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With 2 tables and 3 figures in the text

Parasitological and Molecular Detection of Equine Piroplasmosis in Horses and Donkeys in South Darfur State, Sudan

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ملخص البحث

أجرى هذا البحث فى الفترة من مارس 2009م إلى مارس 2010م لتشخيص داء بابسيات (Piroplasmosis) الفصيلة الخيلية و معرفة مدى إنتشاره فى ولاية جنوب دارفور. أخذت عينات دم مرة واحدة من حيوانات سليمة ظاهرياً من سبع محليات هي: نيالا(69 خيل و 77 حمير) و الضعين (24 خيل) و أبومطارق (24 حمير) و كاس (4 خيل و 21 حمير) و كتيلا(35 خيل و 141 حمير) و رهيد البردى (20 خيل و 31 حمير) و تلس (35 خيل). جُهزت العينات على هيئة مسحات دموية و بقع دموية على ورق ترشيح. أظهرت المسحات الدموية وجود طفيلى البابيزيا الحصانية (*Babesia caballi*) فى الحميربمعدل إنتشار 0.34% (294/1) فى محليات جنوب دارفور المذكورة و عدم وجوده فى 187 عينة من الخيل. كما أظهرت المسحات الدموية وجود طفيلى التيلرية الخيلية (*Theileria equi*) بمعدل إنتشار2.14% (187/4) فى الخيل و2.04% (294/6) فى الحمير و كان معدل إنتشاره فى الخيل 2.9% (69/2) فى نيالا و 5.7% (35/2) فى كتيلا ، و فى الحمير 5.2% (77/4) فى نيالا و 1.42% (141/2) فى كتيلا. بإجراء فحص الحمض النووى للطفيليين بواسطة تفاعل البلمرة المتسلسل (PCR) وجدت البابيزيا الحصانية فى 33.3%(36/12) من الخيل و لم توجد فى الحمير (55). أما الحمض النووي للتيلرية الخيلية فوجد فى 13.9% (36/5) من الخيل و 23.6% (55/13) من الحمير. كان مدى إنتشار الحمض النووى للبابيزيا الحصانية فى محليات الضعين و نيالا و كاس 38.1%، 25% و 33.3% على التوالى بينما كانت معدلات إنتشار الحمض النووى التيلرية الخيلية فى نيالا و أبومطارق و كاس و الضعين 31.82% ، 20.83% ، 20.83% ، و 4.76%على التوالى (معدلات الإنتشار فى الخيل33.3% فى نيالا و4.8% فى الضعين) ، وفى الحمير30% فى نيالا و 23.8% فى كاس و 20.8% فى ابو مطارق). أوضحت المسحات الدموية أن التيلرية الخيلية أكثر إنتشاراً من البابيزيا الحصانية أما تقنية تفاعل البلمرة المتسلسل أظهرت وجود التيلرية الخيلية فى الحميردون البابيزيا الحصانية.

Summary

Four hundred and eighty-one blood smears (187 from horses and 294 from donkeys) and 91 blood spots on filter papers (36 horses and 55 donkeys) were collected from apparently healthy animals in seven localities (Nyala, El-Dein, Abu-Matariq, Kass, Kateila, Rehaid Albirdi and Tulus) during the period from March 2009 to March 2010 and examined using parasitological and molecular techniques, respectively. Blood smears examination revealed that, *Babesia caballi* piroplasms were only seen in a donkey at Nyala locality. *Theileria equi* piroplasms were seen in four (2.14%) and six (2.04%) horse and donkey samples, respectively. *T. equi* was found to be prevalent in Nyala (4.11%) and Kateila (2.27%) but no piroplasms were seen in smears collected from animals in other localities. Using PCR, *B. caballi* DNA was detected in 12 horse samples (33.3%). While, *T. equi* DNA was detected in five horses (3.9%) and 13 donkeys (23.6%). The prevalence of *B. caballi* was 38.1% in El-Dein, 25% in Nyala and 33.3% in Kass. While the prevalence of *T. equi* was 31.82% in Nyala, 20.83% in Abu-Matariq, 20.83% in Kass and 4.76% in El-Dein. In horses the prevalence was 33.3% in Nyala and 4.8% in El-Dein. In donkeys it was 20.8% in Abu-Matariq, 23.8% in Kass and 30%in Nyala. The study concluded that, equine piroplasmosis is prevalent in South Darfur, and *T.equi* is more likely to be diagnosed microscopically than *B. caballi*. By molecular technique, *B. caballi* was more common than *T. equi* in horses and was not detected in donkeys.

Introduction

Equine piroplasmosis, caused by *Babesia caballi* and *Theileria equi*, is considered to be the most important tick-borne disease of horses and donkeys in tropical and subtropical areas (Kuttler, 1988; Mehlhorn and Schein, 1998).

B. equi was assigned to the genus *Babesia* until Mehlhorn and Schein (1998) transferred it to the genus *Theileria* on the basis of life cycle differences. These authors demonstrated that *T. equi*, like other members of the genus *Theileria*, has a pre-erythrocytic developmental stage within the lymphatic cells of their vertebrate host. Genetically, this species had been proved to be more related to the genus *Theileria* than *Babesia* (Allsopp *et al*, 1994). But on genomic analysis, it belongs to a lineage between *Babesia* and *Theileria*; an additional data are needed to determine the final placement of this parasite (Deepak *et al*, 2014)

B. caballi and *T. equi* are transmitted by the Ixodid ticks *Hyalomma*, *Dermacentor*, and *Rhipicephalus*. They can also be directly transmitted between animals by contaminated needles or blood transfusion. Maternal infection of equine foetuses by *Babesia* and *Theileria* species has been reported (de Waal and van Heerden, 2004; Rothschild and Knowles, 2007; OIE, 2008). Transplacental transmission of *T.equi* from carrier mares to asymptomatic foals can occur (Allsopp *et al*, 2007).

In the Sudan, Abdoon (1984) and Salim (2008) reoprted that *H. anatolicum* was responsible for equine piroplasmosis transmission.

B. caballi and *T. equi* have been idetnified as the causative agents of acute, subacute and chronic equine piroplasmosis in equids (de Waal, 1992). Therefore, they are of considerable veterinary importance particularly to the horse breeding industry (Kuttler, 1988).

Diagnosis of equine piroplasmosis could include a combination of clinical signs, identification of the organism using light microscopy, serology, inoculation of blood into a susceptible animal (Frerichs *et al*, 1969; de Waal and van Heerden, 2004), polymerase chain reaction, PCR (Vial and Gorenflot, 2006) and/or xenodiagnosis (Frerichs *et al*, 1969; de Waal and van Heerden, 2004). Microscopic examination is generally reliable in the acute phase of the disease but at low level of parasitaemia which can be found even with acute symptoms, the parasite may not be detected (Bonfini and Semproni, 2006).

Due to the difficulties experienced in detecting low numbers of parasites by light microscopy in subclinically infected or carrier animals, serological methods such as complement fixation test (CFT), indirect fluorescent antibody (IFA) test and enzyme-linked immunosorbent assay (ELISA) have been developed to aid in the diagnostic process (de Waal and van Heerden, 2004; Phipps and Otter, 2004).

The PCR has been applied for detection of many species of *Babesia* and *Theileria* with high sensitivity and specificity compared to serological assays (Buling *et al*, 2007). PCR does not generally detect mixed infections although there are some amplification protocols that can, to some extent, detect mixed piroplasmid infections (Birkenheuer *et al*, 2003). To overcome this problem, reverse line blot (RLB) has been developed for simultaneous detection and identification of equine *Babesia* and *Theileria* species (Daniel *et al*, 2004).

Diagnosis of equine piroplasmosis in the Sudan based on, clinical signs and microscopical examination is routinely done in veterinary clinics. CFT (Abdoon, 1984), ELISA and PCR (Salim, 2008) and capillary electrophoresis genotyping method (Salim *et al*, 2013) have been used to detect the caustive agents of equine piroplasmosis either for treatment purposes or epidemiological studies.

Materials and Methods

Study area

The study was conducted from March 2009 to March 2010 in seven localities (Fig. 1) in South Darfur State (later it was divided to South Darfur and East Darfur States). It is situated in the western part of the Sudan between latituates 8°30' to 13°N and longitudes 23°15' to 28° E bordered by West

Kordofan from the east, West Darfur from the west, and North Darfur from the north. It shares international borders with Republic of South Sudan, Republic of Central Africa and Chad.

Blood smears

Blood from the jugular veins using vacutainers with anticoagulant (EDTA) was collected from 481 randomly selected equines (187 horses and 294 donkeys). Blood smears were prepared according to Preast *et al* (2007). These samples were collected from Nyala (146), El-Dein (24), Abu-Matariq (24), Kass (25), Kateila (176), Rehaid Albirdi (51) and Tulus (35). The blood smears were stained with 10% Giemsa's stain solution. The smears were then examined for erythrocytic stages of *B. caballi* and *T. equi* under a compound microscope using oil immersion objective (x 100 and x 10 eye-piece). About 50 microscopic fields were examined in each slide.

Blood spots

Blood collected from the jugular veins using vacutainers with anticoagulant (EDTA) was spotted on filter papers (Whatman, England) making circles of about 2 cm in diameter, air dried and fixed with absolute methanol. They were individually sealed off in polythene bags and labelled indicating animal species, number, locality, and date of collection prior to storing at 4°C until used.

DNA extraction

A total number of 91 blood spots on filter papers (36 horses and 55 donkeys) was subjected to DNA extraction. These were from Nyala (*n*=22), El-Dein (*n*=21), Abu-Matariq (*n*=24) and Kass (*n*=24). Genomic DNA was extracted from blood spots on filter papers as described by Salim *et al* (2010).

Fig. 1. Map of South Darfur State. Localities **(●)** from which samples were collected. **Up: Map of Sudan showing South and East Darfur States**. **Down: Localities surveyed in ex South Darfur**

PCR amplification

A total number of 91 extracted DNA (36 from horses and 55 from donkeys) was used for detection of *T. equi* and *B. caballi* genomes (Table 2).

***T. equi* detection**

The 18S rRNA gene of *T. equi* was amplified 1.6 kb using forward primer *T. equi* 4F (5′-AAGCCATGCATGTCTAAGTATAAGCTTTT-′3) described by Oosthuizen *et al* (2008) and reverse primer 18SRev-TB (3′-GAATAATTCACCGGATCACTCG-′5) described by Matjila *et al* (2008). Reactions were performed in a total volume of 10 µl which contained 5 µl of 2X GoTaq® Green Master Mix (Promega, USA), 0.5 µl of 10nM of each primer, 2µl template DNA and 2µl deionized water. The cycling conditions were performed as described by (Bhoora *et al*, 2009). Briefly, PCR cycling included an initial denaturation step at 94°C for 2 min, followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 45 s and extension at 72°C for 1 min.

This was followed by a final extension step at 72°C for 7 min. PCR products were electrophoresed in 1.5% agarose in TAE buffer and stained using GelRed dye (Biotium, Inc. Hayward, CA, USA) before being visualized under UV light. *T. equi* positive samples had shown expected bands of approximately 1600 bp.

***B. caballi* detection**

To amplify DNA of *B. caballi*, a multi-copy gene encoding the 48-kDa rhoptry protein of *B. caballi* (Ikadai *et al*, 1999) was used. Forward primer BC48-F(5′-GGCTCCCAGCGACTC TG-′3) and reverse primer BC48-R (5′-GCATCAAG-AGGGCACTTAAG-′3) were used to amplify 610 bp from the *B. caballi* BC48 gene. PCR was performed in a total volume of 10 µl as mentioned for *T. equi*. PCR cycling included an initial denaturation step at 94°C for 2 min, followed by 40 cycles of denaturation at 94°C for 30s, annealing at 58°C for 30s, and extension at 72°C for 1 min. This was followed by a final extension step at 72°C for 7 min. PCR products were electrophoresed in 1% agarose in TAE buffer before being visualized under UV light. The expected band of *B. caballi* positive samples was 610 bp.

Prevalence of piroplasms of *B. caballi* and *T. equi*

Blood smears examination revealed that only one sample (0.34%) was positive for *B. caballi* in a sick donkey at Nyala (Table 1, Fig. 2), and 10 (2.1%) samples were positive for *T. equi*, four (2.14%) in horses (Fig. 3) and 6 (2.04%) in donkeys. In horses, the prevalence was 2.9% in Nyala and 5.7% in Kateila whereas in donkeys it was 5.2% in Nyala and 1.42% in Kateila. But no parasites were seen in equine smears from other localities (Table 1).

Detection of *B. caballi* and *T. equi* DNA using PCR

Using simple PCR, *B. caballi* DNA was detected in 12(33.3%) horse samples (Fig.4). The prevalence in horses recorded 38.1% in El-Dein, 25% in Nyala and 33.3% in Kass (Table 2).

Out of 91(36 from horses and 55 donkeys) blood spots, 18 (19.8%) were positive for *T. equi* DNA, five (13.9%) from horses and 13 (23.6%) from donkeys. A prevalence of 31.82% in Nyala, 20.83% in Abu-Matariq, 20.83% in Kass and 4.8% in El-Dein. In horses, the prevalence was 33.3% in Nyala and 4.8% in El-Dein. In donkeys, it was 20.8% in Abu-Matariq, 23.8% in Kass and 30% in Nyala (Table 2).

Result

Table 1: Prevalence of equine piroplasmosis in South Darfur localities using blood smears technique. March 2009 – March 2010.

Animal species	Locality	No. of animals	*B. caballi*	*T. equi*
Horses	Nyala	69	0	2(2.9%)
	El-Dein	24	0	0
	Kass	4	0	0
	Kateila	35	0	2(5.7%)
	Rehaid Albirdi	20	0	0
	Tulus	35	0	0
	Total	**187**	**0**	**4(2.14%)**
Donkeys	Nyala	77	1(1.3%)	4(5.2%)
	Abu-Matariq	24	0	0
	Kass	21	0	0
	Kateila	141	0	2(1.42%)
	Rehaid Albirdi	31	0	0
	Total	**294**	**1(0.34%)**	**6(2.04%)**
Grand total		**481**	**1(0.2%)**	**10(2.08%)**

Table 2: Piroplasms DNA detected by PCR in horses and donkeys at South Darfur. March 2009 –March 2010.

Animal species	Locality	No. of animals	*B. caballi* (%)	*T. equi* (%)
Horses	Nyala	12	3(25%)	4(33.3%)
	El-Dein	21	8(38.1%)	1(4.8%)
	Kass	3	1(33.3%)	0%
	Total	**36**	**12(33.3%)**	**5(13.9%)**
Donkeys	Nyala	10	0%	3(30%)
	Abu-Matariq	24	0%	5(20.8%)
	Kass	21	0%	5(23.8%)
	Total	**55**	**0%**	**13(23.6%)**
Grand total		**91**	**12(13.2%)**	**18(19.8%)**

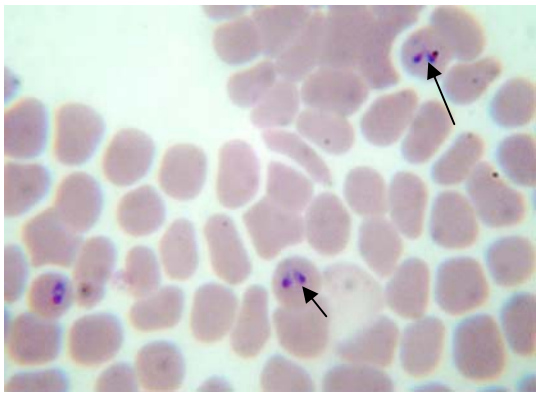

Fig.2. Giemsa-stained blood smear from a donkey. Erythrocytic single and pyriform shapes of *B. caballi*, (arrows). X100.

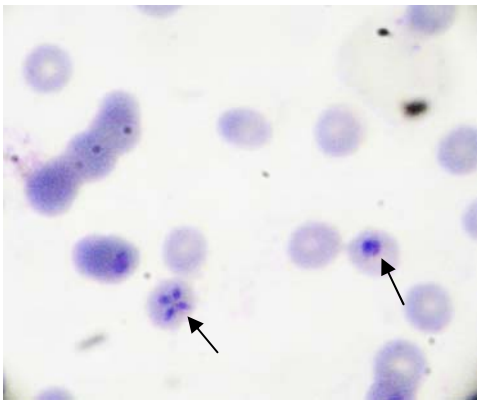

Fig. 3. Giemsa-stained blood smear from a horse. Erythrocytic Maltase cross and ring shape of *T. equi*, (arrows). X100.

Fig.4. 1.5% Agrose gel showing PCR product of *B. caballi* amplified from horses′ blood spots in El-Dein, using *B. caballi* primer, lane (M) molecular marker 100bp, lanes 14,15,16,17,18 and 19 positive *B. caballi* DNA. Lanes 1, 2, 3,…, 13 and 20 negative for *B. caballi* DNA. Size of amplificon = 470 bp.

Discussion

Most studies on equine piroplasmosis have dealt with horses and little is known about piroplasmosis in donkeys. Several direct and indirect detection methods, including blood smears (Retief, 1964), *in-vitro* cultures (Holman *et al*, 1993), DNA probes (Posnett and Ambrosio, 1991), serology (Böse and Peymann, 1994; Brüning *et al*, 1997) and PCR (Salim, 2008), have been used for the diagnosis of *B. caballi* and *T. equi* infections.

T. equi may be found for years in red blood cells, albeit in very limited numbers (Mehlhorn and Schein, 1998). On the other hand, equines may remain carriers for *B. caball* up to four years (Ayele *et al*, 2013). In the present study, out of 481 equine blood smears 10 (2.08%) were positive for *T. equi*, four horses (2.14%) and six donkeys (2.04%) constituting a prevalence rate of 4.11%in Nyala and 2.27% in Kateila. Concerning *B. caballi* piroplasm, an only one (0.34%) sample was positive; this sample was from a clinically sick donkey in Nyala. Previous studies on equine piroplasmosis in the Sudan using blood smears revealed a 20% prevalence rate in Khartoum (Abdoon, 1984); 8.3% for *T. equi*, and none for *B. caballi* (Salim, 2008). *B. caballi* is extremely difficult to detect in blood smears at any stage of the disease except the early acute phase of infection (Frerichs *et al*, 1969). Similarly, Todorovic and Carson (1981) reported that after establishment of the carrier state, there may be a complete absence of circulating parasites in the blood stream. It should be stressed that the microscopic diagnosis of *B. caballi* and *T. equi* by thin blood smears lacks enough sensitivity to give an accurate result in cases of low parasitaemia in carrier horses, which obviously leads to false negative results (Hodgson, 2002). Maltase cross form of *T. equi* was observed in most positive samples.The 'Maltase Cross' appearance in some developmental stages of *T. equi* is regarded as definitive evidence for its diagnosis (OIE, 2000).

In this study, *T. equi* DNA was detected in 18 (19.8%) samples (3.9% and 23.6% from horses and donkeys, respectively), while *B. caballi* DNA was detected in 12 (33.3%) horse samples only. This finding is in line with Salim (2008) in case of *T. equi* (25.2%), but he couldn't detect *B. caballi* DNA in horse samples. This difference could be attributed to the tick spectrum variation between the two study areas and/or differences in persistence of tick vectors transmission and climatic condition between the two study areas.

Transmission of piroplasms is usually influenced by the dynamics of vector populations, and these are directly influenced by climatic conditions. Altogether 12 species of ixodid ticks of the genera *Dermacentor*, *Hyalomma*, and *Rhipicephalus* were listed as vectors for equine piroplasmosis (OIE, 2008). However, the development of *T. equi* in the salivary glands was studied in detail only in *R. e. evertsi*, *R. turanicus*, *R. microplus*, *H. detritum*, and *H. excavatum*. The vector capacity for *B. caballi* was proven for *D. nitens*, *D. marginatus*, *D. albipictus*, *D. silvarum*, and *H. plumbeum* (de Waal and van Heerden, 2004). In west Shoa and Sidamo areas in Ethiopia, two confirmed cases of equine babesiosis in horses transmitted by *R. e. evertsi* were reported by Pegram *et al* (1981). Walker *et al*. (2003) have described *R. e. evertsi* as potential vector of *B. caballi* and *T.equi* of equines and *H. truncatum* as a potential vector of *B. caballi*, and *R. decoloratus* as a vector of *Borrelia theileri* in horses and ruminants. Abdoon (1984) and Salim (2008) confirmed that the biological vector of equine piroplasmosis in Sudan was *H.anatolicum,* the most abundant tick in their study area. In South Darfur State other tick species such as *R. decoloratus*, *R. annulatus*, *R. e. evertsi*, *H. detritum (H. scupense*), *H. truncatum* and *H. excavatum* might be responsible for transmission of equine piroplasmosis mainly *B. caballi* (Osman, 2015).

It is concluded that equine piroplasmosis represents an important disease in South Darfur State. The conventional parasitological technique revealed a very low prevalence rate. This method is useful in detecting the parasites in individual clinical cases and outbreaks as the parasites appear in the blood during the acute stage. Molecular techniques, as clearly indicated by this study, have a better potentiality in revealing the most real prevalence and endemicity of the disease. More

studies are needed to elucidate the association between the tick populations present in the study area, the disease prevalence and hypothesized risk factors to highlight a clear enzootic picture of the disease. These will definitely help in drawing a suitable control strategy.

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