# CHANGES IN SOME URINE PARAMETERS IN VERVET MONKEY MODEL OF TRYPANOSOMIASIS: TOWARDS NON-INVASIVE DIAGNOSIS OF SLEEPING SICKNESS

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## Abstract

The current diagnostic procedures for human African trypanosomiasis (HAT) are invasive. Urine being a non-invasive sample could be an alternative to the current screening that is based on blood sampling and cerebrospinal fluid (CSF). This study was aimed at determining whether urine could be used in the diagnosis of sleeping sickness. Three vervet monkeys were infected with *Trypanosoma brucei rhodesiense (T.b rhodesiense)* while other three served as non-infected controls. Urine samples were collected in plain 50 ml Falcon® at weekly intervals during early and late stage disease. Protein analysis in urine was done using the fortress protein assay kit. Analysis for pH, specific gravity and ketones was done using rapid test strips (ChoiceLine 10, Roche Germany). Genomic DNA was extracted using phenol-chloroform method and the Serum resistant (SRA) gene amplified. There was a significant increase in total protein concentration on day14, 49, 56 and 63 post-infection (PI). Ketone, acidity and specific gravity levels in urine increased significantly ($P < 0.05$) during the acute stage (days 7 and 14 PI). The changes in urine during infection may indicate damage to the glomerulus membrane. There was no amplification of trypanosome and this could be attributed to inhibitors in the urine (DNA degradation by nucleases and, high concentration of metal ions). This study suggests that changes in urine parameters could be used as biomarkers for early and late stage diagnosis of *T.b.rhodesiense* HAT and thus warrants further investigation.

**Introduction**

Human African Trypanosomiasis (HAT) due to *T.b rhodesiense* is a neglected disease that affects poor rural populations across sub-Saharan Africa and is fatal if left untreated (Kennedy *et al.,* 2004). Confirmation of diagnosis is based on detection of parasites in either blood or lymph by microscopy. Staging of the disease is dependent upon presence of trypanosomes and the number of white cells in cerebrospinal fluid (CSF) in accordance with WHO recommendations (WHO, 1998). The current diagnosis of HAT is limited by many factors including painful lumbar punctures, poor compliance when repeated sampling is required, insensitive microscopic examination due to fluctuating and low parasitaemia and delayed examination or exposure of the trypanosomes to sunlight that lead to their lysis (Chappuis *et al.,* 2005). Therefore, this necessitates the use of less invasive methods which are easy to perform and also can lead to rapid detection of parasites.

Urine collection being non-invasive and painless could be an alternative to the current screening that is based on blood sampling and CSF. Urine samples have been shown to have the same sensitivity and specificity just like blood in diagnosis of diseases such as malaria (Mharakurwa *et al.,* 2006), leishmaniasis (Roser *et al.,* 2008) and cystic echinoccosis (Dubey *et al.,* 2007) and therefore their use could improve the patient satisfaction and increase compliance in HAT. Previous studies on American trypanosomiasis (*T. Cruzi*) have indicated presence of antigens in urine of infected mice (Corral *et al.,* 1996). Various urine biomarkers such as elevated levels of lactate have been identified in urine of *T.b. brucei* infected mice (Yulan *et al.,* 2008). In this study, investigations were done to determine the presence of trypanosome DNA and changes in various parameters (total protein, urine pH, specific gravity and ketone) during early(1–7 dpi),  transitional stage (14–28 dpi) and advanced late-stage disease (35–63 dpi) in the vervet monkey model of HAT (Farah *et al.,* 2005).

**Materials and Methods**

**Trypanosomes**

*Trypanosoma b. rhodesiense* stabilate IPR 001 was used. The stabilate was obtained from a CSF sample of a late stage sleeping sickness patient in Bugiri District, Uganda in 2008. The isolate was passaged thrice in mice and cryopreserved in liquid nitrogen.

**Experimental Animals**

Six vervet monkeys of both sexes (four males and two females) weighing between 2.5 and 6 kg were obtained from the monkey colony of the Institute of Primate Research (IPR). Due to animal screening procedures and animal availability, the sex balance was not possible. The animals underwent a 90-day quarantine, during which they were screened for zoonotic diseases and treated for ecto- and endoparasites before being subjected to the experiment. They were maintained on commercial pellets supplemented with fresh fruits and vegetables. Drinking water was provided *ad libitum*.

**Experimental Design**
*Trypanosoma b. rhodesiense* stabilate IPR 001 was used to infect three monkeys (2 males and 1 female). The other three monkeys served as the uninfected controls. At 28day post-infection (dpi), the infected monkeys were treated sub-curatively using Diminazene aceturate (DA) at 5 mg/kg body weight for three consecutive days to induce the late stage disease.

**Sample collection**
Sixty six urine samples were collected using urine receptacles placed at the bottom of the cage in the early morning. For each subject, urine was collected 1 week before infection to provide baseline data and thereafter, they were collected on a weekly basis for a period of 9 weeks. The urine for each subject was stored in two aliquots each of 2ml at -40 degrees C. The end of experiment (63dpi) was guided by ethical guidelines to alleviate extensive suffering of animals.

**Analysis of total protein, pH, specific gravity and Ketones**
Fortress Protein Assay kit (Fortress Diagnostic Ltd, Antrim, UK) was used for analysis of proteins in urine. 20µl of urine and the standard were mixed with pyrogyllol red reagent and incubated for 5 minutes at 37 degrees C. The absorbance of the samples and the standard were measured against the blank at 600nm in a spectrophotometer (Novaspec II, Cambridge, England). Fresh urine was also analysed for pH, specific gravity, and ketones using urine test strips (Choiceline 10, Roche Germany).

**DNA Extraction**
Genomic DNA was extracted from 30 urine samples (from 0dpi-35dpi) using the phenol-chloroform extraction method described by Sambrook *et al.* (1989). Briefly, 1ml of Urine sample was used and prior to extraction, the sample was centrifuged for 10 minutes at 12,000g. The pellet was washed with 200 µl TE, vortexed for 5minutes and centrifuged for 10 minutes at 12,000g. This was repeated 2 times and the supernatant discarded. The pellet was suspended in 200 µl of TE buffer. Protein and RNA were digested using 20 µl of 20mg/ml of proteinase K and 20µl of 20mg/ml RNAse respectively. The extracted DNA was re-suspended in 50µl of TE buffer and then stored at -20 degrees C. The DNA was separated on a 1% (w/v) agarose (Sigma Aldrich, Steinheim, Germany) gel in 1xTBE buffer for thirty minutes and visualized under UV by staining with ethidium bromide (Sambrook *et al.*, 1989).
The DNA was then quantified using UVmini-1240 spectrophotometer (Shimadzu, Japan) with the absorbance at 260nm and 280nm to determine the purity of the DNA. The DNA ratio 1.8 was used in the subsequent polymerase chain reaction.

**Polymerase Chain Reaction**
The SRA gene for the 30 samples was amplified as previously described by Maina *et al.,* 2007. The SRA forward primer sequence was

5'ATAGTGACAAGATGCGTACTCAACGC 3' and the SRA reverse primer sequence was 5'AATGTGTTCGAGTACTTCGGTCACGCT 3'. The negative control was without the DNA template. The positive control was the trypanosome DNA from a *T. b. rhodesience* infected mouse. The presence of a distinctive band (of approximately 284bp) was considered as a positive PCR result for *T.b rhodesiense* DNA.

**Data analysis**

Data was managed using Microsoft excel (Microsoft USA, version 2007) which served as the database. Computation of the means and the standard errors for pH, proteins, ketones and specific gravity was done. The differences between the paired clustered means were compared using the student's t-test. Differences between the means were deemed statistically significant at $p < 0.05$.

**Results**

**Parasitaemia**

The parasites multiplied rapidly, giving a first parasitaemia peak of approximately antilog 9.0 at 9 DPI. Thereafter, the parasitaemia remained high, characterized by minor fluctuations (Figure 1). The trypanosomes were first detected in the CSF on 14 DPI. Treatment with DA, 28 DPI resulted in clearance of the trypanosomes in the blood. The parasites reappeared in blood from 51-56 DPI.
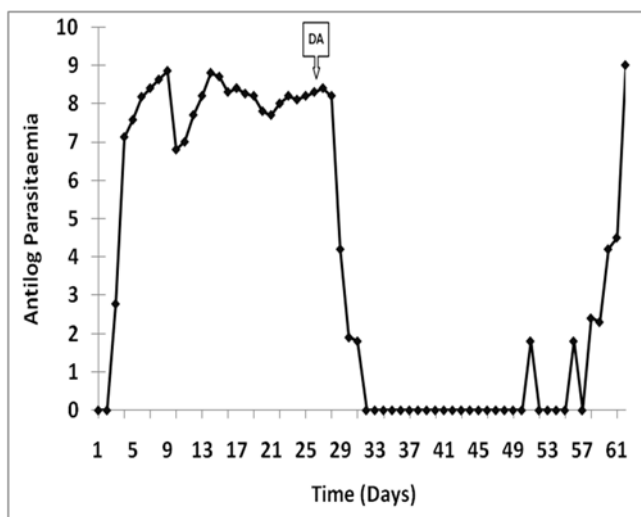


*Figure 1: Mean daily parasitaemia ( ◆ ) of monkeys infected with T. b. rhodesiense IPR001 indicating the point of sub-curative treatment with Diminazene aceturate (DA) to induce late stage*

**Total protein levels**

The mean concentration of proteins in the control group was maintained within the range of 3mg/dl-9 mg/dl and was not significantly different from those of infected animals at 0 dpi. In infected animals, the levels of protein in urine increased

significantly (P<0.05) to 23mg/dl at 14 dpi and then decreased after the acute stage (Figure 2). After the induction of late stage disease, the mean protein concentration also increased significantly (P<0.05) attaining a peak level of 28mg/dl at 63 dpi.

**Urine pH**

The mean pH of the control group ranged from 8 to 9 throughout the experimental period. In the test group, the pH of urine decreased significantly (P<0.05) to 5.5 and 6 on day 7 and 28 PI respectively. After induction of late stage at day 28 PI, the levels increased to 9 on day 49 PI. There was a notable decrease (P<0.05) in pH at day 56 and 63 PI (Figure 3).
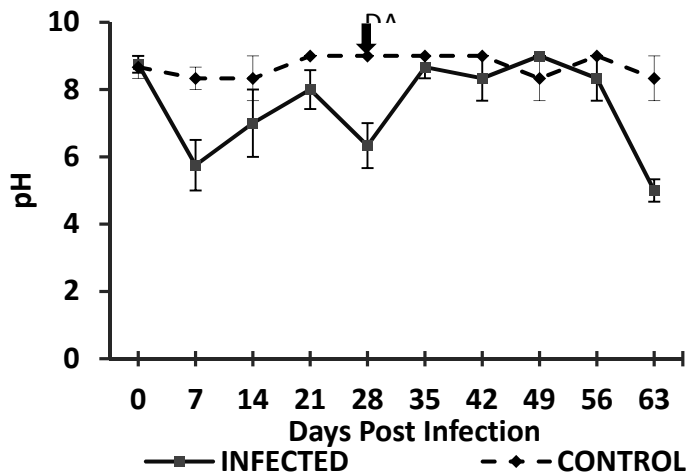


***Figure 3:*** *Mean pH in urine of T.b rhodesiense infected and uninfected vervet monkeys*

**Ketone Levels**

In the control group, the ketone levels were within the range of 0-1.5mg/dL throughout the experimental period. In the infected group, ketones levels in urine increased significantly (P<0.05) after infection to 5mg/dl at day14 and 28 PI and then decreased to 1.5 mg/dL at day 21 PI (Figure 4). After sub-curative treatment with DA, the levels decreased during the entire late stage.
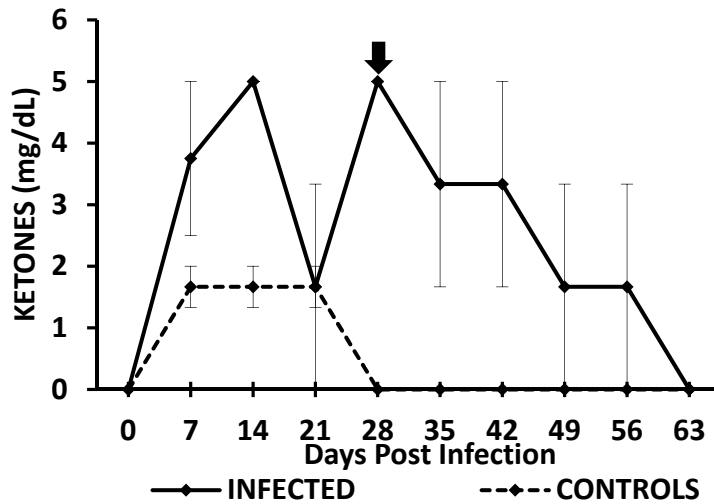
*Figure 4: Mean ketones in urine of T.b rhodesiense infected and uninfected vervet monkeys*

**Specific Gravity**
The specific gravity in the control group was maintained within the range of 1 and 1.01during the experimental group. In infected animals, the urine specific gravity increased significantly (P<0.05) to 1.017 at 7 dpi, and then significantly decreased (P<0.05) to 1.01 at day 21 PI. There was a notable increase in specific gravity (1.016) at day 28 PI (Figure 5). After induction of late stage disease, the levels decreased significantly (P<0.05) and later increased to a peak value of 1.027 at 63dpi.



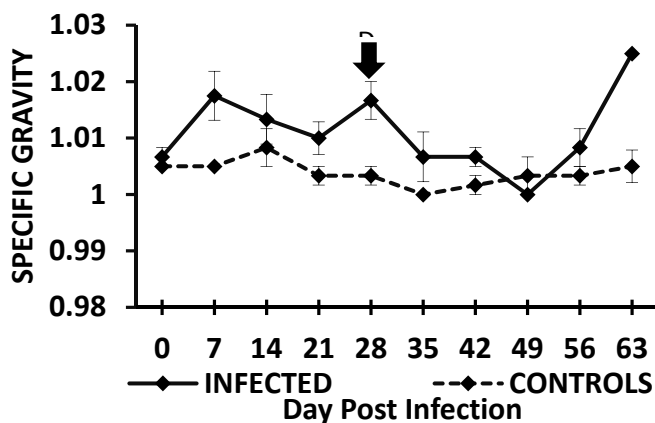*Figure 5: Mean specific gravity in urine of T.b rhodesiense infected and uninfected vervet monkeys*

**Amplification of DNA in Urine Samples**

Genomic DNA for the thirty urine samples was amplified. The expected fragment/ band size was 284bp (Figure 6). There were no amplified products in all the 30 urine samples.
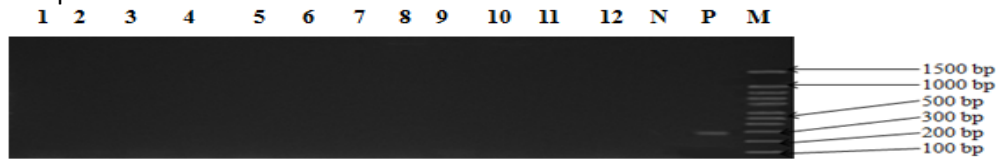


***Figure 6:*** *Amplified PCR products run in 2% (W/V) agarose gel. M- 1500 bp Marker, P- Positive control (T. b. Rhodesiense DNA), N- Negative control and 12urine samples PCR products {samples 1-5(dpi 0), samples 6-10(dpi 7), sample 11-12(dpi 14)}*

**Discussion**

Development of non-invasive tests for HAT is a priority for the implementation of appropriate control measures. The kidneys filter waste products from blood while retaining components the body needs. However, in some diseases and conditions can allow some molecules/metabolites to pass through the filters of the kidneys, causing changes in urine.

The results of this study show that the increase in urine protein concentration was dependent on parasitaemia, increasing significantly in acute stage of infection. The observed proteinuria in infected monkeys could probably be attributed to damage in the kidneys, resulting in increased permeability of the glomerular filter as reported by Simaren (1989). The damage may be as a result of deposition of immune-complexes on the basement membrane (Gray *et al.,* 1985). The greater the damage to the glomeruli, the higher is its permeability of even high molecular weight proteins which are able to pass through (Gray *et al.,* 1985).

In the current study, the acidity of urine increased significantly on day 7 and 28pi.This could be due to excess ketone bodies excreted in urine. The production of ketone bodies leads to an increase in hydrogen ions in plasma, signalling hyperventilation and increased renal hydrogen ion excretion which could explain the increased urine acidity. The increase in ketones during the early disease stage corresponds with previous studies done by Yulan *et al.,* 2008 which showed increased levels of ketones in urine of *T.b.brucei* infected mice during the acute stage of infection. Voorhes (1969) showed that *T. brucei* infection produced a condition resembling type 2 diabetes characterized by increased concentrations of urinary ketogenic acids, together with decreased peripheral glucose utilization. The high specific gravity noted in the infected could be attributed to damage of the glomerulus membrane that would allow molecules such as debris from dead trypanosomes, immunological debris, ketone bodies and other waste products to pass through. Studies have shown that in the early stages of acute nephritis, there is increased pore size due to glomeruli damage (Gray *et al.,* 1985) and this may explain the increase in specific gravity of urine.

A damaged membrane could allow the parasite to pass through. Indeed plasmodium DNA has previously been detected in urine (Mharakurwa *et al.,* 2006). However in

this study *T.b rhodesiense* DNA was not detected in all the urine samples of infected animals. This could be attributed to a number of factors such as DNA degradation by nucleases present in the fluid (Brinkman *et al.,* 2004), high concentration of metal ions in urine (Behzadbehbahani *et al.,* 1997). Bergmann *et al* (2002) reported that storage of frozen urine samples for more than 3 months increased the inhibitory nature of the urine. More sensitive methods of amplification Of DNA such as Loop Mediated Isothermal Amplification (LAMP) should be investigated.

This study suggests that proteins, ketones, pH and specific gravity in urine could be used as biomarkers for early and late stage diagnosis of *T.b.rhodesiense* HAT. Although other diseases and conditions could also lead to proteinuria, ketonuria and acidity in urine, use of these parameters as biomarkers for HAT could be useful in screening large populations of people in an endemic area so that only those people showing these conditions would be subjected to other parasitological diagnostic tests.

**Acknowledgements**

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