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Rapid detection of *Trypanosoma cruzi* by colorimetric loop-mediated isothermal amplification (LAMP): A potential novel tool for the detection of congenital Chagas infection

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ABSTRACT

Early diagnosis of congenital *Trypanosoma cruzi* transmission in newborns is essential because babies show high indices of cure. Conventional diagnosis is based on microscopic examination and serology. Molecular diagnosis is a promising alternative to replace conventional diagnosis, although it is not well suited for adoption in laboratories with limited resources. Isothermal DNA amplification methods have the advantage of not requiring expensive equipment. The aim of this work was to apply loop-mediated isothermal amplification (LAMP) to detect congenital infection in babies colorimetrically. This assay was able to detect all *T. cruzi* discrete typing units and *Leishmania braziliensis*, but not other pathogens. The assay showed a limit of detection of 50 parasites/mL in spiked artificial samples. This assay was tested in 27 blood samples of babies born to *T. cruzi*-infected mothers and showed 100% of concordance with conventional diagnosis. This is the first study to detect *T. cruzi* in clinical samples by LAMP, showing that this assay would be useful in the detection of congenital *T. cruzi* infection. The advantages of this novel tool include the speed with which the assays can be completed, the no-need of trained personnel, and the fact that it can be performed without complex and expensive laboratory equipment.

Chagas disease affects approximately 6–7 million people in Latin America (WHO, 2016). The infection has spread throughout the world via migrations of infected people (Schmunis and Yadon, 2010). The etiological agent, *Trypanosoma cruzi*, is transmitted via different routes, with congenital transmission being of great importance in places where vector populations are under control as well as in non-endemic areas (Carlier et al., 2015). Pharmacological treatment at the acute stage can eliminate infection, so early diagnosis of congenital infection is essential (Altcheh et al., 2014).

Conventional diagnosis of congenital Chagas infection in neonates is complex and requires an algorithm that was developed based on various tests (Carlier et al., 2015). Parasitological diagnosis in neonates involves concentrating parasites via centrifugation using capillary or Eppendorf tubes, followed by a microscopic examination (Carlier et al., 2011). Although these techniques concentrate parasites, they have limited clinical sensitivity due to multiple factors, including the parasitic load, disease prevalence and technical issues (Carlier et al., 2015). Therefore, diagnosis relies on serological methods at 8–10 months of age once maternal antibodies become undetectable (Carlier et al., 2011).

The conventional parasitological diagnosis has many disadvantages: a) it is laborious, b) it requires a trained microscopist, c) its analytical sensitivity depends on the skills of the microscopist and d) its quality control programs are difficult to establish (Linder et al., 2008). Molecular assays are a promising alternative, but are confined to specialized laboratories because they require complex and expensive instruments (Vasoo and Pritt, 2013). Isothermal amplification of DNA (IA) is a molecular method that has the advantage of not requiring any specific instruments. Various IA methods, including strand recombinase polymerase amplification (RPA) and loop-mediated isothermal amplification (LAMP), have been described (Mitsunaga et al., 2013). LAMP relies on auto-cycling strand-displacement DNA synthesis using *Bacillus stearothermophilus* (Bst) DNA polymerase and six primers that allow for amplification of products that contain a stem and loop structure. LAMP can be used to amplify a specific DNA sequence in less than one hour using a simple heating block (Notomi et al., 2000).

Thekisoe et al. (2010) described a LAMP assay that successfully detected *T. cruzi* in *Rhodnius pallescens*, whereas Mikita et al. (2014) used a LAMP assay to detect trypanosomatids as *Leishmania spp.*, *Trypanosoma rangeli* and *T. cruzi*. The aim of this study was to utilize a previously reported LAMP assay with human blood samples to evaluate its usefulness in the diagnosis of congenital Chagas disease (Mikita et al., 2014).

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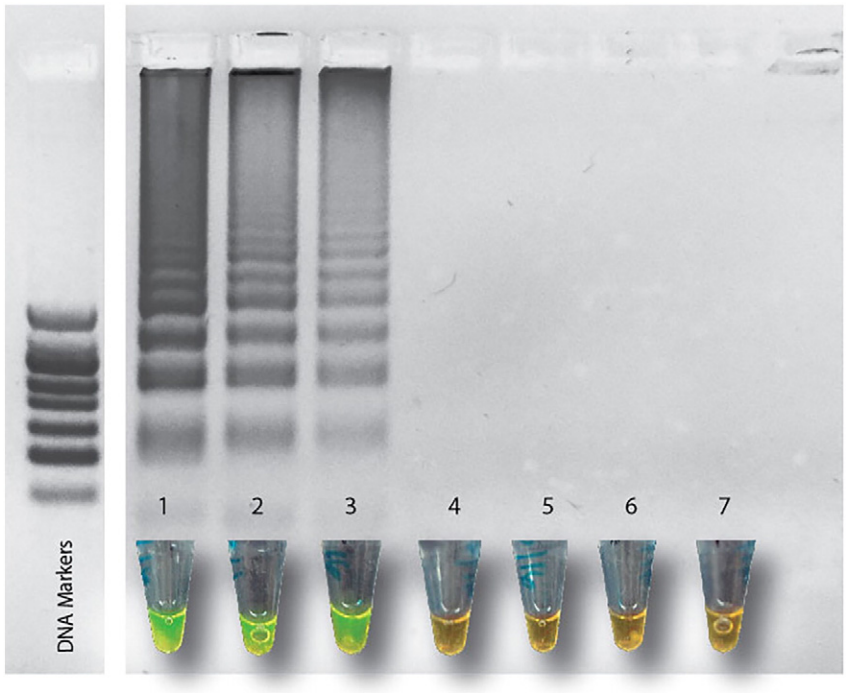

Fig. 1. Colorimetric visualization and limit of detection of the LAMP assay for *Trypanosoma cruzi*. LAMP products on agarose gel (top row); visualization of LAMP using SYBR® Green (bottom row). Green fluorescence indicates a positive reaction, whereas an orange color indicates a negative reaction. DNA Marker: 50 bp DNA ladder; lane 1: 5000 parasites/mL (par/mL); lane 2: 500 par/mL; lane 3: 50 par/mL; lane 4: 5 par/mL; lane 5: 0.5 par/mL; lane 6: non-template control, lane 7: non-template control.

Table 1
Analysis of clinical samples by conventional diagnosis and molecular methods.

Sample ID	Age* (days)	DNA concentration (ng/uL)	Conventional diagnosis		Molecular methods	
			Microscopic examination*	Serology**	LAMP gel*	LAMP SYBR Green*
7100	7	25.3	Neg	NR	Neg	Neg
7174	9	24	Pos	Not done	Pos	Pos
7025	11	35.2	Neg	NR	Neg	Neg
7047	12	24	Neg	NR	Neg	Neg
7005	17	31.2	Neg	NR	Neg	Neg
6634	19	4.4	Pos	Not done	Pos	Pos
7056	24	48.3	Neg	NR	Neg	Neg
7053	29	9.9	Pos	Not done	Pos	Pos
6847	30	21.6	Neg	NR	Neg	Neg
6905	30	12.2	Neg	NR	Neg	Neg
7116	30	33.7	Neg	NR	Neg	Neg
6821	30	22.1	Neg	NR	Neg	Neg
6795	30	32	Neg	NR	Neg	Neg
6894	45	28.3	Neg	NR	Neg	Neg
7050	60	24.6	Neg	NR	Neg	Neg
7069	60	17.9	Pos	Not done	Pos	Pos
7066	60	26.7	Neg	NR	Neg	Neg
7068	60	17.2	Pos	Not done	Pos	Pos
6984	60	28.3	Neg	NR	Neg	Neg
7032	60	13.2	Neg	NR	Neg	Neg
7115	90	4.9	Neg	NR	Neg	Neg
6609	90	0.4	Pos	Not done	Pos	Pos
7027	90	14.2	Neg	NR	Neg	Neg
6921	120	22.1	Neg	NR	Neg	Neg
6866	120	123.5	Neg	NR	Neg	Neg
6814	150	34	Neg	NR	Neg	Neg
6652	180	8.5	Pos	Not done	Pos	Pos

ID = identification; LAMP gel = amplification products were analyzed using agarose gel electrophoresis; LAMP SYBR Green = amplification products were analyzed by the naked eye using SYBR® Green; Neg = Negative; NR = Non-reactive; Pos = Positive.

* At the timee of obtaining the first control, the microhematocrit test was used.

** Between 8 and 10 months of age, the following serological tests were used: hemagglutination test (Wiener Lab, Argentina) and ELISA (Wiener Lab, Argentina).

Mikita et al. (2014) used a LAMP assay targeted to 18S rRNA genes to detect Leishmaniasis. Considering the sequence similarity (83%) present in 18S rRNA genes (GenBank accession n: M80292), we optimized the LAMP assay by using the same primer sequences that were used to detect *T. cruzi*. We evaluated various betaine and magnesium concentrations and melting temperatures (Annex). The optimal reaction conditions were: 0.5 M betaine, 8 mM $MgSO_4$ and amplification at 55 °C for 60 minutes.

The amplification products consisted of multiple bands that are characteristic of LAMP and were visualized by agarose gel electrophoresis or colorimetrically using SYBR® Green, which was added after the amplification cycle (Fig. 1). An immediate change to green fluorescence indicated a positive reaction, whereas an orange color indicated a negative reaction. The gel electrophoresis results were in complete concordance (100%) with the colorimetric visualization results for detecting LAMP products (Table 1). Notably, colorimetric visualization was easy to judge by the naked eye, even when the DNA concentration was low (i.e., Sample 6609, Table 1). Given its speed and ease, colorimetric detection could become the preferred method and thus eliminate the need for agarose gel electrophoresis.

A panel of various pathogens was used to evaluate the analytical specificity of the detection assay. LAMP was unable to detect genomic DNA from *Toxoplasma gondii*, *Treponema pallidum* or *Human herpesvirus 5* (HHV-5), but was able to detect DNA from *Leishmania braziliensis* and *T. rangeli* (Fig. 2). LAMP was positive for all *T. cruzi* discrete typing units (DTUs) (Fig. 2). These results demonstrate that although the primers used were previously designed to detect *Leishmania* species, LAMP is useful for the detection of *T. cruzi* and *T. rangeli* DNA, as was reported by Mikita et al. (2014). To our knowledge, there are no published studies concerning the congenital transmission of Leishmaniasis or *T. rangeli* in humans. Nevertheless, particular attention must be paid to potential false positives in regions where these parasites are prevalent. Even so, LAMP is unable to detect DNA of the other pathogens related to congenital infection (*T. gondii*, *T. pallidum* or HHV-5).

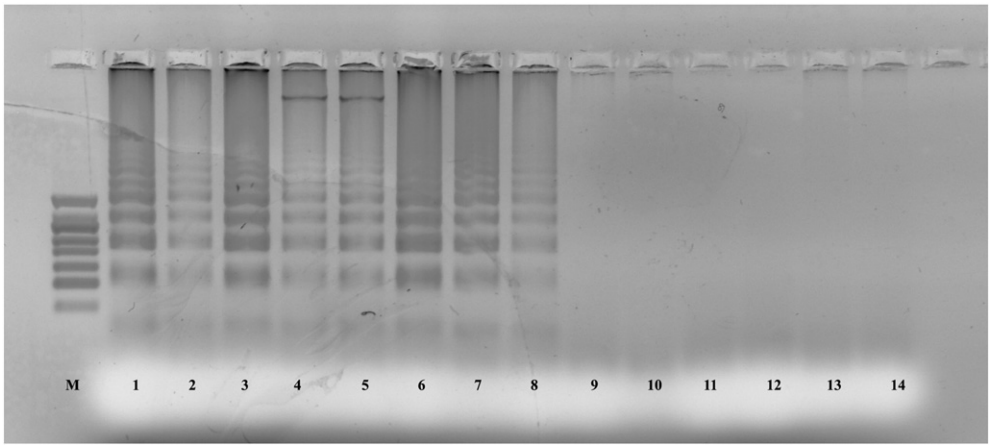

Fig. 2. Analytical specificity of the LAMP assay for the detection of *Trypanosoma cruzi:* DNA from six different DTUs and related pathogens was analyzed. M: 50 bp DNA ladder; lane 1: TcI; lane 2: TcII; lane 3: TcIII; lane 4: TcIV; lane 5: TcV; lane 6: TcVI; lane 7: *Leishmania braziliensis*; lane 8: *T. rangeli*; lane 9: *Toxoplasma gondii*; lane 10: *Human herpesvirus 5* (HHV-5); lane 11: *Treponema pallidum*; lane 12: non-Chagas infected human; lane 13: non-template control; lane 14: non-template control.

The analytical sensitivity of the assay was evaluated using artificial control samples (non-infected human blood spiked with *T. cruzi*). The limit of detection of the LAMP assay was 50 parasites/mL (Fig. 1). The analytical sensitivity was approximately equal to that of the conventional parasitological method (40 parasites/mL) (Torrico et al., 2005).

Since the 18S rRNA gene sequence has been shown to have high specificity for identifying pathogens (Adams and Hamilton, 2008), we used a LAMP assay targeted to this sequence. Nevertheless, this gene contains 110 copies per nucleus in the *T. cruzi* genome (Stothard et al., 2000). It is likely that the analytical sensitivity of LAMP could be improved by designing primers that target regions that have a higher copy number in the parasitic genome. In fact, by using genomic DNA from reference stocks characteristic of the *T. cruzi* DTUs, we observed positive results with dilutions ranging from 100 fg to 10 pg of DNA per reaction, depending on the DTU being analyzed (Supplementary Table S1), similar to those reported for real-time PCR (Cura et al., 2015). PCR targeted to repetitive motifs, such as satellite DNA, allows for the detection of a lower parasitic load (Schijman et al., 2011).

The assay also showed a high reproducibility, in 20 consecutive runs using a positive and a negative control results were 100% reproducible. By contrast, we could not obtain an appropriate reproducibility in tests using the primers reported by Thekisoe et al. (2010) (data not shown). This optimized LAMP was challenged in a proof-of-concept assay using clinical samples from 20 non-infected and 7 infected babies. These 27 samples were selected from a cohort of newborn patients who attended the hospital for congenital Chagas disease diagnosis (Annex). LAMP was able to detect *T. cruzi* DNA in all seven infected babies (Sensitivity: 100%, [CI95] 64.6–100%) and did not detect *T. cruzi* DNA in the 20 uninfected babies (Specificity: 100%, [CI95] 83.9–100%). In summary, there was a 100% concordance between the LAMP and conventional diagnosis methods (Table 1). Interestingly, three samples tested positive for the first 30 days after birth by both the LAMP and the microhematocrit methods. In fact, the positive results obtained by microhematocrit suggest that LAMP detected live parasites and not maternal DNA debris. However, this early detection of parasites will need to be confirmed in a larger number of samples.

To our knowledge, this study is the first to detect *T. cruzi* in clinical samples using an IA method. Future clinical studies will need to be conducted to fully validate its use in a clinical setting. The rapid colorimetric detection was found to perform similarly to conventional diagnosis, highlighting the suitability of LAMP for laboratories without trained microscopists since the procedure can be performed using portable and relatively low-cost devices.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.diagmicrobio.2017.06.012.

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