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Role of Tampan Tick *Ornithodoros savignyi* (Acari: Argasidae) in Transmitting *Trypanosoma Evansi* in Laboratory Animals

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ABSTRACT

Trypanosoma evansi (Kinetoplastida, Trypanosomatidae) is a protozoan parasite recorded to be mechanically transmitted through several ways generating a significant economic impact on livestock. Different species of insects including *Glossina spp.*, *Tabanus* spp., *Stomoxys* spp. and *Cryptotylus* spp. have a role in transmission of *Trypanosoma evansi*. Also, Soft tick species have been suggested as vectors for *Trypanosoma evansi*. The present work was initiated to study the role of *Ornithodoros savignyi* ticks in experimental transmission of *Trypanosoma evansi* to the laboratory animals. The collected *Trypanosoma evansi* from naturally infected camels was propagated in pathogen free Swiss albino mice to be used in the experimental infection. Groups of pathogen free albino rats were inoculated with *Trypanosoma evansi* to be used in experimental infection of *Trypanosoma* free *Ornithodoros savignyi* ticks (laboratory reared on rabbits) by feeding on parasitaemic rats. The experimentally infected ticks were examined by light microscope, histopathological and polymerase chain reaction (PCR) for detection of *Trypanosoma evansi*. Examination of gut of infected ticks by light microscope revealed that the number of parasite/field reached 12-17 parasites after 2 hours of feeding and decreased gradually till reached one parasite / field after 24 hours then disappeared after 48 hr after feeding. Histopathological examination showed *Trypanosoma evansi* between the cells of salivary gland after 28-30 days from feeding of *O. savignyi* ticks on infected rats. Analysis of PCR amplified fragments on agarose gel electrophoresis revealed that 227 bp was detected for *Trypanosoma evansi* in salivary gland and whole body of *Ornithodoros savignyi* ticks. Feeding of the experimentally *T.evansi* infected ticks (*O. savignyi*) on free trypanosoma rats showed negative results as no parasitaemia was detected in the rats up to 30 days after feeding. The study concluded that *O. savignyi* ticks can take *T. evansi* infection that remain for up to 30 days in its body but cannot experimentally transmit it to other animals.

Key words: Ticks, *Ornithodoros savignyi*, gut, salivary gland, *Trypanosoma evansi*, PCR, Histopathological study.

INTRODUCTION

Trypanosomes are unicellular flagellated protozoa belonging to phylum Sarcomastigophora, order of Kinetoplastidae, family of Trypanosomatidae and the genus of *Trypanosoma*. The subgenus Trypanozoon includes the pathogenic species *Trypanosoma evansi*, *T. brucei* and *T. equiperdum* (FAO, 2000). *T. evansi* thought to have evolved from *T. brucei* by adaptation to mechanical transmission enabling it to spread beyond the tsetse belt in Africa (Hoare, 1972).

The variations in the different species of Trypanosomes depend on size, shape of the body, position of nucleus, degree of development of the undulating membrane and flagellum (Smyth, 1996). *T. evansi* is the most widely distributed spp. since they occur in tropical Africa, Northern Africa, Middle East, Southeastern Asia, central and South America (Davila *et al*., 2003 and OIE, 2012).

T. evansi causes 'surra', which is an animal disease. Surra spreads in various geographical areas, widely distributing among domestic and wild animals, such as camels, cattle, buffaloes, horses, donkeys, mules, sheep, goats, dogs, cats, Indian elephants, pigs, tapirs, deer, tiger, capybara, foxes, jackals, hyenas, mongoose, and bears (Muhammad *et al*., 2007). In Egypt, *T. evansi* is a major livestock disease causing significant economic losses in camels (Abdel-Rady., 2009).

T. evansi has several origins, hosts and clinical features, also has multiple and complex means of

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transmission that differ according to the hosts and the geographical area. It is mechanically transmitted in several ways, via sucking, biting insects including *Tabanus, Cryptotylus* and *Stomoxys* species (Vokaty *et al*., 1996). Transmission can also be vertical, horizontal, per-oral and iatrogenic with various epidemiological significances depending on the host species, season and the location (Marc Desquesnes *et al*., 2013). Additionally vampire bats (*Desmodus rotundus*), ticks and triatomine bugs have also been suggested as vectors in the transmission of this *Trypanosoma* spp. The syndromes associated with *T. evansi* infection vary from chronic to acute and fatal with the clinical symptoms including progressive weakness, emaciation, fever, anemia and death (Omer *et al*., 2007 and Saleh *et al*., 2009). Previous attempts were done for detection of *Trypanosoma spp*. in ticks and also to estimate the role of ticks in transmitting trypanosomes (Morzaria *et al*., 1986; Sridhar and Sharma, 1989; Bacellar *et al*., 1990; Wahba *et al*., 2003; Latif *et al*., 2004 and Austen *et al*., 2011). The aim of this study was to determine the role of *Ornithodoros savignyi* ticks in transmitting *Trypanosoma evansi* experimentally to the laboratory animals.

Study include: A: Experimental infection I for propagation, cryopreservation of *T. evansi* and infection of *Ornithodoros savignyi*. B: Experimental infection II to study the role of infected *O. savignyi* in transmitting *T. evansi* to pathogen free rats.

MATERIALS AND METHODS

Animals

In the present study, fifteen pathogen free Swiss albino mice of 6 weeks old and of body weight ranged from (30- 40 g) and sixty eight pathogen free albino rats of body weight ranged from 230-270 g were obtained from animal house, National Research Centre, Dokky, Giza. The mice and rats were kept for adaptation 10 days before experimental *T. evansi* infection under controlled conditions of temperature 25±2°C and humidity 70%.They had free access to commercial diet and water.Three male New-Zealand rabbits of 6–8 weeks old and of body weight ranged from 1.5–2 kg was kept under laboratory condition to be used for laboratory rearing of ticks (*Ornithodoros savignyi*) to obtain naïve colony according to Christrine and Mango 1977; walker *et al*. 1984 and Habeeb, 1996).

Ticks

Four hundred and thirty *O.* savignyi ticks were collected from the ground of camel pens from Shalateen city (Red Sea governorate) and identified according to Walker *et al*., 2003.

Propagation and cryopreservation of *Trypanosoma evansi*

Blood was sampled from jugular vein of camels and examined for *T. evansi* through the microscopic examination of wet and stained blood smears. The infected blood was kept in EDTA tube for propagation in mice. Each of the 15 mice was injected intra-peritoneally with 0.5 ml of the infected blood. All the mice were examined for presence of trypanosomes after 12, 24, 48 and 72 h post infection till appearance of parasitaemia. The presence and degree of parasitemia were estimated for each infected mouse by two methods: direct blood smear from the tail vein and Giemsa-stained blood smear. The blood preparations were examined under a microscope at ×1000. *T. evansi* trypomastigotes were counted in 10 fields and the mean of these counts was estimated to express the level of parasitemia by the number of trypomastigotes in a high-power field according to Uzonna *et al*., 1998. At the first peak of parasitemia between 1.3×10^8 trypanosomes/ml and 2.5×10^8 trypanosomes/ml, the infected mice are euthanized and the blood harvested by cardiac puncture into tubes containing EDTA as anticoagulant. The blood is mixed gently before addition of a cryopreservative at a ratio of 1:1. The samples are suspended in liquid nitrogen vapor for at least 2 h using a cooling jacket before permanent storage in liquid nitrogen at –196°C Dar *et al* 1972. The most commonly used cryopreservative is 20% glycerol in EDTA saline glucose (ESG).

ESG was prepared by dissolving 8.00 g NaCl, 0.30 g KH2PO4, 2 g EDTA (disodium or dipotassium), and 2 g glucose in 800 ml distilled water, adjusting the pH to 8.0, and then topping up to 1 liter with deionized/distilled water. Glycerol is then added to the prepared solution at a ratio of 1:4 to make 20% glycerol in ESG according to Grace *et al*., 2016.

Experimental infection I

A-Infection of rats with *T. evansi*: Sixty pathogen free albino rats (230-270 g) were divided into 2 main groups; group A (49 rats) were inoculated with *T. evansi* strain and group B (11 rats) used as negative control.

At day zero the cryopreserved blood containing *T. evansi* was thawed. Each rat from group A was inoculated with *Trypanosoma evansi* strain by intraperitoneal injection of 0.5 ml of the cryopreserved parasites.The inoculated rats were examined daily for parasitaemia. The degree of parasitemia was estimated by blood smear examination (as shown in Fig. 1). (Da Silva *et al*., 2009; Wolkmer *et al*., 2009).

B-Feeding of ticks on *T. evansi* infected blood

When reaching the peak of parasitaemia (10^6-10^8*Trypanosoma* / ml) in the blood of rats, rats were prepared for ticks feeding by clipping of hair on the back, then fixing plastic capsules. *Trypanosoma* free *O. savignyi* of different stages (nymph and adults laboratory reared on rabbits) were put to feed on the rats for 0.5 to 2 h. (as shown in Fig. 2) (according to Vergne *et al*., 2011).

The fed ticks were kept at 25^0C and 50–60% relative humidity in continuous darkness throughout the experiment (Thekisoe *et al*., 2007) and were divided into 4 main groups as follows:

Group 1: (80 ticks): Were incubated in the lab to be used in the experimental infection II.

Group 2: (100 ticks): Were examined at different intervals by PCR technique.

Group 3: (30 ticks): Were used for histopathological examination.

Group 4: (220 ticks): Were examined for presence of *T. evansi* in haemolymph and gut by wet haemolymph smear and stained film technique after 2,4,6,8,24 and 48 hours after feeding.

Recognition of *T. evansi* in *O. savignyi* gut.

To identify *T. evansi* in the gut contents of *Ornithodoros savignyi* ticks. Ticks fed on infected rats with *T. evansi* were secured ventrally to a petri dish with paraffin and refrigerated for 10 min. Integument were incised with a fine sterile scalpel blade in the lateral sides and smear from the gut contents were done. The smears were fixed and stained with 10% Giemsa stain. Gut content smears were done after 2, 4, 6, 8, 12, 24 and 48 hours after feeding of *Ornithodoros savignyi* respectively and examined by using X40 and oil immersion lens of Olympus microscope.

Histopathological examination

Salivary glands were taken from control negative *O. savignyi* ticks as well as those fed on experimentally infected rats, fixed in 10% formalin and processed according to the method of Bancroft *et al*.1996.

Samples those fed on experimentally infected rats were examined up to 30 days post feeding. Sections were deparaffinized and stained with hematoxylin and eosin for histopathological examination by light microscopy.

Molecular detection of *Trypanosoma evansi*

Extraction kit was used for genomic DNA purification from rat blood and tick tissues **(**Gene JET Genomic DNA Purification kit #K0721) made in (EU) Lithuania.

Extraction of DNA from blood and samples of ticks

Kit of (Gene JET Genomic DNA Purification kit #K0721) was used to extract DNA of blood and tick tissues samples. A final DNA product of 50 µl was eluted. Following extraction, DNA samples were stored at -20 ºC until analyzed by PCR. Polymerase Chain Reaction and Gel Electrophoresis Analysis Multiplex PCR was performed using primers specific for *T. evansi* (Table 1), for preliminarily screening of DNA samples (Njiru *et al*. 2004 and Singh *et al*.2004).

Polymerase Chain Reaction (PCR)

PCR was performed in a thermal cycler programmed to a temperature-step cycle of 94°C at 5 min, 94°C at 1 min, 60°C at 1 min, followed by 2 min extension at 72°C for a total of 30 cycles. The final extension was carried out at 72°C for 7 min, (Njiru *et al*. 2004 and Singh *et al*.2004).

Gel electrophoresis

The amplified DNA samples were electrophoresed on 1.5% agarose gel and stained with ethidium bromide to visualize the amplified DNA fragments under ultraviolet light.

Experimental infection II for *T. evansi* free rats

Group of 8 *Trypanosoma* free rats were used for feeding of infected *O. savignyi*. On each rat 10-15 of the infected *O. Savignyi* of different stages (nymph and adult) were left for feeding from 0.5 to 2 hours and parasitaemia was followed up in the blood of rats by microscopic examination of blood smears for 30 days post feeding in order to estimate its infectivity with *T. evansi* parasite.

RESULTS

Examination of camel blood smear**s** showed monomorphic thin trypamastigote parasite**s** with total body length ranged from 14-28 µm, long free flagellum and thin posterior extremity with subterminal kinetoplas**t** (Fig. 3). The detected parasite was identified as *T.evansi*.

Detection of *T. evansi* in *Ornithodoros savignyi* gut after feeding on experimentally infected rats with *T. evansi.*

Examination of gut content after 2 hours of fed ticks showed that the number of *T. evansi* parasite was 12-17 parasite/microscopic field.

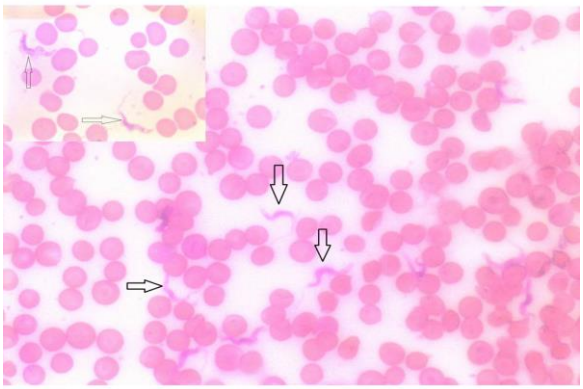

Fig. 1: Rat blood smear showed *T.evansi* parasite between RBCs (arrow).

Fig. 2: Feeding technique of *Ornithodoros savignyi* on the rat Fixed capsule on the back of rat: *Trypanosoma* free *Ornithodoros savgnyi* were put on rat: - Each capsule contain from 10-15 different stages of ticks (nymph and adult): Covering of capsules: Fed *Ornithodoros savgnyi*.

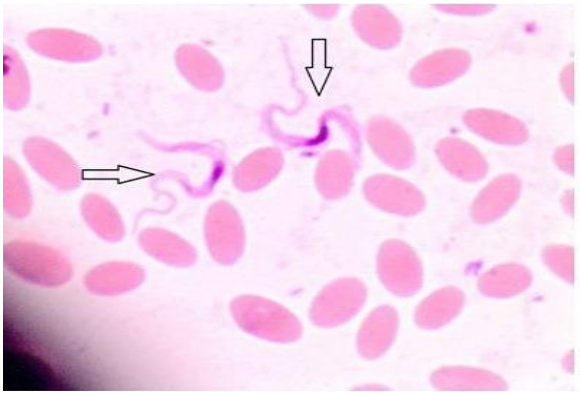

Fig. 3: Giemsa stained camel blood smear showed morphological features of *T. evansi*.

Table 1: *Trypanosoma evansi* species-specific primer pair.

Specificity	Primer name	Primer Sequence	Base pairs	Reference
T. evansi	PMURTTec.F	5'-TGCAGACGACCTGACGCTACT-3'	21	Njiru *et al*., 2004
	PMURTTec.R	5'-CTCCTAGAAGCTTCGGTGTCCT-3'	22	Singh *et al*., 2004

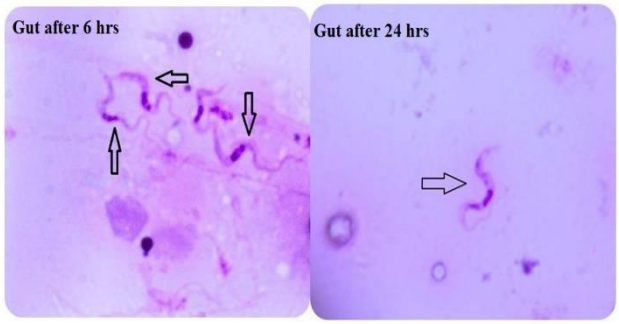

Fig. 4: Showed *T. evansi* in the gut smear of *O. savignyi* ticks experimentally fed on infected rats.

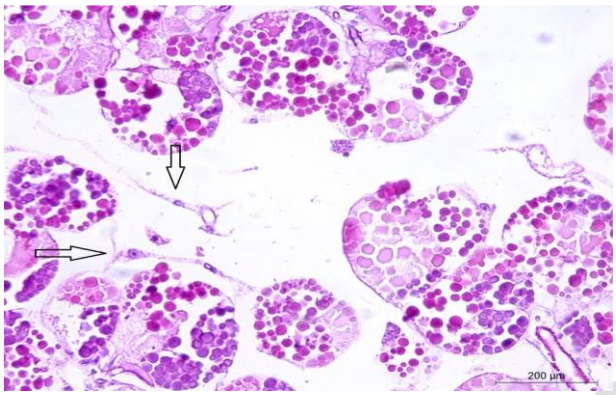

Fig. 5: Showed *T. evansi* in the transverse section of salivary gland of experimentally infected *O. savignyi* ticks (arrow).

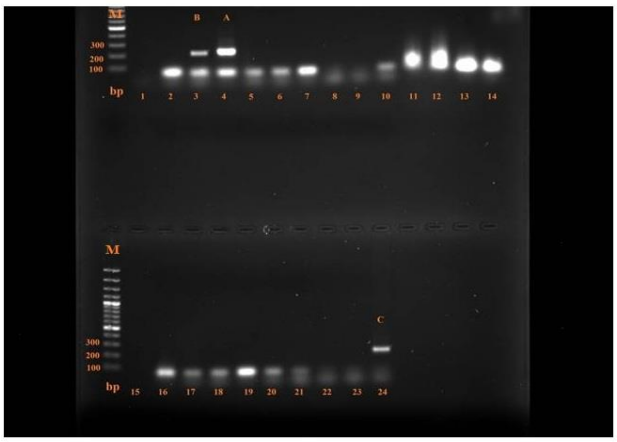

Fig. 6: Agarose gel stained with ethidium bromide showing the PCR amplification products of *Trypanosoma evansi*: -Lane M: 100 DNA bp size marker.-Lane A. positive PCR signal for *T. evansi* in infected blood sample as apositive control (227 bp): -Lane B. positive PCR signal for *T. evansi* in infected *O. savignyi* ticks (227 bp): -Lane C. positive PCR signal for *T.evansi* in salivary gland of infected *O. savignyi* ticks (227 bp).

After 4, 6 and 8 h of feeding of ticks, gut content, the number of parasite/field showed gradual reduction to be 6-10, 4-6 and 3-5 parasite / field respectively then reached one parasite / field after 24 h (Fig. 4) and completely disappeared after 48 h post feeding.

Histopathological examination

Examination of the transverse section of tick salivary glands after feeding on the experimentally infected blood showed presence of leptomonas form of *T. evansi* between the cells of salivary gland of *Ornithodoros savignyi* ticks (Fig. 5).

Trypanosoma evansi was appeared in salivary glands of infected ticks (*O. savignyi*) at 28-30 days after infection of ticks with *T. evansi*.

Molecular characterization of *T. evansi* in experimentally infected *O. savignyi* ticks

Molecular characterization: Analysis of PCR amplified fragment on agarose gel electrophoresis revealed that 227 bp was detected for *T. evansi* in salivary gland and whole body of *O. savignyi* ticks (Fig. 6).

Detection of *T. evansi* infection in rats (after feeding of infected ticks)

Feeding of infected ticks (*O. savignyi*) on rats free from infection showed that negative results (no parasitaemia was detected in the rats through 25-30 days after feeding).

DISCUSSION

Trypanosoma evansi is the most widely geographically distributed pathogenic flagellated haemoprotozoan parasite. It is transmitted mechanically by blood sucking flies (Hoare, 1972, Luckins, 1988, Lun *et al*., 1993 and Reid, 2002).

It infects a large number of animal species, including cattle, buffaloes, donkeys, horses, and camels, causing trypanosomiasis, commonly known as surra, which evokes significant economic loss (Luckins , 1988, Lun *et al.*, 1993 and Li *et al*.,2009).

Tick infestation on animals is of economic importance as these animals are important for meat and milk production, and their health and production are greatly affected by the high tick infestation (Nazifi *et al*., 2011).

In the present study *Trypanosoma evansi* was identified as *T. evansi* according to the description of Hoare (1972). The parasite was recognized in wet blood film by their movement among the red blood cells (RBCs). Examination of the Giemsa stained blood smear showed that *T. evansi* was monomorphic thin trypamastigote parasite with length range from 14-28 µm, long free flagellum and thin posterior extremity with subterminal small kinetoplast as reported previously by Soulsby, 1982 and Wahba *et al* 2003.

In the present study, *Trypanosoma evansi* was propagated in vivo by infecting the lab animals (mice and rats) and feeding of *Ornithodoros savignyi* (soft ticks) on infected lab animals (rats) to take trypanosoma infection.

Our results agreed with el Kady, 1998 and Latif *et al*., 2004 who mentioned that hard ticks of the genera (*Hyalomma* and *Rhipicephalus*) and soft ticks of the genera *Ornithodoros* have been reported to harbor trypanosome parasites.

Also, Thekisoe *et al* 2007 reported that *Trypanosoma KG1* isolate was also isolated from hard tick *Haemaphysalis hystricis* ticks and propagated in vivo by infecting the soft tick *Ornithodoros moubata* ticks and the parasite isolated in the midgut and the salivary glands. This observation suggests that this trypanosome has the ability to infect both the hard and soft tick species.

In our study examination of smears from gut content of *O. savignyi* ticks that experimentally fed on parasitemic rat revealed that, *T. evansi* was present till 24 hours after feeding then disappear. The same results was reported but in naturally infected tick species as in Egypt by Wahba *et al.,* 2003 who recorded leptomonas form of *Trypanosoma evansi* in salivary glands and gut smears of *O. savignyi* and *Hyalomma dromedrii* ticks. Also, in Egypt Elkady, 1998 recorded *Trypanosoma sp.*in the guts of *Hyalomma spp* that collected from Beer El Abd, Nakhel and Dahab as well as in Japan, Thekisoe *et al* 2007 isolated *Trypanosoma KG1* from the hard ticks (*Haemaphysalis hystricis* ticks) and propagated in vivo by infecting the soft tick *Ornithodoros moubata* and so detected the parasite from the midgut and the salivary glands.

In the present study *T. evansi* was not detected in the haemolymph smears of *O. savignyi* experimentally fed on infected rats .On the contrary,Wahba *et al.,* 2003 detected *T. evansi* in the haemolymph smears of the same species of ticks but in naturally infected cases.

Histopathological examination of the salivary glands of infected *O. savignyi* ticks revealed presence of *T.evansi* in-between cells at 28-30 days after infection.

The conducted polymerase chain reaction (PCR) for detection of DNA in blood and ticks, showed 227bp PCR product for the specific detection of *T. evansi* by using specific primers for *T. evansi*, the data which came in agreement with that reported by Singh *et al*., (2004) for positive cases of *T.evansi* infection in camels using the same primer.

The present investigation concluded that PCR is a useful tool for detection and confirmation of the *Trypanosoma spp*, this came in accordance with that provided by Clausen *et al*., (2003), Hilali *et al*., (2006), Rjeibi *et al*., (2015) and Masiga and Nyang'ao, (2001) for successful detection of *T. evansi* infection in horses, water buffalo calves, dogs and camels respectively.

In our results Feeding of infected ticks (*O. savignyi*) on rats free from infection showed that negative results (no parasitaemia was detected in the rats through 25-30 days) after feeding. On the other hand other *Trypanosoma* species have been shown to be transmitted by ticks Latif *et al* 2004 demonstrate the high vectorial capacity of *Hyalomma anatolicum anatolicum* ticks for *Trypanosoma thieleri.* Morzaria *et al.,* 1986 found various developing stages of *Trypanosoma theileri*-like flagellates in the engorged nymphs, freshly moulted adults and mature adults of *Hyalomma anatolicum anatolicum* ticks partially engorged on rabbits. When these ticks were applied to two calves, one calf became infected with the trypanosome. The difference between our results may be due to the different hosts and different *Trypanosoma spp*.

Conclusions

The study concluded that *O. savignyi* ticks can take *T. evansi* infection that remain for up to 30 days in its body but cannot transmit it to other animals.

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