-mail: barghash_7@yahoo.com

ABSTRACT. Neither physiological nor pathological changes following treatments explained why trypanosomes in the same group of experimentally treated animals correlated in virulence. Also, they behaved like each other but not similar to other groups despite the same *T. evansi* injected strain. The current study aims to discuss whether molecular changes might occur to *Trypanosoma evansi* isolates followed treatments are responsible for that difference or not. Ten preserved isolates from *T. evansi* after previous treatments besides the original strain of *T. evansi* that injected before treatments were used in the present study. These isolates were intraperitoneally inoculated in 11 groups of male Wister Albino rats with equal doses. Parasitological findings and the molecular changes accompanied were discussed along with the experiment based on PCR-TR3/TR4 specific-primers. The study also achieved alignments, gene sequence and phylogenetic analysis for submitted and reference strains belong to *T. evansi*, *T. brucei*, *T. b. brucei,* and *T. b. gambiense* deposited in GenBank. The present results assessed molecularly the effectiveness and highly antitrypanosomal activity of human plasmas O^+ and A^+ on *T. evansi* than others, and how their strains drifted from its original sequence to the nearest form of *T. brucei.* At the same time, *T. evansi* in other plant extract groups multiplied progressively like cancer cells and became more virulent and close to reference strains of *T. evansi*. Our data further indicated that *T. evansi* after treatment was a paraphyletic group. It also corroborated the antitrypanosomal activity-specificity and the molecular changes occurring were correlated to the type of treatment.

Keywords: *Trypanosoma evansi*, rats, plant extract, molecular analysis

Introduction

Trypanosoma evansi, the causative agent of camel trypanosomosis (Surra), is affecting mainly camels among other hosts worldwide, with high mortality and morbidity, causing various types of trypanosomoses [1,2]. It has a large diversity of mammalian hosts, and can periodically switch its major variant surface glycoprotein (VSG) producing relapses of parasitemia [3]. *T. evansi* consists of a large number of morphologically identical populations that are known to differ significantly with various biological characters and could be regarded as a natural mutant of *T. brucei* [4]. Many studies have been carried out to examine whether *T. evansi* populations are genetically sub-structured. Whereas macro-heterogeneity has been found in Egypt [5,6], and micro-heterogeneity in stocks from Brazil, Ethiopia, and China, two studies considered *T. evansi* strains as homogeneous taxon [7,8]. Other Kenyan studies reported the predominant VAT RoTat 1.2 in all *T. evansi* isolates except for two types: (a) non-RoTat 1.2 *T. evansi* type A; (b) *T. evansi* type B that lack both RoTat 1.2 genes [9].

To understand why trypanosomosis varies so widely virulence from one set of circumstances to another, the interaction of a wide range of factors and features on the host which determined patterns of trypanosomosis and their epidemiology even at the local scale was previously discussed in Egypt [10]. However, *T. evansi* is described as presenting genotype variability among its isolates according to geographical distribution and suggested some undefined population sub-structuring within *T. evansi* that may have arisen through other modes of genetic variations such as mutation and genetic drift [11]. Besides, phylogenic and molecular analyses showed three genotypes of *T. evansi* in Egypt [12].

Recently, we carried out two studies to evaluate the antitrypanosomal activities of some plant

extracts, human plasmas, and Diminazene aceturate (DA) on *T. evansi* experimental infection in rats [13, 14]. Despite the same injected strain of *T. evansi,* the results revealed highly significant differences between groups of rats after treatments. It was estimated at haematological and biochemical parameters followed by acute pathological changes, but the *in vitro* and *in vivo* studies could not explain why?

The goal of the current study was to assess the molecular changes of *T. evansi* isolates after being treated for trypanosomosis.

Materials and Methods

Ethical approval. The current experiment was conducted in compliance with internationally accepted principles of the European (EU) Directive 2010/63/EU for animal experiments and the National Institutes of Health guide (NIH Publications No. 8023 revised 1978). The sourced human materials were following the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Preserved isolates. Eleven preserved isolates of *T. evansi* from our previous studies, within it; the original strain was used in the present study. They originated from 10 rat groups after infection with *T. evansi* and treated with used: (i) leaves ethanol extracts from five medicinal plants [13] were selected based on information from traditional healers on their curative effect against *T. evansi* and other parasites: *Camellia sinensis* (Green tea), *Thymus vulgaris* (Thymol)*, Mentha longifolia* (Mint), *Azadirachta indica* (Neem), and *Saliva offiicinalis* (Saige); (ii) four human plasmas from four adult females were clinically healthy with different blood groups (O^+, AB^+, B^+, and A^+) [14]; (iii) diminazene aceturate (DA) was selected as the most antitrypanosomal drug for trypanosomosis). Each original group belonged to plant extracts and plasmas administrated doses of 0.5 ml/200 g. B. wt., 2 times along the experiment, for 35 days. Whilst DA was given intraperitoneally (IP) about 200 µl (after serial dilution of 0.5 ml/10 kg body weight) of diminazene aceturate® as a chemical drug (Vial contains: 5250 mg Diminazene aceturate + 6560 mg Phenazone).

Trypanosome propagation in mice. The preserved isolates used in the present study were passages in white Swiss mice (100 µl blood containing trypanosomes/each mouse) in 11 different groups containing 3 mice for each. Parasitaemia level in mice was observed daily post-infection until giving parasitemia of approximately 10^5 Trypanosoma/ml [15].

Experimental animal model. One hundred and ten male Albino Wister rats weighing 200 g. were purchased from Animal House in the Faculty of Science at Ain Shams University, Cairo, Egypt, and kept in well ventilated plastic cages. They were exposed to 12 hours of light and dark cycles fed

Table 1. Status and description of thirteen submitted strains and their originated isolates

Strain	Group	Source of preserved isolate used	Current isolation date
1X	1	Infected with *T. evansi* but not-treated	day-21
2X	2	Infected and treated with Diminazene aceturate	day-35
3X	3	Infected and treated with a human plasma A^+	day-14 (A+)
4X	3	Infected and treated with a human plasma A^+	day-35 (A^{+2})
5X	4	Infected and treated with a human plasma B^+	day-35
6X	5	Infected and treated with a human plasma AB^+	day-35
7X	6	Infected and treated with a human plasma O^+	day-14 (O+)
8X	6	Infected and treated with a human plasma O^+	day-35 (O^{+2})
9X	7	Infected and treated with *Saliva offiicinalis* extract	day-35
10X	8	Infected and treated with *Camellia sinensis* extract	day-35
11X	9	Infected and treated with *Thymus vulgaris* extract	day-35
12X	10	Infected and treated with *Azadirachta indica* extract	day-35
13X	11	Infected and treated with *Mentha longifolia* extract	day-35

Thirteen strains from eleven groups were selected as one strain for each group by the end of the experiment, and two strains from 3^{th}, 6^{th} groups taken at day-14 due to remarkable changes started before other groups

Table 2. The similarity between submitted strains and reference strains deposited in GenBank

Reference Strain/Isolate/Clone	lcl|Query_10109		
	Identity	Accession No.	Ref.
T. evansi VSG gene. isolate: Punjab	100 %	LC008133.1	[18]
T. evansi VSG gene for VSG protein. Clone: Ludhiana.	100 %	AB979445.1	[19]
T. evansi VSG (RoTat 1.2) gene. Unpublished	99 %	JQ653273.1	[20]
T. evansi isolate H4 VSG gene.	99 %	HM209055.1	[21]
T. brucei 221 VSG expression site BAC	99 %	AL671259.1	[22]
T. brucei VO2VSG expression site BAC	99%	AL671259.2	[23]
T. b. gambiense DAL972 chromosome 5.	99 %	FN554968.1	[24]
T. b. brucei strain 927/4 GUTat10.1 chromosome 1.	98 %	AL929603.1	[25]
T. b. gambiense DAL972 hypothetical protein. Unpublished	96 %	XM011774008	[26]
T. brucei ingi retroposon Bam HI internal fragment	94 %	M33484.1	[27]

Sequences identity are numbered according to the original file

with pellets and fresh vegetables, and watered ad libitum throughout the experimental period. The rats were allowed 10-days of acclimatization before they were divided into groups of eight rats per cage.

Experimental design. The experimental design was carried out in the same conditions as our two previous studies [13,14]. Eleven groups of rats (n=10) were distributed among 11 big cages as summarized in Table 1. Of these, five groups were classified into one group for each isolate originated from ethanol leaf plant extracts at its suitable *in vitro* concentration and administration. Besides, four groups containing rats injected with 4 isolates belong to human plasma with different blood groups (O^+, AB^+, B^+, A^+). The tenth group of rats composed of the original strain of *T. evansi* without treatment (control group), whereas the eleventh infected group composed of diminazene aceturate treated isolate.

Sampling. Each group was infected with 0.1 ml blood/rat sourced from the parallel infected mice group exhibited 3×10^5 trypanosomes. After parasites were detected in the bloodstream, rats were observed daily for clinical signs; prepatent period, longevity, and the mortality rate were also observed [16]. The parasitaemia levels in all groups were checked three times a week by wet blood films prepared from tail blood at 40× magnification. About 3 ml of blood samples were obtained from each group by heart puncture in vacutainer tubes with EDTA, two times at 14 and 35 days post-infection when trypanosomes reappeared [11,13,14].

DNA extraction and PCR-amplification. A total of 13 strains out of 11 groups were represented from the injection with the bloodstream forms of the preserved isolates as shown in Table 1. They were selected as one strain for each group by the end of the experiment except for 3th and 6th groups (2 strains/group) taken at days-14, 35 due to remarkable changes started before other groups. The thirteen genomic DNAs were extracted using a commercially available DNeasy Blood & Tissue Kit (QIAGEN Inc., Hilden, Germany), according to the manufacturer's instructions. The molecular characterization was achieved by *T. evansi* DNA species-specific primers (TR3: 5'-GCGCGGATTC TTTGCAGACGA-3' and TR4: 5'-TGCAGACACT GGAATGTTACT-3') (SIGA, Foster City, California, USA). Primers were derived from a trypanosome-specific repetitive nucleotide sequence fragmented that amplified 257 bp [12,17]. PCR amplification reaction was performed in a total reaction volume of 50 µl containing 100 ng of template DNA and 25 µl of commercially available PCR master mix (QIAGEN, Hilden, Germany), and 20 pmol of each primer. Initial denaturation was one cycle at 94°C for 3 min. followed by 35 cycles of denaturation, annealing at 57°C for 1 min. and polymerization, and finally one cycle for the final extension at 72°C for 5 min. The product of each sample was electrophoresed and compared to a standard DNA 100 bp (QIAGEN, Hilden, Germany).

DNA sequencing and phylogenetic analyses. Thirteen PCR products were purified and sequenced in MACROGEN, Inc. (Seoul, South Korea). The nucleotide sequences were aligned with 10 existing sequences belong to *T. evansi*, *T. brucei*, *T. b. brucei* and *T. b. gambiense* in the GenBank databases

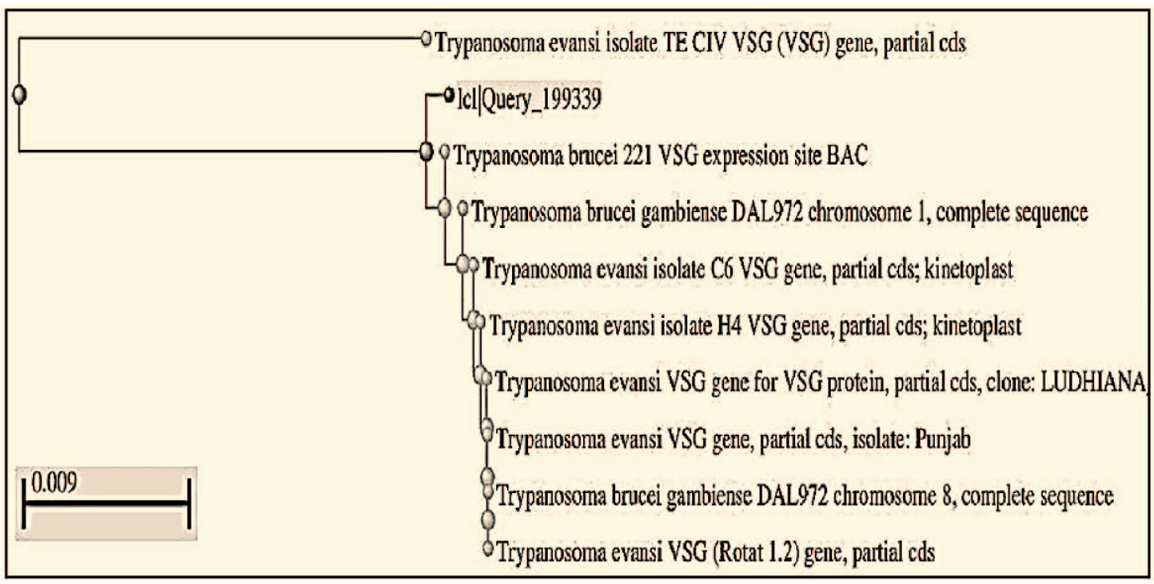

Figure 1. Phylogenetic tree generated based on TR3/TR4 results for the original injected strain of *Trypanosoma evansi* used (IcIQuery_199339 with shadow color), and reference *T. brucei* subspecies/strains showing similarity between them

(Table 2) using Clustal W software and BLASTn using BLAST programs and databases of the NCBI (National Center for Biotechnology Information, Bethesda, MD, USA) (www.blast.ncbi.nlm.nih.gov/Blast.cgi). The presence and absence of genes were manually verified in the alignments using the Integrative Genomics Viewer (IGV). Genes were considered present if there were mapping reads for each nucleobase position in the gene. The resulting file was filtered to retain only SNPs for each strain and the number of heterozygous SNPs was determined and concatenated to a Bioedit sequence. The high mapping quality cut-off prevents repetitive regions or unreliably mapped reads from causing false-positive SNPs. The phylogenetic trees were created for the ascertainment bias inherent in SNP alignments, the nucleic acid sequence Maximum Likelihood method of a molecular clock, version 3.6a2.1, and neighbor-joining (NJ) algorithms, version 2.1 and Bioedit, version 3.6a2.1.

Results

Antitrypanosomal activity and parasitological findings

In the present study, all infected groups gave parasitemia with different values after day 7 post-infection but only a limited set of variable antigenic types have been described in *T. evansi.* Some changes occurred in the behavior, movement, virulence, and morphology of trypanosomes following treatments, especially in groups of plant extracts isolates compared to the infected-untreated group. Whereas morphometric changes of *T. evansi* occurred: the body extends and the undulating membrane became elongated, the flagellum increased in length, besides the behind become wider. Parasitemia was significantly different between groups and three different degrees of *T. evansi* virulence were estimated based on mobility and mortality. It was low in no clinical signs (28–35 days), moderate (21–27 days), whereas it was high in 1st group that increased gradually and rats died directly started from day-17. By the end of the experiment, no rats from *S. offiicinalis*, *C. sinensis*, O^+, A^+, and B^+ groups died within 35 days whereas plasma has significantly increased the longevity of rats.

Regarding plant extracts isolates; *T. evansi* in the groups from eight to eleven were detected and progressed to high numbers throughout the experiment. Four rats died before day-27 in groups 10, 11 as a result of reappearances and progress of parasites. Whereas, the groups of human plasma isolates from three to six taking more days to appear in the bloodstream, then fluctuated in numbers before complete disappearance on day 15 post-infection and returned on day 33 as non-motile. Before the cut of the experiment on day-35, *T. evansi* was detected in all treated groups and the lowest level was reported on 6th group animals. Comparison among all treated groups indicated that 6th followed by 3rd groups exhibited the highest antitrypanosomal effect against *T. evansi* then the

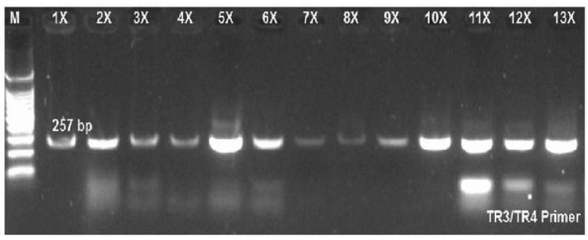

Figure 2. Ethidium bromide stained agarose (1.5%) gel showing amplification products of TR3/TR4 of 13 *Trypanosoma evansi* strains at the same size of 257 bp. Lane M: a 100 bp molecular size marker. Lanes from 1X to 13X correspond to *T. evansi*: infected untreated (lane 1), after treated with Diminazene aceturate (lane 2), after treatments with human plasmas (lanes from 3X to 8X), after being treated with plant extracts (lanes from 9X to 13X).

4th group. In the 2nd group, containing DA isolate, *T. evansi* completely cleared from circulation on day 3 to 10 days post-infection with a prolonging survival period of up to 17 days, and then returned before the cut of the experiment.

Molecular findings

Sequence analysis of the 1X strain injected into all groups of rats and amplified at 257 bp with BLASTn ICI Query_199339 revealed homogeneity with *T. evansi* 100% and maximum identities between 94 and 99 % to other corresponding *T. brucei* spp. in GenBank as shown in Fig. 1. Despite the low number of circulating trypanosomes in 3rd and 6th groups in compared to the others, trypanosomes DNA were amplified and detected with different strengths in the blood of all groups of the same size 257 bp, as shown in Fig. 2. Neighbor-Joining for genetic distance was conducted for only 13 present isolates after treatment (Fig. 3a, b). The generated trees revealed the close relationship between the strains of each type of treatment and its activity. It also confirmed the superiority of DA, and human plasma (O^{+2}, A^{+2}) treatments that cluster together, whereas the other strains from treated groups grouped. Further comparison of the sequences of TR3/TR4 separated the 1X strain in one group. Surprisingly, strains of A^{+} and O^{+} that were taken after 14 days came in contact with leaf plant extracts, not plasmas treated isolates. Although *T. brucei,* as well as all, tested *T. b. brucei* and *T. b. gambiense* strains do not express nor contain RoTat 1.2 VSG gene, they were more similar to human plasma treated strains than ethanol plant extract treated strains of *T. evansi*. In total, 13 submitted strains

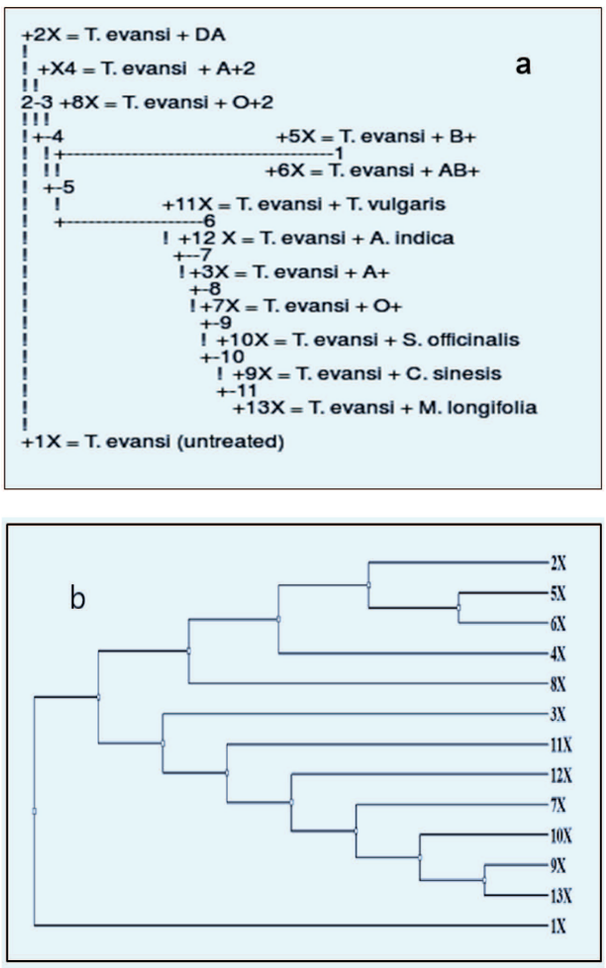

Figure 3 (a,b). DNA parsimony algorithm and Neighbor-joining method trees depicting the phylogenetic relationship for 13 submitted isolates based on TR3/TR4 sequence fragment that amplified 257 bp of *T. evansi* before and after treatment

were segregated into sub-clusters although very related; the isolation is not identical and corroborated the antitrypanosomal activity-specificity of plant extracts and human plasma proteins.

All 23 genomes discussed in the present study were aligned to the *T. evansi,* and the resulting file was sorted by position and variants were investigated between the present and the reference genes. Unique SNPs for *T. evansi* isolated the clusters that were identified and determined. The genome of trypanosome strains belongs to plant extracts that have only one unique SNP variation in time and space at nucleotide positions in 243 although they were isolated from 5 different groups. Whereas a total SNP (231) variation was found within human plasma treatment groups, and the A^{+} and O^{+} strains had the least variation. On the other

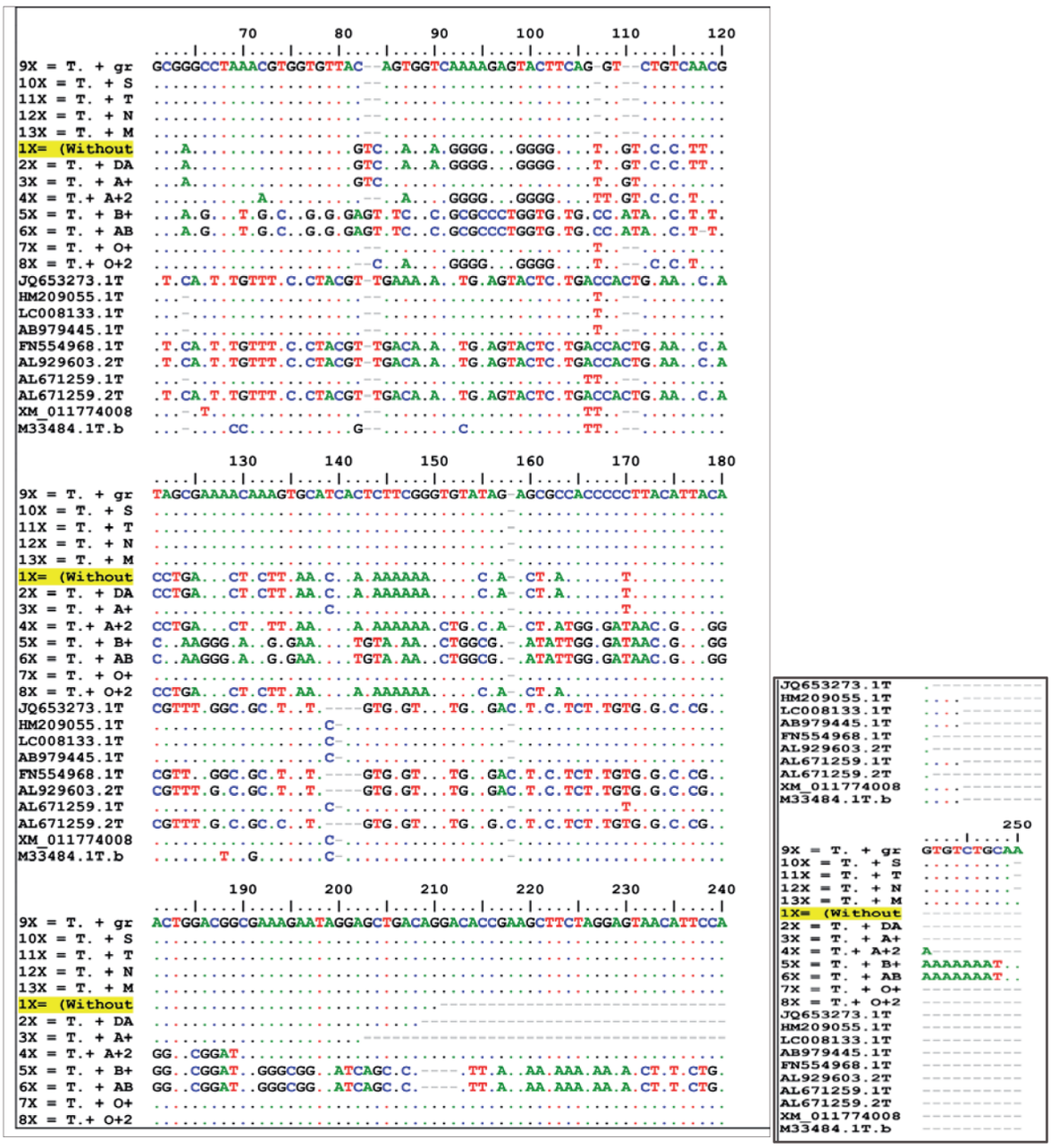

Figure 4. Total nucleotide sequence differences in TR3/ TR4 genes between isolates from the current study and reference sequences of *T. brucei* spp. Dots indicate base identical to those in obtained isolates

hand, the SNP's variation in reference strains was nearer to that observed between human plasmas treated strains than plant extracted plant strains. The complete list of different SNPs for each study group is presented in Fig. 4.

Concerning the DNA parsimony algorithm and the maximum likelihood method of the molecular clock is shown in Fig. 5 a, b. It compared 13 strains of the present study with two *T. b. gambiense*, one *T. b. brucei*, 3 *T. brucei* and 4 different strains from *T. evansi*. Three clades were produced: the first contained only the closely 1X (the original strain) and 2X (the drug-treated strain) with related identity, whereas the second showed identity between 4X, 8X that belong to A^{+2} and O^{+2}, respectively. The third clade contained other strains of treated groups of plant extracts, and the 10 reference *T. brucei* subspecies strains plus the closely related isolates 5X and 6X strains belong to B^{+} and AB^{+}.

Following alignment, genetic distance and phylogenetic analyses revealed a similar evolutionary history for *T. evansi* and *T. brucei* subspecies. The 6 unknowns (1X, 2X, 4X, 8X, 5X,

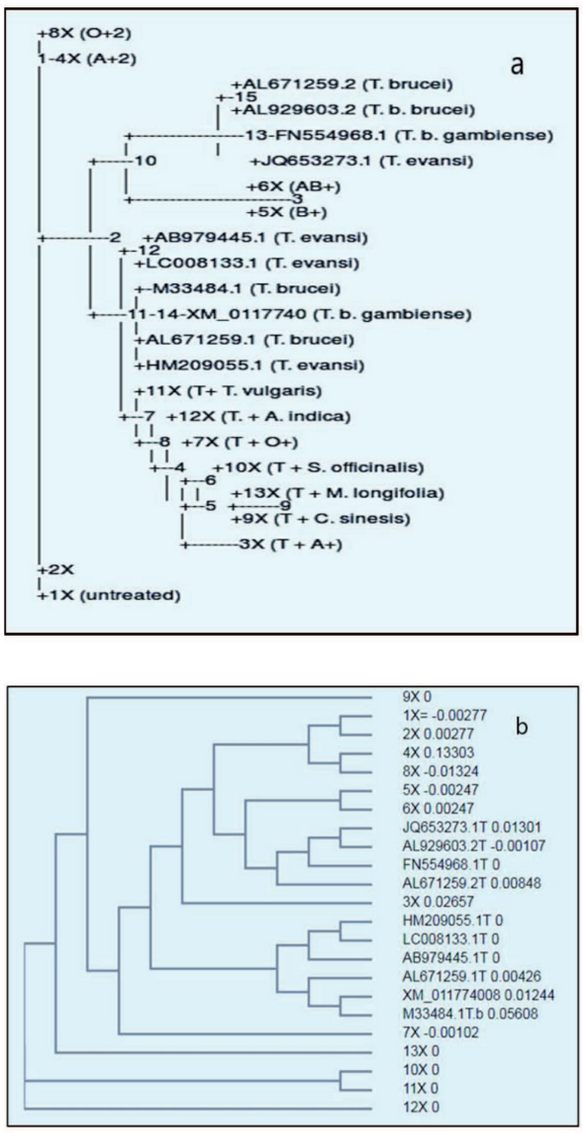

Figure 5 (a,b). Showing the phylogenetic relationship of submitted *T. evansi* strains with other *T. brucei* subspecies stains available in GenBank based on the neighbor-joining method and maximum likelihood trees. The GenBank accession numbers and distance are shown also and transition/ transversion ratio = 2.000000.

6X) are genetically most related to *T. evansi* JQ65 3273.1, *T. brucei* AL671259.1, *T. b. brucei* AL929 603.2, and *T. b. gambiense* FN554968.1 strains. Whereas, *T. evansi* type A: 3X, 7X strains form a monophyletic cluster with closely related *T. evansi* strain HM_209055.1, *T. b. gambiense* XM_011774 008, and *T. brucei* AL_671259.1 than *T. brucei* _M33484.1, *T. evansi* AB_979445.1, and *T. evansi* LC_008133.1. Moreover, the phylogenetic analysis of all 23 genomes from the *Trypanozoon* subgenus provides new insights into the origin of *T. evansi* and *T. brucei* and their relation to the different *T. brucei* spp. It also confirmed the distance relationship between them rather than the differences between reference *T. evansi* deposited in GenBank and that submitted after treatment.

Discussion

Trypanosoma evansi is the most widely distributed trypanosome species, affecting domestic animals, with high mortality and morbidity causing various types of trypanosomiases. It is similar to *T. brucei* multiplies for one or two weeks after infection and in the more advanced stages of the infection, they cross the meningeal barrier [28]. The differences in virulence and/or pathogenicity for *T. evansi* strains or isolates in experimentally infected rats were previously discussed, but a few studies reported this after treatment [11,28]. We previously evaluated and discussed the clinic-pathophysiological parameters and trypanocidal effects against *T. evansi* infection [13,14]. It supported the viability of human blood groups (except for AB^+) followed by ethanol leaf plant extract of *S. officinalis* has the highest antitrypanosomal activity compared to DA.

The current study carried out virulence and their related genotypes after treatment against *T. evansi* infection. Morphometric changes to *T. evansi* occurred after treatment and significant differences in parasitemia were observed between groups of rats despite their close evolutionary relationship. DA was clinically available, but the development of drug resistance was observed along the course of the infection after ten and twenty-seven days post-infection, whereas trypanosomes returned without changeable. The reappearance of the parasites in all treated-groups by the end of the experiment can be explained as the new trypanosomes have evolved different ways of accomplishing what essentially the same developmental transition is in agreement with [13,14].

On the other hand, the present study estimated different degrees of virulence between *T. evansi* isolates based on motility and mortality. This coincides with two recent studies [10,29] assessed three degrees of virulence in 17 strains of *T. evansi* without treatments collected from Surra endemic countries using Swiss white mice. The increase in longevity of plasma-treated rats compared to other groups agrees with the results of two recent studies [29,30] investigated the susceptibility of *T. evansi* to therapy with slight differences using human plasma in experimentally infected rats. Despite the

refractory resistance of rabbits and humans to infection with *T. evansi*, plasma from healthy rabbits used to increase the life expectancy and cure some of the treated rats [31].

The ability of rats to survive was related to the capacity of the various treatments to delay or limit the level of parasitemia related to immunological response [24]. The different strengths of signals produced by TR3/TR4-PCR in 13 strains appear to be in full agreement with the results of the parasitemia state at the time of trypanosomes isolation [32,33]. Notably, most African trypanosomes are lysed by the Apolipoprotein L1 in human serum except for *T. b. gambiense* and *T. b. rhodesiense*. This is because they express specific serum resistance proteins: (TgsGP) in *T. b. gambiense* glycoprotein and serum resistance-associated protein (SRA) in *T. b. rhodesiense* [27].

Although, *T. evansi* was not infectious to humans and the reference strains of *T. brucei* spp. do not express nor contain RoTat 1.2 VSG gene. *Trypanosoma brucei* subspecies came to a close relationship with our submitted strains of human plasma treated groups. This may be due to molecular changes occurring in *T. evansi* DNA, or the infecting trypanosome has diverged from the classic injected form as evidenced by sequencing analysis and alignment. Particularly, the parasitemia was low in these groups correlate with the parasitemia state of *T. brucei* subspecies previously reported [34].

In contrast, all strains of the plant extract treated groups were similar and behaved with a different selection of parasitemia, mortality, appearance, and longevity. We suggest that the treatment with plant extracts may knock down the trypanolytic activity. This allows the trypanosomes to avoid ApoL1-mediated immunity and consequently leads to an increase in the risk of contracting trypanosomes in agreement with [30]. Thus, the clustering of the investigated strains seems to be correlated to the type of treatment. Confirm this offering, many attempts have been made to cultivate different *T. evansi* isolates *in vitro,* but the parasite viability declines within a few days and substantial molecular changes were introduced accompanied by the complete loss of the kinetoplast [35]. Thus, parasite propagation is achieved via experimentally infected animals as reported by [36]. Hence, the experimental infection in rats in the present study was not responsible for the molecular changes, but the treatment was.

On the other hand, *T. b. gambiense*, *T. b. brucei* and their ancestor *T. brucei* dominate in sub-Saharan Africa [37], whereas *T. evansi* (cause of trypanosomosis) dominates mainly in North and East-Africa in two types A and B based on the presence, number, and homogeneity of minicircle [34]. We hypothesize that the small genetic variation between and within *T. evansi* and *T. brucei* spp. refer to *T. evansi* that could have emerged from a West African *T. brucei* ancestor. This is in line with the hypothesis of [38] who suggested that *T. evansi* type A has emerged from a single *T. brucei* strain that originates from West Africa.

In conclusion, the current study evaluated the genomic changes in *T. evansi* isolates used after being treated. It evaluated and discussed how *T. evansi* in some human plasma treated-groups become less virulent than other treated-groups due to genetically drifted from its original sequence of the nearest form of *T. b. gambiense*.

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