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Short communication

DNA detection of *Trypanosoma evansi*: Diagnostic validity of a new assay based on loop-mediated isothermal amplification (LAMP)

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ABSTRACT

Trypanosoma evansi (*T. evansi*) is the most widely spread pathogenic trypanosome in the world. The control of trypanosomiasis depends on accurate diagnosis and effective treatment. Focusing on the presence of *T. evansi* in Asia, we developed a detection assay based on tracing phosphate ions (Pi) generated during LAMP targeting the variant surface glycoprotein (VSG) gene of Rode Trypanozoon antigenic type 1.2 (RoTat 1.2 VSG). The diagnostic potential as well as the use of the assay as a test-of-cure method after berenil treatment, was assessed in mice at different time points of infection. In addition, 67 buffalo blood collected from Tongling county, Anhui province, as well as 42 cattle sera from the Shanghai area, were used to evaluate the diagnostic validity of the test. The detection limit of the novel LAMP assay was determined to be as low as 1 fg of *T. evansi* DNA, while the reaction time for the test was only 30 min. Hence it outperforms both microscopy and PCR. In the test-of-cure assessment, successful berenil mediated cure could be confirmed within 48 h after treatment. This offers a tremendous advantage over conventional antibody-based diagnostic tools in which successful cure only can be confirmed after months. In the cattle and buffalo screening, the LAMP was able to detect a false-negative determined sample, wrongly classified in a conventional microscopy and PCR screening. Finally, no cross-reactivity was observed with other zoonotic parasites, such as *T. evansi* type B, *T. congolense*, *T. brucei*, *Schistosoma japonicum*, *Plasmodium falciparum*, *Leishmania donovani*, *Toxoplasma gondii* and *Angiostrongylus cantonensis*. We conclude that the novel LAMP assay is sensitive, specific and convenient for field use, particularly in areas where infection incidence has become extremely low. The LAMP assay could be used as a tool for trypanosomiasis control and elimination strategies in areas where *T. evansi* Type A infections are causing a threat to livestock farming.

1. Introduction

Trypanosoma evansi (*T. evansi*) is the most widespread pathogenic trypanosomes in the world. It is present in regions ranging from South America, North Africa, the Middle East and Turkey, Russia, the Indian subcontinent including Sri Lanka and the Central Asian territories all the way to Eastern China (Desquesnes et al., 2013). It naturally infects a variety of both wild and domestic animals, including camels, cattle and buffaloes, hence causing significant economic losses. In the past, *T. evansi* has been reported to be widespread throughout China. The distribution has been reported in more than 20 provinces of China with positive serological scores ranging from 5% to 20% using an indirect hemagglutination test. In some places the infection rate of cattle has been reported to be as high as 64.47% (Pan 1997). Important to flag here is that *T. evansi* has also been identified as a potential threat to human health. In 2004, the first case of human infection with *T. evansi* was found in the central India. Here, infection was probably caused by transmission from blood from an infected animal (Joshi et al., 2005), while actual human disease progression in the victim was linked to a genetic artifact of the apolipoprotein L1 (ApoL-1) gene that is supposed

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to confer resistance to non-human trypanosomes. Following occasional reports on other non-Africa human trypanosome infections, a most recent deadly human *T. evansi* infection was reported in Southeast Asia (Van Vinh Chau et al., 2016). In this case, the victims' susceptibility to infection was not related to an ApoL-1 defect. Combined, these reports indicated that the frequent contact with animals infected with *T. evansi* does increases the possibility transmission of parasites to humans, and that most likely the existence of non-African human trypanosomiasis in Asia is vastly under-reported. Due to the human infection aspect, the bio-safety risk class of *T. evansi* has been augmented to the same category as *T. gambiense* that causes human African trypanosomiasis.

Trypanosomiasis control depends on sensitive and accurate diagnostic assay, especially in areas with low-intensity infections. As the prevalence of trypanosomiasis goes down, new diagnostic tests need to have improved sensitivity and specificity in order to prevent re-emergence of the disease due to unpredicted outbreaks in the animal reservoir. There are two main types of *T. evansi* serotypes of type A which expresses RoTat 1.2 VSG gene and type B which devoid of RoTat 1.2 VSG. RoTat 1.2 VSG is an important diagnostic antigen for *T. evansi* type A (Ngaira et al., 2005). To date, antibody screening tests are used in pre-screening procedures. The card agglutination test for trypanosomiasis (CATT/*T. evansi*) has been proposed as a useful field tool in this strategy. However, CATT has severe limitations as it is incapable of distinguishing current from past infections, and cannot be used as test-of-cure. Both are linked to the fact that infection-induced antibodies remain in the circulation long after active parasitemia has been controlled or cured. With the development of molecular techniques, PCR-based methods have been used to detect *T. evansi* DNA (Ngaira et al., 2004, 2005; Sumbria et al., 2015; Sudan et al., 2015; Sharma et al., 2015). However, PCR requires dedicated relatively fragile and expensive equipment and the technique needs long reaction time periods. Together, this makes the technique less suitable for field application, especially in the developing countries. Loop-mediated isothermal amplification (LAMP), firstly reported by Notomi et al. (2000), is a sensitive nucleic acid amplification technique with the advantages of being rapid. LAMP does not require high-end expensive equipment and thus is more suitable for the field application. LAMP assays have been developed to detect parasites that include *Plasmodium* spp. (Poon et al., 2006; Han et al., 2007), *Leishmania* spp. (Takagi et al., 2009), *Toxoplasma gondii* (Sotiriadou and Karanis, 2008; Kong et al., 2012), *Schistosoma japonicum* (Kumagai et al., 2010; Tong et al., 2015), *Trypanosoma* (Thekisoe et al., 2007; Ngotho et al., 2015), *Cryptosporidium* (Karanis et al., 2007) and *Giardia* (Plutzer and Karanis., 2009). Studies by Ngotho et al. (2015) show that LAMP has superior sensitivity when compared to PCR in detection of trypanosome DNA in primate serum, saliva and urine. Indeed, LAMP enables the amplification of a few copies of DNA to 10^9 copies in less than 1 h under isothermal conditions (Notomi et al., 2000). Despite these advantages, so far LAMP assays have also shown their weaknesses. Indeed, there is a high risk of aerosol contamination when using LAMP diagnosis, due to the large amount of amplicons produced in the assay. In order to address the latter, we designed a simplified closed LAMP format. In the present study, we targeted the RoTat 1.2 VSG gene of *T. evansi* type A found in China, and developed a new sensitive and simple assay based on tracing phosphate ions (Pi) generated during LAMP. Diagnostic validity assessment of this assay on animal samples from field sites and indoor slaughterhouses were evaluated, with the aim of providing a potential tool to support animal trypanosomiasis control strategies.

2. Methods

2.1. Ethics statement

Animal experiments were approved by The Institutional Animal Care and Use Committee (IACUC) of the Zhejiang Academy of Medical Sciences, PR China. And the Ethical Clearance Number was ZJAMS20160093.

2.2. Parasites and samples collection and observation

T. evansi (030795-buffalo-Indonesia, metacyclic trypomastigotes) and the DNA of type B *T. evansi*, *T. congolense* and *T. brucei* were kindly provided by Vrije Universiteit Brussel, Belgium. *T. evansi* parasites were propagated in female rats, rat weighing approximately 200 g by infection with 1×10^6 *T. evansi.* After 3-4 days, blood was harvested after rats were anesthetized with chloralhydrate. *T. evansi* parasites were separated from the blood cells by anion-exchange chromatography with DEAE Sefinose™ FF (Sango, Shanghai, China) (Ngaira et al., 2004). To test LAMP performance during experimental infections, imprinting control region (ICR) mice were used, divided into 5 groups of 5 mice each. Mice of group A-C were infected with different amount of *T. evansi* of 2000, 200 and 20 parasites each mouse respectively. Mice of group D were infected with 20 *T. evansi* and were administrated with 0.8 mg of active ingredient of berenil per mouse (Brand VERIBEN, CEVA) at the 6th dpi. Mice of group E were left uninfected and used as controls. Blood was collected every two days post infection or treatment from the vena orbitalis posterior plexus. For on-site LAMP in Tongling, Anhui Province, 67 blood field samples from buffaloes were used, while, 42 additional sera samples of cattle were kindly provided by Shanghai Veterinary Research Institute, Chinese Academy of Agricultural Sciences. The cattle samples without anticoagulant were allowed to clot at room temperature and then centrifuged (1500*g* for 10 min).

For the microscope examination, after centrifugation of the blood, the buffy coat in the middle was examined for the presence of trypanosome parasites.

2.3. DNA extraction

Total genomic DNA from these trypanosomes and all of the collected blood samples were extracted using the DNeasy Blood & Tissue Kit (QIAGEN, Maryland, USA) according to standard methods. Briefly, 200 μL blood or *T. evansi* cultures were dissolved in 400 μL lysis buffer (10 mM Tris-Cl, 100 mM EDTA, 0.5% SDS, pH 8.0) and proteinase K was added to a final con-centration of 200 μg/ml. Samples were then incubated in 55 °C water bath for 20 min. After incubation, proteinase K was inactivated by boiling the samples for 5 min. The following DNA extraction steps were performed according to the manufacturer's instructions.

2.4. LAMP and PCR assay

DNA from *T. evansi* was amplified using both LAMP and PCR methods. Four primers and two additional loop primers for LAMP, were designed targeting the RoTat 1.2 VSG (GenBank: AF317914.1) gene of *T. evansi* using the soft of Primer Explore, while a set of two PCR primers were designed to amplify the fragment containing the region amplified by LAMP targeted the same VSG gene (Table 1). The LAMP assay was carried out according to a previously reported procedure

Table 1
Specific primer of PCR and LAMP used in this study.

Primers	Sequences(5′-3′)
F	CAAAACTAACAGCCGTTGCAGCG
R	AGTTCCGGTACCTTCTCCATTTC
F3	GTAGGAAGCAACACCTGCG
B3	TTGATTAGTGCTGCGTGTGT
FIP	TGCGAGGTGCACCTTGATGTTGAAGCAATAACCGGCAACGAC
BIP	GAAGGCAAAGTTGACGACCAGCTGTGGTGTGCTTTTCCTTGT
LF	GCGATTTTGATCCCGCCG
LB	CAGAACGAGCAGAATTTTCCA