Use molecular techniques as an alternative tool for diagnosis and characterization of *Theileria equi*

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(Received September 23, 2017; Accepted October 14, 2017)

Abstract

The purpose of this study was to determine the prevalence of clinical, subclinical and chronic infection with the equine parasite *T. equi* in some Egyptian localities (Cairo and Giza governorates). A panel of 396 equine blood samples representing 141 horses, 250 donkeys and 5 mules was collected from equines during the period from April 2015 to March 2016 using microscopic examination and conventional PCR. Microscopically a twenty two (5.56%) of 396 were positive for *T. equi* merozoites that appeared as small rounded, pyriform shaped and maltase cross shaped merozoites. Among 8/141(5.67%) horses and 14/250 (5.60%) donkeys were found to have positive for *T.equi*. A one hundred blood samples (45 horses, 50 donkeys and 5 mules) selected randomly were also examined by PCR. The results of PCR showed 30/100(11/45 (24.4%) horses, 18/50 (36%) donkeys and 1/5 (20%) mule) were positive for *T.equi*. When the sequenced PCR amplicons (n=3) were aligned to the reference nucleotide sequences of *T. equi* accessed in Genbank, the horse isolate showed insertion of Thymine (T) base at position 23 and substitution of Thymine (T) base with Cytosine (C) base at position 91, while the donkey and mule isolates have no alterations when compared to the reference sequences. The phylogenetic analysis showed that the sequenced PCR isolates belonged to *T.equi*. The obtained sequences were deposited in the GeneBank database under accession numbers MF192854, MF192855 and MF192856.

Keywords: Equine, *T. equi*, prevalence, PCR, Egypt
Available online at http://www.vetmedmosul.org/ijvs

إستخدام التقنيات الجزيئية كوسيلة بديلة لتشخيص وتوصيف الثيليريا إكواي

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الخلاصة

لغرض من هذه الدراسة هو تحديد معدل الخمج بطفيل الثيلريا الخيلية فى بعض محافظات جمهورية مصر العربية (القاهرة والجيزة). اذ تم جمع عدد ٣٩٦ عينة دم من الفصيلة الخيلية والتي مثلت ١٤١ راساً من خيول محلية و ٢٥٠ من الحمير و٥ من البغال للفترة من نيسان (ابريل) ٢٠١٥ الى أذار (مارس) ٢٠١٦ باستخدام الفحص المجهري واختبار تفاعل انزيم البلمرة المتسلسل التقليدى.عند الفحص المجهري تم ملاحظة الاطوار المختلفة من دورة حياة طفيلي الثيلريا داخل خلايا الدم الحمراء اذ لوحظ الطفيل باشكال الدائري، الكمثري وشكل صليب مالطة فى عدد ٢٢ عينة دم بنسبة ٥,٦٧% من الخيول المحلية وبنسبة ٥,٦٠% من الحمير. باجراء اختبار تفاعل انزيم البلمرة المتسلسل التقليدى على ١٠٠ عينه دم مثلت ٤٥ من الخيول المحلية، ٥٠ من الحمير و ٥ من البغال تم اختيارهم بطريقة عشوائية، وجد ان ٣٠ عينة كانت ايجابية للثيلريا الخيلية منها ١١ من الخيول المحلية و١٨ من الحمير و ١ من البغال. تم عمل التتابع الجينى لعدد ٣

من العينات الايجابية وعمل شجرة العائلة الجينية للتاكد من انها تنتمى بالفعل الى عائلة البيروبلازميدات عن طريق مقارنتها بنظيراتها الموجودة على بنك الجينات NCBI ووجد بالفعل انها تنتمى الى هذه العائلة مع بعض التغيرات الطفيفة فى الخيول المحلية حيث وجد استبدال للقاعدة النيتروجينية الثايمين بالسيتوزين فى الموضع ٩١ وكذلك ادخال لقاعدة ثايمين عند الموقع ٢٣ لكن فى الحمير والبغال فهى مطابقة بنسبة ١٠٠%. واثبتت شجرة العائلة الجينية ان هذه المعزولات تنتمى الى الثيلريا الخيلية وتم نشرها على بنك الجينات بالاكواد الاتية MF192854 و MF192855 و MF192856.

Introduction

Equine theileriosis is a tick-borne disease of horses, donkeys, mules and zebras with worldwide distribution that affects on equine industry, causing economic losses and significantly impairing the international movement of equines (1). The disease is endemic in tropical, subtropical and some temperate areas of the world (2). It is caused by an obligatory intraerythrocytic protozon of the phylum *Apicomplexa* which is *T. equi* (2,3). The prevalence of *T. equi* mainly depends on the prevalence of the tick vector which belongs to the genera *Boophilus, Dermacentor, Hyalomma* and *Rhipicephalus* (4,5). Other sources of infection are infected blood transfusion and the reusing of infected syringes and surgical instruments (6). *T. equi* is responsible for the appearance of most clinical cases and causes severe clinical signs (7). Infected equines may remain carriers of *T. equi* for their lifetime. These carrier animals act as a reservoir of infection and as a source of infection for the tick vector (8,9). The spread of the disease is affected by many factors such as climatic conditions and the international movement of equines, so disease-free countries should direct appropriate control strategies when they import equines (5,10)**.** Usually serological tests are used to supervise the movement of horses across borders (5,11).

The prevalence of *T. equi* was detected in Egypt using microscopical and serological examination. Microscopical examination of stained blood smears has been the standard diagnostic technique for equine theileriosis for several years but it is effective only in acute phase of the infection. In the case of carrier animals, it is not sensitive to detect the infection due to low parasitemia (12). Many serological assays have been developed for the detection of antibodies against *T. equi* such as immunofluorescent antibody technique (IFAT), immunochromatographic test, complement fixation test (CFT) and enzyme-linked immunosorbent assay (ELISA) (13). These methods have proved to be more sensitive although they have some disadvantages related to antibody detection limit and cross-reactivity to other *Babesia* species (14). Polymerase chain reaction (PCR) proved to has higher sensitivity and specificity in detection of the protozoal DNA of *Theileria* species (15,16). The PCR technique has sufficient sensitivity to detect protozoal DNA from a blood sample with parasitemia of 0.000001% (15). The aim of this study was to determine the prevalence of *T. equi* in the equine population in both two Egyptian governorates (Cairo and Giza) based on microscopic examinations and molecular techniques. Further, molecular characterization of *T. equi* was made based on 18s rRNA sequences.

Materials and methods

Sample collection and microscopic examination

A total number of 396 equine blood samples representing 141 horses, 250 donkeys and 5 mules was collected from different localities in Egypt (Cairo and Giza), during the period from April 2015 to March 2016. All samples were collected by the jugular venipuncture method using EDTA-tubes from working equines. Some of these animals was healthy and others were clinically diseased. All tubes were marked by the necessary data (age, sex, location and date of collection) and then samples were sent in an ice box to the Parasitology Department, Faculty of Veterinary Medicine, Kafrelshiekh University, for examination. In the lab, thin blood films were prepared from the whole blood, stained with Giemsa stain. and examined for the presence of *T. equi* merozoites using an oil emersion lens of the light microscope according to (17).

DNA extraction and PCR amplification

Genomic DNA was extracted from 100 whole blood samples (45 horses, 50 donkeys and 5 mules) representing microscopically positive blood samples (n=22) and from randomly selected microscopically negative blood samples (n=78), using Thermo Scientific™ GeneJET Genomic DNA Purification Kit (Cat No #K0722) according to the instructions in the manufacturer's manual. The obtained DNA was stored at -20°C until used in the downstream applications. The PCR technique was applied using primers developed by (15) that specifically detect 392 bp from the 18s rRNA gene of *T.equi*. The assay utilized a universal forward primer (Bec-UF2) with a sequence 5-TCGAAGACGATCAGATACCGTCG-3 and a *Theileria equi* specific reverse primer (Equi-R) with a sequence 5-TGCCTTAAACTTCCTTGCGAT-3. PCR was performed using GoTaq® G2 Flexi PCR Kit (Promega, USA) with a total volume of 25 µl containing 5X Green GoTaq® Flexi Buffer (10 µl), 25mM MgCl2 Solution (2 µl), PCR Nucleotide Mix (dNTPs) 10 mM each (1µl), Primer mix 10 pmol (1µl), 1.25 u GoTaq®G2 Flexi DNA Polymerase

template DNA (5 µl), DNase/RNase free water (7 µl). The thermal profile was 95°C for 5 min, followed by 35 successive cycles of denaturation at 96°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min. Then a final extension was made at 72°C for 5 min, and then holding stage at 4°C for infinite time. 8 µl of the generated PCR products were migrated on 1.5 % ethidium bromide stained agarose gel under a constant volt of 80 V for 40 min. The gel was then visualized using UV-Transilluminator and then photographed by the associated camera (15). *T. equi* positive samples showed a band of 392 bp.

Sequencing and phylogenic analysis

Positive PCR products (n=3) representing 1 horse, 1 donkey and 1 mule were extracted from agarose gel using a thermo scientific gene JET gel extraction kit (Cat No. K0691) and were sent to the sequencing unit at the Animal Health Research Institute, Dokki, Giza for sequencing in a single direction using specific *T. equi* reverse primer (5-TGCCTTAAACTTCCTTGCGAT-3). The sequences were compared with each other and then with the GenBank database by the nucleotide sequence homology search. using the BLAST analysis database that is available at the National Centre for Biotechnology Information (NCBI) (http://blast.ncbi.nlm.nih.gov). All sequence data were edited subsequently by the naked eye, using Bioedit 7.2.5 software (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). A phylogenetic analysis was performed using MEGA version 7 (http://www.megasoftware.net). A phylogenetic tree was produced by applying the Neighbor-Joining technique with using *Hepatozoon canis* (DQ439543.1) as out groups. Sequences produced during this study have been deposited in the GeneBank database under accession numbers MF192854, MF192855 and MF192856.

Results

A total number of 22 (5.56%) of the 396 Giemsa-stained blood smears were harbored *T. equi* merozoites (Table 1). They appeared as small rounded, pyriform shaped and maltase cross shaped merozoites (Figure 1).

The conventional PCR amplification showed that a total number of 30 (30%) out of 100 examined blood samples showed 392 bp fragments in 1.5% ethidium bromide agarose gel which were specific for *T. equi* (Figure 2). The infection rate of blood samples tested by conventional PCR amplification was compared with the infection rate of the same samples found by microscopical examination, as shown in Table 2.

When the sequenced PCR amplicons (n=3) were aligned to the reference nucleotide sequences of *T. equi* accessed in the Genbank, the horse isolate showed the insertion of Thymine (T) base at position 23 and substitution of Thymine (T) base with Cytosine (C) base at position 91; while the donkey and mule isolates had no alterations when compared to the reference sequences (Figure 3). The phylogenetic analysis showed that the sequenced PCR isolates belong to *T. equi* (Figure 4).

Table 1: Prevalence of *T. equi* among examined animals based on microscopical examination of Giemsa-stained blood smears

	No. examined	No. infected	Infection rate (%)
Horses	141	8	5.67
Donkeys	250	14	5.60
Mules	5	--	--
Total	396	22	5.56

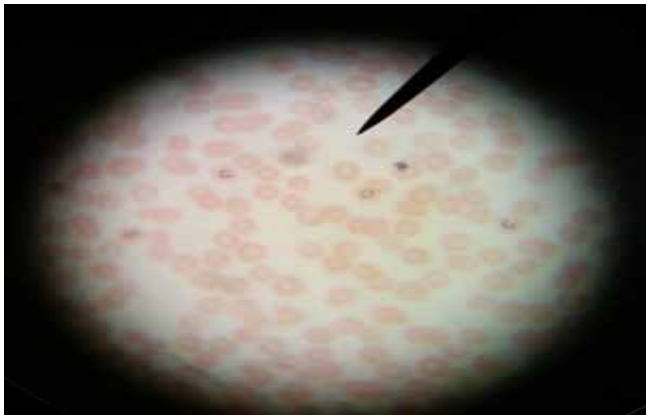

Figure 1: Microscopically stained blood smears showed small rounded merozoites of *Theileria equi* (x100).

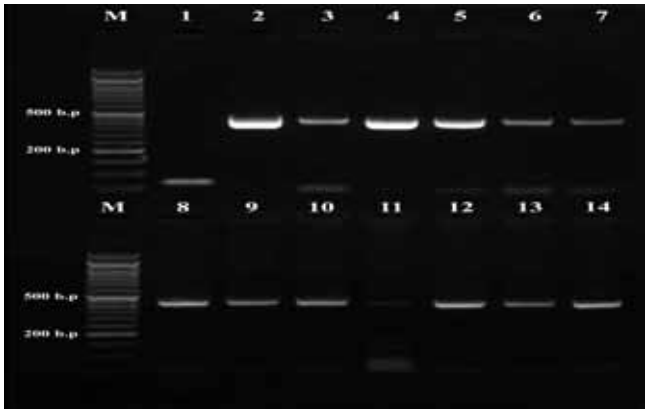

Figure 2: Conventional PCR detection of *Theileria equi* at 392 bp fractionated on 1.5% agrose gel. Lane M: 50 bp DNA ladder, Lane 1: *T. equi* negative control, Lane 2: *T. equi* positive control (392bp), Lane 3-14 *T. equi* positive samples (392bp).

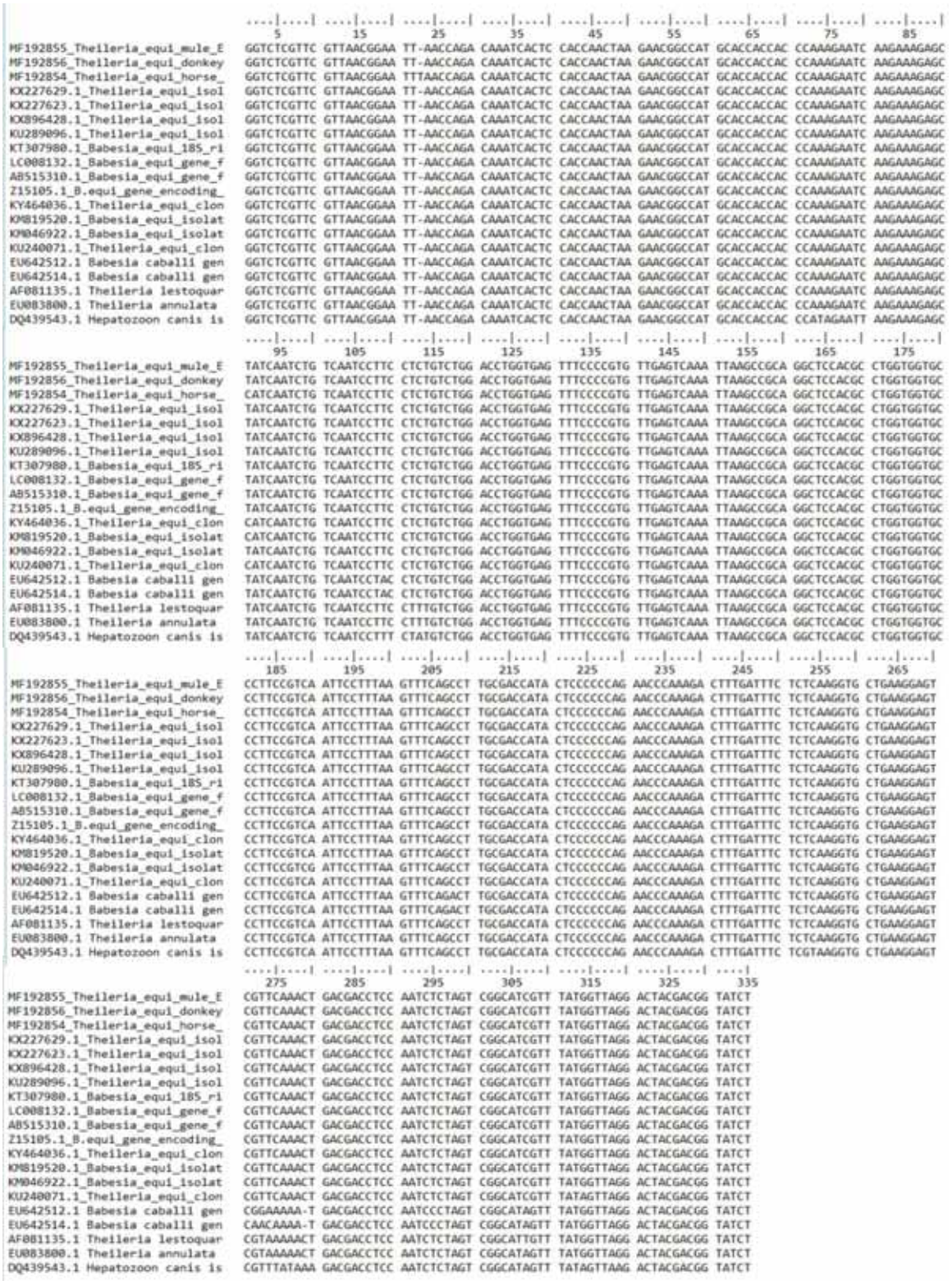

Figure 3: Alignment of nucleotide sequence of *Theileria equi* of isolates obtained from Egyptian equines with reference to sequences of *Theileria equi* accessed in the genbank, in addition to sequences of *Hepatozoon canis* as out group.

Table 2: Infection rates of 100 tested blood samples for *T. equi* using microscopical examination compared with conventional PCR amplification

Animals	Number examined	Microscopical examination		Conventional PCR examination	
		Number infected	Infection rate (%)	Number infected	Infection rate (%)
Horses	45	8	17.8	11	24.4
Donkeys	50	14	28	18	36
Mules	5	--	--	1	20
Total	100	22	22	30	30

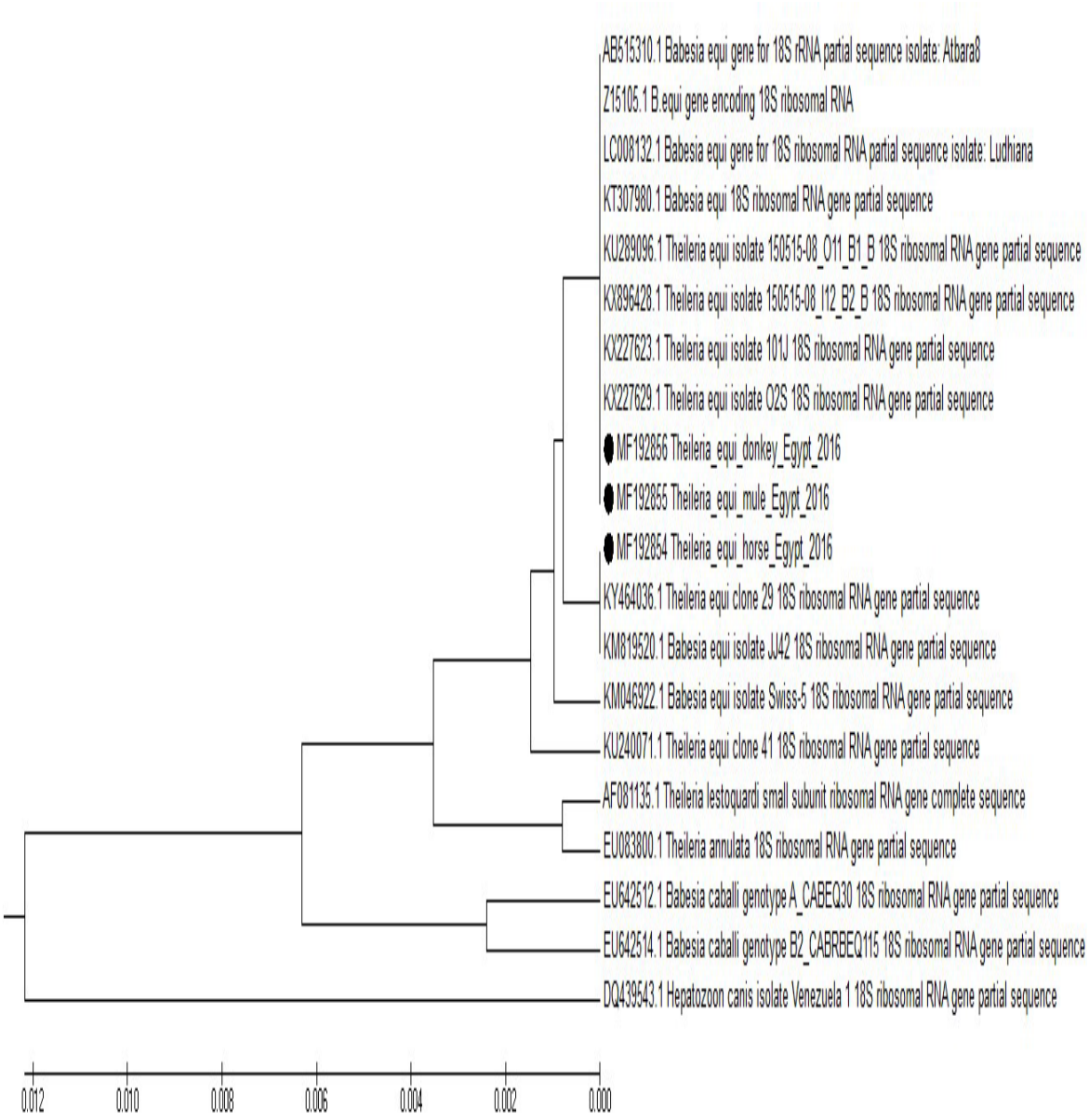

Figure 4: Genetic relationship of *Theileria equi* isolates obtained from egyptian equines with reference to sequences of *Theileria equi* accessed in the genbank. Phylogenetic tree was produced by applying Neighbor-Joining technique of the nucleotide sequence of the 18s rRNA gene with using *Hepatozoon canis* (DQ439543.1) as out group.

Discussion

The objective of this study was to estimate the prevalence of *T. equi* microscopically and by conventional PCR and then to apply the molecular characterization of *T. equi* to specific geographic areas in Egypt (Cairo and Giza governorates).

Microscopic examination has been shown to be insensitive to detect low parasitemia especially in areas where the disease is endemic (18). More-ever, microscopic examination depends on host specificity and this is less useful with such parasites of broader host specificity as *Babesia microti* (19). The conclusion is that, molecular techniques are a more objective tool for the diagnosis of *T. equi* (20).

In this study, the overall prevalence of *T. equi* by microscopical examination of blood samples was (5.56 %). This result agrees with previous studies in Egypt that showed the infection rate of *T. equi* is between 5% and 10% (21–23). The infection rate found in this study was lower than that recorded earlier in Egypt, which showed infection rates of 19.8%, 34%, 13.9%, 18% and 38.9% respectively (24–28). This may be due to differences in the various geographic areas in Egypt and high vector tick activity in the areas of sampling. Lower activity now may be due to a greater awareness by owners of preventive measures, more aggressive treatment of infected animals and increasing efforts of tick control. The time of sampling can make a difference, where samples are collected at the acute or chronic stage of the disease.

The infection rate of *T. equi* was recorded in various other countries, as in Iran 2016, Iran 2014 and in central Ethiopia (9.7%, 9.1% and 12.2% respectively) (7,29,30). The prevalence found was higher than that recorded in Egypt, due to such different environmental conditions as temperature and humidity, which affects tick activity. Also, type of animal, whether racing or working equines. Hygienic measures and vector control also play a role in such differences.

In the present study, there are no marked differences between the infection rate of *T. equi* in horses (5.67%) and donkeys (5.6 %). This agrees with (30)**,** who recorded infection rates (51.2% and 51.6%) of *T. equi* in horses and donkeys.

In this study, molecular techniques showed a higher sensitivity than microscopic examination in the diagnosis of subclinical and carrier animals and this agrees with (9,31,32)**.**

In the present study, by application of conventional PCR, the *T. equi* infection rate was 30%. This result was lower than that recorded in Egypt by (28) (77.80%) and higher than (29,35)**,** who recorded infection rates of 10.83% and 13.90% respectively by PCR amplification.

Other countries recorded higher rates of infection, as in Brazil 96% by nested PCR (34), Egypt 47.7% by nested PCR (26) and Iran 96.8% by conventional PCR (29). The lower prevalence was recorded in (35), Brazil (15.0%) (36) and Turkey (2.96%) (37).

Sequencing and phylogenetic analysis of piroplasms depends mainly on 18s rRNA, due to its low substitution rate; constrained and conserved function and occurrence in multiple copies (38)**.** Sequencing and phylogenetic analysis of the positive PCR product for *T. equi* recorded a 100% similarity with previously published sequences on the GeneBank database for donkey and mule sequences, but a 99% similarity for horse sequences, as illustrated in figure (4)**.**

In conclusion, it is recommended to use PCR as a rapid confirmatory technique for *T. equi* because it has higher sensitivity than microscopic examination in subclinical and chronic phases of the infection and in carrier cases. Also, it is recommended to conduct further studies on *T.equi,* to determine the best method for diagnosis and to illustrate the best control and preventive strategies against this very significant equine parasite.

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