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 محمود عبد النبي الصيفي'، نشوى مجد حلمي'، نجوى مجد الهواري'، شيماء صبحى سرور و أحمد محمود سليمان'

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الخلاصة

لغرض من هذه الدراسة هو تحديد معدل الخمج بطفيل الثيلريا الخيلية في بعض محافظات جمهورية مصر العربية (القاهرة والجيزة). اذ تم جمع عدد 797 عينة دم من الفصيلة الخيلية والتي مثلت 111 راساً من خيول محلية و 701 من الحمير و من البغال المفترة من نيسان (ابريل) 701 الى أذار (مارس) 701 باستخدام الفحص المجهري واختبار تفاعل انزيم البلمرة المتسلسل التقليدي 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 في عدد 701 عينة دم بنسبة 701, من الخيول المحلية وبنسبة 701, من البغال تم اختيار هم بطريقة عشوائية، البلمرة المتسلسل التقليدي على 701 عينه دم مثلت 701 من الخيول المحلية و 701 من الجمار و 701 من البغال تم اختيارهم بطريقة عشوائية، وجد ان 701 عينة كانت إيجابية للثيلريا الخيلية منها 701 من الخيول المحلية و 701 من الحمير و 701 من البغال. تم عمل التتابع الجيني لعدد 701

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 ٢٢ 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 الاتبة MF192854 و MF192855

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 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 crossreactivity 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 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 GoTag® Flexi Buffer (10 µl), 25mM MgCl2 Solution (2 µl), PCR Nucleotide Mix (dNTPs) 10 mM each (1µ1), Primer mix 10 pmol (1µl), 1.25 u GoTaq®G2 Flexi DNA Polymerase template DNA (5 μ l), DNase/RNase free water (7 μ l). 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 μ l 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.	No.	Infection rate
	examined	infected	(%)
Horses	141	8	5.67
Donkeys	250	14	5.60
Mules	5		
Total	396	22	5.56

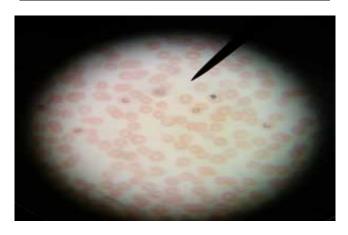


Figure 1: Microscopically stained blood smears showed small rounded merozoites of *Theileria equi* (x100).

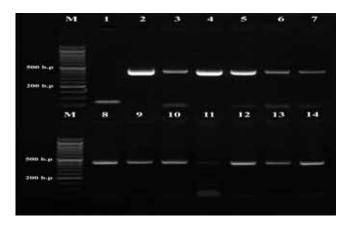


Figure 2: Conventional PCR detection of *Theileria equi* at 392 bp fractionated on 1.5% agrose gel. Lane M: 50 bp DNA ladder, Lane 1: *T. equi* negative control, Lane 2: *T. equi* positive control (392bp), Lane 3-14 *T. equi* positive samples (392bp).

			muland						
	5	15	25	35	45	55	65	75	85
MF192855_Theileria_equi_mule_E								CCAAAGAATC	
MF192856_Thelleria_equi_donkey MF192854_Thelleria_equi_horse_								CCAAAGAATC	
KX227629.1_Theileria_equi_isol								CCAAAGAATC	
KX227623.1_Theileria_equi_isol								CCAAAGAATC	
XX896428.1_Theileria_equi_isol								CCAAAGAATC	
KU289096.1_Theileria_equi_isol								CCAAAGAATC	
KT307980.1_Babesia_equi_185_ri								CCAAAGAATC	
LC008132.1 Babesia_equi_gene_f								CCAAAGAATC	
AB515310.1_Babesia_equi_gene_f Z15105.1_B.equi_gene_encoding								CCAAAGAATC	
KY464036.1_Theileria_equi_clon								CCAAAGAATC	
KM819528.1_Babesia_equi_isolat								CCAAAGAATC	
KM046922.1_Babesia_equi_isolat								CCAAAGAATC	
KU240071.1_Theileria_equi_clon								CCAAAGAATC	
EU642512.1 Babesia caballi gen EU642514.1 Babesia caballi gen								CCAAAGAATC	
AF881135.1 Theileria lestoquar								CCAAAGAATC	
EU083800.1 Theileria annulata								CCAAAGAATC	
DQ439543.1 Hepatozoon canis is								CCATAGAATT	
The state of the s									
	95	105	115	125	135	145	155	165	175
MF192855_Theileria_equi_mule_E								GGCTCCACGC	
MF192856_Theileria_equi_donkey								GGCTCCACGC	
MF192854_Theileria_equi_horse_								GGCTCCACGC	
KX227629.1_Theileria_equi_isol KX227623.1_Theileria_equi_isol								GGCTCCACGC	
KX896428.1_Theileria_equi_isol								GGCTCCACGC	
KU289896.1_Theileria_equi_isol								GGCTCCACGC	
KT307980.1_Babesia_equi_185_ri	TATCAATCTG	TCAATCCTTC	CTCTGTCTGG	ACCTGGTGAG	TTTCCCCGTG	TTGAGTCAAA	TTAAGCCGCA	GGCTCCACGC	CTGGTGGTGC
I,C008132.1_Babesla_equi_gene_f								GGCTCCACGC	
AB515310.1_Babesia_equi_gene_f								GGCTCCACGC	
Z15105.1_B.equi_gene_encoding_								GGCTCCACGC	
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KOM046922.1_Babesia_equi_isolat								GGCTCCACGC	
KU240071.1_Thelleria_equi_clon								GGCTCCACGC	
EU642512.1 Babesia caballi gen	TATCAATCTG	TCAATCCTAC	CTCTGTCTGG	ACCTGGTGAG	TTTCCCCGTG	TTGAGTCAAA	TTAAGCCGCA	GGCTCCACGC	CTGGTGGTGC
EU642514.1 Babesia caballi gen								GGCTCCACGC	
AF081135.1 Theileria lestoquar								GGCTCCACGC	
EU883800.1 Theileria annulata								GGCTCCACGC	
DQ439543.1 Hepatozoon canis is								GGCTCCACGC	
	185	195	205	215	225	235	245	255	265
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MF192856 Theileria_equi_donkey								TCTCAAGGTG (
MF192854_Theileria_equi_horse_	CCTTCCGTCA	ATTCCTTTAA	GTTTCAGCCT	TGCGACCATA	CTCCCCCAG	AACCCAAAGA	CTTTGATTTC	TCTCAAGGTG (CTGAAGGAGT
KX227629.1_Theileria_equi_isol		ATTCCTTTAA					CTTTGATTTC	TCTCAAGGTG (
KX227623.1_Theileria_equi_isol						AAFCCAAAGA			
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KUZ89996.1 Theileria equi isol KT307980.1 Sabesia equi 185 ri LC088137.1 Sabesia equi gene f A5515310.1 Sabesia equi gene f 715185.1 B. equi gene encoding KY464036.1 Theileria equi clon KW819520.1 Sabesia equi isolat KW046922.1 Sabesia equi isolat	CCTTCCGTCA CCTTCCGTCA CCTTCCGTCA CCTTCCGTCA CCTTCCGTCA CCTTCCGTCA CCTTCCGTCA CCTTCCGTCA CCTTCCGTCA CCTTCCGTCA	ATTCCTTTAA ATTCCTTTAA ATTCCTTTAA ATTCCTTTAA ATTCCTTTAA ATTCCTTTAA ATTCCTTTAA ATTCCTTTAA ATTCCTTTAA	GTTTCAGCCT GTTTCAGCCT GTTTCAGCCT GTTTCAGCCT GTTTCAGCCT GTTTCAGCCT GTTTCAGCCT GTTTCAGCCT GTTTCAGCCT GTTTCAGCCT	TGCGACCATA TGCGACCATA TGCGACCATA TGCGACCATA TGCGACCATA TGCGACCATA TGCGACCATA TGCGACCATA TGCGACCATA	CTCCCCCAG CTCCCCCAG CTCCCCCAG CTCCCCCAG CTCCCCCAG CTCCCCCAG CTCCCCCAG CTCCCCCAG CTCCCCCAG CTCCCCCAG	AACCCAAAGA AACCCAAAGA AACCCAAAGA AACCCAAAGA AACCCAAAGA AACCCAAAGA AACCCAAAGA AACCCAAAGA	CTTTGATTTC CTTTGATTTC CTTTGATTTC CTTTGATTTC CTTTGATTTC CTTTGATTTC CTTTGATTTC CTTTGATTTC CTTTGATTTC	TCTCAAGGTG (TCTCA	CTGAAGGAGT CTGAAGGAGT CTGAAGGAGT CTGAAGGAGT CTGAAGGAGT CTGAAGGAGT CTGAAGGAGT CTGAAGGAGT CTGAAGGAGT CTGAAGGAGT
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KUZ89996.1 Theileria_equi_isol KT307980.1 Babesia_equi_BS_ri LC088132.1 Babesia_equi_gene_f A5515310.1 Babesia_equi_gene_f Z15105.1_B.equi_gene_encoding KY464036.1 Theileria_equi_clon KY819520.1 Babesia_equi_isolat KY046922.1 Babesia_equi_isolat KV240071.1 Theileria_equi_clon	CCTTCCGTCA CCTTCCGTCA CCTTCCGTCA CCTTCCGTCA CCTTCCGTCA CCTTCCGTCA CCTTCCGTCA CCTTCCGTCA CCTTCCGTCA CCTTCCGTCA CCTTCCGTCA CCTTCCGTCA	ATTCCTTTAA ATTCCTTTAA ATTCCTTTAA ATTCCTTTAA ATTCCTTTAA ATTCCTTTAA ATTCCTTTAA ATTCCTTTAA ATTCCTTTAA ATTCCTTTAA ATTCCTTTAA	GTTTCAGCCT GTTTCAGCCT GTTTCAGCCT GTTTCAGCCT GTTTCAGCCT GTTTCAGCCT GTTTCAGCCT GTTTCAGCCT GTTTCAGCCT GTTTCAGCCT GTTTCAGCCT GTTTCAGCCT	TGCGACCATA	CTCCCCCAG CTCCCCCAG CTCCCCCAG CTCCCCCAG CTCCCCCAG CTCCCCCAG CTCCCCCAG CTCCCCCAG CTCCCCCAG CTCCCCCAG CTCCCCCAG CTCCCCCAG	AACCCAAAGA AACCCAAAGA AACCCAAAGA AACCCAAAGA AACCCAAAGA AACCCAAAGA AACCCAAAGA AACCCAAAGA AACCCAAAGA AACCCAAAGA	CTITGATTIC	TCTCAAGGTG (TCTCA	CTGAAGGAGT CTGAAGGAGT CTGAAGGAGT CTGAAGGAGT CTGAAGGAGT CTGAAGGAGT CTGAAGGAGT CTGAAGGAGT CTGAAGGAGT CTGAAGGAGT CTGAAGGAGT CTGAAGGAGT
KU289996.1 Theileria equi isoli KT307980.1 Babesia equi 185 ri LC008132.1 Babesia equi gene f A5515310.1 Babesia equi gene f 715105.1 B. equi gene encoding KY464036.1 Theileria equi clon KY854036.1 Babesia equi isolat KV240071.1 Theileria equi clon EU642512.1 Babesia caballi gen EU642514.1 Babesia caballi gen AF081135.1 Theileria lestoquar	CCTTCCGTCA	ATTCCTTTAA ATTCCTTTAA ATTCCTTTAA ATTCCTTTAA ATTCCTTTAA ATTCCTTTAA ATTCCTTTAA ATTCCTTTAA ATTCCTTTAA ATTCCTTTAA ATTCCTTTAA ATTCCTTTAA ATTCCTTTAA ATTCCTTTAA	GTTTCAGCCT GTTTCAGCCT GTTTCAGCCT GTTTCAGCCT GTTTCAGCCT GTTTCAGCCT GTTTCAGCCT GTTTCAGCCT GTTTCAGCCT GTTTCAGCCT GTTTCAGCCT GTTTCAGACT GTTTCAGACT GTTTCAGCCT	TGCGACCATA	CTCCCCCAG	AACCCAAAGA AACCCAAAGA AACCCAAAGA AACCCAAAGA AACCCAAAGA AACCCAAAGA AACCCAAAGA AACCCAAAGA AACCCAAAGA AACCCAAAGA AACCCAAAGA AACCCAAAGA	CTTTGATTIC	TCTCAAGGTG (TCTCA	CTGAAGGAGT
KU289996.1 Theileria_equi_isol KT307980.1 Babesia_equi_BS_ri LC008132.1 Babesia_equi_gene_f AB515310.1_Babesia_equi_gene_f Z15105.1 B.equi_gene_encoding_ KY464036.1 Theileria_equi_clon KM819520.1_Babesia_equi_isolat KW046922.1_Babesia_equi_isolat KW046971.1 Theileria_equi_clon EU642512.1 Babesia caballi gen EU642513.1 Babesia caballi gen EU683800.1 Theileria_lestoquar EU083800.1 Theileria_lestoquar	CCTTCCGTCA	ATTCCTTTAA ATTCCTTTAA ATTCCTTTAA ATTCCTTTAA ATTCCTTTAA ATTCCTTTAA ATTCCTTTAA ATTCCTTTAA ATTCCTTTAA ATTCCTTTAA ATTCCTTTAA ATTCCTTTAA ATTCCTTTAA ATTCCTTTAA ATTCCTTTAA ATTCCTTTAA ATTCCTTTAA ATTCCTTTAA ATTCCTTTAA ATTCCTTTAA	GTTTCAGCCT GTTTCAGCCT GTTTCAGCCT GTTTCAGCCT GTTTCAGCCT GTTTCAGCCT GTTTCAGCCT GTTTCAGCCT GTTTCAGCCT GTTTCAGCCT GTTTCAGCCT GTTTCAGCCT GTTTCAGCCT GTTTCAGCCT GTTTCAGCCT GTTTCAGCCT	TGCGACCATA	CTCCCCCAG	AACCCAAAGA	CTITGATTIC	TCTCAAGGTG TCTCA	CTGAAGGAGT
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KU289996.1 Theileria_equi_isol KT307980.1 Babesia_equi_BS_ri LC008132.1 Babesia_equi_gene_f AB515310.1_Babesia_equi_gene_f Z15105.1 B.equi_gene_encoding_ KY464036.1 Theileria_equi_clon KM819520.1_Babesia_equi_isolat KW046922.1_Babesia_equi_isolat KW046971.1 Theileria_equi_clon EU642512.1 Babesia caballi gen EU642513.1 Babesia caballi gen EU683800.1 Theileria_lestoquar EU083800.1 Theileria_lestoquar	CCTTCCGTCA	ATTCCTTTAA ATTCCTTTAA ATTCCTTTAA ATTCCTTTAA ATTCCTTTAA ATTCCTTTAA ATTCCTTTAA ATTCCTTTAA ATTCCTTTAA ATTCCTTTAA ATTCCTTTAA ATTCCTTTAA ATTCCTTTAA ATTCCTTTAA ATTCCTTTAA ATTCCTTTAA ATTCCTTTAA	GTTTCAGCCT GTTTCAGCCT GTTTCAGCCT GTTTCAGCCT GTTTCAGCCT GTTTCAGCCT GTTTCAGCCT GTTTCAGCCT GTTTCAGCCT GTTTCAGCCT GTTTCAGCCT GTTTCAGCCT GTTTCAGCCT GTTTCAGCCT GTTTCAGCCT GTTTCAGCCT GTTTCAGCCT	TGCGACCATA	CTCCCCCAG	AACCCAAAGA	CTTTGATTIC	TCTCAAGGTG TCTCA	CTGAAGGAGT
KU289996.1 Theileria_equi_isol KT307980.1 Babesia_equi_iBS_ri LC008132.1 Babesia_equi_gene_f AB515310.1_Babesia_equi_gene_f Z15105.1_B.equi_gene_encoding_ KY464036.1 Theileria_equi_clon KY819520.1_Babesia_equi_isolat KY046922.1_Babesia_equi_isolat KY046971.1 Theileria_equi_clon EU642512.1 Babesia caballi gen EU642514.1 Babesia caballi gen EU683800.1 Theileria lentoquar EU083800.1 Theileria lentoquar EU083800.1 Theileria lentoquar	CCTTCCGTCA	ATTCCTTTAA	GITTCAGCCT	TGGGACCATA	CTCCCCCAG CTCCCCCCAG CTCCCCCAG	AACCCAAAGA	CHITGATTIC	TCTCAAGGTG TCTCA	CTGAAGGAGT
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KUZ89996.1 Theileria equi isol KT307980.1 Babesia equi 185_ri LC088132.1 Babesia equi gene f AB515310.1 Babesia equi gene f AB515310.1 Babesia equi gene f Z15106.1 Babesia equi isolat KW464036.1 Theileria equi clon KW819520.1 Babesia equi isolat KW240071.1 Theileria equi clon EU642512.1 Babesia caballi gen EU642514.1 Babesia caballi gen AF081135.1 Theileria lestoquar EU083800.1 Theileria annulata DQ439543.1 Hepatozoon canis is	CCTTCCGTCA	ATTCCTTTAA ATTCCTTAA ATTCCTTTAA ATTCCTTAA ATTCCTTTAA ATTCCTTAA ATTCCTTAAA ATTCCTTAAA ATTCCTTAAA ATTCCTTAAA ATTCCTTAAA ATT	GITTCAGCCT ATTCCTAGT AATCTCTAGT	TGGGACCATA TGCGACCATA TGCGACCATCA TGCGACATCATC TGGCATCGTT CGGCATCGTT CGGCATCGTT	CTCCCCCAG CTCCCCCCAG CTCCCCCCAG CTCCCCCCAG TTCCCCCCAG TTCCCCCCAG TTCCCCCCAG TTCCCCCCAG TTCCCCCCAG TTCCCCCCAG TTCCCCCCAG TTCCCCCAG TTCCCCCCAG TTCCCCCAG TTCCCCCCAG TTCCCCCCAG TTCCCCCCAG TTCCCCCCAG TTCCCCCCAG TTCCCCCAG TTCCCCCCAG TTCCCCCCAG TTCCCCCCAG TTCCCCCCAG TTCCCCCCAG TTCCCCCAG TTCCCCCAG TTCCCCCAG TTCCCCCAG TTCCCCCCAG TTCCCCCAG TTCCCCCAG TTCCCCCCAG TTCCCCCAG TTCCCCCCAG TTCCCCCCAG TTCCCCCCAG TTCCCCCCAG TTCCCCCCAG TTCCCCCAG TTCCCCCCAG TTCCCCCCAG TTCCCCCCAG TTCCCCCCAG TTCCCCCAG TTCCCCCAG TTCCCCCCAG TTCCCCCAG TTCCCCCAG TTCCCCCAG TTCCCCCCAG TTCCCCCCAG TTCCCCCCAG TTCCCCCCAG TTCCCCCCAG TTCCCCCCAG TTCCCCCCAG TTCCCCCCAG TTCCCCCCAG TTC	AACCCAAAGA AACCAAAGA AACCCAAAGA AACCAAAGA AACCCAAAGA AACCCAAAGA AACCCAAAGA AACCCAAAGA AACCCAAAGA AC	CHITGATTIC TAICT TAICT	TCTCAAGGTG TCTCA	CTGAAGGAGT TTGAAGGAGT CTGAAGGAGT CTGAAGGAGT CTGAAGGAGT CTGAAGGAGT CTGAAGGAGT CTGAAGGAGT CTGAAGGAGT CTGAAGGAGT CTGAAGGAGT CTGAAGGAGT CTGAAGGAGT CTGAAGGAGT CTGAAGGAGT
KUZB9996.1 Theileria equi isol KT307980.1 Babesia equi iBS_ri LC088132.1 Babesia equi gene f AB515310.1 Babesia equi gene f AB515310.1 Babesia equi gene f Z15105.1 B. equi gene encoding KY454316.1 Theileria equi isolat KU240071.1 Theileria equi isolat KU240071.1 Theileria equi clon EU642512.1 Babesia caballi gen AF081135.1 Theileria lestoquar EU63800.1 Theileria equi mulata DQ439543.1 Hepatozoon canis is MF192855 Theileria equi donkey MF192856 Theileria equi donkey MF192856 Theileria equi horse EX227629.1 Theileria equi isol	CCTTCCGTCA CCTTCCAACCT CGTTCAAACT CGTTCAAACT CGTTCAAACT CGTTCAAACT CGTTCAAACT	ATTCCTTTAA	GITTCAGCCT ATCCTAGT AATCCTAGT AATCCTAGT AATCCTAGT	TGCGACCATA TGCGACCATC TGGCATCGTT CGGCATCGTT CGGCATCGTT CGGCATCGTT CGGCATCGTT	CTCCCCCAG TTCCCCCAG TATGGTTAGG TATGGTTAGG TATGGTTAGG TATGGTTAGG TATGGTTAGG TATGGTTAGG TATGGTTAGG TATGGTTAGG TATGGTTAGG	AACCCAAAGA ACCCAAAGA ACCCAAGA ACCCAAAGA ACCCAAGA ACCCAAAGA ACCCAAAGA ACCCAAAGA ACCCAAAGA ACCCAAAGA ACCCAAAGA AC	CHIGATHIC THIGATHIC THIGAT	TCTCAAGGTG TCTCA	CTGAAGGAGT TTGAAGGAGT CTGAAGGAGT CTGAAGGAGT CTGAAGGAGT CTGAAGGAGT CTGAAGGAGT CTGAAGGAGT CTGAAGGAGT CTGAAGGAGT CTGAAGGAGT CTGAAGGAGT CTGAAGGAGT CTGAAGGAGT CTGAAGGAGT
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KUZ89996.1 Theileria equi isol KT307980.1 Sabesia equi 185 ri LC088132.1 Sabesia equi gene f AB513310.1 Sabesia equi gene f AB513310.1 Sabesia equi gene f AT51965.1 S. equi gene encoding KY464036.1 Theileria equi clon KW819520.1 Sabesia equi isolat KU240071.1 Theileria equi clon EU642512.1 Sabesia caballi gen EU642514.1 Sabesia caballi gen EU642514.1 Sabesia caballi gen EU643514.1 Sabesia caballi gen EU642512.1 Sabesia caballi gen EU642512.1 Sabesia caballi gen EU642514.1 Sabesia caballi gen EU642514.1 Sabesia caballi gen EU642514.1 Sabesia caballi gen EU642514.1 Sabesia caballi gen EU642515.1 Theileria equi mule E MF192855 Theileria equi mule E EU6227629.1 Theileria equi jool EU627629.1 Theileria equi jool EU6896428.1 Theileria equi jool	CCTTCCGTCA CCTTCCAAACT CGTTCAAACT	ATTCCTTTAA	GITTCAGCCT ATCCTAGT AATCTCTAGT	TGGGACCATA TGGGACCATC TGGGATCGTT CGGCATCGTT	CTCCCCCAG CTCCCCCCAG CTCCCCCAG TATGGTTAGG	AACCCAAAGA ACCCAAAGA ACCCACAGA ACCTACGACGG ACCTACGACGG ACCTACGACGG ACCTACGACGG ACCTACGACGG	CTITGATTIC TTIGATTIC TAICT TAICT TAICT TAICT TAICT	TCTCAAGGTG TCTCA	CTGAAGGAGT
KUZ89996.1 Theileria equi isol KT307980.1 Sabesia equi 185 ri LC088132.1 Sabesia equi gene f AB515310.1 Sabesia equi isolat KV454036.1 Theileria equi clon KV819520.1 Sabesia equi isolat KU240071.1 Theileria equi clon KU642512.1 Sabesia caballi gen KU642514.1 Sabesia caballi gen AF081135.1 Theileria lestoquar EU083800.1 Theileria equi mulata DQ439543.1 Hepatozoon canis is MF192855 Theileria equi donkey MF192855 Theileria equi horse KX227623.1 Theileria equi isol KX227623.1 Theileria equi isol KX896428.1 Theileria equi isol KX896428.1 Theileria equi isol KX896428.1 Theileria equi isol KX8906428.1 Theileria equi isol KX8906428.1 Theileria equi isol KX8906428.1 Theileria equi isol	CCTTCCGTCA CCTTCCAAACT CGTTCAAACT	ATTCCTTTAA	GITTCAGCCT ATCCTAGT AATCCTAGT AATCCTAGT AATCTCTAGT AATCCTAGT AATCTCTAGT	TGGGACCATA TGCGACCATA TGCGACCATC TGGCATCGTT TGGCATCGTT TGGCATCGTT TGGCATCGTT TGGGCATCGTT TGGGATCGTT TGGG	CTCCCCCAG TTCCCCCAG TTCCCCCAG TTCCCCCAG TTCCCCCAG TTCCCCCAG TTCCCCAG TTCCCCCAG TTCCCCCAG TTCCCCCAG TTCCTCAGG TATGGTTAGG	AACCCAAAGA ACCCAAAGA ACCCAAAGA ACCCAAAGA ACCCAAAGA ACCCAAAGA ACTACGACGG ACTACGACGACGG ACTACGACGG ACTACGACGACGACACACACACACACACACACACACACAC	CHIGATHIC THACH TANCT	TCTCAAGGTG TCTCA	CTGAAGGAGT
KUZ89996.1 Theileria equi isol KT307980.1 Babesia equi j85 ri LC088132.1 Babesia equi j8me f A5513310.1 Babesia equi j5me KY464036.1 Theileria equi j5olat KV246097.1 Theileria equi j5olat KV240071.1 Theileria equi j5olat KV240071.1 Theileria equi j5olat KV240071.1 Theileria equi j5olat KV240071.1 Theileria equi j5olat EV083380.1 Theileria equi j6olat EV083380.1 Theileria equi j5olat KV227623.1 Theileria equi j5olat KV230936.1 Theileria equi j5olat KV230936.1 Theileria equi j5olat KV307980.1 Babesia equi j85 ri	CCTTCCGTCA CCTTCCAAACT CGTTCAAACT CGTTCCAAACT CGTTCCAACT CGTTCCAAACT CTTCCATC	ATTCCTTTAA	GITTCAGCCT AITCCTAGT AATCCTAGT	TGGGACCATA TGCGACCATA TGCGACCATC TGGCATCGTT	CTCCCCCAG TACGGTTAGG TATGGTTAGG	AACCCAAAGA ACCCAAAGA ACCCACAGAGA ACTACGACGG ACTACGACGA ACTACGACGG ACTACGACGACGG ACTACGACGG ACTACGACGACGA	CHITGATTIC THACH TANCH	TCTCAAGGTG TCTCA	CTGAAGGAGT
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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	Microscopical	examination	Conventional PCR examination			
	examined	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		

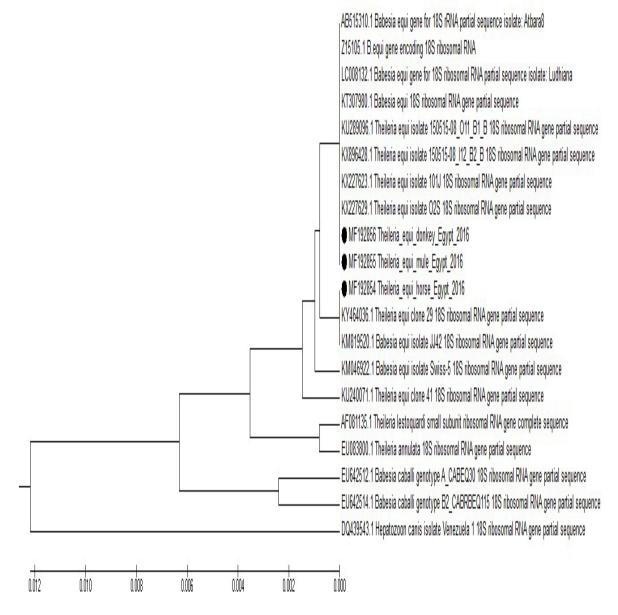


Figure 4: Genetic relationship of *Theileria equi* isolates obtained from egyptian equines with reference to sequences of *Theileria equi* accessed in the genbank. Phylogenetic tree was produced by applying Neighbor-Joining technique of the nucleotide sequence of the 18s rRNA gene with using *Hepatozoon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