

Comparison of microscopy, card agglutination test for *Trypanosoma evansi*, and real-time PCR in the diagnosis of trypanosomosis in dromedary camels of the Abu Dhabi Emirate, UAE

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Abstract

Introduction: Trypanosomosis is an important disease of dromedary camels caused by the pathogenic protozoan *Trypanosoma evansi*. This study aimed to compare three different tests for its diagnosis in this species: conventional microscopy, the card agglutination test for trypanosomosis/*T. evansi* (CATT/*T. evansi*) and real-time PCR. **Material and Methods:** Whole blood and serum samples collected from 77 dromedary camels of Abu Dhabi, United Arab Emirates, were analysed with the test methods stated. Statistical analysis was done using McNemar's chi-squared test, and Cohen's kappa index (κ) was calculated. **Results:** We obtained results with positivity of 18% (14/77) by microscopy, 22% by CATT (17/77) and 60% (46/77) by real-time PCR, with the chain reaction detecting at a respectively three- and two-fold greater rate than the other techniques. Analysis of the data revealed a relative sensitivity of 30.4% and 37.0% for microscopy and CATT, respectively, compared to real-time PCR. The difference between the real-time PCR's sensitivity and those of the other methods was statistically significant, with X^2 values of 30.03 and 20.1, respectively ($df = 1$ and $P = 0.05$ in both cases). Agreement of microscopy results with those of with CATT was good ($\kappa = 0.72$; 95% CI = 0.62–0.82). Cohen's kappa index showed fair agreement of real-time PCR with microscopy ($\kappa = 0.26$; 95% CI = 0.16–0.36) whereas it was in poor agreement with CATT ($\kappa = 0.09$; 95% CI = 0.02–0.15). **Conclusion:** Real-time PCR was found to be more sensitive than microscopy and CATT.

Keywords: trypanosomosis, dromedary camels, diagnosis, microscopy, real-time PCR.

Introduction

Trypanosomosis, also called Surra and caused by *Trypanosoma evansi*, is the most important and serious pathogenic protozoan disease of dromedary camels (9). *Camelus dromedarius*, the one-humped camel, is a widely distributed domestic animal in arid and semiarid regions of Africa, Arabia and Western Asia up to India. The disease has the widest geographical distribution as a camel affliction, being detected in 23 countries, cattle, horse, and dog variants being diagnosed in 16, 13 and 12 countries, respectively (3). The global distribution of *T. evansi* is proved by reports from Asia, Africa, South America, and Europe of the

parasite affecting a large diversity of mammalian hosts (3). It was found that *T. evansi* had spread from Africa into Asia through infected host species, mainly dromedary camels, horses and mules.

Surra impairs productivity gravely and is considered the most economically important disease in dromedary camels. It causes anorexia, weakness and emaciation that lead to low milk and meat yield, poor traction power, increased abortion and death (12); these last two manifestations characterise clinical outbreaks in the Middle East (3). Trypanosomosis is mechanically transmitted by haematophagous flies of the *Stomoxys* and *Tabanus* genera (9). The disease may develop as either an acute form or a chronic form, which may last

for many months or possibly years. Infections with *T. evansi* are usually diagnosed when the animal shows clinical symptoms and diagnosed definitively in the laboratory by the detection of the parasite in the blood or tissue fluids and the detection of antibodies to it. Since diagnosis based on clinical signs alone is inaccurate, laboratory methods are often relied on in assisting the diagnosis of *T. evansi* infection.

While parasitological methods such as the microscopy of stained blood smears and the haematocrit centrifugation technique are fast, inexpensive, and highly specific, their analytical sensitivity is very low (3). These techniques often fail to detect ongoing infections, as the level of parasitaemia is often low and fluctuating, particularly during the chronic stage of the disease (4). Serological antibody detection tests are proven to be useful in epidemiological surveys, but the major drawbacks are cross-reactivity with non-specific antibodies and the persistence of specific antibodies for weeks or months after successful treatment. Detection of DNA based on PCR is advantageous because of its superior sensitivity and accuracy: the chain reaction can identify trypanosomes to the subspecies level and can detect prepatent, chronic and mixed infections (10). Diagnosis of animal trypanosomosis is challenging due to the fluctuations in parasitaemia, aparasitaemic intervals and the difficulty of directly detecting the parasite, especially in the subpatent phase of infection, therefore the use of more sensitive diagnostic tools such as PCR and real-time PCR is necessary (5). The present study aimed to compare the diagnostic performance of microscopy, the card agglutination test for trypanosomosis/*T. evansi* (CATT/*T. evansi*), and real-time PCR in camel trypanosomosis.

Material and Methods

Sampling. During routine diagnosis at the veterinary laboratories of the Abu Dhabi Agriculture and Food Safety Authority (ADAFSA) in 2019, whole blood collected from 77 dromedary camels (from Abu Dhabi and the Al-Dhafra region in the emirates of Abu Dhabi) was subjected to the three diagnostic tests. The camel owners' consent was obtained before inclusion of samples in the study. Blood samples collected in duplicate from the jugular vein into serum clot activator tubes and ethylenediaminetetraacetic acid (EDTA) tubes were brought to the laboratory within 24 h of collection in cool packs. Sera were separated from blood in the clot activator tubes after centrifugation at $2,355 \times g$ for 5 min. Serum and blood samples for DNA extraction were stored at -20°C until further use.

Microscopy. Thin blood smears were prepared from the whole blood samples (with EDTA) within 24 h of collection as per standard laboratory methods, air-dried, fixed in absolute methanol for 2 min and stained with Giemsa stain (1:5 dilution in distilled water) for 30 min. The stained smear was carefully

screened for blood parasites in a minimum of 300 fields using a 100 x oil immersion objective (7, 17).

Card agglutination test for trypanosomosis/*T. evansi*. Detection of *T. evansi*-specific antibodies was carried out by CATT/*T. evansi* (Institute of Tropical Medicine, Antwerp, Belgium) in serum that was prediluted 1:4 in CATT diluent, according to the manufacturer's instructions. Briefly, one drop of camel serum diluted up to 1:4 in CATT buffer was pipetted onto a plastic-coated test card, then one drop of CATT reagent was added, and the reaction mixture was spread out using a clean stirring rod and allowed to react on a card with a manual rotation for 5 min. Blue granular deposits revealed a positive reaction visible to the naked eye.

Extraction of DNA and amplification by PCR. DNA was extracted from the buffy coat after centrifugation of the EDTA blood tube containing the sample at $2,355 \times g$ for 5 min. Extraction was performed manually using a QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany), following the manufacturer's instructions. The extracted DNA was quantified using a Nanodrop-2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and either used immediately or stored at -20°C for later use.

The RoTat 1.2-F (5'GCG GGG TGT TTA AAG CAA TA 3') and RoTat 1.2-R (5'ATT AGT GCT GCG TGT GTT CG 3') primers were previously used in a gel-based PCR specifically to detect the *T. evansi* genome (6). Based on these primers, we further optimised conditions for a real-time PCR based on SYBR green chemistry (QuantiFast SYBR Green PCR Kit; QIAGEN). The optimisation included the constitution of the master mix, the number of cycles and the annealing temperature. The PCR mix consisted of 12.5 μL of 2 x qPCR mix, 1 μL of each primer (10 pmol), 1 μL of the template DNA (100 ng) and 9.5 μL of water to make a total volume of 25 μL . The cycling protocol was one denaturing cycle at 95°C for 5 min followed by 35 cycles at 95°C for 15 s and 58°C for 45 s, and the melting curve was specified at 95°C for 15 s, 60°C for 1 min and 95°C for 15 s to obtain a specific melting temperature of each amplicon. The real-time PCR was performed on the QuantStudio Flex Real-Time PCR System (Thermo Fisher) with a positive (reference *T. evansi* DNA 10 ng/ μL) and negative control (nuclease-free water) included during the DNA extraction as well as the reaction.

Statistical Analysis. Statistical analysis was performed using Microsoft Excel. Test results were analysed to obtain the sensitivity, the positive predictive value (PPV) and the negative predictive value (NPV) along with their respective 95% confidence intervals (95% CI), with real-time PCR considered the gold standard. The test results were compared for statistical significance using McNemar's chi-squared test. Cohen's kappa index (κ) and its 95% CI were calculated to evaluate the agreement between microscopy, CATT, and real-time PCR (2).

Table 1. Prevalence of infection, sensitivity, positive predictive value (PPV), negative predictive value (NPV) and Cohen's kappa index (Kappa) (real-time PCR was taken as the reference standard)

Method	Overall prevalence ^a	Sensitivity ^b	PPV ^b	NPV ^b	Kappa ^c
Microscopy	14/77 (18%)	30.4 (17.1–43.7)	100 (100)	49.2 (38.0–60.4)	0.26 (0.16–0.36)
CATT	17/77 (22%)	37.0 (23.0–50.9)	100 (100)	51.6 (40.5–62.8)	0.09 (0.02–0.15)
Real-time PCR	46/77 (60%)				

^a – Number of positive samples followed by infection prevalence (in %) in parentheses; ^b – Percentage, followed by the 95% confidence interval in parentheses; ^c – Kappa index followed by the 95% confidence interval in parentheses

Results

The overall prevalence of trypanosomosis in the 77 samples ascertained using different diagnostic tests and the sensitivity, PPV, NPV and κ are shown in Table 1. Cohen's kappa index showed moderate agreement of real-time PCR with microscopy, whereas the reaction method was in weak agreement with CATT.

Among the 17 and 14 positive results obtained from CATT and microscopy respectively, there were 12 common positive cases, demonstrating good agreement ($\kappa = 0.72$; 0.62–0.82) between the tests.

All the samples positive by microscopy were also positive by real-time PCR. The chain reaction yielded 46 positive results from 77 samples, whereas microscopy yielded 14 from the same number of samples. The approximate 95% CI for the true difference in the proportions detected by the two methods is 0.306 to 0.526. The real-time PCR detected 60% (46/77) of samples as being infected and microscopy detected 18% (14/77), a sizable difference of 42% emerging between the two tests in favour of real-time PCR (Table 2).

Table 2. Comparison of microscopy and real-time PCR using McNemar's chi-squared test

Real-time PCR	Microscopy		Total
	Positive	Negative	
Positive	14	32	46
Negative	0	31	31
Total	14	63	77

P value < 0.00001 at 0.05 significance level (degrees of freedom = 1)

Table 3. Comparison of CATT and real-time PCR using McNemar's chi-squared test

Real-time PCR	CATT		Total
	Positive	Negative	
Positive	12	34	46
Negative	5	26	31
Total	17	60	77

P value < 0.00001 at 0.05 significance level (degrees of freedom = 1)

Similarly, the approximate 95% CI for the true difference in the proportions detected by real-time PCR and CATT is 0.242 to 0.512. Here also, real-time PCR detected 60% (46/77) of samples as harbouring the parasite, whereas CATT detected 22% (17/77), a significant disparity of 38% appearing between the two tests in favour of real-time PCR (Table 3). Real-time PCR was thus found to be more sensitive than microscopy and CATT by a significant difference ($X^2 = 30.03$, degrees

of freedom (df) = 1, $P = 0.05$, $X^2 = 20.1$, df = 1, $P = 0.05$). Five samples positive by CATT were found to be negative by real-time PCR (Table 3).

Discussion

Molecular methods provide higher detection rates than parasitological methods in detecting trypanosomosis (16). Due to the chronicity of the disease, often a low number of parasites will be circulating in blood which makes microscopy a less sensitive method in the diagnosis of trypanosomosis. In camels, trypanosomosis can be acute, chronic and in healthy carriers subclinical; the chronic infection is known as Surra (rotten) or Tibersa (three years disease) (9). Parasitological diagnosis by microscopic examination has poor sensitivity and, in most situations, *Trypanosoma evansi* is under-diagnosed and the level of infection is frequently higher than reported (12). Similar results were reported earlier in camels in Egypt with *Trypanosoma* detection of 20.24% by microscopy and 67.06% by PCR (12). A study on the prevalence of Surra in 1,005 dromedary camels from the Cholistan Desert, Pakistan, reported an overall prevalence of 0.7% in Giemsa-stained thin smears, 40.1% in a formol-gel test, 47.7% in a CATT/*T. evansi*, 44.2% in an ELISA/variant surface glycoprotein (VSG) RoTat 1.2, 39.9% in immune trypanolysis, 31.9% in a *Trypanosoma brucei* primers 1 and 2 (TBR1/2) PCR and 30.5% in a RoTat 1.2 PCR (25).

The parasitological prevalence assessed by Giemsa-stained thin smear was much lower even in the present study (18%) than the prevalence found by the molecular method (60%), suggesting the high sensitivity of the latter. The use of buffy coat samples which concentrated the *Trypanosoma* for molecular testing in the present study might have increased the probability of molecular detection over that of detection by examination of direct blood smears. The modest level of agreement of microscopy with real-time PCR in the present study ($\kappa = 0.26$) notwithstanding, the very high ratio of real-time PCR detections to blood smear detections suggests the higher sensitivity of the test to detect the low parasitaemia levels typical during the initial incubation period or the chronic stage of infection. In the present study, 12 samples positive by microscopy were positive by CATT and real-time PCR, flagging active infections detected by all the three tests, whereas the five samples positive by CATT but negative by PCR

might signify recently recovered infections where the immune response is active.

Diall *et al.* (10) and Gutierrez *et al.* (15) reported that the sensitivity of the CATT test varied from 86% to 100% when compared to parasitological methods such as Giemsa-stained blood smears and the micro-haematocrit centrifugation technique. The expression of VSG RoTat 1.2 early during infections results in the detectability of anti-RoTat 1.2 antibodies in the early phase of infection, but CATT cannot distinguish current from recovered infections. Abdel-Rady (1) reported detection of anti-trypanosomal antibodies with CATT in 43% of camels, whereas only 4.1% were positive by microscopy. The lower positivity rate of 17% returned by CATT in the present study and the poor agreement with real-time PCR ($\kappa = 0.09$) might be due to the animals having been in the incubation period or having had recently-acquired infections (4, 8) and having been sampled prior to the appearance of RoTat 1.2-specific antibodies. Although CATT/*T. evansi* cannot distinguish between present and past infection, it can be effectively used in correlation with the clinical signs and symptoms of the disease in animals. Being a pen-side test, it has an advantage over conventional parasitological techniques such as microscopy and it is cost-effective, simple and rapid when compared to a molecular diagnosis-based PCR assay which requires a sophisticated laboratory.

Singla *et al.* (24) analysed 264 blood and sera samples from cattle of Punjab, India and revealed respective 1.89%, 34.47% and 51.89% positivity for *T. evansi* by Giemsa-stained blood smear examination, CATT/*T. evansi* and PCR, which is comparable with the present study results. A systematic review and meta-analysis of the published studies on the distribution, host ranges and prevalence of *T. evansi* infection in a subset of 165 publications indicated that the prevalence of *T. evansi* in domestic animals ranged from 14–31%, 6–28% and 2–9% using antibody detection, molecular tests, and parasitological tests, respectively, with camels as the most affected, followed by buffalo and cattle (3). A cross-sectional study conducted in three districts of Kenya to estimate the prevalence of *T. evansi* compared four diagnostic tests: micro-haematocrit centrifugation technique (MHCT), PCR, CATT/*T. evansi*, and mouse inoculation (MI) and reported the overall prevalence of Surra as 5.3% using MHCT, 26.6% using PCR and 45.9% using CATT/*T. evansi*. There was a significant difference ($P < 0.001$) between PCR and the CATT/*T. evansi* test, MHCT and MI in detection of *T. evansi* and an establishment of PCR as more sensitive than other tests (21). Salim *et al.* (22) reported the absence of the VSG RoTat gene in 13 out of 30 *T. evansi* internal transcribed spacer 1 PCR-positive camel samples. This has an implication for the direct CATT/*T. evansi* based on the predominant variable antigen type VSG RoTat 1.2 gene, because a considerable number of positive cases will be missed and the sensitivity of CATT will be low. Singh *et al.* (23) reported PCR as a 100% sensitive test for diagnosis of *T. evansi* infection in camels and

approximately four times as sensitive as parasitological and serological methods.

PCR is more sensitive and able to detect latent infections, is considered superior to parasite and antigen detection tests and provides greater accuracy than conventional methods (11, 12, 19). Mirshekar *et al.* (20) reported a high prevalence of 31.35% (116/370) of *T. evansi* infection in Iranian dromedary camels applying molecular methods by amplifying a fragment of the mini-chromosome satellite DNA of *T. evansi* using TBR1/2 primers. In the present study of 77 samples, the real-time PCR yield of 46 positives set against the respective 14 and 17 from microscopy and CATT and the significant differences in the results of McNemar's chi-squared test for symmetry demonstrate that real-time PCR is the most sensitive diagnostic technique among the trio of chain reaction, serological antibody-based test and microscopy. Molecular methods can detect the parasite at an early stage of infection, in low concentrations in subclinical infections and even in animals in the healthy carrier state. Since a large proportion of infections (50–80%) in the field are chronic and do not develop detectable levels of parasitaemia, diagnosis of trypanosomosis is often challenging using the lower sensitivity of conventional methods (3, 18). The choice of tests to be used for diagnosis depends on their characteristics, and since no method is 100% sensitive and 100% specific, it is important to include sensitive molecular techniques in the testing algorithm for accurate diagnosis of trypanosomosis in dromedary camels.

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Animal Rights Statement: Blood samples used for study were routine samples brought for disease diagnosis from naturally infected camels and their owners' consent was obtained before the study.

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