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Taguchi method for the optimization of three loop mediated isothermal amplification procedures for *Tritrichomonas foetus* detection

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ABSTRACT

Taguchi method was used to optimize loop mediated isothermal amplification tests aimed to amplify segments of the elongation factor 1a1 (tf-ef1a1), the 5,8 ribosomal gene (tf-5,8 r) or the beta tubulin 2 (tf-btub2) from the protozoan parasite *Tritrichomonas foetus*. L9 orthogonal array and quadratic loss functions that penalize deviations from prediction values revealed the effect of amplification reaction components. Analysis of variance (ANOVA) decomposed the contribution of individual factors to a small Ct. Confirmation experiments established that optimum conditions were predictable, verifiable and reproducible. Primers concentration conditioned the non-specific amplification of tf-ef1a1 while betaine and magnesium concentration contributed to accelerate the time to reach a positive threshold in tf-ef1a1, tf-5,8 r and tf-btub2. The general strategy of simple and robust experimental design holds potential as a general optimization protocol for LAMP tests in every diagnostic laboratory.

1. Introduction

Bovine trichomonosis is a worldwide distributed venereal disease caused by the flagellate protozoan *Tritrichomonas foetus*. The infection is related to long intercalving periods and low conception rates (Clark et al., 1983; Mylrea, 1962). The damage predicted in a herd with 20 % prevalence of *T. foetus* would be around 14 % reduction of the annual calf crop (Rae, 1989). There is no vaccine to effectively control *T. foetus* host colonization and it is not advisable to use drugs to treat *T. foetus* carrier animals. Bovine trichomonosis control relies upon the identification of infected bulls followed by their removal from the herd (World Assembly, of Delegates of the OIE, 2018).

Preputial samples from potential carrier bulls are tested through culture and microscopical examination or with quantitative PCR (qPCR). Culture sensitivity is low (~70−80 %) and negative results cannot be delivered before 7 days (Cobo et al., 2007). Mandatory control plans often impose culture tests to be repeated and culling be effective with a single positive result (Yao, 2013). Otherwise contamination of preputial samples and growth of saprophytic protozoa may cause false positive diagnosis (Hayes et al., 2003). Staining techniques or standard PCR help to confirm the growing protozoa are *T. foetus* (Cobo et al., 2007).

Standard PCR detection has a similar overall sensitivity than the culture technique and takes less than five hours from DNA isolation to results analysis (Felleisen et al., 1998). Quantitative PCR combines the high specificity of the end-point PCR with a high sensitivity (McMillen and Lew, 2006). The qPCR technique has gained place in *T. foetus* detection despite requiring expensive amplification equipment, costly methods of DNA purification and highly trained operators.

Loop-mediated isothermal amplification (LAMP) is a technique developed for specific amplification of known DNA sequences (Notomi et al., 2000). 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 (Mori and Notomi, 2020; Notomi et al., 2000). LAMP is carried out at isothermal temperature and generates large amounts of DNA. Thus it is not mandatory to use an expensive thermal cycler and the result can be read through multiple systems, even with the naked eye (Mori and Notomi, 2020). Furthermore, unlike PCR, LAMP tests have shown high tolerance to biological fluids, implying that DNA purification might be dispensable (Oyhenart, 2018).

A LAMP test based on the amplification of the *T. foetus* 5.8 ribosomal DNA sequence (tf-r5.8) was shown to be more sensitive than culture and

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standard PCR and it could be used for direct detection of *T. foetus* in preputial samples (Oyhenart et al., 2013). Another LAMP test targeting the *T. foetus* elongation factor 1 alpha 1 (tf-ef1a1) was 100–1000 times more sensitive than standard PCR and performed well with crude contaminated cervical samples (Oyhenart, 2018). The same tf-ef1a1 LAMP performed better as well as qPCR method with crude samples and it was successfully used in the search for *T. foetus* in cat feces, porcine and bovine swabs (Dąbrowska et al., 2020, 2019). A third LAMP method targeting *T. foetus* beta-tubulin genes was also shown as useful as tf-ef1a1 LAMP (Dąbrowska et al., 2019).

LAMP can be performed in any research or diagnostic laboratory in a water bath, a turbidimeter, a fluorescence reader or a real-time thermal cycler. There are ready to use reactions as well as reaction components that can be separately acquired and mixed in-house. However, implementing a new LAMP protocol or replicating a procedure developed by another laboratory can be difficult. Positive results in absence of DNA template or negative results in tubes with a correct substrate are common in the use of this technique (Kuboki et al., 2003; Nagai et al., 2016; Wang et al., 2015).

LAMP, like any other technique, needs adjustments. LAMP reactions are overall tolerant to temperature variation and most of protocols are set at 65 °C. Primer sequences design can be done through free software available at Eiken (http://primerexplorer.jp/e/). Large resourced laboratories test several couples of primers and keep only those that allowed amplification. Primer concentration should be adjusted as not all of them would behave the same way and synthesis as well as purification are not ideal.

Otherwise changes in reagents concentration can have a strong influence in the results. The LAMP procedure can be influenced by magnesium sulfate ($MgSO_4$), betaine (Trimethylglycine) and deoxyribonucleotides (dNTPs) (Notomi et al., 2000; Wang et al., 2015). To find a most convenient setting for diagnostic reaction conditions may help to reduce the risk of false results more or less common in each diagnostic tool (Oyhenart, 2020; Wang et al., 2015). Furthermore, optimization can help to shorten the detection time which is directly related to higher sensitivity.

Factorial methods can help optimize molecular techniques by evaluating all combinations of all levels of each factor (Thanakiatkrai and Welch, 2012). If several variables are to be analyzed at many levels a factorial design can lead to a very large number of experiments. Taguchi method is an alternative approach that allows to identify more appropriate conditions for a process by minimizing the number of experiments and reducing time and effort (Lin, 2002; Nalbant et al., 2007).

The aim of this work was to determine the impact of some components of the LAMP reaction (namely $MgSO_4$, betaine and dNTPs) on DNA amplification to demonstrate that different sets of primers may require different configurations. By using a Taguchi design, a more convenient environment was found to improve the speed of LAMP reactions that drive the amplification of fragments of three genes from the *T. foetus* genome: the 5.8S ribosomal DNA (tf-5.8 s), the elongation factor 1 alpha 1 gene (tf-ef1a1) and the beta tubulin 2 gene (tf-btub2).

2. Materials and methods

2.1. DNA preparation

T. foetus DNA was obtained from cells growing in liver infusion medium supplemented with 10 % horse serum. Cells were centrifuged by ten minutes at 8,000×*g* and pellets were washed 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 mili-Q water and DNA concentration was estimated through absorbance at 260 nm. DNA from *Tetratrichomonas* spp or *Pentatrichomonas hominis* DNA was obtained through the same procedure and used to test the specificity of LAMP reactions (Oyhenart et al., 2013). *Monocercomonas colubrorum* DNA was also used to test the specificity and it was gently provided by Dr. Vladimir Hampl (Hampl et al., 2007).

Table 1
Primers used in tf-5.8 s, tf-ef1a1 and tf-btub2 loop mediated isothermal amplification reactions.

Primer name	Sequence
tf-5,8 r-F3	5'-CTTGGCTTCTTACACGATGA-3'
tf-5,8 r-B3	5'-TCCTATATGAGACAGAACCCTT-3'
tf-5,8 r-FIP	5'-GTGCATTCAAAGATCGAACTTGTCGAGAACGTTGCATAATGCGATA-3'
tf-5,8 r-BIP	5'-AGCTTGCTAGAACACGCATATATGTTGTTTTTCGCTCTTTTGCTTAA-3'
tf-ef1a1-F3	5'-TCGCTCTGGAAGTTCGAATC-3'
tf-ef1a1-B3	5'-TGACGGCGATGATGACTTG-3'
tf-ef1a1-FIP	5'-CGGCAGCATCAGCTTGTGATGTTTAATCATCGATGCTCCAGGAC-3'
tf-ef1a1-BIP	5'-CAACACGTGGTGGTTTCGAAGCTTCGAGGGTGAAAGCAAGAAGA-3'
tf-ef1a1-LF	5'-CCTGTGATCATGTTCTTGATGAAGT-3'
tf-ef1a1-LB	5'-TGGTATCGCTGAACAGGGC-3'
tf-btub2-F3	5′-AACAATGGACTCAGTCCGTG-3′
tf-btub2-B3	5′- GGAGTGTTCCGAGACCTGA-3'
tf-btub2-FIP	5′-ACCCTTGGCCCAGTTGTTACCTTCTTCCGCCCAGATAACTTCG-3′
tf-btub2-BIP	5′-CCGTAAGGAAGCCGAATCCTGCTTACCTGTTCCACCACCGAG-3′
tf-btub2-LF	5′-GGCACCAGATTGTCCGAAAA- 3′
tf-btub2-LB	5′-CGCCCTCCAAGGATTCCAACT -3′

2.2. Primer sequences

Tf-r5.8 and T-ef1a1 primers were already described and are shown in Table 1 (Oyhenart, 2018; Oyhenart et al., 2013). Tf-btub2 LAMP primers (Table 1) were designed with Primer Explorer v5 software (http://primerexplorer.jp/e/) and their purpose is the amplification of a fragment of the beta tubulin 2 gene (GenBank accession number: AY277787.1). Fig. 1 shows the amplified sequence in the tf-btub2 gene aligned with similar sequences from the *T. foetus* genome and in related genomes.

2.3. Loop mediated isothermal amplification

Standard LAMP conditions were: 1.6 u M FIP and BIP, 0.2 u M B3 and F3, 0.8 u M LB and LF, 4.5 mM MgSO4, 0.8 M betaine, 0.125 mM dNTPs, 10 mM KCl, 11 mM (NH4)2SO4, 0.1 %v/v Tween 20 and 8U Bst DNA polymerase (Mclab, San Francisco, CA, USA) in 20 mM Tris−HCl, pH 8.8. Unless indicated 100 ng of *T. foetus* DNA and 1/100,000 SYBR GREEN I (Sigma-Aldrich) were used. Twenty five (25) ul LAMP reactions were incubated at 65 °C and read for 120 min. The fluorescence signal was recorded every 60 s in a Heal Force Real-time Thermal Cycler X960 (Heal Force, Shanghai, China). Each condition was tested in duplicate with milliQ water as negative control. Drugs were purchased from Merck. Primers were from Eurofins and Integrated DNA Technologies.

2.4. Taguchi method

The primers concentration of tf-ef1a1 was adjusted by using an orthogonal matrix (L9) because false positive results was observed in

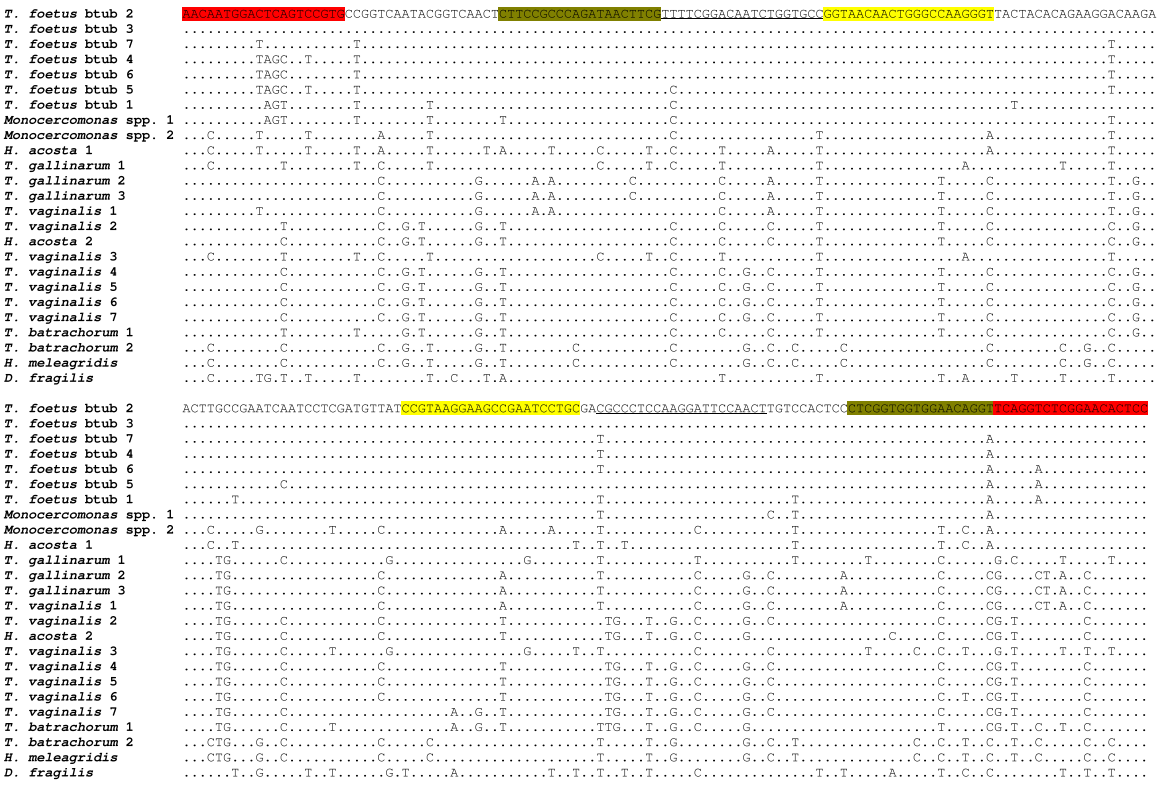

Fig. 1. *Tritrichomonas foetus* tubulin 2 fragment amplified in the tf-btub2 loop mediated isothermal amplification and similar sequences. F3 and B3 primers are depicted in red. F2 and B2 regions contained in the 5'-end of FIP and BIP primers are highlighted in green. F1 and B1 regions contained in the 3'-end of FIP and BIP primers are highlighted in yellow. Loop primers target sequences are underlined. Reference sequences for *T. foetus* and other members of the parabasalia group are: *T. foetus* beta tubulin 2: AY277787.1, *T. foetus* beta tubulin 1: AY277786.1, *T. foetus* beta tubulin 3, 4, 5, 6 and 7 were previously described (Oyhenart and Breccia, 2014), *Monocercomonas* spp. 1: AY277790.1, *Monocercomonas* spp. 2: AY277791.1, *H. acosta* 1: AY277780.1, *H. acosta* 2: AY277779.1, *T. gallinarum* 1: AY277781.1, *T. gallinarum* 2: AY277782.1, *T. gallinarum* 3: AY277783.1, *H. meleagridis:* JN390975.1, *T. batrachorum* 1: AY277788.1, *T. batrachorum* 2: AY277789.1, *D. fragilis*: M186142.1, *T. vaginalis* 1: XM_001582993.1, *T. vaginalis* 2 XM_001321203.1, *T. vaginalis* 3: XM_001311949.1, *T.vaginalis* 4: XM_001284521.1, *T. vaginalis* 5: XM_001300401.1, *T. vaginalis* 6: XM_001579619.1, *T. vaginalis* 7: XM_001318453.1.

Table 2
L9 (3 × 3) orthogonal array. The matrix shows nine reactions with combinations of factors $MgSO_4$, betaine and dNTPs at three different concentrations.

Reaction	Factor		
	$MgSO_4$ (mM)	betaine (M)	dNTPs (mM)
1	2.25	0.4	0.062
2	2.25	0.8	0.125
3	2.25	1.77	0.250
4	4.5	0.4	0.125
5	4.5	0.8	0.250
6	4.5	1.77	0.062
7	9	0.4	0.250
8	9	0.8	0.062
9	9	1.77	0.125

previous experiment with standard primers concentration. In the orthogonal arrangement 3 concentrations of each pair of oligonuclotides (0.4, 0.2 and 0.1 u M of B3 and F3, 0.2, 0.4 and 0.8 u M of LB and LF) were evaluated to verify specific and non-specific amplification in 120 min. The final concentration of the tf-ef1a primers was 0.8 u M of FIP and BIP, 0.2 u M of B3 and F3, 0.2 u M of LB and LF.

An orthogonal matrix L9 (3 × 3) was employed to optimize the concentrations of factors $MgSO_4$, betaine and dNTPs of tf-5.8 r, tf-ef1a1 and tf-btub2 LAMP reactions. A preliminary screening covering a range of 0−15 mM $MgSO_4$, 0−2 M betaine and 0.03−0.5 mM dNTPs showed ranges beyond which DNA amplification could not be observed. Lower-upper limits of betaine were 0.4–1,77 M, of $MgSO_4$ were 2.25−9 mM and of dNTP were 0.062−0.250 mM. Then, three factor levels were calculated by multiplying and dividing the reference concentration by 2. As detailed in Table 2 nine different reactions were evaluated with combinations of each factor at a given concentration.

Taguchi method can use a loss function to estimate the "quality loss" of a process (Ross, 1996; Nalbant et al., 2007; Lin, 2002). The loss function mathematically penalizes small deviations from a theoretical target value and is defined as S/N ratio. According to the objective of the experiment there are three types of S/N ratio: larger is better, smaller is better and nominal is better. Lowest Ct values being "smaller is better" the S/N ratio was obtained with Eq. (1) (Thanakiatkrai and Welch, 2012):

$$\text{S/N ratio} = -10 \times \log_{10} (\Sigma\, y^2/r) \quad (1)$$

Where r = number of replicates and y = response variable (Ct value). S/N ratios were calculated for each LAMP reaction by employing the Ct values of duplicate experiments (Cobb and Clarkson, 1994; Nalbant et al., 2007). In order to perform the calculations, the reactions that did not produce amplification were considered to have a Ct of 120 min. The optimal reactions conditions were those that minimized the Ct value and maximized the S/N ratio.

Analysis of variance (ANOVA) was used to establish which factors significantly affect the response variable (Lin, 2002; Nalbant et al., 2007; Ross, 1996). S/N ratio means were compared at different levels of the factors. The experimental design was evaluated at a confidence level of 95 %. The percent contribution of each factor (x) to the reactions was calculated according to Eq. (2):

$$\text{CP (\%)}: Ss_x/SS_T \quad (2)$$

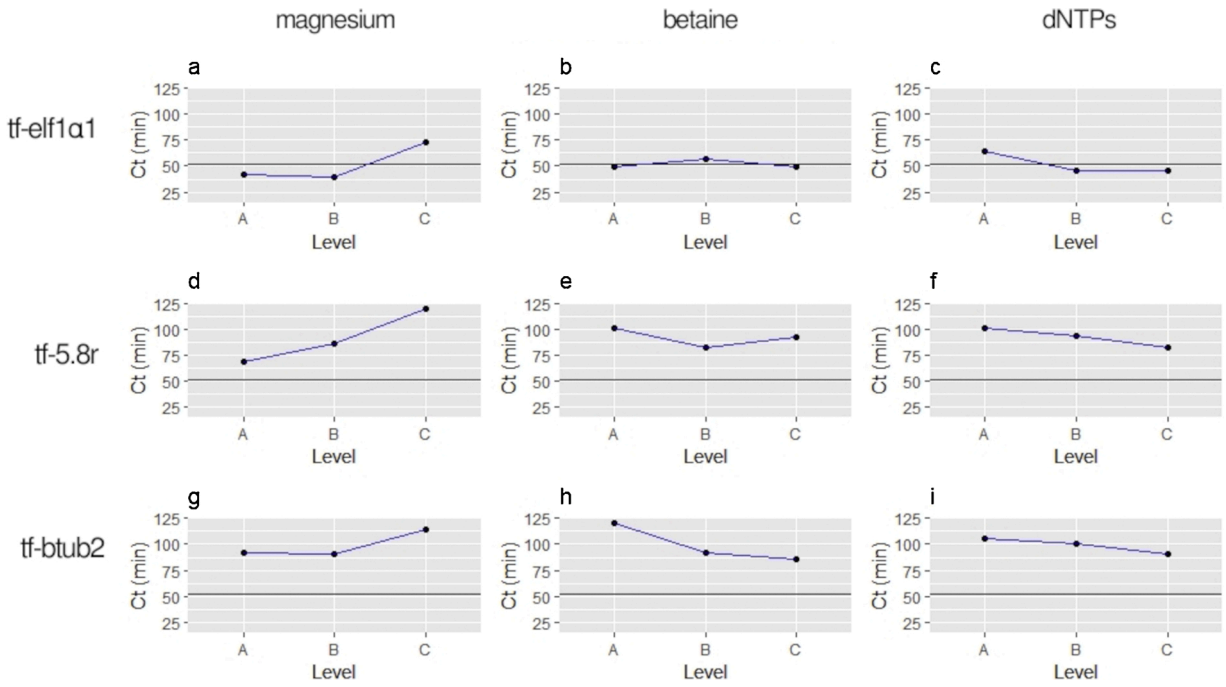

Fig. 2. Main effects of magnesium sulfate, betaine and dNTPs on the speed of LAMP reactions. Tf-elf1α1 LAMP (a, b and c), Tf-r5.8 (d, e and f) and Tf-btub2 (g,h and i). LAMP reactions at different concentrations of magnesium sulfate (a,d and g), betaine (b, e and h) and dNTPs (c, f and i). Values on the x-axis represent three levels for each factor. Response (Ct) values are expressed in minutes on the y-axis. Horizontal lines represent the mean response values.

Table 3
Response table and mean signal to noise ratio for each factor level. Levels A, B and C for each factor are: 2.25, 4.5 and 9 mM magnesium, 0.4, 0.8 and 1.7 M betaine and 0.062, 0.125 and 0.250 mM dNTP. The highest ratios are shown in bold and the optimal levels are shown under heading optimal condition. CP (%): contribution percentage. ** significant at 95% confidence level.

LAMP	Factor	Mean S/N ratio from each level			Delta	Rank	CP (%)	Optimal condition
		A	B	C				
tf-ef1a1	Magnesium	−32,47	**−31,93**	−36,94	5,01	I	69,531	4.5 mM
	Betaine	−33,77	−33,82	**−33,75**	0,07	III	0,429	1.7 M
	dNTPs	−35,34	**−33,00**	−33,01	2,34	II	12,438	0.125 mM
tf-r5.8	Magnesium	**−36,68**	−38,62	−41,58	4,91	I	73,499**	2.25 mM
	Betaine	−39,99	**−37,95**	−38,94	2,05	III	12,596**	0.8 M
	dNTPs	−39,98	−39,06	**−37,84**	2,13	II	13,817**	0.250 mM
tf-btub2	Magnesium	−39,12	**−38,98**	−41,12	2,15	II	29,534	4.5 mM
	Betaine	−41,58	−39,03	**−38,61**	2,98	I	53,254	1.7 M
	dNTPs	−40,31	−39,91	**−39,00**	1,32	III	9,325	0.250 mM

where: SSx is the corrected sum of squares of the S/N ratio of factor x and SST is the total sum of squares.

To verify the improvements in the three LAMP detection systems, confirmation experiments were carried out with optimal levels that maximize the S/N ratio and minimize the Ct values.

2.5. LAMP sensitivity

The analytical sensitivity of LAMP reactions was determined by testing serial dilutions. Pure *T. foetus* DNA (100 ng/uL) was 10-fold serially diluted with milliQ water. *T. foetus* cells from fresh cultures in exponential growth phase were washed three times in buffered saline solution (150 mM NaCl, 50 mM Tris HCl, pH 7.4), counted and 10-fold serially diluted in the same buffer. A volume of 1 ul of each dilution was used as template for the amplification assays. Closed tube reactions included calcein (50 μM) and MnCl2 (0.5 mM). Results are representative of two independent assays.

2.6. Statistical analysis and data format

All datasheets and statistical analysis were performed with R Statistical Software v3.4.4 through the RStudio integrated development environment 4.0.3. Library used for the Taguchi design was qualityTools. The graphics were made with ggplot2, scales and cowplot libraries.

2.7. Ethical statement

The study did not involve the use of human or animal subjects or samples.

3. Results

Tf-r5.8 s, tf-ef1a1 and tf-btub2 LAMP reactions were performed with different concentrations of magnesium sulfate, betaine and dNTPs. Results are resumed in Fig. 2 and Table 3. Increasing magnesium concentration led to a sustained increase in tf-r5.8 Ct values (Fig. 2d). Meanwhile tf-ef1a1 and tf-btub-2 reactions showed similar Ct values

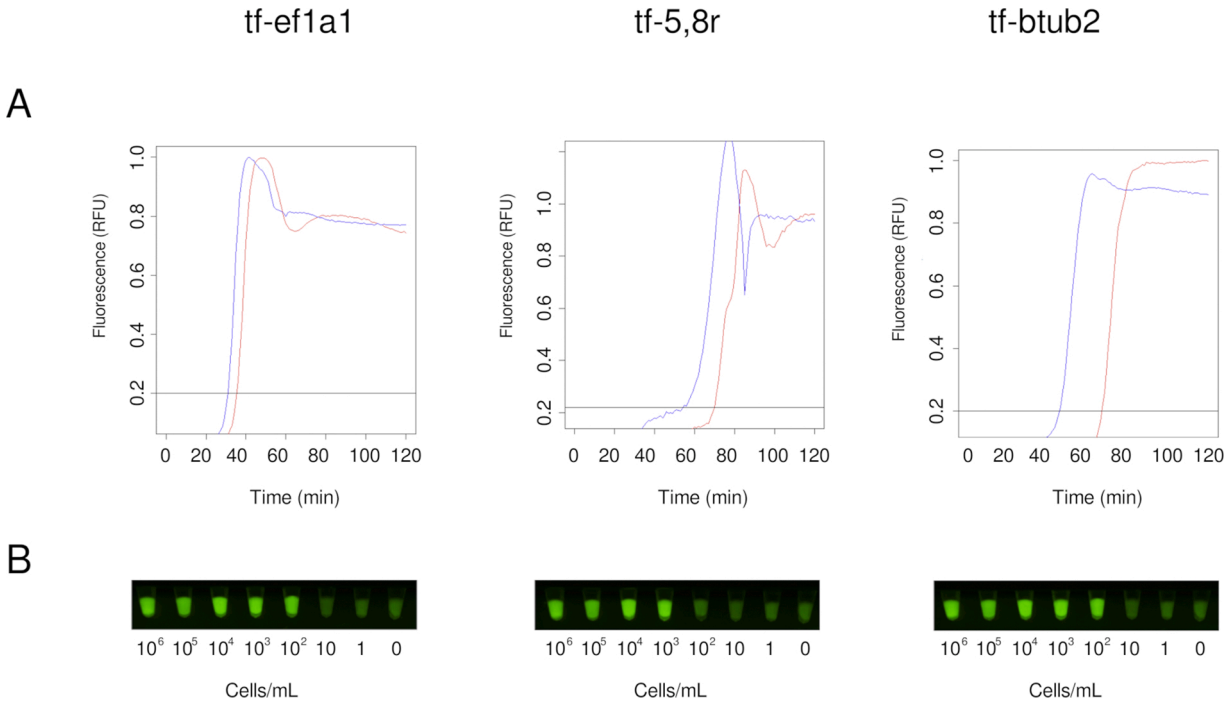

Fig. 3. Improvent in Ct values and sensitivity of *Tritrichomonas foetus* LAMP reactions. A) Change in fluorescent signals with initial (red) and final (blue) conditions are shown for tf-ef1a1, tf-r5.8 and tf-btub2 LAMP reactions. B) Sensitivity of tf-ef1a1, tf-r5.8 and tf-btub2 LAMP assays for saline solution spiked with different concentrations of the parasite. Serial dilutions (10-fold) of *T. foetus* B1 cells were performed and 1 u L sample was tested. Tf-ef1a1 and tf-btub2 reactions were consistently positive with 0.1 cells/reaction (10^2 cells/mL) and tf-r5.8 reactions were consistently positive since 1 cell/reaction (10^3 cells/mL).

with 2–4.5 mM magnesium and were less performant (Ct >100 min) with 9 mM magnesium (Fig. 2a and g).

Tf-ef1a1 reactions showed similar Ct values with 0.4–1.7 M betaine (Fig. 2b). Low betaine concentration (0.4 M) caused that tf-r5.8 and tf-btub2 reactions either to start after 80 min or not amplified (Fig. 2e and h). High betaine concentrations showed a positive effect on the speed of tf-bt2 reactions (Fig. 2h).

Low dNTPs concentrations (<0.125 mM) decreased the kinetics of all LAMP reactions (Fig. 2c, f and i). Tf-ef1a1 reactions presented similar Ct values between 0.125 and 0.250 mM dNTPs (Fig. 2c). Increasing dNTPs concentration showed a positive effect on the speed of tf-r5.8 and tf-btub2 reactions (Fig. 2f and i).

Optimal concentrations of each factor being those that maximized the S/N value delta values were obtained and were used to rank the response variable (Table 3). The optimal conditions for tf-ef1a1 LAMP were 4.5 mM magnesium, 1.7 M betaine and 0.125 mM dNTP, while for the tf-r LAMP were 2.25 mM magnesium, 0.8 M betaine and 0.250 mM dNTP 5.8 and for the tf-btub2 LAMP were 4.5 mM magnesium, 1.7 M betaine and 0.250 mM dNTP (Table 3).

ANOVA was used to quantify the variation issued from each control factor on tf-ef1a1, tf-r5.8 and tf-btub2 LAMP reactions. As shown in Table 3 the variability due to magnesium concentration headed the values in LAMP tf-ef1a1 and tf-r5.8 while betaine had the greater contribution in LAMP tf-btub2. The variability due to magnesium was significantly higher than experimental error in tf-r5.8 and was not significant in LAMP tf-ef1a1 and in LAMP tf-btub2.

To verify the improvement in the response value the reaction conditions predicted through Taguchi were contrasted with initial conditions (2.25 mM magnesium, 0.8 M betaine and 0.125 mM dNTPs). The results obtained showed reductions of Ct values of 4.4 min for tf-ef1a1 (35,9–31,6; 95 % CI = 19.2–43.9), 15.7 min for tf-r5.8 (68,2–52,5, 95 % CI = 51.8–53.2) and 16.5 min tf-btub2 (64,8–48,3; 95 % CI = 43,0–53,5) (Fig. 3A). The sensitivity of tf-ef1a1, tf-r5.8 and tf-btub2 LAMP reactions with pure DNA was around 10 pg/ul. Furthermore, tf-ef1a1 and tf-btub2 reactions showed positive results with 0.1 cells/reaction and tf-r5.8 reactions were consistently positive since dilutions containing 1 cell/reaction (Fig. 3B).

4. Discussion

Taguchi method allows to quickly test buffer components that most likely influence a LAMP reaction. Here in three LAMP reactions for *T. foetus* DNA detection were tested and all three showed improved Ct times and analytical sensitivity. Optimal conditions were established through real-time fluorescence monitoring but can be translated to naked-eye detection.

The three tested *T. foetus* LAMP reactions present themselves with different particularities. The tf-5.8r lacks loop primers and target a region with 37 % GC content which is predicted to have a strong tertiary structure. The tf-ef1a1 amplified region has 48 % GC content and it is part of one the most highly transcribed genes (Huang et al., 2013). It is possible that the high tf-ef1a1 reaction speed comes from amplification of ef1a1 transcripts. The tf-btub2 aimed region has 53 % GC content and it is highly similar to other regions in the same genome (Oyhenart and Breccia, 2014). All three LAMP reactions are expected to react differently to changes but it is not possible to confidently predict the way or the amplitude. It was shown that loop primers greatly help to increase the time for detection (Nagamine et al., 2002). However, from this work it is clear that reactions lacking loop primers can still be improved.

LAMP is a very powerful technique that is gaining place in the infectious diseases detection lab (Mori and Notomi, 2020). Public policies included LAMP as an effective way to detect widely distributed common pathogens (Global Tuberculosis Programme, 2016). Rapid and easy-to-use procedures based in LAMP have been designed to detect microorganisms from different biological samples with high sensitivity and specificity in high or low complexity laboratories. The sars-cov2 pandemic and the need for highly sensitive rapid tests has accelerated the implementation, approval and use of several LAMP-based kits in recent months.

5. Conclusion

The use of the Taguchi design allowed us to improve the kinetics of three LAMP reactions. This study showed that there is not a single appropriate condition for amplification. The inherent characteristics of the LAMP targeted region could strongly influence the output. Also, the use of standard conditions or ready-to-use mixtures can help to set up new reactions but a fast inspection of different settings can help to improve the results of a LAMP reaction.

CRediT authorship contribution statement

Jorge Oyhenart conceived the study. **Mariana Morero** carried out experiments and statistical tests. Manuscript was drafted **Mariana Morero** and **Jorge Oyhenart**. Funding was from **Rosana Ramirez** and **Jorge Oyhenart** projects. All authors approved the manuscript.

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Declaration of Competing Interest

The authors report no declarations of interest.

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