Longevity and Infectivity of *Trypanosoma evansi* Isolated from the Gut of the Stable Fly *Stomoxys calcitrans* (Diptera: Muscidae)

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Abstract. Stable fly *Stomoxys calcitrans* is a blood-sucking fly prevalent in tropical and subtropical countries. The objective of this study was to determine the longevity and infectivity of *Trypanosoma evansi* that isolated at different intervals from the gut of experimentally infected stable fly with *T .evansi.* Two generations of flies were used aged 24 and 48 hours (hr). The flies were fed on blood of mice infected with *T. evansi* through the chicken's skin placed on glass container in a water bath at 37 °C. Ten fed flies were dissected under the stereo-microscope of intervals; immediately, 1, 12, 24, 26, 27, 28, 29 and 30 hr post-feeding (pf). Smears were prepared from the gut's isolated trypanosomes and simultaneously inoculated intraperitoneally into four mice for each interval. Three serological tests were employed to detect the antigenic variations of the *T. evansi*; agglutination, indirect fluorescent antibody (IFA) and gel diffusion. All mice were infected with *T. evansi* for all intervals except 27, 28 and 29 hr pf. Infectivity was decreased with time of feeding. Binary fission was observed at 12 hr pf and the parasites disintegrated at 30 hr pf. IFA test showed cross-reaction of various isolates of *T. evansi* in comparison with agglutination and gel diffusion tests.

Keywords: Longevity, infectivity, *Stomoxys calcitrans*, *Trypanosoma evansi*, serological tests.

1. INTRODUCTION

Stable fly *S. calcitrans* is a blood-sucking fly prevalent in most countries of the world [1-3]. This fly is in close resemblance to house fly and both flies are classified to the family Muscidae. As a result of the widespread distribution of this fly coupled with the nutritional habit of males and females on blood, it considered as an important vector in the transmission of many diseases. The direct effect is anaemia from the feeding of large numbers of the flies, which may lead to loss of body weight and reduce in productivity and in some cases to the death of the host [4]. Furthermore, the indirect effects are due to the pathogens transmitted by the fly like trypanosomes and bacteria of *Bacillus anthracis*, *Staphylococcus*, *Enterobacter sakazakii* and many viruses [5-8].

T. evansi is a hemoflagellate protozoan transmitted mechanically by horse flies (*Tabanus* sp.) and stable flies (*Stomoxys* sp.) in China and Indonesia and tsetse fly in Africa. While, vampire bats serve as vectors and reservoirs in South and Central America [9, 10]. Mostly, *T. evansi* transmitted through intermittent feeding of stable fly [11]. Moreover, *S. calcitrans* failed to transmit *T. evansi* from infected goats to other goats or to camels [12]. In Spain, *S. calcitrans* considered exclusively as the vector in the transmission of *T. evansi* to camels [13]. Furthermore, *T. brucei, T. vivax, T. evansi* and *T. congolense* mechanically transmitted to mice within 3 minutes of interrupted feeding of *S. niger* [14]. The survival time of *T. congolense* in *Stomoxys* spp. up to 210 minutes and *T. evansi* up to 480 minutes after the flies were fed on infected blood and completed their feeding on uninfected mice [9]. The aim of this study is to determine the longevity and infectivity of *T. evansi* isolated from the gut of *S. calcitrans* at different intervals and the antigenic variations that takes place during the passage of the parasite in the fly's gut.

2. MATERIALS AND METHODS

2.1. Rearing of Stable Flies

S. calcitrans flies were collected from the field of the Faculty of Veterinary Medicine, Baghdad University and identified according to [15]. Flies were placed in cage size 30 x 30 cm and fed on sheep's blood in soaked gauze. Fly's Eggs were collected daily by brush and transfers to petri dishes contain horse manure, blood and alfalfa for feeding larvae. Pupa were transferred to other dishes containing horse manure. Life cycle had been conducted in an incubator at 30 °C.

2.2. The Parasite

T. evansi (Iraqi strain) was isolated from infected camel in Baghdad province.

2.3. Laboratory Animals

a- White mice (Balb/c) injected intraperitoneally with 0.1 ml (10^5) *T. evansi* infected blood and these mice

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were used for the purpose of feeding flies. Antigens and antisera for the serological tests were prepared from infected mice.

b- New Zealand white rabbits injected intravenously into the marginal ear vein with 0.5 ml (10^5) *T. evansi* of infected blood. Antisera were prepared from these rabbits for serological tests.

2.4. Collection of Blood

a- Blood collected from mice by cardiac puncture.

b- Rabbit blood was collected from the marginal ear vein.

2.5. Fly

Two groups of flies were used, aged 24 and 48 hr. Flies were fed on the *T. evansi* infected blood of mice through the membrane according to the method [16]. Flies were fed through the chicken's skin, which placed on a lid of 1.0 ml size and connected by rubber thread, placed in a water bath at 37 °C (Figure **1**). Infected blood was injected through the skin by syringe.

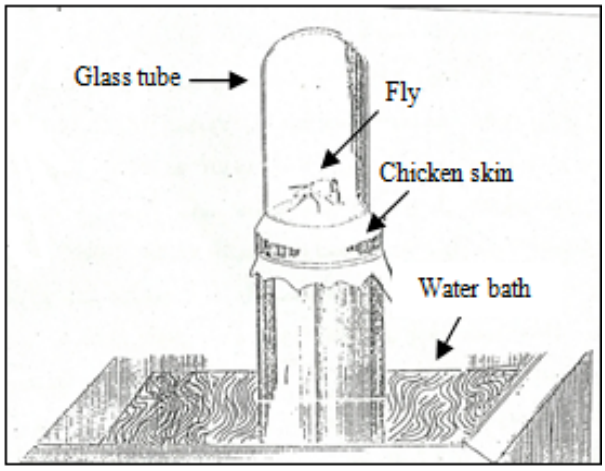

Figure 1: Apparatus for feeding *S. calcitrans* fly consisted of glass tube lid filled with *T. evansi* blood and chicken skin.

Subsequently, one fly in a glass tube was placed on the skin, and left to feed until the abdomen filled with blood. A group of ten flies was dissected at directly, 1, 12, 24, 26, 27, 28, 29 and 30 hr pf. Gut was separated on the slide, dissected and mixed with phosphate buffered saline (PBS) pH 7.2. The gut's contents were examined under the microscope for the presence of trypanosomes. Simultaneously, flies gut contents were injected to 4 mice of each interval.

2.6. Serological Tests

Three serological tests were applied to study the antigenic variations of the *T. evansi* that takes place during the passage of trypanosomes in the gut of the fly:-

i- Agglutination test according the method [17]. Two fold dilutions of sera were used 1:10 to 1:1280.

ii- Indirect fluorescent antibody test (IFA) according the method [18].

iii- Gel diffusion test according to the method [19].

2.6.1. Preparation of Antisera

a- Mice antisera:-

The gut of the infected flies was injected intraperitoneally into five mice. Parasitaemia was detected at 14 days post-infection. One ml of blood was collected from each mouse and serum was separated and kept in -20° C until used.

b- Rabbits antisera:-

Rabbits were inoculated intravenously with 0.5 ml of infected mice's blood. Five ml of blood was collected three days post -infection. Serum was separated and kept at - 20 °C until used.

2.6.2. Preparation of Antigens

Twenty flies of each age group were fed on the infected blood of different intervals. Isolated fly's guts were suspended with PBS, and injected intraperitoneally to ten mice of each interval. Blood of mice was collected in EDTA tube, centrifuged at 5000 rpm for 5 min, buffy coat was separated and mixed with 1.0 ml of PBS and centrifuged as before. The supernatant was discarded and the sediment pellet of the parasites was resuspended in 1.0 ml of PBS. The same procedure was repeated for each interval.

2.6.3. Antigen Preparation for Gel Diffusion Test

Trypanosomes isolated from the blood of mice and the guts of the flies at different intervals were ultrasonicated according to [20].

3. RESULTS

3.1. Infectivity of *T. evansi*

The *T. evansi* isolated from the gut of stable fly showed the ability to infect mice at immediately, 1, 12,

24 and 26 hr pf. No trypanosomes were detected in the fly's gut at 27, 28 and 29 pf. The parasite was degenerated at 30 hr pf (Table **1**).

Table 1: Infectivity of *T. evansi* Isolated from the Gut of Stable Flies in Mice at different Time Intervals

No. of flies	Post feeding hours	No. of mice	Infectivity
10	direct	4	+
10	1	4	+
10	12	4	+
10	24	4	+
10	26	4	+
10	27	4	Nil
10	28	4	Nil
10	29	4	Nil
10	30	4	Nil

3.2. Morphology and Motility of *T. evansi*

Morphological changes and sluggish movement of trypanosomes were observed after one hr pf. It looks longer and thinner (Figure **3A**) in comparison with the blood form (Figure **2**). Motility was decreased at 26 hrs pf although the reproduction was detected at 12 hr pf (Figure **3B**). Likewise, more than one nucleus and flagellum were observed at 24 hr pf (Figure **3C**) and the chromatin granules appeared more pronounced at 26 hr pf (Figure **3D**). There was a decrease in the number of trypanosomes which was inversely proportional to the intervals 24 and 26 hr pf.

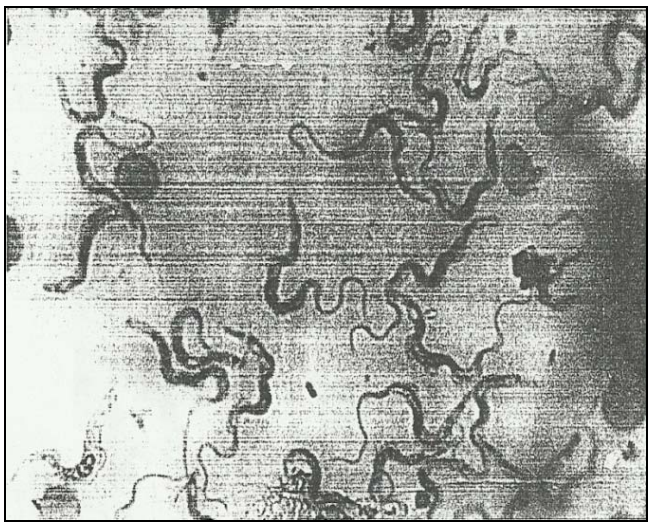

Figure 2: Blood smear of mouse infected with *T. evansi* (×100).

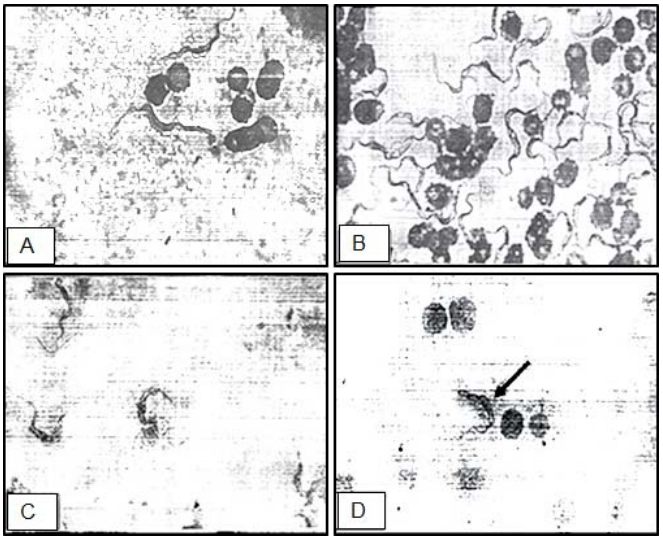

Figure 3: Smears prepared from the gut of *S. calcitrans* fly infected with *T. evansi* at different intervals . A. 1 hr pf. B. 12 hr pf. C. 24 hr pf. D. 26 hr pf (×100).

3.3. Serological Tests

3.3.1. Agglutination Test

Table **2** showed the results of agglutination test of *T. evansi* antigens prepared from the gut of the fly with antisera of different intervals. The titres were high with homologous antigens and anti-sera and low or nil with heterologous antisera. The titres were high with immediately and one hr pf antigen with anti-sera of 12 hr pf and no reaction with anti-sera of 24 and 26 hr pf. The same results were obtained with antigens isolated from blood of mice and guts of flies at different intervals (Table **3**).

Table 2: Maximum Antibody Titres of Antisera with *T. evansi* Antigens Isolated from the Gut of Stable fly by Agglutination Test

Antigen (hr)	Direct	Antisera			
		1 hr	12 hr	24 hr	26 hr
0	1280	1280	10	0	0
1	1280	1280	10	0	0
12	80	320	1280	320	320
24	0	0	80	320	80
26	0	0	80	80	320

3.3.2. IFA

Table **4** showed the interaction between *T. evansi* antigen isolated from the gut of the fly and the blood of infected mice with homologous and heterologous anti-

Table 3: Maximum Antibody Titre of Antisera with *T. evansi* Antigens Isolated from the Blood of Mice by Agglutination Test

Antigen (hr)	Direct	Antisera			
		1 hr	12 hr	24 hr	26 hr
0	*1280 / 1280	1280 / 1280	10 / 320	0 / 80	0 / 0
1	1280 /1280	1280 /1280	10 / 320	0/ 80	0 / 0
12	80 / 80	320 / 320	1280 / 1280	320 / 320	320 / 320
24	0 / 0	0 / 0	80 / 80	320 / 1280	80 / 320
26	0 / 0	0 / 0	80 / 80	80 / 320	320 / 1280

*= The first number represents stable fly antigens and the second one is the mouse's blood antigens.

Table 4: Maximum Antibody Titre of Antisera with *T. evansi* Antigens Isolated from the Gut of Stable Fly and Mouse's Blood by IFAT

Antigen (hr)	Direct	Antisera			
		1 hr	12 hr	24 hr	26 hr
0	*1280 / 1280	1280 / 1280	10 / 320	0 / 0	0 / 0
1	1280 /1280	1280 /1280	80 / 320	0/ 80	0 / 0
12	0 / 320	320 / 320	1280 / 1280	320 / 320	320 / 320
24	0 / 0	0 / 0	80 / 640	1280 / 1280	160 / 1280
26	0 / 0	0 / 0	80 / 640	320 / 1280	1280 /1280

* = The first number represent stable fly antigens and the second one is the mouse's blood antigens.

sera. High titres of homologous antigens and anti-sera while there were low or nil with heterologous one. Moreover, there was high titre between antigens isolated immediately and one hr pf with homologous antisera.

3.3.3. Gel Diffusion Test

Antigens from the gut of the stable fly have no interaction with anti-sera of the periods immediately, 1, 12, 24 and 26 hr pf. While, the test was positive with antigens from the blood of infected mice and homologous anti-sera. Two lines between non-diluted antiserum and dilution 1:2 whereas, there was no precipitation in the other dilutions (Table **5**).

Table 5: Gel Diffusion Test of *T. evansi* Antigens Isolated from the Gut of Stable Fly and Blood of Mice with Rabbit Antisera

Flies / Mice Antigens (hr)	Serum				
	Undiluted	1 :2	1:4	1:8	1:16
0	0 / +	0 / +	0 / 0	0 / 0	0 / 0
1	0 / +	0 / +	0 / 0	0 / 0	0 / 0
12	0 / +	0 / +	0 / 0	0 / 0	0 / 0
24	0 / +	0 / +	0 / 0	0 / 0	0 / 0
26	0 / +	0 / +	0 / 0	0 / 0	0 / 0

4. DISCUSSION

The results of this study showed that the morphology and motility of *T. evansi* in the gut of stable fly is in agreement with the results of [21], how noted that *T. evansi* lose its motility at two hours pf and degraded at 24 hr pf. Sluggish movement of the parasite was observed at four hours off and no movement at 32 hr pf and decomposing all of the parasites at 72 hr pf [22]. The differences in the duration of these intervals in comparison with this study may attribute to the difference of the parasite strains and virulent.

T. evansi isolated from the gut of the stable fly showed ability to infect mice until 26 hr pf, and those in agreement with [22], who noted the infectivity of trypanosomes until 29 hr pf. The results of the current study are different from those of [23] who reported that infectivity of *T. equinum* isolated from the guts of stable and horse flies until 8 hr pf. As well as with the results of [24] that showed that mice were infected with *T. brucei* till 30 minutes pf. The differences in the results may be related to the difference in the species and strain of the parasite. However, the digestive enzymes in the gut's fly and differences in the immune response of the insect itself could play an important role in the survival and reproduction of trypanosomes in the gut of the fly [25, 26]. Furthermore, decomposition of the parasite inside the gut's fly can be attributed to the nature of the blood, digestive enzymes of the gut and quantity of glucose These factors are necessary for the survival of trypanosomes for a long time in the gut of stable fly [27].

The parasites appeared longer, thinner with more than one nucleus and the increases of chromatin granules which could be as an attempt by the parasite to multiply and to stay alive, but the parasite is unable to penetrate the gut wall and get rid of the influence of the gut surroundings and degradation due to the effect of digestive enzymes [22].

It is well known that the cell membrane and the flagellum of trypanosomes are covered with a dense coat called variable surface glycoprotein (VSG) [28]. Variant specific antigens (external antigens) located on this surface coat [29], which were involved in agglutination, precipitation and protection tests [30, 31]. Continuously changing of these antigens at different intervals depends on the species and strain of the parasite [32, 33]. Trypanosomes loss their surface coats, lead to the emerges of other groups of antigens with new coat which differ from the original one and this phenomenon known as antigenic variation [34].

In this study, some of the properties that related to these antigens, include the infection, which depends on the existence of surface coat [22, 28]. Likewise, the infection of mice injected with trypanosomes isolated from the gut of the stable fly up to 26 hours and this attributed to the presence of an external coat of the trypanosomes in the gut of the stable fly. This is contrary to [35], who pointed out that the antigen in the agglutination reaction of the blood phase of trypanosomes which located on the surface coat of the parasite lost in the body of the insect. Trypanosomes inside the gut of the insect will covered with a new coat as a result of entering an environment free of antibodies [36]. This coat is similar to the original coat at the beginning of inoculation to the host. This is in agreement with the findings of [22]. They showed that an interaction between antigen of *T. evansi* that isolated from the gut of the stable fly was one hr pf with rabbit anti-serum in agglutination test and the change may be happened to the coat after one hr pf. In the current study the changes take place after one hr pf. and it may be due to the cross reaction between antigens isolated from the gut of the flies with homologous and heterologous antisera. The presence of surface coat which contains specific antigens that detected by agglutination test [19, 37]. Agglutination test results showed that the titres were high between the variables of the blood phase of *T. evansi* and homologous antisera and little or no reaction with heterologous antisera.

In a fluorescent test, the similar results were obtained by [37]. Furthermore, high antibody titres between the antigens isolated from the animal's blood and homologous antisera, and low or negative with heterologous antisera. Fluorescent antibody test showed higher reaction with different gut's isolates in comparison with agglutination test and this attributed to the common and specific antibodies are involved in IFA [22, 32]. Positive reactions by gel diffusion test were noted between the variables *T. evansi* antigens and rabbits anti-sera [19, 37] which in agreement with the results of current study.

5. CONCLUSIONS

T. evansi was disintegrated in the gut of the stable fly at 30 hr pf. Cross reactions were observed between variant gut's fly antigens with homologous and heterologous antisera. The interaction between variant antigens and anti-sera isolated at different periods, indicate the presence of the external coat of trypanosomes during the passage in the gut of the stable fly.

ABBREVIATIONS

hr = hour

IFA = Indirect fluorescent antibody test

pf = post-feeding

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