



Serological and molecular detection of selected hemoprotozoan parasites in donkeys in West Omdurman, Khartoum State, Sudan

Afraa ELATA^{1,2)}, Ehab MOSSAAD³⁾, Rawan SATTI³⁾, Nadia MATAR³⁾, Yuma OHARI¹⁾, Xuenan XUAN¹⁾, Noboru INOUE⁴⁾ and Keisuke SUGANUMA^{1)*}

¹⁾National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Inada, Obihiro, Hokkaido 080-8555, Japan

²⁾Department of Preventive Medicine and Public Health, Faculty of Veterinary Medicine, University of Khartoum, Khartoum, 13314, Sudan

³⁾Department of Pathology, Parasitology and Microbiology, College of Veterinary Medicine, Sudan University of Science and Technology, Khartoum, P.O. Box 204, Sudan

⁴⁾Obihiro University of Agriculture and Veterinary Medicine, Inada, Obihiro, Hokkaido 080-8555, Japan

ABSTRACT. In Sudan, donkeys are important animals, providing transportation and income possibilities. However, the prevalence of parasites in donkeys in Sudan has not been thoroughly characterized. Accordingly, in this study, we aimed to detect selected hemoprotozoan parasites in donkeys in West Omdurman, Khartoum State, Sudan, wherein people depend mainly on donkeys for their daily life. In total, 198 blood samples collected from donkeys in a local market in West Omdurman, were screened using serological and molecular diagnostic techniques. Serologically, 52 (26.3%), 56 (28.3%), and 19 (9.6%) samples were positive for trypanosomosis using Card Agglutination Test for *Trypanosoma evansi*, *Trypanosoma evansi* crude antigen-based enzyme-linked immuno sorbent assay (ELISA) and recombinant *Trypanosoma evansi* GM6-4r-based ELISA, respectively. ELISA for equine piroplasmosis revealed 156 (78.8%) and 10 (5.1%) *Theileria equi*- and *Babesia caballi*-positive samples, respectively. PCR detected *Trypanosoma congolense*, subgenus *Trypanozoon*, *Theileria equi*, and *Babesia caballi* in 18 (9.1%), 77 (38.9%), 18 (9.1%), and 8 (4%) samples, respectively. Of the 77 *Trypanozoon*-positive samples, 35 (45.5%) were confirmed as *Trypanosoma evansi* type A. To our knowledge, this is the first report of detection of *Trypanosoma congolense* in donkeys outside of tsetse-infested areas in Sudan.

KEY WORDS: donkey, piroplasmosis, Sudan, trypanosomosis

J. Vet. Med. Sci.

82(3): 286–293, 2020

doi: 10.1292/jvms.19-0534

Received: 25 September 2019

Accepted: 10 January 2020

Advanced Epub:

22 January 2020

The donkey (*Equus asinus*) is believed to have originated from Africa and was domesticated from the African wild ass (*Equus africanus*) in Nubia (Northern Sudan) by around 4000 BC [9]. The widespread use of donkeys in rural and urban areas of Africa for transportation and farm work indicates that this animal has an important role in the economies of many developing countries, including Sudan [13]. As home to approximately 7.51 million donkeys, Sudan has the fifth largest donkey population in Africa [12, 30, 42]. These donkeys have a significant presence in agriculture and transport operations in urban areas and in the outskirts of the cities, such as in West Omdurman, where the poor use donkeys as a mean of transportation and source of daily income [47]. Donkeys have often been described as sturdy animals; thus, there is a perception that they do not require much care when they get sick, and their health problems are neglected in most parts of Sudan despite their huge contribution to the improvement of human life [40]. However, donkeys are susceptible to a variety of diseases and are usually asymptomatic carriers [3].

Trypanosomosis (TR) and equine piroplasmosis (EP) are two of the most important hemoprotozoan parasitic diseases in equines. TR is caused by unicellular flagellar protozoa of the genus *Trypanosoma* in the family *Trypanosomatidae*; these organisms live and multiply in the blood and other tissue fluids [4]. The severity of the disease depends on the *Trypanosoma* species and the virulence of individual isolates. Surra and dourine are caused by *Tr. (Trypanozoon) evansi* and *Tr. (Trypanozoon) equiperdum*, respectively, which are widely distributed around the world. *Tr. (Trypanozoon) brucei*, *Tr. (Nannomonas) congolense*, and *Tr. (Duttonella) vivax* are the causative agents of nagana, the tsetse-transmitted trypanosomosis, which occurs in an area of 10 million km² in 37 African

*Correspondence to: Suganuma, K.: k.suganuma@obihiro.ac.jp

(Supplementary material: refer to PMC <https://www.ncbi.nlm.nih.gov/pmc/journals/2350/>)

©2020 The Japanese Society of Veterinary Science



This is an open-access article distributed under the terms of the Creative Commons Attribution Non-Commercial No Derivatives (by-nc-nd) License. (CC-BY-NC-ND 4.0: <https://creativecommons.org/licenses/by-nc-nd/4.0/>)

countries, where tsetse flies live [19]. Donkeys seem to have the greatest resistance to tsetse-transmitted trypanosomosis among equids, and the disease has become a clinical problem when accompanied by precipitating factors, such as the stress of work [47]. In Sudan, in early 1915, trypanosomes were found to cause TR in a group of equines, resulting in 100% mortality, owing to their use as transport animals in tsetse-infested areas [46]. The parasite causing this outbreak was identical to *Tr. brucei*. Thereafter, in the 1930s, *Tr. congolense* was identified as the causative agent of the disease in horses from tsetse-infested areas in Sudan [6]. Infection with mechanically-transmitted *Tr. evansi* was first diagnosed in horses in 1952 [11]. Dourine, a type of sexually transmitted trypanosomosis caused by *Tr. equiperdum*, was identified in a donkey mare in Nyala, Western Sudan in 1961 [44] although World Organization for Animal Health (OIE) data indicate that this disease has never been recorded in Sudan [30]. Since the first report, the disease has not been reported again. Recently, *Tr. brucei* subspecies, *Tr. vivax*, *Tr. simiae*, and *Tr. congolense* have been reported in equines in Sudan [37]. Although TR is usually reported in veterinary clinics in Sudan, its epidemiology is still unclear, particularly in donkeys. Importantly, TR can contribute to a reduction in the strength and survival of donkeys [43]. Moreover, one report described a significant association between trypanosome infection and mean body condition score in donkeys [24].

EP is a hemoprotezoan disease of equids caused by two intra-erythrocytic protozoa of the genera *Theileria* (*Theileria equi*) and *Babesia* (*Babesia caballi*) [32]. EP can occur as acute, subacute, or chronic disease [14]. EP has a worldwide distribution and is endemic in most tropical and subtropical zones and in some temperate climatic zones [7]. This disease is transmitted by ticks or mechanically by improperly sterilized instruments, such as syringes and needles. Moreover, EP is an OIE notifiable disease in Sudan [47]. EP was first reported in Sudan in 1907 as biliary fever, with the conclusion that *Th. equi* was more prevalent than *B. caballi* [31, 38]. Recent studies have reported the occurrence of EP in different parts of Sudan [36]. Microscopic examination of Giemsa-stained blood smears for detection and identification of EP- and TR-causative protozoa is of low sensitivity, particularly in cases with low parasitemia [18, 23, 39]. Thus, serological and molecular techniques have been shown to be more accurate diagnostic methods for detection of EP [33] and TR [15].

Previous studies on EP in Sudan did not include donkeys from Khartoum State [36], and few donkeys from Khartoum North were included in another study on TR in Sudan [37]. Therefore, we conducted this study to provide an update on the prevalence of TR and EP in donkeys in West Omdurman, Khartoum State, Sudan by utilizing serological and molecular diagnostic techniques.

MATERIALS AND METHODS

Study area and sample collection

Samples were obtained from 198 donkeys in a local market in West Omdurman, Khartoum State (Fig. 1), after obtaining consent from the donkey owners. Apparently healthy donkeys, that did not present with typical symptoms or health complaints as indicated by their owners, were selected randomly for sampling. Briefly, 8 ml of blood was drawn from the jugular vein; 3 ml was stored in vacutainer tubes with EDTA (Terumo, Tokyo, Japan) for DNA extraction, and 5 ml was stored in plain vacutainers (Terumo) for serum separation. Sera were separated by centrifugation into 1.5-ml tubes and kept at -20°C until use. Genomic DNA of each sample was extracted from whole blood after loading onto WhatmanTM FTATM Elute Cards (GE Healthcare, Chicago, IL, USA), according to the manufacturer's instructions. Permission for this study was obtained according to the standards of animal experimentation at Obihiro University of Agriculture and Veterinary Medicine (Approval No. 29-2, 18-18, 19-19).

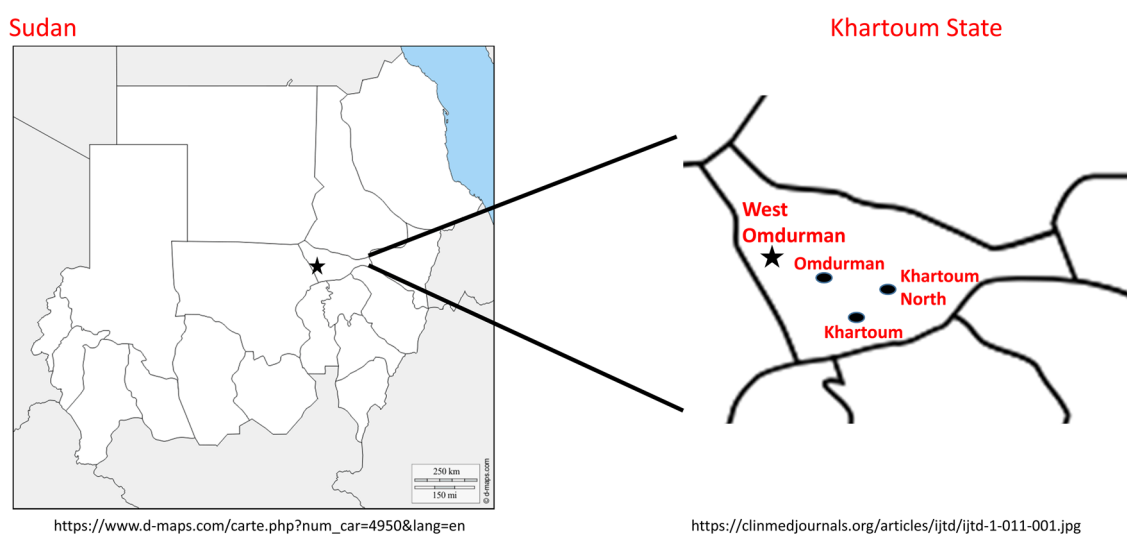


Fig. 1. Map of Sudan showing the sampling location in West Omdurman, Omdurman city, Khartoum State.

Card Agglutination test for *Trypanosoma evansi* (CATT/ *Tr. evansi*)

CATT/ *Tr. evansi* was used for the detection of anti-salivarian trypanosomes antibodies in serum samples according to the manufacturer's instructions (Institute of Tropical Medicine, Antwerp, Belgium) and the OIE manual [29]. Briefly, 25 μ l of serum (diluted 1:4 with CATT diluent) was dispensed onto the reaction zone of a plastic test card. One drop (approximately 45 μ l) of CATT reagent was added to the serum, and the mixture was then spread using a stirring rod and allowed to react on a CATT rotator (Institute of Tropical Medicine) for 5 min at 70 rpm. A sample was considered positive when blue agglutinates were visible [5, 45].

Enzyme-linked immunosorbent assay (ELISA)

All serum samples were tested for TR antibodies with recombinant *Tr. evansi* GM6-based ELISA (rTeGM6-4r- ELISA) and *Tr. evansi* crude antigen-based ELISA (TeCA-ELISA). The rTeGM6-4r antigen was produced, and ELISA was conducted as described previously [27]. *Tr. evansi* cell lysate crude antigen (TeCA) was prepared according to the OIE manual [29], and ELISA was conducted as described previously [27]. For EP, merozoite antigen 2 (EMA-2)- and 48-kDa merozoite rhoptry protein (BC48)-based ELISAs were performed as described previously [16, 25] for detection of *Th. equi* and *B. caballi*, respectively.

The optical density (OD) was assessed at 5 min after the addition of the substrate and immediately after stopping the reaction by measuring the OD₄₅₀ using a Glomax Multi detection system microplate reader (Promega, Madison, WI, USA). Cutoff values (mean \pm 3 standard deviations) for rTeGM6-4r-, TeCA-, EMA-2-, and BC48-based ELISAs were calculated from the OD values of four negative control sera from healthy donkeys, that were commercially obtained (Sigma-Aldrich, St. Louis, MO, USA; Jackson Immuno Research Laboratories, West Grove, Inc., PA, USA; Southern Biotech, Birmingham, AL, USA; Immuno Bio Science Corp., Mukilteo, WA, USA).

Polymerase chain reaction (PCR)-based identification of the parasites

Two different PCR techniques were employed to detect and identify trypanosomes DNA in donkeys, (i) single-step internal transcribed spacer-1 (ITS1)-PCR, which amplifies ITS1 and allows for the simultaneous detection of three major trypanosome species (*Trypanozoon*, *Tr. congolense*, and *Tr. vivax*) [28]; and (ii) RoTat1.2 variable surface glycoprotein (VSG) PCR (*Tr. evansi* type A-specific), which specifically amplifies the RoTat1.2 VSG gene encoding the VSG in *Tr. evansi* type A [8]. All primers sequences used in PCR in this study are listed in Table 1.

ITS1-PCR was performed in a total reaction volume of 10 μ l, including 2 μ l of 5 \times Phusion[®] HF reaction buffer (1.5 mM MgCl₂ was included in the final concentration), 0.8 μ l of 250 μ M dNTPs, 0.1 μ l Phusion[®] DNA polymerase (New England BioLabs, Japan Inc., Tokyo, Japan), 1 μ l each of 10 μ M forward and reverse primers, 5.1 μ l double-distilled water, and 1 μ l DNA sample. The PCR conditions were as follows: an initial denaturation step at 98°C for 30 sec; followed by 35 cycles of amplification with denaturation at 98°C for 10 sec, annealing at 64°C for 30 sec and extension at 72°C for 30 sec and a final extension step at 72°C for 2 min.

RoTat1.2 VSG PCR was also performed in a total reaction volume of 10 μ l containing 1 μ l of 10 \times reaction buffer, 0.3 μ l of 50 mM magnesium chloride, 0.8 μ l of 250 μ M dNTPs, 0.1 μ l Taq DNA polymerase (Invitrogen, Thermo Fisher Scientific Inc., Waltham, MA, USA), 0.5 μ l each of 10 mM forward and reverse primers, 5.8 μ l double-distilled water, and 1 μ l DNA sample. The PCR conditions were as follows: an initial denaturation step at 94°C for 3 min; followed by 40 cycles of amplification with denaturation at 94°C for 30 sec, annealing at 57°C for 30 sec and extension at 72°C for 60 sec and a final extension step at 72°C for 10 min.

Two more PCR techniques were employed to detect and identify piroplasms in donkeys (i) EMA-1 PCR, which specifically amplified the gene encoding *Th. equi* merozoite antigen 1 [2]; and (ii) BC48-PCR, which amplified the gene encoding 48-kDa rhoptry protein of *B. caballi* [2]. All primers sequences used in PCR in this study are listed in Table 1.

All reactions were carried out in a Veriti[™] thermal cycler (Thermo Fisher Scientific). The PCR products were electrophoresed on 2% agarose gels, stained with ethidium bromide, and visualized under ultraviolet light.

Table 1. PCR techniques and primers used for detection of trypanosome and piroplasm DNA in donkeys

PCR	Target gene	Primers	Sequence 5'-3'	Specificity	Size (bp)	References
ITS 1	ITS1	ITS1CF	CCGGAAGTTCACCGATATTG	<i>Trypanozoon</i>	480	[28]
		ITS1BR	TTGCTGCGTTCTTCAACGAA	<i>Tr. congolense</i>	620–700	
				<i>Tr. vivax</i>	250	
RoTat 1.2	RoTat 1.2 VSG	RoTat 1.2F RoTat 1.2R	GCGGGGTGTTTAAAGCAATA ATTAGTGCTGCGTGTGTTTCG	<i>Tr. evansi</i> (type A)	205	[8]
<i>Theileria equi</i>	EMA-1	EMA-1F EMA-1R	GCATCCATTGCCATTTTCGAG TGCGCCATAGACGGAGAAGC	<i>Th. equi</i>	750	[2]
<i>Babesia caballi</i>	BC48	BC48F BC48R	GGCTCCCAGCGACTCTGTGG CTTAAGTGCCCTCTTGATGC	<i>B. caballi</i>	610	[2]

DNA cloning and sequencing

The ITS1, RoTat1.2 VSG, EMA-1 and BC48 amplicons were extracted from agarose gels using a QIAamp gel extraction kit (Qiagen, Hilden, Germany), cloned, and transformed into chemically competent *Escherichia coli* (One Shot Mach1; Thermo Fisher Scientific) using Zero Blunt TOPO (for ITS1) and TOPO TA (for RoTat1.2 VSG, EMA-1 and BC48) cloning kits (Thermo Fisher Scientific), respectively, according to the manufacturer's instructions. After checking several colonies for each PCR cloned product by colony PCR, 18, 7, 4 and 1 positive clones of ITS1, RoTat1.2 VSG, EMA-1 and BC48, respectively, were selected for plasmid DNA purification using a QIAprep Spin Miniprep kit (Qiagen). Approximately 100–200 ng/ μ l pure plasmid DNA was used for sequencing with a Big Dye Terminator kit (Thermo Fisher Scientific). The sequencing PCR conditions consisted of an initial denaturation step at 96°C for 1 min, followed by 25 cycles of amplification with denaturation at 96°C for 10 sec, annealing at 50°C for 5 sec and extension at 60°C for 2 min. The PCR products were ethanol-precipitated and dissolved in 20 μ l Hi-Di formamide solution before DNA sequencing.

Sequencing analysis

The ITS1 sequences of *Trypanozoon* and *Tr. congolense*, the RoTat1.2 VSG, the EMA-1 and the BC48 sequences obtained from donkey DNA samples were aligned with published sequences from the GenBank database using the Basic Local Alignment Search Tool (BLAST), and the phylogenetic trees were constructed using the maximum likelihood method implemented in the Mega software program (version 7). All sequences generated in this study were deposited in the GenBank database under accession numbers LC492114–LC492131, LC493166–LC493172 and LC514705–LC514709.

RESULTS

Seroprevalence of hemoprotozoan parasites

CATT/ *Tr. evansi*: Of the 198 donkeys investigated in this study, anti-salivarian trypanosomes antibodies were detected in 52 (26.3%) samples using CATT/ *Tr. evansi*.

ELISA: Cutoff values for rTeGM6-4r-, TeCA-, EMA-2-, and BC48-based ELISAs were calculated from the OD values of four negative control sera, yielding values of 0.30, 0.56, 0.69, and 1.27, respectively (Fig. 2). Notably, of the 198 donkeys investigated in this study, 19 (9.6%) and 56 (28.3%) were found to be positive for anti-salivarian trypanosomes antibodies using rTeGM6-4r- and TeCA-based ELISA, respectively (Fig. 2A). In contrast, 156 (78.8%) and 10 (5.1%) were found positive for *Th. equi* and *B. caballi*, respectively (Fig. 2B).

Molecular detection of hemoprotozoan parasites

Of 198 samples, using ITS1-PCR, the DNA of *Trypanozoon* (~480 bp) and *Tr. congolense* (~700 bp) was detected in 77 (38.9%) and 18 (9.1%) samples, respectively. Moreover, five (2.5%) samples showed evidence of mixed infection with *Trypanozoon* and *Tr. congolense* (Supplementary Fig. 1A). Additionally, 35 (45.5%) of 77 *Trypanozoon*-positive samples were found to be positive using RoTat1.2 PCR, confirming them as *Tr. evansi* type A (~205 bp) (Supplementary Fig. 1B). Regarding piroplasms, species-specific PCR assays showed that 18 (9.1%) and 8 (4%) of 198 samples were positive for *Th. equi* (~750 bp) and *B. caballi* (~610 bp), respectively (Supplementary Fig. 2). A summary of the PCR results is shown in Table 2.

Sequencing analysis

Confirmation of the ~480- and ~700-bp PCR products as *Trypanozoon* and *Tr. congolense* savannah type, respectively, was achieved by selection, cloning, and sequencing of the ITS1 region of 18 positive samples (nine samples each). The sequence similarity and pairwise distance values among trypanosomes obtained in this study and reference sequences of trypanosomes from GenBank confirmed the presence of the subgenus *Trypanozoon* (Fig. 3; GenBank database accession numbers LC492114–LC492122) and *Tr. congolense* savannah (Fig. 4; GenBank accession numbers LC492123–LC492131). The multiple sequence alignments of ITS1 have been shown in Supplementary Fig. 3A and 3B.

Seven positive ~205-bp PCR products were selected for cloning and sequencing of the RoTat1.2 VSG region, confirming them as *Tr. evansi* type A (GenBank accession numbers LC493166–LC493172). Moreover, selection, cloning, and sequencing of the EMA-1 region of 4 positive ~750-bp and one positive ~610-bp PCR products confirmed the etiology as *Th. equi* and *B. caballi*, respectively (GenBank accession numbers LC514705–LC514709).

DISCUSSION

Donkeys are common livestock animals in Sudan; there are an estimated 7.51 million donkeys in Sudan, with an annual increase of approximately 3% [42]. Donkeys are used for transportation, packing, pulling carts, farming, raising water, and milling [41]. The number of donkeys in Khartoum is declining with urbanization [34]; however, donkeys remain essential for transportation, particularly in the rural areas and margins of the three cities of Khartoum [3]. Although donkeys can acquire a variety of diseases, they are hardy animals and usually remain asymptomatic carriers [3]. In this study, we used molecular and serological techniques to evaluate the status of some important hemoprotozoan parasites in donkeys in Khartoum State owing to the general neglect of these animals despite their huge economic contributions, ultimately allowing individuals and families to increase their income and avoid poverty.

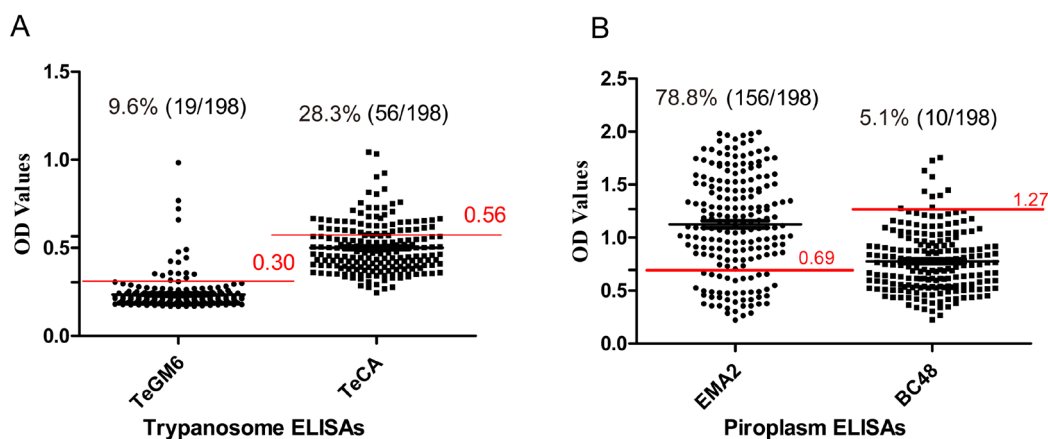


Fig. 2. Detection of anti-salivarian trypanosome antibodies (A) and anti-piroplasm antibodies (B) in donkeys using ELISA. Cutoff values for ELISAs are indicated in red.

Table 2. Prevalence of hemoprotozoan parasites DNA in donkeys by PCR assays

Spp.	<i>Trypanozoon</i> (~480 bp)	<i>Tr. congolense</i> (~700 bp)	Mixed <i>Trypanozoon</i> and <i>Tr. congolense</i>	<i>Tr. evansi</i> type A (~205 bp)	<i>Theileria equi</i> (~750 bp)	<i>Babesia caballi</i> (~610 bp)
No. of positive samples (%)	77/198 (38.9)	18/198 (9.1)	5/198 (2.5)	35/77 (45.5)	18/198 (9.1)	8/198 (4)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1LC492114																	
2LC492115	1.57																
3LC492116	4.09	4.40															
4LC492117	5.97	5.03	4.72														
5LC492118	5.66	4.72	4.40	1.57													
6LC492119	5.66	4.72	4.40	0.94	1.26												
7LC492120	5.66	4.72	4.40	1.57	0.63	1.26											
8LC492121	5.35	4.40	4.72	1.89	0.31	1.57	0.94										
9LC492122	1.57	0.63	4.40	5.03	4.72	4.72	4.72	4.40									
10AB551922_ <i>Tr. evansi</i>	1.26	0.31	4.09	4.72	4.40	4.40	4.40	4.09	0.31								
11KR858267_ <i>Tr. evansi</i>	5.97	5.03	4.72	0.63	1.57	0.94	1.57	1.89	5.03	4.72							
12AF306771_ <i>Tr. brucei</i>	4.40	3.46	3.14	1.57	1.26	1.26	1.26	1.57	3.46	3.14	1.57						
13AF306772_ <i>Tr. brucei</i>	2.20	1.26	4.09	4.72	4.40	4.40	4.40	4.40	1.26	0.94	4.72	3.14					
14KU552352_ <i>Tr. equiperdum</i>	1.89	0.94	4.72	5.35	5.03	5.03	5.03	4.72	0.94	0.63	5.35	3.77	1.57				
15KU552354_ <i>Tr. equiperdum</i>	5.03	4.09	3.14	2.20	1.89	1.89	1.89	2.20	4.09	3.77	2.20	0.63	3.77	4.40			
16JN673389_ <i>Tr. congolense</i>	63.52	64.47	64.15	66.04	66.04	65.09	66.04	65.72	64.47	64.47	65.41	65.41	64.47	63.84	65.72		
17JN673388_ <i>Tr. congolense</i>	63.21	64.15	63.84	65.72	65.72	65.09	65.72	65.41	64.15	64.15	65.72	65.09	64.15	64.15	65.41	6.29	
18KY412803_ <i>Tr. theileri</i>	55.03	54.72	55.66	54.72	55.97	55.03	55.97	55.66	54.72	54.72	55.03	55.35	55.66	54.72	55.97	67.61	68.24

Fig. 3. Sequence analysis and pairwise distance analysis of ~480-bp amplicons detected in the samples taken from donkeys in Khartoum State based on internal transcribed spacer-1 (ITS1) sequences confirmed the parasites as *Trypanozoon*. Accession numbers of the *Trypanozoon* isolates detected in this study have been indicated in blue, boldface font.

The ITS1-PCR method was used in this study. The ITS1 region of rDNA is a preferred target for detection of trypanosomes because of its highly conserved flanking regions and size variability among trypanosomes species [28]. TR has been reported to be more prevalent in horses than in donkeys in Sudan because only *Tr. vivax* was detected in donkeys, whereas *Trypanozoon*, *Tr. congolense*, *Tr. vivax*, and *Tr. simiae* were detected in horses in different parts of the country [37]. Here, we observed prevalence rates of 38.9% for *Trypanozoon* (77/198) and 9.1% for *Tr. congolense* (18/198) in donkeys in West Omdurman, Khartoum State,

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
1LC492123																							
2LC492124	1.84																						
3LC492125	4.36	4.04																					
4LC492126	0.61	2.14	3.72																				
5LC492127	0	1.84	4.36	0.61																			
6LC492128	5.65	5.33	2.46	4.99	5.65																		
7LC492129	6.31	6.63	5.35	5.64	6.31	4.06																	
8LC492130	5.34	5.02	4.05	4.68	5.34	3.1	5.36																
9LC492131	1.52	3.39	5.96	2.14	1.52	7.27	7.94	6.96															
10JN673389_ <i>Tr. congolense</i> savannah, Tanzania	0.61	2.46	5	1.22	0.61	6.3	6.3	5.99	2.14														
11JN673388_ <i>Tr. congolense</i> savannah, Tanzania	3.4	2.77	3.09	3.71	3.4	3.72	5.98	4.36	4.34	4.03													
12JX910374_ <i>Tr. congolense</i> , Burkina Faso	9.06	9.75	9.72	9.03	9.06	10.4	11.2	11.2	10.1	9.74	10.4	30											
13MG255203_ <i>Tr. congolense</i> , Cameroon	5	5.32	5.66	4.99	5	6.98	7.31	6.67	6.61	4.99	5.01	24.6	10.5										
14MG255204_ <i>Tr. congolense</i> , Cameroon	3.71	3.4	2.47	3.08	3.71	3.73	4.69	3.42	5.3	4.35	3.72	22.4	9.07	5									
15MG283145_ <i>Tr. congolense</i> , Cameroon	5.3	5.62	6.62	5.29	5.3	7.94	8.27	7.63	6.91	5.3	5.96	25.3	11.5	2.14	5.95								
16FJ712718_ <i>Tr. congolense</i> , Kenya	3.09	3.4	2.46	3.08	3.09	3.71	5.33	3.4	4.66	3.72	2.45	23.2	9.07	4.37	2.46	5.31							
17MK495745_ <i>Tr. congolense</i> , Cote d'Ivoire	5.3	5.62	6.62	5.29	5.3	7.94	8.27	7.63	6.91	5.3	5.96	25.3	11.5	2.14	5.95	0	5.31						
18MK651119_ <i>Tr. congolense</i> , Algeria	7.38	7.05	4.08	6.7	7.38	5.05	8.44	6.73	9.05	8.05	6.04	25.8	12	8.76	5.4	9.75	5.38	9.75					
19AB742531_ <i>Tr. congolense</i> , Ghana	24.9	24	25.3	24.8	24.9	23.6	27.1	23.6	26.9	25.7	22.3	49.7	31.2	24.1	25.3	25.7	24	25.7	27.8				
20U22319_ <i>Tr. congolense</i> riverine forest, Kenya	25.8	24.9	26.2	25.7	25.8	24.5	28.1	24.5	27.8	26.6	23.2	51.1	32.2	25	26.2	26.6	24.9	26.6	28.8	0.61			
21MK756201_ <i>Tr. congolense</i> , Nigeria	28.7	27.9	28.4	28.7	28.7	25.8	29.4	26.6	30	29.6	26.1	52.9	35	28	28.4	29.6	27.8	29.6	30.1	2.77	2.77		
22U22317_ <i>Tr. congolense</i> kilifi, Kenya	60.7	61.4	64.4	61	60.7	62.4	64.3	63.8	60.7	60.1	62.7	91	67.7	63.5	62	63.8	64	63.8	67	70.8	71.8	71	
23MK756200_ <i>Tr. congolense</i> kilifi, Nigeria	63.3	62.6	64.1	63.6	63.3	62.1	64.1	63.6	63.3	62.6	63.8	90	69	64.7	61.8	65	63.7	65	66.8	70	71	70.3	1.84

Fig. 4. Sequence analysis and pairwise distance analysis of ~700-bp amplicons detected in the samples taken from donkeys in Khartoum State based on internal transcribed spacer-1 (ITS1) sequences confirmed the parasites as *Trypanosoma congolense* savannah. Accession numbers of *Tr. congolense* savannah detected in this study have been indicated in red, boldface font.

Sudan. Other PCR products (~250, ~280, ~400, and ~600 bp, suspected to be *Tr. vivax*, *Tr. godfreyi*, *Tr. simiae* and *Tr. congolense* kilifi, respectively) were also detected by ITS1-PCR (Supplementary Fig. 1). Only the prevalence of *Trypanozoon* and *Tr. congolense* savannah was confirmed by DNA sequence analysis. Sequencing of other PCR products did not result in confirmation of the suspected trypanosomes (data not shown).

Tr. congolense and *Tr. vivax* were previously detected in camels, dogs, and equines in non-tsetse-infested areas in Sudan [21, 22, 37], suggesting a mechanism of mechanical transmission. Mechanical transmission of *Tr. congolense* by African tabanid has been demonstrated experimentally under natural conditions [10]. Moreover, after the referendum of South Sudan from Sudan in 2011, the movement of animals between the two countries has contributed to the spread of trypanosomiasis in tsetse-free areas [1]. These factors may explain the presence of *Tr. congolense*, which we reported for the first time, in donkeys in Khartoum, which is hundreds of kilometers away from the tsetse-infested areas. In Sudan, RoTat1.2 VSG PCR has been used for confirmation of *Tr. evansi* in camels [21, 35]. This is the first report of detection of *Tr. evansi* type A in donkeys using RoTat1.2 VSG PCR in Sudan. Notably, RoTat1.2-negative samples could be *Tr. evansi* type B or *Tr. brucei*.

Various serological tests, including indirect fluorescent antibody test, ELISA, and CATT/*Tr. evansi*, have been introduced for laboratory and field use for detection of trypanosome-specific antibodies. Only CATT/*Tr. evansi* can be used in the field. This method classifies truly infected animals, implying applications in the targeting of individual animals for treatment with trypanocidal drugs. ELISA is suitable for verifying the disease-free status of animals because it can correctly classify uninfected animals [30]. OIE recommends serial testing with CATT and ELISA, followed by retesting of suspect animals to declare a disease-free status. Furthermore, it is preferred to confirm the findings by PCR. In the current study, 52 (26.3%), 19 (9.6%), and 56 (28.3%) serum samples were found positive using CATT/*Tr. evansi*, rTeGM6-4r-based ELISA, and TeCA-based ELISA, respectively. The detection performance of crude and recombinant antigen-based ELISAs was reported to be relatively similar for the detection of trypanosomes antibodies in animals [26]. However, crude antigen-based ELISA showed high cross-reactivity with *Theileria*- and *Babesia*-infected serum samples from water buffalo [27]. In this study, the number of positive trypanosome-infected sera detected with TeCA-based ELISA in donkeys was higher than the number detected with rTeGM6-4r-based ELISA. In contrast, antibodies and DNA of *Th. equi* and *B. caballi* were detected in those donkeys. Thus, the possibility of cross-reactivity may explain the differences in the detection performance of ELISAs.

In this study, out of 198 samples, we found that 8 (4%) and 10 (5.1%) samples were positive for *B. caballi*, whereas 18 (9.1%) and 156 (78.8%) samples were positive for *Th. equi* in West Omdurman, Khartoum State using PCR and ELISA, respectively. These findings indicated that the prevalence of *Th. equi* was higher than that of *B. caballi* in donkeys in Khartoum, as detected

by both serological and molecular techniques. In contrast, the seroprevalence of piroplasms in donkeys in Khartoum, as detected by ELISA, was higher than the prevalence of the parasites detected by PCR, potentially because of chronic infections, causing the parasitemia to be below the detection limit of PCR. The same findings have been reported in Mongolia, where piroplasms were detected in 51.2% and 81.6% of horses by PCR and ELISA, respectively [25]. The low correlation between serological and molecular methods for detection of equine piroplasms could be explained by the observation that these methods detect different entities and therefore differ in principle. Thus, PCR is considered a reliable diagnostic method for active infections, whereas serological tests are reliable for the detection of persistent infections [17, 20]. A similar infection pattern of *Th. equi* higher than *B. caballi* was reported in horses in different parts of Sudan, and the prevalence was higher when using ELISA than when using PCR; however, no donkeys were included in that study [38]. Another molecular surveillance study was conducted in five states in Central, Eastern, and Western Sudan, including Khartoum State, using blood samples from horses and donkeys. In that study, 22% of horses in Khartoum State were PCR-positive for EP, but no donkeys from Khartoum State were examined [36]. The current study targeted donkeys only, and we provided basic information on the prevalence of EP; these findings could help in the development of future control strategies.

In conclusion, we documented high prevalence rates of TR and EP in donkeys in the study area. Different causative agents of these diseases were identified, and some agents, such as *Tr. congolense* savannah and *Tr. evansi* type A, were reported for the first time in donkeys in Sudan. Moreover, the detection of tsetse-transmitted *Tr. congolense* savannah in donkeys in West Omdurman should alert the veterinary authority of the possibility that the parasite could be mechanically transmitted to other susceptible animal species. Thus, donkeys should be included in any control strategies for trypanosomiasis in the future and should be given more veterinary care in general in Sudan.

ACKNOWLEDGMENTS. This work was financially supported by the Japan Society for the Promotion of Science (JSPS): KA-KENHI (grant no. 16K18793; Grants-in-Aid for Young Scientists [B]), and Japan Agency for Medical Research and Development (AMED): International Collaborative Research Program for Tackling the NTDs (Neglected Tropical Diseases) Challenges in African countries (grant no. 18jm0510001h0104).

REFERENCES

1. Ahmed, S. K., Rahman, A. H., Hassan, M. A., Salih, S. E., Paone, M. and Cecchi, G. 2016. An atlas of tsetse and bovine trypanosomiasis in Sudan. *Parasit. Vectors* **9**: 194. [Medline] [CrossRef]
2. Alhassan, A., Pumidomming, W., Okamura, M., Hirata, H., Battsetseg, B., Fujisaki, K., Yokoyama, N. and Igarashi, I. 2005. Development of a single-round and multiplex PCR method for the simultaneous detection of *Babesia caballi* and *Babesia equi* in horse blood. *Vet. Parasitol.* **129**: 43–49. [Medline] [CrossRef]
3. Angara, T. E. E., Ismail, A. A. and Ibrahim, A. M. 2011. The role of donkeys in income generation and the impact of endoparasites on their performance. *U. of K. J. Vet. Med. Anim. Prod.* **2**: 65–89.
4. Aregawi, W. G., Agga, G. E., Abdi, R. D. and Büscher, P. 2019. Systematic review and meta-analysis on the global distribution, host range, and prevalence of *Trypanosoma evansi*. *Parasit. Vectors* **12**: 67. [Medline] [CrossRef]
5. Bajyana Songa, E. and Hamers, R. 1988. A card agglutination test (CATT) for veterinary use based on an early VAT RoTat 1/2 of *Trypanosoma evansi*. *Ann. Soc. Belg. Med. Trop.* **68**: 233–240. [Medline]
6. Bennett, S. C. J. 1936. The treatment of Equine *Trypanosoma Congolense* infections with Surfén C (Bayer). *J. Comp. Pathol. Ther.* **49**: 151–159. [CrossRef]
7. Brüning, A. 1996. Equine piroplasmosis an update on diagnosis, treatment and prevention. *Br. Vet. J.* **152**: 139–151. [Medline] [CrossRef]
8. Claes, F., Radwanska, M., Urakawa, T., Majiwa, P. A. O., Goddeeris, B. and Büscher, P. 2004. Variable surface glycoprotein RoTat 1.2 PCR as a specific diagnostic tool for the detection of *Trypanosoma evansi* infections. *Kinetoplastid Biol. Dis.* **3**: 3. [Medline] [CrossRef]
9. Clutton, B. J. 1999. A natural history of domesticated mammals. Cambridge University, Cambridge.
10. Desquesnes, M. and Dia, M. L. 2003. Mechanical transmission of *Trypanosoma congolense* in cattle by the African tabanid *Atylotus agrestis*. *Exp. Parasitol.* **105**: 226–231. [Medline] [CrossRef]
11. El Karib, A. 1961. Animal trypanosomiasis in the Sudan. *Sudan J. Vet. Sci. Anim. Husb.* **2**: 39–40.
12. F. A. O. Statistical Yearbook. 2014. Food and Agriculture Organization, Rome.
13. Fielding, D. 1988. Pack transport with donkeys. *Appr. Technol. J.* **15**: 11–13.
14. Friedhoff, K. T. and Soulé, C. 1996. An account on equine babesioses. *Rev. Off. Int. Epizoot.* **15**: 1191–1201 (Int Off Epizoot). [Medline] [CrossRef]
15. Gari, F. R., Ashenafi, H., Tola, A., Goddeeris, B. M. and Claes, F. 2010. Comparative diagnosis of parasitological, serological, and molecular tests in dourine-suspected horses. *Trop. Anim. Health Prod.* **42**: 1649–1654. [Medline] [CrossRef]
16. Huang, X., Xuan, X., Yokoyama, N., Xu, L., Suzuki, H., Sugimoto, C., Nagasawa, H., Fujisaki, K. and Igarashi, I. 2003. High-level expression and purification of a truncated merozoite antigen-2 of *Babesia equi* in *Escherichia coli* and its potential for immunodiagnosis. *J. Clin. Microbiol.* **41**: 1147–1151. [Medline] [CrossRef]
17. Kappmeyer, L. S., Perryman, L. E., Hines, S. A., Baszler, T. V., Katz, J. B., Hennager, S. G. and Knowles, D. P. 1999. Detection of equine antibodies to *babesia caballi* by recombinant *B. caballi* rhoptry-associated protein 1 in a competitive-inhibition enzyme-linked immunosorbent assay. *J. Clin. Microbiol.* **37**: 2285–2290. [Medline] [CrossRef]
18. Krause, P. J. 2003. Babesiosis diagnosis and treatment. *Vector Borne Zoonotic Dis.* **3**: 45–51. [Medline] [CrossRef]
19. Luckins, A. G. 1994. Equine trypanosomiasis. *Equine Vet. Educ.* **6**: 259–262. [CrossRef]
20. Mahmoud, M. S., Kandil, O. M., Nasr, S. M., Hendawy, S. H. M., Habeeb, S. M., Mabrouk, D. M., Silva, M. G. and Suarez, C. E. 2015. Serological and molecular diagnostic surveys combined with examining hematological profiles suggests increased levels of infection and hematological response of cattle to babesiosis infections compared to native buffaloes in Egypt. *Parasit. Vectors* **8**: 319. [Medline] [CrossRef]

21. Mossaad, E., Salim, B., Sukanuma, K., Musinguzi, P., Hassan, M. A., Elamin, E. A., Mohammed, G. E., Bakhiet, A. O., Xuan, X., Satti, R. A. and Inoue, N. 2017a. *Trypanosoma vivax* is the second leading cause of camel trypanosomosis in Sudan after *Trypanosoma evansi*. *Parasit. Vectors* **10**: 176. [Medline] [CrossRef]
22. Mossaad, E., Satti, R. A., Fadul, A., Sukanuma, K., Salim, B., Elamin, E. A., Musinguzi, S. P., Xuan, X. and Inoue, N. 2017b. The incrimination of three trypanosome species in clinically affected German shepherd dogs in Sudan. *Parasitol. Res.* **116**: 2921–2925. [Medline] [CrossRef]
23. Muieed, M. A., Chaudhary, Z. I. and Shakoori, A. R. 2010. Comparative studies on the sensitivity of polymerase chain reaction (PCR) and microscopic examination for the detection of *Trypanosoma evansi* in horses. *Turk. J. Vet. Anim. Sci.* **34**: 507–512.
24. Mukiria, P., Mdachi, R., Thuita, J., Mutuku, J., Wanjala, K., Omolo, J., Mulugeta, G., Trawford, A., Ouma, J. and Murilla, G. 2010. Semi-longitudinal study of trypanosomiasis and its vectors in donkeys (*Equus africanus asinus*, Fitzinger) in the Lamu archipelago. Proceedings of the 12th KARI Biennial Scientific Conference.
25. Munkhjargal, T., Sivakumar, T., Battsetseg, B., Nyamjargal, T., Aboulaila, M., Purevtseren, B., Bayarsaikhan, D., Byambaa, B., Terkawi, M. A., Yokoyama, N. and Igarashi, I. 2013. Prevalence and genetic diversity of equine piroplasms in Tov province, Mongolia. *Infect. Genet. Evol.* **16**: 178–185. [Medline] [CrossRef]
26. Nguyen, T. T., Motsiri, M. S., Taiou, M. O., Mtshali, M. S., Goto, Y., Kawazu, S., Thekisoe, O. M. M. and Inoue, N. 2015a. Application of crude and recombinant ELISAs and immunochromatographic test for serodiagnosis of animal trypanosomosis in the Umkhanyakude district of KwaZulu-Natal province, South Africa. *J. Vet. Med. Sci.* **77**: 217–220. [Medline] [CrossRef]
27. Nguyen, T. T., Zhou, M., Ruttayaporn, N., Nguyen, Q. D., Nguyen, V. K., Goto, Y., Suzuki, Y., Kawazu, S. and Inoue, N. 2014b. Diagnostic value of the recombinant tandem repeat antigen TeGM6-4r for surra in water buffaloes. *Vet. Parasitol.* **201**: 18–23. [Medline] [CrossRef]
28. Njiru, Z. K., Constantine, C. C., Guya, S., Crowther, J., Kiragu, J. M., Thompson, R. C. A. and Dávila, A. M. 2005. The use of ITS1 rDNA PCR in detecting pathogenic African trypanosomes. *Parasitol. Res.* **95**: 186–192. [Medline] [CrossRef]
29. OIE 2018. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. World Organization for Animal Health. Chapter 3.1.21. https://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/3.01.21_TRYPANO_SURRA.pdf [accessed on January 15, 2020].
30. OIE 2020. OIE-Listed diseases, infections and infestations in force in 2020. <https://www.oie.int/en/animal-health-in-the-world/oie-listed-diseases-2020/> [accessed on January 15, 2020].
31. Oliver, A. 1907. Annual Report. Khartoum: Sudan Veterinary Department.
32. Onyiche, T. E., Sukanuma, K., Igarashi, I., Yokoyama, N., Xuan, X. and Thekisoe, O. 2019. Review on equine piroplasmosis: epidemiology, vector ecology, risk factors, host immunity, diagnosis and control. *Int. J. Environ. Res. Public Health* **16**: 1736. [Medline] [CrossRef]
33. Persing, D. H. and Conrad, P. A. 1995. Babesiosis: new insights from phylogenetic analysis. *Infect. Agents Dis.* **4**: 182–195. [Medline]
34. Rabeh, A. 2009. Chairman of the Donkey Carts Trade Union-Mayo-Gebel awliya-Khartoum. Personal Communication.
35. Salim, B., Bakheit, M. A., Kamau, J., Nakamura, I. and Sugimoto, C. 2011. Molecular epidemiology of camel trypanosomiasis based on ITS1 rDNA and RoTat 1.2 VSG gene in the Sudan. *Parasit. Vectors* **4**: 31. [Medline] [CrossRef]
36. Salim, B., Bakheit, M. A., Kamau, J. and Sugimoto, C. 2013. Current status of equine piroplasmosis in the Sudan. *Infect. Genet. Evol.* **16**: 191–199. [Medline] [CrossRef]
37. Salim, B., Bakheit, M. A. and Sugimoto, C. 2014. Molecular detection of equine trypanosomes in the Sudan. *Vet. Parasitol.* **200**: 246–250. [Medline] [CrossRef]
38. Salim, B. O. M., Hassan, S. M., Bakheit, M. A., Alhassan, A., Igarashi, I., Karanis, P. and Abdelrahman, M. B. 2008. Diagnosis of *Babesia caballi* and *Theileria equi* infections in horses in Sudan using ELISA and PCR. *Parasitol. Res.* **103**: 1145–1150. [Medline] [CrossRef]
39. Seifi, H. A., Mohria, M. and Sardaria, K. 2000. A mixed infection of *Babesia equi* and *Babesia caballi* in a racing colt: a report from Iran. *J. Equine Vet. Sci.* **20**: 858–860. [CrossRef]
40. Sowar, N. A. M. A. 2006. Investigation of parasites of working donkeys in Khartoum State, Sudan. MVSc thesis. Department of Preventive Medicine and Veterinary Public Health, Faculty of Veterinary Medicine, University of Khartoum, Khartoum.
41. Starkey, P. and Starkey, M. 1997. Regional and world trends in donkey populations. p. 244. In: Donkeys, People and Development (Starkey, P. and Fielding, D. eds.), A resource book of the Animal Traction Network for Eastern and Southern Africa (ATNESA). ACP-EU Technical Centre for Agricultural and Rural Cooperation (CTA), Wageningen. <https://www.atnesa.org/donkeys/donkeys-starkey-populations.pdf> [accessed on January 15, 2020].
42. Statistical Bulletin for Animal Resources No 19. 2009. Information Centre, Ministry of Animal Resources and Fisheries (MARF), Khartoum.
43. Svendsen, E. D. 1997. The Professional Handbook of the Donkey, 3rd ed. pp. 166–182, Whittet Books Ltd., London.
44. Uilenberg, G. 1961. A case of dourine in a donkey mare in the Sudan. *Tijdschr. Diergeneesk.* **86**: 130–133.
45. Verloo, D., Holland, W., My, L. N., Thanh, N. G., Tam, P. T., Goddeeris, B., Vercruyssen, J. and Büscher, P. 2000. Comparison of serological tests for *Trypanosoma evansi* natural infections in water buffaloes from north Vietnam. *Vet. Parasitol.* **92**: 87–96. [Medline] [CrossRef]
46. Webb, C. E. 1915. Trypanosomiasis of donkeys and mules in the Anglo-Egyptian Sudan: Some results of transmission experiments and arsenical treatment. *J. Comp. Pathol. Ther.* **28**: 1–20. [CrossRef]
47. Wilson, R. T. 2017. A retrospective review of 130 years of equine disease in Sudan. *Res. Rev. J. Vet. Sci.* **S1**: 9–22.