Journal of Animal and Veterinary Advances 12 (7): 807-812, 2013
ISSN: 1680-5593

Early Tool for Prediction of *Trypanosoma evansi* Infections Related to Seroprotein Changes of Dromedary in Tunisia

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Abstract: Trypanosomiasis is a haemoprotozoan disease which affects both human and all species of domesticated mammal, especially *Trypanosoma evansi* in dromedaries. In the present study, early tool for prediction of *Trypanosoma evansi* infections related to seroprotein changes of dromedary in Tunisia was studied. Animals were distributed into 3 groups on the basis of their status related to trypanosomiasis by indirect immunoflurorescence-test: Healthy animals with negative serology for *T. evansi* infection, seropositive animals without clinical signs (apparently healthy) and seropositive diseased animals. Quantifiable changes in the electropherogram were apparent in dromedaries infected by trypanosomiasis with a significant hyperprotidemia and an increase of the gamma globulins fraction in seropositive animals with or without clinical signs ($p<0.05$). Moreover, seropositive camels that are not suffering from majors zoonotic bacterial diseases can cause immunoglobulin secretion. The results from this study showed that there was specific pattern in serum protein and electrophorerogram that could be used to distinguish infected with or without symptoms of *T. evansi* infection from uninfected animals. It appears that serum protein electrophoresis can be used by clinicians to predict trypanosomiasis with great ease in outbreak of *T. evansi* infection in combination with serological, clinical and laboratory findings of this disease.

Key words: Dromedary, trypanosomiasis, electrophoresis, indirect immunoflurorescence, Tunisia

INTRODUCTION

Dromedaries thrive in arid zones of many countries in the world and provide food, hides and transport. Trypanosomiasis is a serious threat to human and animal health in most African countries. Indeed, Trypanosomiasis is a serious disease from both economic and animal health because it involves substantial losses by death and decreases animal production. Trypanosomiasis in camels may occur in both acute and subclinical or carriers forms. Infection with *T. evansi* can be difficult to detect because parasites are not always detectable in the blood (Bajyana and Songa, 1992; Dia *et al*., 1997; OIE, 2010). A number of methods have been developed to increase the sensitivity of parasitological tests (microscopic examination of blood using wet or dry preparations, haematocrit centrifugation test and inoculation in laboratory mice). Serological tests to detect antibody or antigen have been developed because parasitological tests have poor sensitivity (Dia *et al*., 1997; OIE, 2010). Some of these tests are expensive and cumbersome to perform which is important as the disease is found mainly in poor countries with limited facilities (Chaudhary and Igbal, 2000). Nucleic acid based tests are generally sensitive, although they are not always reliable. False negatives or positives, DNAse or other inhibition can limit routinely the use of molecular tests in addition to their high cost (OIE, 2010). The effective control and treatment of parasitic diseases require rapid, reliable and sensitive diagnostic tests. Serum protein electrophoresis is a simple, reliable and rapid method of separating different protein fractions. Protein electrophoresis can be performed by using commercially available kit which allows good performance (Kaneko *et al*., 1997; Karen, 2003). It is relatively inexpensive, requires only microliters of serum and provides results within a few hours. In addition, multiple samples can be analyzed simultaneously and overall

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changes in the different protein sera fractions can be quantified. Although, this technique provides very useful information about the quantitative alterations of the serum protein fractions related to the disease of other animal, it is not commonly used in dromedary medicine.

The aim of this study was to evaluate a change of the serum electrophoretic pattern and electropherogram by electrophoresis to improve detection and investigation of primaries immune responses of animals infected by *T. evansi* with clinical symptoms and subclinical infection of dromedary without sings for disease. Also, to confirm *T. evansi* infection related to change in seroprotein, a dromedary immunological statute has been analyzed for proving that animals are free of tuberculosis and brucellosis, described amongst major zoonotic bacterial diseases for a dromedary in Tunisia (OIE, 2010).

MATERIALS AND METHODS

Animals: One hundred and twenty six male camels from semi-extensive breeding in Southern Tunisia were tested. Marked with numbered collars, these animals are aged between 4 and 7 years. The animals are well maintained by the staff of the regional tourism office, kept isolated from other breeding groups in the region. The animals are regularly treated with classic internal and external antiparasitic drug. All animals have undergone clinical examination.

Blood samples: For each animal, blood samples were taken by puncture of the jugular vein. Blood is collected in dry tubes (type vacutainer) and sera were then separated by centrifuging at 3000 rpm for 10 min within 2 h after collection. These samples were then divided into two aliquots of 2 mL and stored at -20°C until analysis within no more than a month.

Analytical methods: Indirect immunofluorescence test. The indirect Immunofluorescence test (IFI) was performed by the technique adopted by Katende *et al.* (1987). The conjugate and Evans blue were used at the dilution of 1/100. The antigens consist of freeze-dried trypanosomes parasite diluted at 1/4 as recommended by the ILRAD in Nairobi (Katende *et al.*, 1987). The reading was made using an immunofluorescence microscope Leitz type. The positivity threshold was set at 1/80.

Enzyme Linked Immunosorbent Assay (ELISA) test: For measurement of serum IgG antibodies by in house ELISA for tuberculosis caused by *Mycobacterium bovis*: Ninety six well polystyrene microtiter plates (Nunc) were coated overnight at 4°C with 100 mL of antigen solution in 0.05 M carbonate buffer (pH 9.6) in the following same concentrations of the two antigen Purified Protein Derivative (PPD) and Early Secreted Antigen Target 6 kDa (ESAT-6) either 0.50 μg per well. The plates were subsequently washed 3 times with Phosphate Buffer Saline (PBS) pH 7.20 plus 0.05% Tween 20 and were blocked overnight at 4°C with carbonate buffer plus 2% bovine serum albumin (pH 9.6). The serum samples were diluted to 1:100 in PBS plus 1% Bovine Serum Albumin (BSA) and 0.20% Tween were added to the wells and were incubated for 1 h at room temperature. After another wash with PBS-Tween, the plates were incubated for 1 h at room temperature with Horseradish peroxidase-conjugated mouse anti-human IgG monoclonal antibodies (BD Pharmingen; catalogue No. 555788) at a 1:1000 dilution. Enzyme activity was assayed by incubation for 30 min at room temperature with 100 mL of TMB plus substrate per well. To stop the reaction, 100 mL of 0.20 M sulphuric acid was added and the Optical Density (OD) was measured at 450 nm and at 620 nm for background subtraction. Sera were considered reactive when OD results were greater than the cut-off value. Positive and negative sera controls were also tested. Negative sera had OD values of <0.06 (mean = 0.04) whereas positives had mean OD values of 0.151.

For serological diagnosis of dromedary Brucellosis by Indirect ELISA Method, indirect-ELISA test kit (IDEXX-laboratories, France) has been involved in the study. Lipopolysaccharide-coated wells of *Brucella abortus* were used. ELISA test was performed according to procedure of manufacturer.

Serum total protein electrophoresis: The serum total proteins were assayed by the colorimetric test, the Biuret reaction (Karen, 2003). Researchers have used Biomaghreb kit. Measurements of absorbance were performed using a spectrophotometer UV-Visible (Schimadzu). The serum protein electrophoresis was performed on a base of cellulose acetate gel at pH 8.60 in veronal buffer and supplied by a generator (Helena) at a voltage of 175 volts for 13 min. After Ponceau staining, the electropherograms were quantified using a Helena Process u-24, at 520 nm by hydrometer.

Statistical methods: All results were expressed as mean±standard deviation. Statistical calculations were performed using the software stat view. Significances of differences were analyzed by Mann-Whitney and t-tests. A value of $p<0.05$ was considered as statistically significant.

RESULTS AND DISCUSSION

The Indirect Immunofluorescence test (IFI) has allowed many informations; firstly, confirming the clinical diagnosis for ill camels by *T. evansi* infection

(seropositive). Secondly, the techniques allowed to distinguish between healthy animals (seronegative) and subjects apparently healthy (seropositive) (Table 1). Serological analysis by IFI allowed us to divide them into three different groups. The first group is healthy (45 camels seronegative). The second group contains 55 seropositive, apparently healthy camels. The final group contains 26 seropositive, clinically ill camels.

Most cases develop recurrent episodes of fever. Infected animals show progressive anemia, marked depression, dullness and loss of condition. Some camels develop oedema in their dependent parts of the body. However, no clinical signs were observed to either apparently healthy animals but seropositive by IFI or healthy dromedary (seronegative). Also, none of these seropositive animals to trypanosomiasis by IFI was detected seropositive by ELISA test whether for tuberculosis or brucellosis, amongst major's bacterial zoonotic diseases for dromedary in Tunisia.

A total of 5 protein fractions were obtained in dromedary: albumin and α1, α2, β and γ globulins (Fig. 1). The Albumin/Globulin ratio in average was about one in healthy dromedary (Table 1). However, this ratio decreased significantly in seropositive with or without symptoms of trypanosomiasis in dromedary (Table 2). The electropherogram of serum proteins of dromedary infected with *T. evansi* (Fig. 2) differs easily from that of healthy animals (seronegative, Fig. 1). In fact, the electropherogram is characterized by an increase in the area of gamma globulins for animals with a positive serology in both dromedaries with or without clinical signs of trypanosomiasis (Fig. 2). In addition to the statistical analysis indicated in Table 2, significant differences ($p<0.05$) for two parameters were studied

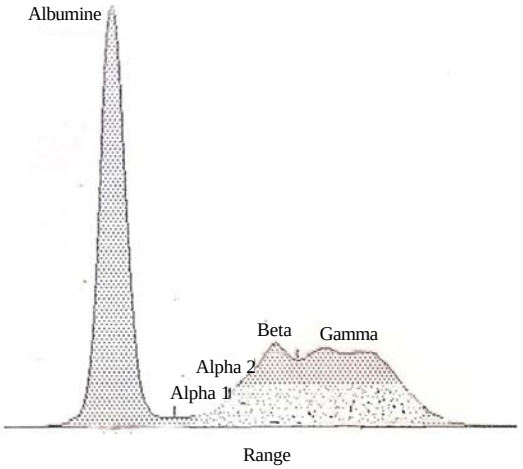

Fig. 1: Electropherogram of normal serum proteins in healthy camels on cellulose acetate

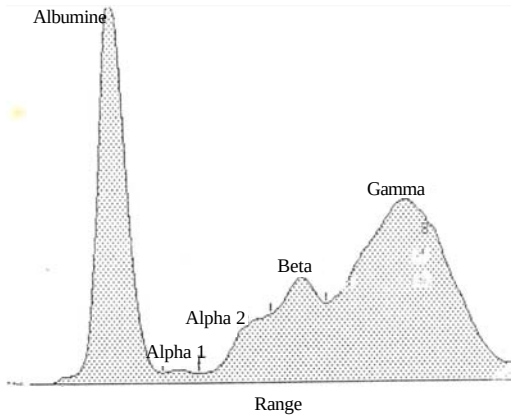

Fig. 2: Electropherogram of serum proteins in camels suffering from trypanosomiasis to *Tyrpanosoma evansi* with or without clinical signs

Table 1: Serum total proteins concentrations (g/L), their fractions (g/L) and Albumin/Globulin ratio (Alb/Glob) from the healthy dromedary and change of these parameters in trypanosomiasis to *Trypanosoma evansi* (Mean±SD)

Dromedary	Total protein	Albumin	Alpha 1 globulin	Alpha 2 globulin	Beta globulin	Gamma globulin	Alb/Glob
Seronegative1*							
Healthy (n = 45)	61.1±9.00	30.8±4.6	1.8±0.7	3.0±0.9	8.4±2.50	17.1±3.9	1.1±0.2
Seropositive[1]*							
Without symptoms (n = 55)	104.6±18.2	48.1±9.9	3.0±1.2	5.2±1.8	13.6±4.60	34.6±8.4	0.9±0.2
With symptoms (n = 26)	103.2±18.7	40.0±9.0	2.4±1.4	5.0±1.5	12.8 ±4.9	43.1±8.4	0.7±0.2

[1]Serological status of camels is obtained by the indirect immunofluorescence test. *Results between dromedary seronegative and seropositive are significantly difference (t-test, $p<0.05$)

Table 2: Percentage of the serum total proteins fractions in seronegative (Healthy) dromedary and seropositive group animals (App.: Apparently or ill) infected by *T. evansi*

Dromedary	Albumin	Alpha 1 globulin	Alpha 2 globulin	Beta globulin	Gamma globulin
Seronegative*					
Healthy (n = 45)	50.4±7.5[a]	2.9±1.1[a]	4.9±1.4[a]	13.7±4.1[a]	27.9±6.4[a]
Seropositive*					
Without symptoms (App. Healthy, n = 55)	45.9±9.4[a]	2.8±1.1[b]	4.9±1.7[b]	13.0±4.4[b]	33.1±8.0[a]
with symptoms (Ill, n = 26)	38.7±8.7[a]	2.3±1.3[c]	4.8±1.4[c]	12.4±4.7[c]	41.7±8.1[a]

For each parameter, values indicated with the same superscript letters are significantly different (Mann-Whitney test, $p<0.05$). *Serological status of camels is obtained by the indirect immunofluorescence test

(serum total protein and gamma globulin) between healthy and infected animals by *T. evansi* with or without symptoms.

Trypanosomiasis is an immunological disease that affects most of the body's organs with more severe disease developing over time. Several serological tests are used to confirm the disease and to detect infected animals such as the ELISA and the Card Agglutination Test for Trypanosomosis (CATT) (Bajyana and Songa, 1992; OIE, 2010). In this study, the immunoflourescence indirect test was chosen because it remains the most sensitive tests for the diagnosis of trypanosomiasis camelina by *T. evansi* (OIE, 2010). In the study, all seropositive animals have showed a specific polyclonal antibody increase. In fact, no antibody secretion was detected against ESAT-6 specific antigen for virulent strains of *Mycobacterium tuberculosis* or anti *Brucella abortus* responsible for a tuberculosis or brucellosis respectively, among major bacterial zoonotic described in camels (OIE, 2010; Wernery *et al*., 2007; Lyashchenko *et al*., 2011). This result contradicts with the hypothesis of the existence of other concomitant diseases that can cause a hypergamma gobulinemiae.

Advent of serum protein electrophoresis has greatly enhanced diagnosis accuracy in different disease processes (Karen, 2003). It appears that in the South of Tunisia, the results show the average concentration of serum total protein in healthy male camels aged between 4 and 7 years was 61.1 g L^{-1}. The normal electropherogram in dromedary is characterized by the existence of five fractions as shown in Fig. 1. The average relative percentage for each fraction was as follows: 50% for albumin, 3% for alpha, 5% for alpha 2, 13% for beta and 28% for gamma globulins (Table 2). The electropherogram of the dromedary and its distribution (Fig. 1) were similar to those of a dog, goat and man. However, they present differences from those of cattle and horses (Katende *et al*., 1987; Bourdoiseau *et al*., 1997; Dia *et al*., 1997; Crivellente *et al*., 2008; Alberghina *et al*., 2010). During the investigation, the results show the values of electrophoretic pattern and relative percentage are generally consistent with the literature under similar conditions such as age, physiological status, diet and season which can change the under dromedary herd (Bourdoiseau *et al*., 1997; Dia *et al*., 1997; Salman and Afzal, 2004; Al-Busadah, 2007).

In camels with positive serology for *T. evansi*, there was a hyperprotidemia whether with or without clinical signs of the disease. Only for albumin and gamma globulin studied, statistical analysis showed a significant change ($p<0.05$) in infected camels compared to seronegative healthy subjects (Table 2). During the investigation, the increase in serum total protein was detected before the onset of symptoms and thus may be considered as precocity in trypanosomiasis diagnosis. Earlier study showed that this hyperprotidemia is more pronounced with the deterioration of general status of the animal which is more important in the case of the most advanced states which are manifested by clinical symptoms (Boid *et al*., 1980a, b; Anosa, 1988; De La Rue *et al*., 1997; Chaudhary and Igbal, 2000). Instead, Sazmand *et al*. (2011) have advanced a hypoprotidemia in camels infected with *T. evansi*. This could be explained by the stage of disease progression in infected animals studied in their study. Indeed, in the chronic phase of the disease, liver and kidney alterations would result in a lower rate of serum proteins.

Moreover, the increase in the albumin concentration in absolute value may be explained by dehydration because no pathological processes associated to the production of albumin have been described (Kaneko *et al*., 1997; Piccione *et al*., 2011). But in relative percentage, in infected dromedary with clinical symptom, a significant decrease was observed. This may explain the oedema noted in trypanosomiasis by the decline in oncotic pressure related in decrease of the serum albumin.

The α-globulin fractions are known as acute stage proteins because their concentration increases immediately after an inflammation or a wound (Stockham and Scott, 2002; Carapeto *et al*., 2006). Although, this increase is due to tissue damage and inflammatory processes, elevation of the α-globulin fraction may be observed in diseases that are not linked to inflammation such as what occurs in cases of hepatic or renal damage (Kaneko *et al*., 1997; Carapeto *et al*., 2006). However, in relative percentage, any significant difference was observed between the different animal groups (Table 2).

The results of β-globulin sub-fractions in camel with trypanosomiosis were compared with those obtained in the apparently healthy group that did not reveal significant differences (Table 2). It is important to note that changes in this fraction depend on different factors such as the progress of the disease (acute or chronic) and the course or severity of the haemolytic anaemia. In addition an increase in its concentration related to acute hepatic damage has been described as a consequence of the increase in the transferrin concentration (Kaneko *et al*., 1997). In the interpretation of serum protein electrophoresis, most attention focuses on the gamma region which is composed predominantly of antibodies of the IgG type. In pathological conditions, there is some increase in serum globulin as a result of increased production especially in inflammatory

processes. An increased concentration of gamma globulins is usually associated with the increase production of antibodies following the infection. In this context, De La Rue *et al.* (2000) have observed a slight increase in lymphocytes number, probably due to hosts reaction against parasites. This could explain the origin of the increase in gamma globulins since it is secreted by plasmocyt. Thus, the increase in the gamma globulin concentration also contributes to the increase in the total proteins concentration in dromedary seropositive with or without symptoms, respectively ill or apparently healthy animals. This increase observed in the serum gamma globulin concentration (Fig. 2) is related to the humoral immunity activated by the parasitical infestation of the animals studied.

Although, the immune response in some animals may produce an increase of immunoglobulin which is too small to be detected through routine electrophoresis techniques (Stockham and Scott, 2002). These findings are in correlation with those of Boid *et al.* (1981) and their increased gamma globulin was reported in camels infected with *T. evansi*. Many factors can cause an increase in the gamma region. Several disease stages cause a spike-like peak in a focal region of the gamma-globulin zone (Fig. 2). An increase in gamma globulin during both acute and chronic *T. evansi* infections in camels has been reported (Boid *et al.*, 1981). However, this increase in gamma globulin is not specific to the infection with *T. evansi*, according to the report of the OIE (2010), based on several biochemical tests that can be applied to help diagnosis such as flocculation, formol-gel, mercuric chloride precipitation and thymol turbidity tests. These tests may still have some use in the field because they are simple to perform, rapid and not expensive.

Finally, an albumin/globulin ratio is lower than 1 with a statistically significant difference between different animal groups (Table 1 and 2). This may be due to the increase in the albumin concentration observed and sum of all globulin fractions. Using serum protein electrophoresis as a prediction for diagnostic tool of *T. evansi* infection for the dromedary is beneficial as it has been described previously for companion animals, other large animals, birds and exotic species (Kaneko *et al.*, 1997; Zaias and Cray, 2002; Ganheim *et al.*, 2007; Crivellente *et al.*, 2008). Serum protein pattern has been indicated in the pathogenesis of various parasitic infections of dogs and horses including leishmaniosis (De La Rue *et al.*, 2000; Lobetti *et al.*, 2000; Zaragoza *et al.*, 2000; Carapeto *et al.*, 2006). However, the electropherogram has not been reported previously in camels naturally infected with *T. evansi*. Characterization of the electropherogram in clinical context of dromedaries (*Camelus dromadarius*) may greatly benefit epidemiological studies.

Rarely and solely diagnosis of a particular disease, protein electrophoresis is an excellent method for the detection of acute and chronic inflammatory processes and stimulation of humoral immunity.

CONCLUSION

It is essential that the normal electrophoretic pattern has to be determined in adult dromedary because this reflects the response to changes in homeostasis or disease. This study provides values for serum protein fractions in dromedary, through the use of Cellulose Acetate Electrophoresis Method. The separation of serum protein fractions by electrophoresis revealed 5 protein fractions. The electropherogram of healthy dromedary is different to that of dromedary which has IFI test positive with or without symptoms of *T. evansi* infections. Therefore, during a trypanosomiasis, there was a marked increase in serum total protein with essentially a hyper gamma globulin reflecting a primary immune response during the disease. Thus, the determination of serum total proteins and their fractions, particularly gamma globulin, could be evolved into important diagnostic aid. Indeed, all of the results allow considering the application of serum protein electrophoresis as a tool that help for an early diagnosis in outbreak before the onset of symptoms and monitoring of trypanosomiasis camelina by *T. evansi* in combination with serological, clinical and laboratory findings of a disease.

ACKNOWLEDGEMENT

Researchers would like to thank the University of La Manouba for financial contribution.

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