

Full Length Research Paper

# Application of loop-mediated isothermal amplification (LAMP) of the random insertion mobile element (*RIME-LAMP*) to diagnose camel *Trypanosomiasis* in Sudan

Mubarak M. Abdelrahman<sup>1\*</sup>, Ehab A. Frah<sup>1</sup>, Dianah Katiti<sup>2</sup>, Enock Matovu<sup>2</sup> and Intisar E. Elrayah<sup>1</sup>

<sup>1</sup>Tropical Medicine Research Institute, Khartoum, Sudan.

<sup>2</sup>Faculty of Veterinary Medicine, Kampala, Uganda.

Accepted 6 April, 2011

Control of *Trypanosomiasis* is dependent on accurate diagnosis and treatment of infected patients or animals. Highly sensitive tests are available for serological screening but the sensitivity of parasitological confirmatory tests remains insufficient and needs to be improved. Ongoing research is opening perspectives for a new generation of field diagnostics. This study aimed to apply and investigate new diagnostic technique loop-mediated isothermal amplification (LAMP) of the random insertion mobile element (*RIME-LAMP*) to diagnose camel *Trypanosomiasis* in Sudan. Extracted DNA from camels was used to detect *Trypanosoma evansi* infection in camels using *RIME-LAMP* technique. A total of 368 DNA samples positive reactor with card agglutination test for *Trypanosomiasis* (CATT *T. evansi*) was tested using *RIME-LAMP*. A total of 208 samples reacted positive (56.52%) using *RIME-LAMP* whereas only 17 samples were positive (6.34%) using parasitological methods. *RIME-LAMP* helps increase the probability of detecting the positive cases of camel *Trypanosomiasis* and it was found to be one useful and alternative molecular diagnostic tool for the detection of *T. evansi* infections.

**Key words:** Sudan, loop-mediated isothermal amplification (LAMP) - random insertion mobile element (*RIME-LAMP*), camel, *Trypanosoma evansi*.

## INTRODUCTION

Sudan has a very big population of camels reared for milk, meat, transport and racing. With a camel population of 4.4 million, Sudan is only second to Somalia (7 million camels; FAO, 2008). Camel rearing is mainly practiced by nomadic pastoralists in a belt running East-West across the country. Camel *Trypanosomiasis* is the most important single cause of economic losses in camel rearing areas, causing morbidity of up to 30% and mortality of around 3% (Ngerenwa et al., 1993; Egbe and Chaudry, 1994; Pacholek, et al., 2001; Njiru et al., 2002). In Sudan, surra (camel *Trypanosomiasis*) locally known as Guffar, is considered by farmers as the most important of the parasitic diseases that affect their animals, leading

to considerable economic loss. This comes in terms of low milk and meat productivity and death of the animals due to the disease. *Trypanosoma evansi* is the causative agent of surra which is endemic in all camel rearing areas of the country.

Diagnosis of camel *Trypanosomiasis* remains unsatisfactory to date. There are no pathognomonic signs of surra; laboratory diagnosis has to be carried out to confirm infection. Traditionally, this involves serological and parasitological diagnosis.

Currently, serological diagnosis of *T. evansi* infection in camels is limited. Indirect fluorescent antibody test (IFAT) is one of the serological tests which is a binding assay of the antibody antigen reaction and is measured directly and bound antibodies are visualized using anti-host species immunoglobulin (anti bovine immunoglobulin) conjugated to a fluorescent dye, which may be observed using an ultraviolet microscope (Luckins and Mehlitz,

\*Corresponding author. E-mail: muba73@yahoo.com. Tel: +249 (0) 912148160. Fax: +249 (0) 183 781845.

1978). Enzyme linked immunosorbent assay (ELISA), another primary binding assay, was developed in the early 1970s and has since, in various forms, become one of the most widely used techniques in biomedical science. In the ELISA, in its original form, antigens are immobilized by passive adsorption on to a solid phase and frequently, 96 well polystyrene microtitre plates were detected using specific antibody labeled with an enzyme that catalysis the conversion of a colourless substrate to a visible colored product. A more useful variant, the indirect ELISA, is analogous to the IFAT in that specific antibody in a test sample, if present, binds to the immobilized antigen and is itself detected by means of enzyme labeled anti-species (anti-bovine) conjugate and conversion of a substrate chromogen mixture to a colored product. In practical terms, the attraction of the ELISA is twofold. Firstly, the test may be performed without specialized equipment, so that it may be adapted to inexpensive versions for use in the field or under-resourced laboratories. Secondly, with suitable equipment, various degrees of automation are possible, allowing a high throughput of test samples in suitably equipped laboratories (Luckins, 1992).

Current serological diagnosis of *T. evansi* infection in camels is based on the native variable antigen type RoTat 1.2. Card agglutination test for trypanosomiasis (CATT)/*T. evansi* is one of the serological tests for detection of antibodies against *T. evansi*. Infection with *T. evansi* results in production of circulating antibodies against several surface antigens of the parasite. Such antibodies can be demonstrated in the serum of the infected host by direct agglutination.

The CATT/*T. evansi* antigen is a freeze dried suspension of purified, fixed and stained blood stream form trypanosomes, expressing a predominant variable antigen type of *T. evansi* (Ro Tat 1.2).

Parasitological diagnosis is mainly carried out by the direct microscopic examination of blood or Buffy coats and/or sub-inoculation of camel blood into rodents such as mice and rats. However, the parasitological tests have a generally poor sensitivity, often less than 50% (Monzon et al., 1990; Nantulya et al., 1989; Nantulya, 1990; Luckins, 1992; Yadvendra et al., 1998). The most sensitive amongst the parasitological methods is the mini anion exchange centrifugation technique (mAECT). This is particularly so when trypanosomes are less than 100 ml, in which in the case they may be missed. Moreover, parasite detection can be rather labor-intensive and require specialized technicians to detect the typically few trypanosomes in circulation. Failure to demonstrate parasites therefore, does not necessarily exclude infection (Chappius, 2005). The implication of this is that, in most situations, *T. evansi* is under-diagnosed and the level of infection is greater than frequently reported. On the other hand, serological techniques, for example, immunofluorescent antibody test (IFAT), enzyme linked immunosorbent assay (ELISA) and the card agglutination

test for trypanosomiasis (CATT), although sensitive, cannot distinguish current from cured infections (Luckins and Mehlitz, 1978). Besides, only the CATT is available for serology since the others require a sophisticated laboratory not commonly present where diagnosis is most needed.

The loop-mediated isothermal amplification (LAMP) is a novel method that can amplify a few copies of DNA to  $10^9$  copies in less than an hour under isothermal conditions. The test was initially reported as simple and easy to perform, as it required only four specific primers, *Bst* DNA polymerase and a simple laboratory heat block or water bath for the reaction (Notomi et al., 2000). An added advantage of LAMP methods is that DNA amplification can be monitored spectrophotometrically and/or with the naked eye with or without the use of dyes (Noritaka et al., 2003). Recently, Njiru et al. (2008) reported on a LAMP using 6 primers that target the random insertion mobile element (*RIME*) that is present in all *Trypanozoons*. This opens a way for sensitive diagnosis of *T. evansi* that belongs to this sub-genus. In this study, we aimed to evaluate the *RIME* - LAMP for the detection of *T. evansi* in naturally infected camels in Sudan.

## MATERIALS AND METHODS

### Study design

Cross sectional study was conducted to investigate the application of *RIME* - LAMP in the field of diagnosis of camel *Trypanosomiasis* in Sudan.

### Study area

Four different surveys were conducted during the year 2008, in four different Sudanese states: Gadarif and Kassala states (Eastern Sudan), Gezira State (Central Sudan) and North Kordofan State (mid-Western Sudan) which are famous in camels rearing.

### Sample size

Case finding method was used to determine the sample size of the study. 1186 blood samples were collected from the four Sudanese States. The number of samples of each state was 200 from Gadarif state, 428 from Kassala state, 300 from Gezira state and 228 from North Kordofan state.

### Sample collection

The permission for the collection of camels' samples was obtained from the camels' owners after the permission of the veterinarian authorities in each state. Blood was collected from the jugular vein from each camel using 5 ml disposable syringe. Three drops of the fresh collected blood were immediately placed in FTA card (Whatman classic FTA® card) and the rest was placed in 5 ml plastic tube for serum separation.

### Serum separation

Blood was put into 5 ml plastic tube with tight cover. Tubes were left

**Table 1.** Total number of samples used in this study and their positive percentage.

| Test                  | Negative | Positive | Percentage | Total number |
|-----------------------|----------|----------|------------|--------------|
| CATT <i>T. evansi</i> | 0        | 368      | 100        | 368          |
| Parasite detection    | 351      | 17       | 6.34       | 368          |
| RIME LAMP             | 160      | 208      | 56.52      | 368          |

in room temperature for 3 to 5 h before centrifugation for 10 min at 5000 rpm. The serum was then collected by a pipette using new tip for each sample and stored in a freezer at -20°C until use.

#### Thin and Thick smears

Two drops of the fresh collected blood were put into a clean slide with label area. One drop of blood was spread all around the top quarter of the slide making thick smear. The second drop was placed into the middle of the same slide and spread gently using another slide to make a thin smear. The slides were marked and allowed to dry. Thin smears were fixed with 100% methanol. Both thin and thick smears were stained by Giemsa stain following standard procedures.

#### CATT *T. evansi* test

The kit was composed of: CATT antigen (2.5 ml/vial) freeze dried sustention of purified, fixed and stained trypanosomes of VAT RoTat 1.2 and preservative of sodium azide (0.1%); CATT buffer (30 ml /vial), phosphate buffer saline (ph 7.2) and preservative of sodium azide (0.1%); positive control (0.5 ml/vial) freeze dried goat antiserum and preservative of sodium azide (0.1%); and negative control (0.5 ml/vial) freeze dried solution of bovine albumin and preservative of sodium azide (0.1%).

The best was performed on a plastic card. 25 µl of diluted serum or plasma was mixed with one drop (45 µl) of reconstituted antigen. When antibodies are present in the test sample, trypanosomes agglutinate with a 5 min rotation at 70 rpm (Magnus et al., 1978).

After separation of serum, the first test performed on the samples was the CATT test. 25 µl of serum was mixed with 45 µl of the reconstituted CATT reagent on the test card, and then the card was rotated for 5 min. After 5 min rotation at 70 rpm, result was read before removing the card from the rotator as follows: very strong agglutination was considered as strongly positive reaction (+++ve); strong agglutination was considered as positive reaction (++ve); moderate agglutination was considered as weakly positive result (+ve); and absence of agglutination was considered as negative result (-ve).

#### Blood spotted on FTA cards

One drop of fresh blood was placed into the FTA card (Whatman classic card) and was allowed to dry in the shade. Dried FTA cards were later put separately into a tightly closed container with silica gel and were transported to the laboratory, where DNA extraction was done.

#### DNA extraction

A single 3 mm diameter circle punch was obtained from each sample using a manual plier one-hole punch, then the sample spot was fixed by overlaying with methanol; the first time at room temperature for 20 min and the next two times incubated at 37°C

for 40 min each (Helene et al., 2009). Genomic DNA was then eluted from the paper in a heat incubation step run on a thermal cycler, then the dry sample spots were transferred to a thin-walled (0.2 ml) PCR tube containing 5 µl, 10× PCR buffer and 45 µl water. The tube was subjected to one cycle of 60°C for 30 min, 99.9°C for 10 min and then cooled to 4°C. The FTA paper was left in the tube with the eluant and stored at -20°C till used (Helene et al., 2009).

#### Loop-mediated isothermal amplification (LAMP) of the random insertion mobile element (RIME)

RIME – LAMP was carried out as described by Njiru et al. (2008). This was in a total reaction volume of 25 µl containing final concentrations of 2 µM of each FIP and BIP, 0.8 µM each of LF and LB, 0.2 µM B3 and F3 primers, 0.8 M of betaine, 200 µM dNTPs and 8 U of *Bst* polymerase and 2 µl of template. The reaction temperature was set at 62°C for one hour in a 2720 thermocycler (Applied Biosystems); there after, the temperature was increased to 80°C for 4 min so as to terminate the reaction and finally was decreased to 4°C as the holding temperature. Two negative control tubes and one positive control tube were included in every run. The negative control tubes contained double distilled water, while the positive control tube contained DNA extracted from *T. b. brucei* GVR 35 strain. Visualization of the results was by the addition of 3 µl of 1/10 dilution of SYBR Green1 to the 25 µl amplicons; the positive samples turned green, while the negative samples remained orange.

#### Statistical analysis

Difference between RIME-LAMP and parasitological detection method was calculated using two by two analyses. Significant difference was calculated using Chi-square (McNemar) test.

## RESULTS AND DISCUSSION

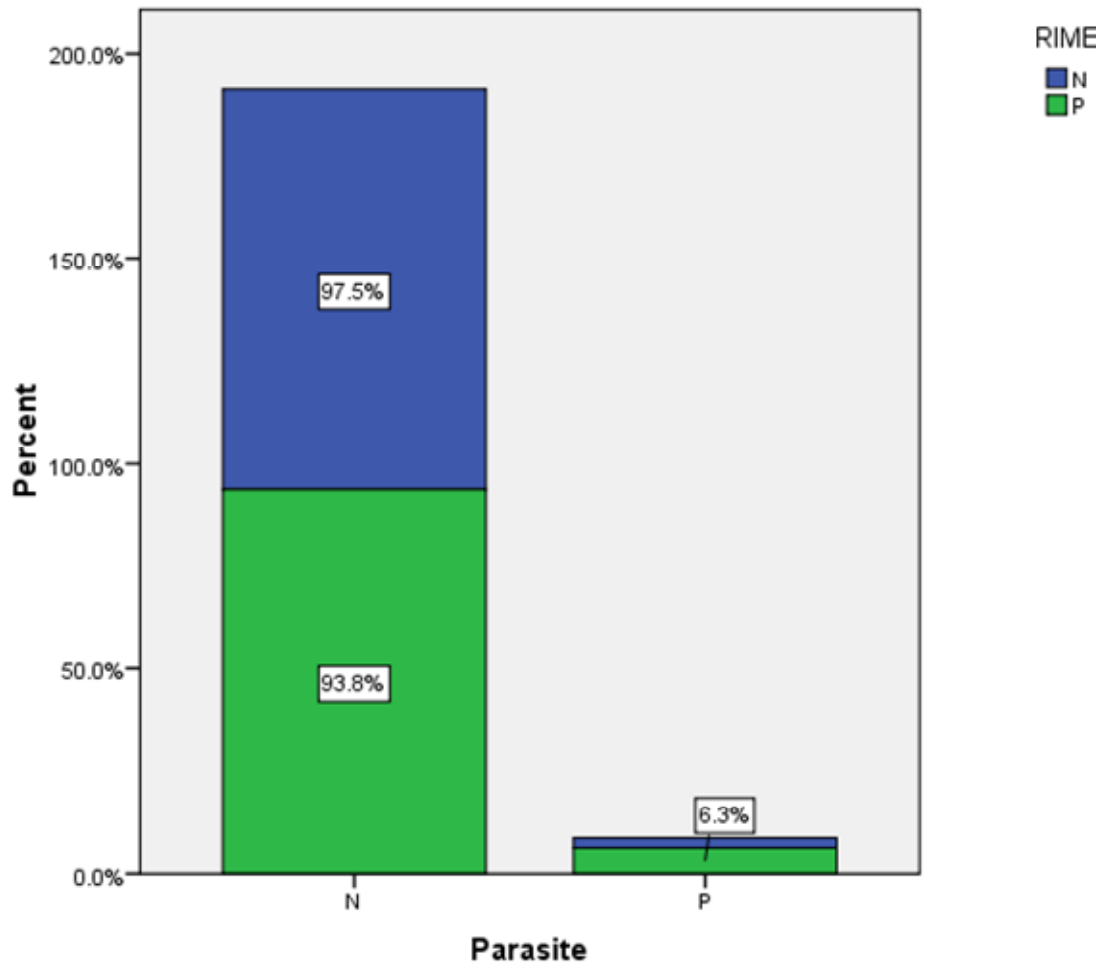
All the samples used in this study (368) reacted positive using CATT\ *T. evansi* (100%). Parasitological positive samples were found to be 17 samples (6.34%) from the total CATT positive reactors; whereas positive samples using RIME-ALMP were found to be 160 samples (56.52%) as shown in Table 1. In Table 1, the parasitological methods detected only 6.34% of the samples as positive, while RIME- LAMP detected 56.52% of the samples as positive.

In Table 2, it can be seen that parasitological method detected only 17 positive cases. When RIME – LAMP was used, the number increased significantly (P = 0.000) to 208 positive case. Figure 1 shows the increasing percentage of detection of positive cases of camel *Trypanosomiasis*; from 6.3% when parasitological

**Table 2.** Comparison between *RIME* – LAMP and parasitological detection of positive cases of camel *Trypanosomiasis*.

| Technique          | Status | <i>RIME</i> – LAMP |     | Total |
|--------------------|--------|--------------------|-----|-------|
|                    |        | +ve                | -ve |       |
| Parasite detection | +ve    | 13                 | 4   | 17    |
|                    | -ve    | 195                | 156 | 351   |
|                    | Total  | 208                | 160 | 368   |

+ve, Positive; -ve, negative

**Figure 1.** Comparison between *RIME* - LAMP and parasitological method for the detection of the true positive samples.

methods were used to 93.8% when *RIME*- LAMP was used.

Proper diagnosis of infections needs specific and sensitive diagnosis techniques. Diagnosis of camel *Trypanosomiasis* with *T. evansi* infection is dependent on a number of factors; those related to host-parasite interaction including levels of parasitaemia and presence or absence of trypanosomal antibodies and those relating to the type of diagnostic test used particularly its

detection limits. The most specific diagnosis methods are the parasitological methods, but it lacks high sensitivity. Serological diagnostic methods are more sensitive than the parasitological methods in revealing the true extent of *Trypanosomiasis* in camel herds (Ngaira et al., 2003), but unfortunately, less specific and molecular diagnosis methods are more sensitive and specific most of the times than all the previously mentioned methods. Delafasse and Doutouin (2004) using Buffy coat technique

(BCT) and CATT revealed a prevalence rate of *T. evansi* of 5.3 and 30.5%, respectively, in Chad. However, non-validation, standardization, application and deployment are factors militating against their use in the field.

In this study, parasitological methods (thin and thick smears stained with Giemsa) were just able to detect 17 positive samples, whereas the number of the positive samples increased significantly ( $P = \text{value}; 0.00$ ) showing high significant difference when RIME-LAMP used; 208 positive samples were detected.

LAMP offers a better alternative in the diagnosis of animal *Trypanosomiasis* because it detects infections at much higher level and possesses advantages such as simplicity and the ability to produce extremely large amounts of amplified products that can be easily detected by visual inspection of the turbidity or fluorescence of the reaction mixer (Notomi et al., 2000; Mori et al., 2001).

## Conclusion

There is great need for the application of validated and standardized diagnostic tests (particularly sensitive and specific tests) at both local and regional levels, in such studies of LAMP technique; with proper primer design, the LAMP method can be exploited as a useful and alternative molecular diagnostic tool for the detection of infections. In this study, RIME-LAMP was found to be one of these tests. More investigations in different locations in Africa and the wide world are needed to give a comprehensive sight of view regarding this test in the field of *Trypanosomiasis*.

## ACKNOWLEDGEMENTS

Special thanks go to Tropical Medicine Research Institute (TMRI), National Centre of Research (NCR), Khartoum, Sudan and BecANet (Bio-science Eastern and Central Africa Net) for their financial support for this work. Our deep gratitude goes to the Faculty of Veterinary Medicine, Makerere University, Uganda; all the RIME-LAMP work was done in their laboratories under the supervision of Dr. Enock Matovu.

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