Loop mediated isothermal amplification of 5.8S rDNA for specific detection of *Tritrichomonas foetus*

Jorge Oyhenart*, Florencia Martínez, Rosana Ramírez, Marcelo Fort, Javier D. Breccia

INCITAP – CONICET – Facultad de Ciencias Exactas y Naturales, Universidad Nacional de La Pampa, Av. Uruguay 151, 6300 Santa Rosa, La Pampa, Argentina

ABSTRACT

Tritrichomonas foetus is the causative agent of bovine trichomonosis, a sexually transmitted disease leading to infertility and abortion. A test based on loop mediated isothermal amplification (LAMP) targeting the 5.8S rDNA subunit was designed for the specific identification of *T. foetus*. The LAMP assay was validated using 28 *T. foetus* and 35 non-*T. foetus* trichomonads strains. It did not exhibit cross-reaction with closely related parasites commonly found in smegma cultures like *Tetratrichomonas spp.* and *Pentatrichomonas hominis*. Bovine smegma did not show interferences for the detection of the parasite and, the sensitivity of the method (4×10^3 CFU/mL, approximately 10 cells/reaction) was slightly higher than that found for PCR amplification with TFR3 and TFR4 primers. The LAMP approach has potential applications for diagnosis and control of *T. foetus* and, practical use for low skill operators in rural areas.

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1. Introduction

Trichomonosis (or bovine trichomoniasis) is a worldwide distributed venereal disease of cattle caused by the flagellate protozoan *Tritrichomonas foetus*. In bulls, the parasite colonizes the preputial cavity with slight or not clinical manifestation (Clark et al., 1974). The infection in females may cause vaginitis, placentitis, uterine discharge, pyometra and abortion (Parsonson et al., 1976).

Economic losses caused by bovine trichomoniasis are significant and currently, there is no effective treatment available (Clark et al., 1983). Vaccines are not effective to prevent cows infection against *T. foetus* and chemotherapy, depending on the country, is not recommended or prohibited due to the limited efficacy and high toxicity (Cobo et al., 2002, 2004; Kvasnicka et al., 1992; Guest and Solomon, 1993). Although outbreaks are reduced with the use of artificial insemination, in extensive farming the control of trichomonosis relies on the search for contaminated bulls prior to the breeding season and the exclusion of positive animals (Bondurant, 2005).

The diagnosis of *T. foetus* is based on the culture of preputial samples and examination under light microscope (Lun et al., 2000; Clark and Diamond, 2002; Schönmann et al., 1994). Negative results are not reported before 7 days of incubation and the method often renders false-positive results (Sarah Parker et al., 2003; Corbeil et al., 2008). Contamination of prepucia with fecal flora is a source of saprophytic protozoa such as *Tetratrichomonas sp.* and *Pentatrichomonas hominis* that grow in culture and mistakenly led to positive diagnosis (Taylor et al., 1994; Hayes et al., 2003; Dufernez et al., 2007). Staining techniques or phase contrast illumination may help in differentiating *T. foetus* from contaminant flagellated protozoa but such methods are prone to observer error (Lun et al., 2000; Taylor et al., 1994).

* Corresponding author. Tel.: +54 92954549591.
E-mail address: jorgeoyhenart@gmail.com (J. Oyhenart).

The presence of *T. foetus* can be detected through polymerase chain reaction amplification of the ribosomal DNA sequences (rDNA) (Riley et al., 1995; Felleisen et al., 1998; Gookin et al., 2002). Primers TFR1 and TFR2 anneal to sequences found in members of the Trichomonadidae family (Felleisen, 1997). The reaction yields a product of variable size that might help to differentiate *T. foetus* from other trichomonads, usually with assistance of a post-amplification technique such as restriction fragment length polymorphism (Hayes et al., 2003). The primer pair TFR3-TFR4 allows specific amplification of the 5.8S gene and flanking sequences ITS1 and ITS2 from *T. foetus* (Felleisen et al., 1998). Sensitivity of PCR detection of *T. foetus* was found similar to the culture technique and takes less than 5 h from DNA isolation to result analysis (Cobo et al., 2007; Mutto et al., 2006). However, the increase in sensitivity entails an increment in costs and higher technical skills.

Loop-mediated isothermal amplification (LAMP) is a technique developed for specific amplification of known DNA sequences (Notomi et al., 2000). The procedure employs a strand displacement DNA polymerase and 4–6 primers that recognize 6–8 regions on the target DNA respectively, thus providing a high level of specificity (Notomi et al., 2000; Nagamine et al., 2002; Imai et al., 2007). The LAMP test produces large amounts of DNA under isothermal conditions (60–65 °C) and it can be performed in a simple water bath. In addition, the high amount of DNA generated makes possible to evaluate the results by the naked eye (Dukes et al., 2006; Hill et al., 2008; Parida et al., 2005; Goto et al., 2009; Iwamoto et al., 2003). Unlike PCR tests, LAMP has shown high tolerance to biological fluids, implying that DNA purification might be dispensable (Poon et al., 2006; Kaneko et al., 2007).

Rapid and easy-to-use procedures based in LAMP technique have been designed and successfully used to detect pathogenic bacteria (*Salmonella* sp., *Legionella* sp., *Listeria monocytogenes*, verotoxin-producing *Escherichia coli*, *Campylobacter* sp.), viruses (norovirus, severe acute respiratory syndrome corona virus, influenza A and B) and parasites (*Plasmodium* sp., *Trypanosoma cruzi*, *Babesia* sp., *Taenia* sp.) from different biological samples (eggs, blood, stools, urine, nasopharyngeal aspirates, etc.) (Mori and Notomi, 2009 and references therein). The aim of this study was to develop a LAMP based assay for the rapid, sensitive and specific diagnosis of *T. foetus* helping to circumvent many drawbacks of the culture and PCR tests.

2. Materials and methods

2.1. Identification of parasite isolates

Trichomonads isolates were obtained from routine diagnosis cultures from bull smegma performed by two local veterinary laboratories. The isolates, cultured on Diamond's modified liquid medium (Lun et al., 2000) supplemented with 10% horse serum (Internegocios SA, Argentina), were periodically examined using an optical microscope to recognize *T. foetus* and non-foetus trichomonads isolates. The isolates were identified by specific PCR amplification using primers TFR1-TFR2 and TFR3-TFR4 (see Sections 2.4 and 2.5, and Fig. 1). The DNA amplicons from every isolate were bi-directionally sequenced using primers TFR1 and TFR2 (see Section 2.8). The isolates were finally identified as 28 *T. foetus*, 22 *Tetratrichomonas spp.* and 13 *P. hominis*.
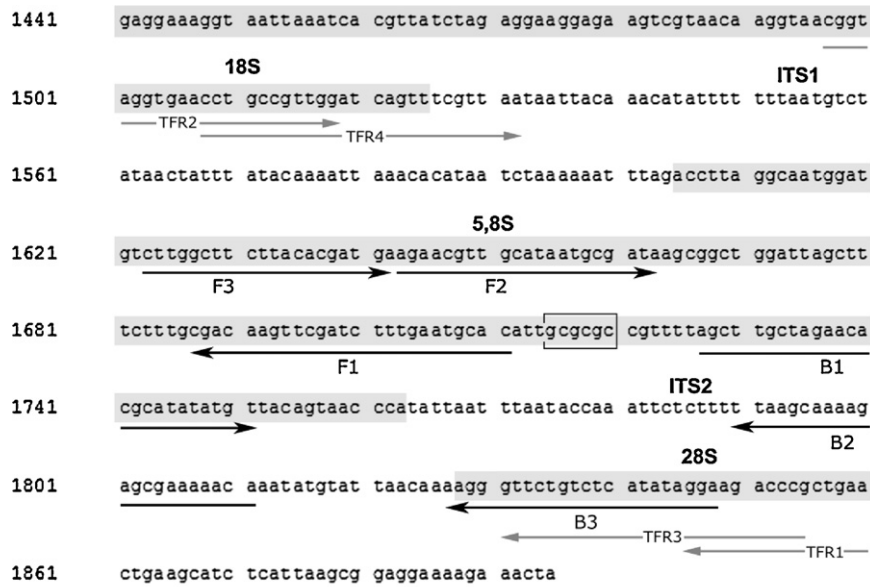

Fig. 1. Schematic representation of the primers used in this study. Nucleotide sequences of 5.8S rDNA used for primers design (GenBank accession number AF466749.1). Highlighted in gray are 18S, 5.8S and 28S rDNA sequences. Primers recognition sequences are shown in capital letters. A right arrow indicates that a sense sequence is used for the primer. The left arrow indicates that the complementary sequence was used for the primer. F1, F2 and B1, B2 are the sequences used in the construction of FIP and BIP primers and they included a TTT linker. TFR1–TFR4 are PCR reported primers (Felleisen et al., 1998; Hayes et al., 2003). The squared sequence depicts recognition and cutting sequences for PauI enzyme.

2.2. Animal fluids

Bovine smegma from bulls was obtained with a 20-cm long catheter attached to a rubber bulb. Fifty milliliters of sterile physiological solution (150 mM NaCl) pre-warmed to 30–37 °C was repeatedly flushed into the preputial cavity then aspirated and placed into sterile test tubes. The absence of *T. foetus* in the smegma samples was checked by culture, PCR and LAMP assays. For DNA extraction, 1 ml sample was centrifuged and the precipitate was used. Another sample (1 mL) was inoculated in Diamond's modified medium at 37 °C and microscopically inspected for the presence of parasite for 10 days.

2.3. Template DNA preparation

Unless indicated in the text, samples were centrifuged 10 min at $7500 \times g$, the pellet was washed twice with Tris-buffered saline solution (150 mM NaCl in 50 mM Tris HCl, pH 7.4). Genomic DNA extraction was performed throughout proteinase K digestion followed by extraction with phenol/chloroform and precipitation with ethanol (Sambrook et al., 2001). Pellets were suspended in 400 μl of milli-Q water and the concentration was estimated through the absorbance at 260 nm using an Ocean Optics USB4000 spectrophotometer.

2.4. Primer sequences

PCR primers TFR1–TFR4 (Fig. 1) were previously reported (Hayes et al., 2003; Felleisen et al., 1998). LAMP forward and backward inner primers (FIP, BIP), and outer primers (F3, B3) were designed by using the Primer Explorer v3 software (http://primerexplorer.jp/lamp, Eiken Chemical Co. Ltd.) based on the GenBank accession number AF466749.1 sequence of the *T. foetus* ribosomal gene (Fig. 1). LAMP and PCR primers were synthesized by Operon (Huntsville, USA).

2.5. Polymerase chain reaction

The primer pairs TFR1-TFR2 were used for amplification of DNA from the trichomonad group and TFR3-TFR4 for specific amplification of *T. foetus* DNA. PCR reactions were performed in 25 μl containing 10 mM Tris–HCl, pH 8.3, 50 mM KCl, 2.0 mM MgCl$_2$, 0.5 μM each primer (TFR1 and TFR2, or TFR3 and TFR4), 140 μM each dNTP and 2 U of Taq DNA polymerase (Fermentas). Fifty nanograms of genomic DNA or 2.5 μl of serially diluted samples (Section 2.10) were used as templates. Amplification comprised 36 cycles of denaturation at 94 °C for 30 s, annealing at 67 °C for 30 s and extension at 72 °C for 90 s, with a final extension step of 15 min at 72 °C. At the end of amplification 6 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) were added and 8 μL of the sample were resolved in 2% agarose gels at 10 V/cm for 30 min and stained with ethidium bromide (0.5 μg/ml). Products were also resolved in 5% polyacrylamide gels [39:1 methylene-bis-acrylamide in Tris–borate EDTA (TBE)] (Sambrook et al., 2001). Band sizes

were calculated by using the Fragsize free sofware (Oliveira de Carvalho, 2002).

2.6. LAMP reaction

Several parameters such as chemicals concentration, temperature, and time were optimized for LAMP reaction by using DNA from the *T. foetus* isolate GM0032. FIP and BIP primers concentration was varied between 0.8 and 2.4 μM, 100 and 400 μM dNTPs, 0 and 1.6 M betaine (N,N,N-trimethylglycine), and 0–4 mM MgSO$_4$. LAMP reactions were carried out at 58–66 °C for 0–120 min in a Multigene gradient thermal cycler (Labnet) or in a water bath.

2.7. Detection of amplified LAMP products

LAMP DNA amplification was assessed through agarose gel electrophoresis or by direct visualization after addition of SYBR Green I (Sigma–Aldrich). For agarose gel electrophoresis 5 μL of loading buffer were added to 20 μL of reaction product and 5 μL were analyzed on 2% agarose gels as indicated above (Section 2.5). For visual inspection, 2 μL of 1/100 dilution of SYBR Green I (S9430, Sigma–Aldrich) were added to 25 μL LAMP reactions. An orange to green color change under natural light or a dark to fluorescent signal under ultraviolet illumination was indicative of positive reaction.

2.8. DNA sequencing

Gel bands from PCR or LAMP reactions were sliced and DNA was purified by using Wizard SV Gel and PCR clean up system (Promega). Pure DNA was sequenced with the Big Dye Terminator system (Applied Biosystems) in an ABI/Hitachi Genetic Analyzer 3130. The sequences were aligned with the target one using the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990).

2.9. LAMP specificity

A total of 63 parasite strains were tested in order to determine the specificity of the LAMP assay. Positive LAMP reactions were precipitated with 2 volumes of ethanol and suspended in 50 μL of 1X PauI (Fermentas) restriction buffer. Digestion was performed with 1 U of PauI at 37 °C overnight. After inactivation of the enzyme, 1–5 μL of product was analyzed on 2% agarose gels as indicated above or on 10% polyacrylamide gels stained with silver nitrate. For silver staining, gels were submerged in fixing solution (10% ethanol, 0.5% acetic acid) and were subsequently stained with 35 mM AgNO$_3$. After brief washing in H$_2$O, gels were incubated in developing solution (3% NaOH, 0.25% formaldehyde) and reaction stopped submerging the gel again in the fixing solution. The specificity of LAMP reactions was also tested by bidirectional sequencing of low molecular weight bands extracted from agarose gel, using F3 or B3 primers.

2.10. LAMP sensitivity

The analytical sensitivity of LAMP was determined assaying serial diluted pure DNA and 2 crude DNA preparations from *T. foetus*. One crude DNA preparation performed with boiled cells in saline buffer (50 mM Tris HCl, pH 7.4, 150 mM NaCl) and the other with boiled cells of *T. foetus* in bovine smegma suspension. Pure DNA from *T. foetus* (100 μg/μL) was 10-fold serially diluted with milli-Q water. Crude DNA samples were performed from a cell suspension (2.5×10^6 *T. foetus* cells/mL) serial diluted (5-fold) in saline buffer and boiling them for 10 min. The smegma was diluted 1:10 with milli-Q water and passed (10 times) through a 25-gauge needle, this suspension was used to prepare cell dilutions (5-fold) starting with 2.5×10^6 parasites/mL and then boiling the samples for 10 min. Samples were kept at $-20\,^\circ$C until use. A volume of 2.5 μl of each dilution was used as template for the amplification assays. Results are representative of three independent assays.

3. Results

3.1. Trichomonads identification

Trichomonad isolates were identified by DNA amplification and sequencing of ITS1, ITS2, 5.8S ribosomal DNA using the primers TRF1 and TRF2. The 63 isolates were identified as 28 strains of *T. foetus*, 22 of *Tetratrichomonas spp.* and 13 of *P. hominis*.

3.2. Detection of T. foetus by LAMP

Incubation of *T. foetus* DNA with *Bacillus stearothermophilus* (*Bst*) DNA polymerase (New England Biolabs) and four primers designed to target the 5.8S rDNA gene produced a characteristic ladder of multiple bands in agarose gels (Fig. 2A). The reactions were performed in a gradient of temperatures (58–66 °C), obtaining the higher signal at 65 °C. The time course of the LAMP reaction showed detectable products of amplification in agarose gel after 90 min of incubation. However, consistent results with any detectable amount of DNA were obtained after 120 min reaction (Fig. 2B). LAMP mixtures containing amplified fragments turned green after addition of SYBR Green I, whereas solutions with no DNA amplification retained the original orange color of the reagent (not shown). Under UV light positive samples containing SYBR Green I showed strong fluorescence while negative reactions were colorless (Fig. 2C).

LAMP amplification products have predictable repeated stem-loop DNA structures with inverted repeats (Notomi et al., 2000). In order to confirm such structure, amplified products were digested with PauI (BssHII), which cuts between F1 and B1 (Fig. 1). PauI digestion generated bands of approximately 110 and 135 bp which was in agreement with predicted fragments of 113 and 135 bp (Fig. 2D). Further confirmation was obtained through direct sequencing of low molecular weight bands with primers F3 and B3, which showed 100% identity with the *T. foetus* targeted 5.8S rDNA sequence (not shown).
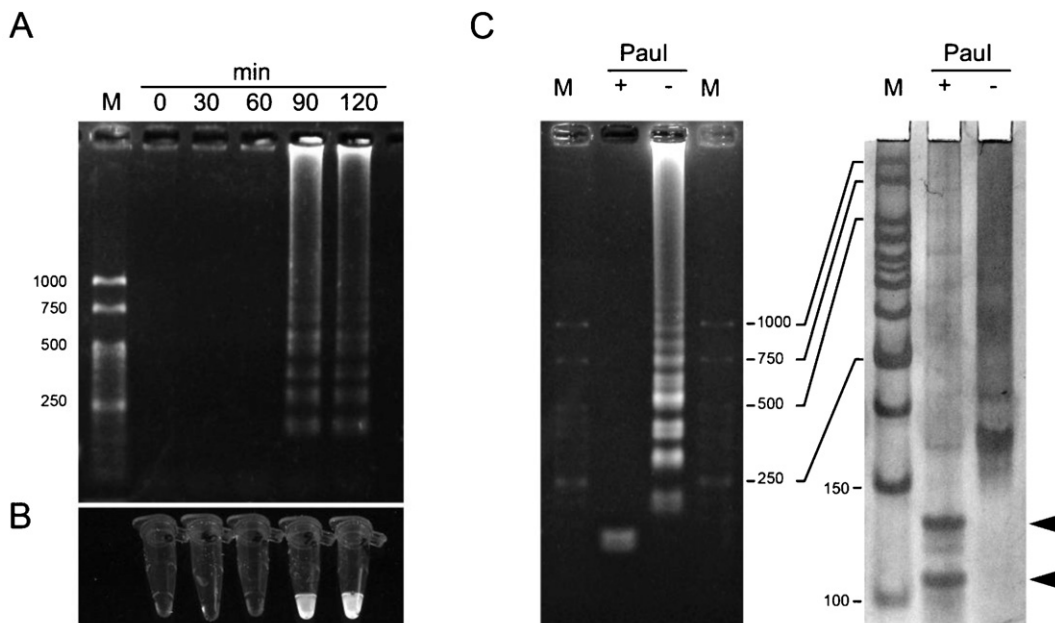

Fig. 2. Loop mediated isothermal amplification of *T. foetus* DNA. (A) Timecourse of LAMP reaction: lane M, 50-bp ladder; the following lanes show LAMP products at 0, 30, 60, 90 and 120 min. (B) Visual inspection: test tubes under UV light containing the same amplification products as in (A). Positive reactions turned bright green upon addition of SYBR Green I, while the negative ones remained dark under UV light. (C) Digestion of *T. foetus* LAMP amplification products with PauI (BssHII) developed in 2% agarose gel (left) and silver stained 10% non-denaturing PAGE (right). M, 50-bp ladder; (+) restriction products (1 μl); (−) non digested product (5 μl). Arrowheads point to digestion products with expected sizes of 113 and 135 base pairs.
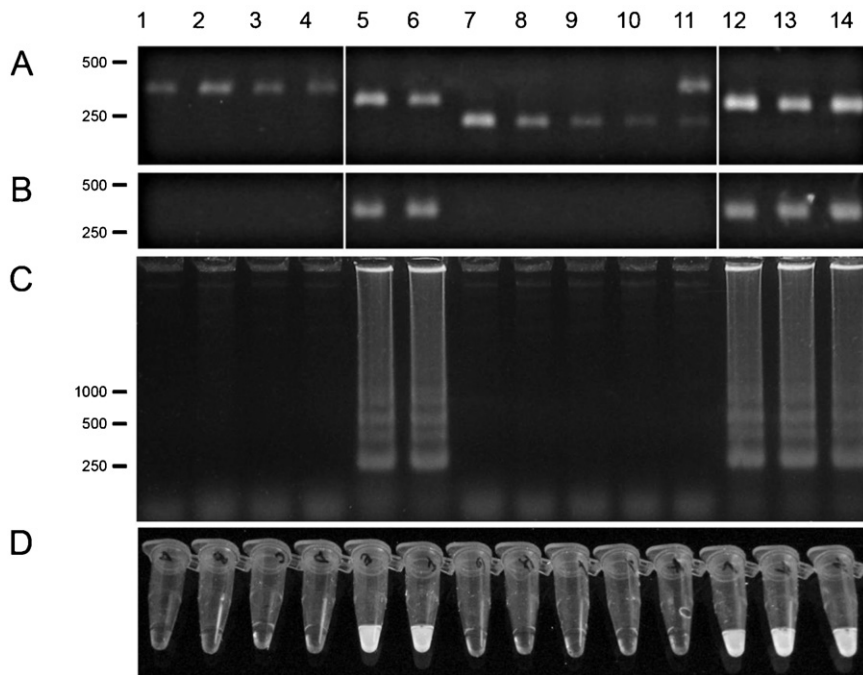
Fig. 3. Specificity of *T. foetus* LAMP. (A) DNA from Trichomonads strains were amplified through PCR (primers TFR1 and TFR2 for genus recognition) and developed on 5% non-denaturing PAGE. Lane M, 50-bp ladder used as a size marker; lanes 1, 2, 3 and 4 *Tetratrichomonas spp.* strains GM018, GM019, GM020 and GM022 respectively; lanes 5, 6, 12, 13 and 14 *T. foetus* strains GM031, GM032, GM050, GM051 and GM052 respectively; lanes 7–10 *Pentatrichomonas hominis* strains GM033, GM034, GM035 and GM036 respectively; lane 11 mixed culture of *Tetratrichomonas spp.* GM022 and *Pentatrichomonas hominis* GM035. (B) The same samples as in (A) but with primers TFR3 and TFR4 (specific for *T. foetus*). (C) Agarose gel analysis of LAMP specific for *T. foetus* 5.8S ribosomal gene. Lanes follow the same order as in (A) and (B). (D) LAMP reactions as in (C) after addition of SYBR green I.

3.3. Specificity of T. foetus rDNA LAMP

LAMP control reactions (without *T. foetus* DNA) performed in absence of betaine showed slight fluorescence and the reaction with *T. foetus* DNA showed lower signal. In order to increase the contrast between positive and negative samples, betaine concentration was optimized using a factorial design, varying it between 0 and 1.6 M. Although low betaine concentrations were enough to eliminate unspecific signals, the lower concentration with the higher signal to noise ratio, for *T. foetus* specific amplification, was found at 0.8 M betaine (not shown). The specificity of 5.8S ribosomal DNA LAMP was evaluated with the 28 strains of *T. foetus*, 22 *Tetratrichomonas spp.* and 13 *P. hominis*. No false negative were found with *T. foetus* strains and no positive results were detected with the non-*T. foetus* trichomonads. The same results were obtained after three independent replication experiments by agarose gel electrophoresis or through visual inspection after addition of SYBR green I in the tubes (Fig. 3).

3.4. Sensitivity of T. foetus rDNA LAMP

Dilutions of *T. foetus* DNA were used to assess both LAMP and PCR sensitivity. Minimum DNA concentration detected with LAMP reaction was 10 pg/mL, either analyzed on agarose gel or by visual inspection (Fig. 4A). Such limit did not improve by preheating the template DNA (data not shown) and it was similar to that obtained with PCR method using primers TFR3-TFR4 (Fig. 4A bottom).

The LAMP approach was then evaluated with disrupted *T. foetus* cells diluted in saline buffer. LAMP and PCR presented similar analytical detection limit of ≈10 cells per reaction (4×10^3 cells/mL) (Fig. 4B).

Finally, LAMP method was tested for its capacity to detect DNA from *T. foetus* cells in raw samples of bovine smegma spiked with the parasite. LAMP assay rendered clear positive results from a dilution close to the detection limit found for the neat buffer dilution of the parasite (4×10^3 cells/ml), whereas PCR showed a faint band at the same concentration (Fig. 4C).

The optimized conditions for LAMP reaction contained 1.6 μM of each FIP and BIP primers, 0.2 μM of each F3 and B3 outer primers, 200 μM each dNTP, 0.8 M betaine (Sigma), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% (v/v) Triton X-100, 8 U *Bst* DNA polymerase in 20 mM Tris–HCl, pH 8.8. The time for the reaction was set at 120 min at 65 °C and the enzyme was inactivated by increasing the temperature (80 °C) for 10 min at the end of the reaction.

4. Discussion

The control of trichomonosis relies on accurate identification of *T. foetus* through culturing and observation of the protozoa from smegma samples. This microbiological procedure depends on pathogen viability and takes over a week to deliver conclusive results. Since contamination is not uncommon, especially with non-pathogenic
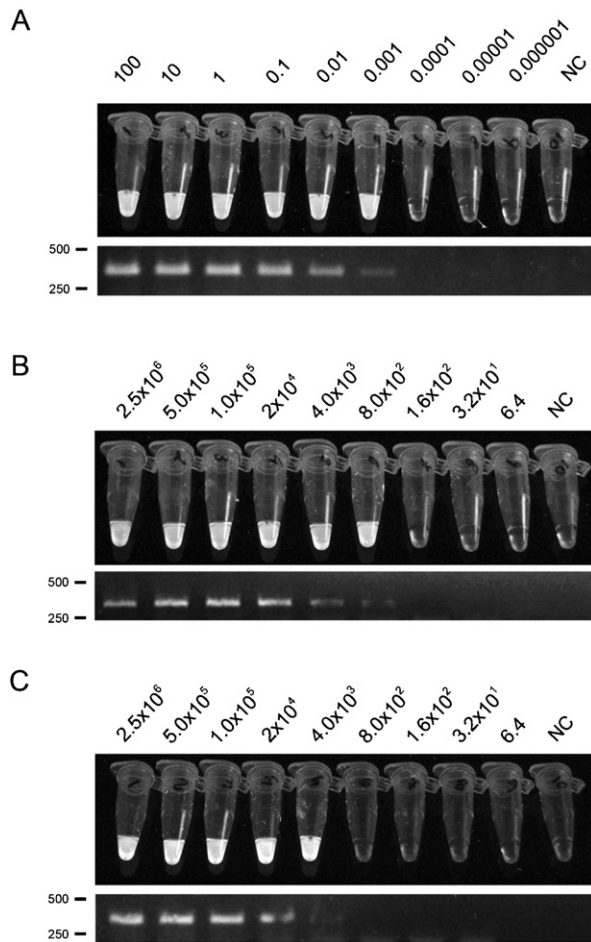
A

B

C

Fig. 4. Analytical sensitivity of *T. foetus* 5.8S rDNA LAMP. (A) DNA from *T. foetus* GM032 at different concentrations used for both LAMP (top) and PCR (bottom) assays. Detection limit $\approx$10 pg. (B) Sensitivity of LAMP assayed with crude DNA consisting of lysates of *T. foetus* cells in saline solution. Detection limit $\approx$4 $\times$ 10^3 cells/mL. (C) Sensitivity of LAMP assay for smegma spiked with different concentrations of *T. foetus* GM032. Detection limit $\approx$4 $\times$ 10^3 cells/mL. DNA (ng) and organism concentration (cells/mL) used for LAMP and PCR are indicated in the top of the gels. NC: negative control without DNA or without cells. The position of the molecular markers (from a 50 bp marker) are indicated in the left side of the gels. The results corresponded to three independent experiments were performed by triplicate.

trichomonads, it often results in false-positive diagnosis (Clark and Diamond, 2002). PCR approach based in the amplification of rDNA sequences helps for the rapid discrimination of *T. foetus* from other trichomonads (Felleisen et al., 1998). However, PCR requires precision instruments for the amplification and the sensitivity of the simplest test for *T. foetus* detection is overall comparable to the culture method (Cobo et al., 2007).

The 5.8S rDNA sequence was chosen because it is the only well characterized genomic sequence among the trichomonad group. Thus we were capable to control the presence of specific and non-specific target sequences. Four primers lead to specific amplification of *T. foetus* DNA in less than 2 h. Faster reactions can be obtained by adding loop primers that would initiate further DNA amplification from the stem-loop region (Nagamine et al., 2002). Nevertheless, we tested manually designed loop primers, without detecting any improvement in sensitivity or reducing the reaction time of the system. These results might be due to the already stem loop rich structure of ribosomal sequences. The knowledge of the parasite genome would make possible the design of new LAMP protocols based in other specific sequences.

The analytical sensitivity of the LAMP assay for *T. foetus* was comparable to that obtained with PCR, when tested against purified DNA or crude DNA from cells suspended in buffer. In a complex fluid (smegma), PCR amplification showed a dim band at 4.0×10^3 cell/mL, while LAMP amplification was positive without decreasing the signal strength (Fig. 4). Such effect is a usual finding in LAMP reactions and might be explained by the high amount of DNA obtained by LAMP reactions, as it was previously reported, where isothermal amplification yielded almost 100 times more DNA than a PCR (Mori et al., 2001). On the other hand, sensitivity of PCR for *T. foetus* has been enhanced through enzyme linked assay (Felleisen et al., 1998), nested-PCR (Gookin et al., 2002; Fernandes et al., 2008) and real time PCR (McMillen and Lew, 2006; Dufernez et al., 2007). Nevertheless, LAMP for *T. foetus* detection is a technique conceived to perform DNA amplification with minimal equipment and the high sensitivity achieved ($\approx$10 parasites/reaction) could be used for direct diagnosis with minimal sample preparation. Up to now, LAMP is rapid, sensitive and specific as most molecular methods and it has found useful applications in diagnosis of virus, several pathogenic microorganisms and few parasites (Mori and Notomi, 2009).

The high amount of DNA generated is, to our advice, the major potential pitfall of LAMP as it is extremely easy to contaminate a laboratory by simply opening a test tube. However, several methods have been designed to obtain the results without even opening tubes, including turbidity measurements (Mori et al., 2001), calcein or hydroxy naphthol blue color changes (Tomita et al., 2008; Goto et al., 2009). *T. foetus* isothermal amplification was clearly visualized after addition of SYBR Green I. An interesting approach was recently reported where the fluorescent compound is entrapped into wax beads that melt at 80 °C and it is released in the last step of the reaction, without interfering during the amplification (Mao et al., 2012).

Diagnosis of trichomonosis has gained sensitivity by increasing the frequency of bull sampling as well as combining microbiological culture and PCR methods, as the best way to identify every infected animal (Mendoza-Ibarra et al., 2012; Cobo et al., 2007). Since the control of *T. foetus* does not seem to evolve toward more simplified tests, the LAMP approach could offer an interesting alternative. Particularly, providing a point of care test for small labs and rural locations.

Acknowledgments

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