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5 ORIGINAL RESEARCH ARTICLE

6 Use of colorimetry in a new LAMP test for the visual detection 7 of *Chlamydia abortus* in domestic ruminants

8 ***Uso de colorimetría en una prueba nueva de LAMP para la detección visual de***
9 ***Chlamydia abortus en rumiantes domésticos***

10 ***Utilização de colorimetria em um novo teste LAMP para a detecção visual de Chlamydia***
11 ***abortus em ruminantes domésticos.***

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22

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Abstract

Background: *Chlamydia abortus* is the etiological agent of ovine enzootic abortion (OEA), a worldwide disease that significantly impacts to cattle, sheep, and goat production. This bacterium causes abortions during the last third of gestation and its zoonotic. The lack of prior symptoms before an abortive event complicates the clinical diagnosis of OEA and its differentiation from other infections, highlighting the need for an effective diagnostic tool. Some conventional diagnostic methods could be accurate, but in some cases require expensive equipment and highly trained personnel, limiting their field application. Under this context, the loop-mediated isothermal amplification (LAMP) emerges as an accessible and efficient alternative, capable of operating at a constant temperature and simplifying the interpretation of results visually through colorimetry. **Objective**: To validate a new LAMP assay using colorimetry for the visual detection of *Chlamydia abortus* in domestic ruminants. **Method**: Fifty-six DNA samples collected from ruminants with abortion history were used as biologic control of reference. LAMP specific primers were designed for the TARP gene of *C. abortus.* A colorimetric assay was validated for the LAMP test of C. abortus (crLAMP-Chla). The concordance (K), sensibility (Se) and specificity (Sp) parameters of crLAMP-Chla were calculated considering real time PCR as reference. **Results**: The crLAMP-Chla detected the pathogen from 15 minutes at 65 °C, even at low DNA concentrations. The K, Se and Sp values were 1, 100% and 100% (P=0.05) respectively. **Conclusion**: This methodology represents a significant advancement for the control of *C. abortus* in ruminants, providing an accessible and efficient diagnostic tool to improve public and animal health.

Keywords: *Chlamydia abortus; colorimetry; diagnostic; isothermal; LAMP; molecular; optimization; validation; zoonotic.*

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Resumen

Antecedentes: *Chlamydia abortus* es el agente etiológico del aborto enzoótico ovino (OEA), una enfermedad mundial que impacta significativamente la producción bovina, ovina y caprina. Esta bacteria provoca abortos durante el último tercio de la gestación y es zoonótica. La falta de síntomas previos antes de un evento abortivo complica el diagnóstico clínico del OEA y diferenciación de otras infecciones, lo que destaca la necesidad de una herramienta de diagnóstico eficaz. Algunos métodos de diagnóstico convencionales pueden ser precisos, pero en algunos casos requieren equipos costosos y personal altamente capacitado, lo que limita su aplicación en campo. Bajo este contexto, la amplificación isotérmica mediada por bucle (LAMP) surge como una alternativa accesible y eficiente, capaz de operar a temperatura constante y simplificar la interpretación de resultados visualmente a través de colorimetría. **Objetivo**: Validar un nuevo ensayo de LAMP utilizando colorimetria para la detección visual de *Chlamydia abortus* en rumiantes domésticos. **Método:** Como referencia en los controles biológicos se utilizaron 56 muestras de ADN colectado de ruminates con historial de aborto. Se diseñaron cebadores específicos LAMP para el gen TARP de *C. abortus*. Se validó un ensayo colorimétrico para la prueba LAMP de *C. abortus* (crLAMP-Chla). Se calcularon los parámetros de concordancia (K), sensibilidad (Se) y especificidad (Sp) de crLAMP-Chla tomando como referencia PCR en tiempo real. **Resultados:** El crLAMP-Chla detectó el patógeno a partir de los 15 minutos a 65 °C, incluso a bajas concentraciones de ADN. Los valores de K, Se y Sp fueron de 1, 100% y 100% (P=0.05) respectivamente. **Conclusiones:** Esta metodología representa un avance significativo para el control de *C. abortus* en rumiantes, proporcionando una herramienta diagnóstica accesible y eficiente para mejorar la salud pública y animal.

Palabras claves: *Chlamydia abortus; colorimetría; diagnóstico; isotérmica; LAMP; molecular; optimización, validación; zoonosis.*

Antecedentes: *Chlamydia abortus* é o agente etiológico do aborto enzoótico ovino (OEA), uma doença mundial que impacta significativamente a produção de bovinos, ovinos e caprinos. Esta bactéria causa abortos durante o último terço da gestação e é zoonótica. A falta

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de sintomas prévios antes de um evento abortivo complica o diagnóstico clínico da OEA e a sua diferenciação de outras infecções, destacando a necessidade de uma ferramenta diagnóstica eficaz. Alguns métodos de diagnóstico convencionais podem ser precisos, mas em alguns casos requerem equipamentos caros e pessoal altamente treinado, limitando a sua aplicação em campo. Neste contexto, a amplificação isotérmica mediada por loop (LAMP) surge como uma alternativa acessível e eficiente, capaz de operar a temperatura constante e simplificar visualmente a interpretação dos resultados através da colorimetria. **Objetivo**: Validar um novo ensaio LAMP utilizando colorimetria para detecção visual de *Chlamydia abortus* em ruminantes domésticos. **Métodos:** Cinquenta e seis amostras de DNA coletadas de ruminantes com histórico de aborto foram usadas como controles biológicos de referência. Primers LAMP específicos foram projetados para o gene TARP do C. abortus. Foi validado um ensaio colorimétrico para o teste LAMP de *C. abortus* (crLAMP-Chla). Os parâmetros de concordância (K), sensibilidade (Se) e especificidade (Sp) do crLAMP-Chla foram calculados usando a PCR em tempo real como referência. *Resultados:* O crLAMP-Chla detectou o patógeno após 15 minutos a 65 °C, mesmo em baixas concentrações de DNA. Os valores de K, Se e Sp foram 1, 100% e 100% (P=0,05), respectivamente. **Conclusões:** Essa metodologia representa um avanço significativo para o controle de *C. abortus* em ruminantes, fornecendo uma ferramenta de diagnóstico acessível e eficiente para melhorar a saúde pública e animal.

Palavras-chave: *<u>Chlamydia</u> <u>abortus</u>; colorimetría; diagnóstico; isotérmica; LAMP; molecular; otimização; validação; zoonose.*

Introduction

Chlamydia abortus is the causative agent of the Ovine Enzootic Abortion (OEA), a disease with a high economic impact on ruminants and endemic in Mexico since 2016 (DOF, 2018). This pathogen causes abortions during the last third of gestation or generates the birth of weak offspring that will probably die within the next 48 hours (Aldama et al., 2022). Besides,

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113 *C. abortus* is also a zoonosis, generating severe complications such as spontaneous abortions
114 or premature births (Turin et al., 2022).

115 Animals acquire the microorganism mainly through contact with contaminated materials
116 from aborted fetuses or by ingesting infected food. This bacterium has been found in
117 calostrum, milk, vaginal secretions, urine, and feces (Martínez-Serrano et al., 2022; Ramos,
118 2023). Furthermore, in comparison with other abortive diseases, females with OEA may birth
119 to apparently healthy offspring, complicating the detection of the disease via clinical signs
120 (Aldama et al., 2022).

121 The conventional diagnostic techniques for *C. abortus* detection include bacterial culture,
122 serology, and the polymerase chain reaction (Barati et al., 2017). While bacterial culture is
123 precise, it is a laborious technique that requires facilities with high levels of biosecurity (Bush
124 and Everett 2001; Aldama et al., 2022). The most sensitive and specific techniques for the
125 molecular detection of this pathogen is PCR. Nonetheless, it requires a laboratory that implies
126 highly trained personnel, so its uses in outdoors is limited and the cost for equipment and
127 reagents could be considerable (Fakruddin, 2011).

128 In recent years, the loop-mediated isothermal amplification (LAMP) technique has increased
129 in popularity as an alternative diagnostic method capable of overcoming the aforementioned
130 limitations. The LAMP technique is fast, efficient, and do not require thermocyclers; it is
131 performed at a constant temperature, facilitating the pathogen detection on field conditions
132 (Notomi et al., 2015).

133 In México, LAMP was also executed by LAMP for the molecular detection of *C. abortus*
134 (Chla-LAMP) and the advances in the study demonstrated accuracy and great potential as a
135 simpler technique in comparison to conventional methods. However, the visualization of
136 results was based on agarose gels that required a laboratory (Aragón et al., 2024). The
137 versatility of LAMP could allow the incorporation of other assay as the colorimetric
138 detection, enabling the visual interpretation of results without the need of additional
139 equipment as electrophoresis (Alhamid et al., 2022). Therefore, the objective of this study
140 was to validate a new LAMP assay using colorimetry for the visual detection of *Chlamydia*
141 *abortus* in domestic ruminants.

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Materials and methods

Ethical considerations

The present study did not use animals directly, only biological samples donated from a previous experiment (Limón-González et al., 2024). However, in the report No. 2024-04 the Animal Welfare and Research Ethics Committee of the Instituto Tecnologico de Sonora approved the procedures of the main project.

Study location

The study was conducted in the Laboratory of *Desarrollo e Innovacion en Biotecnologia Veterinaria* of the *Departamento de Ciencias Agronómicas y Veterinarias* of the *Instituto Tecnológico de Sonora* (ITSON), in Sonora, México.

Biologic material

From a previous study (Limón-González et al., 2024), fifty-six DNA samples were used as biologic controls and provided by the Centro de Investigación Nacional Interdisciplinaria en Salud Animal e Inocuidad (CENID) of the Instituto Nacional de Investigaciones Forestales Agrícolas y Pecuarias (INIFAP) in México. The DNA extraction were from vaginal swabs samples collected from ovine and bovine with abortion history. The positive and negative biologic control were previously identified in real time PCR (qPCR) by Limón-González et al. (2024). Also, were tested by LAMP in agarose gels (Chla-LAMP) through the conditions previously reported by Aragón et al. (2024).

Design of LAMP primers and synthetic positive

To design the LAMP primers for *C. abortus*, the *Translocated Actin Recruiting Phosphoprotein* (TARP) gene was selected as the appropriate target. The numbers of access of the reference sequences for primer design were: *CP158097.1, CP070224.1*, and *LS974600.1*. LAMP primers were designed using the *NEB LAMP Primer Design Tool platform* version 1.4.1 (https://lamp.neb.com/#!/), generating six primers [F3, B3, FIP

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170 (F1c+F2) and BIP (B1c+B2), LF, LB] capable of recognize a total of eight regions of the
171 TARP gene (Table 1). The specificity of the oligonucleotides was confirmed by *in silico*
172 analysis in the Primer-BLAST GenBank® sequence alignment software of the NCBI
173 (National Center for Biotechnology Information) database (https://www.ncbi.nlm.nih.gov/).

174 In order to synthesize the positive control (DNAs+), the BLAST tool of NCBI database was
175 used, where the CP158097.1 sequence was obtained from the alignment using the primers
176 downloaded in FASTA format. A total of 50 bp upstream from F3 and 50 bp downstream
177 from the first B3 where the oligonucleotides were aligned.

178

179 **Table 1.** Sequences primer sets designed for the detection of *C. abortus*.

Pathogen	***Target Gene***	***Primer***
C. abortus	*TARP*	F3:GCCCATGGAATTCCAAGGAA B3:TCACAGGTTGAGCATAAGGC FIP:CAGACCACCAACATCGCCCCACCTTCTGTATGGC GTCGA BIP:CAAAGACCCAACGAGGGCATGGACAGGTTGTGG TTGCTGTT LF: TGGCTCCAGATTCACATTTAGTTCC LB: TTCTCTAGAGAAACTTCCTCCCCCA

180

181 *Optimization of colorimetric LAMP assay conditions (crLAMP-Chla)*

182 For optimization of colorimetric LAMP conditions for *C. abortus* (crLAMP-Chla*)*, primer-
183 specific amplification was initially confirmed by a conventional PCR assay (PCR-Chla)
184 using external primers including Cb-B3 and Cb-F3, which amplify a ~219 bp fragment of
185 the target gene. PCR amplification was performed with GoTaq® Green Master Mix
186 polymerase (Promega, Madison, WI, USA) and 1 ng/μL of DNAs+, following the
187 manufacturer's concentrations adapted to a final volume of 25 μL. Cycling conditions were:
188 one step of 95 °C for 2 min, followed by 35 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72
189 °C for 30 s. The final extension cycle was 72 °C for 5 min.

190 The crLAMP-Chla reaction was performed using DNAs+ of the target gene. To optimize
191 reaction times, the test was conducted in a 20 μl final volume containing the Cb-LAMP
192 oligonucleotides in a 10X mixture with the WarmStart® Colorimetric LAMP 2X Master Mix

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193 kit with UDG (New England Biolab, USA) and 1 ng of DNAs+, at a temperature of 65 °C
194 for a maximum time of 40 mins. Within such time, the reaction was visually inspected to
195 determine color changes of the phenol red dye. Negative reactions remained pink while
196 positive reactions changed to yellow. To confirm the reaction process, 10 µl of the PCR
197 product, and 5 µl of the crLAMP-Chla product, and 1 µl of 6X loading buffer (Invitrogen™,
198 USA) were taken to perform electrophoresis on a 1.5% agarose gel stained with ethidium
199 bromide.

200

201 *Analytical sensitivity of crLAMP-Chla assay*

202 According to the optimized protocol, DNAs+ was used as a template to determine the
203 sensitivity of the crLAMP-Chla system based on the lower limit of detection. Ten serial
204 dilutions were performed in a concentration range of 1 ng/µl to 1 ag/µl in order to corroborate
205 the sensitivity of each of them.

206

207 *Comparison of crLAMP-Chla with other molecular tests using biological samples*

208 According to the established conditions in the optimized crLAMP-Chla protocol, the fifty-
209 six DNA isolates from the biologic controls were used to validated the crLAMP-Chla test,
210 including DNAs+. Finally, the results were visualized colorimetrically as described in the
211 interpretation section and compared with the results of PCR-Chla analyses and the LAMP
212 test protocol for *C. abortus* based on agarose gels (Chla-LAMP) described by Aragn et al.
213 (2024).

214

215 *Statistical analysis for the concordance, sensibility and specificity of crLAMP-Chla*

216 To measure the capability of crLAMP-Chla in the detection of the biological control samples
217 previously detected by real time qPCR (Limón-González et al., 2024)), the degree of
218 concordance was calculated by the Cohen's Kappa coefficient (K) as follows:

219 $K = (Po – Pa) / (1 - Pa)$

220 Where:

221 Po: Proportion of observed relative agreements between tests.

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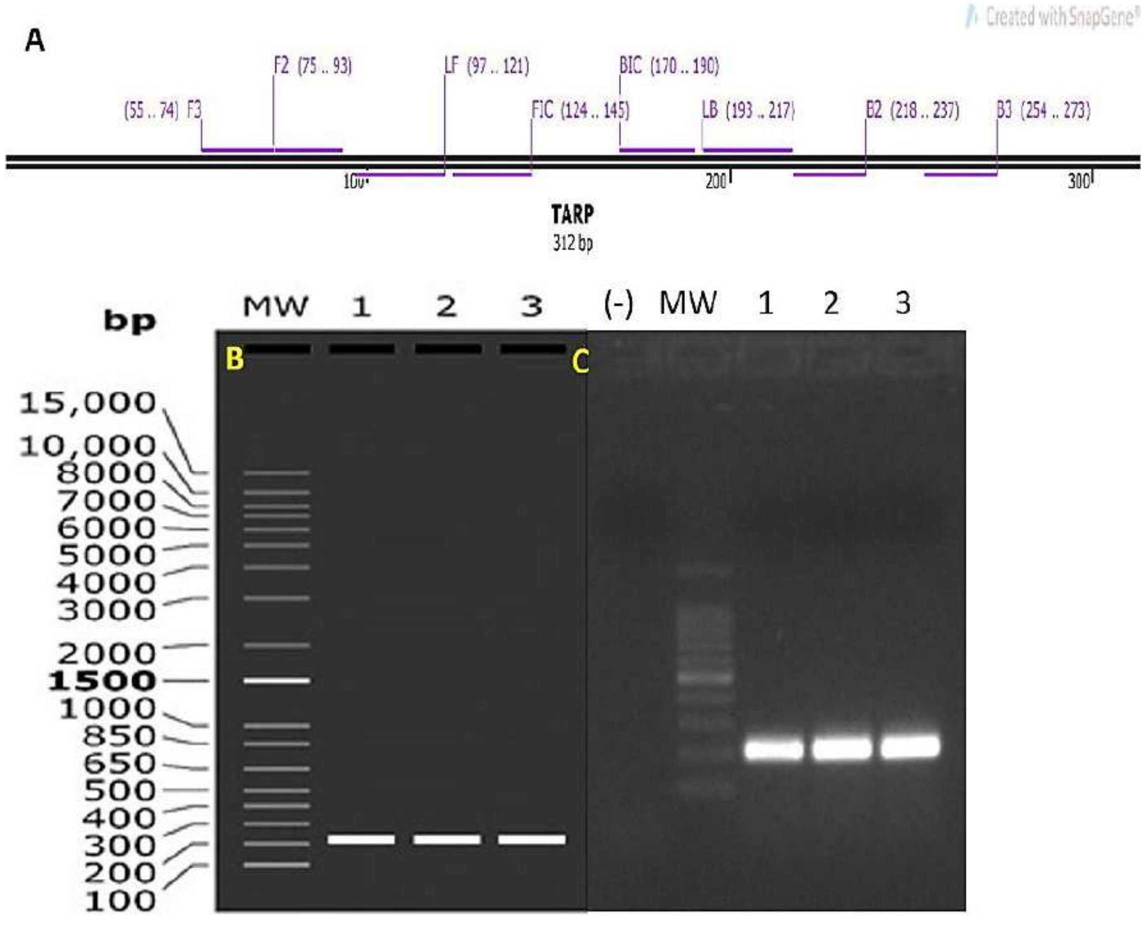
Pa: Hypothetical proportion of random agreements

Also, the sensibility (Se) and specificity (Sp) were calculated for crLAMP-Chla. The K, Se y Sp analyses were performed with 95% confidence in the WinEppi online platform (http://www.winepi.net/sp/index.htm), using a table arrangement of positives and negatives frequencies of qPCR and crLAMP-Chla tests according to Azzimonti (2005). The K value was interpretated as Landis and Koch (1977).

Results

Design of LAMP primers and DNAs+

The *in silico* analysis and the PCR-Chla utilizing primers Ca-F3 and Ca-B3 with the DNAs+ showed a band of approximately 219 bp on both electrophoresis methods (*in silico* and agarose gel, figure 1A-1C), confirming the specific binding of the primers to the target gene.

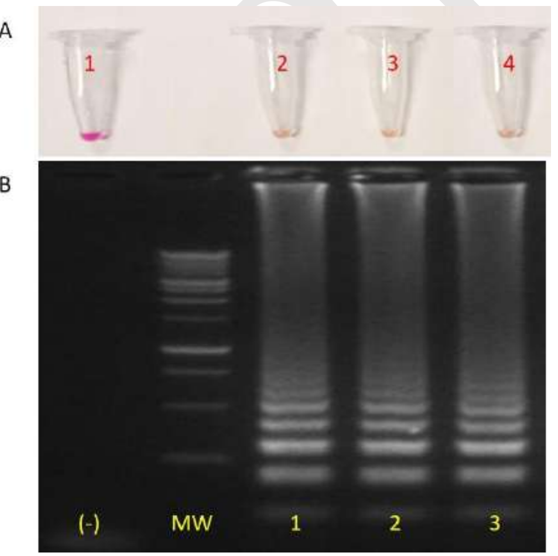


236 **Figure 1.** A) *In silico* primer alignment B) 1.5% agarose gel simulation. C) 1.5% agarose gel
237 electrophoresis. (-) = Negative Control, MW = Molecular weight, 1, 2 and 3 positive band
238 (~219 bp).

239

240 *Optimization of colorimetric LAMP assay conditions (crLAMP-Chla)*

241 The colorimetric crLAMP-Ca assay conditions were optimized with 1 ng of DNAs+ from
242 the TARP gene, showing a color change from pink (negative) to yellow (positive) at a
243 temperature of 65°C. The time required for the amplification of 1 ng of *C. abortus* DNAs+
244 was 15 minutes; although the sample was removed after 40 mins as indicated previously in
245 the protocol. Subsequently, ladder scans (characteristic of LAMP) were observed by
246 electrophoresis in the same samples, which changed to yellow, matching the results. For
247 confirmation purposes, the comparison was performed by triplicate (Figure 2)



248

249 **Figure 2.** Colorimetry and agarose gel electrophoresis with positive results with DNAs+ for
250 the detection of *C. abortus*. A). In crLAMP-Chla, tubes 1: negative (pink and no band
251 pattern), tubes 2, 3 and 4 are positives (yellow and band pattern). B). crLAMP-Chla in
252 agarose gel electrophoresis. Bands in track, (-) = Negative Control; MW = Molecular weight;
253 1, 2 and 3 positive bands.

254

255 *Analytical sensitivity of crLAMP-Chla assay*

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256 Sensitivity of the crLAMP-Chla assay was analyzed by colorimetry after incubation to
257 different concentrations of DNAs+ or different copy numbers in a nuclease-free solution. In
258 the crLAMP-Chla colorimetric reactions, the change of color from pink to yellow was visible
259 directly in the first tube in a 1 ng concentration in 15 minutes, finishing the colorimetric
260 change with 0.01 fg in 45 min, with a limit of 10 copies, approximately (Figure 3).

261

262 *Comparison of crLAMP-Chla with other molecular tests using biological samples*

263 From the four negative and twenty-six positive samples analyzed by qPCR, the crLAMP-
264 Chla and Chla-LAMP detected the 100% respectively. Conventional PCR only detect the
265 60% and 40% of the true positive and negative biologic controls of qPCR. A visual
266 comparison of the crLAMP-Chla, Chla-LAMP and PCR is shown in figure 4.

267

268

269 **Figure 3.** Sensitivity analysis of crLAMP-Chla. Tubes 1: negative; 2: 1 ng (+); 3: 0.1 ng (+);
270 4: 0.01 ng (+); 5: 1 pg (+); 6: 0.1 pg (+); 7: 0.01 pg (+); 8: 1 fg (+); 9: 0.1 fg (+), 10: 0.01 fg
271 (+).11: 1 ag negative result.

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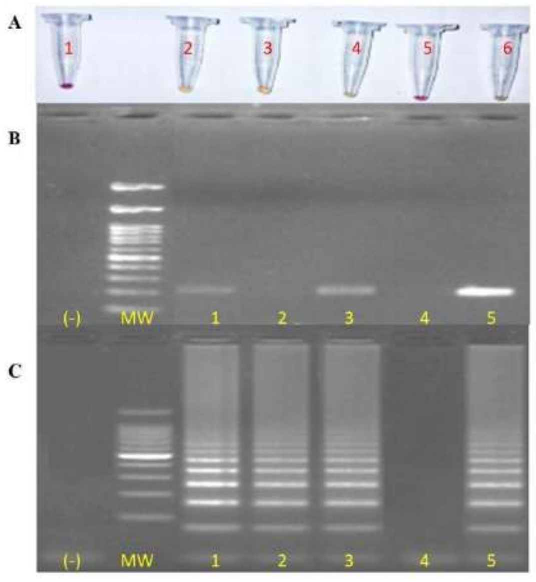

Figure 4. Comparison of crLAMP-Chla with other molecular tests. A) Colorimetry of crLAMP-Chla with biological samples. Tubes 1: Negative control; 2: Positive; 3: Positive; 4: Positive; 5: Negative; 6: DNAs+. B). Agarose gel electrophoresis of PCR-Chla with primers F3 and B3 in biological samples. Bands in track (-) = Negative Control; MW = Molecular weight; 1, 3: positive samples; 2,4: negative samples; 5: DNAs+. C). Agarose gel electrophoresis of Chla-LAMP with biological samples. Bands in track, (-) = Negative Control; MW = Molecular weight; 1,2,3: positive samples; 4: negative samples; 5: DNAs+.

Concordance, sensibility, and specificity analysis

The concordance between qPCR and crLAMP-Chla was K=1, and Se and Sp for were 100% and 100% respectively (P=0.05).

Table 2. Concordance (K), sensibility (Se), and specificity (Spe) of crLAMP in relation to qPCR as the reference test.

Diagnostic	qPCR	crLAMP-Chla	K	Se	Esp
Concord (+)	4	4	1	100%	100%
Incon (+)	0	0			
Concord (-)	52	52			
Incon (-)	0	0			
Total	56	56			

Con= Concordance, Incon= Inconcordance, K= concordance, Se= test sensitivity, and Esp= test specificity. Confidence level at 95%. Interpretation of K value: <0.00=no agreement; >0.00–0.20=negligible; 0.21–0.40=discrete; >0.41–0.60=moderate; 0.61–0.80=substantial; 0.81–1.00=almost perfect

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Discussion

The BLAST alignment of the sequence amplified by the external primers (F3-B3) showed 100% homology to the C. abortus genomic sequence and allows the synthesis of DNAs+ that reduces the standardization time for other diagnostic tests. Furthermore, it avoids the direct contact with contagious pathogens for the personnel. Additionally, it has been reported with higher sensitivity in molecular diagnostics (Aragón-López et al., 2021; Aragón et al., 2024). Also, it has been demonstrated that it is possible to use synthetic fragments as positive controls for the standardization of tests designed to identify microorganisms that are difficult to culture and require a long time for their process. Also, the synthesized sequences as positive controls assure the standardization of bioassays when the biological isolated it is not available.

Comparing the sensitivity of the crLAMP-Chla assay with other LAMPs for *C. abortus*, important differences were observed in the detection capacity, as well as in the applicability of the methods and the detection time. For instance, Lin et al. (2012) reported that a LAMP assay targeting the MOMP gene (a different gene in this study) had a sensitivity equivalent to a nested PCR and superior to an isolation in chicken embryo. Nonetheless, it was not precisely specified the quantity of detectable DNA as the crLAMP-Chla assay of this study (10 DNA copies). Aragón et al. (2024) described an isothermal test for the detection of *C. abortus* and also a LAMP technique and reported an analytical sensitivity of 30,893 DNA copies. This indicates that previous tests have an adequate analytical sensitivity but may not reach the same level of accuracy as the crLAMP-Chla assay. Another important difference in the crLAMP-Chla assay is the use of colorimetry to interpret the results with naked eye, a suitable feature application under field conditions test without sophisticated infrastructure or additional equipment to interpret results.

Previous to 2016, *C. abortus* was considered an exotic disease in Mexico by the country's health authority. Due to several reports from research centers and universities, it was possible to change the sanitary status to endemic disease (DOF, 2018). In this sense, it is necessary a diagnostic tool for an accurate identification of clinical cases. Considering the +/- controls from the reference test (qPCR), the concordance between qPCR and crLAMP-Chla according to Landis and Koch (1977) was almost perfect (K=1). Also, Se and Sp for crLAMP-Chla were 100% and 100% respectively, demonstrating high capability to identified genetic

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material of *C. abortus*. The LAMP technique is therefore an emerging and alternative tool for the molecular detection of pathogens with relevance in veterinary and public health. It is fast and could be portable; has a high specificity and sensitivity than conventional PCR or similar in relation to qPCR. The incorporation of colorimetry into the LAMP technique has accrued more popularity to this method in veterinary diagnostics, since it allows for a simple visual interpretation based on a color change, eliminating the need for sophisticated equipment for detection. Studies performed with different microorganisms (such as *Bovine alphaherpesvirus* or *Mycoplasma bovis*) using colorimetry-based LAMP assays have demonstrated the effectiveness of this technique with a variety of pathogens that affect different animal species (Peltzer et al., 2020; Sierra et al., 2023).

Conclusion

The crLAMP-Chla assay demonstrated the same capability of qPCR to identified by colorimetry with naked eye the absence or presence of genetic material of *C. abortus.* This positions the crLAMP-Ca assay as an effective and practical diagnostic tool for the rapid and accurate detection of *C. abortus* in resource-limited settings.

Declarations

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343 microorganisms: *Chlamydia spp., Brucella melitensis, Leptospira spp.* and *Coxiella*
344 *burnetiiin* sheep and goats".

345 *Conflicts of interest*

346 The authors declare they have not conflicts of interest related to this report.

347 *Authors contribution*

348 Conceptualization and design of the study: Aragón-López, Morales-Pablos, Leyva-Corona.
349 Wrote the first draft: Aragón-López, Leyva-Corona. Laboratory procedures: Aragón-López,
350 Rojaz-Arzaluz, Palomarez-Reséndiz. Edited and review the manuscript: Aragón-López,
351 Rojaz-Arzaluz, Palomarez-Reséndiz, Luna-Nevarez, Leyva-Corona, Sánchez-Castro,
352 Morales-Pablos. Search for project funding: Leyva-Corona, Morales-Pablos. All authors
353 were agree with the last edition of the manuscript.

354 *Use of artificial intelligence (AI)*

355 During the preparation of this work no AI or AI-assisted technologies were used.

356

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