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Original Article

Molecular Study of Sheep Malignant Theileriosis at Barka Region in the Sultanate of Oman

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ABSTRACT

Background: We used the PCR technique based on the abovementioned primer pair and sequencing to demonstrate the *Theileria* infection in the sheep samples collected from Sultanate of Oman.
Methods: According to the frame work of "integrated control of ticks and tick borne diseases in globalized world managed by EU-ICTTD-3 project, the samples from blood, liver, spleen, lymph node and lung were sent to the laboratory of Iranian Research Center for Ticks and Tick-borne Diseases (IRCTTD). Samples from blood smear and impression smears from liver, spleen, lymph node, and lung were analyzed by Geimsa staining. The DNA was extracted from the abovementioned samples and analyzed by PCR technique using specific primers derived from the nucleotide sequences of 18S rRNA gene of *T. lestoquardi*, which can amplify the common region in other *Theileria* and *Babesia* spp. Subsequently the amplified DNA was sequenced.
Results: The analysis of blood smears of the sheep was negative for piroplasmosis performed through the Giemsa staining. The impression smears prepared from liver, spleen, lymph node, and lung showed suspicious structures mimicking *Theileria* schizonts in some cells. The results showed an expected PCR product of 428 bp in length, which is specific for *Theileria spp*. The PCR products were subsequently sequenced. The corresponding nucleotide sequence is registered under accession number **JF309152** in GenBank. The sequence alignment in GenBank showed that the PCR products had 99% homology to the known *T. lestoquardi* registered under accession number AF081135 in the GenBank.
Conclusion: Oman sheep are highly susceptible for *Theileria* infection and the infected sheep mostly die before the microschizonts or erythrocytic form of *Theileria* appears in the nucleated or erytrocytic cells respectively.
Keyword: Theileria lestoquardi, *DNA, Tick borne disease, PCR,* Theileria china I, II, *Oman*

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Introduction

The tick-borne diseases of livestock constitute a complex of several diseases with the different etiological agents, such as protozoa, rickettsia, bacteria, and viruses. The only common feature between these diseases is that they can all be transmitted by ticks. Theileriosis and Babesiosis belong to this complex and cause diseases in the livestock with high morbidity and mortality and therefore they give rise to the high economical looses worldwide (1-5). For the long time, it was assumed that *T. lestoquardi* is the only pathogenic parasite in small ruminants (6). However, recently a previously identified parasite has been described as a *T. china* I and II *spp*., which are pathogenic for small ruminants as well, causing a fatal diseases of small ruminants distributed in North China (6-10). Interestingly, the purpose of reviews of tick-borne diseases has been increasingly recognized on the world as public health problems. The Geimsa stained of blood smear is the most used method for the identification and characterization of these piroplasms, which accompanied with some technical problems causing false morphological diagnosis and in some cases, needs special diagnostic knowledge. In certain cases serological methods such as the immuno fluorescence antibody test (IFAT) or immunoperoxidase test, have also been applied (4,11-13).

Shayan and Rahbari (2005) showed the DNA extracted from infected blood smear with *Theileria* spp. and *Babesia* spp. could be amplified with the common primer pair derived from hyper variable region V4 of 18S rRNA. They showed the theilerial hypervariable region V4 of 18S rRNA consists of more nucleotides than in *Babesia* spp. Therefore, the application of the reported primer pair could easily and simultaneously differentiate between *Theileria* spp. and *Babesia* spp. (14). The PCR product of *Theileria* spp. and *Babesia* spp. will be then 426-430 bp and 389-402 bp respectively. The difference of ca. 30 bp in the length of PCR product will be easily determinable in 1.8% agarose gel.

In the present study, we used the PCR technique based on the abovementioned primer pair and sequencing to demonstrate the *Theileria* infection in the sheep samples collected from Sultanate of Oman.

Material and Methods

Collection of samples

After lethal diseases in a sheep herd (11dead from 53 sheep) at Rumais (Barka) region in the sultanate of Oman, samples were prepared from the alive and dead sheep. Small pieces of lung, spleen, liver, and lymph node were cut and transferred into the tubes containing 70% ethanol. Additionally, impression smears from the above-mentioned organs were also prepared. The ethanol fixed samples were sent to the Iranian Research Center for Ticks and Tick-borne Diseases for molecular analysis and the impression smears were stained in 10% Geimsa solution in phosphate-buffered saline (PBS, pH of 7.2) at the Sultan Qaboos University, College of Agricultural and Marine Sciences, Department of Animal and Veterinary Sciences. Blood smears were also stained with Geimsa.

DNA extraction

DNA was extracted from lung, liver, spleen and lymph node using DNA extraction kit (MBST, Tehran, Iran) and extracted DNA was stored at -20 ºC until subsequent analysis. Briefly, we cut 3×3 mm from each infected organ (lung, liver, spleen and lymph

node) and was first lysed in 100 µl lysis buffer and the proteins were degraded with 10 µl proteinase K for 10 minutes at 55˚C. After addition of 270 µl Bindings buffer and incubation for 10 minutes at 70˚C, 320 µl ethanol (100%) was added to the solution and after vortexing, the complete volume was transferred to the MBST-column. MBST column was first centrifuged and was then washed twice with 500 µl washing-buffer. Finally, DNA was eluted from the carrier with Elution buffer.

PCR amplification

Two pairs of primers were designed based on the nucleotide sequences of 18S rRNA gene of *T. annulata/lestoquardi* primer sense (5' CAC AGG GAG GTA GTG ACA AG 3') was derived from nucleotide 431 to 450 of complete sequence of 18srRNA gene of *T. lestoquardi* (Accession number AF081135). This area was common from nucleotide 274 to 293 in *T. ovis* (Accession number **GU726904**), from nucleotide 419 to 438 in *B. ovis* (Accession number **AY260178**). Primer antisense (5' CTA AGA ATT TCA CCT CTG ACA3') was derived from nucleotide 838 to 858 of complete sequence of 18srRNA gene of *T. lestoquardi* (Accession.number **AF081135**). This area was common from nucleotide 685 to 705 in *T. ovis* (Accession number **GU726904**), from nucleotide 789 to 809 in *B. ovis* (Accession number **AY260178**).

The PCR was performed in a total reaction volume of 100 µl containing 10 µl of 10X PCR buffer, 3 µl MgCl2 (50 mM), 2 µl of dNTP (10mM each), 0.5 µl Taq DNA polymerase (5 U, Fermentas), 2 µl of each primer (20 µM), 79/5 µl dH2O and 1µl of template DNA (approximately 100 ng). The reaction was repeated for 35 cycles under the following conditions: 5 min at 94°C, 45" at 94°C, 45" at 56°C, 45" at 72°C and finally, PCR was completed with the additional extension step for 10 min. Distilled water was used as negative control in each PCR reaction and the DNA from *T. annulata* was used as positive control. PCR products were separated on 1.8% agarose gel in 0.5× Tris–borate–ethylenediamine tetraacetic acid (EDTA) buffer and visualized using ethidium bromide and an UV illuminator.

PCR product extraction from Agarose gel

The PCR product isolated from the agarose gel using the gel extraction Kit (MBST, Iran) according to the manufacturer's instructions. Briefly, the DNA bands were cut from the gel under UV illumination and dissolved in the binding buffer at 60°C. The dissolved agarose was transferred into the MBST column. After washing twice with wash buffer, the bounded DNA to the carrier was eluted using 20 µl Tris–EDTA buffer. The extracted PCR product from the agarose gel was sequenced by Kawsar Company in sanger method.

Results

During a lethal disease in a sheep herd consisting of 53 sheep at Rumais (Barka) region in the Sultanate of Oman, 11 sheep were died. One of these sheep died suddenly without any clinical signs, whereas the other ten showed anorexia and anemia before they were died.

According to the frame work of "integrated control of ticks and tick borne diseases in globalized world managed by EU-ICTTD-3 project, blood smears, the samples from, liver, spleen, lymph node and lung were sent to the laboratory of Iranian Research Center for Ticks and Tick-borne Diseases (IRCTTD). In blood smears no schizonts or piroplasm form was detectable, whereas schizonts like structures was detected in the impression smears of lymph node, lung, liver and spleen (fig. 1A, B and C).

The DNA was extracted from the sent samples and amplified using common primers designed from 18S rRNA gene of *Theileria* spp. These primers can amplify simultaneously *Theileria* spp. and *Babesia* spp. The PCR products of *Theilera* spp. and *Babesia* spp. were 426-430 bp and 389-402 bp, respectively. The PCR analysis showed an expected PCR product of 428 bp in length in DNA prepared from liver, lung, and lymph node (Fig. 1 D).The PCR product was purified and sequenced and was registered under accession number **JF309152** in GenBank.

Sequence analysis showed 99% homology to the registered in GenBank for *T. lestoquardi* under accession numbers: gb|**AY260185**.1, gb|**AY260184**.1, gb|**AY260183**.1, gb|**AF-081135**.1|**AF081135**). The *Theileria* sp. isolated in Barka region in the Sultanate of Oman differed from the above-mentioned sequences only in one nucleotide at position 254. The nucleotide sequence at position 254 in *Theileria* sp. isolated in Barka region in the Sultanate of Oman was A whereas in other sequences for *T. lestoquardi* was G. The above mentioned nucleotide sequence also showed 99 percent homology to the *T. annulata* sequence data registered under accession numbers gb|**GU233775**.1, gb|**GU233773**.1, gb|**GU2-24091**.1, gb|**GU-129923**.1, gb|**GU129921**.1, gb|**FJ426369**.1, gb|**EU083801**.1, gb|**EU08-3800**.1, gb|**EU-083799**.1, gb|**EU407241**.1, gb|**AY524666**.1, gb|**AY508473**.1 in GenBank. The sequence differed from the mentioned genes only in one nucleotide sequence at position 225 (T/C). The Oman sequence had 94% homology to the *T. ovis* registered also in GenBank under accession numbers gb|**FJ752026**.1, gb|**FJ603460**.1, gb|**EU-622911**.1, gb|**EF-092453**.1, gb|**EF09-2452**.1, gb|**AY533144**.1, gb|**AY260173**.1, gb|**AY-260172**.1, gb|**AY-508461**.1, gb|**AY50-8460**.1, gb|**AY508459**.1, gb|**AY508458**.1. The Oman sequence also had 92% homology to the *T. china*1 and *T. china* 2 sequence registered in GenBank under accession numbers **AY262119** and **AY262121**.

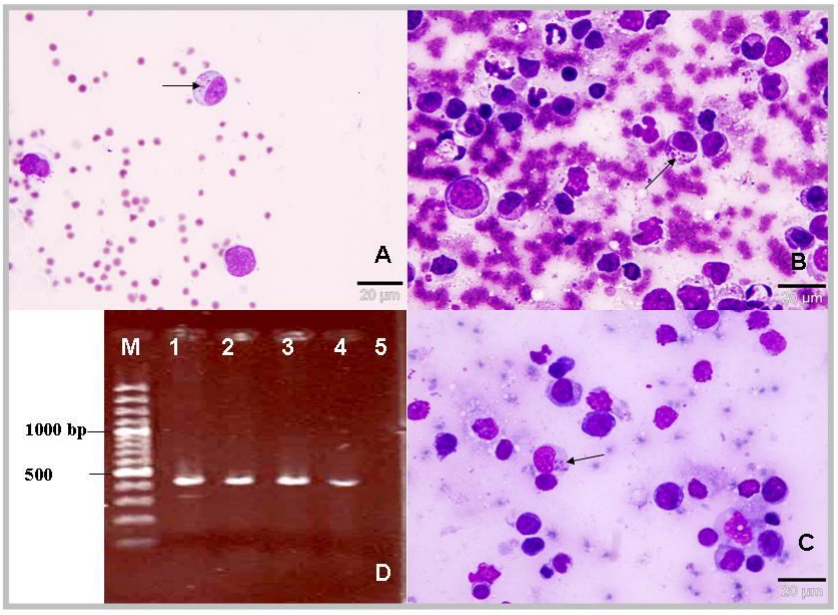

Fig. 1: Giemsa staining from impression smears were prepared from lung (A), spleen (B) and lymph node (C). DNA was extracted from the mentioned organs and amplified with primers derived from 18S rRNA gene (lung D1, liver D2, lymph node D3, spleen D4 and negative control D5).

Discussion

For the long time it was assumed that *T. lestoquardi* is the only pathogenic parasite in small ruminants (6). Recently, however, a previously identified parasite has been described as the species of *T. china* 1 and *china* 2 , which are pathogenic for small ruminants causing fatal diseases (6-9). There are different methods to diagnose theileriosis. The Geimsa stained of blood smear is the common method for the identification and characterization of these piroplasms in Iran, which accompanied with some technical problems causing false morphological diagnosis, and in some cases needs special diagnostic knowledge. In certain cases, serological methods such as the immuno fluorescence antibody test (IFAT) or immunoperoxidase test have also been applied (11-13). Detecting antibodies against *Theileria* using serological tests is a useful technique in epidemiological surveys, but false positive and negative results due to cross-reactions or weak specific immune response are some disadvantages of these tests (15, 16). The gold standard method for the characterization of *Theileria* species is the method of polymerase chain reaction (PCR).PCR or PCR-RFLP was used for the identification of piroplasms in blood by many investigator (8, 14, 17-22). Shayan and Rahbari (2005) used the stained blood smears as the sources for DNA extraction and applied it for the characterization of *Theileria spp*. and *Babesia spp.* simultaneously (14).

According to the framework of "integrated control of ticks and tick borne diseases in globalized world managed by EU-ICTTD-3 project" the members try to expand their activity as network in the boundary countries. In order to such cooperation activity, samples from liver, spleen, lymph node and lung were sent from Sultan Qaboos University, College of Agricultural and Marine Sciences, Department of Animal and Veterinary Sciences to the laboratory of Iranian Research Center for Ticks and Tick-borne Diseases (IRCTTD).

Eleven sheep from complete 53 sheep at Rumais (Barka) region in the Sultanate of Oman were died. One of these sheep died suddenly without any clinical signs, whereas the other ten showed anorexia and anemia before they were died. In accordance to the pathology of theileriosis, the dead animal showed icterus, splenomegaly, hepatomegaly, mottled and yellowish color in the liver and congestion in lung. Although the blood smears did not show any piroplasmic or schizont stage in the corresponding cells, the schizont as structures were detectable in the impression smears prepared from lymph node, liver, and lung. The PCR analysis of DNA extracted from liver, lung, and lymph node revealed the *Theileria* specific amplicon of approximately 428 bp product.

Tageldin et al. (2005) reported that Oman sheep are highly susceptible for *Theileria* infection and the infected sheep mostly die before the microschizonts or erythrocytic form of *Theileria* appear in the nucleated or erytrocytic cells respectively. The same phenomenon was observed with the 11 out of 53 sheep (23). The schizonts were but observed in the different examined organ and therefore it is to speculate that the sheep could be died under toxic shock caused by induction of cytokine production such as TNF-α, IL-1 and IL-6 in infected cells (24, 25).

The nucleotide sequence analysis of 18S rRNA gene of Oman *Theileria* showed 99 percent homology to the *T. annulata* and *T. lestoquardi*. The nucleotide sequence of Oman *Theileria* differed only in one nucleotide at position 254 with the corresponding nucleotide sequence of *T. lestoquardi* and

differed in only one nucleotide at position 225 with the corresponding nucleotide sequence of *T. annulata*. The sequence analysis showed 94 and 92 percent homology to the corresponding nucleotide sequence of *T. ovis* and *T. china* 1 and 2 respectively. Since it is believed that *T. annulata* most probably is not infective for sheep, we believe that the Oman *Theileria* belong to the *T. lestoquardi* species.

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