Molecular diagnosis of *Trypanosoma evansi* infection in *Camelus dromedarius* from Eastern and Western regions of the Sudan

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Abstract: *Trypanosoma evansi* infection is the most important disease of Camel in the Sudan. The present work was carried out to evaluate a simple PCR-based technique for field diagnosis of *T. evansi* infection in camels from Eastern and Western regions of the Sudan. A representative number of 600 camels (*Camelus dromedarius*) from different areas of Gedarrif State (Eastern) and North Kordofan State (Western) were examined from May 2005 to July 2007 for *Trypanosoma evansi* infection. The tests used were parasitological (Wet Smear Film, WSF; Thin Smear Film, TSF; Buffy Coat, BC), serological (Card Agglutination Test/T. evansi, CATT), and DNA amplification by polymerase chain reaction (PCR). The prevalence of *T. evansi* infection in camels was detected in 36 (out of 40), 100 (out of 210), 36, 22, 10 (Out of 600); by PCR, CATT, TSF, BC and WSF with sensitivity of 90%, 47.6%, 6%, 3.7% and 1.7%, respectively. PCR revealed a specific 200 bp band in positive samples. The intensity of PCR bands was variable in different test samples depending upon the level of infection in the test samples. The history of intermittent fever, emaciation, oedema, poor body condition significantly correlated with positive serological status in CATT as well as trypanosome DNA detection by PCR. As there are no previous studies in the Sudan on the molecular characterization of the parasite, this research is useful in formulating strategic control programmes.

Key words: *Trypanosoma evansi*, camels, molecular diagnosis, Sudan

التشخيص الجزيئى للإصابة بتريبانوسوما إفانساى فى إبل شرق و غرب السودان

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الملخص: اجرى البحث الحالى فى الفترة من مايو 2005م إلى يوليو 2007م لدراسة تقييم البوليميريز التفاعلى التسلسلى (PCR) كإختبار تشخيصى حقلى للعدوى بالتربانسوما إيفانساى فى الإبل فى كل من شرق وغرب السودان مقارنة مع الاختبارات بالطرق الباراسيتولجية والسيرولجية. وهو من أهم الامراض التى تصيب الإبل فى السودان. أهم اهداف هذه الدراسة تقييم مقدرة كفاءة البوليميريز التفاعلى التسلسلى فى تحديد طفيل التربانسوما ومعرفة الخصائص الجزيئية للطفيل المستخدم من القضارف شرقاً و كردفان غرباً. اجريت اختبارات تشخيصية مجهرية للمرض شملت المسحة الرطبة و المسحة المصبوغة و الغشاء الابيض. اجرى المسح بغرض التعرف على الطفيل المسبب للعدوى بمرض التربانسوموس في المضيف الطبيعى (الإبل) و قد أختيرت العينات عشوائياً و جمعت فى أوراق الترشيح ليتم إستخلاص الدى إن اى (DNA) ليستخدم فى تحليل البوليميريز التفاعلى التسلسلى. و يتم تمييز خصائص طفيل التربانسوما إيفانساى بإستخدام أنواع متخصصة من البوادى. وهنا يتم تفكك عشوائى للتركيب الشكلى لل الدى إن اى ليستخدم للمقارنة في التركيبة الوراثية للتربانسوما إيفانساى التى تم عزلها و المستجلبة من عدوى طبيعية للإبل. وقد اجرى التشخيص لعدد 600 من الإبل و قد أظهرت النتائج حساسية عالية و تخصص نوعى لفحص البوليميريز التفاعلى التسلسلى (90%) و بدرجة اقل حساسية للإختبارات السيرولجية (CATT) والمجهرية: المسحة المصبوغة (TSF) والغشاء الابيض (BC) والمسحة الرطبة (WSF). تناولت الدراسة أيضاً تقصى حقلى (إستبيان) شمل العلامات التشخيصية العيارية ـ الأمراض الأخرى المتواجدة فى المنطقة. هذا البحث يعطى صورة عامة عن مستوى حدوث المرض خاصة وانه لا توجد اى دراسة جينية مؤسسة و مستحدثة عن الطفيل فى السودان. كما إنه يؤطر لصياغ استراتيجية وبرنامج مكافحة كيموثيربيوتيكية (دوائية). وعموماً فإن هذه الإختبارات الجزيئية تمكن من التشخيص المبكر للعدوى بالتربانسوما وهذا ما لا تستطيعه طرق التشخيص الطفيلية المجهرية و السيرولوجية. كما ان هذا الإختبار لايستخدم للتشخيص الجزيئى فحسب بل بصورة مثلى فى الدراسات الوبائية، تصميم البرامج العلاجية وبرامج السيطرة على المرض.

Introduction

Trypanosomosis are a large group of diseases caused by obligated flagellate blood parasites; that infect members of every vertebrate class (Hoare, 1972). The Trypanosomes are found in blood and / or tissues of vertebrate host causing Trypanosomosis in human and his animals (FAO, 1994). "Surra" is an animal disease occurring in Africa, Asia, and Latin America, caused by *Trypanosoma evansi*. The parasite is transmitted by biting flies such as Tabanidae and Stomoxys species, as well as by Vampire bats including *Desmodus rotondus* (Hoare, 1972).

T. evansi is thought to have been evolved from *T. brucei* on the northern extremity of the tsetse belt in Africa. It is indistinguishable morphologically from *T. brucei* and also undergoes antigenic variation. However, it differs fundamentally in that it cannot complete normal cyclic development in the tsetse fly and is instead mechanically transmitted by another blood sucking flies. As a result *T. evansi* has spread outside the tsetse belt in Africa to large parts of North Africa, the Middle East, Asia and Latin America. Significantly, strains of *T. evansi* were found to lack maxicircles in their kDNA networks. Since transmission through the tsetse fly requires a functioning mitochondrion, this would explain the blockage of the process. Moreover, in marked contrast to the situation in *T. brucei*, the minicircles of *T. evansi* show a great deal of homology in strains from different parts of the World, suggesting restrictions on the ability of *T. evansi* to mate, whereas in *T. brucei*, the highly heterogenous minicircles are postulated to have arisen from much more frequent exchanges of kDNA at the insect stage (Van der Ploeg et al., 1985; Borst et al., 1987).

In Sudan, Trypanosomosis was first reported in 1904 in cattle arriving to Khartoum from Upper Nile (Karib, 1961). The disease causes significant morbidity and mortality in Camels in Sudan which has population of over 3 million Camels (Omer et al., 2004). The disease occurs both in chronic and acute form (Gutierrez et al., 2000). The chronic form of the disease is most common and is likely to be associated with secondary infection due to immuno-suppression (Njiru et al., 2004). Clinical signs and pathological lesions caused by *T. evansi* in camels are unreliable for definitive diagnosis (Chaudhary and Igbal, 2000). Haematocrit centrifugation technique and ELISA were adopted once or twice during a limited survey to determine the prevalence of the disease in Eastern Sudan (Elamin et al., 1998). In Southern Sudan the card agglutination test (CAT) was used for detection of *T. b. gambiense* infections (Chappuis et al., 2002).

Molecular markers are required to detect accurately *T. evansi* infections in the early stages. Therefore, the aim of this study is to evaluate a simple PCR-based technique for field diagnosis of *T. evansi* infection in camel, to compare between conventional, serological and molecular techniques in diagnosis of *T. evansi* in camels and to detect infections in camels in the very early stage.

Materials and Methods

Study area

El-Showak is a research station that belongs to the Camel Research Centre (CRC). It is a focal point for camel pastoralists in Butana area. Being a collection point, it becomes an important camel market in the region. Butana is situated well within the arid zone of the Eastern Sudan and occupies an area of approximately 120000 km^2 and lies between latitude 13° 4' N to 17° 50' N and longitude 32° to 36° E. Most of the Butana is series of flat easily flooded plains interspersed by few hills. The prevailing climate is warm in summer which extends most of the year (March-October) and includes the rainy season (June-September). The vegetation composed of *Aristida spp.* (Gow) *Cymbopongon nervatus* (Nal); *Acacia mellifera* (Kitir); *Calotropis procera* (Usher); *Capparis deciduas* (Tunduub) and a variety of grasses (Abdalla, 1985). Normally the camels and their owners move / migrate in search for water and grasses eastward to the Ethiopian borders, where the Tsetse flies *Glossina fucipes* is reported (Kheir et al., 1995).

El-Obied is the capital of Northern Kordofan. It is about 400 Km West of Khartoum. It is one of the largest animal wealth centers in the Sudan. Besides being a trade market it holds the various veterinary services that include the Regional Veterinary Laboratory (RVL) which is the research laboratory that serves disease surveillance, diagnosis and vaccine production. The region is dry with sandy soil and the *Acacia spps.* are the dominant trees. The nomads in the dry season (summer) migrate southward in search of water and grasses and in the wet season (autumn) with the muddy conditions and the flies they move northward. Therefore, in the hot season will be in the Tsetse area south of Bahr el Arab and thus will be subjected to the Tsetse bites (Abdalla, 1999).

Sample Size

The Eastern and Western regions hold about 2/3 of the animal wealth in Sudan. Out of 3 million Camels in Sudan, there are about 2 million in these two areas. Consequently sample size was sufficient enough to cover large number. A number of 500 Camels (*Camelus dromedaries*) from Eastern region (El-Showak – Gedarif State) and 100 Camel from Western region (El-Obeid – North Kordofan State) were selected randomly in this survey during the period 2005 - 2007 for *T. evansi* infection. Those camels were in the sex ratio female: male; 3: 1. All samples were divided into 3 age groups; Group (A) age < 2 years (16.7%); Group (B) 2 < age < 10 (50%); Group (C) age ≥ 10 years (33.3%). Parasitological techniques were used for prevalence study, while the three methods were used on the same samples in order to evaluate the sensitivity of the PCR in the diagnosis of Trypanosomiosis.

Parasitological Techniques

Initially all the parasitological methods were used for the all 600 samples. Then based on the clinical signs in order to have insight on the disease prevalence. The a number of 210 and 40 of suspected samples were used for the CATT and PCR method; respectively. Wet Smear Film (WSF) was prepared by taking a

drop of blood from peripheral ear vein of camel and examined microscopically. Stained Thin Smear Film (TSF) was prepared by spreading a drop of blood on slide, fixation with absolute methanol, air drying, and staining with 1% Giemsa before microscopically examination. Buffy Coat (BC) was prepared by taking a drop of blood from peripheral ear vein of camel in heparinized capillary tube then was placed in a microhaematocrit centrifuge (Japan) and was centrifuged for 5 minutes at 15.000 rpm at room temperature as described previously (Murray et al., 1977). The parasites in a drop of the buffy coat were parasitologically identified by microscopic examination and have been maintained by inoculation into experimental rats.

Experimental Infection of Rat

T. evansi Infected blood was inoculated intraperitoneally (IP) into each of 3 rats, the rats were examined daily. When the rats became parasitaemic three days post infection, TSF were prepared and were examined microscopically.

Card Agglutination Test (CATT) for Trypanosomiasis

CATT was performed by taking 10 ml blood from the jugular vein of each Camel using a sterile plain glass vacutainer with a tube holder and two-way needle (Henk-Sass, Germany). The blood samples were centrifuged at 1500 rpm for 10 minutes at room temperature to separate serum. The CATT/T. evansi kit was used (Institute of Tropical Medicine, Antwerp, Belgium) and the test was performed as per manufacturer. A number of 170 samples were tested from Eastern camels and 40 samples were tested from Western camels.

Isolation of Trypanosome DNA by Chelex-100 from Rat and Camel blood

DNA isolation by Chelex-100 was performed with a modification of the methods described by Walsh and co-workers (1991) and Wooden and his colleagues (1993). A volume of 10 μl aliquot of the frozen (Camel / Rat) blood was added to 1ml of 0.15% Saponin (Sigma, USA) in phosphate-buffered saline

(PBS pH 7.2) in a 1.5 ml microfuge tube, and was incubated 10 min on ice. Parasites were collected by centrifugation at 10000 rpm and room temperature for 1 min and were then washed once with cold PBS and recentrifuged. The resultant cells pellet was added to 200 μl of 5% Chelex-100 (Bio- Rad, USA) in distilled water, incubated at 56°C for 15 min, and subjected to boiling water for 8 min. The Chelex was removed by centrifugation at 10000 rpm at room temperature for 1 min, and the supernatant was saved in a fresh tube. A volume of 6 μl aliquot of the supernatant was used for PCR amplification. For dried blood samples from rats and camels, a small piece (about 5 mm^2) of filter paper (Whatman N0. 41) with a 10 μl blood spot was initially soaked in 0.15% Saponin in PBS and processed as described above. A number of 36 samples were tested from Eastern camels and 4 samples were tested from Western camels.

PCR amplification and gel electrophoresis

A set of Oligonucleotides primers TBS-01 (5'-CGAATGAATAATAAACAATGCGCAGT-3') and TBS-02 ('5-AGAAGGATTTATTAGCTTTG TTGC-3') (MWG-Biotech AG, Germany) were designed based on the *T. brucei* sequence which corresponds to conserved regions of *T. brucei* and *T. evansi* genome and were used for the amplification of *T. evansi* DNA in camels blood samples. The PCR reaction mixture in a final volume of 25μl contained 6 μl purified DNA, 1x PCR buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM MgCl$_2$), 200 μM each of dNTP, primers at 0.25 mM, 5 units of Taq polymerase **(**Institutes of Endemic Diseases, U of K, Sudan). After 3 min initial denaturation at 94^{0}C, amplification was performed using 35 cycles of denaturation at 94°C for 1 min, annealing at 58^{0}C for 1 min, and extension at 72^{0}C for 1 min in a DNA Thermal Cycler (Mj RESEARCH, PT-200, USA). The amplified products were analyzed by electrophoresis in 1.5% agarose gel, stained with ethidium bromide (Ali et al., 2003; Ali et al., 2010; Gomes et al., 2010). PCR products were visualized under UV illumination and documented by photography.

Statistical analysis

The estimates of Trypanosomosis prevalence, sensitivity, specificity and predictive values or the probabilities for true positive, true negative, false positive, and false negative and 95% confidence intervals were calculated for each technique using the Clinical Research Calculator 1. The null hypothesis that all techniques can give same prevalence was tested using Chi square test.

Results

Prevalence of *T. evansi* in Camels

The prevalence of *T. evansi* infection in camels was found to be as shown by figure (1): Out of 600 tested camels; 10, 22, and 36, were found positive using WSF, BC and TSF; respectively. Out of 210 (randomly selected from the parasitological- negative of the total 600) camels tested 100 were found positive using CATT. Among 40 (randomly selected from the 110 CATT-negative samples) camels tested by PCR 36 were found positive. Some samples found negative by parasitological methods but were positive by PCR, unlike the CATT method when used for the same samples. The PCR were positive in all CATT positive samples (data not shown).
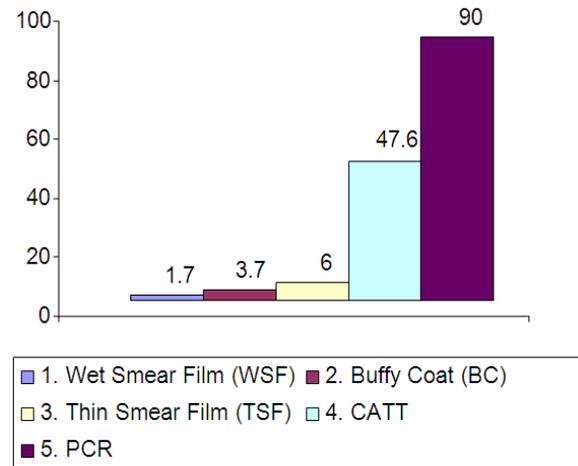

Figure 1. *T. evansi* infection rates among camels examined by parasitological, serological and molecular techniques.

The statistical analysis of the results (Table 1) indicates that the highest estimated value of prevalence is shown by the PCR and the lowest estimated value of prevalence is shown by the

WSF technique. The CATT technique has the highest lower limit value for the 95% confidence interval for the sensitivity. Both the PCR and the TSF have the same lower limit value for the 95% confidence interval for the sensitivity. While the WSF technique has the lowest lower limit value for the 95% confidence interval for the sensitivity. The WSF technique has the highest lower limit value for the 95% confidence interval for the specificity, while the PCR technique has the lowest lower limit value for the 95% confidence interval for the specificity.

Table 1. Statistical analysis using Clinical Research Calculator 1.

	Estimated Value	95% Confidence Interval Lower Limit	Upper Limit
Prevalence (PCR)	0.9	0.754042	0.967474
Prevalence (CATT)	0.47619	0.407355	0.545911
Prevalence (TSF)	0.06	0.042945	0.082895
Prevalence (BC)	0.036667	0.02367	0.055878
Prevalence (WSF)	0.016667	0.008491	0.031469
Specificity (PCR)	1	0.395774	1
Specificity (CATT)	1	0.957947	1
Specificity (TSF)	1	0.991567	1
Specificity (BC)	1	0.991769	1
Specificity (WSF)	1	0.991936	1
Sensitivity (PCR)	1	0.879934	1
Sensitivity (CATT)	1	0.953899	1
Sensitivity (TSF)	1	0.879934	1
Sensitivity (BC)	1	0.815025	1
Sensitivity (WSF)	1	0.655464	1

Chi square $(X2) = 0.547$, Significant at $p < 0.05$

Result of the Card Agglutination Test (CATT) for the surveyed Camels

Eight of the tested samples produced very strong agglutination i.e. highly positive (Figure 2). Nine of the tested samples produced strong agglutination. Fourty of the tested samples produced moderate agglutination. A number of 43 of the tested samples produced weak agglutination, while a number of 110 of the tested samples didn't produce agglutination.
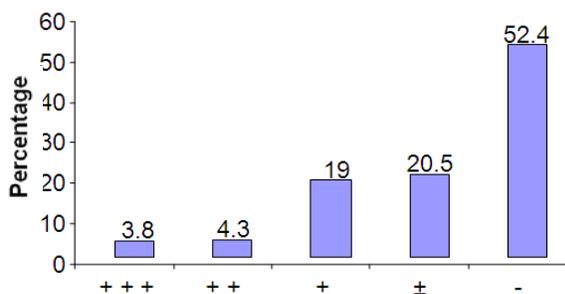

Figure 2. The intensity of the agglutination activity of studied camel blood samples using CATT.

Eight of the CATT positive samples have very strong reaction, 9 have strong reaction, 40 have moderate reaction, and 43 have weak reaction, while 110 have no reaction.

Result of the Polymerase Chain Reaction (PCR) for surveyed Camels

A 200 bp DNA fragment was amplified by PCR from DNA extracted from suspected samples collected from both Eastern and Western regions. TB-01 (Forward) and TB-02 (Reverse) set of Oligonucleotides primers were used in this PCR analysis. Some of the samples collected from Eastern region (E13-E28-E29-E30-E31) were loaded on 1.5% TBE agarose gel and visualized with 10 mg/ml ethidium bromide under the UV light as shown in figure (3). While some of the samples collected from Western region (W49-W39-W43-W50) were loaded on the same gel for sake of comparison. DNA extracted from experimentally infected Rat blood (originally from Eastern sample E20)

was used as positive control. A small size DNA fragment approximate 100 bp was appeared nearly on all analyzed samples those gave positive result with PCR. All trials of PCR optimization were unable to get rid of it, thus it may not be a non-specific binding or primers dimer. This could be due to that the amplified region present in two copies in the genome of the *T. brucei* or *T. Evansi* i.e as a repeat sequence. This suggestion could be tested by Southern blott analysis, however this beyond the scope of this work.

PCR amplified fragment of 200 bp resulted from samples collected from Al-Showak region (E13-E28-E29-E30-E31) were loaded on lanes (1-3-4-5-6) respectively. Samples collected from Al-Obeid region (W49-W39-W43-W50- positive control- DNA marker- negative control) were loaded on lanes (2-7-8-9-10-11-12) respectively.
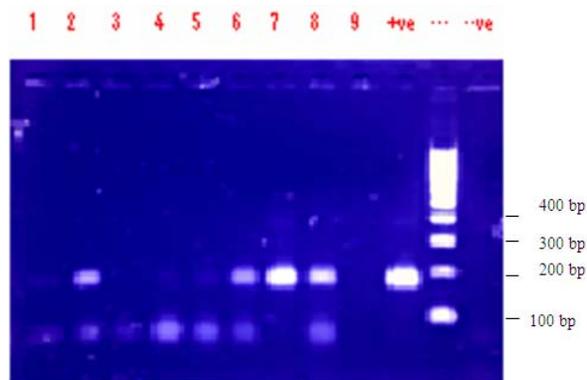

Figure 3. PCR analysis of some of the suspected samples.

The signs of Trypanosomosis observed in the tested animals are shown in figure (4). The most common signs were dullness, urine odour and hanging head. Less common were Diarrhea, Fever and paleness in mucous membrane. However, most of the signs described here were found correlated with the PCR positive result. Moreover the severity of symptoms is correlated with the specificity of the PCR, or on other words the high number of symptoms observed in a camel, the high intense was the PCR band.
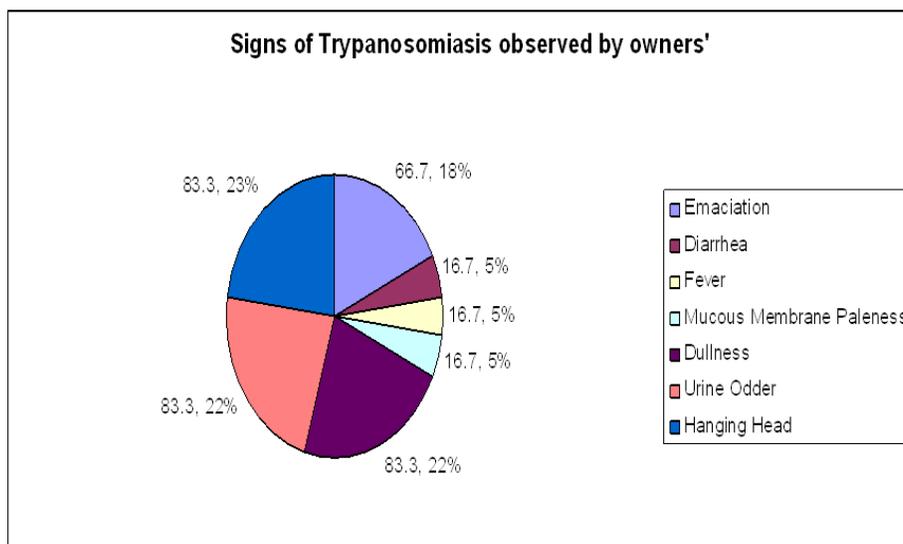

Figure 4. Clinical Signs of surveyed camels.

Some of the signs observed in the 600 surveyed animals were also stated by camel owners. In this study, the occurrence of most of symptoms was observed in camels which their samples were tested with parasitological methods, whereas fewer symptoms were observed in camels which their samples were tested with PCR.

Discussion

According to this study, parasitological techniques frequently used for diagnosis of *T. evansi* infections had low sensitivity. The serological tests identified the animals with sub-patent infections which could not be detected by parasitological methods. Moreover, its sensitivity is high (83.3%) as 30 out of 36 of blood samples from animals with parasitological negative result were positive by CATT. This finding confirms the usage of CATT instead of parasitological methods for more reliable result.

PCR has been used in detection of infection with *T. evansi* in buffaloes (Omanwar, 1999; Holand et al., 2000), horses (Clausen et al., 2003) and in Camels (Masiga and Nyang'ao, 2001). There was no comprehensive data on the use of PCR for detection of infection in Sudanese breed of dromedary Camels (*Camelus dromedarius)* (Hunter, 1986; Aradaib and Magid, 2006).

When using molecular method (PCR) in this study 40 camels were tested from parasitologically and serologically negative samples and 90% of them were found to be positive. Therefore, PCR was the more specific and sensitive method of the all methods used during this study.

There are several lines of evidence of the high sensitivity of PCR in diagnosis of *Trypanosoma* infection. Clausen and his colleagues (1998) compared the sensitivity and specificity of the PCR with (HAT) and (MAECT) for diagnosis of *Trypanosoma* infections in Livestock (cattle). They found that the detection rate of PCR was about x 2 higher. In another study to detect *T. evansi* in Camels using PCR and CATT/T. evansi in Kenya, the PCR was found the more sensitive method (Njiru et al., 2004).

During this study, all samples those were found positive by parasitological methods were positive as well by PCR, unlike the CATT method when used for the same samples. Some sample were negative by parasitological methods but were found positive when using PCR (Data not shown). PCR was able to detect infections in camels in the very early stage.

Therefore, PCR (90%) was the more specific and sensitive method of all methods used in this study. From the statistical analysis (p > 0.05), we conclude that there is a significant difference in the prevalence when using different diagnostic techniques.

In spite of the fact that the survey was conducted at different seasons of the year on the two study areas, the prevalence of Camel Trypanosomosis in Eastern and Western Sudan seems to be similar and no significant difference was reported. This can be attributed to similar conditions are exist on the two regions such as; establishment of surrounded circumstances, lack of health care, and lack of veterinary services. Drug resistance may arise as a result of absence of diagnostic measures and the ethno-veterinarians, such as sub-dosing, high Trypanosomosis challenges, faulty administration of drugs and efficacy of the available drugs. Moreover, the seasonal migration of camels in Western region, deep Southwards for water and grass in area of high Savannah where, relatively high challenge biting flies such as Tabanidae and Stomoxyinae is exist. Whereas camels in the Eastern region are always driven in all seasons during drought in search of water and grazing higher up in the Ethiopian plateau at the Sudanese-Ethiopian borders. In this movement they brought in close contact with the Tsetse fly belt. However, the possibility of having other Trypanosome infections is weak as their owners' were examined for diseases and were found healthy.

According to this study, there is a relationship between the clinical manifestation of Trypanosomosis and the diagnostic method used, the more accurate the method used the less symptoms observed. This finding is useful in early detection of infection and thus early treatment of the diseased camels.

This new molecular approach will make it possible to detect infections in Camels in the very early stages where microscope examination is unclear and to monitor groups of animals after trypanocidal treatment. Furthermore, this will not only be beneficial for diagnosis but also useful for epidemiological

study and designing rational Trypanosomosis control program.

Conclusion

This study recommends that the PCR technique should be adopted as a routine method for diagnosis of *T. evansi* infection in camels in all research centres of the Sudan. This implies that all the research centres should be supplied with all the facilities to conduct PCR analysis.

Given that no diagnostic method can be considered 100% sensitive, serology, parasitology and molecular techniques should be performed in order to exclude the infection from an animal. Isolation of trypanosomes to confirm the species identification is also possible by rat inoculation.

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