

Seroprevalence of Camel (*Camelus dromedarius*) Trypanosomiasis, with special Reference to Gene Sequencing of *Trypanosoma evansi* in Sharkia Governorate

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Abstract

A total of 330 camels (*Camelus dromedarius*) of both sexes and with different ages, at different localities in Sharkia Governorate, Egypt, were examined clinically from April 2013 to March 2015. Some adult camels showed emaciation, edema of legs and abdomen which were suspected to be infected by *Trypanosoma* species. This study aimed to determine the seroprevalence of trypanosomiasis using indirect ELISA test and the results showed that 174 (52.7%) were infected. The infection rate was higher in males (54.8%) than females (42.2%), also, in camels more than 2-years old and in summer and spring seasons. Thirty blood samples from the examined animals by ELISA test (15 positive and 15 negative) were subjected to PCR. The results revealed that 18 samples were positive for *T. evansi* by PCR. Two positive samples for RoTat1.2 VSG encoding gene were chosen for DNA sequence analysis, one of them was obtained from newly imported camel in a camel farm at Belbais city and the other one belonged to a camel from one of Belbais villages at Sharkia Governorate. Nucleotide sequence alignment of RoTat1.2 VSG gene variants from Egyptian *T. evansi* showed some heterogeneity with other *T. evansi* isolates from Egypt. In conclusion, PCR technique is more sensitive and specific than ELISA for the diagnosis of *T. evansi* infection in camels.

Key words: Camels, *Trypanosoma evansi*, Seroprevalence, ELISA

Introduction

Camels are considered important sources of meat and milk production, moreover, they have a special position in Egypt because they are used for transportation, drought power and other farm works, besides the utilization of their hair, wool and hides [1]. Worldwide, there are 14 million camels with 90% being dromedaries, and 110,000 camels belonging to one humped species *Camelus dromedarius* are present in Egypt [2]. *Trypanosoma evansi* multiplies in blood, body fluids and tissues resulting in a disease commonly known as "Surra". This disease is a wide spread wasting disease affecting a range of wild species and domestic livestock population [3]. In Egypt, *T. evansi* is enzootic in camels and is the foremost cause of impairment in dromedary camel productivity [4].

Mechanical transmission of *T. evansi* by haematophagous flies such as *Tabanus* and Stable flies (stomoxys) has resulted in the

widespread nature of the disease [5]. Affected camels have fever and show anorexia, marked generalized edema, rapid deterioration and death. The chronic form of *T. evansi* infection is characterized by progressive loss of body weight, intermittent high fever, marked generalized muscular atrophy, pale mucous membranes and occasionally abdominal edema [6]. Moreover, a characteristic odor of the urine could be attributed to increase of urinary ketones [6].

The chronic form may be associated with secondary infections due to the immunosuppression following *T. evansi* infection, which may complicate the clinical analysis [7]. The actual prevalence of the disease is underestimated because detection of the parasites in blood is difficult due to discontinuous parasitemia [8]. Although, parasitological techniques are easy, rapid and economic, they are not sufficient for the diagnosis of *T. evansi* in camels due to low

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parasitemia and also in the chronic forms of the disease [9].

Serological tests such as enzyme linked immunosorbent assay (ELISA) have been used for analysis of trypanosomiasis in camels due to the simple application of the assay [10]. In addition, ELISA is straightforward, and can be used for large-scale screening [11]. The nature of the antigen used in ELISA was specific enough to distinguish between infections with different trypanosome species, therefore, ELISA is considered the antibody-detection test of choice for diagnosis of trypanosomiasis [12].

Molecular diagnostic methods especially polymerase chain reactions (PCR) are promising for the diagnosis of trypanosomal diseases by the detection of trypanosomal DNA in the blood samples. Different primers for the amplification of DNA from subgroups of trypanosomes have been reported [13]. The used primers and the repetition of the target sequence in the genome determine the sensitivity of the PCR assay [14].

This work was conducted to estimate the seroprevalence of clinically suspected camel trypanosomosis by indirect ELISA in the different localities at Sharkia Governorate and to compare the diagnostic effectiveness of ELISA and PCR. In addition, amplification and sequencing of the Rhode Trypanozoon antigenic type (RoTat) Variant surface glycoprotein (VSG) associated gene.

Material and Methods

Animals

A total of 330 camels (*Camelus dromedarius*), of both sexes (259 male and 71 female camels) and different ages (197 adult animals >5 years, 117 young animals 2-5 years and 16 calves <2 years) were included in the present study. The animals were from different localities at Sharkia Governorate during the period from April 2013 to March 2015 (Table 1). Some adult camels were clinically suspected to be infected with *T. evansi* (They showed emaciation, odema of legs and abdomen, conjunctivitis and sometimes diarrhea).

Blood samples

From each camel, two blood samples (10 mL) were collected by the jugular vein puncture. The first sample was collected in a tube containing EDTA while the second one was collected without anticoagulant for serum collection. The blood samples collected on EDTA were preserved at -20°C for extraction of trypanosome DNA for PCR assay.

Enzyme linked immunosorbent assay (ELISA)

The indirect ELISA test was conducted using a commercially available kit *T. evansi* indirect ELISA kits (Lilli Test, Central Veterinary Research Laboratory, CVRL-UAE) [15]. The corrected optical density (O.D.) of the examined samples and controls were figured by subtracting the mean O.D. of the two antigen negative wells from the mean O.D. of the two relating antigen containing wells. These corrected O.D.s were expressed as percentage of the O.D. obtained with the strong positive control included in each plate (percent positivity, P.P.). If for a given sample the difference between the two raw O.D.s was more than 25 % of their mean, the outcomes were rejected and the sample retested [16].

Polymerase chain reaction (PCR)

Thirty blood samples from 30 camels (15 positive and 15 negative by ELISA test) were chosen and subjected to PCR assay.

DNA extraction and purification

Extraction of DNA from whole blood samples was performed using blood DNA extraction kit spin-column (BioTeke), following the manufacturer's instructions. The concentration of DNA in µg/mL was measured at 260 and 280 nm by ultra-violet spectrophotometer (Shimadzu, Japan). The PCR assay was carried out for the amplification of 164 bp of the highly repeated mini-chromosome satellite sequence using the primer TBR1: 5'-GAA TAT TAA ACA ATG CGC AG-3' and TBR2: 5'-CCA TTT ATT AGC TTT GTT GC-3') [17]. The reaction conditions were: an initial cycle at 94°C for 1 min and then 30 cycles of denaturation at 94°C for 30 sec, annealing at 60°C for 1 min and extension at 72°C for 30s, and finally one cycle at 72°C for 2 min. Moreover, 488 bp fragment

targeting RoTat 1.2 VSG gene was amplified using the primers ILO7957: 5'-GCC ACC ACG GCG AAA GAC-3' and ILO8091 5'-TAA TCA GTG TGG TGT GC-3' [18]. The PCR amplification was performed as an initial cycle at 94°C for 3 min and then 40 cycles of denaturation at 94°C for 30 sec, annealing at 52°C for 30 sec and extension at 72°C for 1 min, and finally one cycle at 72°C for 5 min. Electrophoresis of the amplicons was carried out through 1.5% agarose gel containing ethidium bromide (0.5 g/mL) and the image of the amplified DNA was captured using a gel documentation system (Bio spectrum UVP, UK) [19].

Sequence analysis of *T. evansi* RoTat 1.2 VSG gene

Two positive samples by PCR technique were chosen for DNA sequence analysis, one of them was obtained from newly imported camel in a camel farm at belbais (sample No. 16) and the other one was belonged to a camel from one of Belbais villages at Sharkia governorate (sample No. 21). The RoTat 1.2 VSG PCR product was purified using the QIA quick PCR Purification Kit (Qiagen)) and sequencing reaction was carried out using BigDye® Terminator v3.1 Cycle Sequencing Kit. Thermal profile (Fast PCR Machine) for *T. evansi*. Alignment of the sequences was carried out by the DNA BaserV3 software. The nucleotide sequences were compared with other sequences available in GenBank using the Basic Local Alignment Search Tool (BLAST)(<http://www.ncbi.nlm.nih.gov/BLAST>) [20].

Table 1: Seroprevalence of trypanosomiasis in different localities at Sharkia Governorate during the period April 2013 to March 2015, by ELISA

Localities	No. of examined camels	Positive samples	Percentage
Belbais villages	62	27	43.5%
Veterinary Clinic, Faculty of Veterinary Medicine, Zagazig University	19	8	42,1%
Menia El-Kamh	17	8	47%
Abou-Hamad	94	42	44,6%
Mashtol Veterinary Clinic	63	38	60.3%
El-Qurain	36	25	57.1%
Inshas Veterinary Clinic	39	26	69,4 %
Total	330	174	52.7%

Results

Out of 330 camels clinically suspected for trypanosomiasis, 174 (52.7%) were positive by ELISA test for circulating antibodies to *T. evansi*, (Table, 1). Seroprevalence of *T. evansi* infection in camels revealed that 142 (54.8 %) male and 30 (42.2%) female camels were positive by ELISA test respectively, (Table, 2). Regarding animal age, the test revealed that 56.4%, 51.2% and 31% of young, adult and calf camels were positive by ELISA test, respectively (Table, 2). Concerning seasonal variation, the results showed that at summer, autumn, winter and spring 61.8%, 27.2%, 31.3% and 58.7% of the examined camels were positive by ELISA test (Table, 2).

Results of examination by PCR using TBR primers revealed that 18 (60%) samples were positive for infection Figure (1). All samples which were positive to *T. evansi* infection by ELISA (15 samples) were positive by PCR while the remaining positives by PCR (3 samples) were negative by ELISA assay. Amplification of the RoTat 1.2 VSG encoding gene in all TBR positive samples was carried out. The results revealed the amplification of the expected 488 bp fragment from all *T. evansi* confirmed samples. Two samples were chosen for sequencing, the first one was belonged to newly imported camel in a camel farm at Belbais (sample No. 16) and the second sample was belonged to a camel from one of Belbais villages at Sharkia Governorate (sample No. 21).

Table 2: Seroprevalence of *Trypanosoma evansi* infection in camels in relation to animal sex, age and seasonal variations

Criteria		No. of examined camels	Positive samples	Percentage
Gender	Male	259	142	54.8%
	Female	71	30	42.2%
Age	>5 years (adult)	197	101	51.2%
	2.5 years (young)	117	66	56.4%
	< 2 years (calf)	16	5	31%
Seasons	Summar	152	44	61.8%
	Autumn	22	6	27.2%
	Winter	64	20	31.3%
	Spring	92	54	58.8 %

Nucleotide sequence alignment of RoTat1.2 VSG encoding gene from the first isolate which belonged to newly imported camel (sample No. 16) showed 100% similarity between different *T. evansi* isolates from Kenya (AF317914), India (EF495337 and KU589274.1), and from Egypt (JX888091 and Egy 3 isolate [4]). Whereas, the same isolate (sample No. 16) showed 99% similarity with *T. vivax* isolate from India (JX134605). On the other hand, the Egyptian isolates Egy 2 isolate [4] showed difference in 3 bp substitutions (Figure 2).

Nucleotide sequence alignment of RoTaT1.2 VSG encoding gene from the second isolate which was belonged to a camel from one of Belbais villages at Sharkia Governorate (sample No. 21) showed 100% similarity to *T. evansi* isolates from India (JX134605), and 99% similarity with *T. vivax* isolates from Egypt (JX888091), India (EF495337) and Kenya (AF317914). On the other hand, the Egyptian isolates Egy 2 and Egy 3 [4] showed difference in 2-4 bp substitutions revealing lower similarity.

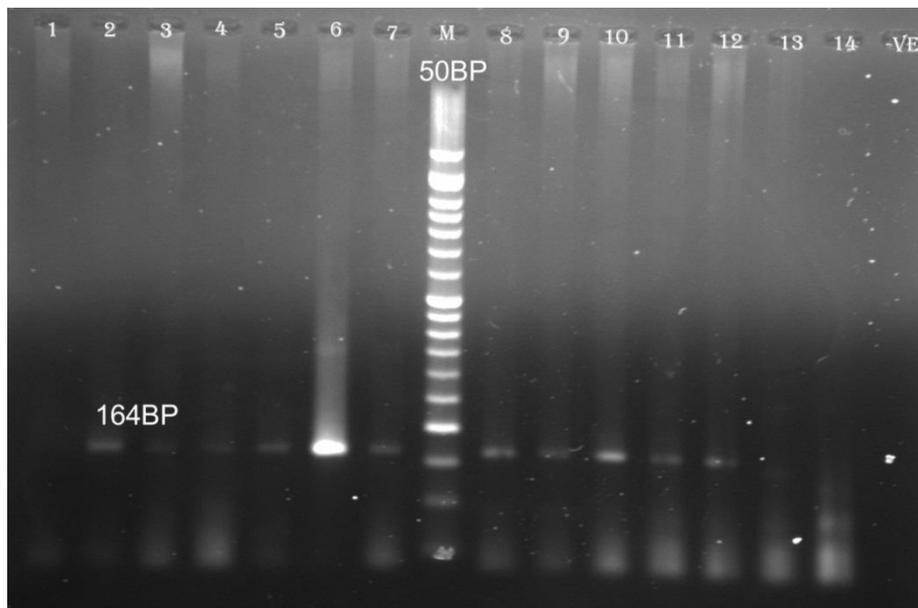


Figure 1: Agarose gel electrophoresis of amplified *T. evansi* DNA (164 bp PCR products). M: 50 bp DNA ladder as a standard marker, Lane (2, 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12): Positive PCR products from field blood samples. Lane (1, 13, and 14): Negative PCR products from field blood samples.

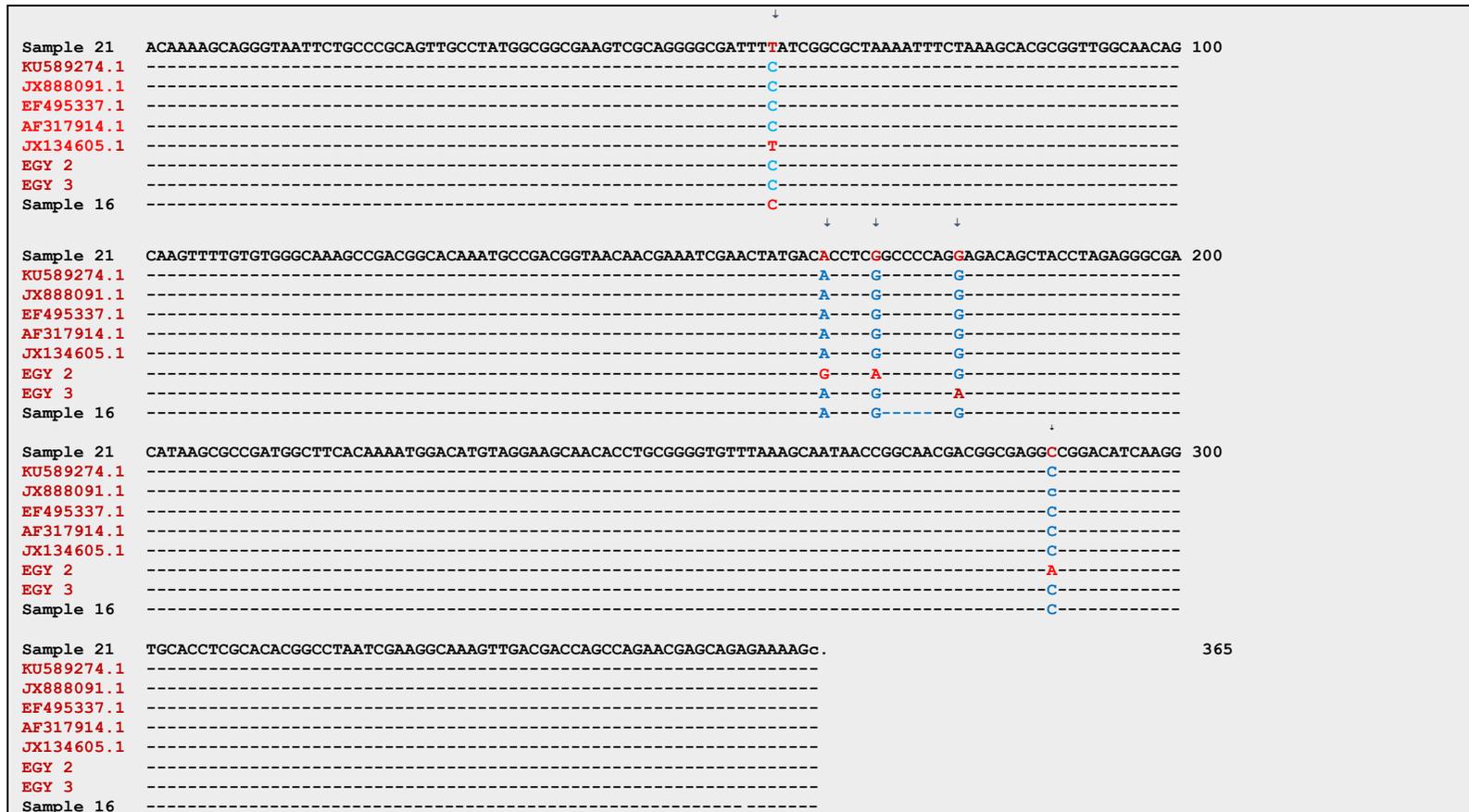


Figure 2: Nucleotide sequence alignment of RoTaT1.2 VSG encoding gene variants from Egyptian (Sharkia) *T. evansi* (This study, sample 16 and sample 21) with other VSG *T. evansi* isolates from Egypt EGY 2 and EGY 3 [4] and JX888091; from India (EF495337, JX134605 and KU589274.1) and from Kenya (AF317914). Identical bases are shown as dashes and pointed to variations site with an arrow.

Discussion

Indirect enzyme linked immunosorbant assay (ELISA) revealed that 52.7% of the examined samples were positive. This finding coincides with 55.9% [21], 64.3% [22] and 44.2% [23] reported in other studies in Egypt and Pakistan. The authors concluded that ELISA is sensitive and specific for the detection of trypanosomes' antibodies. However, another study reported lower prevalence rate of antibodies (26%) among camels among camels in Cairo during 2011 [24]. Moreover, Fikru *et al.* [25] concluded that ELISA although specific for trypanosomes, the test cannot discriminate between active infections and those which persist after infections because antibodies could be detected for at least 100 days after trypanosomal treatment.

Seroprevalence of *T. evansi* antibodies in male camels was higher than in female camels. This could be attributed to that male camels might be in stress because of weakness due to physical work, travelling in terms of searching for food and water and consequently more exposure to vectors. Bogale *et al.* [26] reported higher prevalence in male than female camels in agreement with this study, while, Ngaira *et al.* [27] reported no differences in *T. evansi* seroprevalence in both sexes. On other hand, Tahseen *et al.* [23] recorded higher prevalence in females than males, which was correlated to pregnancy and lactation.

Regarding the age of camels, antibodies against *T. evansi* were detected in 56.4%, 51.2% and 31% of young, adult and calf camels, respectively. Higher prevalence rates in adult and young population than calf is in agreement with the results of Singh *et al.* [28] who reported that the highest seroprevalence was in young camels up to 5 years of age (21.7%). Maternal immunity waning and being the new lot in the herd could explain the increased susceptibility of young animals to infection. In contrary, Dially *et al.* [29] recorded higher seroprevalence in adult camels, these variations may be due to various factors such as overestimation of disease owing to persistence of antibodies following treatment, chronic nature of infection, stress, poor management, draught and preference by

vectors because of large surface owing to severity of disease.

Data concerning the seasonal variation of trypanosomiasis determined by ELISA showed that 61.8%, 27.2%, 31.2% and 58.8% of the examined camels were positive during summer, autumn, winter and spring seasons, respectively. These findings are in agreement with other reported studies [8,30-31]. The highest seasonal seroprevalence of *T. evansi* in summer and spring could be due to the increased activity of the vector during these seasons. In addition, studies of tabanus in the different tropical regions have demonstrated a clear relationship between the seasonal outbreaks of *T. evansi* infections and the increase in the numbers of tabanus during the downpours [32].

PCR by using TBR primers revealed that 18 samples were positive out of 30 samples. All samples, those were positive to *T. evansi* infection by ELISA test (15 samples) were positive by PCR, while, the remaining positive by PCR (3 samples) were negative by ELISA test. The PCR results demonstrated an identification rate higher than with the ELISA. These findings are in accordance with those reported by Clausen *et al.* [33] and Konnai *et al.* [34], who concluded that PCR has been developed and used for surveys with high sensitivity and specificity for detection of trypanosomes.

The TBR primers were selected in the present study due to the reported higher sensitivity and specificity compared to other primer sets for the detection of *T. evansi* and were able to detect low parasitemia in blood [14]. The high detection was attributed to the high repetition of the sequences in the genome. Consequently, for early diagnosis and rapid treatment of acute and chronic infections, routine use of TBR primers can be suggested [14,35].

The existence of RoTat 1.2 VSG encoding gene in *T. evansi* is considered a useful tool to differentiate this species from other *Trypanozoon* members [19]. In addition, the highly conserved property of this sequence was documented [36]. The obtained results showed that *T. evansi* isolates contained RoTat 1.2 VSG encoding gene, this is in agreement

with other reported studies in Egypt [4,37]. Sequencing analysis of the amplicon from two representative isolates are shown in Figure 2. Only one base pair substitution was observed in sample No. 21 compared to other sequences in Kenya and India. Similar observation was reported in Egypt by Amer *et al.* [4] indicating heterogeneity. The authors attributed the heterogeneity to the long persistence of the parasite in camels and the existence of geographic and host effect [4].

Similar results were also reported by Elhaig *et al.* [37] where 100% similarity of *T. evansi* RoTat 1.2 VSG sequence compared with other sequences from Egypt [4], Kenya (AF317914) and India (EF495337) was observed. While, 99% identity with an isolate from India (JX134605) was reported [37].

Conclusion

This study affirms that ELISA is the test of decision to deploy during surveys and in routine diagnostic practice of trypanosomiasis in camels. The PCR technique is sensitive and specific for the detection of the chronic infection and low parasitaemia in infected camels by trypanosomiasis. The Nucleotide sequence RoTaT1.2 VSG gene Egyptian (Sharkia) *T. evansi* showed heterogeneity among the Egyptian isolates.

Conflict of interest

All the authors have no conflict of interest to declare.

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الملخص العربي

دراسة وبائية سيروولوجية لمرض التريبانوسوما ايفانساى فى الجمال ذات السنام الواحد وتحليل التسلسل الجينى

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أجريت هذه الدراسة على ٣٣٠ جمل (من الجمال ذات السنام الواحد) من مختلف الأعمار ومن الجنسين وفى فصول السنه المختلفه من مناطق مختلفه داخل محافظة الشرقيه وذلك خلال الفتره من أبريل ٢٠١٣ حتى مارس ٢٠١٥. هذه الجمال لوحظ فيها الضعف العام والورم فى الأرجل و أسفل البطن مما أعطى الشك فى أصابتها بالتريبانوسوما. وكان الهدف من هذه الدراسه هو القيام بدراسه وبائيه سيروولوجيه لمرض التريبانوسوما فى الجمال باختبار الأليزا. وأوضحت نتائج الدراسه الوبائيه السيروولوجيه أن هناك ١٧٤ عينة كانت ايجابية للأصابة وكان ذلك بنسبة اصابة ٥٢.٧%. وأوضحت النتائج أيضا أن نسبة الأصابة فى الجمال الذكور كانت ٥٤.٨% بينما كانت فى الإناث ٤٢.٢%. وأظهرت نتائج الدراسه الوبائيه السيروولوجيه لمرض التريبانوسوما تبعا لعمر الجمل ان معدلات الأصابة كانت أكثر فى الجمال الأكبر من عامان وفى فصلي الصيف والربيع. وتم استخدام إختبار تفاعل البلمرة المتسلسل على عدد ٣٠ عينة مختبرة سابقاً باختبار الاليزا (تشمل ١٥ عينة إيجابية و ١٥ سلبية) وأوضحت النتائج وجود عدد ١٨ عينة إيجابية للإصابة بمرض التريبانوسوما. تم أختيار عينتين إيجابيتين باختبار تفاعل البلمرة المتسلسل وذلك لاجراء تحليل التسلسل الجينى لطفيل التريبانوسوما فى هاتين العينتين (عينه من جمل حديث الأستيراد فى مزرعه من مزارع الجمال ببلييس والأخري من جمل من إحدى قرى مركز بلييس أيضا) وأوضحت النتائج تماثلا بدرجة عاليه بين التسلسل الجينى لهاتين العينتين مع التسلسل الجينى للتريبانوسوما ايفانساى فى المناطق الجغرافيه المختلفه مثل كينيا والهند ولكنها أظهرت أختلافات واضحه مع بعض الأنواع للتسلسل الجينى لطفيل التريبانوسوما المصريه. ومن الدراسه يستخلص أن تفاعل البلمرة المتسلسل يعتبر أكثر حساسية وتحديدًا من الاليزا لتشخيص التريبانوسوما فى الجمال.