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By

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Abstract

Trypanosoma evansi is the most widespread of the pathogenic salivarian trypanosomes and cause a serious disease called (surra) that is affect the domestic animals such camels and horses in Tropical and subtropical countries and often leads to reduced productivity and economic losses. Therefore, the objectives of the present study were to determine the prevalence rates of trypanosomiasis using polymerase chain reaction (PCR) among camels from five different regions of Saudi Arabia and to sequence and characterized the *T. evansi* from these animals. In the current study, 832 camel blood samples collected from five different regions of Saudi Arabia for detecting *T. evansi*. A generic ITS1-PCR and RoTat 1.2 VSG gene were applied in this study to analyze camels' blood samples. Molecular analysis was performed using ITS1-PCR which showed that the highest prevalence of trypanosomes was observed in Al-Qaseem province (50.1%) followed Riyadh province (49%), whereas in Hail and the Northern Borders, there were fewer infections with trypanosomes (28.4% &17.6%), respectively. PCR amplification was carried out targeting RoTat 1.2 VSG gene on TS1-positive samples and some of them were negative for RoTat1.2. The test negative in RoTat 1.2 PCR but ITS1 PCR positive could suggest *T. evansi* type B. Presence of *T. evansi* type B is interest to the international community, as this has a message to redesign the existing molecular and serological diagnostic markers. However, to our knowledge this the first study demonstrating *T. evansi* type B out of Africa.

Keywords: *Trypanosoma evansi,* Camels, ITS1 rDNA , RoTat 1.2, PCR, Saudi Arabia.

Introduction

Trypanosoma evansi is a protozoan parasite of both intra and extra vascular fluids of mammals causing the disease surra throughout tropical and subtropical regions of the world (Luckins, 1988). It has a large diversity of mammalian hosts. It is transmitted mechanically by hematophagous flies (*Tabanus*, *Chrysops, Atylotus*, *Lyperosia*, *Haematopota* and *Stomoxys*) causing production losses, anemia, weight loss, abortion and is fatal in a range of domestic and wild species (Luckins and Dwinger, 2004; Desquesnes *et al*, 2013). However, the disease is still a serious problem in camel husbandry that causes significant economic losses in many camel farms worldwide causing reduction in meat and milk (Derakhshanfar *et al*, 2010). Nevertheless, camels are particularly susceptible to surra (OIE, 2010). Several parasito-

logical tests were applied for diagnosis of Surra such as haematological and microscopic examinations (Ravindran *et al*, 2008; Shyam *et al*, 2013), or serological tests such as the card agglutination test (CATT/*T. evansi*) (Songa and Hamers, 1988; Njiru, *et al*, 2004), enzyme-linked immunosorbent assay (ELISA) (Olaho-Mukani *et al*, 1996; Ngaira *et al*, 2003; Aslam *et al*, 2010), the latex agglutination test (Verloo e*t al*, 1998), the immune trypanolysis (TL) assay (Van Meirvenne *et al*, 1995), and immunofluorescence antibody test (IFAT) (Katende *et al*, 1987). However, these tests cannot differentiate between past and recent infections and lack sensitivity and specificity.

Polymerase chain reaction (PCR) assays appear to be a promising technique for the diagnosis of trypanosomal infection based on the detection of trypanosomal DNA in the blood samples due to its sensitivity and specificity in detecting all the stages of an infection (Clausen *et al*, 1998). Various target sequences such as ribosomal DNA, internal transcribed spacer region (ITS), kinetoplast DNA and VSG genes are reliable targets for detection of *T. evansi* (Urakawa *et al*, 2001; Verloo *et al*, 2001; Cox *et al*, 2005; Njiru *et al*, 2005; Sengupta *et al*, 2010; Salim *et al*, 2011; Birhanu *et al*, 2015; Sumbria *et al*, 2015; El Wathig *et al*, 2016; Tehseen *et al*, 2017).

In Saudi Arabia, the camel populations are estimated at nearly 1,356,7290 heads. Besides, thousands of live camels imported annually from neighboring countries such as Sudan, Somalia, Djibouti and Arabian Gulf countries (General Authority for Statistic, 2015). They are mainly used as a source of meat, milk, skin, leathers and load carrying, which is an important role in transportation in the desert and rural areas. However, there are lack of information on trypanosomiasis infections in Saudi animals particularly at the molecular levels and the most of the previous studies conducted in camels from Saudi Arabia were epidemiological, serological, biochemical and haematological studies (Kasim, 1984; Hussein *et al*, 1991; Omer *et al*, 1998; Al-Khalifa *et al*, 2009; Al-Afaleq *et al*, 2015; El Wathig *et al*, 2016).

The objectives of the present study were to determine the prevalence rates of trypanosomiasis using PCR among camels from five different regions of Saudi Arabia and to sequence and characterized the *T. evansi* from these animals.

Materials and Methods

Study areas: The investigation was conducted over a year from April 2016 to June 2017 in five different regions of Saudi Arabia; in the Central Region (Riyadh), the Eastern Province, Al-Qaseem Province, Hail and Northern Borders (Fig. 1).

Sampling and blood collection: 832 camels (271 males & 561 females) with ages 0≤4 year to >4 years old) were examined for trypanosomiasis. All animals were apparently clinically healthy at the time of blood collections. Blood samples collected from each animal (5-10ml) from jugular vein into vacutainer EDTA tubes (BD Vacutainer® Tube, Gribbles Pathology, VIC, Australia) and transported to Parasitological Laboratory, Department of Biological Sciences, Faculty of Science and Humanities, Shaqra University for DNA extraction.

DNA extraction: Total genomic DNA (gDNA) was isolated using the DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany) and eluted in 50µl or 100µl of elution buffer as per the manufacturer's instruction. An aliquot between 50µl-100µl of gDNA from each of the samples was stored at -80 °C prior to being sent to the Molecular Laboratory, School of Biological and Marine Sciences, Plymouth University for PCR analysis. At the Molecular Labora-tory, gDNA was stored at -20°C for up to a month prior to molecular diagnostics.

*Trypanosoma evansi PCR:*A Two-step PCR protocols were used to analyze *T. evansi* in DNA samples. In the first step, DNA samples were used to amplify the 250-270bp region of ITS gene using forward primer ITS1 CF (5'-CCGGAAGTTCACCG

ATATTG-3') and reverse primer ITS1 BR (5'-TGCTGCGTTCTTCAACGAA-3'), the primers are specific for parasites belonging to the genus *Trypanosoma* and amplifying a 250 bp, 400 bp,480bp & 700bp for region of ITS1 of *T. vivax, T. simiae, T. brucei* subspecies and *T. congolense* savannah, respecttively (Njiru *et al*, 2005). Then positive ITS1 *T. evansi* isolates were further subjected to PCR test specific for *T. evansi*, in which a primer set that amplifies 151bp of the *T. evansi* RoTat 1.2 VSG gene fragment was used (Konnai *et al*, 2009), this was TeRoTat920F (5'-CTGAAGAGGTTGGAAATG GAGAAG-3') and TeRoTat1070R (5'-GT TTCGGTGGTTCTGTTGTTGTTA-3'),and then all PCR reactions were performed in a final volume of 50µl containing 25µl of Dream Taq DNA polymerase Master Mix 2X (Thermo Scientific™, UK), 0.4µM (1µL) of each primer and 2µL of DNA template. The reaction was brought to 50µL total volume with PCR grade water (Invitrogen, UK). Positive and negative controls were included in all assays. Thermal cycling conditions for *Trypanosoma* species were consisted of an initial 2 min incubation at 95°C, followed by 40 cycles of denaturation at 95°C for 30 sec), primer annealing (58 °C for 30 sec and extension at 72°C for 1min. and a final extension step at 72°C for 5min and the samples held at 4°C. Aliquots of 10µL PCR product was electrophoresed on a 1.5% agarose gel containing 10µL/mL Syber safe (Thermo Scientific™, UK) in Tris-acetate–EDTA buffer at 100 V for 45 min and photographed under UV imaging system (ImagQunat Laz4000, GE Healthcare Life Science, UK). Size of each product was estimated by comparison with a Gene Ruler 100bp DNA Ladder Marker (Thermo Scientific™, UK).

Sequencing and phylogenetic trees analysis: To determine the *T. evansi*, positive samples were sent to Macrogen Europe (Netherlands) for sequencing ITS1 region using forward ITS1 CF and reverse ITS1 BR primers and the results were compared with the sequences available at GenBank data-base using BLAST (http://blast.ncbi.nlm.nih.gov/). Phylogenetic analysis was constructed by comparing identified sequence in this study with the related sequences from GeneBank using the neighbor-joining method with the distance algorithms available in the Molecular Evolutionary Genetics Analysis package (MEGA7).

Statistical analysis: Statistical analyses were performed with the statistics package SPSS (Version 17.0; IBM, New York, USA). Animals were divided into two age groups, animals up to 4 years (young) and animals more than 4 years old (mature). The association between the prevalence of *T. evansi* and risk factors such as location, sex and age was determined using the chi-square test of significant *P* value ($p < 0.05$).

Results

Based on ITS1-PCR results, the prevalence of trypanosomiasis *evansi* in the camels was 42.3% (Fig. 2). The PCR amplification was carried out targeting RoTat 1.2 VSG gene on TS1-positive samples and some of them were negative for RoTat1.2 (Fig. 3). The test negative in RoTat 1.2 PCR but ITS1 PCR positive could suggest *T. evansi* type B.

The phylogenetic data and blast results of ITS1 showed that *T. evansi* is closely related to *Trypanosome evansi* isolate SANSTEFANO-HS, accession number MG564285 isolated from tick infesting *Canis lupus familiaris* in Egypt (Fig. 4). The phylogenetic tree inferred from the ITS1 nucleotide sequences (490 bp) was unable to show inter- and intraspecific genetic diversity of the parasites. In the current study, the sequences of new isolates of ITS1 from positive *Trypanosoma evansi* isolated from Saudi camels deposited in the GenBank (National Center for Biotechnology Information, NCBI) database under accession numbers MH087230.

The highest prevalence of trypanosomosis was in Al-Qaseem Province (50.1%) followed by Riyadh Province (49%), whereas in Hail and the Northern Borders, there were

fewer infections (28.4% &17.6%), respectively (Tab. 1). The results show that female camels to be the most infected with *T. evansi* (47%), while male camels infected with *T. evansi* (32.5%). Most *T. evansi* infections were recorded in camels over four years old (49.1%), while infections in young camels constituted (30.5%). The results show no significant effects of age and sex on the prevalence of *T. evansi* in camels (p=0.333 and 0.333), respectively.

Table 1: Number and prevalence of Trypanosomiasis in camels.

Factor		No of Camels	Trypanosomiasis	Trypanosomiasis	P-value
		examined	Positive	Negative	
Sex	Male	271	88 (32.5%)	183 (67.5%)	0. 333
	Female	561	264 (47%)	297 (53%)	
	Total	832			
Age	< 4 years	305	93 (30.5%)	212 (69.5%)	0.333
	> 4 Years	527	259 (49.1%)	268 (50.9%)	
	Total	832	352 (42.3%)	480 (57.7%)	
P > 0.05 = Non significant					

Table 2: Effect of sex and age factors on prevalence of trypanosomiasis in camels.

Location	No of Camels	Trypanosomiasis	Trypanosomiasis	P-value
	examined	Positive	Negative	0.00000
Riyadh	237	116 (49%)	121 (51%)	
Eastern province	221	106 (48%)	115(52%)	
Al-Qaseem	156	79 (50.1%)	75 (49.4%)	
Hail	116	33(28.4%)	83 (71.6%)	
Northern Boarders	102	18 (17.6%)	84 (82.4%)	
Total	832	352 (42.3%)	480 (57.7%)	
P < 0.05 =significant				

Discussion

In an attempt to explore the status of trypanosomosis in Saudi Arabia and the *T. evansi* that causes it, we carried out this study and applied the generic ITS1-PCR test to analyze 832 samples collected from camels in five regions of Saudi Arabia. The PCR test is increasingly used in surveys to study trypanosomosis in different countries (Njiru *et al*, 2005; Ravindran *et al*, 2008; Bhutto *et al*, 2010; Salim *et al*, 2011; Elhaig *et al*, 2013; Hussain *et al*, 2016; Tehseen *et al*, 2017). However, this method has drawbacks on field, DNA samples have a problem of specificity not sensitivity of the ITS1 CF and BR primers, which range between 10pg (100 trypanosomes) for Trypanozoon, *T. vivax* and *T. congolense* clades to 100pg (1000 trypanosomes) for *T. simiae* and *T. godfreyi* (Njiru *et al*, 2005). It also cannot differentiate between the different species of *T. brucei* subgroup at its sub-species level. Based in ITS1 region, Njiru *et al*. (2005) and Cox *et al*. (2005) documented specific PCR product length corresponding to each *Trypanosoma* species, which was the base of differentiation among *Trypanosoma* species. For example, the region of ITS1 PCR product size is 250bp, 400bp, 480bp and 700bp for *T. vivax, T. simiae, T. brucei* subspecies and *T. congolense* savannah, respectively. The variable surface glycoprotein of trypanosomes RoTat 1.2 VSG is a predominant variant antigen supposed to be expressed in all *T. evansi* examined so far (Verloo *et al*, 2001). Then, the low positive numbers of *T. evansi* detected based on RoTat 1.2 VSG gene compared with other PCR targets in this study agreed with previous studies (Njiru *et al*, 2005; Salim *et al*, 2011; Elhaig *et al*, 2013; Barghash *et al*, 2014; Tehseen *et al*, 2015). Nevertheless, mixed infection by *T. evansi* type A and type B is also possible since they identified from Kenya (Ngaira e*t al*, 2004; Njiru *et al*, 2006), Sudan (Salim *et al*, 2011) and Ethiopia (Fikru *et al*, 2015; Birhanu *et al*, 2016) .This study reported for the first time *T. evansi* type B out of Africa and could be the reason related to exporting livestock from east Africa particularly from

the Sudan to Saudi Arabia.

Historically, camel trypanosomiasis have spread to Saudi Arabia from the neighboring countries since it has been reported in all the countries bordering Saudi Arabia (Boid *et al,* 1985). However, the present study is the first molecular survey nationwide in Saudi Arabia that showed that the prevalence rate of *T. evansi* detected by PCR was 42.3 % in camels. This rate is higher than those reported in other studies in different locations in Saudi Arabia. For example, El Wathig *et al.* (2016) examined 195 camels from Al-Jouf Province, Northern part of Saudi Arabia, and they found that 25% of camels were infected with *T. evansi.* Other studies on the *T. evansi* infection in camels were microscopy or serological studies. For example, Kaism (1984) reported an overall incidence of 2% of camel trypanosomiasis in Saudi Arabia by using the direct stained thin blood smear examination technique. However, a higher incidence rate of 13.2% was detected (Hussain *et al,* 1991). Omer *et al*. (1998) reported a prevalence of 5.5% among camels in Al-Qaseem region. Al-Khalifa *et al.* (2009) documented the incidence rate of *T. evansi* in camels from 0 to 40% in different regions in Saudi Arabia. In contrast, Afaleq et al., (2015) reported the prevalence of *T. evansi* infection among camels in different regions of Saudi Arabia was 0.8%. Serologically, Omer *et al*. (1998) found that 19.7% of the sampled camels in Al-Qaseem region were positive for trypanosomes by the passive hemagglutination test and 13.8% by Ag-ELISA. But, El-Wathig and Faye (2013) and Afaleq *et al*. (2015) reported that the prevalence of *T. evansi,* using CATT/*T. evansi* test was 43.8% and 39.4% respectively.

Studies in neighboring countries also recorded different results. For example, in Iraq, Al-Amery et al., (2017) recorded the prevalence of *Trypanosoma* spp. by blood smears as 31.87 %, Al-Rawashdeh *et al*. (2000) reported that *Trypanosoma* was 33% in Jordan. In Egypt, studies showed that *Trypanosoma* spp. prevalence was 4.5% -74.4% (Abdel-Rady 2006; Zayed *et al*, 2010; Elhaig *et al*, 2013; Barghash *et al*, 2014). On the other hand, Haridy *et al*. (2011) stated that the mortality rate was up to 20% and fatality rate might reach up to 100% in untreated camels and reported the first Egyptian human case which was successfully treated as indicated clinically, parasitologically and serologically. In Sudan, *Trypanosoma* spp prevalence rate and infection pattern were also performed with varied estimates of prevalence at 5.4% using parasitological examination and 1.3% with ELISA (Elamin *et al*, 1998). The overall prevalence by using molecular epidemiological tools ranged from 33.9 to 42.1%, respectively (Salim *et al*, 2011). The differences might be attributed to the differences of the management and the environment in which these studies were performed, sampling size of tested animals or methods of PCR or microscopic and serological methods that used, animals natural immunity and local climatic conditions that had an effect on tabanid flies distribution (Al-Khalifa *et al*, 2008).

The present results showed that the pattern of prevalence of *T. evansi* in camels differed according to the different locations because of the variations of the environment conditions. The data agreed with others in Saudi Arabia (Al-Khalifa *et al*, 2009; El Wathig *et al*, 2016) and other countries (Barghash *et al*, 2014; Tehseen *et al*, 2015). The environment conditions favor the distribution of biting flies vectors of *T. evansi* (Schillinger and Rottcher, 1986).

The current results showed no significant effect of sex where by female were more infected than males (P>0.333). These data agreed with studies from Nigeria (Joshua *et al*, 2008), Egypt (Barghash *et al*, 2014; El-Naga and Barghash, 2016), Pakistan (theseen *et al*, 2015). But, disagreed with Bogale *et al.* (2012); Hussain *et al.* (2016) and Al-Amery *et al.* (2017) who reported that male camels was more infected than females in Ethiopia, Pakistan and Iraq, respectively. The higher prevalence in females in this

study might be due to the fact all samples were from female camels (67.4%). Another reason could be due to pregnancy. A pregnant female with weak immunity that led to reduce resistance compared to male camels (Bhutto *et al*, 2010).

In addition, the present results showed no significant effect of age on the rate of *T. evansi* infection (P>0.333) and that most *T. evansi* infections were present among animals with ages equal to or more than four years. These results agreed with previous studies (Diall *et al*, 1993; Bogale *et al*, 2012; Tehseen *et al*, 2015; Al-Amery *et al*, 2017). The high infection rate in adult camels could be attributed to several factors such as the movements and travel of camels where the fly burden is high, poor management, stress, drought, poor silage and preference by biting flies (Njiru *et al*, 2004; Bhutto *et al*, 2010; Tehseen *et al*, 2015).

Conclusion

The outcome data showed that PCR is a more specific, sensitive and reliable tool for the detection of *T. evansi* in camel blood samples than the other methods used previously in Saudi Arabia. This study showed that only *T. evansi* was detected in camels in Saudi Arabia and no other trypanosomes were noticed. Also, some samples tested negative by RoTat 1.2 VSG-PCR and positive by ITS1-PCR. Therefore, ITS1-PCR is recommended for general trypanosomiasis detection but no specific for *T. evansi.*

Competing interests: The authors declared that they neither had competing interests nor received fund

Authors' contributions: ADA and PR developed the study design. MS and MSA coordinated the study design and the related activities. ADA, IOA, SAA and ZSA carried out the field and laboratory investigations of all the experiments. AES and IOA, carried out the statistical analysis, PR and ADA analysis phylogenetic tress. ADA, HIA and PR prepared and revised the manuscript. All authors read and approved the final manuscript.

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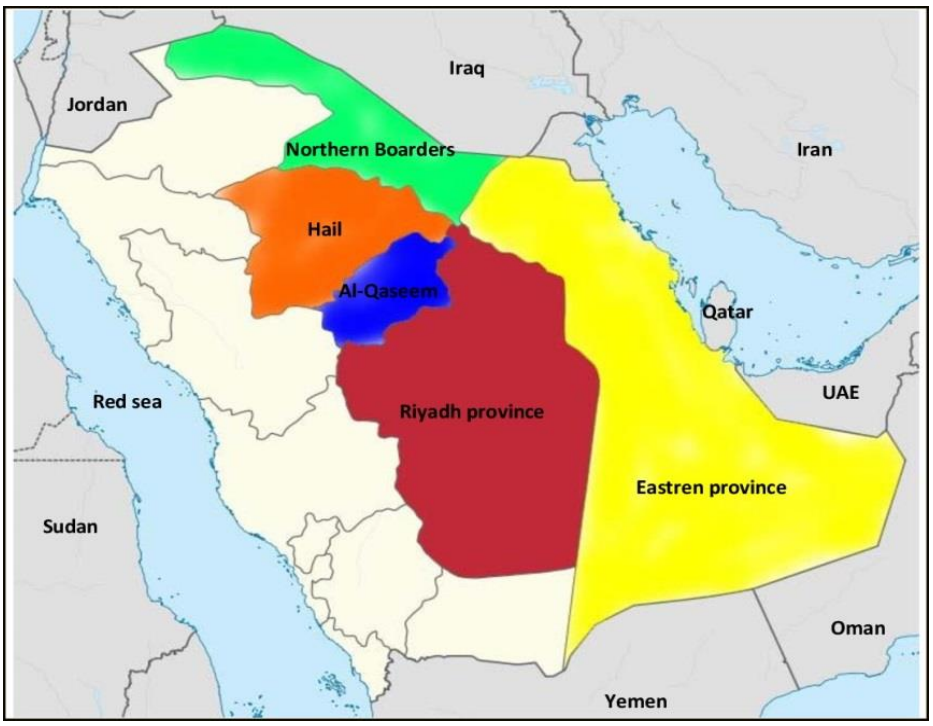
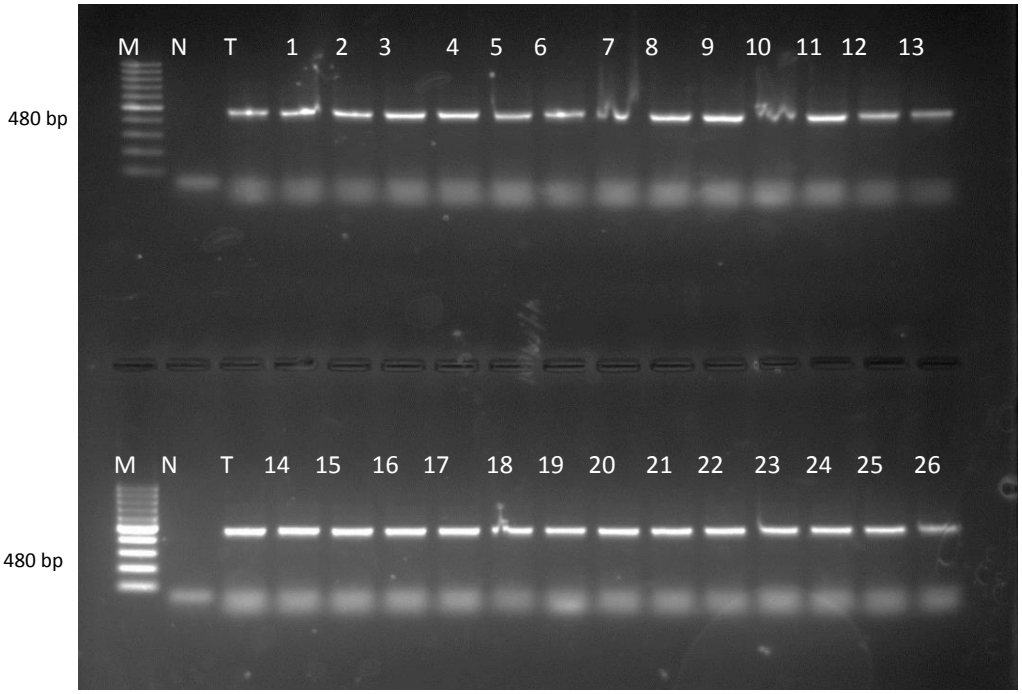
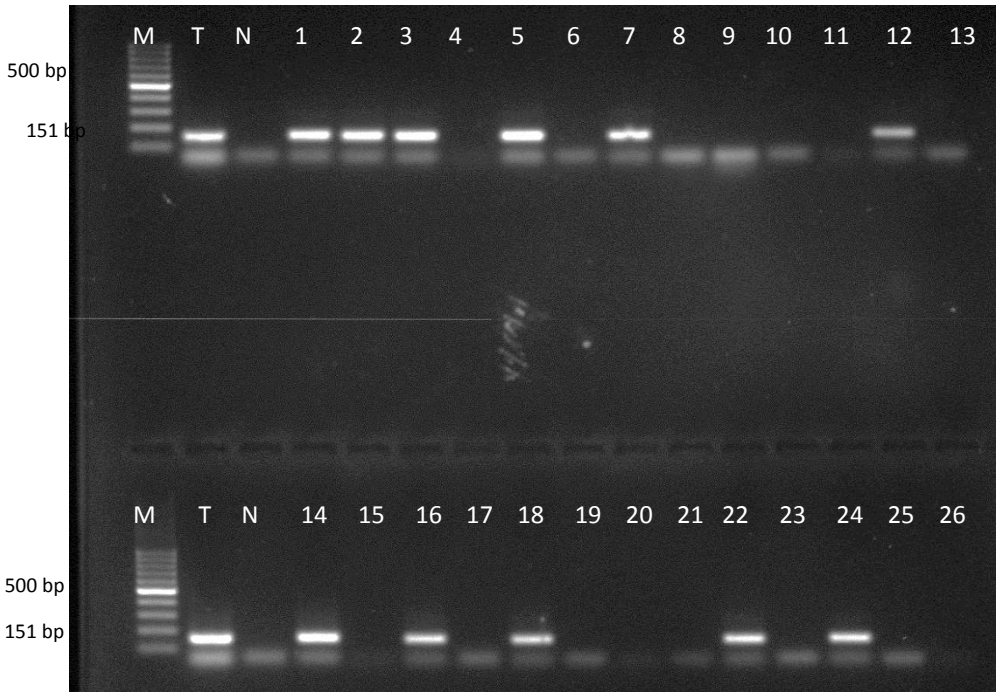
Explanation of figures

Fig. 1: Map showing the location of the study areas in Saudi Arabia.
Fig. 2: Agarose gel electrophoresis (1.7%) of amplified DNA from collected samples using ITS1 CF / ITS1 BR. M, 100 bp molecular size marker (Generuler); Lane N, negative PCR control (water); T. *T. evansi*-positive control DNA; lanes 1-26 template DNA isolated from camels blood sample from different locations.
Fig. 3: Agarose gel electrophoresis (1.7%) of amplified RoTat 1.2 VSG gene of *T. evansi* using specific primers TeRoTat920F and TeRoTat1070R. M, 100 bp molecular size marker (Generuler); Lane T.e , *T. evansi*-positive control DNA; N, negative PCR control (water); Samples (1-26) shown here from geographically different areas; Lane: 1 and 6 from Riyadh ; Lane; 7-13 from Eastern province; Lane: 14 and 18 from Al-Qaseem province; Lane: 19-23 from Hail; Lane: 24-26 from Northern Borders. Arrows indicate 151 bp amplicons specifically generated using TeRoTat920F and TeRoTat1070R primer set.
Fig. 4: Phylogenetic relationships of Saudi isolates of *Trypanosoma* parasites with other salivarian trypanosomes based on ITS 1 region of rRNA. GenBank accession numbers given in parentheses, Evolutionary history inferred by using Maximum Likelihood method based on Tamura-Nei model. Evolutionary analyses were conducted in MEGA7.



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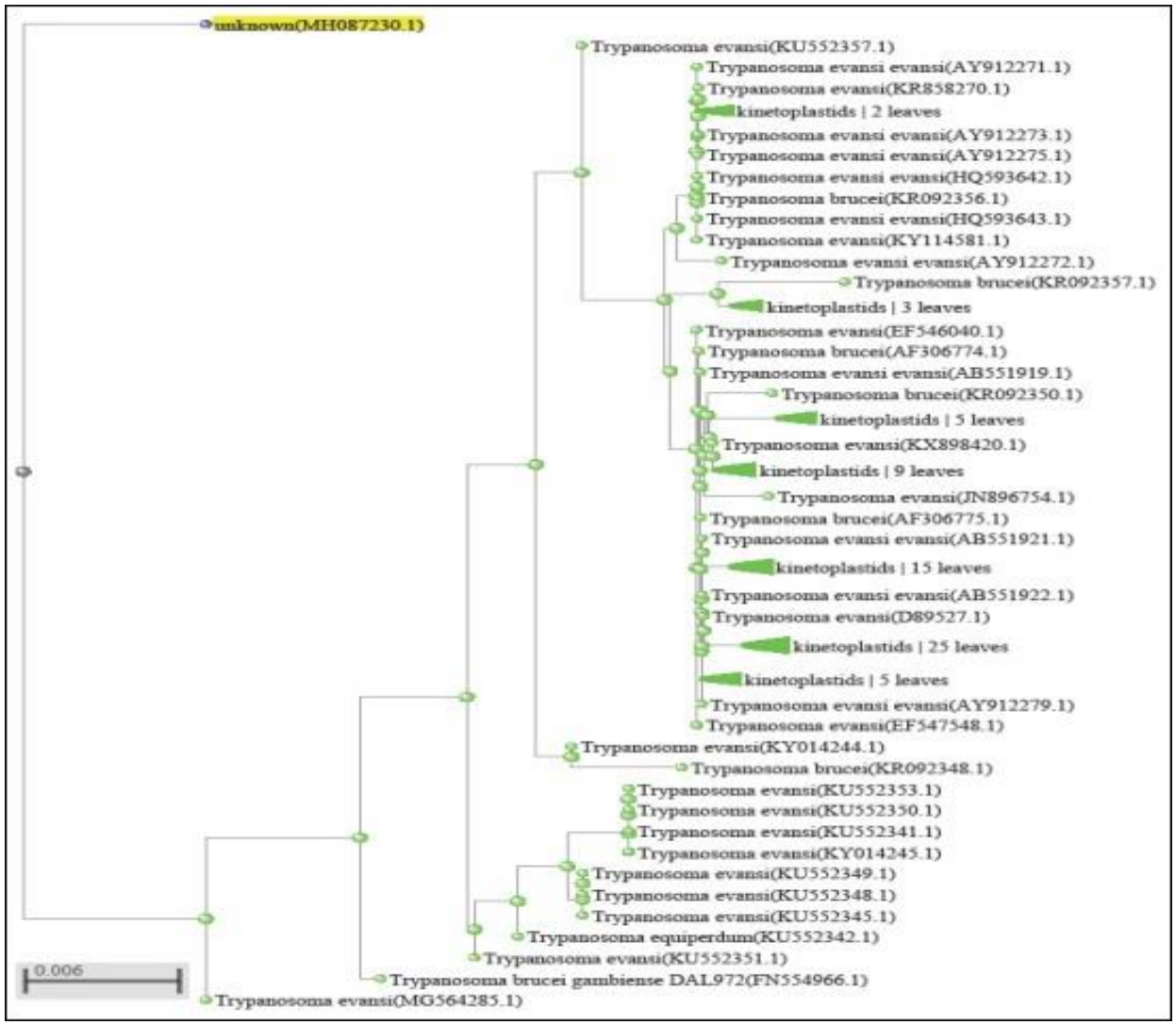
151 bp
M T N 14 15 16 17 18 19 20 21 22 23 24 25 26
500 bp
151 bp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Trypanosoma evansi(KU552357.1)
Trypanosoma evansi evansi(AY912271.1)
Trypanosoma evansi(KR858270.1)
kinetoplastids | 2 leaves
Trypanosoma evansi evansi(AY912273.1)
Trypanosoma evansi evansi(AY912275.1)
Trypanosoma evansi evansi(HQ593642.1)
Trypanosoma brucei(KR092356.1)
Trypanosoma evansi evansi(HQ593643.1)
Trypanosoma evansi(KY114581.1)
Trypanosoma evansi evansi(AY912272.1)
Trypanosoma brucei(KR092357.1)
kinetoplastids | 3 leaves
Trypanosoma evansi(EF546040.1)
Trypanosoma brucei(AF306774.1)
Trypanosoma evansi evansi(AB551919.1)
Trypanosoma brucei(KR092350.1)
kinetoplastids | 5 leaves
Trypanosoma evansi(KX898420.1)
kinetoplastids | 9 leaves
Trypanosoma evansi(JN896754.1)
Trypanosoma brucei(AF306775.1)
Trypanosoma evansi evansi(AB551921.1)
kinetoplastids | 15 leaves
Trypanosoma evansi evansi(AB551922.1)
Trypanosoma evansi(D89527.1)
kinetoplastids | 25 leaves
kinetoplastids | 5 leaves
Trypanosoma evansi evansi(AY912279.1)
Trypanosoma evansi(EF547548.1)
Trypanosoma evansi(KY014244.1)
Trypanosoma brucei(KR092348.1)
Trypanosoma evansi(KU552353.1)
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0.006