Pathogenicity of Ethiopian *Trypanosoma evansi* Type A and B in Swiss Albino Mice Model

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Abstract: Surra caused by *Trypanosoma evansi* is one of the important pathogenic parasitic diseases of camels, equines, other domestic and wild animals. *T. evansi* type A is endemic to Africa, Latin America, and Asia while *T. evansi* type B is so far identified only in Ethiopian and Kenyan camels. Little is known about the pathogenicity of *T. evansi*. This study was conducted to determine the pathogenicity of *T. evansi* type A and B in Swiss albino mice colony. We genetically characterized two *T. evansi* type A and two *T. evansi* type B isolated from camels in Tigray and Afar. Six mice were infected by each of the isolates and compared with 6 uninfected mice (control). Parasitemia was followed on Matching Method. Weight and PCV of each mouse were measured pre-infection and after 6 days post-infection. Each mouse was examined for visible clinical signs. Highly parasitaemic mice were euthanized on diethyl ether to collect vital organs for gross and histopathologic examination. Major clinical signs in infected mice were rough hair coats, pale mucous membranes of the eye, and incoordination. Compared to the control, there were no significant reductions in the body weight of mice (*T. evansi* type A, p= 0.493, *T. evansi* type B, p=0.299), but there was significant reduction in the mean PCV values in both *T. evansi* type A (p= 0.0001) and *T. evansi* type B (p= 0.0008) stocks. Splenomegaly, hepatomegaly, edema and pneumonia were the prominent lesions observed at necropsy. Microscopic lesions seen in vital organs were congestion, capillaries distended with red blood cells, cellular infiltration, accumulation of hemosiderin, necrosis and degenerative changes. The clinical signs and gross and histopathologic lesions were comparable between mice infected by *T. evansi* type A and B. In conclusion, *T. evansi* type A and B showed similar *in vivo* pathogenicity. As a result, a special model for comparative pathological study on host-trypanosome interaction is essential.

Keywords: *Pathogenicity, Swiss albino mice, Trypanosoma evansi type A and type B*

Introduction

Trypanosoma evansi (surra) is the number one protozoan disease of camels and horses. Infected camels and equines may die within 3 months. Moreover, cattle, buffalo, pigs, goat, and sheep infected with *T. evansi* suffer from immune suppression, resulting in increased susceptibility to other diseases or vaccination failure against classical swine fever and *Pasteurella multocida* (Holland *et al*., 2001; Holland *et al*., 2003; Desquesnes *et al*., 2013a). *T. evansi* has no maxicircle but has minicircles (Borst *et al*., 1987; Schnaufer *et al*., 2005; Lai *et al*., 2008). Based on the restriction digest on the minicircle kDNA pattern, *T. evansi* is divided into *T. evansi* type A and *T. evansi* type B that largely differ in the genetic makeup (Borst *et al*., 1987). *T. evansi* isolates with minicircle type A have the RoTat 1.2 variable surface glycoprotein (VSG) and are the most abundant in Ethiopia, East and West Africa, Latin America and Asia (Songa *et al*., 1990; Ou *et al*., 1991; Lun *et al*., 1992; Njiru *et al*., 2006; Birhanu *et al*., 2015). In contrast, the occurrence of type B minicircle is not common and so far has only been detected in camels from Kenya and recently Ethiopia (Borst *et al*., 1987; Ngaira *et al*., 2005; Njiru *et al*., 2006; Birhanu *et al*., 2015; Birhanu *et al*., 2016).

The parasitemia causes a large number of red blood cells (RBCs) to be removed from circulation by cells of the mononuclear phagocytic system (MPS) in the spleen, bone marrow and haemal lymph nodes of the host. The removal of a large number of RBCs leads to a fall in packed red cell volume (PCV) to below 25% or even to as low as 10% (Habila *et al*., 2012). Abortions (Shaapan, 2016), immuno suppression, and infiltration and dissemination of *T. evansi* in the central nervous system (CNS) with fatal clinical symptoms in horse and different animals were documented (Berlin *et al*., 2009; Saleh *et al*., 2009; Holland *et al*., 2001; Holland *et al*., 2003).

In experimental infection of goats, the observable gross lesions are liver with petechiations and pale muscle. In the spleen, depletion of red pulp and presence of extramedullary hematopoiesis characterized by the presence of megakaryocytes (Cesta, 2006; Elmore, 2006) while in the lungs, pulmonary edema, hemosiderosis and congestion (Ellman and Gee, 1951), petechiation of liver and paleness of muscles are documented (Dargantes *et al*., 2005). Postmortem examination in *T. evansi* infected aborted and stillborn camel fetuses was characterized by subcutaneous edema, presence of a moderate amount of dark red hemolysed blood in the thoracic and abdominal cavity,

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bronchopneumonia, hepatic necrosis and acute congestion in all vital organs. Histopathological changes observed in an aborted fetus of dromedary camels were severe congestion, thickening of the bronchial and alveolar wall and mononuclear infiltration in the fetal lung, necrotic and degenerative changes in the liver, nephritis along with severe congestion and tubular necrosis in the kidneys and necrotic and degenerative changes and congestion of capillaries in the brain (Narnaware *et al*., 2016).

Trypanosoma evansi has a considerable direct and indirect economic impact on livestock productivity of the globe. Direct estimated costs include risk of infection, costs of diagnosis, alternative treatments, collecting animals for treatment, cost of controlling the disease and costs of animal losses (Seidl *et al*., 1998). However, *T. evansi* indirectly affect by reducing milk yield, decreasing reproductive performance, reduction in growth, reduction in draught power, and the bodyweight of a different animal population (Dávila and Silva, 2000; Salah *et al*., 2015; Kumar *et al*., 2017).

Trypanosoma evansi has a broad spectrum of infectivity for small rodents. Mouse is a preferred animal model to reveal subclinical infections in domestic animals and to study host pathogenicity (El Rayah *et al*., 1999; Eisler *et al*., 2001; Gillingwater *et al*., 2010; Verdillo *et al*., 2012). Information on the *in vitro* pathogenicity of *T. evansi* was recently published (Birhanu *et al*., 2015); but, no literature evidence is available about the *In vivo* pathogenicity of Ethiopian *T. evansi* type A and B. This study was, therefore, designed to determine comparative pathogenicity of *T. evansi* type A and type B in Swiss albino mice model.

Materials and Methods

In Vivo Pathogenicity of T. evansi

The four *T. evansi* stocks (2 *T. evansi* type A and 2 type B) were obtained from samples collected by Birhanu and colleagues in 2013 from Afar and Tigray National Regional States of Ethiopia. Isolation of the field samples was made in Swiss albino mice colony and incorporated in liquid nitrogen cryobank collections of the Mekelle University College of Veterinary Medicine (Birhanu *et al*., 2015). Characterization through species-specific PCRs and genotyping was made at the Institute of Tropical Medicine, Belgium, World Animal Health (OIE) reference center for diagnosis of Surra (Birhanu *et al*., 2016) (Table 1).

Table 1. *T. evansi* stocks used in the study

***T. evansi* stock code**	**Origin**	**RoTat 1.2 PCR**	**Type B PCR**	**Kinetoplast**	**Reference**
MCAM/ET/2013/MU/04	Megale, Afar	Pos	neg	Kinetoplastic	Birhanu *et al*., 2015;
MCAM/ET/2013/MU/09	Kukufto, Tigray	Pos	neg	Akinetoplastic	Birhanu *et al*., 2016
MCAM/ET/2013/MU/10	Awash-fentale, Afar	Neg	pos	2% akinetoplastic	
MCAM/ET/2013/MU/14	Awash-fentale, Afar	Neg	pos	2% akinetoplastic	

Ethical Considerations

Handling and use of experimental mice followed expedited approval of Mekelle University College of Veterinary Medicine (CVM-CRC/21/08) and were in accordance with the Institutional Review Board of the Ministry of Science and Technology of Ethiopia.

Experimental Setup and Laboratory Animal Model

Swiss albino mice colonies with initial body weight of 25-30 g, 6-8 weeks old, were used. Inclusion of mice as a study subject was following the standard guidelines for single-dose mice test that state each experiment to consist 6 mice (Eisler *et al*., 2001). Each mouse was stained with picric acid stain on the tail, back and head region for identification purposes. Before inoculation with trypanosomes, body weight and packed cell volume (PCV) of each mouse was recorded. Variation in body weight among study subjects was kept to a maximum of 10% through the inclusion of mice that have comparable body weight.

Trypanosomes Infection and Estimation of Parasitemia

To infect mice, stabiletes from the cryo-collection were thawed in a water bath with a temperature of 37 °C for 5 min and mixed with an equal volume of phosphate-buffered saline glucose (PBSG, 7.5 g/l $Na_2HPO_4.2H_2O$, 0.34 g/l $NaH_2PO_4.H_2O$, 2.12 g/l NaCl, 10 g/l D-glucose, pH 8). To check the presence of motile trypanosomes, 5 µL of the diluted stabilete was transferred into a glass slide covered with a coverslip (24x24 mm) and examined under x640 (40x16) magnification. Following confirmation of viability, stabiletes were inoculated into two mice for each *Trypanosome* strain (eight mice) to check the quality/infectivity of the isolate. Three to four days later, when the parasitemia was at a peak, the mice were anesthetized by diethyl ether and euthanized, blood was collected on heparin by heart puncture, diluted in PBSG to a concentration of 2 trypanosomes per field in 5 µL of diluted sample, Matching Method (MM) log 6.9, nearly $8x10^7$ trypanosomes/ mL (Herbert & Lumsden, 1976). Each stock was inoculated into six mice, therefore for all the four strains, 24 mice were inoculated and additional six mice were used as controls. All together 30 mice were used for the experiment. The experimental mice were inoculated with 0.2 mL of diluted blood with a parasitemia of either of the two trypanosomes per field in MM (Herbert & Lumsden, 1976).

Examination for Pathological Changes

Six days post-infection, each mouse was checked for parasitemia in 5 µL of tail blood. When the parasitemia of the mice was at least 4 trypanosomes per field, weight and PCV were measured and any visible clinical sign was registered (Herbert and Lumsden, 1976). For PCV measurement, tail blood-filled ¾ of the capillary tube was centrifuged at 12000 rpm for 5 min, and PCV was read on the microhematocrit reader. Thereafter, the control group received no strain; however, the parasite inoculated mice were monitored for visible clinical signs. Three mices from each group were euthanized 6 days post-infection for subsequent postmortem and histopathologic examination. After weighing, the gut was opened with scalpel blood and spleen, heart, liver, brain, stomach, intestine and lung were examined grossly. For histopathologic examination, samples were collected and fixed in a jar containing 10% buffered formalin (Verdillo *et al.*, 2012). The tissue samples were dehydrated in alcohol embedded in paraffin wax, sectioned at 4-6 µm thickness, stained with Haematoxylin- Eosin and examined using a compound light microscope (Biswas *et al.*, 2001).

Data Analysis

Data entry was made in the Microsoft Excel spreadsheet version 2016. Stata version 11 was used for data analysis (StataCorp, 2013). Descriptive statistics such as frequency, percentage, chart and mean ± standard deviations (SD) were used to report responses. Independent paired t-test was used for comparison of group mean of PCV values and weight of mice while two-way ANOVA was used to compare within and among pathogenicity (postmortem vital organ weight). Multiple comparisons using a post-hoc test was used to compare the mean difference among the groups. At all levels, a *p-value* of 0.05 was used as a cut-off value to test the level of statistical significance.

Results

Effect of T. evansi Strains on PCV and Weight of Mice

Compared to the control, there were no significant reductions (*T. evansi* type A, p= 0.493, *T. evansi* type B, p=0.299) in weight of mice (Table 2), but there was a significant reduction in the mean PCV values in both *T. evansi* type A (p= 0.0001) and *T. evansi* type B (p= 0.0008) stocks (Table 3).

Table 2. Mean weight variation before and after 6 days parasitemia

***T. evansi* type**	**Mean and SD of weight of mice**		**Mean diff**	**t-calc-value**	**P-value**
	Pre-infection	**Post-infection**			
A	26.50± 3.60	26.97± 3.32	-0.46	-0.69	0.49
B	32.47± 8.69	31.13± 6.24	1.35	1.04	0.30

Table 3. Comparison of mean PCV values of mice pre and 6 days post-infection of *T. evansi* type A and *T. evansi* type B stocks

***T. evansi* type**	**Stock**	**Mean and SD of PCV**		**Mean diff**	**t-calc**	**P-value**
		Pre-infection	**Post-infection**			
A	MCAM/ET/2013/MU/04	52.02±3.82	45.48±6.59	6.534	4.619	0.000
	MCAM/ET/2013/MU/09	52.98±3.65	52.08±2.70	0.896	0.966	0.033
	Total	**52.45±3.74**	**48.47±6.13**	**3.98**	**4.034**	**0.0001**
B	MCAM/ET/2013/MU/010	51.67±5.21	47.07±10.14	4.605	2.491	0.001
	MCAM/ET/2013/MU/014	52.33±2.95	49.5±3.96	2.833	3.146	0.003
	Total	**51.96±4.34**	**48.14±8.06**	**3.82**	**3.444**	**0.0008**

Clinical Signs of Mice Infected with T. evansi Strains

The major clinical signs observed in *T. evansi* infected mice were rough hair coat, paleness of mucus membrane of the eye, depression, shallow breathing, snoring sounds, abdominal breathing, nervousness with losing balance, in-coordination, recumbent with unable to stand and paralysis of hind limbs with walking by pulling (Figure 1).

Post-mortem and Histopathology Lesions of Mice Infected with T. evansi

Postmortem lesions of mice infected with *T. evansi* strains: After peak parasitemia (6 days' post-infection), mice in parasitemia (6 mice in each cage) and control group (6 mice) were euthanized and examined for postmortem lesions. The major gross pathological findings were highly enlarged spleen, enlarged and congested liver and slightly edematous and congested brain. Enlargement in size and increase in weight of spleen and liver from *T. evansi* infected mice was significantly higher compared to non-infected mice (Table 4).

Histopathology lesions of mice infected by *T. evansi:* Microscopic examination of tissues from mice sacrificed 6 days post-infection and/or died mice infected with *T. evansi* revealed various histopathological changes in the brain, kidney, spleen, and liver. In the brain, the most frequent and common microscopic abnormalities were edema and neuronal necrosis with shrunken angular neurons, acidophilic

cytoplasm and a contracted/pyknotic nucleus, which are characteristic of ischemic necrosis of neurons. Dilatation of the blood vessel lumen by trypanosome parasites, erythrocytes, proteins, lymphocytes and other inflammatory cells were also observed. The histological section of the kidney of mice infected with *T. evansi* revealed various degrees of tubular degeneration and necrosis with the accumulation of acidophilic proteinaceous material in the cytoplasm of epithelial cells of tubules. Some of the glomeruli were shrunken and degenerated/necrosis. Besides, the infiltration of mononuclear inflammatory cells and the accumulation of blood, hemosiderin pigment and clumps of parasites were also examined in the renal tubules and glomeruli.

Microscopic examination of spleen from infected mice revealed infiltration of activated macrophages, plasma cells and multinucleated giant cells in most of the sections of spleen tissue. Congestion and hemosiderosis with severe disruption of the structure of spleen were also observed. The histological section of the liver of mice infected with *T. evansi* showed that the centrilobular veins and sinusoids of the liver were distended with red blood cells, hemosiderin and clumps of trypanosome. Various degrees of fatty degeneration, coagulative necrosis and dissociation of hepatocytes surrounding the central vein were also noted. Hepatic cells lose their polyhedral shape and become relatively swollen and round.

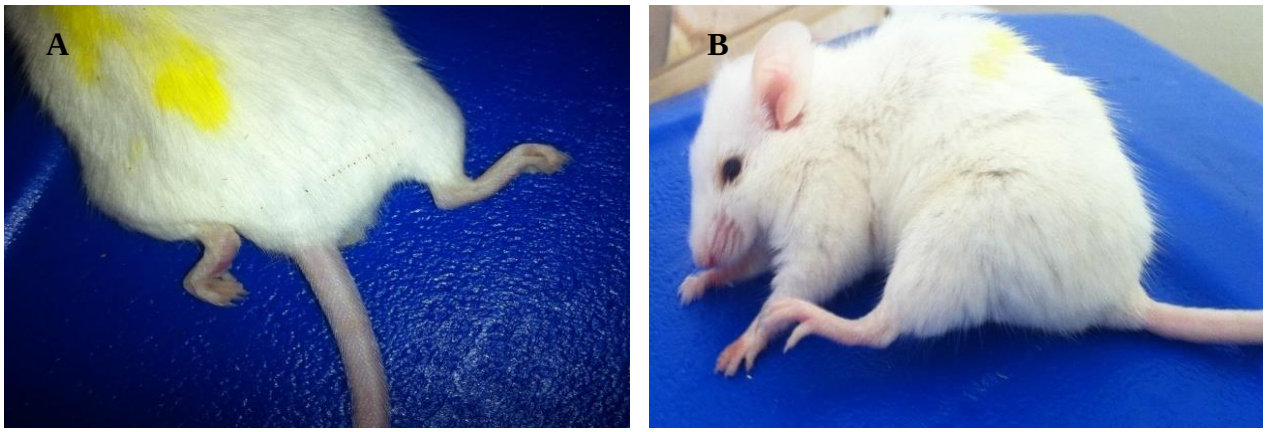

Figure 1. Mice infected by *T. evansi* type B MCAM/ET/MU2013/MU/010. (A). paralysis of the hind limbs and (B) nervousness and in-coordination.

Table 4. Weight of spleen and liver of mice infected by *T. evansi* type A and B compared with non-infected (control) mice

Organ	Mean and SD of postmortem organ weight (gram)			P-value
	T. evansi type A	*T. evansi* type B	Control	
Spleen	0.36 ± 0.06	0.36±0.14	0.12± 0.02	0.0002
Liver	2.28± 0.24	2.60±0.37	1.65± 0.10	0.0000

Discussion

Similar body weight, but lower PCV in mice infected with *T. evansi* type A and *T. evansi* type B stocks 6 days-post-infection agreed to Verdillo *et al.* (2012). The fluctuation observed in the bodyweight of mice infected with *T. evansi* type B may be due to loss of appetite (Verdillo *et al.*, 2012).

The major clinical signs observed in *T. evansi* infected mice were similar to previous reports (DeMenezes *et al.*, 2004; Bal *et al.*, 2012; Verdillo *et al.*, 2012). Interestingly, though they are non-specific, these clinical presentations are observed in domestic susceptible hosts such as camel, horse, swine and goat (Diesing *et al.*, 1986; Payne *et al.*, 1990; Silva *et al.*, 1995; Dargantes *et al.*, 2005; Tamarit *et al.*, 2010; Desquesnes *et al.*, 2013b).

The most common abnormalities on gross examination in *T. evansi* infected mice agreed with previously reported results such as splenomegaly, hepatomegaly, edema in brain, congestion in brain and edema and congestion in kidneys (Biswas *et al.*, 2001; Bal *et al.*, 2012; Verdillo *et al.*, 2012). Similarly, at necropsy *T. vivax* infected young zebu cattle were characterized by an enlarged spleen, enlarged lymph nodes, pneumonic and emphysematous lung, enlarged liver, and hemorrhages on the brain and intestine (Shimelis *et al.*, 2015).

In *T. evansi* infected mice, the histological section of the liver showed fatty degeneration, coagulative necrosis and dissociation of hepatocytes surrounding the central vein. Besides, congestion, hemosiderin and trypanosome parasite in the centrilobular veins and sinusoid of the liver were observed. These observations agreed with various previous studies (Biswas *et al.*, 2001; Bal *et al.*, 2012; Verdillo *et al.*, 2012). Liver injuries/abnormalities in trypanosome infection may be caused by the parasite itself or its toxic products and the presence of hemosiderin due to lysis of blood cells. Consumption of oxygen and glucose by trypanosomes for their multiplication lead to hypoxemic state as a result of which animal tissues/cells are deprived of oxygen and glucose and it results in degenerative and

necrotic changes in all the vital organs (Biswas *et al.*, 2001; Habila *et al.*, 2012).

The splenomegaly and other microscopic lesions observed in this and other studies are due to the fact that spleen is one of the sites where parasitized, senescent and other aberrant red blood cells are destroyed to remove erythrocytes from circulation. In addition, the presence of parasite and infiltration of inflammatory cells also contribute to the increase in the size of spleen (Biswas *et al.*, 2001; Bal *et al.*, 2012; Habila *et al.*, 2012; Verdillo *et al.*, 2012; Narnaware *et al.*, 2016). The microscopic lesions of kidneys include shrunken glomeruli infiltration of inflammatory cells around glomeruli and tubules, congestion, necrosis, tubular degeneration, cells filled with acidophilic proteinaceous material, blood and hemosiderin filled tubular lumens. These were in agreement with the findings in *T. vivax* infected zebu calf and *T. evansi* vertical transmission in camel fetus. Besides, edema and ischemic neuronal necrosis and dilatation of the blood vessel lumen by trypanosome parasites, erythrocytes, proteins, lymphocytes and other inflammatory cells were observed (Shimelis *et al.*, 2015; Narnaware *et al.*, 2016). Lesions of kidney and brains observed in this study might be associated with the damage of tissues by the presence of parasites in the brain and kidneys, hypoglycemia arising from reduction of glucose level and anemia/hypoxia (Batista *et al.*, 2007; Rodrigues *et al.*, 2009; Batista *et al.*, 2011; Habila *et al.*, 2012).

In general, mice experiment seems to be a good model to study the pathogenicity of *T. evansi* infection. However, we are not able to conclude whether the mouse model we used is a good model to study the comparative pathology of the different *T. evansi* strain infections. No variations were observed in PCV while gross and histopathologic lesions were difficult to compare. Therefore, differences in the pathogenicity of the two strains in the preferred host (camels) could not be concluded from the present study. To rule out the existence of variations in pathogenicity and clinical presentation of camels infected by *T. evansi* type A and type B, the experiment shall be repeated in camels. In a previous field study, camels infected with *T. evansi* type B showed a mean PCV of 22% as compared to a mean PCV value of 26% in camels infected by *T. evansi* type A. However, conclusions could not be made as the number of camels infected by *T. evansi* type B were only 4 and this difference could have happened by chance (Birhanu *et al.*, 2015).

Conclusion

Mice were used to study the pathogenicity of *T. evansi* type A and *T. evansi* type B. Similar pathology was observed but the model might not be a good one to conclude on comparative pathogenicity. For differential pathological study on host-trypanosome interaction between the two *T. evansi* types, we recommend further studies to be conducted in camels.

Conflict of Interests

The authors declare that they have no competing interests.

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