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REGULAR ARTICLE

Epidemiological surveys of camel trypanosomosis in Al-jouf, Saudi Arabia based on PCR and ELISA

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ABSTRACT

Trypanosomosis due to *Trypanosoma evansi* (surra) is a major enzootic disease of the dromedary camel. The present study was conducted to determine the prevalence of camel trypanosomosis in the northern part of Saudi Arabia with different methods of diagnosis (ELISA, PCR) and to compare the results to whose obtained previously with Card Agglutination Test for Trypanosomiasis (CATT/T.*evansi*). A total of 195 blood samples and 118 serum samples were used for molecular and serological investigation respectively. After analyses, 25% (49/195) and 3% (4/118) samples were positive using PCR and ELISA respectively. The variability of trypanosomosis was highly significant to the factor moving, location, breed and clinical signs with PCR. The discrepancy between PCR, CATT test and ELISA is likely due to antibodies degradation on spotted papers maintained several weeks at ambient temperature. This is the first molecular diagnosis report which gives a picture of camel trypanosomosis in Al-jouf, Saudi Arabia.

Keywords: *Trypanosoma evansi*; Camels; PCR; ELISA; Risk factors; Al-jouf

INTRODUCTION

Trypanosomosis in camel, due to *Trypanosoma evansi* is probably the main enzootic disease for this species widely present in all countries where camels are reared (Eyob and Matios, 2013). In Saudi Arabia, the prevalence was assessed to be 5.5% using direct observation of the parasites in blood but it is known that sensitivity of direct parasitological observation is very low. With passive hemagglutination test and with Ag-ELISA (Antigene Enzyme Linked Immuno-Sorbent Assay) the prevalence was 19.7 and 13.8% respectively (Omer et al., 1998), but those tests are not the one recommended for *T. evansi* diagnostics by the World Organization for Animal Health (OIE, 2012). The use of a combination of convenient diagnosis tools is of high importance to get a clear epidemiological status of the camel flock in a given country (Desquesnes et al., 2009b), and thus propose efficient control methods.

In a previous survey achieved in Al-Jouf area, in the northern part of Saudi Arabia (El-Wathig and Faye, 2013), a prevalence of 43.8% was reported by using CATT/*T. evansi*® CATT test (Card Agglutination Test for Trypanosomiasis) (Bajyana-Songa and Hamers, 1988). The sensitivity and specificity of CATT/*T.evansi* were respectively estimated around 72-85% and 84–100% (Dia, 1997; Delafosse and Doutoum, 2000; Pathak et al., 1997; Luckins et al., 1999). In human African trypanosomosis (sleeping sickness), Lejon et al (2010) reported that CATT/*T. gambiense* sensitivity decreases between primary cases and retreatment cases. The objective of the present study was thus to assess the infectious status of the camels sampled by return back on this previous survey by using other diagnosis tools for estimating prevalence of trypanosomosis. We chose to use PCR-TBR and ELISA/*T. evansi* as recommended by OIE (2012) and Pruvot (2010). Moreover, analysis of risk factors associated to apparent prevalence was performed.

MATERIAL AND METHODS

Samples procedure

According to the availability of the camel herds as well as willingness of the owner, non-probability sampling method (convenience sampling) was employed in the study site as described by Thrusfield (1996). As the whole, 194 sera were collected in 25 farms. In each farm, animals presenting symptoms potentially linked to trypanosomosis were sampled, and for each sick animal, one healthy camel with

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similar characteristic (breed, sex and age) was sampled in the same time. Blood samples with EDTA were collected during February to October 2011 from both clinically healthy and suspected camels with trypanosomosis and kept at -20°C. Sera were separated from buffy coat and red cells after centrifugation at 5000 x g for 15 min. Due to limited budget, ELISA test was applied on 118 samples only selected randomly among 194.

Survey in camel farms

Each sample was described by the date of sampling, the location (different location in Al-jouf), the age status (young male, adult male, lactating female, non-lactating female) and the breed of the animal (Hamrah, Malha, Safrah and Wadha), the moving status (urban moving, desert moving, urban indoor and desert in-door), the clinical signs at sampling time (healthy, emaciation, general clinical signs) and treatment (not treated, treated with antibiotics, treated with antiparasitic, treated with anti-trypanosomes).

Laboratory analysis

The analysis regarding the CATT test was achieved in Al-Jouf, Camel and Range Research Center according procedures described in El-Wathig and Faye, (2013). All analyses regarding PCR and ELISA-test were achieved at the laboratory of UMR INTERTRYP at CIRAD Montpellier, (France) since the necessary equipment were not available in Al-Jouf. For that, samples (whole blood and smear) were spotted on 4M Whatman paper, dried, maintained at ambient temperature and used 12 weeks after spotting for ELISA and PCR.

Protocol for ELISA/*T.evansi*

One hundred eighteen sera samples were analysed. Spotted sera were eluted using 200µl of sterilized water.

T. evansi procedure for ELISA test is derived from a previously described technique (Desquesnes et al., 2009). Briefly, Microtest 96-wells Polysorp Nunc1 immunoplates (Nunc, Roskilde, Denmark) were coated with 100 µl/well of *T. evansi* soluble antigen at 5 mg/ml protein concentration in carbonate buffer (pH 9.6) and incubated overnight at 4° C. The antigen solution was discarded and the plates were blocked with 150 µl/well of blocking buffer, PBS–5% skim milk (powder (ref: 190-12865 -0.1% Tween 20 (ref: P1379, Sigma Aldrich), Wako Pure Chemical Industries Ltd., Osaka, Japan) with permanent shaking (300 rpm) for 30 min at 37° C. The blocking buffer was discarded. Sera diluted 1:100 in blocking buffer were transferred in duplicate on the ELISA plate. After 30 min in a shaker-incubator at 37° C, 300 rpm, the plates were washed five times with PBS 0.1% Tween 20 (Sigma–Aldrich) (washing buffer, WB). Then 100 µl of conjugated anti-protein A (A5295, Sigma–Aldrich), diluted 1:10,000 in blocking buffer, was added and the plates incubated for 30 min at 37° C with permanent shaking (300 rpm). After washing five times with washing buffer, 100 µl of the complex substrate/chromogen K blue substrate(Neogen Europe Ltd., Scotland, UK) was added. The plates were incubated in a dark room for 30 min. Optical density (OD) was measured at 620 nm in an ELISA reader (Perkin Elmer-Wallac Victor11420 Multilabel counter, Perkin Elmer, Waltham, MA, USA). A sample was called positive if it presented a relative percent of positivity (RPP) larger than a cut-off value of 30%, according to Desquesnes et al., (2009a).

DNA Extraction

195 samples were used for PCR diagnostic, in the laboratory, each blood impregnated Whatman paper was placed in 1.5ml tube and 1ml of 5% chelex-100 solution was added. The tubes then were heated at 56°C for one hour and 30 minutes at 95°C. After centrifugation at 14,000rpm for 3 minutes, the supernatants containing the DNA were diluted 1/3, 1/10, and 1/50 before to be used as template for PCR (Biotechiques, 1991. No 4, P: 506).

PCR analysis

Five microliters of three different dilution of chelex supernatant were added to the 45 µl of master mix. PCR with specific primers for Trypanozoon TBR1 and TBR2 according to the technique described by Moser et al., (1989) were conducted.

Briefly TBR1/2 primers were used to amplify a 173 bp highly repeated sequence of mini chromosome satellite DNA, it was so far the gold standard since it was the first primer set and largely used for detection of Trypanozoon DNA. Thermocycling profile started with initial hold for 3 minutes at 95° C, followed by 40 cycles at 95° C for 30 sec, 55° C for 30 sec and 72° C for 1 minutes and final extension step of 5 minutes at 72° C. PCR products were migrated 45 minutes at 100 V in 2% agarose gels, together with Marker Phi X 174/Hae III. (Eurogentec, Seraing, Belgium), stained with ethidiumbromide and visualized under UV light.

Statistical analysis

The relationships between the diagnosis tests were assessed by chi² test on cross table CATT*ELISA, CATT*PCR and ELISA*PCR by using the procedure <<contingency table>> in XLstat, 2013 (Addinsoft ©)

The relationships between positive PCR in one hand, and positive ELISA in another hand with the variation factors (CATT test, moving, location, breed, season, pre-treatment, lactation and clinical signs) were explored by Multivariate analysis. Multiple component analysis with result of test

(ELISA or PCR) in supplementary variable- followed by classification using hierarchical Ascending classification were achieved by using MCA and HAC procedure in XLstat, 2013.

RESULTS

Among the 194 sera, 103 were negatives to CATT test and 91 positives.

Relationships between tests used

Among the camels negative to CATT test, 23.3 % (25/103) were positive with PCR whereas among camels positive to CATT test, 26.3 % (24/91) were positive with PCR. This difference was not significant. Regarding ELISA test, similar trait was observed but with lower positive prevalence: 3% of the sera were positive among CATT negative (3/99) *vs* 5.2% among positives (1/19). The proportion of positive ELISA was similar among positive PCR (5.3%) and negative PCR (5.2%).

Relationships with farm data

Association between apparent prevalence and herd factors was tested only for PCR results, as ELISA results are doubtful in this study (see discussion part).

To describe the relationships between all farm's factors, an automatic classification was performed. Regarding to classification it appeared that, the PCR is highly significant to the factor moving, location, breed and clinical signs (Fig. 1).

The significant farm factors were Desert moving chi^2=5.991 (P<0.009), Wadha breed (with white coat) chi^2=7.815 (P<0.017), clinical signs (emaciation and weakness) chi^2=5.991 (P<0.007) and location (Sakaka, Gara and Moghera) chi^2=15.507 (P<0.001).

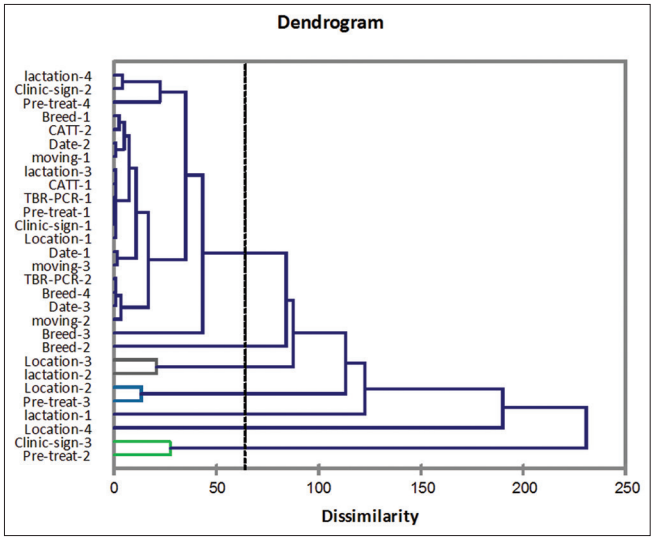

Fig 1. Dendrogram issued from automatic hierarchical classification for PCR.

DISCUSSION

The present study was designed to compare the PCR using TBR1/TBR2 primer set specific for subgenus *Trypanozoon* and ELISA/*T.evansi* with CATT test. There are several lines of evidence of high sensitivity of PCR in diagnosis of trypanosome infection. Njiru et al (2004) found that the PCR was more sensitive than CATT/T. *evansi* in Kenyan's Camel. In other study, Nahla et al (2011) recommended that the PCR technique should be adopted as a routine method for diagnosis of *T.evansi* infection in camels. In any case, because PCR targets an active infection with circulating parasites, whereas CATT and ELISA test target antibodies (IgM and IgG respectively), these diagnostic tests are complementary and are likely to provide different results.

The apparent relationships between the methods used were weak. In our study, negative PCR observed in case of positive CATT-test may be due to the fact that infected animals harboring antibodies may exhibit parasitaemia below the PCR level (from 1 to 20 trypanosomes/ml) as stated by Desquesnes., et al (2009a) and Pruvot (2010). The same authors reported that the persistence of antibodies remained for 4-5 months after elimination of parasite in treated or naturally cured animals. Conversely, 14% of CATT negative camels were positive in PCR that is in line with the estimated CATT sensitivity (around 80%, Dia, 1997; Delafosse and Doutoum, 2000; Pathak et al., 1997; Luckins et al., 1999) and the fact that positive CATT test requires the triggering of agglutinating antibodies.

The Ab-ELISA method for diagnosing animal trypanosomes has been used for long time (Gray and Luckins, 1977; Zweygarth et al., 1986). In our study, we found a surprisingly low rate of Ab-ELISA positive tests, in comparison to CATT and PCR results. This discrepancy is likely due to a failure for this specific experiment to detect circulating IgG, since this test gave previously robust results (Desquesnes et al., 2009a, Desquesnes et al., 2009b, OIE, 2012). In this experiment, sera were frozen, thawed, then spotted and let 2 to 12 weeks on the spot before performing the ELISA. Studying stability of antibodies directed against malaria antigens, Corran (2010) demonstrated a decrease in antibodies titers when spots were kept at ambient temperatures for several weeks, which is the case in this analysis. Moreover, tests performed in our laboratory showed that ELISA optic densities decreased after 2 weeks of storage at ambient temperature (data not shown). It must be also noticed that camelids have a specific structure in

antibodies (Hamers-Casterman et al., 1993), in comparison with humans or livestock species, and this could contribute to their quick degradation. In conclusion, the fact that sera remained on Whatman paper several weeks before ELISA likely resulted in immunoglobulin degradation, damaging the performance of the ELISA.

According to the farm factors, moving, breed and location were correlated with a higher prevalence assessed by PCR. Indeed, herd movements under nomadic system lead to higher risk of being exposed to *T. evansi* compared to camels in ranching system (Ngaira et al 2002). Delafosse and Doutoum (2004) reported that, the prevalence was higher in transhumant herd compared to settled herds. Njiru et al., (2002) reported a double prevalence of trypanosomosis in ranch compared to traditional systems.

The effect of breed is not clear, because Saudi camel breeds are differentiated by coat colors (Abdallah and Faye, 2012). It is widely admitted that trypanosome vectors as *Glossina* sp. have preference for some colors, but the preference of tabanides for some colors is not clear (Mekuria and Gadissa, 2011). In our preliminary survey, it was observed a decreasing prevalence from breed with white coat (49%) to breed with dark color as Malha (40%) or Safra (30%) (El-Wathig and Faye, 2013). The higher prevalence for white-color camel (Waddah breed) was confirmed on PCR-positive camels (3 times higher than for other breeds on average). However, a recent publication (Almathen *et al*., 2012) reported that the different phenotypes based on the coat color did not differ regarding their genotypes. In addition, Pathak and Khanna (1995) reported that all camels were equally susceptible to trypanosome infection regardless of breed and age.

The pattern of prevalence of *T. evansi* differed according to different location due to the ecology of study area which has a direct effect on the distribution of biting flies responsible for mechanical transmission of *T. evansi* (Schillinger and Rottcher, 1986).

The factor "clinical signs" was linked to high prevalence only with PCR, these signs were clearly the symptoms of trypanosomosis (emaciation and weakness). Swai et al. (2011) stated that a higher infection might be due to lower body resistance because nutritional stress or other infection, and therefore rendering them more susceptible to *T. evansi* infection.

CONCLUSION

The present study showed that, the trypanosomosis is prevalent in some locality in Al-Jouf at significant level. Farm factors as breed, clinical sings and movement pose a great risk of *surra* to camel breeding. More longitudinal study would be useful to better understand incidence dynamic according to herd's factors and biting insects' populations.

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Author contributions

M.E.W: field research and writing; B.F.: supervision and writing; S.T, S.R. and G.B.: lab analysis

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