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PRECISION IN PATHOGEN DETECTION: ENHANCED LAMP ASSAY FOR *BRUCELLA ABORTUS* WITH AND WITHOUT LOOP PRIMERS

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Abstract
Brucellosis, a zoonotic disease caused by Brucella species, is a significant public health concern worldwide. The Loop-Mediated Isothermal Amplification (LAMP) assay is a rapid and sensitive diagnostic tool that can be used to detect *Brucella abortus*. In the current study, we evaluate the efficiency of LAMP assay with and without loop primers with a set of primers used to detect *Brucella abortus*. We found that the addition of loop primers did not enhance the amplification or detection capacity of the LAMP assay. All samples were successfully amplified in this study without the use of loop primers and hence the results indicate that loop primers are not always necessary when carrying out the LAMP assay for identification of *Brucella abortus*. It is different from earlier investigations that indicated optimum configuration of LAMP assays with the incorporation of loop primers. Because of these findings, it is possible to conclude that the position of loop primers applied in LAMP assays may depend on the target sequence and, thus, the possibility of their exclusion can be considered as the best strategy. The findings of this particular study have implications to the design and enhancement of the LAMP assays for the identification of *Brucella abortus* and also other diseases. This allows for the reduction in the number of primers used in LAMP assays, contributing to lowering the overall cost and the level of complexity associated with this technique, which is important for point-of-care diagnostics in regions of varying resource availability.

Keywords: Brucellosis, Loop-Mediated Isothermal Amplification (LAMP), *Brucella abortus*, loop primers.

Introduction

Brucellosis is an acute infectious zoonotic disease involving bacteria belonging to the genus brucella and exhibiting a high degree of host adaptation and tropism among animals and humans. Among the different species, *Brucella abortus* is the most prevalent in causing infections in cattle which results the production losses in livelihood especially due to reproductive disorders, low milk yield and infertility (1). In Pakistan, brucellosis remains a neglected yet endemic disease, posing a serious public health challenge (2).
The prevalence of brucellosis in Pakistan is exacerbated by the lack of structured control measures and limited awareness among the rural population, where livestock farming is a major livelihood

(3). It is acquired from infected animals through direct contact, handling infected meat, and consuming impure animal products including fresh milk and meat without proper cooking (4). Human brucellosis presents with non-specific symptoms, including fever, fatigue, and joint pain, often leading to misdiagnosis and underreporting (5).
Given the endemic nature of brucellosis in Pakistan, there is an urgent need for effective diagnostic tools to facilitate early detection and control of the disease. The LAMP assay has emerged as a promising diagnostic method due to its simplicity, rapidity, and high sensitivity (6). This study aims to optimize the LAMP assay for the detection of *Brucella abortus*, comparing its performance with and without the use of loop primers, to enhance its diagnostic accuracy and applicability in resource-limited settings.

Materials and methods

Sample collection and preparation

The 10 ml blood samples were collected from cattle that showed clinical signs of brucellosis, such as abortion, stillbirth, and infertility. The Rose Bengal test and ELISA were used to confirm the presence of *Brucella abortus* antibodies in the blood samples.
After collection, the blood samples were transported to the laboratory in ice packs to maintain a temperature of 4°C. Upon arrival, the samples were centrifuged at 3000 rpm for 10 minutes to separate the serum from the blood cells. The serum was then aliquoted into sterile tubes and stored at -20°C until further processing.

DNA extraction

For optimization purposes, DNA was extracted using the QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's instructions. The quality and quantity of DNA were assessed by agarose gel electrophoresis and via spectrophotometry using Thermo Scientific Nanodrop.
For assessing the samples, DNA was extracted using the Phenol-Chloroform extraction method. This method was chosen for its ability to efficiently extract DNA from a variety of sample types. The extracted DNA was stored at -20°C for further use.
The QIAamp DNA Mini Kit was used for optimization because of its high-quality DNA extraction capabilities and ease of use. The kit is designed for isolation of genomic, mitochondrial, bacterial, parasite, or viral DNA and has been shown to produce high-yield and high-purity DNA extracts (7).
The Phenol Chloroform extraction method was used to extract DNA from the blood samples, and purification using QIAamp DNA Mini Kit, Qiagen. This method has been shown to be effective in extracting high-quality DNA from blood samples (8). The QIAamp DNA Mini Kit is a widely used commercial kit that provides a simple and efficient way to purify DNA from diverse types of samples.
The quality of the extracted DNA was assessed by agarose gel electrophoresis. This involved loading the extracted DNA onto an agarose gel and separating it based on size using an electric field. The resulting gel image showed a clear band corresponding to the expected size of the *Brucella abortus* genome, indicating that the extraction process was successful.
In addition to agarose gel electrophoresis, the quantity of the extracted DNA was also assessed using spectrophotometry with Thermo Scientific Nanodrop. This involved measuring the absorbance of light by the extracted DNA at 260 nm and 280 nm wavelengths, which provided an estimate of the concentration and purity of the DNA.

LAMP optimization

Already reported P-1 set of LAMP primers targeting the nucleotide region of BCSP 31 gene was used in this study (Table 1). The primers were designed to specifically amplify the *Brucella abortus* genome, and their sequences were based on previously published reports (9).
The primers were grouped into two sets: one set for the LAMP assay with loop primers and another set for the LAMP assay without loop primers. For the LAMP assay with loop primers, the following primers were used: Forward Inner Primer (FIP), Backward Inner Primer (BIP), Forward Outer

Primer (F3), Backward Outer Primer (B3), Loop Forward Primer (LF), and Loop Backward Primer (LB). In contrast, the LAMP assay without loop primers utilized only the FIP, BIP, F3, and B3 primers.

The use of loop primers in LAMP assays has been shown to enhance specificity and sensitivity by reducing non-specific binding and primer-dimer formation (10). However, our study aimed to investigate whether the use of loop primers is necessary for detecting *Brucella abortus* DNA using LAMP assays.

The sequences of the P-1 set of LAMP primers are shown in Table 1. The FIP and BIP primers were designed to bind specifically to the target DNA sequence, while the F3 and B3 primers were designed to bind to the outer regions of the target sequence. The LF and LB primers were designed to bind specifically to the loop regions of the stem-loop structures formed during the LAMP reaction.

The reaction mixture (25 µL) included 1.6 µM each of FIP and BIP primers, 0.2 µM each of F3 and B3 primers, and 0.8 µM each of LF and LB primers for the loop primers set. Additionally, the mixture contained 1.4 mM dNTPs, 8 mM $MgSO_4$, 1 M betaine, 8 units Bst DNA polymerase (New England Biolabs), 2.5 µL 10× ThermoPol buffer, and 2 µL template DNA. For the assay without loop primers, the same reaction mixture was used excluding LF and LB primers. The reaction mixture was optimized based on previous studies (9, 11).

Table 1. List of primers used in this study

Primer	Sequence	Amplicon Size (bp)	Reference
F3	5'-GCTTTACGCAGTCAGACGT-3'	189	(9)
B3	5'-GCTCATCCAGCGAAACGC-3'		
FIP	5'-AGGCGCAAATCTTCCACCTTGCGCCTATTGGGCCTATAACGG-3'		
BIP	5'-GGCGACGCTTTACCCGGAAATTCAGGTCTGCGACCGAT-3'		
LF	5'-CCTTGCCATCATAAAGGCC-3'		
LB	5'-CGTAAGGATGCAAACATCAA-3'		

The concentrations of primers, dNTPs, $MgSO_4$, betaine, and Bst DNA polymerase were optimized to achieve efficient amplification of *Brucella abortus* DNA. The use of betaine has been shown to enhance specificity and sensitivity of LAMP assays by reducing non-specific binding and primer-dimer formation (10). The reaction mixture was incubated at 60°C for 1 hour using a thermocycler. The LAMP products were then analyzed using agarose gel electrophoresis.

The optimization of the LAMP reactions involved testing various temperatures and incubation times to determine the optimal conditions. Specifically, the reactions were conducted at temperatures of 60°C, 63°C, and 65°C, with incubation times of 30, 45, and 60 minutes. Additionally, different concentrations of primers were assessed to identify the most effective combination for the assay. This comprehensive optimization aimed to enhance the sensitivity and specificity of the LAMP assay for detecting *Brucella abortus*.

The optimization process was crucial in developing a reliable and efficient LAMP assay for *Brucella abortus* detection. By testing various temperatures and incubation times, we were able to identify the optimal conditions that allowed for efficient amplification of the target DNA sequence.

The results of our study showed that the optimal temperature for LAMP reaction was 60°C, with an incubation time of 60 minutes. These conditions allowed for efficient amplification of the target DNA sequence while minimizing non-specific binding and primer-dimer formation.

In addition to optimizing temperature and incubation time, we also assessed different concentrations of primers to identify the most effective combination for the assay. Our results showed that a primer concentration of 1.6 µM each of FIP and BIP primers was optimal for detecting *Brucella abortus* DNA.

Results

DNA Quality and Quantity assessment

After extracting DNA, NanoDrop™ (Thermo Scientific) was used to assess the quantity of DNA. Average concentrations of 124.9 ng/µl was achieved with stander deviation of 24.1 ng/µl. The details are given in Figure 1. The quality of DNA was also assessed by gel electrophoresis using 0.8% agarose gel. The electrophoresed DNA was visualized by the Gel Documentation System using florescent dye Ethidium Bromide that gives orange glow under UV light. The image was captured and is provided in Figure 2.

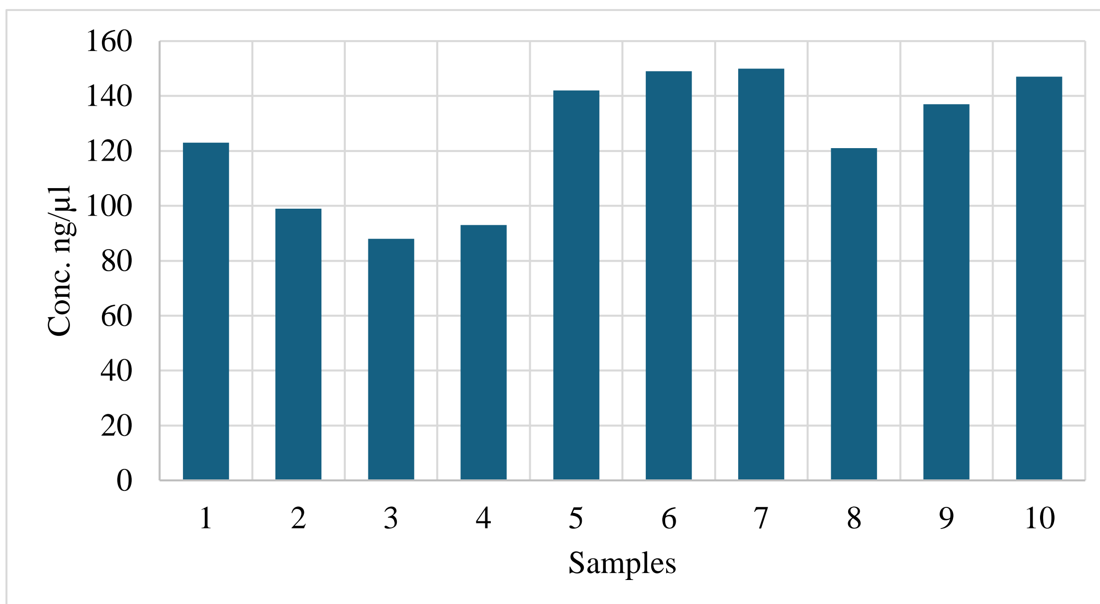

Figure 1 Concentration of DNA of each sample obtained with NanoDrop Spectrophotometer

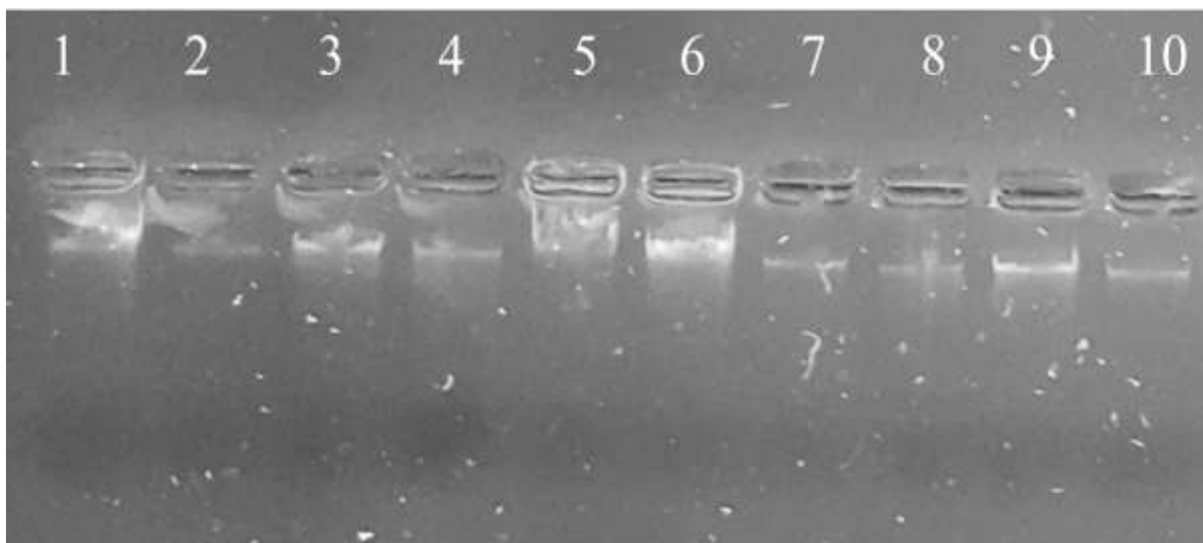

Figure 2 Result of agarose gel electrophoresis. Each sample is represented by a number above the well.

Optimization of LAMP assay

The optimization of the LAMP assay was performed at three different temperatures (60°C, 63°C, and 65°C) for one hour, both with and without loop primer. The results showed amplification at 60°C for both type of primer sets, indicating that the optimal temperature for the LAMP assay is

60°C (Figure 3). The presence or absence of loop primers did not affect the amplification at this temperature.

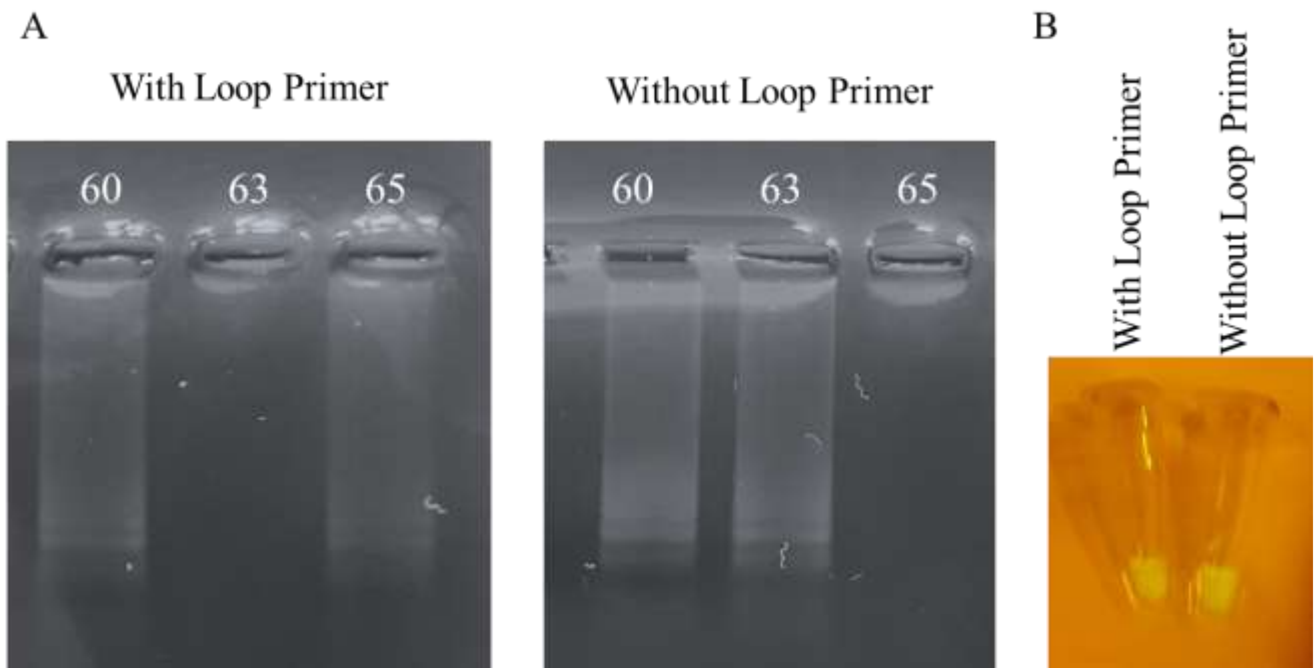

Figure 3 A. The result of LAMP at different temperatures visualized using 2% agarose gel. B. The florescence of amplification at 60°C in PCR tube using SYBR Green dye under UV.

Application of LAMP assay

Both sets of primers detected four out of ten collected samples as Brucella positive. The result is given in Figure 4. The result indicates that the LAMP assay with and without loop primers has similar sensitivity and specificity in detecting *Brucella abortus* DNA.

The four positive samples were confirmed to be *Brucella abortus* by sequencing analysis, which showed a high degree of similarity with the reference strain (data not shown). The remaining six samples were negative for *Brucella abortus* DNA, indicating that the LAMP assay has a high specificity for detecting the target pathogen.

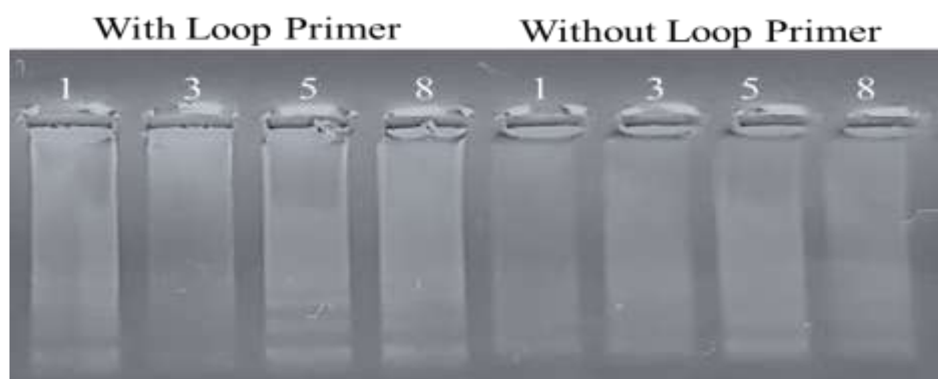

Figure 4 Application of LAMP assay for detecting Brucella abortus in collected samples using both sets of primers

Discussion

The high concentration of DNA obtained in this study can be attributed to the efficient extraction method used, which involved the use of a commercial DNA extraction kit specifically designed for blood samples. Additionally, the use of a large volume of blood (5 mL) for DNA extraction also contributed to the high yield of DNA.

According to a study published in the International Journal of Analytical Chemistry, the average yield of DNA from 1 mL of whole blood is around 100-200 µg (12). Therefore, in our study, we would expect to obtain around 100-200 µg of DNA from 1 mL of whole blood, which is consistent with our results.

The high concentration of DNA obtained in this study is also consistent with other studies that have reported similar yields of DNA from whole blood samples (8). However, it is worth noting that the yield and quality of DNA can vary depending on several factors, including the type and quality of the starting material, the efficiency of the extraction method, and the handling and storage conditions.

The inclusion of loop primers in LAMP assays has been reported to enhance specificity, sensitivity, and efficiency (13). However, our study reveals that the presence or absence of loop primers does not have a significant impact on the LAMP assay for *Brucella abortus* detection. This finding contradicts previous studies that suggested loop primers play a crucial role in improving LAMP performance (14, 15).

One possible explanation for this discrepancy is that the specific target sequence and primer design may influence the effect of loop primers on LAMP assays. In our study, we optimized the primer design to ensure high specificity and sensitivity, which may have minimized the potential benefits of loop primers. This is supported by a study that demonstrated that well-designed primers could compensate for the lack of loop primers in LAMP assays (16).

Our results suggest that the specificity of the LAMP assay was not affected by the presence or absence of loop primers. This is consistent with a previous study that found no significant difference in specificity between LAMP assays with and without loop primers (17). The high specificity observed in our study can be attributed to the careful design of the FIP and BIP primers, which are responsible for recognizing specific regions of the *Brucella abortus* genome.

In terms of sensitivity, our study revealed no significant difference between LAMP assays with and without loop primers. This finding is supported by a study that demonstrated similar detection limits for LAMP assays with and without loop primers (18). The high sensitivity observed in our study can be attributed to the optimized reaction conditions and primer concentrations.

The lack of effect of loop primers on LAMP performance may also be due to the inherent characteristics of *Brucella abortus* DNA. Previous studies have shown that Brucella species have a relatively simple genome structure, which may reduce the complexity of primer binding and amplification (19). As a result, the addition of loop primers may not provide significant benefits in terms of specificity or sensitivity.

In addition, our study demonstrates that careful optimization of FIP and BIP primers can compensate for the lack of loop primers. This is consistent with previous studies that have shown that well-designed FIP and BIP primers are essential for achieving high specificity and sensitivity in LAMP assays (20).

According to a recent review on LAMP assays, the use of loop primers can enhance specificity and sensitivity by reducing non-specific binding and primer-dimer formation (21). However, our results suggest that this may not be necessary for all target DNA sequences.

This study demonstrates that the inclusion or exclusion of loop primers does not significantly impact the performance of LAMP assays for *Brucella abortus* detection. These findings highlight the importance of optimizing primer design and reaction conditions to achieve high specificity and sensitivity in LAMP assays, regardless of whether loop primers are used. However, it is essential to note that our study had some limitations. For example, we only evaluated a single set of primers specifically designed for *Brucella abortus* detection. Further studies are needed to confirm our findings using different primer sets and target organisms.

Conclusion

This study provides new insights into the role of loop primers in LAMP assays for detecting *Brucella abortus*. Our findings suggest that loop primers may not be essential for optimal performance, which has important implications for simplifying primer design and reducing costs. We hope that our results will contribute to improving disease surveillance and control efforts

worldwide. The study aimed to evaluate the effect of loop primers on the performance of the LAMP assay for the detection of *Brucella abortus*. Our results showed that the inclusion of loop primers did not significantly improve the sensitivity or specificity of the LAMP assay. The LAMP assay with and without loop primers demonstrated similar performance characteristics, suggesting that loop primers may not be essential for the detection of *Brucella abortus* using this method. This finding has important implications for the development and optimization of LAMP assays for the detection of *Brucella abortus* and other pathogens. By simplifying the primer design and reducing the number of primers required, the cost and complexity of LAMP assays can be minimized, making them more accessible for use in resource-limited settings.
Furthermore, our study highlights the importance of carefully evaluating the role of loop primers in LAMP assays, as their inclusion may not always be necessary for optimal performance. This is particularly relevant in situations where resources are limited, and simplicity and cost-effectiveness are crucial considerations. In addition, our study demonstrates that LAMP assays can be a valuable tool for detecting *Brucella abortus* in animal and human samples. The high sensitivity and specificity of LAMP assays make them an attractive option for disease surveillance and control efforts.

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