New approach for diagnosis of *Trypanosomes evansi* in camel (*Camelus dromedaries*) by ELISA

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Abstract: In this study, Enzyme linked immune sorbent assay was used for the diagnosis of *Trypanosoma evansi* infections in camels in Abusinple region and Darwa city at Aswan governorate-Egypt in order to evaluate their ability to discriminate between infected and non-infected camels which exported from Sudan. ELISA was used for the detection of trypanosomal antibodies, one using prepared conjugated goat anti-camel IgG alkaline phosphatase and the second by using a non-specific Horseradish peroxidase- labeled staphylococcal Protein. The result demonstrated that, the application of alkaline phosphatase conjugated goat anti-camel IgG were 100 % positive for *trypanosome evansi* while the application of commercial protein-A conjugate were 69.2% positive for *trypanosome evansi* (This means, the application of goat anti- camel IgG gave 100% sensitivity.
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1. Introduction

Pathogenic trypanosomes cause disease in all species of domesticated livestock throughout many of the tropical and subtropical regions of the world. *Trypanosoma evansi* is a widely distributed disease of domesticated livestock throughout many of the tropical and subtropical regions of the world. In Asia, Africa, South America, and recently in Europe (Spain and France), affecting especially camels (camelids) **Luckins, (1988).** It is transmitted mechanically by several different species of haemotophagous biting flies, *Trypanosoma evansi* caused a trypanosomosis which also known as surra, The disease is the most important single cause of economic losses in camel rearing areas, causing morbidity of up to 30.0% and mortality around 3.0% **(Ngerenwa *et al.,* 1993; Egbe-Nwiyi and Chaudry, 1994; Pacholek *et al.,* 2001; Njiru *et al.,* 2002).** The disease is of great concern to many developing countries such as Sudan, where its large camel population estimated at over 4.6 million heads is at risk. Acute disease is characterized by anemia, weight loss, abortion and, if not treated, possibly death. Animals that survive are often infertile and of low productivity. In some instances, infected animals show no overt signs of disease but can succumb if stressed, for example, by work, pregnancy, milking or adverse environmental conditions **Luckins (1988)**. In Africa, it is principally a parasite of camels and horses, present in the tsetse belt, where it is transmitted mechanically by biting insects **Hoare (1972).** The aim of present study was determined to compare between protein –A- as non-specific commercial product and prepared anti-camel conjugated with alkaline phosphatase as conjugate for diagnosis of *Trypanosoma evansi* infection in camel.

2. Material and Methods

Camels

In Abusinple region and Darwa city at Aswan governorate, a total number of 894 imported camels (*Camelus dromedaries*) of different ages and sex were subjected to clinical examination according to the methods described by **Higgins (1983)** and **Radostits, *et al.* (1994)** to detect the clinical manifestation that may be related to *Trypanosoma evansi* infections.

Parasitological examination

Collected blood sample were taken from the jugular vein into Ethylene diamine tetra acetic acid (EDTA)was used as anticoagulant at a concentration of 1.0 mg/1ml of blood for parasitological examination. The hematocrit centrifugation technique **Woo (1970)** was performed on all collected blood samples for the identification of infecting trypanosomes.

Serum samples

Total of 231 serum samples were collected from camels (*Camelus dromedaries*) showed clinical signs of fever, anemia, weakness and loss of appetite and weight. Negative control sera were collected from five camels free from trypanosomosis that give negative results in blood smears were examined for detection of circulating. *Trypanosoma evansi* antigens using suratex test. The all serum samples were stored at -20^{o}C until used for ELISA.

Reagents for use in ELISA

Trypanosoma evansi antigen:

Ten mice infected with *Trypanosoma 2qw evansi* and, after a fulminating parasitaemia had developed, blood were collected. Blood passed through a column of di-ethyl-amino-ethyl cellulose (Whatman, DE52) as described by **Lanham & and Godefrey (1970),** in order to separate parasites from blood elements. The trypanosome suspension was centrifuged at 3200 g for 15 min., the supernatant discarded, and the resulting pellet washed three times in 100Mm phosphate buffered saline, pH 7.2 (PBS) by centrifugation at 2500 g for 15 min. The trypanosomes were resuspended to 1.0 ml in PBS and the antigens prepared using the method described by **Luckins (1986)**. The protein content of each antigen preparation was determined using BCA protein assay reagent. Antigens were subsequently stored at -70°C until used.

Horseradish peroxidase- labeled staphylococcal Protein –A

Conjugated protein A was obtained commercially (Sigma) in a lyophilized form. After reconstitution according to the manufacturer's instructions, the conjugate was divided into 50 µl amounts and stored at -20°C until used.

Alkaline phosphatase-conjugated goat anti-camel IgG

Goat anti-camel IgG was prepared by precipitation of camel Igs using ammonium sulphate, reconstitutions in phosphate buffer saline and then extensive dialysis against the same buffer. The protein concentration of the immunoglobulin fraction was determined using agar gel precipitation test and BCA protein assay reagent, separation of camel IgG by affinity chromatography, immunization of goats and finally purification of goat anti-camel. Alkaline phosphatase enzyme (Sigma) was conjugated to the goat anti-camel IgG**,** 3 mg of alkaline phosphatase were added to 1.5 mg of goat anti- camel IgG in 10X phosphate buffer saline, gluteraldehyde was added to 0.2% with gentle vortex. The mixture were incubated for 2 hours at room temperature, desalt by chromatography on sephadex G-25 and finally stored at 4°C after sterile filtration or addition of sodium azide **El-Hewairy *et al.*, (2004).**

Serological test

Application of prepared conjugated goat anti-camel IgG and comparison with commercial protein-A conjugate.

Sera from suspected camels trypanosomosis and negative control sera which collected from five camels free from trypanosomosis were subjected to examination by Enzyme Linked Immune Sorbent Assay (ELISA). The conjugate was used as goat anti-camel IgG labeled with alkaline phosphatase which compared with Horseradish peroxidase- labeled staphylococcal Protein-A. The substrate was applied as P. nitrophenyl phosphatase in assay for alkaline phosphatase and ABTS for peroxidase.

For each of the enzyme immunoassays, optimal antigen, antibody and conjugate, dilutions were established by chequerboard titrations using negative control sera from uninfected camels and positive control sera selected from camels with parasitologically proven *Trypanosoma evansi* infections. The assay using to compare between commercially available staphylococcal Protein A labeled with Horseradish peroxidase- **Zweygarth (1986)** and conjugated goat anti-camel IgG alkaline phosphatase for detecting trypanosomal antibodies by Enzyme Linked Immune Sorbent Assay (ELISA).

3. Results

Clinical examination of camels:

Examination of 894 camels revealed 663 (47.2%) were apparently healthy while in 231 (25.8%) camels showing some clinical abnormalities (Figure 1), These include weakness, depression, rough coat, emaciation, atrophy of the hump and some camels remained in sterna recumbence.

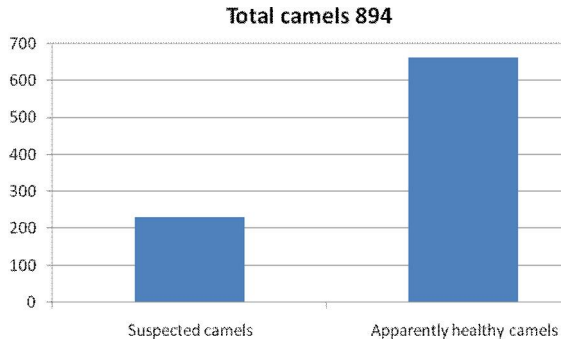

***Figure (1): showed results of clinical examination of camels.**

Parasitological examination:

The results of the Parasitological examination of blood smears from 231 suspected camels revealed that 39 (16.9%) have patent infection and 192 (83.1%) did not have patent infection (Figure 2). The highest infection rate of *Trypanosome evansi* was in summer.

Table (1) : Seasonal prevalence of *T. evansi* infecting camel.

Number of Suspected Camels	SEASSON	Number of Examined camels	Parasitological diagnosis (Blood film)	
			+ ve	- ve
231	Summer	51	12 23.5%	39 76.5%
	Autumn	54	13 24.1%	41 75.9%
	Winter	62	5 8.1%	57 91.9%
	Spring	64	9 14.1%	55 85.9%
	Total	231	39 16.9%	192 83.1%

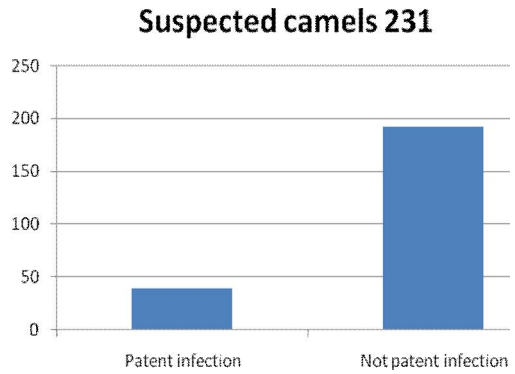

***Figure (2): showed results of parasitological examination.**

Comparison between parasitological examination and prepared conjugated goat anti-camel IgG :

Blood sera of 231 camels including 39 that give positive results by parasitological examination and prepared conjugated goat anti-camel IgG as well as 192 that give negative results in blood smears were examined by using ELISA (using conjugated goat anti-camel IgG). The result revealed that, the application of alkaline phosphatase conjugated goat anti-camel IgG were 74 (32%) positive for *Trypanosome evansi* (table 2).

Serological tests (ELISA):

Application of prepared conjugated goat anti-camel IgG with commercial protein-A conjugate.

Of the 39 serum samples (previously examined by parasitological diagnosis) were confirmed by prepared conjugated goat anti-camel IgG with alkaline phosphatase enzyme and commercial protein-A conjugate. The result revealed that, the application of alkaline phosphatase conjugated goat anti-camel IgG were 39 (100 %) positive for *Trypanosome evansi* while the application of commercial protein-A conjugate were 27 (69.2%) positive for *Trypanosome evansi (Figure 3).* This means, the application of goat anti- camel IgG gave 100% sensitivity. In contrast, the application of commercial protein-A conjugate were (69.2%), also the mean OD reading was higher in goat anti-camel IgG (0. 594) than OD reading of commercial protein-A conjugate (0.299).

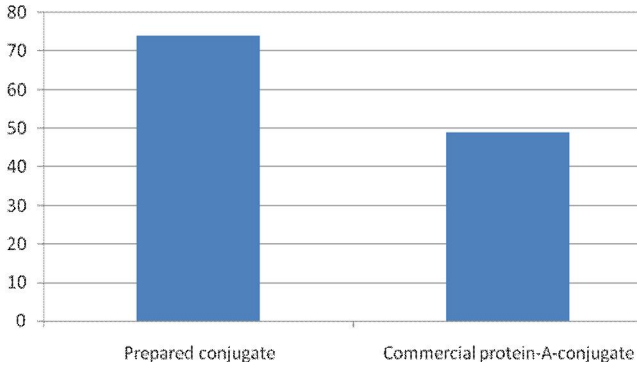

***Figure (3): Comparison between two conjugate (prepared conjugated goat anti-camel IgG and commercial protein-A conjugate). for diagnosis of camel trypanosomiasis by ELISA.**

Table (2): Comparison between blood film and ELISA for diagnosis of *Trypanosoma evansi* in camel.

Number of examined camels	Parasitological examination				Serological test(ELISA)			
	+ve	%	-ve	%	+ ve	%	-ve	%
231	**39**	**16.9**	**192**	**83**	**74**	**32**	**157**	**68**

4. Discussion

Trypanosoma evansi is a Trypanozoon parasite originating in Africa where it most probably developed from tse tse transmitted *Trypanosoma brucei*, by deletion of kinetoplasticmaxicircles **(Lai *et al.*, 2008; Lun and Desser, 1995)**. In Africa, it is principally a parasite of camels and horses, present in the tse tse belt, where it is transmitted mechanically by biting insects such as tabanids and stomoxes **(Hoare, 1972).**

Camel trypanosomosis, also known as surra, is a disease of camels caused by *Trypanosoma evansi*. Surra is widespread in different parts of the world and poses a major constraint to camel productivity **(Elamin *et al.*, 1999; AL-Rawashdeh *et al.*, 2000; Pacholek *et al.*, 2001; Njiru *et al.*, 2002)).** Available information on the prevalence of surra caused by *Trypanosoma evansi* in many countries of the world as reported, are: Nigeria (27%), Chad (30%) **(Losos, 1980);** Mauritania (24%) **(Dia *et al.*, 1997);** Niger (29%) **(Pacholek *et al.*, 2001);** Kenya (28%) **(Njiru *et al.*, 2001);** Ethiopia (21%) **(Zeleke and Bekele, 2001);** Jordan (33%) **(AL-Rawashdeh *et al.*, 2000);** India (22%) **(Pathak *et al.*, 1993);** Sudan (33%) **(Elamin *et al.*, 1999);** Iran (10%) **(Zarif-Fard and Hashemi 2001),** South America (27% in carrier state in capybras) **(Franke *et al.*, 1994),** 35.4 and 43.3%, respectively, in the Tafilalet and Ouarzazate provinces of Morocco **(Rami *et al.*, 2003).** Severe outbreaks, which occurred in different parts of the world where several thousand animals died in the 1970s and, of late, in 1994 and 1995, for instance, in Pantamal, Brazil, have also been well documented **(Luckins, 1998).**These epidemics pose a major constraint to camel productivity given their importance as a source of meat, milk production, transportation and draught power, as well as by-products (wool, hair, skin and hides). In addition, they also provide foreign currency to their owners from their export **(Elamin *et al.*, 1999).**

Trypanosoma evansi can infect a variety of hosts and causes a species-specific pathology. The following descriptions are taken from the accounts of **Mahmoud and Gray (1980) and Luckins (1998)**. In camels the disease is manifested by elevation of body temperature which is directly associated with parasitaemia. Infected animals show progressive anemia, marked depression, dullness, loss of condition, and often rapid death. Anemia was observed to be a major clinical finding in camel trypanosomosis in Morocco **(Rami *et al.*, 2003)**. Milder cases develop recurrent episodes of fever. Some camels develop edema in their dependent parts of the body, urticaria plaques and petechial hemorrhages in serous membranes. Death finally ensues if untreated. However, some may harbor trypanosomes for 2-3 years thus constituting reservoirs of infection to susceptible camels and hosts. Other well documented field reports are death **(Tuntasuvan *et al.*, 1997);** abortion **(Lohr *et al.*, 1986);** weight loss, reduced draught power **(Luckins, 1998)** and nervous signs like circling movement and trembling, unusual aggressiveness, running aimlessly and sudden collapse in severely stressed and over worked animals **(Manuel, 1998).** At post mortem, necrotic foci in the liver and spleen as well as generalized lymphoid tissue hyperplasia are common in camels suffering from surra **(Rottcher *et al.*, 1987**).

There are no pathognomonic signs of surra and so laboratory diagnosis has to be carried out to confirm infection. Traditionally, this involves parasitological and serological diagnosis. Parasitological diagnosis is mainly carried out by the direct microscopic examination of blood or buffy coats and/or sub-inoculation of camel blood into rodents such as mice or rats. However, the test has a poor sensitivity, often less than 50% **s Nantulya *et al.*, 1989; Nantulya, 1990; Luckins, 1992; Yadvendra *et al.*, 1998)**. The implication of this is that in most situations *Trypanosoma evansi* under-diagnosed and the level of infection is greater than frequently reported. On the other hand, serological techniques, e.g. immunfluorescent antibody test (IFAT), Enzyme Linked Immune sorbent Assay (ELISA) and the Card Agglutination Test for Trypanosomosis (CATT), although sensitive, cannot distinguish current from cured infections **(Luckins, 1988)**. In the present work, prevalence of *Trypanosoma evansi* was 32% and this result agree with **Aml M. A. Ra′ouf (2008)** which recorded the prevalence of *Trypanosoma evansi* was 29.17% at darwa quarantine, Aswan, Egypt. Also in the present study goat anti-camel IgG was labeled by alkaline phosphtase enzyme binded by gluteraldehyde using affinity chromatography technique gave high sensitivity in comparison with protein –A, the result revealed that 100% sensitivity with alkaline phosphatase, in contrast the sensitivity was 69% with protein A.

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