Original Research Article

Prevalence ,FirstMolecular Identification and Characterization of *Theilerialestoquardi* in Sheep in Alhuda National Sheep Research Station, AL Gezira State, Sudan

ABSTRACT

Aims: The present study investigated the presence of *T. lestoquardi*in AlhudaNational Sheep Research Stationusingthree diagnostic techniques, identified and characterized the parasite.

Methodology: Forty-five animals from three Sudanese sheep ecotypes were selected randomly during September 2013. Blood and sera samples were examined by microscopy, indirect fluorescent antibody test (IFAT) and polymerase chain reaction (PCR). The PCR product was sequenced to confirm the identity of the parasite.

Results: Microscopic examination (ME) revealed the presence of *Theileria*spp. piroplasms in 31 animals out of 45 (68.9%).Sixteen sera samples out of the 45 were tested for antibodies against T. les , fourteen of these (87.5%) were found positiveby IFAT. PCR targeting*Theilerialestoquardi*merozoite/piroplasm surface antigen (TIms) was positive in 4/45 (8.9%). Prevalence of the disease was significantly higher (P<.05) in females and in >1 year age group by ME. Sequence analysis confirmed the species identity when compared with reference sequences retrieved from GenBank Database and indicated 99% homology. Multiple sequence alignment of Alhuda strain with other *T. lestoquardi*sequences obtained from Sudan and Iran revealed five silent base pair substitutions. Phylogenetic tree showed clustering of Alhuda strain (accession no. KY965145) with other *T. lestoquardi* sequences in one distinct clade.

Conclusion: To the best of our knowledge this is the first report on molecular identification and characterization of *T. lestoquardi*from Al Gezira State, Sudan. The disease could adversely affect the animal population in the state therefore epidemiological surveys are required to build up data necessary for designing efficient control programs.

Keywords:Theilerialestoquardi; sheep; molecular characterization; prevalence; Sudan.

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1. INTRODUCTION

Ticks and tick-borne diseases are considered to be a major obstacle to livestock production in many countries [1]. Theileriasis are important intra-cellular pathogens transmitted by ixodid ticks, which infect wild and domestic ruminants [2]. Several species of *Theileria*infect sheep of these *Theilerialestoquardi*, *Theileriauilenbergi* and *Theilerialuwenshuni*are considered pathogenic while infection with*Theileria. ovis, Theileria. recondita* and *Theileria. Separate* results in low or mild disease. *Theilerialestoquardi*is the causative agent of malignant ovine theileriosis(MOT).In the Sudan the disease is economically important, as sheep play a vital role in the national economy and in the livelihood of farmers.High morbidity and mortality rates were recorded during outbreaks [3 ,4].*Hyalommaanatolicom,* the most efficient transmitting vector of (MOT) [5],has adapted itself to the desert condition of the Sudan and had been reported throughout the year in different parts of the country [6 ,7]..
Infected animals show clinical symptoms which include fever ,enlargement of superficial lymph nodes, respiratory manifestations andjaundice.
Diagnosis of the disease depends on detection of piroplasmsand schizontsin blood smear and lymph node biopsy respectively.In addition sensitive serological and molecular techniques such as IFAT and PCR have been applied for more accurate diagnosis of *T. lestoquardi*infection [9,10].
The aims of the present study were to investigate the presence of *T. lestoquardi*in ANSRSand to elucidate the different risk factors associated with the infection in order to formulate a sound control program of sheep theileriosis in Alhuda Station.In addition molecular identification of the parasite was carried out.

2. MATERIAL AND METHODS

2.1. Study area

Alhuda National Sheep Research Station is located in Alhuda town (Fig. 1) which lies at approximately 14°55'Nlatitude, 32°91 'Elongitude andabout150 kmSouthofKhartoum Capital of Sudan.In general the climate in Al Gezira is dry with a short rainy season from July to September (Mohamed, 2013). Suliman et al., (1990) reported that the mean maximum temperature recorded was 36.7℃ and a mean minimum temperature was 18.2℃.The total area of the station is about 150 hectarsof clay plain, of which 100 hectares are exploited for growing fodder.The station is mandated to undertake research on three ecotypes of Sudan desert sheep,. The three ecotypes are separated to prevent cross breeding and are divided into four functional groups; breeding ewes, suckling ewes with lambs,weaned lambs and flock with rams.Suckling and breeding ewes are usually fenced and fed in pens specially concentrate, while the other two groups graze on fodders and natural range(suliman et al,1990).

2.2. Samples and animals sampled

In September 2013, ANSRS was surveyed for T.lest, and samples of blood were collected from the three ecotypesi.eDubasi ,shogur and Watish.Animals were selected randomly to include both sexes and two age groups(<1year and >1 year). Blood samples (45) were drained in both EDTA and plain tubes for blood smears and sera preparation respectively. Blood spotted on (Whatmann #1)filter paper was immediately prepared in the field for PCR.

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2.3. Microscopic examination (ME)

Thin blood smears were air dried, fixed in absolute methanol for three minutes, stained with10% Giemsa's stain for 45 minutes and examined at 100X oil immersion lens (Olympus CX21). At least 50 fields were searched for the presence of *Theileria*piroplasms.

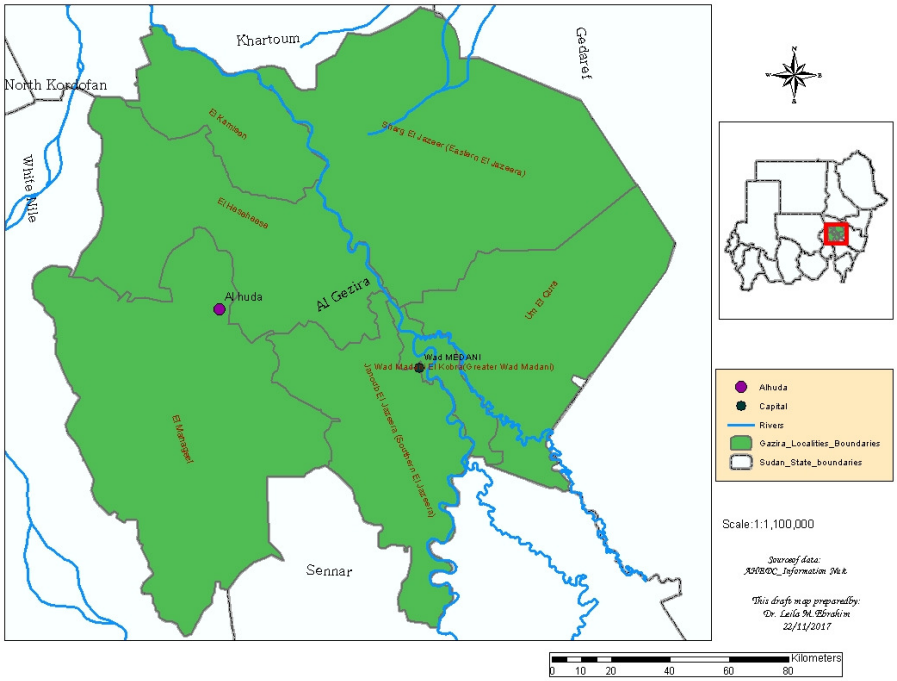

Fig.1.Map of Al Gezira State showing Alhuda town location.

2.4. Indirect fluorescent antibody test (IFAT)

The test was performed according to procedure described by FAO and Salih *et al.*[11,9]. Blood was drained from the jugular vein of animal using sterile syringe, collected in sterile plain vacutainer tube and stored at 4 ℃ overnight. The clotted blood was centrifuged at 2000 rpm for 10 minutes to separate the sera which were transferred to sterile eppendorf tubes these were kept at -20℃ until used. IFA test was carried out following the method described by FAO (1984) and .salih, 2003.In brief schizont antigens were prepared from *T.lestoquardi* cell line (CVRL soba, passage 85). Small drop of the antigen suspension was applied to each well of the IFA slide (Immune fluorescein antibody slide, Germany) .The slides were allowed to dry, fixed in acetone for 10 minutes, and then wrapped as individual slide in tissue paper.and store at -20℃ for later use. Serum from sheep experimentally infected with *T.lestoquardi* at Central Veterinary Research Laboratory (Soba) was used as control ,while negative control was obtained from the same sheep before infection.

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First the antigen slides were allowed to thaw at 4℃ for 30 minutes, then placed in room temperature for another 30 minutes. Each well was labeled with animal number and twowells were assigned for positive and negative controls. All sera were diluted 1/80 in PBS buffer, then added to the slides and incubated for 30min at 37℃.The slide then washed two times in PBS for 10 minutes in a shaker. Anti-sheep immune gamma globulin IgG conjugate was diluted 1/10 in PBS and mixed with 0.1% Evans blue were added to each well and incubated at 37℃ for 30 minutes in dark and washed with PBS as above. Each well was mounted by 10 µl glycerol covered by cover slide and examined under 40X by immune fluorescent microscope (Germany).

2.5. Deoxyribonucleic acid (DNA) extraction and Polymerase Chain Reaction (PCR)

DNA was extracted from blood spotted on filter paper by phenol/chloroform extraction method as described bySambrook, *et al.* [12] and stored at -20 ºC until used. DNA quality and quantity were assessed by Nano drop spectrophotometer (ND1000, spectrophotometer, Nanodroptechnologies, I NC.)

To amplify the Tlms gene, PCR reaction was as follows: 10 pmol*T. lestoquardi* specific forward primer TLF 5′GTGCCGCAAGTGAGTCA 3′and reserve primer TLR 5′-GGACTGATGAGAAGACGAT GAG-3′ [10] were added to Maxime PCR Premix (i-taq-iNt RONBIOTECHNOLOGY, Korea). Five µl genomic DNA and 150µg/ml of enhancer (Bovine serum albumin acetylated) were added to final reaction volume of 20µl. The reaction was performed in a thermal cycler (Applied Bio system, USA) and the thermal cycling conditions were as described by Taha*et al.* [13]. Five µl of each PCR product were visualized in 1.5% ethidium bromide stained gel and run in 1X TBE buffer in electrophoresis chamber.

2.5.1. Sequence analysis

PCR product which showed sharp band was sent to Macrogen Company, Korea, for sequencing. The sequence was compared with GenBank database by nucleotide sequence homology search made at the network server of the National Centre for Biotechnology Information (NCBI) using BLASTn. Sequence has been deposited at GenBank and given the accession number KY965145. Multiple sequence alignment was performed using CLUSTAL multiple sequence alignment by MUSCLE (3.8) online tool.

2.5.2. Phylogenetic analysis

Phylogenetic analysis of the Tlms gene of Alhuda strain and other sequences registered in the GenBank was conducted with MEGA 6.0 software. The Neighbor-Joining Method was used to generate a phylogenetic tree [14,15]. The tree was tested using a bootstrapping method (1000 replicates).

2.6. Statistical analysis

Data were analyzed using statistical package for social sciences (SPSS) version 20.
The differences between variables were checked using the Chi-Square test the probability value less than 0.05 was considered signicant.

3.Results

Theileria spp. piroplasms were detected in 31 out of 45 animals (68.9%) using ME (Table, 1). On the other hand, 14 out of 16 (87.5 %) samples were found positive for T. lestoquardi antibodies by IFAT (Table, 2). Regarding PCR results, T. lestoquardi DNA was detected in 4

out of 45 animals (8.9%), (Table, 2). Microscopic examination showed high prevalence (P < .05) of Theileria spp. in females and adult sheep (>1year), while no significant differences (P > .05) in T. lestoquardi prevalence rates were recorded between sexes and age groups by IFAT and PCR. The three sheep subtypes included in this study showed no significant difference in the prevalence of the disease using the three diagnostic tests.

Blasting AlhudaTlms sequence confirmed the identity of the parasite. Multiple sequence alignment of Alhuda sequence with selected Tlms sequences showed high a similarity to both Sudan and Lahar strains(99.13%) and to Kamalabad and vaccine strains(98.79%). Also the alignment revealed five base pair substitutions in Alhuda sequence (shown in Fig. 2)however these substitutions did not result in amino changes.

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Table, 1: Prevalence of *Theileria spp*. using microscopic examination of blood smears during October/2013

Risk factors	NO. of animal examined	NO. of animals positive for Theileria spp.	% Theieriaspp positive animals
Animal sex			
Female	24	20	83.3
Male	21	11	52.4
Total	45	31	68.9
Animal age			
<1year	16	6	37.5
>1 year	29	25	86.2
Total	45	31	68.9
Animal ecotype			
Dubasi	12	10	83.3
Shogur	21	13	61.9
Watish	12	8	66.7
Total	45	31	68.9

Sex (X^2=5.007 , df= 1, P<.05) , Age (X^2= 11.4136 , df= 1 , P< .05) and ecotype (X^2= 3.048, df= 2, P >.05

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A phylogenetic tree was constructed using the sequence obtained in this study with selected sequences of previously identified merozoite antigens gene of T. lestoquardi and other Theileria species. The structure of the tree derived by neighbor joining analysis (Fig. 3) indicated that T. parva, T. annulata and T. lestoquardi originated from a common ancestor with T. annulata and T. lestoquardi closely related. Also the tree showed clustering of Alhuda strain with other T. lestoquardi sequences in one distinct clade.

Table 2: Prevalence of *Theilerialestoquardi* antibodies using IFA test; and *T. lestoquardi* DNA using PCR

	No. of animals examined	No. of *T.lestoquardi* Positive animals	% *T.lestoquardi* positive animals	No. of animals examined	No. of *T.lestoquardi* Positive animals	*T.lestoquardi* Positive animal%
Animal sex						
Female	10	10	100.0	24	3	12.5
Male	6	4	66.7	21	1	4.8
Total	16	14	87.5	45	4	8.9
Animal age						

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<1year	7	6	85.7	16	1	6.2
>1year	9	8	88.9	29	3	10.3
Total	16	14	87.5	45	4	8.9
Animal ecotype						
Dubasi	4	3	75	12	0	0
Shogur	9	9	100	21	2	9.5
Watish	3	2	66.7	12	2	16.7
Total	16	14	87.5	45	4	8.9

IFIFA test :sex (X2= 1.3402, df=1 , P>.05), age (X2= .0363, df= 1, P>0.05) and ecotype (X2= 7.213 , df= 2 , P>.05). PCR : sex (X2= 0.8281 , df= 1 , P>.05), age (X2= 0.2135 , df= 1, P>.05) and ecotype (X2= , df= , P>.05)

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Alhuda          AACTAGTTGTCAAAGACGGCTTCCGTTTCAAGACCCTTAAGGTTGGAGACAAGACCTTAT
Sudan           AACTACTTGTCAAAGACGGCTTCCGTTTCAAGACCCTTAAGGTTGGAGACAAGACCTTAT
Lahr            AACTACTTGTCAAAGACGGCTTCCGTTTCAAGACCCTTAAGGTTGGAGACAAGACCTTAT
vaccine         AACTACTTGTCAAAGACGGCTTCCGTTTCAAGACCCTTAAGGTTGGAGACAAGACCTTAT
Kamalabad       AACTACTTGTCAAAGACGGCTTCCGTTTCAAGACCCTTAAGGTTGGAGACAAGACCTTAT
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Alhuda          ACAATGTAGACACTTCAAAACATACCCCAGTACGGGCATTCAAACTTAAGCATGATTCCG
Sudan           ACAATGTAGACACTTCAAAACATACCCCAGTACAAGCATTCAAACTTAAGCATGATTCCG
Lahr            ACAATGTAGACACTTCAAAACATACCCCAGTACAAGCATTCAAACTTAAGCATGATTCCG
vaccine         ACAATGTAGACACTTCAAAACATACCCCAGTACAAGCATTCAAACTTAAGCATGATTCCG
Kamalabad       ACAATGTAGACACTTCAAAACATACCCCAGTACAAGCATTCAAACTTAAGCATGATTCCG
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Alhuda          AGGAGTGGTTCAGACTTGATCTTCACGCTGCCCAGCCAAAGATGTTCAAGAAGACTGGAG
Sudan           AGGAGTGGTTCAGACTTAATCTTCACCCTGCCCAGCCAAAGATGTTCAAGAAGACTGGAG
Lahr            AGGAGTGGTTCAGACTTAATCTTCACCCTGCCCAGCCAAAGATGTTCAAGAAGACTGGAG
vaccine         AGGAGTGGTTCAGACTTAATCTTCACCCTGCCCAGCCAAAGATGTTCAAGAAGACTGGAG
Kamalabad       AGGAGTGGTTCAGACTTAATCTTCACCCTGCCCAGCCAAAGATGTTCAAGAAGACTGGAG
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Fig.2. Part of alignment of Alhuda sequence with Sudanese and Iranian strains using CLUSTAL W.*represents identity, dot represents a gap and bold underlined letter represents substituted nucleotide identity, dot represents a gap and bold underlined letter represents substituted nucleotide

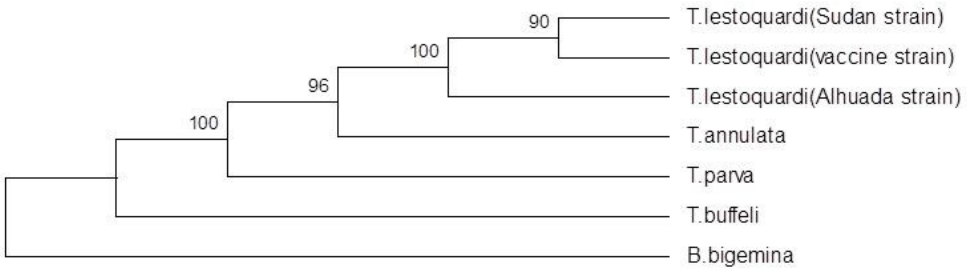

Fig.3. Phylogenetic tree inferred by the neighbor joining analysis. The tree is based on Tlms homologues of: T.Lestoquardi sequences: Alhuda strain, KY965145, Sudan strain AF004775.2, vaccine strain EF092917, T.annulata Z48739.1, T.buffeli D87207.1, T.parva L47209.1. The tree is rooted with the B.bigemina (KC894416.1) sequence. Numbers at each node represent the percentage replicate tree in bootstrap test (1000 replicates).

Discussion

In this survey, *Theileria* spp. were detected in 31 animals out of 45 (68.9%) by ME, which was similar to the rate reported by Gadelrb [16] in Khartoum State, Central Sudan (68%) but higher than that found by Osman et al. [17], South-Darfur State, western, Sudan (4.9%). Different prevalence rates were recorded in different parts of the world; 29% in Iran [18], 4.8% in Tunisia [19] and 1.2% in Maghreb [20]. The high prevalence rate determined in the current study compared to other regions may be attributed to differences in climatic conditions and sample collection times as observed by Yaghfoori [21]. Additionally, microscopic examination might not reflect the real prevalence rate of T. lestoquardi, as these piroplasms are difficult to be distinguished on morphological basis and need much expertise to differentiate between them [22]. A previous survey of T. lestoquardi, in Sudan included Wad-Medani town (Gezira State) using Reverse Line Blot documented a high occurrence of T. ovis [23], this may explain the high prevalence of piroplasms observed in our study.

Several serological surveys of T. lestoquardi were conducted in Sudan using IFAT; Salih et al. [9] reported an overall prevalence rate of 16.2% in main grazing areas of the Sudan. Other studies by Hassan et al. [24] and Osman et al. [17] determined 20.6% and 41% infection rates in Khartoum and South Dafur States respectively. However a high prevalence (87.5%) was recorded in the present study, this may indicate the endemic nature of the infection in this area. PCR confirmed the presence of T. lestoquardi in Gezira state, albeit at lower prevalence rate compared to IFAT. This could be due to the fact that antibodies might represent previous infections [25, 26]. Moreover the low numbers of infected animals detected by PCR could be attributed to the control measures applied before sample collection, in the form of acaricides and treatment with antitheilerial drugs (station animal records).

In the present study, the three Sudanese sheep ecotypes investigated showed no significant differences ($p > 0.05$) in the prevalence rate of T. lestoquardi, while an experimental study by Elimam et al., [4] indicated differences in the susceptibility among Desert, Garaj and watish ecotypes with Desert being the most susceptible ecotype. In this study a higher piroplasm prevalence rate was observed in the age group of >1 year compared to <1 year group. Similarly other studies indicated an increase in the prevalence of Theileria spp. with age [27,

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28]. The low prevalence of theileriosis in young animals could be attributed to the passive transfer of maternal immunity to lambs [27], or to the less exposure time to ticks to produce detectable antibody titers [24]. In the current study females were found significantly prone to infection than males. Similarly Hassan et al., [24] observed that females were more susceptible to infection than males and suggested that this could be due to conditions which might reduce female immunity such as pregnancy and lactation. On the other hand other studies found that age and sex did not statistically affect prevalence of T. lestoquardi infection [29, 30].

Alhuda strain showed close similarity with other Sudanese and Iranian strains, this is in agreement with Katzar et al., [31] who reported a high similarity at nucleotide and amino acid level of T. lestoquardimerozoite surface antigen.

Conclusion

The results of this survey indicate the occurrence of T. lestoquardi in ANSRS for the first time and highlighted the contribution of three risk factors in the existence of the parasite under study. Nevertheless, further studies should be undertaken to clarify other parasites coexisting with T. lestoquardi and to evaluate their effect on animal health. The application of molecular methods in form of specific primers targeting TIms confirmed the identity of the parasite and the conserved nature of T. lestoquardimerozoite gene/antigen; this may suggest its usefulness for inclusion in a recombinant vaccine to control malignant ovine theileriosis in the Sudan. Lastly, survey of tick and tick-borne diseases and T. lestoquardi in particular in the highly animal populated areas such as Gezira State is of great importance for controlling these diseases to improve sheep production in the Sudan.

Competing interests

There is no conflict of interest

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