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Original papers

Molecular identification of different *Theileria* and *Babesia* species infecting sheep in Sudan

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ABSTRACT. The epidemiological aspects of sheep piroplasmosis in Sudan are poorly studied, and further investigations using sensitive and precise techniques are required. In this study, the Reverse Line Blot (RLB) hybridization assay was used to detect and simultaneously differentiate between *Theileria* and *Babesia* species. DNA was extracted from blood collected on filter paper (n=219) from apparently healthy sheep from six different geographical localities in Sudan. Results indicated that *Theileria ovis* (88.6%), *T. separata* (20.1%), *T. lestoquardi* (16.4%) and *T. annulata* (16.4%) DNA could be detected in the blood samples. Single and mixed *Theileria* infections were detected in 74 (33.8%) and 124 (56.6%) respectively and *T. ovis* being the most prevalent species in the country. *T. ovis* and *T. separata* were reported for the first time in sheep in Sudan.

Key words: Reverse Line Blot hybridization assay, Sudan, *Theileria annulata*, *Theileria lestoquardi*, *Theileria ovis*, *Theileria separata*

Introduction

Piroplasms are protozoan parasites of the phylum Apicomplexa, which are differentiated into two genera, *Theileria* and *Babesia*. Piroplasmosis caused by the different species of *Theileria* and *Babesia* are a major constraint of small ruminant production in Asia, Africa and Southern Europe [1]. The pathogenic *Theileria* spp. infecting small ruminants are *Theileria lestoquardi* [2], *T. luwenshuni* and *T. uilenbergi* [3] and other low or non-pathogenic are, *T. ovis, T. recondita* and *T. separata* [4].

Ovine babesiosis is caused by *Babesia ovis* [5,6], *B. motasi* [5,7], *B. crassa* [8,9], *B. foliata*, *B. taylori*, *Babesia* sp. (China) [10] and *Babesia* sp. (Xinjiang) [11]. In Sudan, the reported piroplasms infecting sheep are *T. lestoquardi* and *T. annulata* and were widespread in different localities with variable prevalence rates [12–14].

The method traditionally used for detection and identification of sheep piroplasmosis is based on the clinical findings and microscopic examination of blood and lymph node smears stained with Giemsa's stain [15,16]. These methods are reliable for the detection of acute cases but have limited value particularly in subclinical or chronic cases, where low parasitaemia and/or mixed infection exist. The immunofluorescence antibody test (IFAT) has been described to determine subclinical infections [12,15,17]. However, the vast limitations of IFAT hinder its routine use in epidemiological surveys [18]. These limitations are mainly due to its tediousness, the subjectivity of the results obtained

Table 1. Localities of sheep blood samples collection

Locality	No. of samples	E (degree/minute)	N (degree/minute)
Atbara	57	34° 02’	17° 40’
Khartoum	36	32° 32’	15° 38’
Kosti	30	32° 40’	13° 10’
Medani	35	33° 30’	14° 31’
Damazin	33	34° 18’	11° 52’
Nyala	28	24° 55’	12° 05’
Total	219		

E = East, N = North

and the cross-reactivity against other tick borne-diseases [14] especially in regions where animals are infected with different piroplasms [19]. Alternatively, enzyme linked immunosorbent assay (ELISA) have been developed [20], but its false results and cross-reactivity with other pathogens cannot be ruled [21]. Conventional polymerase chain reaction (PCR) is commonly used to detect ovine theileriosis in epidemiological studies [21,22] but impractical to discriminate between mixed infections and less sensitive to detect subclinical infections [23]. In order to overcome these limitations a reverse line blot (RLB) hybridization assay was developed for simultaneous detection and differentiation between different piroplasms [24], rickettsia and protozoa [25–28]. The small subunit ribosomal RNA gene (18S rRNA) has been successfully used to improve the detection, identification and the classification of *Theileria* and *Babesia* species [29–34].

To date, few PCR-based techniques are described in Sudan for research purpose, but the field application of these techniques is not documented to diagnose small ruminant piroplasmosis.

The objective of the present study was to detect, identify and to discriminate between different *Theileria* and *Babesia* spp. infecting sheep using the RLB hybridization assay.

Materials and Methods

Blood samples. On filter paper, blood spots were collected from 219 apparently healthy sheep from six different ecosystems in the Sudan (Table 1). The filter papers were air-dried, labelled (indicating locality, date of collection and sheep number), separately placed in nylon bag and were stapled. The samples were, then, stored at 4°C until used.

DNA extraction. DNA was extracted from the blood spots using the QIAamp DNA Extraction Kit (QIAGEN, Southern Cross Biotechnologies), following the manufacturer’s protocols. The extracted DNA was eluted and stored at –20°C. The DNA purity and concentration was determined by agarose gel electrophoresis and spectrophotometer.

Reverse Line Blot (RLB) hybridization assay. For the amplification of the V4 hyper variable region of the parasite 18S rRNA gene, the following PCR protocol was performed: In a 0.2 ml PCR tube, 5.0 µl DNA, 12.5 µl UDG-mix (Platinum® Quantitative PCR SuperMix, Invitrogen™ , USA), and 20 pmol of each primer (Isogen, Maarssen, The Netherlands): RLB F_2 (5’-GAC ACA GGG AGG TAG TGA CAA G-3’) and biotin labeled RLB R_2 (5’-Biotin-CTA AGA ATT TCA CCT CTA ACA GT-3’) were used [35,36] and made up to total volume of 25 µl using nuclease-free water. Amplification was performed in an automated thermocycler according to the *Babesia/Theileria* touchdown PCR programme [32]. A *T. parva* positive buffalo DNA sample, 102 [37], and nuclease-free water were used as positive and negative controls, respectively. After PCR amplification, 5 µl of the PCR product was examined on a 2% agarose gel stained with ethidium-bromide and visualized on an ultra-violet transilluminator.

In-house membrane, the relevant *Theileria* and *Babesia* genus- and species-specific probes (Table 2) was prepared. The final PCR products were then analysed using the RLB hybridization technique as previously described [38].

Results

The RLB results obtained from 219 sheep blood samples collected from six different localities in the Sudan are demonstrated in Table 3. Single and mixed *Theileria* infections were detected in 74 (33.8%) and 124 (56.6%), respectively. The

Table 2. List of genus- and species-specific probes used in the RLB hybridization assay

Oligonucleotide probe	Sequence (5'–3')	Source
Theileria/Babesia genus-specific	ATT AGA GTG TTT CAA GCA GAC	[a]Nijhof (unpublished)
Theileria genus-specific	TAA TGG TTA ATA GGA **RCR** GTT G	Gubbels et al.,1999[24]
Babesia genus-specific 1	ATT AGA GTG TTT CAA GCA GAC	[a]Nijhof (unpublished)
Babesia genus-specific 2	ACT AGA GTG TTT CAA ACA GGC	[a]Nijhof (unpublished)
Babesia bicornis	TTG GTA AAT CGC CTT GGT C	Nijhof et al., 2003[35]
Babesia bigemina	CGT TTT TTC CCT TTT GTT GG	Gubbels et al.,1999[24]
Babesia bovis	CAG GTT TCG CCT GTA TAA TTG AG	Gubbels et al.,1999[24]
Babesia caballi	GTG TTT ATC GCA GAC TTT TGT	Butler et al., 2008[60]
Babesia canis	TGC GTT GAC GGT TTG AC	Matjila et al., 2004[61]
Babesia divergens	ACT **R**AT GTC GAG ATT GCA C	Nijhof et al.. 2003[35]
Babesia felis	TTA TGC GTT TTC CGA CTG GC	Bosman et al.. 2007[62]
Babesia gibsoni	CAT CCC TCT GGT TAA TTT G	Zahler et al., 2000[63]
Babesia leo	ATC TTG TTG CCT GCA GCT T	Penzhorn et al., 2001[64]
Babesia major	TCC GAC TTT GGT TGG TGT	Georges et al.,2001[25]
Babesia microti	G**R**C TTG GCA TC**W** TCT GGA	Nijhof et al., 2003[35]
Babesia occultans	CCT CTT TGG CCC ATC TCG	Oosthuizen et al.,2008 [31]
Babesia rossi	CGG TTT GTT GCC TTT GTG	Matjila et al., 2004[61]
Babesia sp. *(sable)*	GCG TTG ACT TTG TGT CTT TAG C	Oosthuizen et al., 2008[31]
Babesia vogeli	AGC GTG TTC GAG TTT GCC	Matjila et al., 2004[61]
Theileria annae	CCG AAC GTA ATT TTA TTG ATT TG	Matjila et al., 2008[65]
Theileria annulata	CCT CTG GGG TCT GTG CA	Gubbels et al.,1999[24]
Theileria bicornis	GCG TTG TGG CTT TTT TCT G	Nijhof et al.,2003[35]
Theileria buffeli	GGC TTA TTT CGG **W**TT GAT TTT	Gubbels et al., 2000[66]
Theileria equi	TTC GTT GAC TGC GYT TGG	Butler et al., 2008[60]
Theileria lestoquardi	CTT GTG TCC CTC CGG G	Schnittger et al.,2004 [28]
Theileria mutans	CTT GCG TCT CCG AAT GTT	Gubbels et al.,1999[24]
Theileria ovis	TTG CTT TTG CTC CTT TAC GAG	Schnittger et al.,2004 [28]
Theileria parva	GGA CGG AGT TCG CTT TG	Gubbels et al.,1999[24]
Theileria separate	GGT CGT GGT TTT CCT CGT	Schnittger et al.,2004 [28]
Theileria sp. *(buffalo)*	CAG ACG GAG TTT ACT TTG T	Oura et al., 2004[67]
Theileria sp. *(kudu)*	CTG CAT TGT TTC TTT CCT TTG	Nijhof et al., 2005[36]
Theileria sp. *(sable)*	GCT GCA TTG CCT TTT CTC C	Nijhof et al., 2005[36]
Theileria taurotragi	TCT TGG CAC GTG GCT TTT	Gubbels et al.,1999[24]
Theileria velifera	CCT ATT CTC CTT TAC GAG T	Gubbels et al.,1999[24]

[a]Dr. Ard M. Nijhof (Institut für Parasitologie und Tropenveterinärmedizin (IPTVM), Freie Universität Berlin, Germany); symbols in bold indicate degenerate positions: R=A/G, W=A/T

prevalence of the detected *Theileria* species are: *Theileria ovis* 194/219 (88.6%), *T. separata* 44/219 (20.1%), *T. lestoquardi* 36/219 (16.4%) and *T. annulata* 36/219 (16.4%). All probes bound only to their respective target species, except probes positive to *T. lestoquardi* that 100% contempo - raneously reacted with *T. annulata* and *T. lestoquardi* (Fig. 1) and these were detected in two localities (Atbara and Khartoum). *Theileria ovis* was detected in all the localities, whereas, *T. separata* was detected in four localities: Damazin 23/219 (10.5%), Khartoum 17/219 (7.8%), Kosti 2/219 (0.9%) and Medani 2/219 (0.9%). No *Babesia* species were detected in any of the samples.

Discussion

The RLB assay is a powerful tool and a practical assay since it is able to detect extremely low levels of parasitemia (10^{-6} %; corresponding to 3 parasites per ml of blood) [24] and simultaneously discriminate *Theileria* and *Babesia* species [24,28]. In this study, the RLB assay is used for the first time in Sudan to detect, identify and discriminate different ovine *Theileria* spp. The cross reaction of T. *lestoquardi* specific probe with *T. annulata* probe had been previously reported [29,38–41]. In fact, *T. lestoquardi* and *T. annulata* are exhibited a strong serological cross-reactivity [15], similar morphology [42,43], share the same *H. anatolicum* vector [44,45] and parasitize the same cell phenotypes of

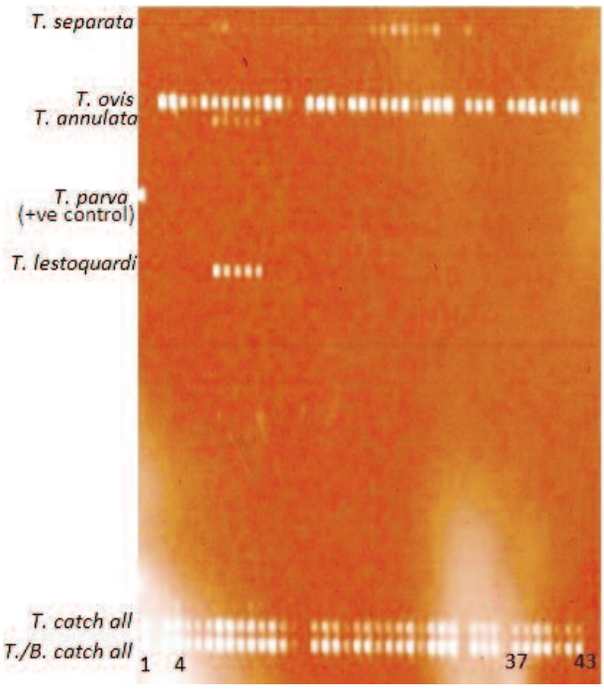

Fig. 1. The X-ray image from the plotting RLB membrane showed *T. annulata* and *T. lestoquardi* cross reactions and *T. ovis* mixed infections

their respective hosts [46]. In addition, *T. annulata* is capable of infecting and transforming ovine and caprine peripheral blood monocytes *in vivo* [47] and *in vitro* [48]. In most cases, their geographic distribution tends to overlap [44]. Moreover, the phylogenetic analyses based on small subunit ribosomal RNA gene and sporozoites surface antigen inferred their close (99.7%) identity [49]. Thus, the present study confirmed the existence of cross-reaction between *T. lestoquardi* and *T. annulata* and, also, pointed to a closer antigenic relationship. However, Taha et al. [14] recently reported on the occurrence of natural *T. annulata* infection in sheep in Atbara area; hence some of the amplicons detected herein could represent authentic *T. annulata* sequences. Therefore, the current results demonstrated that at least three distinct ovine *Theileria* species (*T. ovis*, *T. lestoquardi* and *T. separata*) are detected in sheep in the Sudan. Particularly, *T. lestoquardi* infection is expected, since this parasite had been previously reported in the Sudan [12,14,17,50,51]. While detection of *T. separata* and *T. ovis* in field samples from apparently healthy sheep are reported for the first time in the country. In this respect, the differentiation between malignant and benign *Theileria* spp. by conventional microscopic examination is very difficult. Therefore, the prevalence and surveillance of *T. lestoquardi* in the Sudan by conventional microscopy examination of blood smears from small ruminant is not reliable and is subjective. Thus, sensitive and specific

Table 3. Occurrence of *Theileria* and *Babesia* species infections in sheep blood samples from Sudan as determined by the RLB hybridization assay (n = number of samples)

	Atbara (n = 57)	Khartoum (n = 36)	Kosti (n = 30)	Medani (n = 35)	Damazin (n = 33)	Nyala (n = 28)	TOTAL (n = 219)
Single infections	**0 (0%)**	**0 (0%)**	**14 (46.7%)**	**30 (85.7%)**	**3 (9.1%)**	**27 (96.4%)**	**74 (33.8%)**
T. annulata	0	0	0	0	0	0	0 (0%)
T. lestoquardi	0	0	0	0	0	0	0 (0%)
T. ovis	0	0	14	30	3	27	74 (33.8%)
T. separata	0	0	0	0	0	0	0 (0%)
Mixed infection	**57 (100%)**	**36 (100%)**	**4 (13.3%)**	**2 (5.7%)**	**25 (75.7%)**	**0 (0%)**	**124 (56.6%)**
T. annulata	19	17	0	0	0	0	36 (16.4%)
T. lestoquardi	19	17	0	0	0	0	36 (16.4%)
T. ovis	57	36	2	2	23	0	120 (54.8%)
T. separata	0	17	2	2	23	0	44 (20.1%)
Negative/below detection limit	**0 (0%)**	**0 (0%)**	**12 (40%)**	**3 (8.6%)**	**5 (15.2%)**	**1 (3.6%)**	**21 (9.6%)**

laboratory tests could clearly differentiate and discriminate between pathogenic and non-pathogenic ovine *Theileria* species are essential. Although the RLB assay described here could be useful technique especially in mixed infections but it requires sophisticated laboratory equipment, complex protocol and involves hybridization to achieve higher sensitivity.

Theileria ovis is considered to be widely distributed in Africa, Asia and in Europe, partially corresponding with that of *T. lestoquardi*, whereas *T. separata* has been reported from more limited areas, including several countries in Southern and Eastern Africa [52]. The high prevalence of *T. ovis* (88.6%) in the present investigation was not surprising, since high prevalence of this *Theileria* spp. was detected in Spain (18.9%) [53], Turkey (54.0 to 67.9%) [21,54] and Iran (7%) [55]. On the other hand, there is no *Babesia* species detected in all examined samples, this could be explain by the fact that, we used 15 oligonucleotide probes not specific for *Babesia ovis*, *B. motasi*, *B. crassa*, *B. foliata, Babesia* sp. (China), *Babesia* sp. (Xinjiang) and *B. taylori* which are previously reported in sheep [5–11].

Small ruminants are invariably exposed to *H. anatolicum* infestations in Sudan and *T. lestoquardi* and *T. annulata* infections are widespread with variable prevalence rates [12–14,17]. Since the 1980s, this vector tick has been thriving in semi-desert conditions in Northern Sudan [56]; its distribution is alarming and has attracted increasing attention in recent years [57]. In addition *Rhipicephalus evertsi*, the vector of *T. ovis* and *T. separata* is ubiquitous in Sudan [58,59], suggesting that these parasites are more common than previously thought.

Conclusions

The RLB has been used to detect, identify and to discriminate *T. lestoquardi, T. ovis* and *T. separata* in the Sudan, the latter two species are reported for the first time and mixed infections were frequently detected. The available probes based on the 18S rRNA gene used in the current RLB indicate the cross-reaction between *T. lestoquardi* and *T. annulata.*

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