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Original Article

Field investigation and comparative evaluation of diagnostic tests of *Trypanosoma evansi* in camels in Egypt

Henidy, S. A; Ashmawy, K.I; Abou-Akkada, S.S; El Shanat, S.; Deweir, A.W

*Department of Parasitology, ,Faculty of Veterinary Medicine, Alexandria University Abis, Alexandria, Egypt Post Code: 21944

*Author of correspondence: safeyahenidy@gmail.com

Abstract:

Trypanosomosis is a chronic disease of camels caused by *Trypanosoma evansi* which is transmitted mechanically by *Tabanus* and *Stomoxys* spp. It has a worldwide distribution. In the present study, 295 male camels admitted to Kom-Hammada abattoir, Behera province were examined parasitologically for presence of *T.evansi* by Giemsa stained blood smears (GSBS), microhaematocrit centrifugation technique (MHCT) and polymerase chain reaction (PCR). The study revealed that total prevalence of *T.evansi* was 0.68% (2/295) using GSBS and 0.96% (2/209) using MHCT. While molecular examination of 100 samples by PCR gave a prevalence of 63%. Our results revealed that, using of PCR technique in diagnosis of trypanosomosis is more sensitive and specific especially in low infection rate cases as well as it is of potential role in epidemiological studies.

Key words: camels, Trypanosomosis, Giemsa stained blood smear, microhaematocrit centrifugation technique (MHCT), polymerase chain reaction (PCR).

Introduction

Camels consider as an important source for low cholesterol meat, milk, high quality wool and skin. Camels are known for their adaptation to hard environmental conditions, so they can live in desert areas **(Faye, 2015)**. Trypanosomosis (Surra) is one of the most important parasitic diseases affecting camels caused by *T.evansi*.

Surra disease has immunosuppressive effect and predispose to other diseases especially with the absence of veterinary care in rural areas where camel pastoralists exist, eventually an obstacle to animal husbandry **(Kôhler-Rollefson et al., 2003)**. The general clinical signs of surra disease are intermittent fever (associated with parasitemia), progressive anemia **(Habila et al., 2012)**, loss of condition, enlargement of lymph nodes and spleen **(Brun et al., 1998)** which are not sufficiently pathognomonic for diagnosis. Moreover, the disease usually takes the chronic form and examination of blood

smears is not enough sensitive to detect the infection. Therefore, other sensitive and specific diagnostic techniques are required **(Nantulya., 1990).**

T.evansi affects a large number of wild and domestic animals in tropical and subtropical areas **(Eyob and Matios, 2013; Aregawi et al., 2019)**. *T.evansi* is a monomorphic, dyskinetoplastic, hemoflagellate protozoan resemble *T.brucei* slender form **(Urquhart et al., 1987)**. *T.evansi* occurs wherever camels are kept **(Fassi-Fehri., 1987).** It has a worldwide distribution as it is transmitted mechanically by *Tabanus* and *Stomoxys spp.* Besides its biological transmission by Vampire bats in South America **(Desquesnes et al., 2013)**.

PCR technique is more sensitive than conventional parasitological techniques especially in detecting chronic infection and can be used as a diagnostic tool for epidemiological studies **(Holland et al., 2004; Abdel-Rady, 2008; Ashuor et al., 2013** and **Bal et al., 2014)**.

The controversial diagnosis of Trypanosomosis using conventional approaches brought our attention to evaluate the sensitivity and specificity of PCR against these approaches. Moreover, assessing the seasonal prevalence of *T.evansi* infection in camels *(Camelus dromedarius)* in Behera province, Egypt was one of our targets, in addition to determining the relationship between age, sex, season and the prevalence of *T.evansi* in camels.

Material and methods

Study area:

A cross-sectional survey was conducted from August 2017 to July 2018. A total of 295 male camels of different ages, admitted to Kom –Hammada abattoir at Behera province for slaughtering, were randomly selected for this study.

Collection of the samples:

Blood samples were collected seasonally as explained in Table (1). Two ml blood was collected during slaughtering into tubes containing ethelyne diamine tetra-acetic acid (EDTA) as anticoagulant (50µl/ml blood) for parasitological examination and DNA extraction. The samples were kept in a cool box and transported to the laboratory. A fresh film is made from each sample. Then, samples were stored at -20°c until DNA extraction.

Smears preparation and staining:

A drop of blood was drawn to make a thin blood film. Film is air dried, then fixed in absolute methanol for 5 minutes and stained in Giemsa stain for 30 minutes. Stained films were examined under microscope (Hahn, 1994).

Microhaematocrit centrifugation technique (Woo′s technique, 1969):

Plain capillary tubes were filled with whole blood from 209 samples then centrifuged at 10000 rpm for 5 min in a Heraeus CHRIST haemofuge (Germany). Buffy coat was used for preparation of thin smears then stained with Giemsa stain. Smears were examined under microscope as blood films.

Molecular diagnosis (PCR):

DNA extraction:

Whole blood sample was used for extraction of total genomic DNA using G-spin^{TM} Total DNA Extraction kit (iNtRON Biotechnology, Inc. Korea) according to the manufacturer′s instructions. Extracted genomic DNA stored at -20°c till used.

Polymerase chain reaction protocol:

PCR was carried out for amplification of 164 bp by using minichromosome satellite DNA, subgenus trypanozoon specific primers, TBR 1 ⁄2(TBR 1:5′ GAATATTAAACAATGCGCAG-3′and TBR2:5′-CCATTTATTAGCTTTGTTGC-3′).

Hundred samples were tested using PCR amplification method. The PCR amplification was performed in a total reaction volume of 25 µl containing: 12.5 µl Dream Taq Green master mix (Thermo Scientific), 1.5 µl DNA template, 1 µl each primer (10 pmol) and complete with

nuclease free Water. The samples were thermo cycled in ^{3} Prime thermal cycler (TECHNE, UK) with: initial denaturation at 95° c for 3 min. then 35 cycles of denaturation at 95° c for 30 s, annealing at 50° c for 30 s and extension at 72° c for 30 s with a final extension at 72° c for 7 min. After amplification, 10 µl of PCR product were loaded in 1% agarose gel stained with ethidium bromide (0.5 µg/ml), electrophorized for 1h in electrophoresis unit with 100 bp DNA ladder from NIPPON Genetics (EUROPE GmbH, 100 µg /ml).as a size marker and visualized by gel documentation system UVP PhotoDoc-it^{TM} Imaging System (analytikjena, USA).

Sequencing and BLAST analysis:

After gel electrophoresis, positive bands were cut, purified using PCR purification kit and sequenced in 3500 genetic analyzer (applied biosystem, Germany). The nucleotide sequences were aligned with existing sequences of *T. evansi* in GenBank databases using BLAST programs.

Statistical analysis:

The statistical analysis of results were carried out using SAS software (2004).

Result

Blood film examination:

The microscopic examination of 295 blood films revealed two positive samples (0.68%) for *T.evansi* (Fig,1) and (Table,2). The identification and measurement of parasites were carried out according to **(Otify, 2013)**.

Microhaematocrit centrifugation technique:

Out of 209 examined buffy coat films two infected camels were revealed (0.96%) (Table, 2).

Polymerase chain reaction, PCR technique:

The examination of 100 samples by PCR detected 63 (63%) molecularly positive (Fig, 2) and (Table, 2) which gave a significant difference when compared with two different approaches P<0.0001*- Chi-square value (327.42). The PCR products from TBR 1/2 primers were of 164 bp multiple bands due to tandem repeat nature of the target gene. BLAST analysis of each band showed 89-99% an identity to *T.evansi* (Table, 3).

Regarding seasonal dynamic of the parasite, the highest prevalence was recorded in summer season and the least was in winter and autumn seasons (Table, 4) which showed no significant difference. Concerning the age of examined camels, the highest prevalence of infection was in camels aged between 5-8 years (Table, 5) which was of no significant value.

Table 1: Numbers of collected samples according to different seasons.

Season	Number
Summer	83
Autumn	48
Winter	89
Spring	75
Total	295

Table 2: Total prevalence of *T.evansi* in examined camels with different diagnostic techniques.

Diagnostic technique	No. examined	Positive		P-value & Chi-square value
		No.	%	P<0.0001* Chi-square value (327.42)
Thin blood films	295	2	0.68	
Buffy coat	209	2	0.96	
PCR	100	63	63	

Table 3: Sequencing results of each band of PCR product and BLAST results of nucleotide sequence with identity percent to *Trypanosoma evansi.*

Band	Nucleotide sequence	bp	Identity %
1	F: AATCACCCATACTTTTATGTAGTGCCATATTAATTACAAGTGTGCAACATTAAATACAAGTGTGTAACATTAATTTGCAAGTTTGCAACAATGTTCTTTAGTGTTTAATGGGTGCAACAAAGCTAATAAATGGA R: CATAAGAACATTGTTGCAACTTGCAATTAACGTTACACACTTGTATTTAATGTTGCACACTTGTAATTAATATGGCACACATTAAAAGTTATTGTGTATAATAGCGTTAACTGCGCATTGTTTAATATTCA	164	99.22-88.42
2	F: AAAACAAATACTTTTATGTGTGCCATATTAATTACAAGTGTGCAACATTAAATACAAGTGTGTAACGTTAATTTGCAAGTTTGCAACAATGTTCTTTAGTGTTTAATGGGTGCAACAAAGCTAATAAATGGACCTTATACAAACGAATATTAAACAATGCGCAGTTAACGCTATTATACACAATAACTTTTAATGTGTGCCATATTAATTACAAGTGTGCAACATTAAATACAAGTGTGTAACATTAATTTGCAAGTTTGCAACAATGTTCTTTAGTGTTTAATGGGTGCAACAAAGCTAATAAATGGA R:	309	99.21-89.11

	TAGGACTTGTTGCAACTTGCAATTAACGTTACACACTTGTATTTAATGTTGC ACACTTGTAATTAATATGGCACACATTAAAAGTTATTGTGTATAATAGCGTT AACTGCGCATTGTTTAATATTCATTCGTATAAGGACCATTTATTAGCTTTGTT GCACCCATTAAACACTAAAGAACATTGTTGCAAACTTGCAAATTAACGTTAC ACACTTGTATTTAATGTTGCACACTTGTAATTAATATGGCACACATTAAAAG TTATTGTGTATAATAGCGTTAACTGCGCATTGTTTAATATTCAAGAG		

Table (4): Seasonal dynamic of *Trypanosoma evansi* using PCR technique

Season	No. examined	No. positive	Prevalence (%)	P –value &Chi-square value
Summer	25	17	68	P>0.05 NS Chi-square value (0.47)
Autumn	25	15	60	
Winter	25	15	60	
Spring	25	16	64	
Total	100	63	63	

Table (5): Effect of age on the prevalence of *Trypanosoma evansi* using PCR technique

Age group	No. examined	No. positive	Prevalence (%)	P –value
1-4 years	28	15	53.6	P>0.05 NS Chi-square value (1.59)
5-8 years	41	28	68.3	
More than 8 years	31	20	64.5	
Total	100	63	63	

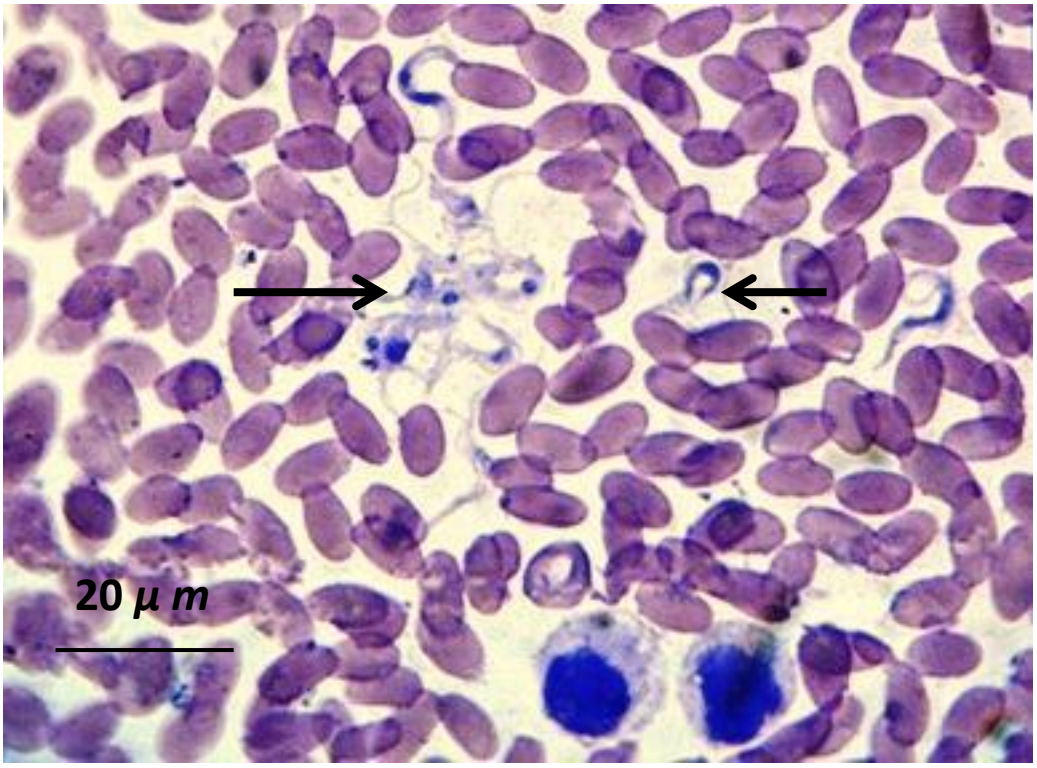

Fig. (1): Blood smear from camel showing *T.evansi* (Arrow) , Giemsa stain, ×100.

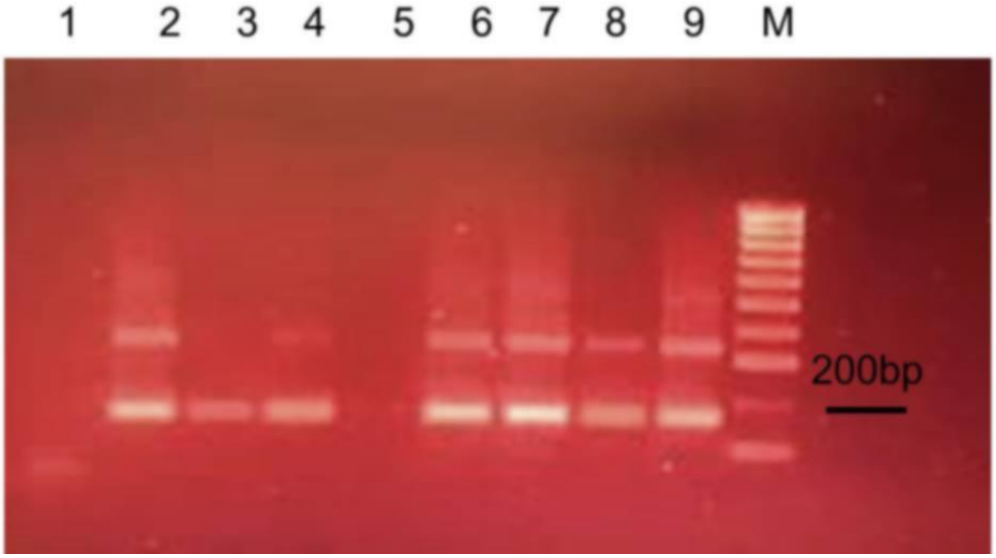

Fig. (2): Ethidium bromide stained agarose gel of PCR for *T.evansi* showing band at 164 bp (M=100 bp DNA ladder, lane 1=control negative, Lane2=control positive, Lane3,4= positive samples,Lane5= negative sample, Lane6-9=positive samples at 164 bp. Dimers at 309 bp due to the repetitive nature of the target gene.

Discussion

The high prevalence (63%) of *T.evansi* in camels indicates that *T.evansi* is endemic in Egypt as reported by several studies such as (**Barghash et al., 2014)** who reported a prevalence of 74.7%; **(Abou El-Naga** and **Barghash, 2016)** recorded a prevalence of 67.06% and **(Elhaig and Sallam 2018)** reported a prevalence of 71.4%. However, the prevalence in the current study was lower than those reported by **(Hegazy, 2017)** who reported a prevalence of 90% this may be attributed to variation in seasonal collection of the samples and low number of samples (10) examined by (**Hegazy, 2017).**

The low prevalence of *T.evansi* using parasitololgical methods (0.68% and 0.96%) is due to the chronic nature of the parasite in camels, intermittent parasitaemia and low sensitivity of parasitological methods **(Nantulya, 1990)**. . The prevalence recorded in this study (0.68% and 0.96%) using parasitological techniques (GSBS and MHCT) and 63% using PCR method revealed that PCR has higher sensitivity in diagnosis of chronic Trypanosomosis in camels. These results are in agreement with the prevalence of 4.1% by GSBS and 56% by PCR recorded by **(Abdel-Rady, 2008)**. **(Barghash et al., 2016)** reported a prevalence of 22.22 and 74.36% using stained blood smears and PCR, respectively. The agreement in results may be due to the same ecological condition in Egypt as well as it ensures the sensitivity and specificity of PCR. A study conducted in Iraq also showed a prevalence of 28, 90% using blood films and PCR, respectively **(Aboed and Faraj, 2017)**, the higher prevalence may be due to difference in distribution of the vector in addition to incrimination of different vectors.

In the present study, the PCR product showed multiple bands this might be returned the tandem repeat nature of TBR 1/2 gene. This is in agreement with **(Herrera et al., 2005**), in the Brazilian Pantanal, who revealed that the amplification of the same DNA segment resulted in the production of 164 bp specific for Trypanozoon species and production of dimers and trimers. **(Bal et al., 2014),** in India, revealed that the results of PCR amplification using TBR1/2 primer showed multiple bands.

Variation in results between different studies may be attributed to different strains of the parasite, concentration of DNA, different PCR protocols and difference in primer concentration.

Our results indicated that the highest prevalence of infection was detected in summer season (68%) while the lowest prevalence (60%) was detected in winter and autumn seasons. Moreover, the two positive cases by blood film; one was detected in summer season and the other in spring season. These results are partially agree with those of **(Sobhy et al., 2017)** who assessed the seasonal prevalence of *T.evansi* using PCR in a descending order as 77.17% in spring, 63.26% in summer, 55.34% in autumn and 52% in winter season. This higher prevalence during summer season may be due to the overspreading of vector during summer season as reported by **(Barghash, 2005).** However, this is disagreeing with (**Bala et al., 2018),** in Sudan, who found a higher prevalence in winter season (52%) than in summer season (40%). **(Bala et al., 2018)** stated that vector population is higher during winter season which explained the higher prevalence. So, further studies must be conducted to evaluate the prevalence of *T.evansi* in both summer and winter seasons and distribution of biting flies to conclude the effect of biting flies distribution on the prevalence of trypanosomosis in dromedary camels.

In the current study, higher prevalence of the disease (68.3%) was recorded in camels (5-8 years) and a lower prevalence (53.6%) was reported in camels (1-4 years) this is may be due to stress caused by work and low number of camels (1-4

years) examined during this study, however this difference is not statistically significant (p>0.05). This findings is in agreement with **(Hegazy, 2017)** who reported a prevalence of 2.2% in camels 5-10 years and a prevalence 0% in camels 1-5 years and camels more than 10 years. In contrary, **(Kassa et al., 2011),** in Ethiopia, found a higher prevalence (7.7%) in young aged camels and a low prevalence (4%) in adult camels.

Regarding sex, as all examined camels were males because there were no slaughtered females available during the period of samples collection, therefore we could not assess the effect of sex on the prevalence of *T.evansi.*

In conclusion, *T.evansi* is a chronic disease of camels in Egypt with a higher prevalence during summer season. PCR is a useful technique for surveillance studies of *T.evansi* with high sensitivity and specificity.

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الملخص العربي

دراسة حقلية وتقييم مقارن للاختبارات التشخيصية لمثقبيات ايفانسى فى الجمال فى مصر

صفية عبدالحميد هنيدى،كرم امام عشماوى،سمية سيف ابوعقادة،شريف كمال الشناط، أميرة وحيد دوير

قسم الطفيليات

كليه الطب البيطرى– جامعه الإسكندريه

أبيس، الإسكندريه

الرمز البريدى:21944

*للمراسله:safeyahenidy@gmail.com

يعتبر مرض التربانوسوما ايفانسى من اهم الامراض التى تصيب الجمال والتى تؤدى الى خسائر اقتصادية كبيرة. تم اجراء الدراسة الحالية لايضاح نسبة انتشار طفيل التربانوسوما ايفانسى(تربانوسوما الجمال) فى الجمال فى محافظة البحيرة بمصر فى الفترة من اغسطس 2017 الى يوليو 2018. وقد اجريت الدراسة للمقارنة بين الاختبارات التشخيصية المختلفة والمستخدمة فى التعرف على الطفيل. تم تجميع عدد295 عينة دم من الجمال اثناء الذبح من مجزر كوم حماده بمحافظة البحيرة وتم فحصهم عن طريق عمل مسحات من الدم الخفيفة المصبوغة بصبغة الجيمسا، فحص طبقة خلايا الدم البيضاء و تفاعل انزيم البلمرة المتسلسل.

وقد اوضحت الدراسة اصابة اثنين من الجمال عند فحص عدد 295 من مسحات الدم الخفيفة بمعدل اصابة 0.68% واصابة اثنين من الجمال عند فحص عدد 209 طبقة من خلايا الدم البيضاء بمعدل اصابة 0.96% وكذلك اصابة 63 جمل عند فحص عدد 100عينة باستخدام تفاعل انزيم البلمرة المتسلسل. وقد بينت الدراسة وجود نسبة اصابة اعلى اثناء فصل الصيف بلغت 68% وفى الاعمار بين 5-8 سنوات بلغت 68.3%. وقد اكدت الدراسة ان تفاعل انزيم البلمرة المتسلسل هو الافضل فى تشخيص الاصابة المزمنة بطفيل التربانوسوما فى الجمال.