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Standardization of an isothermal test as a novel diagnostic tool in México for the molecular detection of *Chlamydia abortus* in small ruminants

Estandarización de una prueba isotérmica como una nueva herramienta diagnóstica en México para la detección molecular de *Chlamydia abortus* en pequeños rumiantes

Carlos Eduardo Aragón-López[1], Erika Gabriela Palomares-Reséndiz[2], Sergio Cuevas-Tellechea[1], Javier Rolando Reyna-Granados[1], Beatriz Arellano-Reynoso[3], Miguel Angel Sánchez-Castro[1], José Clemente Leyva-Corona[1], Marcela Ivone Morales-Pablos[1]*✉

[1] Departamento de Ciencias Agronómicas y Veterinarias del Instituto Tecnológico de Sonora, 5 de febrero 818 south. ZC 85130. Obregon City, Sonora, Mexico.

[2] Centro de Investigación Nacional Interdisciplinaria en Salud Animal e Inocuidad of the Instituto Nacional de Investigaciones Forestales Agrícolas y Pecuarias (INIFAP). ZC 05110, Mexico City.

[3] Facultad de Medicina Veterinaria y Zootecnia de la Universidad Nacional Autónoma de México (FMVZ-UNAM). ZC 04510, Mexico City.

ABSTRACT

Ovine Enzootic Abortion (OEA) is caused by *Chlamydia abortus* and has recently been recognized as endemic in Mexico. Due to the impact of OEA on intensive lamb production, it is necessary to establish a sanitary control plan based on an efficient diagnosis. The loop-mediated isothermal amplification technique (LAMP) is a molecular test that can specifically identify *C. abortus*. The aim of this study was to standardize the LAMP technique for the specific detection of *C. abortus* in ovine vaginal exudates. Primers were identified and modified from an electronic database. The effective conditions of the technique were determined using synthetic DNA and positive biological samples by PCR as references. The assay detection limit was 1×10^{-5} of genetic material, equivalent to 8.5 copies. The standardized LAMP test is proposed as a novel molecular tool for the rapid and specific diagnosis of *C. abortus* infection from ovine vaginal exudate samples in Mexico.

Keywords: *Chlamydia abortus*, ompA gene, LAMP, Ovine Enzootic Abortion.

RESUMEN

El Aborto Enzoótico Ovino (AEO) es causado por *Chlamydia abortus* y ha sido reconocido recientemente como endémico en México. Debido al impacto del AEO en la producción intensiva de corderos, es necesario establecer un plan de control sanitario basado en un diagnóstico eficiente. La técnica de amplificación isotérmica mediada por bucle (LAMP) es una prueba molecular que puede identificar específicamente a *C. abortus*. El objetivo de este estudio fue estandarizar la técnica de LAMP para la detección específica de *C. abortus* a partir de exudado vaginal ovino. Los cebadores fueron identificados y modificados a partir de una base de datos electrónica. Las condiciones efectivas de la técnica se determinaron con ADN sintético y de muestras biológicas positivas por PCR como referencia. El límite de detección de la prueba fue de 1×10^{-5} de material genético, equivalente a 8.5 copias. Se propone el test de LAMP estandarizado como una nueva herramienta molecular para el diagnóstico rápido y específico de la infección por *C. abortus* a partir de muestras de exudado vaginal ovino en México.

Palabras clave: *Chlamydia abortus*, *ompA* gene, LAMP, Aborto Enzótico Ovino.

INTRODUCTION

The most common cause of infectious abortion in sheep flocks is Ovine Enzootic Abortion (OEA), which has significant economic and productivity implications in intensive lamb production. The causative agent is *Chlamydia abortus*, an obligate intracellular gram-negative bacterium (Selim, 2016). Abortive diseases in small ruminants pose a major problem in sheep farming (Benavides Ortíz, 2009), as abortion cases contribute to economic losses due to failed calving (Palomares-Reséndiz *et al*., 2020). Besides being a zoonotic disease, OEA is the most common infectious cause of abortion in sheep worldwide, leading to productivity losses (Selim, 2016). Therefore, OEA is recognized as a constraint to the expansion of the sheep industry and international trade of sheep products (Benavides Ortíz, 2009). The first report of abortive chlamydiosis in small ruminants in Mexico was in 1996, and in 2016, OEA was recognized as an endemic disease (Escalante *et al*., 1996; DOF, 2016). During this time, the disease spread throughout the country due to animal movements for genetic improvement purposes. In 2020, an individual and herd-level seroprevalence of 10.92 % and 43.34 % for *C. abortus*, respectively, was reported in Mexico (Palomares-Reséndiz *et al*., 2020). The background and epidemiological behavior of the disease highlight its relevance in sheep production if it is not controlled in the coming years.

*Author for correspondence: Marcela Ivone Morales-Pablos
e-mail: marcela.morales@itson.edu.mx
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To prevent, control, and reduce economic losses from abortive diseases in lamb production, good management practices, as well as early and accurate pathogen detection, are necessary (Yeh *et al*., 2005). As the sheep industry expands, laboratories must be equipped with diagnostic tools to differentiate abortifacient diseases (Benavides-Ortíz, 2009). However, the intracellular lifestyle of *C. abortus* poses challenges for isolation, culture, and identification (Bush and Everett, 2001). Additionally, serological detection in animals is limited by non-specificity caused by cross-reactivity with other *Chlamydia* species (Selim, 2016). Conventional diagnostic methodologies such as cell culture and serology have limitations in early detection for optimal epidemiological disease control. Due to the lack of specificity and low sensitivity of these methods, we are unable to accurately determine the magnitude of the problem.

Molecular assays can discriminate between different bacterial causes of abortions in ewes, even to distinguish among *Chlamydia* species (Reisberg *et al*., 2013). Implementing a more reliable diagnosis in flock biosecurity programs for the sanitary control of *C. abortus* requires the standardization of molecular tests. For instance, Polymerase Chain Reaction (PCR) requires sophisticated and costly tools for amplification and detection of the target DNA (Fakruddin, 2011). PCR is also susceptible to contaminants from different sources, and sample preparation is needed to remove amplification inhibitors (Corless *et al*., 2000).

As an alternative, Loop-Mediated Isothermal Amplification (LAMP) is a recent molecular method for the identification of microorganisms. This technique is based on the principle of strand displacement activity, allowing amplification of the target DNA using a set of specially designed primers with high specificity and efficiency, even at low concentrations of genetic material (Saharan *et al*., 2014; Hardinge and Murray, 2019). Furthermore, its isothermal feature eliminates the need for expensive thermal cyclers used in PCR, making it a potentially useful and cost-effective method for diagnosing infectious diseases in faraway or vulnerable sectors. In México, sheep production is a system that requires technical advancements to improve efficiency (Hernández-Marin *et al*., 2017), and animal health is essential for sustainability. However, standardizing a molecular diagnostic technique is crucial as factors such as temperature, primer ratio, reaction time, and concentrations of $MgCl_2$, deoxynucleotide triphosphates (dNTPs), and betaine significantly affect the reaction (Ihira *et al*., 2004; Yeh *et al*., 2005). LAMP offers optimization in terms of time and resources required, making it an efficient and cost-effective test for molecular detection of *C. abortus*. Therefore, it is necessary to standardize the conditions for a reliable and feasible LAMP test to ensure specific detection of *C. abortus* from vaginal exudate.

MATERIALS AND METHODS

Synthesis of positive control

For the positive control (CTRL+) design, the primers for *C. abortus* (Chla-LAMP) previously proposed by Lin *et al*. (2011) were selected. The primers (Table 1) were visualized in SnapGene Viewer software and aligned to different sequences of the *ompA* gene that encodes for the *Major Outer Membrane Protein* (*MOMP*), published in different strains of *Chlamydia abortus* (GenBank® accession numbers DQ471955.1, HQ622433.1, EU086705.1, and EF202609.1).

The specificity of the primers was validated *in silico* by the Primer-BLAST (GenBank® software) from the National Center for Biotechnology Information (NCBI) database. For the CTRL+ synthesis, the DQ471955.1 sequence was downloaded in FASTA format as a result of the alignment using the selected primers. With the SnapGene Viewer visualization software, the generated sequence from the alignment was selected considering 50 base pairs (bp) upstream of the Chla/F3 primer and 50 bp downstream of the Chla/B3 primer from the CTRL+. This sequence and the selected primer set, were synthesized at IDT (Integrated DNA Technologies, USA).

Table 1. Primers for amplification of the *C. abortus* ompA gene.
Tabla 1. Cebadores para la amplificación del gen ompA de *C. abortus*.

Primers	Sequences
Chla/F3	ACCTCTAACAGCTGGTACTG
Chla/B3	TGGGTTCCATGTGGTCAAG
Chla/FIP	GAGAGCGCTAAACCAACTTGC-CAACTGACACTAAGTCGGC
Chla/BIP	GGCATAAACTGGTCACGAGCA-ATTTAGGTTGAGCGATGCGG

LAMP reaction standardization

The setting of the LAMP reaction for the detection of the *C. abortus ompA* gene (Chla-LAMP), was carried out with the primer set as described in table 1 in a 25 µL reaction system. The reagent concentrations used were as follows: Chla/F3 and Chla/B3 at 0.1 mM; Chla/FIP and Chla/BIP at 0.8 mM; F2 and B2 at 0.4 mM; 0.2 M betaine; 0.8 mM $MgSO_4$; 0. 2 mM dNTPs; 1 X BST regulator; 0.8 units of BST (2.0 WarmStart DNA Polymerase) enzyme of final concentration, and 10 ng of DNA template were added at the end of the reaction mixture. Afterward, the temperature and time conditions were standardized and determined in a mini incubator (Bio-Rad™) at 62 °C for 30 to 60 m. The visualization of amplified products was analyzed by electrophoresis (1.5 % agarose gel) through visual observation using ethidium bromide (Sigma Aldrich®). Once the results were satisfactory, the protocol was validated in triplicate.

To corroborate the standardization from CTRL+, Chla-LAMP was tested with five DNA samples previously confirmed by PCR as positive for *C. abortus*. The positive samples were 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 Mexico City.

Assay for the analytical sensitivity

From the Chla-LAMP standardization results the analytical sensitivity assay was assessed subjecting the CTRL+ to diffe-

rent gradients of the concentration. Six 1:9 serial dilutions of CTRL+ (5 to 10 e^{-7} ng/µL dilution) were performed to determine the assay's limit of detection.

Detection of *C. abortus* in biological samples

Sampling of the experimental population

In a commercial flock with a history of abortions and suggestive signs to *C. abortus* (Eg. tearing, weak in offspring, stillbirths, placentitis) located in the Yaqui Valley, Sonora, Mexico, 48 ewes (>12 months) were randomly selected. A sterile swab was introduced into the vagina of each ewe and with circular movement the vaginal wall was rubbed to obtain the maximum quantity of exudate. Then, the swab was placed into 1.5 mL vials with phosphate-buffered saline and shipped at 5 °C to the laboratory.

During sampling, the animals' integrity was not compromised at any time and this procedure was approved by an ethics committee in accordance with the Mexican Official Standard NOM-062-ZOO-1999.

DNA extraction

The samples were first homogenized by agitation and inversion. Afterward, the DNA extractions were carried out by the taco™ Nucleic Acid Automatic Extraction System (GeneReach Biotechnology Corp.) using the taco™ DNA/RNA Extraction Kit according to the manufacturer's method. The final extracted product of nucleic acids was quantified and counted with UV spectrophotometry (BioSpect-Nano, Shimadzu®). The integrity of the extracted DNA was verified by electrophoresis in a 1.5 % agarose gel stained with ethidium bromide. All the extractions were stored in 1.5 mL sterile vials at -20 °C to be subsequently processed for the Chla-LAMP technique.

Detection of *C. abortus* in biological samples

Once the genetic material was extracted, *C. abortus* detection was performed following the Chla-LAMP protocol as previously described in the standardization section. The products were visually detected by electrophoresis in 1.5 % agarose gel using a load buffer (Blue-orange®, Promega) and ethidium bromide.

RESULTS

Positive control synthesis

The product of the CTRL+ synthesis yielded a sequence of 316 bp corresponding to a fragment of the *ompA* gene that codifies for the MOMP protein of *C. abortus*, and was positioned between the 687 and 938 nucleotides from the start codon of the reading frame of the gene (Figure 1). The CTRL+ had 100 % of homology to the sequence reported in NCBI GenBank® and was recognized with the identifier DQ471955.1.

Chla-LAMP standardization

The LAMP assay to detect *C. abortus* with the primers that recognize the targeted *ompA* gene displayed conditions of

Figure 1. Diagram of primers and their alignment in specific *C. abortus* ompA sequence.
Figura 1. Diagrama de cebadores y su alineación en secuencia específica de *C. abortus* ompA.

Fuchsia-coloured arrows indicate the direction and site recognized by the primers in the sequence of the synthetic positive control (CTRL+) and the name. The letters in fuchsia indicate the name of the primers.

optimal amplification. The reaction was visualized after 30 minutes, but for better visualization in agarose gel, it was determined at 40 min (Figure 2). The amplification products were observed on agarose gels in different sizes, which are distinctive for LAMP assays (Figure 3).

Analytical sensitivity

The limit of detection in the Chla-LAMP assay was 5×10^{-5} ng of the synthesized genetic material (Figure 4). The amount of acid nucleic represented 8.5 genomes equivalent to 30,893 copies of the sequence (316 bp of an *ompA* gene fragment) that codifies for the MOMP protein of *C. abortus*.

Detection of *C. abortus* in biological samples

The Chla-LAMP reaction to detect *C. abortus* DNA from vaginal swabs was carried out under optimal conditions. The best reaction was achieved at 62 °C for 40 min, which detected 81.25 % (39/48) of the animals with the presence of genomic material of *C. abortus* (Figure 5).

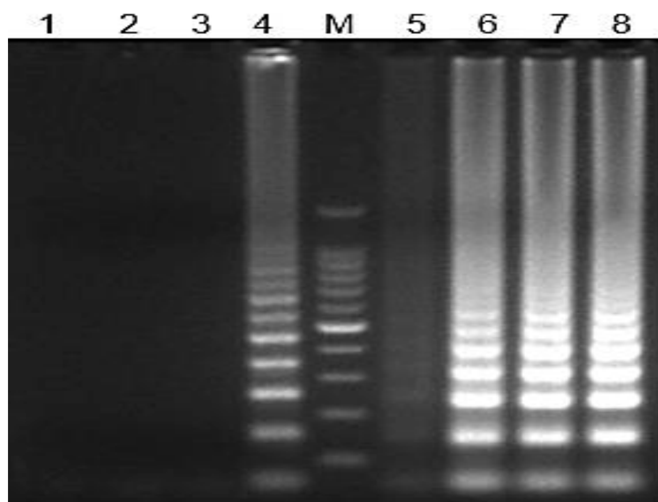

Figure 2. Visualization of LAMP assay for *C. abortus* detection using ompA gene primers at different amplification timepoints.
Figura 2. Visualización del ensayo LAMP para la detección de *C. abortus* utilizando cebadores del gen ompA en diferentes momentos de amplificación.

Tracks 1= negative control at 30 min, 2 = negative control at 40 min, 3 = negative control at 50 min; negative control at 4 = 60 min, M = kb molecular marker 1 kb, 5 = CTRL+ at 30 min, 6 = CTRL+ at 40 min, 7 = CTRL+ at 50 min, and 8= CTRL+ at 60 min.

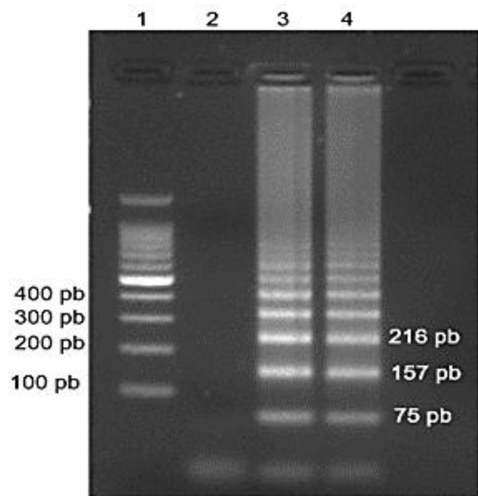

Figure 3. Agarose gel with LAMP product for the amplification of the synthetic DNA and ompA gene.
Figura 3. Gel de agarosa con producto LAMP para la amplificación del ADN sintético y gen ompA.

Tracks 1 = 1 kb molecular marker; 2 = Negative control; 3 = Positive control (CTRL+); 4 = Biological sample of *C. abortus.*

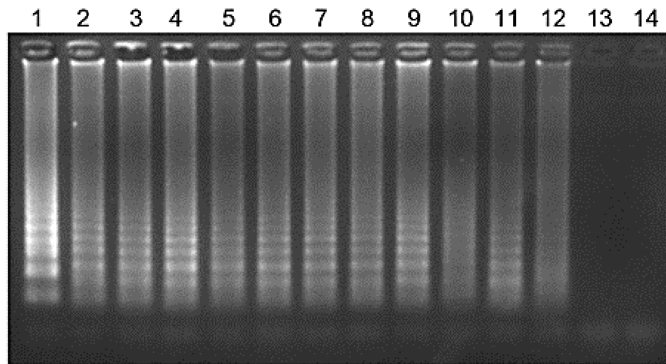

Figure 4. Sensitivity analysis of Chla-LAMP assay by DNA concentration gradient.
Figura 4. Análisis de sensibilidad del ensayo Chla-LAMP por gradiente de concentración de ADN.

DNA concentration in tracks from 1 to 13 = 5.00E00, 1.00E00, 5.00E-01, 1.00E-01, 5.00E-02, 1.00E-02, 5.00E-03, 1.00E-03, 5.00E-04, 1.00E-04, 5.00E-05, 1.00E-05 and 5.00E-06 ng/µL respectively. Track 14 = negative control.

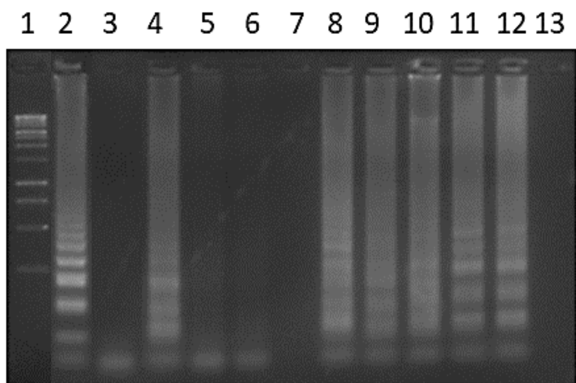

Figure 5. Chla-LAMP amplification of *C. abortus* DNA from vaginal samples.
Figura 5. Amplificación de Chla-LAMP de ADN de *C. abortus* de muestras vaginales.
Tracks 1= 1 kb molecular marker, 2= CTRL+, 3= negative control; from 4 to 13= LAMP product with biological samples.

DISCUSSION

The multiple alignment sequences of the Chla-LAMP external primers (F3/Chla and B3/Chla) in the Primer-BLAST tool, exhibited high *in silico* homology with sequences matching *Chlamydia abortus*. A lower homology was observed for *Chlamydia psittaci*, an isolated pathogen in poultry and other birds (Li *et al*., 2016). No homology was identified with other organisms, ensuring that the primers in the current assay specifically target *C. abortus* DNA. The *ompA* gene sequence chosen as the target in this study can discriminate serotypes, species, and antigens specific to the genus and species within the Chlamydiaceae family (Conlan *et al*., 1988; Salti-Montesanto *et al*., 1997). Primers designed from pmp genes are reported to improve the molecular diagnosis of *Chlamydias* in sheep (Laroucau *et al*., 2001). However, these genes exhibit short-term variability (Read *et al*., 2003), leading to the hypothesis that primers designed from these sequences may require recurrent updates compared to *ompA* genes. In contrast, *the ompA* gene has nearly 100 % conservation of its *ompA* sequences (Everett *et al*., 1999), suggesting that these primers will be more durable and enable the discovery of new strains of *C. abortus*.

Additionally, like the *ompA* primers, the designed primers for *C. abortus pmp* genes are also proposed as a tool for the detection of *C. psittaci* (Laroucau *et al*., 2007). However, it should be noted that the use of *ompA* primers may indicate a lack of specificity for *C. abortus*. Even with nucleotide alignment search tools, the Chla-LAMP primers showed alignment with both organisms. Therefore, it is recommended to explore alternative sequences or molecular markers that can provide better specificity for identifying *C. abortus*. This can help ensure accurate and reliable detection of *C. abortus* without potential cross-reactivity with other *Chlamydias*.

The use of synthetic sequences as positive controls is crucial for the detection of microorganisms. This allows for standardization, and the determination of detection limits in diagnostic panels, ensuring accurate detection and minimizing false negative results. Recently, the use of synthetic fragments has been reported for the detection of zoonotic bacteria such as *Escherichia coli*, *Enterococcus faecalis*, and *Legionella pneumophila* (Cardenas, 2018). Also, synthetic sequences have been used as standards in the multiplex PCR detection of *Taenia spp*. (Ng-Nguyen *et al*., 2017) and for the molecular identification of *Ehrlichia canis*, *Anaplasma platys*, and *Rickettsia rickettsii* (Aragón-López *et al*., 2021). Therefore, the use of synthesized positive sequences in the standardization of molecular assays enhances the efficiency of bioassays for detection, especially when biological isolates are not readily available.

During the standardization of the Chla-LAMP assay, the reaction was visualized on agarose gels within the first 30 min. However, at 40 min the visualization became sharper and more stable. Reactions longer than 60 m sometimes result in false positives due to the impact of template concentration on amplification time. Additionally, prolonged reactions with low-purity primers may also yield false positives

(Hardinge and Murray, 2019). Therefore, it is recommended to work with a positive synthetic control to minimize template concentration variations.

In another study comparing LAMP with conventional nested PCR for *Chlamydia* detection and chick embryo isolation, the assay exhibited the same sensitivity with a limit of detection of 4 genomic copies and higher to the chick embryo isolation (Lin *et al*., 2011). Other analyses reported sensitivity limits of up to 10 and 25 genomes per reaction (Halse *et al*., 2006; Appelt *et al*., 2019). However, Chla-LAMP demonstrated equal or better sensitivity compared to Ashraf *et al*. (2018), who reported detection limits of $1e^{-04}$ and $1e^{-05}$ ng of genetic material from pure cultures and DNA extractions from milk samples, respectively.

CONCLUSIONS

A LAMP assay was standardized for the fast and accurate detection of the *ompA* gene of *C. abortus*. This test was successfully validated, using DNA extracted from the vaginal exudate of ewes from flocks with a history of *C. abortus*-induced abortions, and confirmed by PCR. The standardized Chla-LAMP assay demonstrated specificity, sensitivity, and accuracy in detecting *Chlamydia abortus* in infected sheep, with no risk of cross-contamination with other *Chlamydia*s. LAMP is considered a novel methodology that could be valuable for biosecurity protocols in intensive lamb production.

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CONFLICTS OF INTEREST

The authors of this study declare no conflict of interest.

DECLARATION

For the ethical standards and statement of animal rights in this study, the Research Committee of the Department of Agronomic and Veterinary Sciences of the Instituto Tecnologico de Sonora approved the procedures used in this study, which were in accordance with the Mexican Official Standard NOM-062-ZOO-1999 for the production, care, and use of experimental animals.

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