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APPLICATION OF MOLECULAR TECHNIQUES IN DIAGNOSIS OFTHEILERIOSIS OF CATTLE AND CAMELS IN COMPARISON WITH CONVENTIONAL METHODS IN BEHERA GOVERNORATE

NASHAWA M. HALMY[1] and REDA S. FADLY [2]
[1] Department of Biotechnology, Animal Health Research Institute, Dokki, Giza.
[2] Department of Parasitology, Animal Health Research Institute, Dokki, Giza.

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ABSTRACT

This study was carried out to determine the rate of *Theileria* infection in cattle and camels in Behera governorate by microscopic examination, confirm and identify the species by polymerase chain reaction (PCR). The prevalence of *Theileria* infection in cattle and camels was 20% (15/75) and 28% (21/75) respectively, by microscopic examination (ME) of blood smears. Five positive samples by ME (3camels and 2 cattle) in addition, four negative samples were subjected to semi-nested PCR. The PCR result (6 out of 9) examined blood samples (4 camels and 2 cattle) were PCR positive using *Theileria* species specific primer at 430bp. All positive samples for *Theileria* sp. Using Tbs-S/Tbs-A primers were used as a template and amplified by using Ta-S/ Tbs-A primers specific for *Theileria annulata*. All examined samples (6/6), 2 from cattle and 4 from camels were positive for *Theileria annulata* at 193 bp. In conclusion, the PCR especially semi-nested PCR (snPCR) are highly specific and sensitive methods for identifying the species of *Theileria annulata* and screening the carrier cattle and camels in the epidemiological surveys. On the other hand, Giemsa staining method is not suitable for detecting the carrier or chronic phases of theileriosis, although it is an easy and fast diagnostic technique for detecting this infection in acute phase with clinical signs.

Key words: *Camels, cattle, Theileriosis, Blood smears, Semi-nested PCR.*

INTRODUCTION

Theileriosis is one of the most common tick-borne diseases, which have been studied and described in a wide range of ruminants such as cattle, sheep, and goats. In camels, only few literatures were published (El-Refaii *et al.,* 1998; El Kammah *et al.,* 2001 and El-Fayoumy *et al.,* 2005). *Theileria* are tick-transmitted, obligate intracellular parasites that are important pathogens of livestock in the tropical and subtropical regions of the world. *Hyalomma* spp. (H.spp.), especially *Hyalommadromedarii* and *Hyalommaannatolicum excavatum* are the common ticks infesting camels in Egypt (Abd El-Baky 2001). The disease threatens estimated 250 million cattle and acts as a major constraint on livestock production and improvement in many developing countries. *Theileria* parasites enter the bovine host during tick feeding as sporozoites, which rapidly invade mononuclear leukocytes. Here, they mature into macroschizonts and induce proliferation of the host cell. Macroschizonts develop further into microschizonts and ultimately into merozoites, which are released from the lymphocyte. The merozoites invade erythrocytes and develop into piroplasms. Tropical theileriosis is a lymph proliferative disease in its early phases and is accompanied by enlargement of lymph nodes. On development of pyrexia, a lymphdestructive phase which is associated with a pronounced leukopenia is initiated. The disease is further characterized by a marked anemia. Diagnosis of clinical *T. annulata* infection in cattle is usually based on the detection of macroschizonts in Giemsa-stainedlymph node biopsy smears. After recovery, a long-lasting carrier state occurs, in which low numbers of erythrocytes remain infected with *Theileria* piroplasms. Such carriers are important contributes to the infection within *Hyalomma* Ticks. Hence, detection of piroplasms in carrier animals is an important epidemiological parameter. However, *Theileria* piroplasms may be difficult to find in stained blood smears. More important, it is generally not possible to discriminate *T. annulata* from nonpathogenic *Theileria* species that may occur simultaneously within the same bovine host. Diagnosis of the disease based on microscopic examination of Giemsa stained blood smears and lymph node biopsy is disadvantageous in case of low

Corresponding author: Dr. REDA S. FADLY
E-mail address: dr_redafadly@yahoo.com
Present address: Department of Parasitology, Animal Health Research Institute, Dokki, Giza.

parasitemia and carrier state animals (Kundave *et al.,* 2014 and Khatoon *et al.,* 2015). However, the molecular based assays such as PCR have proven to be the most reliable tool for detecting *Theileria* parasites in clinicaland subclinical cases of theileriosis (Aktas *et al.,* 2006 and Khatoon *et al.,* 2015).

The aim of this study is to confirm and identify the *Theileria* sp. infecting cattle and camels by using polymerase chain reaction (PCR) in comparison to microscopic examination of thin blood.

MATERIALS AND METHODS

Samples:
A total of 150 blood samples on anticoagulant (EDTA) were collected cattle (75) and camels(75). Samples were collected from different localities in Behera Governorate.

Microscopic examination:
Thin blood smears were prepared immediately after drawing the blood samples, fixed with methanol, stained with Giemsa and examined by light microscopy at 1000x magnification as described by (Coles, 1986). The stained blood smears were examined for *Theileria* piroplasms and schizonts as described by (Kelly, 1979). Each blood smear was examined twice before being considered negative.

Samples for PCR:
Nine blood samples divided into five positive samples for *Theileria* infection (2 from cattle and 3from camels) and four negative samples for *Theileria* infection (2 from cattle and 2 from camels) were subjected to PCR using *Theileria* species specific primer.

DNA extraction from whole blood samples
DNA was extracted from whole blood samples by using Thermo scientific kits (Gene JET Genomic DNA Purification Kit #K0721, #K0722).

Oligonucleotide primers:
For conventional PCR, two set of oligonucleotide primers were synthesized and designed Tbs-A/ Tbs-S. and Tbs-A/ Ta-S according to (Hoghooghi *et al.,* 2011). The sequences of primers used in this study are listed in the Tbs-S/Tbs-A primer set was used for PCR amplification of 18SrRNA of *Theileria* sp. and the amplified sequence weight by this primer set for *Theileria* sp. was 426-430 bp. The amplified product of all PCR positive samples for *Theileria* species using Tbs-S/Tbs-A primers were used as a template and amplified by using Ta-S/Tbs-A primers. The Ta-S/Tbs-A primer set derived from the 18SrRNA encoding gene specific for *Theileria annulata*. The amplified sequence by this specific primer set was 193 bp.

Polymerase chain reaction
DNA amplification was done in 25 ul reaction volume containing12.5 ul of 2× Taq PCR Master Mix (Tiangen, Cat No. Cat. no. KT201), 10 pmol of each oligonucleotide primers Tbs-S/Tbs-A, 5 ul of DNA template and fill up to 25 ul with DNA se and RNA se free water. The reaction was performedin an automatic DNA Thermo cycler (Biometra) with the following program: 5min incubation at 95°C to denature double strand DNA, 34 cycles of 45s at 94°C, 90 s at 55°C, 45s at 72°C and finally, PCR was completed with the additional extension step for 5 min. Samples were considered positive for species when a single band of DNA at 426- 430 bp, was evident in the ethidium bromide stained gels, compared with the molecular size marker (100bp DNA ladder.

Semi-nested PCR (snPCR)
In order to show that the Tbs-S/Tbs-A PCR product was *Theileria annulata*, it was amplified in 25 ul reaction volume containing 12.5 ul of 2× Taq PCR Master Mix for PCR (Tiangen, Cat No. Cat. no. KT201), 10 pmol of each oligonucleotide primers Ta-S/Tbs-A primer, 5 ul of the first PCR amplicon as template and fill up to 25 ul with DNAse and RNAse free water. The optimized cycle program for the second PCR was 5 min at 95 C to denature double strand DNA, Two cycle of 94°C for 45 s.55°C for 90 sand 45 s in 72°C. followed by 35 cycles of 45 s at 94°C, 45 s at 55°C and 72°C for 45 s. Finally, Semi-nested PCR (snPCR) was completed with the additional extension step at 72°C for 5 min. according to (Hoghooghi *et al.,* 2011).

Electrophoresis of PCR product:
After amplification 10 ul of the reaction product was mixed with 2 ul of 6X gel loading dye and subjected to electrophoresis on 1.5% agarose gel in TBE buffer (Tris, Boric acid, EDTA buffer) at 100V for 30 min. Gel were stained with ethidium bromide and photographed on UV trans illuminator. Samples were considered positive for *Theileria* species by using Tbs-S/Tbs-A primers and positive for *Theileria annulata* by using Ta-S/Tbs-A when a single band of DNA at 426-430 bp and 193 bp respectively were evident in the ethidium bromide stained gels, compared with the molecular size marker (100 bp DNA ladder, Jena Bioscience Cat. No. M-214) as shown in (Fig 6) and (50 bp DNA ladder, Gene Dire X Cat. No. DMO12-R 500) as shown in (Fig 7).

RESULTS

Microscopical examination:
Blood smears showed that, overall, the prevalence of *Theileria* infection in cattle and camels was recorded as 20% and 28% respectively (Table 2). The stained blood films revealed the presence of ring forms of *Theileria* were found in erythrocytes of cattle (Fig 1),

camel (Fig 5) and schizonts were detected in the lymphocytes with two forms (macro and micro) stages were detected in blood smears of both cattle and camels (Figs. 2,3 and 4).

PCR results:

(Table 3): Showed that, all samples proved to be positive by thin blood smears examination were also positive by (snPCR) after using *Theileria* species specific primer. Whereas, only one camel sample was negative by microscopical examinations were proved to be positive by (snPCR). Six out of nine blood samples (2cattle and 4 camels) were PCR positive using *Theileria* species specific primer at 430 bp (Fig.6). All 6 positive samples for *theileria* species by PCR were amplified by semi nested PCR with *Theileria annulata* specific primer at 193 bp. (Fig. 7).

Table 1: Oligonucleotide primer sequences specific for *Theileria sp*. and *Theileria annulata.*

***Theileria* sp. primer**	**Gene**	**Nucleotide sequences (5'–3')**	**Amplified product**	
Tbs-A	**18S rRNA**	***5*-'CTA AGA ATT TCA CCT CTG ACA G-3'**		**(Hoghooghi, *et al.*, 2011).**
Tbs-S	**18S rRNA**	**5'-CAC AGG GAG GTA GTG ACA AG-3'**	**430 pb**	**(Hoghooghi, *et al.*, 2011).**
Ta-S	**18S rRNA**	***5*-ACG GAG TTT CTT TGT CTG-3'**	**193 bp**	**(Hoghooghi, *et al.*, 2011).**

Table 2: Prevalence of *Theileria* infection in cattle and camels by microscopical examination.

Animals	No.ofanimal	+ve	%
cattle	75	15	20
camels	75	21	28

Table 3: The results of PCR in comparison with microscopical examination (ME).

No. of animal	**Animals**	**ME**	PCR	
			***Theileria* species**	***T.annulata***
1	**cattle**	+	+	+
2	**cattle**	-	-	-
3	**cattle**	+	+	+
4	**cattle**	_	-	-
5	**camel**	_	-	-
6	**camel**	+	+	+
7	**camel**	+	+	+
8	**camel**	+	+	+
9	**camel**	_	+	+
Total	9	5	6	6

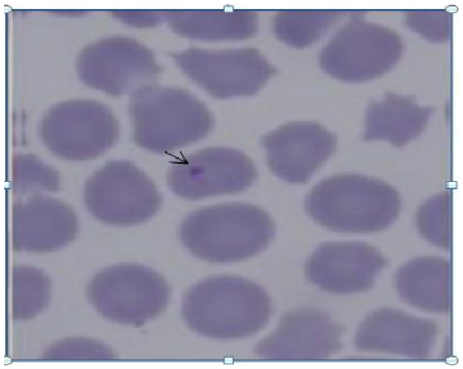

Fig. (1): Gimsa stained blood smear in Cattle Showing intra –erythrocytic piroplasms of *T. annulata* (X 1000).

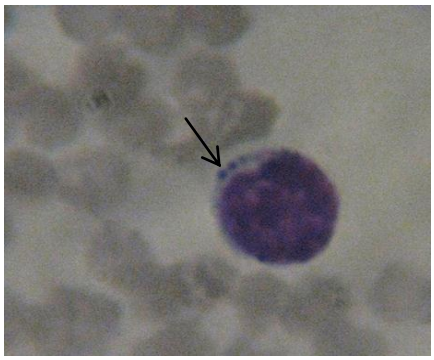

Fig. (2): Gimsa stained blood smear macro schizonts (X 1000)

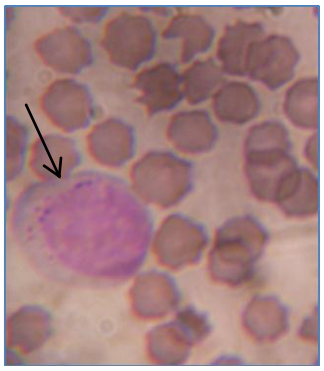

Fig. (3): Gimsa stained blood smearmicroschizonts in cattle (X 1000)

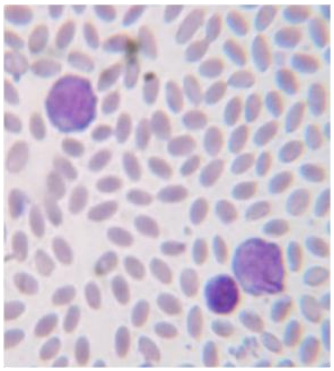

Fig. (4): Gimsa stained blood Smear macro & microschizonts in camel (X 1000)

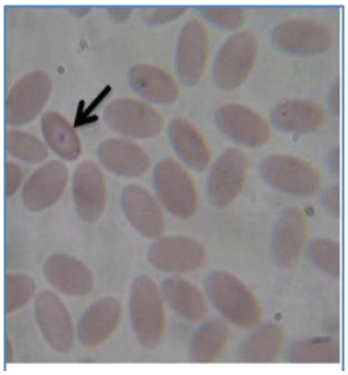

Fig. (5): Gimsa stained blood smear showing ring form of *T. annulata* in camel (X 1000)

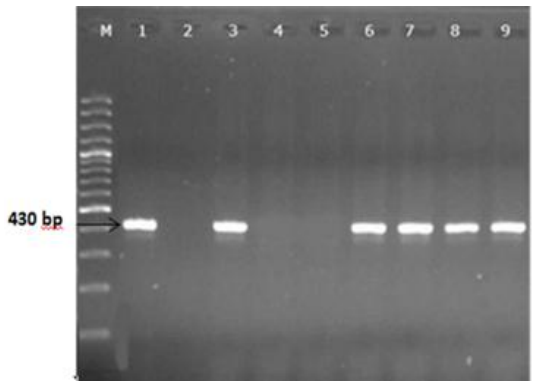

Fig. (6): Ethidium bromide stained 1.5% agarose gel electrophoresis showed PCR amplified fragment of expected size 430 bp (Lane 1, 3,6,7,8,9) resulted from amplification of DNA extracted from blood samples of cattle (1-4) and camel (5-9) using Tbs- S /Tbs-A primers specific to *Theileria* spp. Lane M : 100 marker, Jena Bioscience.

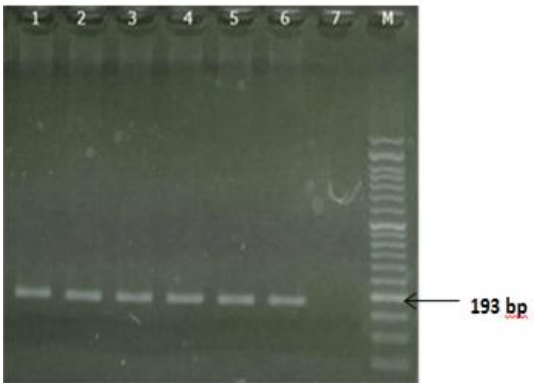

Fig. (7): Ethidium bromide stained 1.5% agarose gel electrophoresis showed PCR amplified fragment of expected size 193 bplane 1-2 contained PCR products from cattle and (lane3-6) contained PCR products from camel resulted from amplification of the first PCR amplicon using Ta-S /Tbs-A primers specific to *Theileria annulata* Lane M:50 bp marker, Gene Dire X.

DISCUSSION

Theileriosis is an important parasitic disease of cattle and camels in Egypt which causes great economic losses of animals and their products (Al–Gaabary, 1995 and AL-Hosary, 2009). In the present study *Theileria* spp. was 20 % in cattle by Giemsa stain blood films (Table 2), this result was relatively similar to Reda (2012) in Behera Province was 23% and Acici (1995) in Turkey was 17 % lower prevalence rates reported by Adel (2007) in Gharbia Governorate was 11.31 % and El Bader *et al.* (2009) in Southvally was10.3%. However higher prevalence rates were reported in Egypt, among them El Bahy (1986), who revealed that prevalence rates was 65%, Gamal El Dien (1993) in Behera province, who found that the prevalence of *T. annulata* was 65.4 % using stained blood films and Hosny *et al.* (2010) in Assiut Governorate whose recorded that the incidence of theileriosis among cattle was 31.58 %. Variation in prevalence rates could possibly be attributed toan abundance of the vectors as a result of high temperature and humidity, age groups evaluated, sample size and health condition of animal.

In the present study the prevalence of *Theileria* infection in one-humped camel was detected at rate of 28% by microscopic examination of blood smears for *Theileria* sp. Similar result recorded by Shereen *et al.* (2015) who recorded that the incidence of theileriosis among camels were (30.86 %) in Egypt. While our result lower that detected by El-Refaii *et al.* (1998); El-Fayoumy *et al.* (2005) and Abd El-Wahab (2009) were 62.1, 44.8 and 44.23% respectively. On the other hand lower percentage of infection reported by Azizi *et al.* (2008); Maha *et al.* (2011) and Osman *et al.* (2015) who recorded that the incidence of theileriosis among camels were 8.1%, 6.75% and 9% respectively. These variations in the different results may be attributed to different localities, population density of camels, environment, hygienic measures, as well as the age variation of examined animals and camel management.

It is imperative to develop sensitive tools for the effective diagnosis of theileriosis in order to reduce the economic losses incurred as result of the disease. A number of conventional and modern techniques are used for the detection of *Theileria* spp. in host animals. The most commonly used is the microscopic examination of blood smears stained by Giemsa, which is typically adequate for detection of acute infections. Due to its low sensitivity, this technique cannot be used for the detection of carrier animals due to low parasitemia. Serological tests have several disadvantages like the presence of antibodies for long period even after treatment and cross-reactivity (Leemans *et al.*, 1999). The use of molecular methods (such as PCR) for the detection and identification of different microorganisms has gained popularity among scientists in recent years. This is because molecular methods are more specific and sensitive than other traditional diagnostic techniques (Altay *et al.*, 2005).

Concerning to PCR assay, this study showed that complete concordance between microscopical examination and PCR assay results in cattle, while in camel PCR results don't coincide with microscopical examination Whereas, one camel blood sample was negative by microscopical examinations were proved to be positive by PCR (Table 3), six cases were positive for *Theileria* sp. Two of cattle and four of camels were positive for *Theileria* species at 430 bp using Tbs-S/Tbs-A derived from 18srRNA encoding gene and they were amplified by semi-nested PCR with *Theileria annulata* specific primer set (Ta-S/Tbs-A) derived from 18srRNA encoding gene and they were positive for *Theileria annulata* and give 193 bp. These results especially in camels may be due to microscopical examinations does not detect positive animals in the early stage of infection and the long-term carrier status, conditions in which parasitemia is very low. Results are in agreement with a previous report on *T. annulata* (Hoghooghi *et al.*, 2011). Our results demonstrate that this PCR assay discriminate *T. annulata* from non-pathogenic *Theileria* species. The result of this study coincided with (Abd El-Wahab, 2009 and Randa *et al.*, 2014) which mentioned that the PCR assays are more sensitive and specific than conventional diagnostic techniques in the diagnosis only few literatures were published. Also Hoghooghi *et al.* (2011) reported that Semi-nested PCR based on detecting *Theileria annulata*, was capable of showing the cases which their Giemsa stained blood smears were false negative or false positive in visual examination under light microscope.

CONCLUSION

The PCR especially semi-nested PCR are highly specific and sensitive methods for identifying the species of *Theileria annulata* from non-pathogenic *Theileria* species. PCR technique being used as confirmatory test with microscopical examination especially on endemic area. On the other hand, Giemsa staining method is an easy and fast diagnostic technique for detecting *Theileria* infection in acute phase while not suitable for detecting the carrier or chronic phases of *Theileria* infection. *T.annulata* is one of *Theileria* spp. highly prevalent and potentially a major problem in cattle and camels in Behera Governorate.

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تطبيق الاختبارات الجزيئية فى تشخيص الثيليريا في الأبقار والإبل بالمقارنة بالطرق التقليدية في محافظة البحيرة

نشوى محمد حلمى ، رضا سمير فضلى
E-mail: dr_redafadly@yahoo.com Assiut University web-site: www.aun.edu.eg

يعتبر مرض ثيليريا الماشيه من أهم الامراض الأقتصادية التي تصيب الماشيه من جميع السلالات والاعمار ويؤدى الى خسائر أقتصادية فادحة. أستهدفت هذه الدراسة الى أستخدام الطريقة التقليدية متمثلة فى المسحات الدموية المصبوغة بصبغة الجيمسا بالاضافة الى تفاعل البلمرة المتسلسل لتشيخص الاصابة بمرض الثيليريا فى الابقار والجمال. أوضحت الدراسة ان طريقة المسحة الدموية مازلت تتمتع بأهمية تشخيصية بينما تفاعل البلمرة المتسلسل هى الأكثر حساسية. أجريت هذه الدراسة على عدد ١٥٠ عينة دم مقسمة (٧٥ من الأبقار و ٧٥ من الجمال). أظهر الفحص الميكروسكوبى أن نسبة الأصابة بمرض الثيليريا فى الابقار والجمال (٢٠ % و٢٨%) على التوالى. بينما اظهر فحص تفاعل البلمرة المتسلسل عند فحص عدد ٩ عينات دم مقسمة الى ٥عينات من دم الجمال (٣ ايجابية بالفحص الميكروسكوبى و٢ سالبه) وعدد ٤عينات من دم الأبقار (٢ ايجابية بالفحص الميكروسكوبى و٢ سالبة) تأكيد نسبة الاصابة فى جميع العينات الايجابية بالفحص الميكروسكوبى وظهرت عينة فى الجمال كانت سالبة بالفحص الميكرسكوبى لوجود الثيليريا وبذلك ارتفعت نسية عدد العينات الايجابية فى الجمال الى ٤ عينات بدلا من ثلاث عينات. أثبت الفحص ان عدد ٦ عينات دم (٢ من الأبقار و ٤ من الجمال) كانت ايجابية لأنواع الثيليريا عند٤٣٠ زوج من القواعد النيتروجينة. جميع هذه العينات الايجابية من الابقار والجمال كانت ايجابية للثيليريا انيولاتا عند ١٩٣ زوج من القواعد النيتروجنية بالفصل الكهربى على الاجاروز جيل اليكتروفوريسيس.