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Highlights

- A novel dry-LAMP method targeting the RoTat1.2 VSG gene of *Trypanosoma evansi* is developed.
- This new diagnostic tool was validated using Surra infected camel blood from Sudan.

**Development and validation of direct dry loop mediated isothermal amplification
for diagnosis of *Trypanosoma evansi***

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Highlights

- A novel dry Loop Mediated Isothermal Amplification is developed.
- A method that diagnose *Trypanosoma evansi* infection targeting the RoTat1.2 VSG gene.
- This new diagnostic method was validated using Surra infected camel blood from Sudan.

Keywords: Surra, LAMP, diagnosis, Camel and Trypanosomal

Abstract

Non-tsetse transmitted *Trypanosoma evansi* infection (Surra) is one of the most important diseases of camels in north and east Africa and of buffalo and cattle in Asia. Early, accurate and feasible diagnosis is a crucial step towards the control of Surra. Dry format of loop-mediated isothermal amplification (LAMP) diagnostics for the detection of *T. evansi* was developed, where the detection limit was determined as to equivalent to one parasite per reaction. The assay was validated by testing blood from 48 camels clinically diagnosed to have Surra, which all tested negative microscopically and revealed 43 (89.6%) to be positive for *T. evansi* when tested by the dry-LAMP. Furthermore, DNA extracted from a randomly selected subset of 20 of these blood samples were then subjected to RoTat1.2-PCR (TaKara Ex Taq), with 14 matching results, with six that were positive by dry-LAMP and negative by PCR. The kappa value of dry-LAMP applied to direct blood was 0.4211, indicating moderate agreement to RoTat 1.2-PCR. In addition, 103 genomic DNA extracted from camels' blood were tested by both dry-LAMP and RoTat1.2-PCR revealed 67 matching results and 31 positive by dry-LAMP and negative by PCR and a further five positives by PCR and negative by dry-LAMP. This novel dry-LAMP method is more sensitive than conventional PCR, direct (without DNA extraction step), is user friendly and does not require cold chain or highly trained personnel.

Introduction

Trypanosoma evansi infects a wide range of animal hosts, including camels and horses with buffaloes and cattle are also affected. The incidence of infection increases significantly following the rainy season when numbers of the biting flies that act as vectors for trypanosomal infection greatly increase (Singh et al. 1993). Camel raising in Africa and buffalo production in Asia are severely affected (Lukins et al. 2004; Salim et al. 2011; Mossaad et al. 2017). Normally humans are resistant to infection with *T. evansi* due to the trypanolytic activity of APOL-1 in the blood (Wheeler 2010), however, this species is known to be a potential emerging zoonotic parasite as reported in India and Vietnam (Johsi et al. 2005; Van Vinh Chau et al. 2016). The full mechanism and frequency of human infection cases are unknown.

T. evansi is a member of *Trypanozoon* subgenus, and the genetic difference compared with the *T. brucei* group is minimal (Carnes et al. 2015; Cuyper et al. 2017). The only known specific marker for *T. evansi* type A is the RoTat1.2 VSG gene (Urakawa et al. 2001), however, RoTat1.2 VSG-negative *T. evansi*, known as type B, is also reported in some east African countries. Identification of *T. evansi* type B relies on different markers targeting the nuclear-encoded VSG JN 2118HU gene and the EVAB gene in the minicircles or F1-ATP synthase γ subunit gene (Njiru et al. 2006; Salim et al. 2011; Birhanu et al. 2016; Cuypers et al. 2017).

Several attempts to diagnose tsetse and non-tsetse transmitted trypanosomes have been published; however, most rely on the PCR technique after successful DNA extraction in the laboratory. Unfortunately, most trypanosome-affected countries are developing ones where laboratory infrastructure and resources are often limited. Recently, we published an innovative dry Loop Mediated Isothermal Amplification (dry-LAMP) system for diagnosis of human African trypanosomiasis (Hayashida et al. 2015), and another test for detecting falciparum and non-falciparum malaria directly from blood without DNA extraction (Hayashida et al. 2017). This dry-LAMP is a promising tool for diagnosis of Surra in the field as this technique requires no cold chain or laboratory DNA extraction. In this study we applied dry-LAMP for the direct detection of *T. evansi* in blood samples from camels clinically diagnosed with Surra.

Methods

LAMP primer design

Several sets of LAMP primers that consist of forward and backward outer primers (F3 and B3), forward and backward inner primers (FIP and BIP), and loop forward and loop backward primers (LF and LB) were designed targeting the RoTat1.2 gene of *T. evansi* DNA as described elsewhere (Notomi et al. 2000). The primers were designed using the online PCR primer design tool Primer3 program (Koressaar and Remm 2007; Untergasser et al. 2012). Thereafter, LAMP primers were assessed by observing the reaction speed, optimum amplification, primer multimer formation, cross-reactivity and optimum reaction temperature via real-time LAMP using a Rotor-Gene 300 thermocycler (Corbett Research, Sydney, Australia) in order to select the best performing primer set. The best performing LAMP primer set was found to amplify approximately 220 base pairs of DNA. Their nucleotide sequences are listed in Table 1 and depicted in Fig 1.

Preparation of the Dry-LAMP reaction

The dry-LAMP reaction mixes were prepared as described previously (Hayashida et al. 2015; 2017). In brief, 8 U of Bst 2.0 WS DNA polymerase, 35 nmol each of dNTPs and 3.0 μ mol of Trehalose were mixed and 1.5 μ l of the mixture were carefully placed onto the inner side of the lid of a 0.2 ml microtube and was left to dry to form a pellet. Later, 3.2 pmol of FIP and BIP, 0.4 pmol of F3 and B3, 1.6 pmol of LF and LB primers and ColoriFluorimetric Indicator (CFI; 3.0 nmol hydroxyl-naphtol blue with 3.5 nl GelGreen™ [10,000x Sol]) were mixed and 1.5 μ l of the mixture was placed on top of the above dried pellet and left to dry. Purified genomic DNA prepared from a culture of *T. evansi* (Tansui strain; isolated in Taiwan) was used as a positive control. A solution consisting of 23 μ l of 1 $\times$ LAMP reaction buffer (20 mM Tris-HCL [pH 8.8], 10 mM KCl, 7 mM MgSO₄ and 0.1% Triton X-100), plus 2 μ l of extracted DNA was added to form a 25 μ l reaction mix that was added to the dry-LAMP reaction tube. To reconstitute the dried pellet on the lid, tubes were turned upside down 5 to 7 times. Then, the reaction tubes were incubated at 64 °C for 40 min and the results were visualized by a handmade battery powered LED illuminator designed for field used (Hayashida et al. 2015). Two independent observers

recognized the colour change; positive reactions showed as a bright fluorescent green colour under transilluminator.

Sensitivity of RoTat1.2 Dry-LAMP and PCR

The detection limit of the RoTat1.2 dry-LAMP was determined using a 10-fold serial dilution of purified genomic DNA from *T. evansi* (Tansui strain), and using a 10-fold serial dilution of lysed parasite by 0.1%-Triton X DDW. The sensitivity of the RoTat1.2 dry-LAMP was compared with the known PCR system targeting RoTat 1.2 VSG gene (Urakawa et al. 2001) using two different PCR enzyme systems: TaKara Ex Taq (Takara) and GoTaq Green (Promega).

Field samples:

Forty-eight camel blood samples were collected in EDTA vacutainer tubes from around El-Gadarif city in eastern Sudan. The procedure was reviewed and approved by the University of Khartoum, Sudan (Approval no. 2018BS), and informed consent was sought from animal owners. These samples were collected from camels symptomatic for Surra from three different herds that showed all or some of the symptoms. Symptoms included weight loss, drooping hump, rough coat and the characteristic smell of urine known as a pathognomic sign for diagnosing Surra. Giemsa-stained thin blood smears were prepared and examined microscopically. The blood sample was diluted 10 times with 0.1%Triton X-DDW and 2 μ l was directly used as the template for RoTat1.2 dry-LAMP.

Additional blood samples were obtained from 103 camels from Kassala states in Eastern Sudan. Samples were collected after obtaining consent from the camels' owners. The procedure was approved by Sudan University of Science and Technology, Khartoum, Sudan (Approval no.28-46). A volume of 5-7 ml of blood was drawn from the jugular vein into plain vacutainer tubes (Terumo, Japan), and placed at 4°C prior transporting to the laboratory where genomic DNA was extracted using a QIAamp DNA Blood Mini Kit (Qiagen, Germany) in accordance with the manufacturer's instructions.

Data analysis

Cohen's kappa coefficient was calculated using VassarStats: Website for Statistical Computation (<http://www.vassarstats.net/kappa.html>); the results were interpreted according to the method previously described by Viera and Garrett (Viera et al. 2005).

Results and Discussion

Validation of the *T. evasni* Dry-LAMP

All 48 camel blood samples from clinically symptomatic camels revealed negative results for the presence of trypanosomes by Giemsa blood smear examination. However, the dry-LAMP detected 43 (89.6%) positive cases with varying degree of positivity. To ensure that no false positives were detected, 20 of the 48 camel blood samples were subjected to DNA extraction and RoTat1.2-PCR, with 14 matching results plus 6 samples positive by dry-LAMP and negative by PCR (Table 2). Dry LAMP showed a high sensitivity of 100% and indicates moderate agreement with PCR with Kappa value of 0.4211.

Comparison between dry-LAMP and PCR targeting RoTat1.2

The results of the two RoTat1.2 PCRs using purified DNA revealed varying limits of detection; the TaKara Ex Taq PCR system detected down to one parasite, equivalent to 0.1 pg DNA, but with a faint band (Fig 2). On the other hand, the detection limit of dry-LAMP in purified DNA was demonstrated to be 10 parasites equivalent to 0.001 ng DNA, and the positive reaction could be observed within 100 min (Fig 2). When a known number of lysed parasite were used, dry-LAMP detected up to 1 parasite with results clearly via the LED light visualization system visualized as fluorescent under transilluminator (Fig 3). Therefore, direct dry-LAMP showed comparable performance to PCR in terms of sensitivity and less time. Extracted DNA from the blood of 103 camels subjected to both dry-LAMP and RoTat1.2 –PCR revealed 67 matching results, plus 31 positive by dry-LAMP but negative by PCR and 5 positive by PCR but negative by dry-LAMP (Table 3). Dry LAMP showed high sensitivity of 82.67% and indicates fair agreement with PCR with Kappa value of 0.326. The failure in the detection of parasites by dry-LAMP could be partly attributed to the mutations in the target regions since LAMP primers anneal the longer sequences compared to PCR primers (154 bp for LAMP vs 36 bp for PCR) and thus the chances of having mismatches in the target regions are accordingly higher in LAMP compare to PCR.

Conclusions:

Direct, fast and easy to use dry-LAMP that can detect RoTat 1.2-positive *T. evansi* type A was developed, and validated using field samples. As most camels with trypanosomiasis are in the chronic phase of the disease, a sensitive molecular technique is required, and our developed dry-LAMP will be promising tool for diagnosis of Surra.

Abbreviations

RoTat1.2: Rode Trypanozoon Antigenic Type 1.2

VSG: Variant Surface Glycoprotein

LAMP: Loop Mediated Isothermal Amplification

Declarations

Ethics approval and consent to participate

The procedure was reviewed and approved by the University of Khartoum, Sudan and informed consent was sought from animal owners.

Consent for publication

Not applicable

Availability of data and material

All data generated or analysed during this study are included in this published article

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Conceptualization: Bashir Salim, and Chihiro Sugimoto
Data curation, investigation, methodology and formal analysis: Bashir Salim, Ryo Nakao and Kyoko Hayashida
Funding acquisition: Bashir Salim and Chihiro Sugimoto
Supervision: Kyoko Hayashida and Chihiro Sugimoto
Validation: Bashir Salim and Ehab Mossaad
Writing and editing – All authors
All authors read and approved the final version of the manuscript

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Table 1: Dry-LAMP primer sequences designed to detect RoTat1.2 of *T. evansi* in this study

F3	GCCAACTATGACACCTCGG
B3	TTCGATTAGGCCGTGTGC
FIP	CGCTGCAACGGCTGTTAGTTTT-CCCAGGAGACAGCTACCT
BIP	CACCTGCGGGGTGTTTAAAGCA-TTGATGTTGCTGGTCGCG
LF	ATAAGCGCCGATGGCTTC
LB	CCGTCGTTGCCGGTTAT

Table 2: The number of cases that were positive (+) or negative (-) for *Trypanosoma evansi* when evaluated using dry Loop Mediated Isothermal Amplification versus Rotat 1.2 VSG PCR in 20 randomly selected samples from the 48 blood samples collected from camels clinically diagnosed to have Surra.

	Dry-LAMP		PCR	
			(+)	(-)
<i>T. evansi</i>	(+)	17	11	6
	(-)	3	0	3

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Table 3: The number of cases that were positive (+) or negative (-) for *Trypanosoma evansi* when evaluated using dry- Loop Mediated Isothermal Amplification versus Rotat 1.2 VSG PCR for 103 extracted genomic DNA samples from camels

	LAMP		PCR	
			(+)	(-)
<i>T. evansi</i>	(+)	55	24	31
	(-)	48	5	43

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Fig 1: Dry- Loop Mediated Isothermal Amplification primers design for *T. evansi* RoTat.1.2 nucleotide detection. Nucleotide sequence alignments for reference sequences and their accession numbers appeared in the left were aligned using MUSCLE implemented in Mega 7 software. Primer recognition sites are indicated with primer names.

Fig 2: Detection limit of the PCR (a) using different polymerases namely TaKara Ex Taq and GoTaq, and dry Loop Mediated Isothermal Amplification (b) targeting RoTat1.2 from purified *Trypanosoma evansi* IL3354 DNA.

Fig 3: Sensitivity of *Trypanosoma evansi* dry Loop Mediated Isothermal Amplification with Triton lysed parasite. The reaction can be detected by naked eyes as color change of HNB (upper panel), or under LED light as fluorescent (lower panel).

Fig. 1

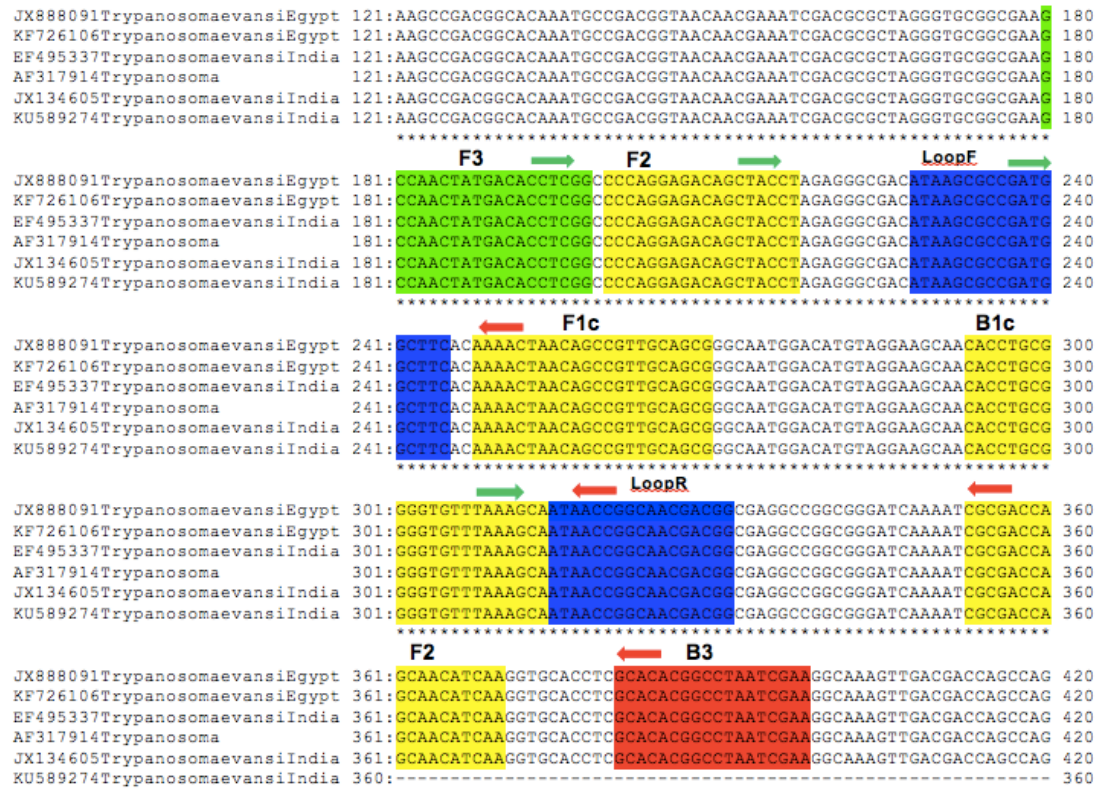


Fig. 2

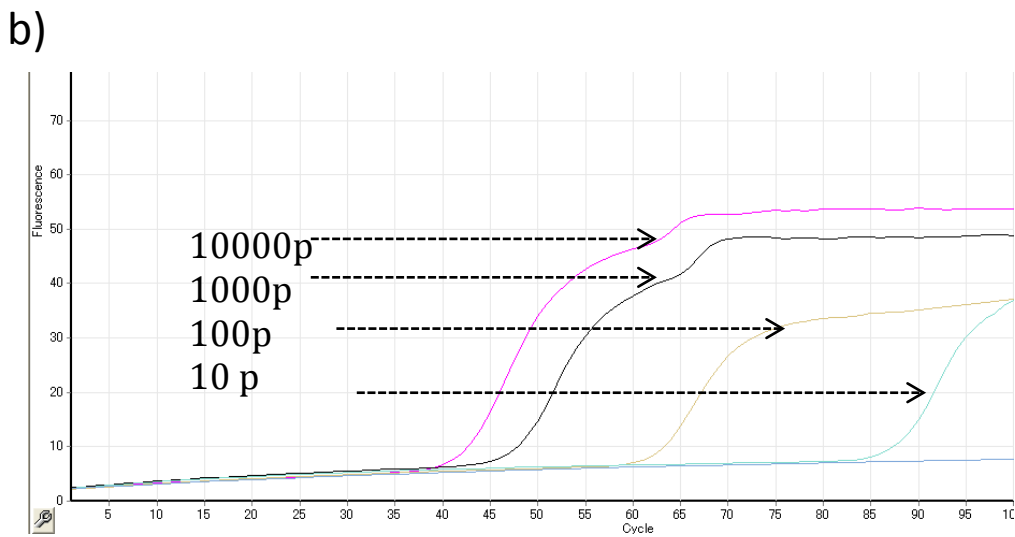
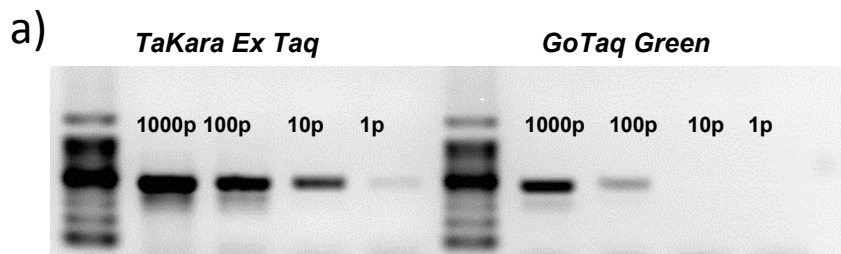


Fig. 3

