Identification of Camel Theileria Annulata and Theileria Spp. Via Microscopic and Molecular Techniques Via the use of Certain Genes: Cytochrome B as An Example in Al-Diwaniyah Province, Iraq

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Abstract
The current investigational work was conducted to recognize the causative agents that induced disease conditions, which are similar to those caused by blood protozoa, in camels that lived in some areas in Al-Diwaniyah Province, Iraq, using a specific gene other than 18S rRNA gene that may result in a final detection as Babesia as stated by previous studies. According to this aim, 125 females (125 blood samples) with suspected protozoal signs, such as weakness and paleness of mucus membranes were included for this investigation. Microscopy and a polymerase chain reaction (PCR) technique, which targeted the cytochrome b gene to detect Theileria annulata and the 18S rRNA gene for identifying other Theileria species, were employed for the current work. The microscopy revealed that Theileria spp. was discovered in 69 (55.2%) of the total samples. For the PCR, T. annulata was located in 9 (10%) while Theileria spp. was uncovered in 16 (17.77%), both of the total number of samples. Blood protozoa, especially Theileria annulata and other species, probably were responsible of the protozoal-induced signs in the tested camel females from Al-Diwaniyah Province, Iraq.
Keywords: Camels, protozoa, Theileria annulata.

1. Introduction

Camels have been documented throughout the globe to the sum of 27 million. Camels have long been regarded as a vital investment for transportation, as well as a supplying source of milk, meat, and wool, since the ancient ages [1].

Despite sensitive to a broad range of pathogenic agents, camels' physical capabilities and the distinctive physiological characteristics of their body systems when dealing with the severe climate of desert places allow them to be important animals for human ability to survive [2]. Camel parasites have been reported to cause anemia, body-weight-wasting, and ultimately death in certain circumstances [3]. Theileria species, such as T. camelensis was initially identified in Russia, where it was given the name [4]. Theileria spp. is a tick-borne protozoan that affects agricultural and wild animals [5]. Based on the protozoan species and the geographical region, it may cause moderate to severe symptoms in domesticated mammals, making it one of the most serious conditions livestock face. Camels may also be infected by T. dromedarii, an additional species found in India [4].

It is common for camels to exhibit no clinical indications of sickness while being infected with Theileria. As a primary vector for Theileria dissemination in camels, Hyalomma dromedarii ticks have been shown to contain several forms of the protozoan [6].

A direct blood smear feature via the utilization of the microscopic examination is currently the most common and most effective way to diagnose and rule out T. camelensis and T. dromedarii [7] presence of the microorganism as determined by the microscopic methods [8]. The precise species that may infect camels has so far escaped most molecular investigations, especially when using PCR techniques that depended on the use of the 18S rRNA gene only [9-11]; therefore, the current study was conducted to identify the species based on the use of a PCR method that targeted the cytochrome b gene to detect Theileria annulata and the 18S rRNA gene to recognize other Theileria species in camels from Al-Diwaniyah Province, Iraq.

2. Materials and methods

Blood samples

From September, 2017 till the final day of March, 2018, this study was conducted, which used 125 camels (sex: females) that were directed to a slaughterhouse in Al-Diwaniyah City, Iraq, with signs of weakness and paleness of mucus membranes plus other animals with asymptomatic characteristics. One and twenty-five blood samples were jugular-vein-collected at 5ml/female. EDTA-sterile-tubes were utilized and quickly transported in an icebox to the Laboratory of Parasitology, College of Veterinary Medicine, University of Al-Qadisiyah, Al-Diwaniyah City, Iraq.

Microscopic evaluation

Giemsa-stained Thin-blood smears on slides were performed for detecting protozoal presence according to morphological features as detailed by Soulsby [12]. In

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brief, a slide was employed to place a tiny amount of blood, spreading it out on the slide using a different slide for doing a smear with a very thin layer. Then, for the smear-drying, the slide was placed on a bench open to air and then fixed with a highly concentrated methanol for 5mins. Later, the stain was applied for 30mins. After that, washing with water followed by a drying process were applied to the stained slides. At the end, a light microscope was recruited to examine the smears utilizing a lens with oil immersion property.

Polymerase chain reaction

Extraction of genomic Theileria DNA

Genomic Theileria DNA was extracted via the use of Column-pure blood Genomic DNA Mini Kit (Applied Biological Materials (Abm), Canada) and by using the protocol steps of the kit. The final DNA product was NanoDrop-evaluated for estimating the DNA purity and concentration.

PCR reaction

The study primers (Table 1) that the targeted the cytochrome b gene for identifying Theileria annulata and the 18S rRNA gene for recognizing other Theileria species [13].

Table 1: The work primers.

Gene and Theileria species		Sequence (5'-3')	PCR product Size (bp)	Reference
Theileria annulata Cytochrome b	F	ACTTTGGCCGTAATGTTAAAC	312	13
	R	CTCTGGACCAACTGTTTGG		
Theileria spp. 18ribosomal RNA gene	F	AGTTTCTGACCTATCAG	1100	13
	R	TTGCCTTAAACTTCCTTG		

The mono-plex PCR ingredients are listed in table 2.

Table 2: The monoplex PCR ingredients (25µl of total volume)

Ingredients of PCR reaction		Volume (µl)
Master mix	(2x)	12.5
Primer	F	1.25
	R	1.25
Genomic DNA		5
PCR water		5

The multiplex PCR ingredients are shown in table 3.

Table 3: The multiplex PCR ingredients (25µl of total volume).

Ingredients of PCR reaction		Volume (µl)
Master mix	(2x)	12.5
Primer (1)	F	0.75
	R	0.75
Primer(2)	F	0.75
	R	0.75
Genomic DNA		5
PCR water		4.5

Table 4 shows the steps regarding the thermocycler times and temperatures.

Table 4: Program of the PCR thermocycler.

Theileria species	Denaturation (Initial)	Cycle repeats= 40 for Theileria annulata and 30 for Theileria spp.			Extension (Final)
		Denaturation	Annealing	Extension	
Theileria annulata	94°C/280s	94°C/120s	56/60s	72°C/60s	72°C/60s
Theileria spp.	94°C/280s	94°C/120s	56/60s	72°C/60s	72°C/60s

3. Results

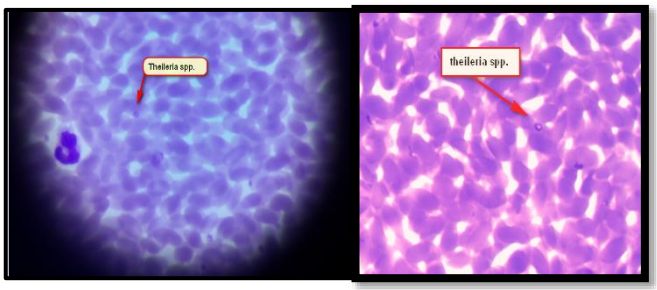

Figure 1: Images of microscopic examination of camel female blood samples that display Theileria spp in the inside of red blood cells. 100x

The microscopy revealed that Theileria spp. was discovered in 69 (55.2%) of the total samples (Figure 1).

For the PCR, T. annulata was located in 9 (10%) while Theileria spp. was uncovered in 16 (17.77%), both of the total number of samples (Figure 2).

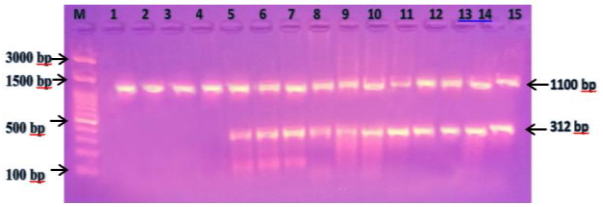

Figure 2: Image of electrophoresed agarose gel of camel female blood samples that displays Theileria annulata cytochrome b gene (312bp lanes) and Theileria spp. 18S rRNA gene (1100bp lanes). M: PCR ladder (100-3000bp).

4. Discussion

Theileria spp. are known to cause theileriosis in different domestic and wild animals, and ticks play a major action in transmitting these protozoa among animals [10]. Studies that looked for the presence of blood protozoa in camels are limited and with certain limitations. The current work intended to discover the presence of Theileria species in the blood of camel females using not only the 18S rRNA gene but also employing the cytochrome b gene as a strong target to differentiate between Theileria species. It was reported that the 18S rRNA gene may introduce unreliable results to tell that Babesia spp. is present in a sample but the reality Theileria spp. is the actual pathogen that occurred in the specific

sample [9, 11, 14]. Therefore, the current study focused on the use of cytochrome b gene as a target to overcome this problem and identify Theileria species in a more accurate method of PCR.

The study outcomes regarding the microscopy method revealed that Theileria spp. was uncovered to be present in about 55% of the tested blood specimens. The present finding agrees with that by Ismael et al. [15], who reported that Theileria spp. was seen in 38.73% of the blood specimens they examined by microscopy. Youssef et al. [16] revealed that Theileria spp. was shown in 30.86% of blood smears from outbreak of theileriosis in camels in Saudi Arabia. The present outcomes from the present study disagree with those in Egypt by Hamed et al. [17], who found that 6.75% of the tested blood specimens were invaded with Theileria spp. Moreover, Hekmatimoghaddam et al. [18] detected that only about 16% of the blood specimens they tested carried Theileria spp. in camels from Iran.

The PCR outcomes revealed the presence of T. annulata in 10% of the tested specimens of camel females. Qablan et al. [19] reported that T. equi was seen in camels from Jordan. Moreover, Mazyad and Khalaf (20) documented that T. ovis was discovered in camels from Egypt. A'aiz et al. [20] reported that only 4.97% blood samples had Theileria annulata as confirmed by their gene sequencing. The low percentages in these studies could be attributed to the fact that most of these studies used the 18S rRNA gene as a target for molecular techniques, which might disrupt the results and direct the findings toward the identification of Babesia spp. instead of Theileria spp., as stated above, and that is why the current study findings revealed a higher percentage because the utilization of the cytochrome b gene as molecular target.

5. Conclusion

Blood protozoa, especially Theileria annulata and other species, probably were responsible of the protozoal-induced signs in the tested camel females from Al-Diwaniyah Province, Iraq.

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