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Tissue Enzymes as Markers of Cardiac Damage during *Trypanosoma brucei rhodesiense* Infection of Sheep

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Abstract: ***This study was done to determine markers of cardiac and CNS involvement during HAT using sheep where eight sheep were intravenously inoculated with 1 × 10[4] T. b. rhodesiense. Two were uninfected. Jugular blood vein samples were used for parasitological analyses and determination of serum concentrations of total protein, LDH and CK and their isoenzymes. There was an increase in total protein, total LDH and total CK activities in both serum and CSF. A flip over pattern LDH1:LDH2 ratio was observed. It was concluded that serum LDH1/LDH2 ratio, has a potential diagnostic value in determination of cardiac damage.***

Keywords: Trypanosomiasis, LDH, CK, CSF, Serum

1. Introduction

Tissue enzymes have been used as sensitive diagnostic markers of CNS infection involvement during bacterial meningitis [1] and also in determining the severity of CNS infection [2]. Indeed CK BB isoenzyme produced in the CNS during disease is able to cross the BBB and therefore can be measured in blood thus enabling the avoidance in the used of CNS invasive procedures to collect cerebral spinal fluid (CSF) in the detection of brain tissue damage [3; 1]. In addition CK and LDH have been used in other disease conditions to determine cardiac involvement [4;5]).

2. Literature Review

Increases in body fluid levels of the tissues enzymes creatine kinase (CK) and lactate dehydrogenase (LDH) have been demonstrated in trypanosome infected animal models. Indeed increases have been demonstrated during experimental animal trypanosome infections [6;7] and infected humans [8]. There is therefore a need to develop a cost-effective model for studies on *T. b. rhodesiense* CNS trypanosome infection and also evaluate the possible use of tissue enzymes CK and LDH as reliable enzymatic markers of heart lesion and staging markers in CNS trypanosome infection.

A good number of HAT patients during the haemolymphatic and also the CNS stage have cardiac damage due to destruction of myocardial cells [9;10] resulting in the loss of membrane integrity leading to enzymes diffusion to extra cellular spaces and drainage into blood vessels. The appearance in the serum of the various cytoplasmic enzymes has formed the basis of a variety of clinical diagnosis procedures for the detection of tissue damage of the heart and CNS using CK and LDH, respectively [11;1]. In *T. b. rhodesiense* infection, its more acute course, pancarditis with congestive heart failure pericardial effusion and pulmonary oedema can cause fatalities at the early stages [12]

3. Statement of the Problem

Currently used markers of cardiac damage during disease are expensive and not easily adaptable to field conditions at the same time this procedure requires qualified staff to perform. Thus there is a need to identify cardiac and CNS tissue specific markers that are sensitive, specific, reliable and easy to perform at the point of trypanosoma infection care. Serum biochemical changes such as total and/or isoenzymes profiling of tissue enzymes have been shown to be more sensitive and reliable, since the levels increase with minimal tissue damage, does not need very qualified staff to assay and is reliable. A number of diagnostic methods like demonstration of trypanosomes, increased white cell count and protein levels in CSF exist for the CNS trypanosome disease. However, these methods have challenges and drawbacks especially the unreliability of white blood cell count and protein analysis methods. Both white blood cells and proteins only increases in later stages of CNS involvement and show wide range of variation. The collection of CSF requires a lumber puncture procedure which is a painful and patient unfriendly in addition to posing ethical considerations.

4. Materials and Methods

Experimental Animals: Ten male indigenous Maasai breed sheep aged two years and weighing between 15 and 27kg were acquired from Naivasha district-Kenya a trypanosomosis free area were used for the study. The sheep were initially housed in a fly proof house as part of quarantine for 30 days for purposes of their acclimatization. During that time the animals were de-wormed using albendazole® at a dose of 25gm/kg bwt and sprayed with acaricide amitraz (triatic®) to ensure that they were free from endo-parasites and ecto-parasites. The sheep were fed twice daily on ratio consisting of hay plus minerals and protein supplements. Water was provided *ad libitum*. Each of the sheep was ear-tagged for identification and transferred to experimental wards to acclimatize for a further two

weeks. During the acclimatization period the animals were weighed weekly and rectal temperature recorded on a daily basis.

Trypanosomes and Preparation of Inoculum: *T. b. rhodesiense* (KETRI 2537) was used in this study. The parasite was a derivative of EATRO 1989 which was isolated from an infected human patient in Uganda and prepared by the direct inoculation of blood and lymph node aspirate into mice [13] and later cryopreserved. The trypanosomes from the cryobank were propagated in cyclophosphamide immunosuppressed donor mice at a dose of 300mg/kg bwt intra-peritoneally for 3 consecutive days [14] before being used to infect the sheep. The stabilate from the Trypanosomiasis Research Centre (TRC) cryobank was thawed and inoculated in three immunosuppressed donor mice. The donor mice were monitored daily for parasitaemia in their blood using tail snips followed by the examination of the blood under the light microscope. At the first peak of parasitaemia, the blood was obtained by cardiac puncture from the mice and the blood diluted in phosphate-buffered saline to a concentration of $1x10^4$ trypanosomes per ml and was used to intravenously (IV) infect eight animals. Two sheep were not infected and served as control.

Following infection of the 8 sheep with trypanosomes all the sheep were monitored on a daily basis and were treated intravenously with 5mg/kg bwt diminazene aceturate (Veriben®) at 42 dpi when the animals showed deterioration in their health status. Thereafter the sheep were monitored for a period of 140 days when the experiments was terminated.

Blood sampling for parasitological analyses: Beginning day one post infection, blood samples from each infected sheep was daily collected from the ear vein into heparinised capillary tubes, centrifuged in a microhaematocrit centrifuge (Hawksley, England) and the buffy coat examined for the presence of parasites [15]. While 2 mL of blood sample from each infected sheep was collected into an EDTA containing vacutainer for the estimation of parasitaemia levels using a rapid approximation method [16].

Blood sampling for determination of serum protein, LDH and CK profiles: 10 mls blood samples each from each sheep in the infected and the uninfected group was collected aseptically from the jugular vein at pre-determined time interval namely weekly up to 42 dpi then every two weeks up to 70 dpi but thereafter every 3 weeks up to the end of the experimental period of 140 dpi. Serum for protein and enzyme assays was prepared by allowing whole blood to coagulate for thirty minutes at room temperature followed by another twelve hours at 4^0C, after which it was separated by centrifuging at 3000 revolutions per minute (rpm). Serum samples harvested were then dispensed into labelled sterile screw-capped vials and stored at -80^{o}C until required for analyses.

CSF sampling for determination LDH and CK profiles: CSF samples were collected every two weeks from each animals anaesthetized using a mixture of diazepam (Rotexmedica, Trittau Germany) and ketamine hydrochloride (Rotexmedica, Trittau, Germany) at a dose of 1mg/kg bwt and 10-15mg/kg bwt, respectively. A volume of 1-2ml of free flowing CSF was obtained aseptically by lumber puncture from lumber vertebral spaces 1 or 2 using a sterile 23 gauge needle [17]. At the same time two drops of free flowing CSF was collected into a capillary tube and the content immediately transferred onto a haemocytometer chamber and presence of trypanosome noted. If trypanosomes were not seen in the counting chamber, approximately 1ml of CSF was collected into a pipette whose tip had been sealed by heating. The content was then centrifuged and the sealed end of the tube examined for trypanosomes using a microscope as described by Gould and Sayer [18].

Biochemical Analyses

Total Serum and CSF protein determination: Total serum protein levels from individual 10 sheep on different date of sampling were determined by the biuret method [19]. Absorbencies were measured at 540nm using an ultra violet (UV) visible spectrophotometer (Cecil-CE 2021-2000 series, England). Bovine serum albumin (BSA) (BIO RAD) was used as a standard

Total Serum and CSF LDH determination: The total LDH activity of both the infected and the control sheep was measured specrophotometrically using commercial diagnostic kit "Lactate dehydrogenase NAC-Kinetic" (Chrono Lab, Switzerland) in a UV-visible spectrophotometer (Cecil CE 2021-2000 Series, England) by measuring by a decrease in absorbance at a wavelength of 540 nm.

LDH Isoenzyme Analyses: Serum sample aliquots from each sheep at different points during infection were separated by subjecting the samples to horizontal electrophoresis on a 12% starch gel. Electrophoresis was carried out at 10^0C for 3 hours at 350 volts, 70 milliamps (mA) using 70ml tris buffer. Isoenzyme bands on the electrophoretic gel were located using a modified method described by Tanzer *et al.* [20]. The stained gels were then incubated at 37^0C in the dark for one hour or until sufficient activity was present indicated by bluish purple colour change of the bands. The isoenzyme bands on the gels were then scanned using a mustek1248B scanner (Mustek Systems, Inc, China) and saved on a computer for later quantification. The proportion of the individual isoenzyme bands were determined with a UN-SCAN-IT gel software program(Silk Scientific, Inc. Utah USA) on a gel and graph digitizing software windows edition version 6.1 which works like a gel densitometer.

Total Serum and CSF CK determination: Total CK for both CSF and serum samples was undertaken in UV-visible spectrophotometer using a CK NAC-Kinetic AG diagnostic kit (Chrono Lab, Switzerland) based on a modified method by Oliver [21].

Statistical Analysis

Biochemical analyses data obtained from the infected and uninfected sheep were done using Microsoft excel 2007 data analysis tool. T-test was used to determine the significant differences between the means of infected and uninfected

groups of all the parameters. Significance was considered at $p< 0.05$. Data are presented as Mean ±standard error of mean (SEM).

5. Results

Parasitaemia: The infected sheep developed parasitaemia at different times post infection .The first sheep was blood positive 4dpi while all infected sheep were trypanosome positive in blood by 7 dpi. This gave a mean pre-patent period of 5.12 dpi with a range of 4-7 days. On the other hand the eight sheep infected with trypanosomes, only two sheep were consistently CSF positive during infection period before treatment while the rest were intermittently positive in CSF by direct microscopy or following centrifugation at different point pre-treatment.

Serum total protein levels: The changes in the mean serum total protein level in infected and un-infected control sheep are presented in Figure 1. The mean pre-infection total protein level was 102±2.25 mg/l and 103.57±14.55mg/l for the infected and uninfected sheep, respectively. Following infection the mean serum total protein level rose to a peak of 124.18±1.24mg/l at 7dpi. Thereafter the mean total protein level declined markedly to reach lowest value of 93.12±0.8mg/l on 28 dpi. After the low levels on 28 dpi, the levels started to increase gradually towards the un-infected values of 104.40±2.4 at 56 dpi. Thereafter, the mean total protein levels started to gradually decline from 107.11±2.77 by the 70 dpi reaching levels of 99.44±2.91mg/l at 126 dpi.

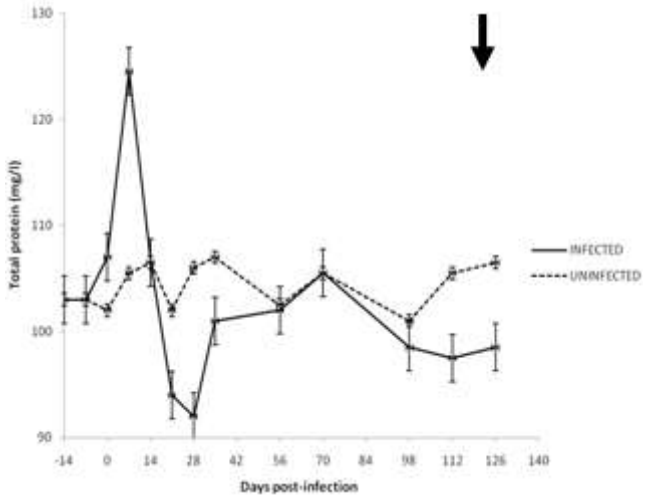

Figure 1: Mean serum total protein concentrations (±SEM) in *T. b. rhodesiense* infected sheep and uninfected control. ↓ = Treatment day of the infected sheep with diminazene aceturate

The mean CSF protein levels of infected and uninfected control sheep as shown in Figure 2. The mean CSF protein levels of pre-infected sheep and control group at 0 dpi were 1.01±0.22 mg/l and 1.08±0.04 mg/l, respectively. Following infection, the CSF protein levels in the infected group of sheep increased from 7 dpi to a first peak of 2 gm/ml at day 21 dpi. Thereafter, the levels declined to 0.91±0.17mg/l by 42 dpi when the sheep were treated with diminazine aceturate. Following treatment the protein levels increased significantly to give a second peak of 2.5±1.42 mg/l at 56dpi. The protein levels however declined to control levels by day 70 dpi at which they remained except for the minor fluctuations up to the end of the experimental period.

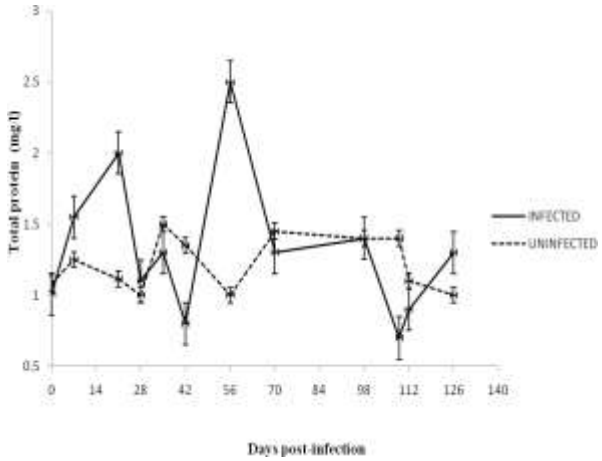

Figure 2: CSF protein (±SEM) levels in sheep infected with *T. b. rhodesiense* and uninfected control. ↓ = Treatment day with diminazene aceturate.

Serum Biochemical Profiles

Serum total LDH enzyme levels: The mean total LDH enzyme activity in infected and control sheep serum is presented in figure 3. The LDH activity in the serum of infected sheep increased significantly from 14 dpi to reach the first peak on 28 dpi. However this increases showed fluctuations resulting in several peaks on 28, 56 and 98 dpi, respectively. The LDH values were significantly higher ($p<0.05$) in infected sheep from 14 dpi when compared to the control group.

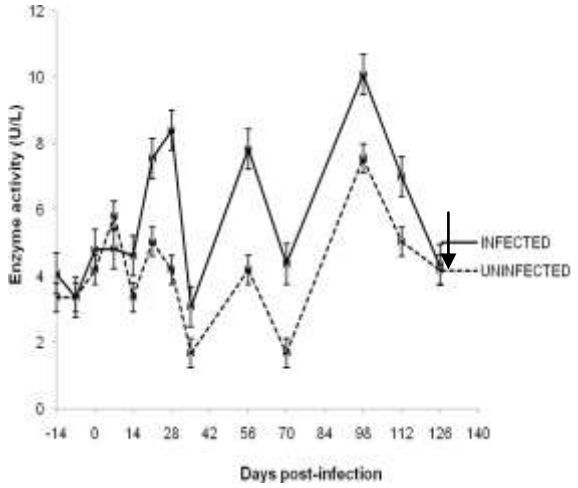

Figure 3: The mean serum total LDH enzyme activity in *T. b. rhodesiense* infected and control sheep

Serum LDH isoenzymes pattern

Following electrophoresis of sheep serum and incubation of gels with the substrate, only four distinct bands were visible corresponding to bands of LDH1, LDH2, LDH3 and LDH4 with the fractions occurring in the order of migration farthest towards the anode. However, only two bands corresponding to LDH1 and LDH2 out of the four LDH isoenzymes observed were clearly visible as strong bands. The respective band intensities were directly proportion to enzyme activity in the serum samples at a given day after infection as observed in UN-SCAN IT densitometry. LDH 1

was more pronounced in intensity at the early stages of infection while LDH2 became more intense at later stages of infection as tabulated in table 1 and plotted in figure 4

Table 1: The % and LDH1/LDH2 ratio in serum of sheep infected with *Trypanosoma b. rhodesiense*

Day after infection (dpi)	LDH1 Concentration (%) (±SEM)	LDH2 Concentration (%) (±SEM)	LDH1/LDH2 ratio
0	28.3 ±1.4	36.4 ± 1.3	0.77
7	35.28 ±1.2	33.89 ±0.4	1.04
14	27.49 ±1.4	25.39 ±1.8	1.08
21	34.76 ±0.7	34.74 ±0.8	1.03
28	36.79 ±0.6	35.66 ±0.3	0.97
35	29.69 ±2.3	49.49 ±3.0	0.59
56	45.87 ±2.3	53.7 ±1.3	0.85
70	19.5 ±1.1	22.1 ±1.4	0.88
98	14.5 ±0.6	28.88 ±4.2	0.50
112	30.11 ±2.8	29.17 ±3.2	0.10
126	16.05 ±0.72	16.07 ±0.7	0.98

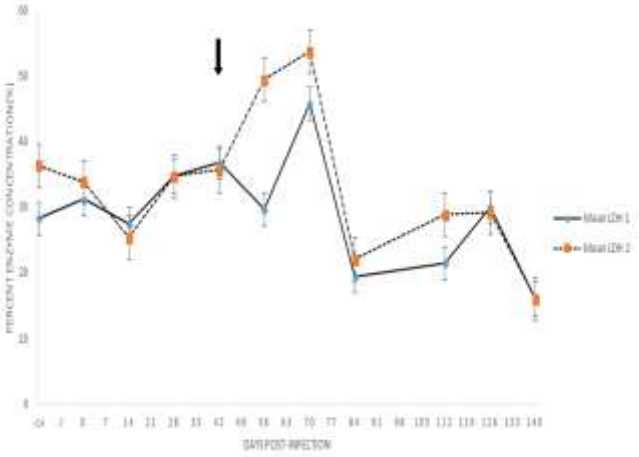

Figure 4: percentage concentrations (%±SEM) of serum LDH1 and LDH2 following infection of sheep with *T. b rhodesiense*. ↓ =Treatment day with diminazine aceturate

Mean serum total Creatine kinase levels

The mean total serum CK enzyme activity in infected and control sheep are presented in figure 5. The mean total serum CK levels in infected sheep increased from 22U/L on 56 dpi to reach peak levels of 102U/L on 98 dpi. Thereafter the levels declined to reach control levels 112 dpi at which they remained till termination of the experiment. The CK enzyme levels in infected sheep were significantly different ($p<0.05$) from control between 70-105 dpi.

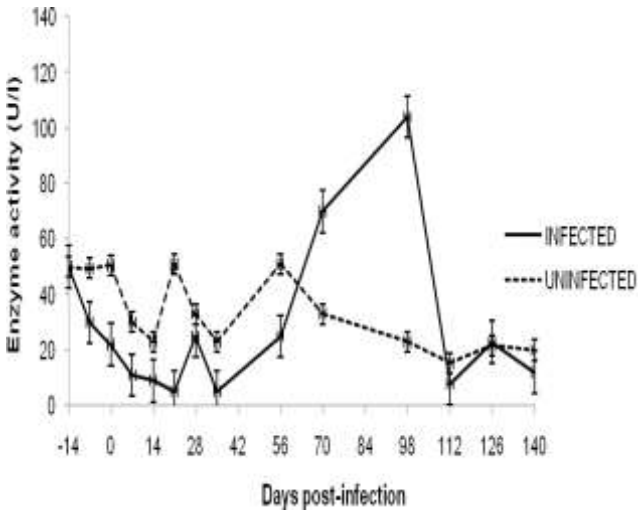

Figure 5: Mean total serum CK enzyme activity in *T. b. rhodesiense* infected and control sheep

6. Discussion

The sheep were parasite positive with human infective trypanosome on the 4dpi. The parasitaemia levels were characteristic of the KETRI 2537 stabilate as observed in monkey [22]. The fluctuation in parasitaemia levels are similar to those observed in humans [23] and rodent animal models infected with trypanosomes [24]. The parasitaemia fluctuation are as a result of immune response to the trypanosomes glycoprotein coat (VSG/clones) [25]. The IgM then produce an immune destruction of trypanosome resulting to a decline in parasitaemia.

The results on parasitaemia showed that not all sheep were not consistently CSF positive. This observation of intermittent appearance of trypanosome in CSF was similar to observations made by Thuita *et al.*, [26]. Trypanosomes are infrequently seen in CSF even when a concentration technique such as double centrifugation is used to improve sensitivity detection [12]. Indeed improve diagnostic sensitivity of trypanosome detection in CSF has also been demonstrated in humans by concentrating trypanosomes by centrifugation using microhaematocrit centrifugation [27]. Trypanosomes have also been demonstrated to appear in a cyclical manner in CSF of rats [28], mice [29] monkey [30;17] and in human [31] infected with trypanosomes.

Although trypanosomes appear in CSF early, establishment of infection in the CNS does not occur at those early times. These trypanosomes that appear in the CNS do not survive and establish CNS infection until later on. This has led to the conclusion that even when trypanosomes cross the blood brain barrier they do not lead to brain infection [28]. Indeed others studies have shown that trypanosomes injected into the CSF cannot permanently settle in the CSF [32; 28] and are thus easily cleared. Other studies have also shown that when trypanosomes are directly placed intrathecally into the striatum, they did not lead to a manifest of infection for until 14 day whereas those placed in the ventricle system did [32], thus suggest that certain changes have to first occur in CNS/CSF before trypanosomes can establish a permanent infection.

In the present study, increase in total protein levels observed have been reported in other animal models of trypanosomosis including rabbits infected *T. brucei* [33], rats infected with *T. brucei* [34], goats infected with *Trypanosoma congolense* [35;36] and monkeys [37]. The same has been observed in *T. b. rhodesiense* infection of man [38]. The initial increase in total protein could be due to increase in the immunoglobulins/gammaglobulinaemia which is a prominent feature of trypanosomosis primarily due to increase in IgM. Indeed trypanosome antigen stimulation and antibody production especially of IgM [39-41] is consistent with the reports of other studies [39;42]. Another possible cause in the increase of the total protein levels could be due to increased synthesis of positive acute proteins. Indeed positive acute proteins such as haptoglobin [43a], lipopolysaccharide binding protein and serum amyloid P [44-47] are known to increase in serum of trypanosome infected animals.In contrast, hypoprotenemia has also been observed in trypanosome infected monkeys [37;44], mice [48;49] and also in humans [50]. The hypoproteinemia could be due to the parenchyamatous disorder observed in the liver during trypanosome infection in animal models and also in humans [51] resulting in reduced synthesis of various proteins since the liver is the main source in the synthesis of most plasma proteins [52]). Indeed inflammation induced liver damage during trypanosomosis and other sustained inflammatory responses leads to decreased synthesis of essential biomolecules such as albumin [52].

The current study was setup to determine the usefulness of serum total CK and LDH including their isoenzymes as markers of determining cardiac damage during *Trypanosome b. rhodesiense* infection in the sheep model. The elevated levels of blood total CK and LDH of infected animals are in agreement with earlier reports observed in trypanosome infected monkeys [44] and humans [8;40;50]. Tissue enzymes are found in the cytoplasm and mitochondria of mammalian cells and are released into body fluids and circulation due to changes in cell membrane permeability or frank necrosis [7]. In addition tissue enzyme profiles indicated the presence and elevated levels of the isoenzymes CK MB and a flipped over pattern of LDH1 and LDH2 ratio both indicative of cardiac involvement. The changes in tissue enzyme levels and isoenzyme profiles were however reversed following treatment. Moreover, the pathological changes in cardiac tissue in were observed in dogs [43], monkeys [30] and humans [53]) infected with trypanosomes. Severe damage of other organs could explain the increased enzyme concentration in plasma of infected sheep [54]. Indeed *Trypanosoma brucei brucei* infection in dogs if untreated results in death due to heart failure from the severe pancarditis [55;43b]. At the same time a good number of HAT patients during the haemolymphatic and also the CNS stage are observed to develop cardiac damage due to destruction of myocardial cells [9;10] resulting in the loss of membrane integrity leading to enzymes diffusion to the extra cellular spaces and drainage into blood vessels thus elevation levels of tissue enzymes in blood.

Monitoring changes in the serum tissue enzymes has been used as a diagnostic value in judging the presence and extent of tissue injury in pathological conditions in animals [56] and humans [57;7;58]. In particular, the isoenzymes of lactate dehydrogenase and Creatine kinase in combination with more specific markers of cardiac injury such as the cardiac troponins, can provide information on the relative severity, extent or duration of myocardial injury [59;60]. At the same time results in the current study show a flipped over pattern in the ratio of LDH1:LDH2 accompanied by increase in isoenzyme CK MB, both of which has been shown to correlate with severity and duration of cardiac injury [61].

The analysis of tissue enzyme CK and LDH plus their isoenzymes yielded very low levels of the total enzymes compared to that of serum. Indeed no isoenzymes bands for both tissue enzymes were observed following gel electrophoresis of CSF. This observation could be due to the low concentration of both enzymes resulting from minimal damage of the CNS by the trypanosomes. Indeed it has been observed that infection of the CNS has to be well established before any observable pathological damage/changes can be seen [62]. In addition, the sub curative treatment with diminazine aceturate of the infected of the sheep in the current study resulted in clearance of the trypanosome from the CNS, clearly indicating that infection had not been established thus explaining the minimal increase in tissue enzymes. This is further collaborated by the results indicating the absence or the presence of low number of trypanosomes in CSF and minimal increases in CSF protein levels. It is likely that infection of the sheep resulted in a more acute infection necessitating their humane treatment before the establishment of the infection in the central nervous system. Indeed animals with trypanosomes in the CNS/CSF have been shown to exhibit no or minimal CNS pathology which however develops with progression of infection [63]).

7. Conclusion

From the current study, it may be concluded that serum LDH1/LDH2 ratio is a promising biomarker of cardiac involvement during *T.b rhodesiense* infection and therefore, has a potential diagnostic value in determination of cardiac damage.

8. Future Scope

Since the sheep developed an acute infection resulting in humane treatment of the sheep resulting in cure, further work is needed to address the question raised on the unsatisfactory LDH and CK total and isoenzyme separation and quantification in CSF. This study recommends a lower than 1×10^4 dose of parasites be used to infect sheep HAT model as this might slow down disease virulence and therefore prolonging animal's life thus allowing adequate time for parasites to be well established in CNS

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