PARASITOLOGICAL AND MOLECULAR DETECTION OF *THEILERIA EQUI* IN DONKEYS IN ASSIUT GOVERNORATE

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Abstract

Theileria equi is a tick-borne hemoprotozoan parasite and one of the causative agents of equine piroplasmosis. Data on *T. equi* infection in donkeys are scarce in Assiut governorate, Egypt. This study was conducted on the prevalence of *T. equi* infection in donkeys by using micropscopic examination and polymerase chain reaction (PCR) assay. Out of 50 donkeys, blood samples examined for *T. equi,* 14% (7/50) were positive by microscopic examination and 38% (19/50) by PCR. Prevalence of *T. equi* had high significant differences between microscopic examination and PCR assay. Prevalence of *T. equi* in male donkeys was 13.6% (3/22) by microscopic examination and 36.4% (8/22) by PCR. In female donkeys it was 14.3% (4/28) by microscopic examination and 39.3% (11/28) by PCR. No significant difference between the prevalence rate of infection in males and females were recorded. Microscopic examination of donkeys' blood smears stained with Giemsa stain revealed forms of theilerial schizogony in lymphocytes (Koch's blue bodies) and intra-erythrocytic relatively small, less than 2 µm long pear-shaped and ring shaped merozoites of *T. equi***.** The B1 gene specified for *T. equi* was detected by PCR in 19 blood samples. All positive samples with microscopic examination were also positive with PCR. The sensitivity, specificity and accuracy of PCR with respect to blood film examination were 61.3%, 100% and 82%, respectively. So, PCR was found to be more sensitive, specific and accurate than blood film examination.The present results suggest that *T. equi* parasite is widely spread in Assiut governorate due to high exposure to ticks.

Keywords: *Theileria equi* - microscopic examination - PCR - Assiut governorate

INTRODUCTION

Principally, donkeys still considered as important animals in farms and have been trained and used in farming and transportation in many countries. Since donkeys are frequently kept outdoors, they are exposed to a high risk of tick bites and consequently to infection with blood parasites. *Babesia caballi* and *T. equi* have been already recognized as a serious problem of major economic importance in donkey husbandry, since the affected animals display loss of appetite and decreased working capacity (Machado *et al.,* 2012).

Mehlhorn and Schein (1998) redescribed the horse-parasitizing species *Babesia equi* as *Theileria equi* and thus transferred from one valid genus to another. It was reclassified as *Theileria species* because of the transstadial transmission in the vector and because sporozoites do not infect red blood cells but penetrate lymphocytes (or macrophages) where they develop into schizonts. The merozoites are released from the schizonts then enter the red blood cells where they grow into piroplasms (Schein *et al.,* 1981). Further support for the close relation of *T. equi* with *Theileria spp*. also comes from the homology found between 30 and 34 kDa *T. equi* surface proteins and similar sized proteins of various *Theileria spp*. (Knowles *et al.,* 1997). Genetically, this species had been proved to be more related to the genus *Theileria* than *Babesia* (Allsopp *et al.,* 1994).

Equine theileriosis caused by *T. equi* considered to be the most pathogenic tick-borne piroplasm of horses and was clinically characterized by fever of up to 40 ºC, anemia, depression, anorexia, icterus, enlarged lymph nodes, hepatomegaly, splenomegaly, hemoglobinuria, bilirubinuria, edema of distal limbs, pulmonary edema, nasal discharge, colic and petechial hemorrhages of the mucous membranes (Salib *et al.,* 2013 and Sumbria *et al.,* 2015).

Erythrocytic stages of *T. equi* were small in size (less than 2 µm) and were spherical, ovoid or cross shaped. They may be found either single, in pairs or in tetrads (Schein, 1988). Transplacental transmission of *T. equi* from carrier mares to asymptomatic foals can occur which becomes a serious economic problem for horse farmers (Allsopp *et al*., 2007).

Diagnosis of equine piroplasmosis can be performed by direct diagnosis includes demonstration of intraerythrocytic forms in Giemsa stained blood smears or by molecular techniques (OIE, 2014). Detection of parasite DNA by PCR has been known as a powerful tool both in the early phase of infection and in carrier animals (Rampersad *et al*., 2003).

Conventional techniques are the gold standard method to detect piroplasms in infected equids with acute signs (Böse *et al.,* 1995). Although this method is simple, it is not very sensitive in cases of low parasitemia and does not permit diagnosis of mixed infections (Krause, 2003). So, molecular techniques have been developed in recent years for the detection of *T. equi*. These methods are based on species-specific PCR assays (Adaszek *et al.,* 2011). The PCR method has been described as a molecular technique for sensitive, specific and reliable genomic detection of *Babesia* and *Theileria* parasites (Alhassan *et al.,* 2005 and Davitkov *et al.,* 2015). Diagnosis by PCR is sensitive enough to detect parasite DNA from 2.5 µl blood sample with parasitemia of 0.000001% (Alhassan *et al.,* 2007 and Sumbria *et al.,* 2016).

In Egypt, the data available on *T. equi* in donkeys is scarce and it is restricted to only few areas and to small numbers of samples. More studies using sensitive and specific diagnostic techniques are still required (Mahmoud *et al.*, 2015). So, this study aimed to determine the prevalence of *T. equi* in donkeys in Assiut governorate by microscopic examination and PCR.

MATERIALS AND METHODS

Sampling: A total number of 50 blood samples were collected randomly from apparently healthy 50 donkeys in Assiut governorate. Blood samples were obtained from jugular vein of each donkey. The sex was recorded for each animal. The blood samples were collected in vacutainer tubes containing EDTA anticoagulant. Blood smears prepared and stained with Giemsa stain. The blood samples in vacutainer tubes stored at -20°C until used for molecular analysis (PCR) (Sgorbini *et al.*, 2015).

Microscopic examination: The prepared blood films were dried and fixed with absolute methyl alcohol for 7-10 min. These blood smears were stained with Giemsa stain according to Coles (1986) and examined with light microscope for the presence of haemoprotozoan parasite (*T. equi*).

Polymerase Chain Reaction (PCR)

1. DNA extraction (Kim *et al.,* 2008): Accurately 100 µl of blood samples were diluted in the same volume of phosphate-buffered saline (PBS). DNA was extracted following the manufacturer's instructions of a commercial DNA blood Mini Kit (Qiagen, Valencia, CA, USA). All DNA extracts were stored at -20 ºC until used. This product was used as a template for PCR.

Primer sequences of *T. equi* used for PCR identification system: Application of PCR for identification of B1 gene specified for *T. equi* was performed essentially by using primers (Biosearch Technologies, Inc., USA) as shown in the following table:

Primer	Oligonucleotide sequence (5′ → 3′)	Productsize (bp)	Reference
Bec-UF2	5′ TCGAAGACGATCAGATACCGTG ′3	392	Pitel *et al.* (2010)
Equi-R	5′TGCCTTAAACTTCCTTGCGAT ′3		

2. DNA amplification reaction (Alhassan *et al.,* 2005): The amplification was performed on a Thermal Cycler (Master cycler, Eppendorf, Hamburg, Germany). The PCR mixture was represented by 50 ml of a mixture (10 mM Tris-HCl [pH8.3], 50 m MKCl, and 1.5 m M $MgCl_2$) containing 3 ml of the template DNA, 2.5 pmol of the primers, 0.2 mMdNTP mixture, and 2.5U of *Taq* DNA polymerase. The

mixture was heated for 10 min at 96ºC to activate the *Taq* DNA polymerase and 40 cycles of the following conditions were repeated: denaturation for 1 min. at 96ºC, annealing for 1 min at 60.5ºC, extension for 1 min at 72ºC and a final extension for 10 min at 72ºC. The amplified products were run in 1.5% agarose gel electrophoresis (Applichem, Germany, GmbH) in 1x TBE buffer stained with ethidium bromide (Bioshop ® Canda Inc, Lot No: 0A14667) and captured as well as visualized on UV transilluminator. Two control samples were used for each PCR cycle including *T. equi* DNA as positive and distilled water as negative control. A 100 bp plus DNA Ladder (Qiagen, Germany, GmbH) was used to determine the fragment sizes.

Statistical analysis:

Chi-square test was used to compare the differences in results between microscopic examination and PCR methods and the infection rates among sex. Results were analyzed using SPSS (Version 17; SPSS Inc., Chicago, USA). A value of $p < 0.05$ was considered as statistically significant (Malekifard *et al.*, 2014).

The sensitivity, specificity and accuracy of PCR assay with respect to blood film [gold standard test] (Ibrahim *et al.,* 2011 and Sumbria *et al.,* 2015) were calculated as follows:

$$\textbf{Sensitivity of PCR with respect to blood film} = \frac{\text{No. of true positive}}{\text{No. of true positive} + \text{No. of false negative}} \times 100$$

$$\textbf{Specificity of PCR with respect to blood film} = \frac{\text{No. of true negative}}{\text{No. of true negative} + \text{No. of false positive}} \times 100$$

$$\textbf{Accuracy} = \frac{\text{No. of true positive} + \text{No. of true negative}}{\text{No. of true positive} + \text{No. of false positive} + \text{No. of false negative} + \text{No. of true negative}} \times 100$$

RESULTS

Out of 50 blood samples of donkeys examined for *T. equi,* 14% (7/50) were positive by microscopic examination and 38% (19/50) by PCR (Table 1). All positive samples with microscopic examination were also positive with PCR. There was a high significant difference between the prevalence of *T. equi* by microscopic examination and PCR assay. The prevalence of *T. equi* in male donkeys was 13.6% (3/22) by microscopic examination and 36.4% (8/22) by PCR, while in female donkeys was 14.3% (4/28) by microscopic examination and 39.3% (11/28) by PCR (Table 2). No significant difference was recorded between the prevalence rate of infection in males and females.

Microscopic examination of Giemsa stained blood smears from donkeys revealed forms of theilerial schizogony in lymphocytes (Koch's blue bodies), being irregular shaped structure microschizonts vary from 4-6 µm contain small chromatin granules, 0.41 - 0.71µm in diameter (mean 0.56 µm) and intra-erythrocytic relatively small, less than 2 µm long pear-shaped and ring shaped merozoites of *T. equi* (Figures 1 and 2). The B1 gene specified for *T. equi* was detected by PCR in 19 blood samples (Figures 3 and 4). The sensitivity, specificity and accuracy of PCR with respect to blood film examination were 61.3%, 100% and 82%, respectively. So, PCR was found to be more sensitive, specificic and accurate than blood film examination.

Table 1. Prevalence of *T. equi* in examined donkeys by using microscopic examination and PCR.

Detection methods	No. of examined animals	No. of Positive samples	Prevalence (%)	Chi^2	P
Microscopic examination	50	7	14	7.484	< 0.01
PCR	50	19**	38		

** High significant statistical variation between microscopic examination and PCR.

Table 2. Prevalence of T. equi in examined donkeys according to sex by using microscopic examination and PCR.

Sex	No. of examined animals	No. of Positive by microscopic examination	Prevalence (%)	No. of Positive by PCR	Prevalence (%)	Chi^2	P
Male	22	3	13.6	8	36.4	0.585	0.444
Female	28	4	14.3	11	39.3		
Total	50	7	14	19	38		

No statistical variation between sexes of animals.

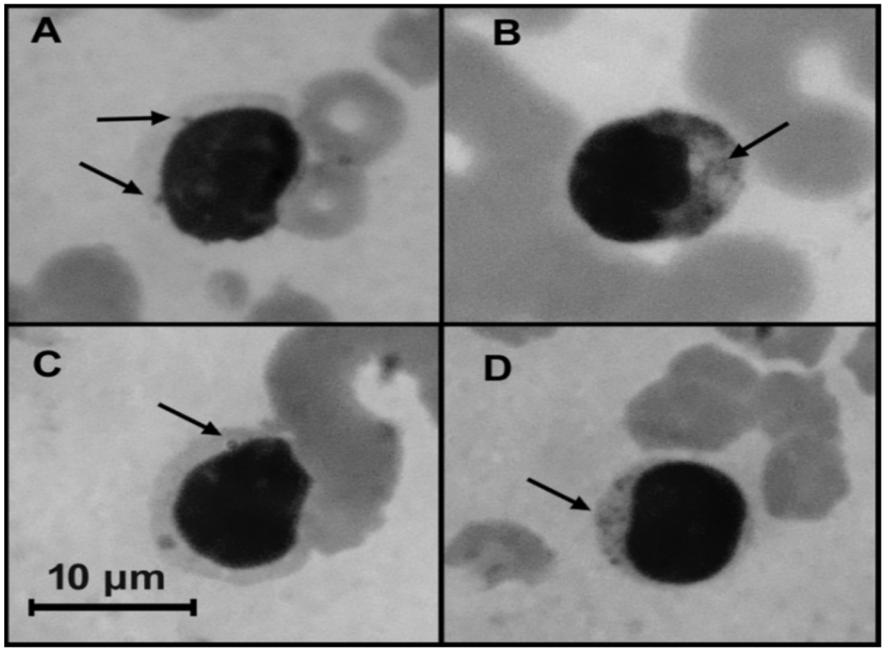

Figure 1. Giemsa stained blood films of donkeys showing forms of theilerial schizogony in lymphocytes. A, B, C, D, showing lymphocytes containing microschizonts of *Theileria* (Koch's blue bodies) (arrows) (scale bar 10 µm).

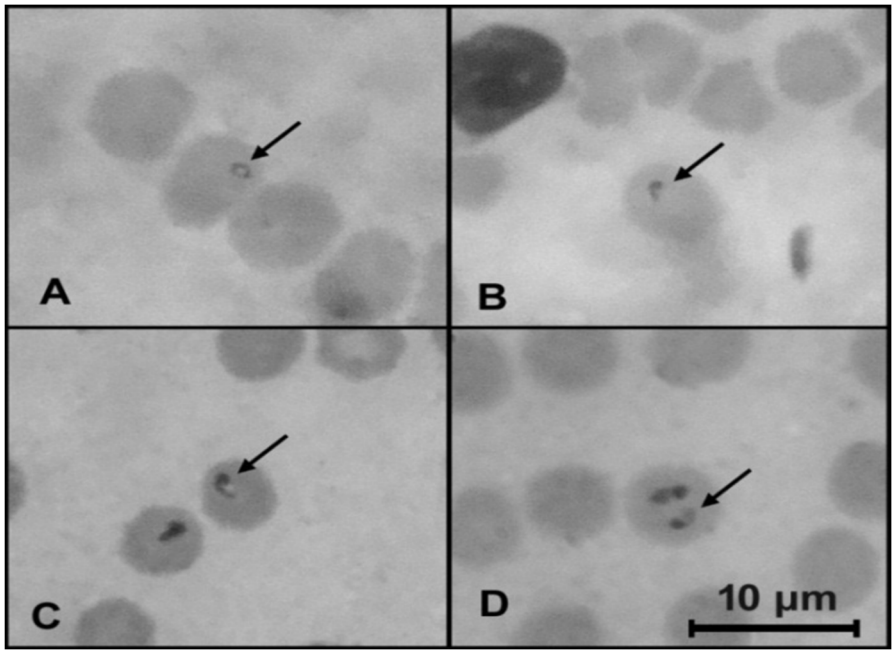

Figure 2. Giemsa stained blood films of donkeys showing intra-erythrocytic pear and ring shaped *Theileria equi.* A, B, C showing single intra-erythrocytic *Theileria* while D showing multiple intra-erythrocytic ones (arrows) (scale bar 10 µm).

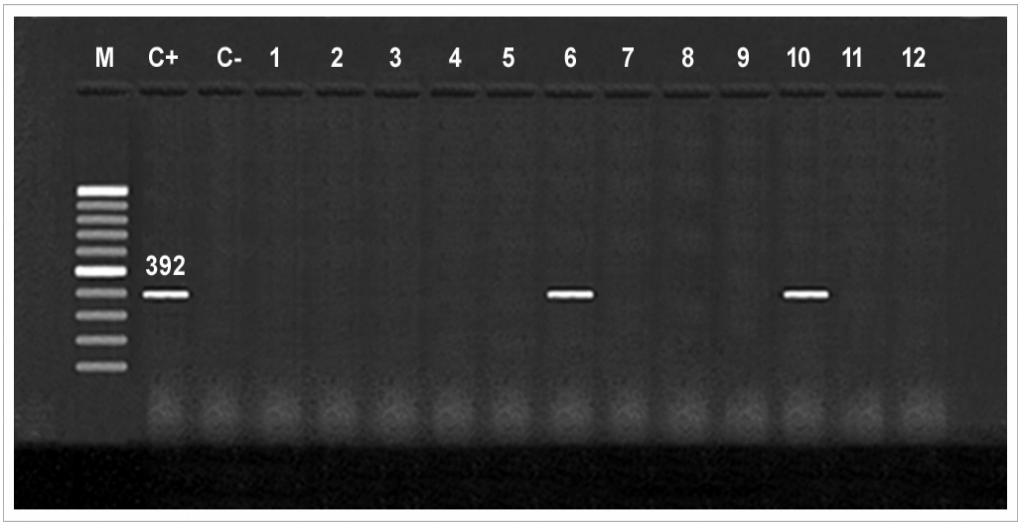

Figure 3. Agarose gel electrophoresis of PCR of Bec-UF2 (392 bp) specific for characterization of *Theileria equi*. Lane M: 100 bp DNA marker. Lane C+: Positive control for *T. equi* Bec-UF2 gene. Lane C-: Negative control. Lanes 6 and 10: Positive *T. equi* DNA. Lanes 1, 2, 3, 4, 5, 7, 8, 9, 11 and 12: Negative *T. equi* DNA.

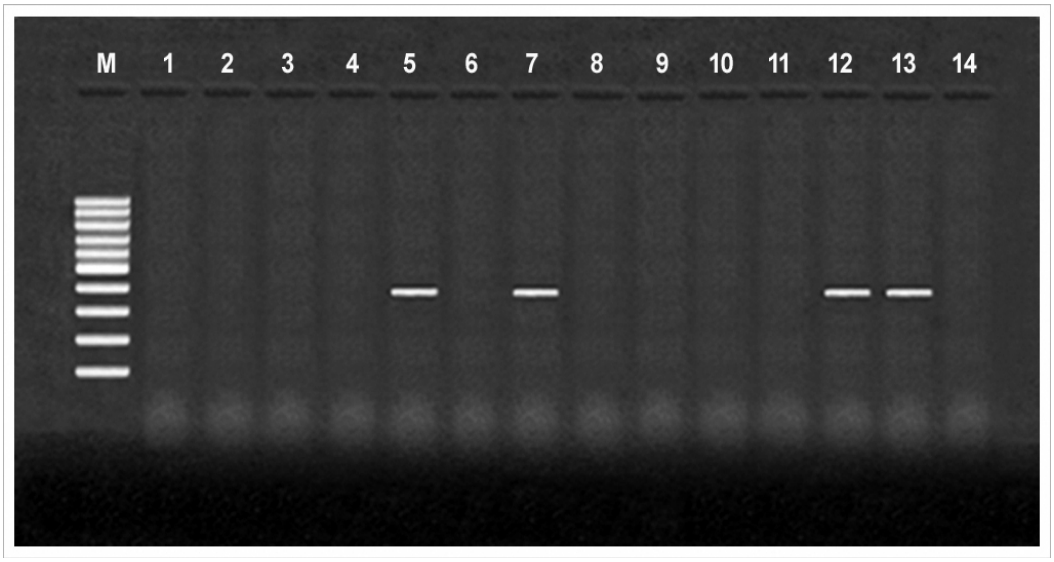

Figure 4. Agarose gel electrophoresis of PCR of Bec-UF2 (392 bp) specific for characterization of Theileria equi. Lane M: 100 bp DNA marker. Lanes 5, 7, 12 and 13: Positive T. equi DNA. Lanes 1, 2, 3, 4, 6, 8, 9, 10, 11 and 14: Negative T. equi DNA.

DISCUSSION

Donkeys usually remain asymptomatic carriers of equine theileriosis with positive antibody titre throughout life. The disease is diagnosed by peripheral blood smear examination, but in carrier donkeys it is very difficult to demonstrate the parasite in stained blood smears as the parasitaemia is extremely low (Salib *et al*., 2013). As donkeys/mules were mainly kept in open yard under poor management condition, hence they are at more risk with respect to vector resulting in haemoparastic infection (Sumbria *et al.,* 2015).

Equine theileriosis, an OIE list disease, caused by tick borne *Theileria equi*, is responsible for important economic losses in equine industry. The disease can occur in peracute, acute and chronic forms (OIE, 2008). It renders negative effects on the health of equids, decreasing their productivity and work efficacy. *T. equi* infection can be peracute with death occurring in 1-2 days, or can be chronic, lasting for weeks (Taylor *et al.,* 2007).

Out of 50 donkeys blood samples examined for *T. equi,* 14% (7/50) were positive by microscopic examination (ME) and 38% (19/50) by PCR. Prevalence of *T. equi* had high significant differences between micropscopic examination and PCR assay. Also, Habibi *et al.* (2016) and Mahdy *et al.* (2016) found significant differences between ME and PCR on the detection of *Theileria equi*. The low positive rate for *T. equi* observed in the present study using direct microscopic identification of the parasite in blood smears compared with PCR was in agreement with Ibrahim *et al.* (2011). Also, the high prevalence of *T. equi* in the present study could be explained by presence of heavy ticks infestation among donkeys in Assiut and donkeys are kept outdoors under poor living condition for daily transport and farm activities, thereby being more exposed to ticks (Sumbria *et al.,* 2015 and 2016). Also, Farah *et al.* (2003) and Salib *et al.* (2013) found high prevalence of *T. equi* in Egypt, which is consistent with the current lack of control measures.

In the present study, the prevalence of *T. equi* of donkeys blood samples examined was 14% by microscopic examination. Our results revealed prevalence of *T. equi* by microscopic examination lower than that reported by Mahdy *et al.* (2016) as they were 24.8% and 27.4% of donkeys and horses, respectively in Giza and Cairo; Salib *et al.* (2013) 41.61% of horses in Giza governourate; Ibrahim *et al.* (2011) 18% of horses in Egypt. On the other hand, the present results higher than that reported by Habibi *et al.,* (2016) 9.67% of horses and mules in Iran; Osman *et al.* (2016) 2.14% of horse and 2.04% of donkey in Sudan; Sumbria *et al*. (2015) 0.93% in Indiaand Tefera *et al.* (2011) 2.08% of donkeys in Ethiopia. These differences in

infection rates may be related to management practices and due to a difference in the prevalence of tick vector for *T. equi* between different regions, where climatic factors such as temperature, humidity and rainfall influence the habitat for ticks (Oncel *et al.*, 2007).

In the present study, the prevalence of *T. equi* was 38% of donkey's blood samples examined by polymerase chain reaction (PCR). It is lower than that reported by Habibi *et al.* (2016) in Iran, 96.77% of healthy horses and mules; Mahdy *et al.* (2016) in Giza and Cairo governorates together 50.4% and 61.9% of donkeys and horses, respectively; Mahmoud *et al.* (2016) in Egypt, 36.4% and 43.1% of horses and donkeys, respectively and Sgorbini *et al.* (2015) in Italy, 41% in horses. The present results are higher than that reported by Davitkov *et al.* (2016) in central Balkan, 22.5% of horses; Osman *et al.* (2016) in Sudan, 13.9% of horses and 23.6% of donkeys; Sumbria *et al.* (2016) in India, 14.14 % of horses, donkey and mules; Laus *et al.* (2015) in Italy, 17.4% of donkeys; (Malekifard *et al.,* 2014) in Iran, 10.83% of horses and (Ibrahim *et al.*, 2011) in Egypt, 26% of horses. This difference could be attributed to the tick spectrum variation between the study areas and/or differences in persistence of tick vectors transmission and climatic condition between the study areas (Osman *et al.,* 2016).

This study showed a high exposure of donkeys to *T. equi*. None of the donkeys included in the study presented clinical signs of theileriosis at time of blood sampling. Similar findings reported by Machado *et al.* (2012). Under a high and constant exposure to tick borne pathogens (TBPs), donkeys may acquire high immunity, which makes them chronically infected carriers and takes under-control their clinical symptoms. However, they may still act as a source of TBPs for ticks, enhancing the transmission and facilitating the spread of these agents (Kumar *et al.,* 2009).

In the present study, prevalence of *T. equi* in male donkeys was 13.6%, while in females was 14.3% by microscopic examination. Also, prevalence of *T. equi* in male donkeys was 36.4% while in females was 39.3% by PCR. Therefore, no significant difference between the prevalence rate of infection in males and females were recorded. Similar results were reported by Malekifard *et al.* (2014) and Salem and El-Sherif (2015) who found no significant difference between the prevalence rate of *T. equi* in males and females, which could be due to the condition of enzootic stability of equine theileriosis in the studied area. In contrary, Munkhjargal *et al.* (2013) and Salib *et al.* (2013) reported higher infection rate in males than females, While males were significantly less affected than mares (Davitkov *et al.,* 2016 and Sumbria *et al.,* 2016) which most likely is due to different management and housing conditions in these countries.

In the present study, microscopic examination of Giemsa stained donkey's blood smears revealed forms of theilerial schizogony in lymphocytes (Koch's blue bodies) and intra-erythrocytic relatively small, less than 2 µm long pear-shaped and ring shaped merozoites of *T. equi*. This was in agreement with that described by Salib *et al.* (2013); Malekifard *et al.*(2014) and Salem and El-Sherif (2015). Also, Soulsby (1982) described *T. equi* as small *Babesia* being 2 µm in length (<2.5 µm), pyriform or comparatively rare round or amoeboid with acute angle in pyriform. The morphological characteristics observed in current study were in agreement with the findings of Soulsby, (1982).

The sensitivity, specificity and accuracy of the PCR with respect to blood film examination were 61.3%, 100% and 82%, respectively. PCR was found to be more sensitive, specificic and accurate than blood film examination. Similar results were recorded by Malekifard *et al.* (2014); Sumbria *et al.* (2015); Habibi *et al.* (2016) and Mahdy *et al.* (2016) who showed PCR was more sensitive than microscopic inspection in diagnosis of *T. equi* infection. Also, Ibrahim *et al.* (2011) recorded that PCR was found to be highly sensitive and more accurate than blood film examination in diagnosis of *T. equi* infection. The microscopic diagnosis of *T. equi* by thin blood smears lacks enough sensitivity to give an accurate result in cases of low parasitaemia in carrier horses as the parasite has a periodically cryptic nature which obviously leads to false negative results (Bashir *et al.,* 2014 and El-Sayed *et al.,* 2015).

It is concluded that the *T. equi* is prevalent among donkyes in Assiut governourate. Conventional parasitological technique revealed a very low prevalence rate. Molecular techniques, as clearly indicated by this study, have a better in revealing the most real prevalence and endemicity of the disease. Donkeys persistently infected with *T. equi* may act as a source of infection for ticks; therefore, animals should be periodically tested and prophylactically treated. We also recommend the eradication of ticks by the use of acaricides through national program.

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الكشف الطفيلى والجزيئى لطفيل الثيليريا إكواي فى الحمير بمحافظة أسيوط

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تعتبر الثيليريا إكواي من طفيليات الدم التى تنتقل عن طريق القراد. البيانات المتعلقة بنسب الإصابة بالثيليريا إكواى في الحمير نادرة في محافظة أسيوط بمصر .لذلك أجرينا هذه الدراسة عن مدى انتشار عدوى الثيليريا إكواى فى الحمير باستخدام الفحص الميكروسكوبى واختبار تفاعل البلمرة المتسلسل. تم فحص ٥٠ عينة من دم الحميرلوجود الثيليريا إكواى وكانت نسبة الإصابة ١٤٪ (٧ / ٥٠) بالفحص المجهري ، ٣٨ ٪(١٩ /٥٠) بواسطة اختبار تفاعل البلمرة المتسلسل. كان فروق مدى انتشار الثيليريا إكواى ذات دلالة إحصائية عالية بين الفحص المجهري واختبار تفاعل البلمرة المتسلسل . كان مدى انتشار الثيليريا إكواى في ذكور الحمير ١٣,٦٪ (٢٢/٣) بالفحص المجهري ، ٣٦,٤٪ (٢٢/٨) بواسطة اختبار تفاعل البلمرة المتسلسل بينما في إناث الحمير ١٤,٣٪ (٢٨/٤) بالفحص المجهري ، ٣٩,٣٪ (٢٨/١١) بواسطة اختبار تفاعل البلمرة المتسلسل ولم تسجل أي فروق بين معدل انتشار الإصابة في الذكور والإناث .تبين بالفحص المجهري لمسحات دم الحميروجود أشكال تكاثر إنشطاري للثيليريا في الخلايا الليمفاوية (أجسام كوخ الزرقاء) والميروزويت لطفيل الثيليريا إكواى داخل كريات الدم الحمراء صغيرة نسبيا ، طولها أقل من ٢ ميكرون على الشكل الكمثرى و الدائرى. كما تم الكشف عن الجين B1 المحددة للثيليريا إكواى بواسطة اختبار تفاعل البلمرة المتسلسل في ١٩ عينة من دم الحمير. كانت جميع النتائج الإيجابية بالفحص المجهري إيجابية أيضا بواسطة اختبار تفاعل البلمرة المتسلسل. كانت الحساسية والنوعية ودقة اختبار تفاعل البلمرة المتسلسل بالنسبة للفحص الميكروسكوبى ٦١,٣ ٪ ، ١٠٠٪ ، ٨٢٪ على التوالي. ثبت أن اختبار تفاعل البلمرة المتسلسل أكثر حساسية ، نوعية ودقة من الفحص الميكروسكوبى. وبذلك تشير النتائج إلى أن طفيل الثيليريا إكواى منتشرعلى نطاق واسع بمحافظة أسيوط بسبب التعرض الكبير للقراد.