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DETECTION OF *TRYPANOSOMA EVANSI* IN CAMELS USING *SYBER GREEN1* REAL TIME PCR ASSAY

(With One Picture and 2 Figures)

By

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الكشف عن طفيل التريبانوسوما إيفانساى فى الجمال باستخدام تفاعل البلمرة المتسلسل ذو الزمن الحقيقي

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يسبب مرض التريبانوسوما فى الجمال خسائر ملحوظة فى العديد من مناطق تربية الجمال فى العالم. وهذا المرض يسببه طفيل من نوع تريبانوسوما إيفانساى. فى هذه الدراسة تم جمع عدد 75 عينة دم من الجمال يتوقع إصابتها بمرض سارا (التريبانوسوما فى الجمال) وتم عمل شرائح من هذة العينات وصبغها بصبغة الجمسا وفحصها تحت الميكروسكوب، وأظهرت النتائج إصابة عدد 9 من 75 عينة (12%) بطفيل التريبانوسوما إيفانساى فى حين تم اكتشاف وجود الحامض النووي الخاص بطفيل التريبانوسوما ايفانساى لعدد 72 من 75 عينة بنسبة 96% وذلك باستخدام تفاعل البلمرة المتسلسل ذو الزمن الحقيقي باستخدام بادئ تتابع وراثي خاص بهذا الطفيل. كما كانت نقطة التعاظم للعينات الإيجابية تظهر إتحادها عند درجة حرارة 85 درجة مئوية. يعد استخدام تفاعل البلمرة المتسلسل ذو الزمن الحقيقي اختبار يمكن إجراؤه للكشف المباشر لطفيل التريبانوسوما إيفانساى في دماء الجمال المصابة وكذلك يمكن إستخدامة لدراسة وبائية المرض بين الجمال والأنواع الأخرى من الحيوانات التى يمكن أن تصاب بهذا الطفيل.

SUMMARY

Camel Trypanosomasis caused by *Trypanosoma evansi* is still a serious problem in camel husbandry which causes considerable losses in many camel-rearing regions of the world. In the present study, 75 camels clinically suspected for Surra were examined parasitologicaly by Giemsa stained blood smear and for DNA amplification by real time PCR. Giemsa stained blood smears on microscopical examination detected only 9 out of

75 camels (12%) as positive for the presence of *T. evansi*. While, Out of 75 DNA samples prepared from camel blood, 72 were positive by real time PCR assay (96%). Primers (TR3, TR4) derived from nuclear repetitive gene of *T.evansi* and SYBER Green1 fluorescent dye has been used. The melting peak chart of the positive samples showed one single peak at an average Tm of 85.0^{o}C. Application of this real time PCR to clinical samples resulted in rapid, direct detection of *T. evansi* in blood of naturally infected camels. The described real time PCR provides a valuable tool to study the epidemiology of *T.evansi* infection in camels and other susceptible animal population.

Key words: *Camels, Real time PCR, SYBER Green 1, Trypanosoma evansi.*

INTRODUCTION

Camel is the principle domestic animals in Saudi Arabia and its meat and milk still constitute a vital source of animal proteins to nomads and city dwellers (Banaja and Ghandour, 1994). Camel Trypanosomasis, Surra, is a disease of camels caused by *Trypanosoma evansi*. The disease is the most important single cause of economic losses in camel rearing areas, causing morbidity of up to 30.0% and mortality of around 3.0% (Ngerenwa *et al.,* 1993, Egbe-Nwiyi and Chaudry, 1994; Pacholek *et al.,* 2001; Njiru *et al.,* 2002). Trypanosomasis is the most important and serious pathogenic protozoal disease of camel caused by *Trypanosoma* species. This parasite has a wide range of distribution throughout tropical and sub-tropical regions of the world. *T. evansi* was reported originally from India, where the term 'Surra' is used to describe the disease (Shah *et al.,* 2004).

The causative agent, *Trypanosoma evansi,* was discovered by Griffith Evans in 1880 in infected camels and equids in the Dara Ismail Khan District of Punjab (Evans, cited by Indrakamhang, 1998). Since then, studies have shown that the parasite can infect all species of domesticated livestock, although the principal host varies geographically (Indrakamhang, 1998; El-Sawalhy and Seed, 1999; Al-Rawashdeh *et al.,* 2000; Diab *et al.,* 1984) recorded the first report *T. evansi* in imported and indigous camels in the Eastern and Southern region of Saudi Arabia.

Camelid trypanosomasis can be acute, but it is usually chronic. It mainly results in wasting, anemia, edema and abortions. Mortality is high in untreated cases (Itard, 1989; Luckins, 1992).

The diagnosis of disease is problematic because the commonly used tests have important limitations. Clinical signs are variable and non specific

while parasitological examination frequently fails to detect latent infection when parasitaemia is scanty in peripheral blood, especially in chronic form (Chaudhary and Iqbal, 2000). Early detection of *T.evansi* plays an important role in the epidemiology and animal health (Viseshakul and Panyim, 1990).

Both of the conventional parasitological methods and serological techniques lack sensitivity and specificity. Therefore molecular techniques, especially polymerase chain reaction (PCR) have been developed in order to overcome the problems faced by conventional and serological techniques. In addition, it was reported that PCR is a reliable method for diagnosis and epidemiological studies (Omanwar *et al.*, 1999).

Polymerase chain reaction assay (PCR) amplifying genetically defined regions of genome parasites including trypanosomes are currently of great interest (Wuyts *et al.*, 1994). Molecular tests for *T .evansi* could potentially improve diagnostic accuracy by using techniques such as PCR to detect *T. evansi* in blood (Wuyts *et al.*, 1995). Detection of minute amounts of trypanosomal DNA using PCR procedure is a possible mean of identifying animals with active infections, and could have the sensitivity and specificity required. The PCR has used been in surveys to determine the prevalence of *T.evansi* in camels from different regions in Kenya (Njiru *et al.*, 2004) and in buffaloes in Vietnam (Holland *et al.*, 2004).

However, real time PCR has not been systematically used for detection of *T.evansi* in camels in Saudi Arabia. In this study, we described a simple, rapid, sensitive and specific assay for detection of *T. evansi* in blood of suspected camels in Saudi Arabia using the real time PCR - SYBER Green assay.

MATERIALS and METHODS

Collection of blood samples:

Seventy five blood samples were collected from suspected clinical camels through jugular vein. Blood samples were collected in clean sterile vacutainers, containing ethylene diamine tetra acetic acid (EDTA) was preserved at -20°C for extraction of total nucleic acids for use as target DNA for PCR amplification. Blood from animals identified positive by microscopy were used as positive control. Blood from healthy camels (proved negative by wet blood film and mouse inoculation test) were used as negative controls.

Microscopy:

75 thin blood smears were also prepared at the time of collection of

blood. They were air dried, fixed in methanol for 2 minutes and allowed to dry, then stained by Giemsa stain 10% and were examined under oil immersion lens (100x).

Extraction of DNA from blood samples:

Automated extraction of total genomic DNA from the whole blood by BioRobot EZ1 workstation (Qiagen®) using EZ1 DNA blood kit (Qiagen®) according to the manufacturer's instructions.

Primers:

T.evansi repetitive DNA primers were designed and synthesized in Biolegio BV®, Belgium to amplify a single band of 257bp PCR product according to Chansiri *et al.* (2002):
TR3 (5′ GCGCGGATTCTTTGCAGACGA 3′)
TR4 (5′ TGCAGACACTGGAATGTTACT 3′)

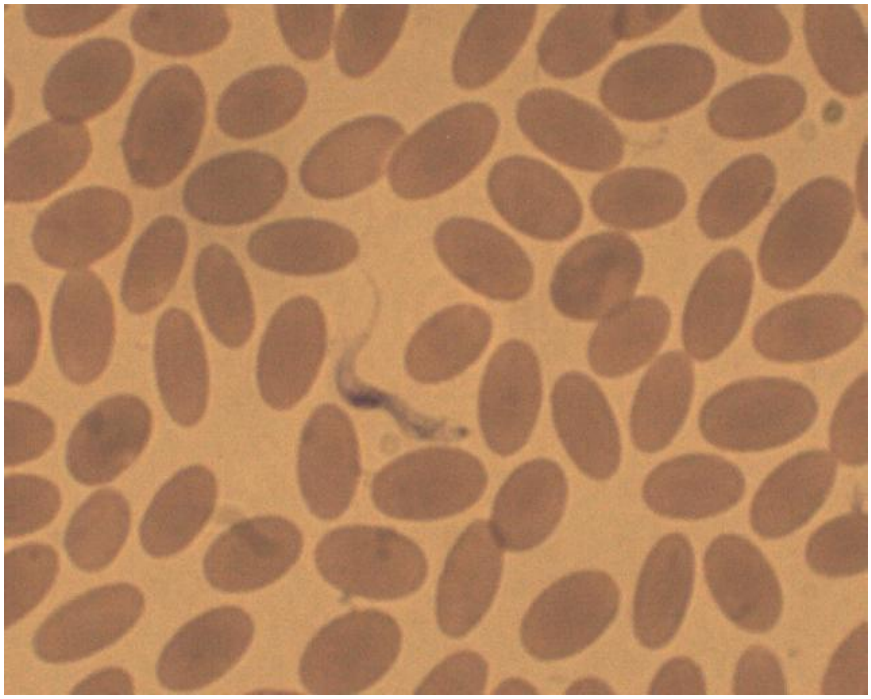
Real times PCR assay:

Polymerase chain reaction was carried out in LightCycler 2.0 (Roach®).The PCR mixture (20 µl) contained 15 µl of reaction mixture containing Fast start DNA Master ^{plus} SYBER Green 1(Roach®) and 10 *pmol*/*µ*l concentration of each primer and 5 *µ*l of genomic DNA. The PCR profile was performed as following; pre-heated for 1 cycle at 90 ºC for 10 min and then denatured at 94 ºC for 5 sec, annealing at 55 ºC for 10 sec and extension at 72 ºC for 10 sec. The PCR amplification was performed for 45 cycles. Fluorescence data were acquired at the end of each cycle in a single step. Once the plateau phase of the PCR had been reached, amplification was stopped and a standard melting curve analysis was performed (95ºC for 0 second, 65ºC for 10 seconds, and a 0.1ºC/second rise to 95ºC) with continual fluorescence measuring. PCR data were analyzed using LightCycler2 software version 4.05.

RESULTS

Microscopic Examination:

Giemsa stained blood smears on microscopical examination detected only 9 out of 75 camels (12%) as positive for the presence of *T. evansi* (Picture 1).



Picture 1: Positive Giemsa stained blood smear, x100.

Real time PCR assay:

1 - Optimizing of PCR

The optimization of the real time PCR reaction components and cycling parameters were performed respectively. Different annealing / elongation temperatures and times were evaluated. The optimized concentration of reaction components and cycling parameters were established as described previously (Figure 1).

2 - Melting curve analysis:

DNA melting curve analysis was performed to identify DNA products from the melting temperature(Tm) of each sample. The melting peak chart of the positive samples showed one single peak at an average Tm of 85.0^{o}C. Neither amplification nor primers dimmers formation occurred without *T.evansi* DNA template (Figure 2).

The result showed the infection in most suspected samples. Out of 75 DNA samples prepared from camel blood, 72 were positive by real time PCR assay therefor the efficiency of detection by this technique was 96%**.**

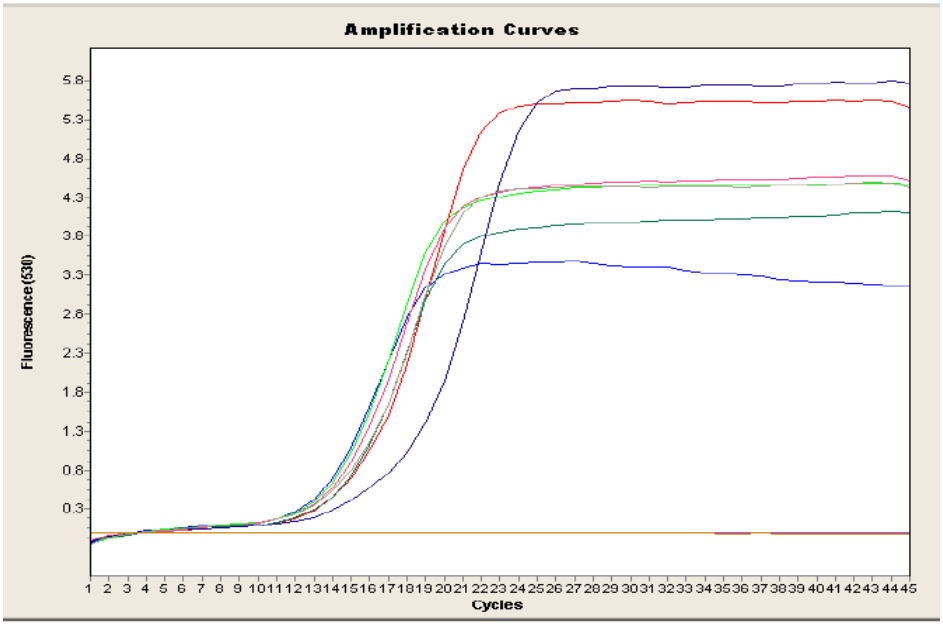


Fig. 1: Amplification curve of real time PCR

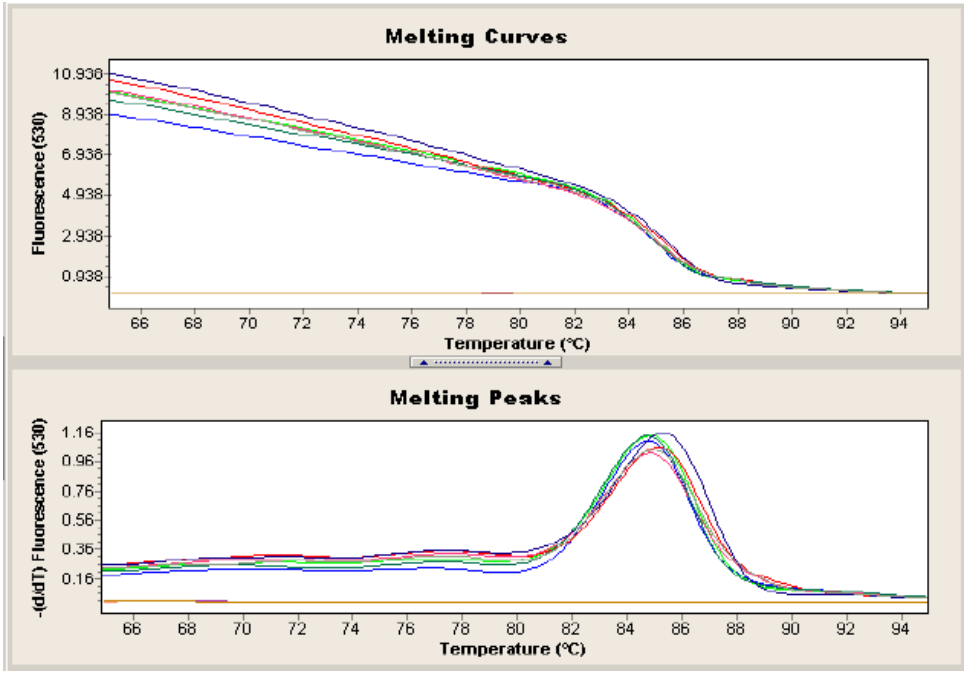


Fig. 2: Melting curve analysis of amplicon obtained from real time PCR.

DISCUSSION

Because of the limitations of immunological techniques (Pathak *et al.,* 1997) and the difficulty of demonstrating the parasite in blood smears due to the intermittent nature of parasitaemia (Nantulya, 1990), So there is an urgent need for sensitive and unequivocal detection of acute disease conditions and chronic carrier states.

In the present study only 9 animals were identified positive by microscopy out of 75 samples (12%) tested. These results agree with Paris *et al.* (1982) who found the sensitivity of microscopic examination from Giemsa stained thin blood film was 10^5 trypanosomes per ml of blood. So, blood smear examination proved to be of limited value in diagnosis of subacute or chronic cases.

On the other hand, the diagnostic sensitivity of the present real time PCR assay using primers derived from nuclear repetitive gene of *T.evansi* and SYBER Green1 fluorescent dye was 96%. This sensitivity is higher than the sensitivity recorded by other assays. El-Metanawy *et al.* (2009) found that the application of PCR using *T.evansi* specific primers was the highest method of detection (93.8%) followed by card agglutination test, mouse inoculation (55.4%), buffy coat technique(18.5%) and wet blood film (13.8%). Also, Wasana *et al.* (2000) concluded that the efficiency of detection *T.evansi* by PCR assay was 93.55%.

The real time PCR assay presents several important advantages over other methods of detection of *T.evansi*. First, the real time PCR is performed in a closed tube with no post-PCR manipulations, thereby reducing post-PCR processing time. Second, the assay is quick; results can be conformed within 1hour.

In conclusion, the real-time PCR assay is very sensitive and specific assay that can detect *T evansi* in blood samples of naturally infected camels and can be recommended for inclusion in survey and control programs.

Application of this real time PCR on practical scale to determine the prevalence of *T.evansi* in naturally infected camels in different regions of Saudi Arabia is in progress.

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