RESEARCH ARTICLE

Investigation of Drug Susceptibility in Rats Experimentally Infected with *Trypanosoma evansi* Isolated from Camels in Sudan

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ABSTRACT- A number of 18 adults male outbred albino rats, weighing between 133-137g were used to investigate the drug susceptibility of *Trypanosoma evansi* strain isolated from naturally infected dromedary camels in Umbadir area, North Kordofan State, Sudan. The rats were divided into 3 groups (C, D and F) of 6 animals each. Group C and D were infected intraperitoneally with *T. evansi* (Umbadir stabilate) with 1×10^4 *Trypanosome* for the inoculum. Group D rats were given quinapyramine sulphate (20 mg/Kg bwt) after parasitaemia was evident. Group F was left as healthy uninfected control for the stabilate. When parasite counts were one or more parasites per field, counting in haemocytometer were used for exact number of parasite per cubic millimeter using Neubaeur's counter. Parasites from tail blood were first fixed, stained and diluted in trypanosome diluting reagent. The parasites were diluted to the level that can be easily counted in WBC counting chamber in the haemocytometer. The total number of parasites was expressed as $\log_{10}$ number of parasites per ml of blood. The presence and degree of parasitaemia were determined daily for each rat by examining tail blood. The identity of the local stabilates of *Trypanosoma evansi* was confirmed through adopting PCR where primers that target the internal transcribed spacer one (ITS1) of the ribosomal DNA were used. There was significant reduction in serum glucose and potassium as well as significant increase in total protein, urea, calcium, albumin and cholesterol in group C. The Umbadir stabilate showed low mortality and high sensitivity to quinapyramine sulphate.

Key-words- Drug susceptibility, *T. evansi*, Dromedary camels, Sudan

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INTRODUCTION

Trypanosoma evansi has a wide range of hosts and is pathogenic to most of the domestic and laboratory animals. Besides causing *Surra* disease in all the principal species of domestic animals [1-2]. *T. evansi* is also highly pathogenic to laboratory animals (rats, mice and rabbits) [3-6]. In New Zealand, about 24 white cross breed rabbits were challenged with strain of *T. congolense*. The infections were characterized by intermittent pyrexia, undulating parasitaemia, anorexia and emaciation [7]. The major plasma biochemical changes included hypoglycaemia,

elevated total protein and plasma cholesterol. There were significant elevation of alkaline phospatase (ALP), aspertate aminotransferase (AST), total bilirubin and fluctuating changes in the levels of plasma alanine aminotransferase (ALT) and urea [7].

The rats infected with *Trypanosoma evansi* resulted into significant reduction in serum glucose and phosphorus; compared to significant increase in Glutamate Oxaloacetate Transaminase (GOT), Glutamate Pyruvate Transaminase (GPT) and total protein. Microscopically, the brain tissues of the infected rats revealed acute congestion of the meningeal capillaries, perivascular oedema, neuronecrosis (vaculation), gliosis and trypomastigotes in dilated capillaries. The lung revealed oedema, congestion, multifocal alveolar emphysema, hyperplasia of the peri-bronchiolar lymphoid tissues and haemorrhages. The spleen showed extensive haemorrhages, haemosiderosis and aggregation of histiocytes resulting in multinuclear giant cells formation. The kidneys showed acute congestion of the glomerular tufts [8].

MATERIALS AND METHODS

Ethics statement

The study protocol was approved by the Faculty of Veterinary Medicine, Sudan University of Science and Technology, according to their guidelines for sampling domestic animals in Sudan and in compliance with the animal welfare of Sudan.

Study area

The *Trypanosome* strain used in this study was isolated from a dromedary camel in Umbadir Area, North Kordofan State, Sudan, while the rest of the experiment events were carried out in the premises of the College of Veterinary Medicine, Sudan University of Science and Technology, Khartoum North, Sudan.

Preparation of the inocula

A strain of *T. evansi* originated from a naturally infected camel from Umbadir in North Kordofan State was used in this study. One albino rat was infected intraperitoneally with blood that was cryopreserved in liquid nitrogen, containing 1×10^4 parasites/animal to obtain a large amount of the parasite for blood inoculation of experimental groups.

Parasitemia in the inoculated rat was regularly monitored by collecting blood from the tail vein and analyzing it by light microscopy. Blood samples showing actively motile organisms with characteristic flagellar movement were considered as positive for the presence of *T. evansi*. At the peak of parasitemia, the rat was anesthetized with chloroform inhalation and with the help of a disposable syringe; blood was collected aseptically in EDTA anticoagulant by cardiac puncture. Using Neubaeur's counter the trypanosome titre was determined in order to be diluted to $1X10^4$ trypanosoma for the inoculums [9].

Experimental animals

Eighteen (18) adult male outbred Albino rats, weighing between 133 to 137 g were used in this study. The rats were divided into 3 groups (C, D and F) each containing 6 rats and were kept in a cage in the same environment with controlled temperature (25–30°C) and relative humidity around 60-70%.

Experimental design and grouping

The experimental rats were distributed into 3 groups of 6 rats each. Group C, the control group as infected with *T. evansi* (Umbadir stabilate) and left without treatment. Group D was infected with *T. evansi* (Umbadir stabilate) and was treated with the quinapyramine sulphate (20mg/kg bwt), after the parasite was seen (at the patency). Group F was uninfected healthy control for Umbadir Stock.

Trypanosome sub-inoculation

Sub-inoculation of the experiment group C and group D was carried out intraperitoneally with the use of a sterile insulin syringe. Rat blood containing 1×10^4 trypanosomes in 0.2 ml volume was inoculated in each rat individually at day zero. The numbers of inoculated flagellates were estimated by Neubauer Chamber and the dilutions to obtain the titre of the inoculum were made in sterile phosphate buffer saline with glucose (PSG).

Table 1. The experimental design of the Umbadir stabilate and protocol of treatment with Quinapyramine sulphate

Group	Stabilate	Parasite	Treatment protocol
C	Umbadir	*T. evansi*	Infected not treated
D	Umbadir	*T. evansi*	Infected and Treated with Q.S. (20mg/kgbwt)
F	Uninfected Healthy Control for Umbadir Stock		

Estimation of parasitaemia

All infected rats were bled daily, as recommended by Eisler *et al.* [10], from the tip of the tail for trypanosomes detection using the following parasitological diagnostic methods:

Wet preparation

A drop of blood was mounted on a microscope slide and covered with 22x22 mm glass cover slip. Counts of parasite per field or per preparation were determined.

Haemocytometer count

The presence and degree of parasitaemia were determined daily for each rat by examining tail blood. A drop (5 µl) of blood was collected from the tail and mixed with trypanosome counting reagent (45 µl). Parasitaemia was counted as for WBC count using Neubeaur counter and the result was designated as a number of parasites per ml of blood. Parasitaemia was counted using 40 × magnifications during the 64 days of experiment.

Drug Dosages

Quinapyramine sulphate was used at a dose rate of 20mg/kg bwt and dissolved in sterile water such that the required dose was contained in 0.2ml of water for each rat and then inoculated intra-peritoneally.

Biochemical analysis

Blood for sera was collected in plain containers from the retro-orbital plexus. Serum samples were collected at four days intervals and were kept at -20°C until needed for biochemical analysis. All parameters were measured using commercial kits (Spinreact S.A./S.A.U. Ctra. Santa Coloma, Spain), except the sodium and potassium where commercial kits (BioMed Schiffgraben 41, 30175 Hannover, Germany) were used. The values obtained were read with a spectrophotometer (Jenway 6305 U.V./vis. Spectrophotometer, UK) at appropriated wavelengths and the values were calculated using standard formulae [11].

Polymerase Chain Reaction (PCR)

DNA extraction

For DNA extraction from blood using protein precipitation method, 5 ml of blood were collected in EDTA tube, 300 µl of blood were placed in a 1.5 Eppendorf tube, then 1000 µl of RCLB were added and mixed by inversion and centrifuged at 9000 rpm for 5 min. The supernatant was discarded and the pellet (white blood cells) was washed with 1000µl of RCLB, then 300 µl of WCLB were added followed by 1 µl of proteinase k and the solution was incubated at 37 ^{o}C overnight. 100 µl of protein precipitation solution (6M NaCl) were added on the next day and mixed by vortexing gently and 200 µl of cold chloroform were added, centrifuged at full speed for 6 min, the equeous phase was then transfered into a clean Eppendorf tube. Double volume of cold Ethanol was then added to precipitate the DNA, centrifuged at 14000 rpm for 5 min, then the supernatant was poured off without disturbing the precipitate, washed with 70% Ethanol (600 µl), air dried at room temperature, resuspended in 100 µl of TE buffer or ddH_2O and then left to be dissolved. The DNA was stored in -20^{o}C.

Amplification

The extracted genomic DNA was subjected to a PCR that amplifies the ITS1 region of the rDNA gene of *T. evansi* by using TeRoTat 920 F 5'-CTGAAGAGGTTGGAAATGGAGAAG-3' and TeRoTat 1070 R, 5'-GTTTCGGTGGTTCTGTTGTTGTTA-3' primers set. To obtain the expected 151 bp PCR product, (maxime PCR premix kit) Master Mix, 2X in a 20 µl total volume was deployed. Each reaction includes, 1 µl of 10 mM of each primer, 4 µl extracted DNA and 14µl of ddH_2O. Thermocycling profile that starts with an initial hold for 2 min. at 95°C, followed by 35 cycles of 95°C for 30 sec., 64°C for 30 sec. and 72°C for 1 min and final extension step of 5 min at 72°C was adopted. PCR products were subjected to electrophoresis in 2% agarose (Vivantis Technologies, Malaysia) in Tris-borate EDTA buffer and was visualized under UV light. The ITS1-PCR detection method constituted a powerful molecular diagnostic tool for *T. evansi* detection as well as discrimination from other trypanosomes in one PCR.

Data analysis

Data were presented as mean ± standard error of mean (SE). The statistical analysis was performed using independent T- test and Statistical Package for the Social Science (SPSS) software. P-values less than 0.05 were considered statistically significant.

RESULTS

The overall mean of parasitaemia

The overall mean of parasitaemia in group C was 5.9 ±2.01 while in group D, it was 0.07 ±0.36 (Table 2).

Table 2. Overall means and Std. Deviation of parasitaemia levels in rats infected-not-treated (C group) Umbadir stabilates and rats infected-treated (D group)

Treatment	Strains	Mean	Std. Deviation	N
Not treated	Umbadir	5.9	2.01	61
Treated	Umbadir	0.07	0.36	61

The response of Umbadir stabilate to Quinapyramine Sulphate in group (C)

Rats inoculated by 1X10^4 of Umbadir stabilate of *Trpanosoma evansi* but were not treated with Quinapyramine Sulphate (group C) inflicted low mortalities during the experiment period where one died at day 30 post infection (pi), one at day 50, one at day 51 and one at day 54, with a mean survival period of 46.3±11 (Table 3).

Table 3. Comparison between rats infected with *T. evansi* (Umbadir Stabilate) treated by Quinapyramine Sulphate at a dose rate of 20mg/kg bwt (after patency Group D) and rats infected-not-treated control (Group C)

Time to death	Control of 6 Rats	Infected Treated of 6 Rats
Day 30	1 rat n= 5 rats	All rats survived until the end of the study period
Day 50	1 rat n=4 rats	
Day 51	1 rat n=3 rats	
Day 54	1 rat n= 2 rats	
X=46.3 ±11		

The response of Umbadir stabilate to Quinapyramine Sulphate in group (D)

All rats survived until the end of the study period. Treatment of rats in group (D) was commenced at day 4 when the parasitaemia level was $\log_{10}$ 2.2. By day 5, all treatment group were negative. Treated rats remained negative until the end of the study period. Up to day 4, there was no significant difference between parasitaemia levels in both treated and control groups. By day 18, the treated group recorded a mean parasitaemia of $\log_{10}$ 0 while that of the control was $\log_{10}$ 6.8 which was significantly higher than the treatment group ($p<0.05$). In the control rats, by day 31, the parasitaemia fluctuated between $\log_{10}$ 7.3 to $\log_{10}$ 8.0 until the end of study period (Fig. 1).

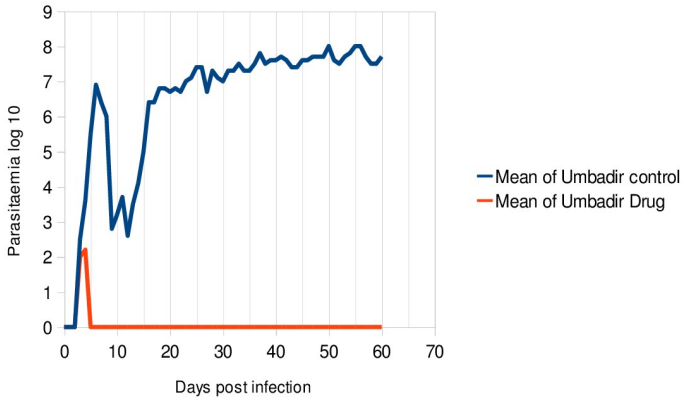

Fig. 1. Comparison of the means of parasitaemia levels ($\log_{10}$) between rats infected with *T. evansi* (Umbadir Stabilate) treated by Quinapyramine Sulphate at a dose rate of 20mg/kgbwt and rats infected-not-treated control

Serum biochemical changes

Serum total protein

The mean serum values of total proteins in group C were increased during the study. The statistical analysis showed a means of 8.2±1.9 g/dl while group D has showed no changes at all days of the experiment. The statistical analysis showed a means of 6.4±0.84 g/dl (Table 4). The normal ranges of some serum biochemical parameters of rats are showed in Table 5.

Table 4. Mean serum levels of biochemical changes in rats infected with *T. Evansi* infected-not-treated control and infected-treated with Quinapyramine Sulphate at a dose rate of 20mg/kg bwt

Parameters	Units	Group C	Group D
Total proteins	g/dL	8.2±1.9	6.4±0.84
Glucose	mg/dL	45±18.4	74.9±19.8
Urea	mg/dL	29.8±8.3	19.6±1.7
Albumin	g/dL	4.7±0.88	4.3±0.54
Calcium	mg/dL	10.7±5.8	8.7±3.3
Cholesterol	mg/dL	118.6±21.6	98.8±24.8
Sodium	mEq/L	147.6±4.7	148.8±3.5
Potassium	mEq/L	4.4±1.3	6±0.62

Values were expressed as Mean±SD

Table 5. Rat Biochemical Reference Normal Ranges

Parameters	Ranges Values	units
Total proteins	5.6 -7.6	g/dl
Glucose	50 – 135	mg/dl
Urea	15 – 21	mg/dl
Albumin	3.8 - 4.8	g/dl
Calcium	05/03/13	mg/dl
Cholesterol	40-130	mg/dl
Sodium	143 - 156	mEq/l
Potassium	05/04/13	mEq/l

Serum glucose

The mean serum values of glucose in group C were decreased. The statistical analysis showed a means of 45±18.4 mg/dl while group D was normal at all days of the experiment. The statistical analysis showed a means of 74.9±19.8 mg/dl (Table 4).

Serum Urea

The mean serum values of urea in group C were increased during the study. The statistical analysis showed a means of 29.8±8.3 mg/dl. While group D were showed no changes at all days of the experiment. The statistical analysis showed a means of 19.6±1.7 mg/dl (Table 4).

Serum Albumin

The mean serum values of albumin in group C were increased. The statistical analysis showed a means of 4.7±0.88 g/dl. While group D were normal at all days of the experiment. The statistical analysis showed a means of 4.3±0.54 mg/dl (Table 4).

Serum calcium

The mean serum values of calcium in group C were increased. The statistical analysis showed a means of 10.7±5.8 mg/dl while group D was normal at all days of the experiment. The statistical analysis showed a means of 8.7±3.3 mg/dl (Table 4).

Serum cholesterol

The mean serum values of cholesterol in group C were elevated during the study. The statistical analysis showed a means of 118.6±21.6 mg/dl while group D was normal at all days of the experiment. The statistical analysis showed a means of 98.8±24.8 mg/dl (Table 4).

Serum sodium

The mean serum values of sodium in group C and group D showed normal levels at all days of the experiment. The statistical analysis in group C showed a means of 147.6±4.7 mEq/l while group D showed a means of 148.8±3.5 mEq/l (Table 4).

Serum potassium

The mean serum values of potassium in group C were decreased. The statistical analysis showed a means of 4.4±1.3 mEq/l. while group D was normal at all days of the experiment. The statistical analysis showed a means of 6±0.62 mEq/l (Table 4).

Confirmation of the identity of the test Trypanosoma by PCR

The stabilate of Trypanosoms used in this study were confirmed to be *Trypanosoma evansi* by PCR using specific primers that specifically target the ITS1 region of the rDNA gene of *T. evansi.* Using this specific technique, the DNA extract from whole blood of rat infected with the *Trypanosome* yielded an amplicon of the size 151 bp; a PCR product size expected for this species of the *Trypanosome* (Fig. 2).

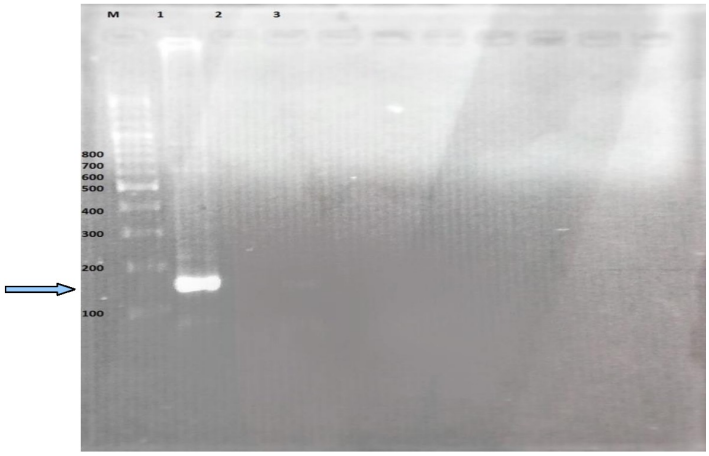

Fig. 2. The resultant amplicon of the ITS1 region of the rRNA gene of *Trypanosoma evansi* as (arrow) 151 bp detected in the DNA harvest of the whole blood of rats infected by this species of Trypanosome in samples 1

DISCUSSION

In this study of Umbadir stabilated *T. evansi* which was isolated from a camel at Umbadir, North Kordofan, Sudan (named as Umbadir stock; sensitive to Quinapyramine Sulphate) were investigated and studied. During this study, the local isolate of *T. evansi* stock was compared in experimentally infected rats. Rats inoculated by $1X10^4$ of the Umbadir stabilate of *T. Evansi*, but were not treated with Quinapyramine sulphate (group C), showed a prepatent period of 3-4 days post infection which is similar to the result reported by Da silva *et al*. [12]. The low mortalities recorded during the experiment in group C were in agreement with Faye *et al*. [13] while Desquesnes [14] reported that *T. evansi* (Sokoto isolate) was pathogenic to donkeys with low mortalities and high morbidity (100%). The result also was in agreement with those reported by Njiru *et al.* [15] and Tekle and Abebe [16] who encountered low mortality and high morbidity among camels infected with *T. evansi* in Ethiopia. In the rats infected-treated with Quinapyramine Sulphate after the patency at a dose of 20 mg/kgbw (D group), a perpatent period of 3-4 days post infection was recorded which is the similar to the result reported by Da silva *et al*. [12] where all rats remained negative and survived until the end of the study period which was attributed to the effect of the drug used.

The increase in serum urea in group C is in agreement with the result reported by Sivajothi *et al*. [17]; Ajakaiye *et al.* [18]; Arora and Pathok [19] and Samia *et al*. [20] who found a similar increase in the concentration of urea in rats experimentaly infected with *T. evansi.* Megahed *et al*. [21] reported similar results when they found that the concentration of urea was increased in pregnant camels infected with *T. evansi* compared to healthy pregnant camels. More studies had similar results among which those conducted in *T. b. brucei* infected rabbits [22] and goats [23] and *T. gambiense* infected vervet monkeys [24]. The elevated serum urea levels had been associated with kidney diseases such as glomerulonephritis, urinary tract obstruction and excessive protein catabolism associated with severe toxic and febrile conditions [25].

In the present study, the increase in the serum calcium in group C was similar to the result of the study conducted in sheep infected with *T. congolense* [26]. However, the levels of calcium were not changed in camels infected by the *Trypanosome* parasite as reported by Chaudhary and Iqbal [27] and Schenk *et al.* [28]. On the other hand, the result reported by Egbe-Nwiyi *et al*. [29] showed that the level of calcium was decreased in the rats infected with *T. congolense.*

In the present study, the serum sodium levels in the group C were found normal unlike the result reported in sheep infected with *T. congolense* where it increased as reported by Tella [30]. The same also was found with the result reported by Arora and Pattok [19]; Samia *et al.* [20] and Wolkmer *et al.* [31] who found that the concentration of sodium was depressed in rats experimentally infected with *T. evansi.*

In the present study, the serum potassium levels in group C were decreased which is in line with the result reported in sheep infected with *T. congolense* where it decreased as reported by Tella [30] as well as in sheep infected with *T. brucei* [32]. Moreover, Arora and Pattok [19]; Samia *et al.* [31] also found that the concentration of potassium was depressed in rats experimentally infected with *T. evansi,* unlike the result obtained by Ikejiani [33] who found that serum potassium levels increased in *T. equiperdum* and *T. brucei* infection of rats; and also with the result reported by Moon *et al*. [34] in *T. rhodesiense* infected mice which had the normal level of potassium.

In the present study, the serum cholesterol in group C was

increased which is in agreement with the findings reported by Megahed *et al*. [21] who found that the concentration of cholesterol has increased in pregnant camels infected with *T. evansi* compared with healthy pregnant camels. Similar result was also reported by Sivajothi *et al*. [17] who found that cholesterol was increased in rats infected with *T. evansi.* However, the result reported by Egbe-Nwiyi *et al*. [29] in rats infected with *T. congolense* and that reported by Barghash [35] in rats infected with *T. evansi*, both showed that the level of cholesterol was decreased which is not in line with our findings in this study.

The serum total proteins in group C were increased progressively during the study which disagreed with the results reported by Hussain *et al*. [36]; Sivajothi *et al*. [17]; Biryomumaisho *et al*. [37]; Katunguka-Rwakishaya [38], Allam *et al.* [39] and Megahed *et al.* [21]. Moreover, the result recorded in this study had contradicted the observations recorded in sheep infected with *T. brucei* studied by Taiwo *et al.* [40]. This increase of total protein was in agreement with the result reported by Arora and Pathok [19] and Samia *et al*. [20] who found that the concentration of total protein was increased in rats experimentally infected with *T. evansi.* Also, the increase in serum total proteins recorded in this study was in agreement with the result reported by Orhue *et al*. [41]; Ekanem and Yusuf [42] and Sow *et al*. [43], who found that the concentration of total protein was increased in rats experimentally infected with *T. brucei*. and *T. brucei*-infected rabbits. The increase in protein levels during the chronic phase of the infection is usually due to the increase in globulin levels, as a result of the immune response by the animals to the infection [44-46]. In the present study, the serum glucose in group C has decreased during the study, which is in line with the result reported by Sivajothi *et al*. [17]; Sinha *et al*. [47]; Arora and Pathok [19] and Samia *et al.* [20] who found that the concentration of glucose was decreased in rats experimentally infected with *T. evansi.* This situation could be explained by the parasites' need for glucose for their cellular metabolism through their glycolytic pathway [48]. However, this finding was not in agreement with that reported by Youssif *et al*. [49] who found that goats infected by *T. evansi* had increased level of glucose.

The increase of serum albumin reported in group C disagrees with the results reported by Arora and Pattok [19] and Samia *et al*. [20] who found that the concentration of albumin was depressed in rats experimentally infected with *T. evansi.* Also that result reported by Megahed *et al.* [21] found that the concentration of albumin was decreased in pregnant camels infected with *T. evansi* compared with healthy pregnant camels and, also, a decrease of albumin in camels infected by *T. evansi* was further reported by Hussain *et al.* [36].

The further confirmation of the identity of the candidate trypanosome by PCR through using primers that specifically targeted the ITS1 region of the rDNA gene of *T. evansi* that is performed in the present study, is similar to the result reported by Croof [50] who used molecular method (PCR) in his study of 40 camels which were tested parasitologically and serologically where 90% of them were found to be positive. PCR has been used in detection of infection with *T. evansi* in buffaloes [51-52], in horses [53] and in camels [54]. There was no comprehensive data on the use of PCR for detection of infection in Sudanese breed of dromedary camels (*Camelus dromedarius*). Hunter [55] and Aradaib and Magid [56] suggested the use of the reliable, easy to perform and less time-consuming PCR for accurate classification of trypanosome species in Sudan where the morphological feature of the trypanosome is the main tool used for its classification.

CONCLUSIONS

18 adult male outbred albino rats were used to investigate the drug susceptibility of *Trypanosoma evansi* strain isolated from naturally infected dromedary camels in Sudan. The rats were divided into 3 groups (C, D and F). Group C and D were infected intraperitoneally with *T. evansi* (Umbadir stabilate) with 1×10^4 trypanosoma for the inoculum. Group D rats were given quinapyramine sulphate (20 mg/Kg bwt) after parasitaemia was evident. Group F was left as healthy uninfected control for the stabilate. Parasites from tail blood were first fixed, stained and diluted in trypanosome diluting reagent to the level that can be easily counted in WBC counting chamber in the haemocytometer. The total number of parasites was expressed as $\log_{10}$ number of parasites per ml of blood. The presence and degree of parasitaemia were determined daily for each rat by examining tail blood. The identity of the local stabilate of *Trypanosoma evansi* was confirmed through adopting PCR where primers that target the internal transcribed spacer one (ITS1) of the ribosomal DNA were used. There was significant reduction in serum glucose and potassium as well as significant increase in total protein, urea, calcium, albumin and cholesterol in group C. The strain used in the study (Umbadir stabilate) showed low mortality and high sensitivity to quinapyramine sulphate.

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