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SHORT COMMUNICATION

Field detection of *Tritrichomonas foetus* through loop mediated isothermal amplification with CELDA

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Abstract
An affordable, portable, and easy-to-build instrument was implemented to perform loop-mediated isothermal amplification (LAMP) in the field. Controlled by Arduino, CELDA heats the reaction tubes, reads the intensity of green fluorescent light and stores data in a computer. In the laboratory and in the field, CELDA was successfully used for the detection of *Tritrichomonas foetus* DNA in less than 60 min and helped distinguish positives with less difficulty than visual inspection.

Keywords Instrument · Diagnostic · LAMP · Trichomonas · DNA · PCR · Culture

Introduction

Loop-mediated isothermal amplification (LAMP) is a technique designed for the amplification of known DNA sequences in a sample that has many advantages in the diagnosis of microorganisms (Mori and Notomi 2020). LAMP is based on a very ingenious design that includes two primers that initiate DNA polymerization from the boundaries of the target sequence and two primers designed to successively hybridize to both strands of DNA, forcing the DNA polymerase to synthesize in a continuous loop (Notomi et al. 2000). LAMP is inexpensive, requires little sample processing, is fast, and highly sensitive.

LAMP produces large amounts of DNA, which makes the results easy to read (Mori and Notomi 2020). A positive reaction may appear cloudy because insoluble salts are generated, show a change in color or in fluorescence intensity if a dye is included that binds DNA or ions that accumulate during synthesis. Positive LAMP tubes can become a source of contamination, so it is advisable to use techniques that allow the results to be read with the tube closed (Liang et al. 2013).

LAMP can be performed in a simple water bath and the results inspected visually. There are also many instruments to evaluate results like turbidimeters and fluorescence readers that have been specifically designed to perform LAMP (Tomita et al. 2008). Real-time PCR thermal cyclers greatly assist in performing LAMP, too. However, these instruments are expensive and transporting them for field diagnostics is challenging and difficult.

There is a great need for inexpensive and portable devices for LAMP testing in diagnostic laboratories, educational settings and for field diagnosis. Field detection is not only of interest in research but also because of cost reduction and sample conservation (Yao 2021). Inexpensive and easy-to-use microelectronic circuits can help design such equipment that is not available on the market. Mechanical, optical and electronic components that are found in a large amount of electronic waste produced in each region can also be used.

Here, an affordable, portable, and easy-to-use instrument was implemented to perform LAMP. CELDA, from Spanish for heater-exciter-and-amplification-reader (Calentador-Excitador-Lector-De-Amplificación) is a basic tool that allows to incubate 4 reaction tubes, reading the increase of green fluorescence light and store data in a computer. We examined the suitability of CELDA for the amplification of *T. foetus* DNA through previously validated tests with artificially contaminated samples and in the field.

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Materials and methods

CELDA

The mechanical chassis was a dustproof plastic junction box. A hole was practised in the lid, on top of which a heater for 4 PCR tubes was adhered. Underneath, the chassis of a CD player with a blue light emitting diode (LED) and a light and color sensor was fixed. The base of the box housed an Arduino Uno controller (Arduino), a l293d chip allowing control of the reader's stepper motor, and a relay that helps turning the sample heater on–off.

The heater was built with 2 aluminum plates, one with 4.5 mm holes, the other with 2 mm holes. After keeping both plates separated and aligned with 4 PCR strip tubes, a nichrome resistor was placed on both sides of the tubes and the space between the plates and resistors was filled with silicone. An additional hole was used to house an lm35 temperature sensor.

The reader was mounted on the optical pick-up of a cd player. A blue LED was fixed in the laser extremity and a Sparkfun APDS9960 RGB sensor was glued in place of the center lens. The blue LED was oriented in a 45° angle to excite the calcein in the tube immediately on top of the reader. The RGB color reader was to measure intensity of green light.

The code, the wiring and the appearance of the instrument are provided in the Supplementary File 1. The schematic wiring diagram was performed with Fritzing. The code can be adapted to other boards of the Arduino family. Here it allows to simultaneously control the heater, the exciter LED, the color reader and the motor that moves the exciter-reader between the tubes. The temperature is maintained at 62–65 °C with the lm35 sensor and a relay. After pressing a start button, the motor drives the pick-up upstream until it finds the end-of-run switch button on the chassis of the CD player. Then it goes to tube 1, initializes the color reader and writes the headers to the serial monitor. In tube 1, the blue LED lights up, the green signal is collected and the measurement is written on the serial monitor. The pick-up then goes to tubes 2, 3 and 4 to collect their respective measurements. Then it returns to the position of tube 1 to restart the cycle, which is repeated indefinitely.

In the laboratory the energy was provided by an ATX power source. In the field the energy needed for heating and driving the stepwise motor was taken from the car cigarette lighter socket. The cost of CELDA materials was U$S 18 and recycled materials would add less than U$S 15. Provided that all individual components are in place the assembly of the CELDA (including software setup) should take less than 5 h for a person with no previous experience.

Instrument calibration

The amplitude of the signal collected by the instrument was optimized by contrasting results of LAMP reactions containing DNA (2 ng) or water in presence of varying concentrations of calcein and manganese chloride.

Well-to-well variation was measured with aliquotes of the same LAMP reaction in the presence-absence of DNA. Differences in the time required to overcome the basal fluorescence signal and the maximum fluorescence signal of each well were used to correct the measurements of each well.

Cells and DNA preparation

Tritrichomonas foetus cells were grown in liver infusion medium supplemented with 10% horse serum (Oyhenart et al. 2013). Cells were passaged every 2–3 days. DNA was obtained from 24 h cell cultures. Tubes were centrifuged 10 min at 8000 xg and pellets were washed 3-times with Tris-buffered saline solution (150 mM NaCl, 50 mM Tris–HCl, pH 7.4). Genomic DNA extraction was performed through CTAB (cetyl trimethylammonium bromide) method (Doyle 1991). Ethanol insoluble pellets were suspended in 400 ul of milliQ water and DNA concentration was estimated through absorbance at 260 nm.

Animal fluids

Cervical vaginal mucus (CVM) samples were collected using a sterile Cassou pipette. Approximately 0.5 mL CVM was distributed in 1.5 mL sterile tubes and directly used for detection or frozen at − 20 °C until LAMP tests were performed (Oyhenart 2018). Before LAMP, CVM samples were fluidified through treatment with proteinase k (1 ug/ml) for 10 min and heat-inactivated 2 min in boiling water.

Bovine smegma was obtained with a 20 cm long catheter attached to a 10 mL syringe. Ten milliliters of sterile physiological solution (150 mM NaCl) pre-warmed to 30–37 °C was flushed into the preputial cavity. After a gentle massage that ensured an even wash of the cavity the liquid was aspirated and placed into sterile test tubes (Oyhenart et al., 2013). One milliliter aliquotes were centrifuged at 7500 xg for 5 min and the supernatant was stored at − 20 °C until used.

Field detection was performed on CVM from 12 virgin Aberdeen Angus heifers obtained 45 days post infection. Heifers 18–24 months old and weighting 330–380 kg were kept under conditions of extensive grazing in a herd free of brucellosis, campylobacteriosis and trichomonosis, were oestrous synchronized and artificially contaminated with *T. foetus* as elsewhere described (Martinez et al. 2018). For estrus synchronization heifers were given vaginal

progesterone (500 mg) releasing devices (Zoovet, Santa Fe, Argentina) and 2 mg estradiol benzoate intramuscularly. Seven days later the progesterone releasing devices were extracted and 500 µg D-cloprostenol injected. The next day animals had 1 mg estradiol benzoate and 24 h later they were infected through instillation of 1 mL of PBS containing 10^6 T. *foetus* B1 motile cells (> 99% viability) in the cranial vagina by using a Cassou pipette. Heifers used in this work were included in a vaccine trial (unpublished results) and were handled by trained personnel according to standards of good practices and conditions approved by the Animal Ethics Committee of the National Institute of Agricultural Technology (Martinez et al. 2018).

Loop mediated isothermal amplification

Loop mediated isothermal amplification primers that allow the amplification of segments of the elongation factor 1a1 (tf-ef1a1) or the beta tubulin 2 (tf-btub2) from *T. foetus* were already described (Morero et al. 2021; Oyhenart 2018). The primers concentration were: 0.8 uM of FIP and BIP, 0.2 uM of B3 and F3, 0.2 uM of LB and LF for tf-ef1a1 reaction and 1.6 uM FIP and BIP, 0.2 uM B3 and F3, 0.8 uM LB and LF for tf-btub2 reaction. LAMP conditions were: 4.5 mM $MgSO_4$, 0.8 M betaine, 0.125 mM dNTPs, 10 mM KCl, 11 mM $(NH4)_2SO_4$, 0.1% v/v Tween 20 and 8U Bst DNA polymerase (Mclab, San Francisco, CA, USA) in 20 mM Tris–HCl, pH 8.8. Calcein and manganese chloride concentration were varied between 25–50 uM and 0.25–2 mM. An optimal signal was observed with 0.5 uM calcein and 0.5 mM manganese chloride. More than 1 mM manganese inhibited the amplification. Unless otherwise indicated, 2 ng of *T. foetus* DNA was used per reaction. Each condition was tested in duplicate with milliQ water as negative control. Twenty five (25) ul LAMP reactions were incubated at 62 °C in a dry bath or CELDA for 120 min. A drop of mineral oil was added on top of every reaction.

LAMP reactions containing calcein were followed up with CELDA and, after 120 min, they were visually inspected under white light or ultraviolet (470 nm) light. Two µL of LAMP products were mixed with 2 µL of loading buffer (50% Glycerol, 11 mM EDTA, 0.017% SDS, 0.015% Bromophenol Blue in 3.3 mM Tris–HCl pH 8.0), resolved in 1.2% agarose gels at 70 V/cm for 60 min and stained with GelRed (Sigma Aldrich).

The LAMP limit of detection was determined by testing serial dilutions of *T. foetus* cells or DNA. *T. foetus* cells in exponential growth phase were washed three times in buffered saline solution (150 mM NaCl, 50 mM Tris HCl, pH 7.4), counted and tenfold serially diluted in the same buffer. Serial dilutions were boiled for 2 min and stored at – 20 °C or they were directly tested. Pure *T. foetus* DNA (1000 pg/uL) was tenfold serially diluted with milliQ water. Samples from serial dilution tubes were used for artificial contamination of bovine smegma or CVM. Contaminated CVM samples were diluted with 0.5 ml of milliQ water containing 20 µg of proteinase K, incubated at 65 °C for 60 min, inactivated 10 min at 80 °C and were stored at – 20 °C until use. A volume of 2 ul of each dilution was used as template for the amplification assays.

Data format and results analysis

Statistical analysis were performed with R Statistical Software v3.4.4 through the RStudio integrated development environment 4.0.3 (“RStudio | Open source and professional software for data science teams,” n.d.). The graphics were made with ggplot2, scales and cowplot libraries.

Results

The analytical sensitivity and the time required for tf-ef1a1 and tf-btub2 DNA amplifications with pure DNA in CELDA was comparable to that obtained in a dry bath. Detection limit was around 20 pg/reaction and the time to reach a peak signal was 35–52 min. In the detection of *T. foetus* DNA detection in artificially contaminated smegma or CVM both tef1a1 and tf-btub2 showed positive results with animal fluids spiked with ~ 10 cells/uL. Figure 1 shows the results obtained after analyzing contaminated smegma.

CELDA was finally used for detection of *T. foetus* DNA in CVM samples from infected cows. Samples were deliberately taken 45 days from artificial infection because culture tests start to show negative results by this time. As detailed in Table 1, 8 samples out of 12 were positive with both tf-btub2 and tf-ef1a1. Seven out of these 8 samples were positive after culture test inspection performed over 1 week and one tf-btub2 and tf-ef1a1 positive sample was negative in culture. Direct detection of amplification products with the naked eye (three observers) occasionally yielded indeterminate results under white light (Table 1).

Discussion

CELDA is a portable, affordable and helpful instrument for sample test evaluation through LAMP. It is a simple instrument that allows a complete diagnosis as it heats the sample to a stable temperature, excites a fluorescent dye and collects a visible signal in a different channel and sends the reading data to a computer.

CELDA was built with on-the-shelf and recycled electronic components. The use of a recycled CD player chassis has limited the number of samples to 4. This, like other

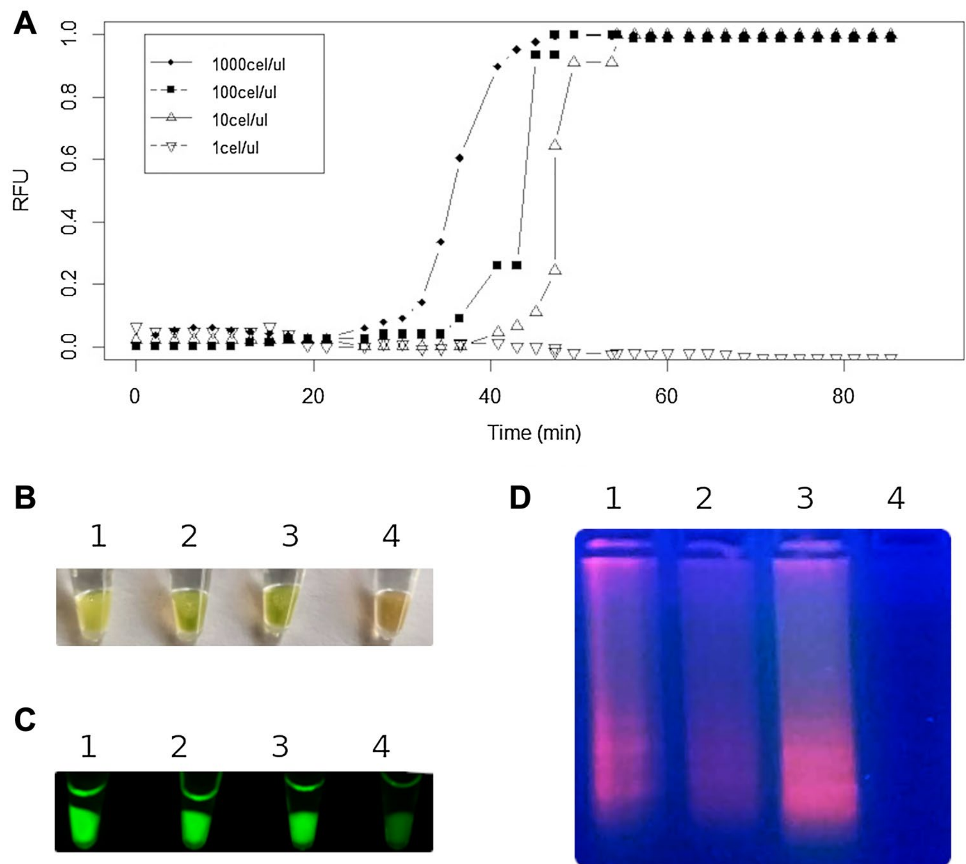

Fig. 1 CELDA detection of *Tritrichomonas foetus* in smegma. Tf-btub2 LAMP results are shown as: **A** Amplification plot obtained with 1, 10, 100 or 1000 cells per uL. **B** Direct visualization of the same tubes after 120 min under daylight. **C** Direct visualization of the same tubes under UV (470 nm) light. **D** Samples of the same reactions amples were resolved in gel. Tf-btub2 reactions were consistently positive with 10 cells/uL or 20 cells/reaction

Table 1 CELDA performance in the field. CVM samples from contaminated cows were tested for *T. foetus* contamination with tf-ef1a1 LAMP and tf-btub2 LAMP for 60 min

Sample	LAMP						Culture
	CELDA		Visual (white light)		Visual (UV light)		
	Tf-ef1	TF-btub2	Tf-ef1	TF-btub2	Tf-ef1	TF-btub2	
1	+	+	+	+	+	+	+
2	+	+	+	+	+	+	+
3	+	+	+	+	+	+	+
4	+	+	+	+	+	+	+
5	−	−	±	±	−	−	+
6	+	+	+	+	+	+	+
7	+	+	+	+	+	+	+
8	+	+	+	+	+	+	+
9	+	+	+	±	+	+	−
10	−	−	−	−	−	−	−
11	−	−	−	−	−	−	−
12	−	−	−	−	−	−	−

Aliquotes of CVM were seeded in liver broth and followed under the microscope for 7 days

aspects of the instrument, may vary depending on the needs and availability of materials.

That CELDA provides a solution for incubation and basic time-precise monitoring of LAMP tests was shown through tf-ef1a1 LAMP and the tf-btub2 LAMP. Both methods

allowed DNA amplification in less than an hour, in the laboratory and in the field, with samples with minimal treatment.

Conclusion

A highly sensitive technique such as LAMP can be performed in the field. A simple instrument was used for the diagnosis of bovine trichomonosis. The same principle is applicable to any other disease.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00203-022-02777-1.

Author contributions JO: CELDA conception and design, material preparation, draft of the manuscript. MM: data collection and analysis, comments on versions of the manuscript. All authors read and approved the final manuscript.

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Declarations

Conflict of interest The authors declare that there is no conflict of interest.

References

Arduino (WWW Document), n.d. URL https://www.arduino.cc/ (Accessed 9.21.21)

Doyle J (1991) DNA protocols for plants. In: Hewitt GM, Johnston AWB, Young JPW (eds) Molecular techniques in taxonomy, NATO ASI series. Springer, Berlin, Heidelberg, pp 283–293. https://doi.org/10.1007/978-3-642-83962-7_18

Fritzing (WWW Document), n.d. URL http://fritzing.org/ (Accessed 10.13.21)

Liang C, Cheng S, Chu Y, Wu H, Zou B, Huang H, Xi T, Zhou G (2013) A closed-tube detection of loop-mediated isothermal amplification (LAMP) products using a wax-sealed fluorescent intercalator. J Nanosci Nanotechnol 13:3999–4005

Martinez FG, Fuchs L, Ramirez R, Breccia J, Oyhenart J (2018) Evaluation of *Tritrichomonas foetus* infection clearance in heifers immunized with a single intravaginal dose of formaldehyde fixed strain B1 cells. Vet Parasitol 255:32–37. https://doi.org/10.1016/j.vetpar.2018.03.019

Morero M, Ramirez MR, Oyhenart J (2021) Taguchi method for the optimization of three loop mediated isothermal amplification procedures for *Tritrichomonas foetus* detection. Vet Parasitol 295:109462. https://doi.org/10.1016/j.vetpar.2021.109462

Mori Y, Notomi T (2020) Loop-mediated isothermal amplification (LAMP): expansion of its practical application as a tool to achieve universal health coverage. J Infect Chemother 26:13–17. https://doi.org/10.1016/j.jiac.2019.07.020

Notomi T, Okayama H, Masubuchi H, Yonekawa T, Watanabe K, Amino N, Hase T (2000) Loop-mediated isothermal amplification of DNA. Nucleic Acids Res 28:E63

Oyhenart J (2018) Direct detection of Tritrichomonas foetus in cattle genital fluid trough loop mediated isothermal amplification of elongation factor 1 alpha 1. Vet Parasitol 261:67–72. https://doi.org/10.1016/j.vetpar.2018.08.011

Oyhenart J, Martínez F, Ramírez R, Fort M, Breccia JD (2013) Loop mediated isothermal amplification of 5.8S rDNA for specific detection of *Tritrichomonas foetus*. Vet Parasitol 193:59–65. https://doi.org/10.1016/j.vetpar.2012.11.034

RStudio | Open source and professional software for data science teams (WWW Document), n.d. URL https://rstudio.com/ (Accessed 10.13.21)

Tomita N, Mori Y, Kanda H, Notomi T (2008) Loop-mediated isothermal amplification (LAMP) of gene sequences and simple visual detection of products. Nat Protoc 3:877–882. https://doi.org/10.1038/nprot.2008.57

Yao C (2021) Control and eradication of bovine trichomonosis in Wyoming, USA by testing and culling positive bulls. Vet Res 52:129. https://doi.org/10.1186/s13567-021-00996-w

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