FUTURISTIC BIOTECHNOLOGY
https://fbtjournal.com/index.php/fbt
ISSN (E): 2959-0981, (P): 2959-0973
Volume 4, Issue 2 (April-June 2024)

Original Article

# Development and Validation of Loop-Mediated Isothermal Amplification (Lamp) Field Assay for the Detection of *Brucella abortus*

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## ARTICLE INFO

***Keywords:***
*Brucella abortus*, BSCP31 Gene, Serum Screening, Rose Bengal Plate Test

***How to Cite:***
Jamil, M., Ali, S., Hasan, A., Abbas, Y., Raza, M. A., Aslam, A., & Hasan, A. (2024). Development and Validation of Loop-Mediated Isothermal Amplification (Lamp) Field Assay for the Detection of *Brucella abortus*: Detection of *Brucella abortus*. Futuristic Biotechnology, 4(02). https://doi.org/10.54393/fbt.v4i02.119

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Received Date: 3rd May, 2024
Acceptance Date: 20th June, 2024
Published Date: 30th June, 2024

## ABSTRACT

Currently, a number of techniques are available for detection of *Brucella abortus (B. abortus)* but these techniques are costly and specialized equipment are needed. Therefore, the development of a rapid, accurate, sensitive, and cost effective technique for identification of *Brucella* species is of high priority. **Objective:** The current research study was designed to detect *Brucella* species more rapidly. The current study area was conducted in district Lodhran, Punjab, Pakistan. **Methods:** A total 100 blood samples (50 cattle and 50 buffaloes) were collected. Serum samples were screened against *B. abortus* antibodies using Rose Bengal plate test (RBPT). The specific gene was designed by using NCBI website and whole genome of *Brucella* species. The primers were designed from Gene accession number 20404. Following primers were designed F3, B3, FIP, BIP, LF, LB, B4, and B5. The LAMP technique for BSCP31 gene was developed by using many concentrations of components and conditions. **Results:** The development and validation of LAMP assay for detection of *B. abortus* from bovine blood in the present study proved helpful in early detection of said pathogen in animal and humans. **Conclusions:** This study will be helpful in prevention and control of animal and human brucellosis in Pakistan.

## INTRODUCTION

Brucellosis is a zoonotic ancient disease, which was reported in Egypt. The causative agent of Brucellosis is Brucella species. The Brucella species are Gram-negative cocco-bacilli bacteria have worldwide distribution [1-3]. David Bruce in 1887 isolated Brucella melitensis in British soldier's spleen that died due to Malta fever which is common in the location of Malta station. After Malta fever discovery, 20 years later it was considered as a vector-borne disease, accidently Themistocles Zammit revealed that Malta fever is a zoonotic disease in 1905, when he isolated *B. melitensis* in milk of goat. It was the time when considered that the goats were not the source of disease because the goats were not ill from inoculation of Brucella cultures. In epidemiology discovered new thing that healthy goats were the carriers of brucellosis [4, 5]. Brucellosis outcomes are very important in economic point of view because of reproductive impairment in cattle cause stillbirth, abortion, infertility and neonatal mortality which can affect country's economics [6]. Recently, there are ten species but each specie has characteristics according to their host, *B. melitenesis* (sheep and goats), *B. pinnipedialis* (pinnipeds), *B. neotomae*, *B. microti* (rodents-Microtusarvalis), *B. ceti* (cetacean), *B. canis* (dogs), *B. inopinata* (Breast infections in females), *B. abortus* (cattle), *B. ovis* (rams) and *B. suis* (pigs) [7, 8]. Brucellosis causes febrile disease having the broad spectrum indications

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**DOI:** https://doi.org/10.54393/fbt.v4i02.119

which were fatal in many cases in humans [9, 2]. Humans are usually infected through the consumption of contaminated food of infected animals, especially unpasteurized milk [10]. Brucellosis species infection has been reported in more than 170 countries every year and about 1/5- 1/6 of world population, the expected loss due to brucellosis in many billions dollars every year [11]. Specific diagnosis normally requires confirmation by isolating the causative agent from clinical samples. Methods of direct Brucella-DNA detection and validation may minimize these drawbacks. Previous researches have cleared the benefits of direct PCR method for Brucella detection [12-14]. The main problem of serological method such as (RBPT) Rose Bengal Plate Test and (STAT) Standard Tube Agglutination Test which are not specific for antibodies test and can affect other Gram-negative microorganisms for example *Yersinia enterocolitica.* At initial stages of infection, antibodies growing period such tests are not of great value in detecting brucellosis. Now a days PCR technique is used for detecting the disease, such commonly used techniques are sensitive and easy to use but require special equipment and skills for detecting the brucellosis, and also require special post protocol for clarification. Anyhow, these above mentioned tests are time consuming, require skilled approach and are not straight forward, whereas, DNA analyses based studies are widely used for diagnosing brucellosis. As these techniques are costly and specialized equipment are needed, so they are not appropriate. Whereas, the development of rapid, accurate, sensitive, cost effective and identification of Brucellosis species is of high priority. All the bacteriological techniques used in past require many expertise and a lot of time to isolate the organism because the growth period of organism requires 2-3 days. Other drawbacks of these techniques are low sensitivity when we load the low amount of the sample pathogen and because brucellosis can spread from animals to human rapidly, so class 3 bio safety cabinets are required to handle its causative agent [15]. LAMP technique is a novel gene amplification which requires 4-6 primers. The target identifies specific regions at constant temperature (60-65ºC) in less than an hour without any special reagent [16]. Accurate development and validation technique for brucellosis is required for achieving accurate and reliable results. LAMP technique is one of such techniques, having all facilities and qualities for diagnosing of brucellosis. LAMP technique is more specific than PCR and more valuable tool for field level assay [17]. The anti- *B. abortus* antibody was reported because of the presence of brucella in semen [18].

The current research study was designed to detect *Brucella* species more rapidly. The current study area was conducted in district Lodhran, Punjab, Pakistan.

# METHODS

### Sample Collection and Serology

The current study was conducted in District Lodhran, Punjab, Pakistan. A total of 100 blood samples (50 cattle and 50 buffaloes) were collected. Serum samples were separated after centrifugation at 3000 rpm for 5 minutes. These serum samples were screened for B. abortus antibodies using Rose Bengal plate test RBPT [19].

### Primer Designing

The specific gene was taken by using NCBI website and whole genome of Brucella species. The primers were designed from Gene accession number 20404. Following primers were designed F3, B3, FIP, BIP, LF, LB, B4, and B5 as shown in figure 1.

```
<P-1>
 781  tcgtcgaatg  gctcggttgc  caatatcaat  gcgatcaagt  cgggctct  ggagtccggc

 841  tttacgcagt  cagacgttgc  ctattgggcc  tataacggca  ccggcctta  tgatggcaag
            F3                   FIP (F2)                      LF
 901  ggcaaggtgg  aagatttgcg  ccttctggcg  acgctttacc  cggaaacgat  ccatatcgtt
              FIP (F1c)                  BIP (B1c)        Sau3 AI
 961  gcgcgtaagg  atgcaaacat  caaatcggtc gcagacctga  aaggcaagcg  cgtttcgctg
             LB                      BIP (B2)                   B3
1021  gatgagccgg  gttctggcac  catcgtcgat  gcgcgtatog  ttcttgaagc ctacggcctc

<P-2>
 961  gcgcgtaagg  atgcaaacat  caaatcggtc  gcagacctga  aaggcaagcg  cgtttcgctg

1021  gatgagccgg  gttctggcac  catcgtcgat  gcgcgtatcg  ttcttgaagc  ctacggcctc
          F3                                    FIP (F2)           LF
1081  acggaagacg  atatcaaggc  tgaacacctg  aagccgggac  cggcaggcga  gaggctgaaa
            Eco RV            FIP (F1c)                          BIP (B1c)
1141  gatggtgcgc  tggacgccta  tttctttgtg  ggcggctatc  cgacgggcgc  aatctcggaa
                              LB
1201  ctggccatct  cgaacggtat  ttcgctcgtt  ccgatctccg  ggccggaagc  ggacaagatt
       BIP (B2)             B3
```

**Figure 1:** Primer Design for LAMP to Detect *Brucella* DNA. Nucleotide Sequence of BCSP31 Gene (GenBank accession no M20404), Used to Design LAMP Primers. Underlining Indicates the Positions of Targeting Sequences

### Bacterial DNA Extraction

Extraction of DNA was done using the PureLink™ Microbiome DNA Purification Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to manufacturer's instruction.

### DNA Extraction from Whole Blood

Extraction of DNA was performed by organic DNA extraction method involving PCI [20]. The extraction of all 50 samples was performed by the following protocol.

### Loop Mediated Isothermal Amplification Assay

The LAMP technique for BSCP31 gene was developed by using various concentrations of components and conditions. The constant temperature 60-68ºC was provided.

### LAMP Products Detection

The amplified outcomes of LAMP were observed through naked eye. The color of product changed from violet to sky blue in positive result with no change in negative result. The result was observed by agarose gel documentation apparatus.


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DOI: https://doi.org/10.54393/fbt.v4i02.119

### LAMP Assay

The loop-mediated isothermal amplification assay was processed in 25µl of reaction mixture consisting 40 pmol $l^{-1}$ of forward inner primer(1µl) and 40 pmol $l^{-1}$ of BIP (1µl), 5 pmol $l^{-1}$ of F3(0.5µl) and 5 pmol $l^{-1}$ of B3(0.5µl), 20 pmol $l^{-1}$ of LF(1µl) and 20 pmol $l^{-1}$ of LB(1µl), betain (2.5µl), 20 mmol $l^{-1}$ Tris–HCl, 10 mmol $l^{-1}$ KCl, 10 mmol $l^{-1}$ (NH4)2SO4, 8 mmol $l^{-1}$ MgSO4 (1.5µl), 0.1% Tween 20,1.4 mmol $l^{-1}$ each deoxynucleoside triphosphates, 8 units of Bst DNA polymerase (2µl) isothermal lamp buffer (2.5µl) (New EnglandBiolabs, Berverly, MA, USA), 2µl of template DNA and 8.5µl distilled water. The reaction mixture was incubated at 63ºC for 35 min. Heated to 95ºC for 2 minutes to end the reaction. The loop-mediated isothermal amplification amplicon was identified as the dimension of fluorescence. An aggregate of 2µl was analyzed by electrophoresis in 2% agarose gel [16].

## RESULTS

A total of 100 samples (50 samples from buffalo and 50 from cows) were collected from district Lodhran. Sixty samples out of 100 were found seropositive for brucellosis based on RBPT.

### Intensification of BCSP31 by Loop-Mediated Isothermal Amplification

Loop-mediated isothermal amplification reaction for LAMP assay was modified and ended at 95ºC for 2 minutes. We attained the same results from this modified protocol. By using primers, P-1 (Table 1),

**Table 1.** Sequences Set of Primers (Primer-1) Used for LAMP

| Primer | Sequence |
|---|---|
| F3 | 5'-GCTTTACGCAGTCAGACGT-3' |
| B3 | 5'-GCTCATCCAGCGAAACGC-3' |
| FIP | 5'-AGGCGCAAATCTTCCACCTTGCGCCTATTGGGCCTATAACGG-3' |
| BIP | 5'-GGCGACGCTTTACCCGGAAATTCAGGTCTGCGACCGAT-3' |
| LF | 5'-CCTTGCCATCATAAAGGCC-3' |
| LB | 5'-CGTAAGGATGCAAACATCAA-3' |

FIP, forward inner primer; F3, forward outer primer; BIP, backward inner primer; B3, backward outer primer; LF, forward loop primer; LB, backward loop primer.

The loop-mediated isothermal amplification assay successfully amplified the target sequence of BCSP31 of *B. abortus* DNA at 63ºC for 35 minutes. After intensification, items were likewise specifically seen by the bare eye with a fluorescent location reagent. The pattern was affirmed by gel electrophoresis (Figure 2).

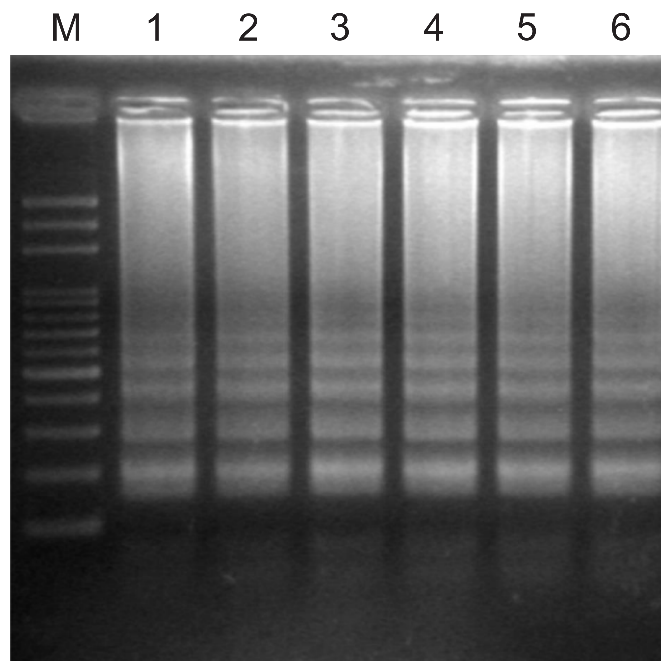


**Figure 2:** Result of LAMP PCR on Gel Electrophoresis. Lane M = DNA Ladder of 100-bp; Lane 1-6 = DNA samples.

### Specificity of the Loop-Mediated Isothermal Amplification examine with different Sorts of Bacterial Species

To assess the specificity of the loop-mediated isothermal amplification primers, *Brucella* species and other non *Brucella* species were used. Huge and explicit enhancement of deoxyribonucleic acid was observed after 35 minutes of all *Brucella* species used, consisting reference, antibody, clinical segregates, while other non-*Brucella* species demonstrated no intensification (Table 2)

**Table 2.** Bacterial Strains Used in This Study and Results OF LAMP Amplification

| Sources No. | | | | | |
|---|---|---|---|---|---|
| ***Brucella* Species** | | | **Non *Brucella* Species** | | |
| **Species** | **Source** | **LAMP Result** | **Species** | **Source** | **LAMP Result** |
| *Brucella abortus* | Microbiology Laboratory, UVAS, Ravi campus, Pattoki | Positive | *Escheri-chia coli* | Microbiology Laboratory, UVAS, Ravi campus, Pattoki | Negative |
| *Brucella melitensis* | Microbiology Laboratory, UVAS, Ravi campus, Pattoki | Positive | *Staphyl-ococcus aureus* | Microbiology Laboratory, UVAS, Ravi campus, Pattoki | Negative |

The figure 3 illustrated the specificity of the loop-mediated isothermal amplification (LAMP) assay when tested on various species.


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DOI: https://doi.org/10.54393/fbt.v4i02.119

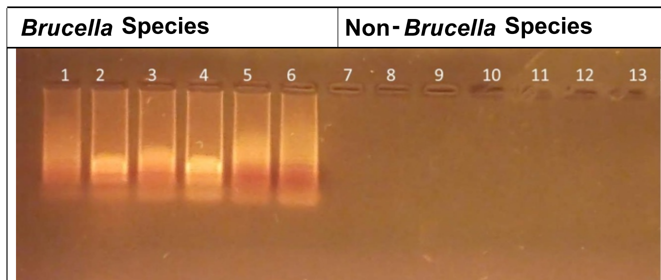


**Figure 3:** Specificity of the Loop-Mediated Isothermal Amplification Assay with Different Types of Species. Lane 1-6 are Bacterial Species and 7-13 are non-brucella Species Per Products Were Identified on the Base of Specific Primers Used for Identification.

## DISCUSSION

This study is a principal report of use of loop-mediated isothermal amplification for recognition of *Brucella* species. Brucellosis has great importance in human health, in both veterinary medicine and public health because it is a zoonotic disease [21]. Different *Brucella* species influence steers, deer, elk, sheep, goats, pigs, hounds and a few different living organisms, including marine warm blooded animals [21, 22]. In people, the infection shows exceedingly assorted side effects, for example, fever that forms into a chronic sickness influencing different parts of body. Epidemic breakout of brucellosis in lab labors have additionally been reported [23-25]. As medication treatment is drawn out and powerful anti-infection agents are restricted, a dependable and early finding of brucellosis is of real significance for starting satisfactory treatment. However advantageous serological diagnostic techniques, for example, Rose Bengal method for identification of *Brucella-explicit* antibodies were accessible. The helpfulness was restricted by a great commonness of *Brucella-explicit* antibodies in disease territories of brucellosis, low amount in the intense stage, and cross-responses with different gram-negative microscopic organisms, for example, Y. *enterocolitica* O:9 [26, 27]. Likewise, developing an assay for the identification of *Brucella* bacteria involves creating a test that can accurately and reliably detect the presence of this pathogen [28, 29]. Biochemical identification is an economical approach, even due to its slow developmental rates (of *Brucella spp). Brucella spp* has a potential natural causative agent of brucellosis [30, 10]. By and large, a quick, explicit, straightforward and safe identification framework for *Brucella spp.* should be set up. The loop-mediated isothermal amplification measure is beneficial as a result of its basic task, quick response and simple detection [16]. A straightforward and economical mechanical assembly, for example, a water bath that gives a steady of 63ºC is adequate for test, not at all like polymerase chain reaction, reaction is specifically seen with exposed vision refuting requirement for agarose gel electrophoretic examination. In addition, loop-mediated isothermal amplification test can be processed nearby equipment for example, a PCR machine is not required. By utilizing our loop-mediated isothermal amplification test, 10 fg of *Brucella* DNA is effectively processed inside 35 minutes, and assessed to relate to 2.8 DNA duplicates per response [31]. The specificity of Brucella loop-mediated isothermal amplification was relatively equivalent to that of real time PCR recently detailed by [32]. Consequently, the specificity of the loop-mediated isothermal amplification measure was better than that of real time PCR. At the point when melt cover examination was incorporated, continuous PCR took around 50 min, while the Brucella loop-mediated isothermal amplification can be done for 35 minutes. We likewise assessed the loop-mediated isothermal amplification through which *Brucella* was identified. In the defected spleen, loop-mediated isothermal amplification distinguished as few as 8.2x102 CFU of *B. abortus.* These outcomes propose that the loop-mediated isothermal amplification measure would be valuable for quick finding of brucellosis at beginning of disease and also for diagnosis of microorganisms. *Brucella* loop-mediated isothermal amplification strategy created in this examination is a quick, fast and very explicit technique that can be substituted for polymerase chain reaction or constant polymerase chain reaction tests. It is a valuable technique for determination and reconnaissance of brucellosis. Dairy cattle entire blood tests were handled by regular techniques for DNA extraction and henceforth, PCR inhibitors may exist, yet the LAMP test is a solid examine as the compound Bst polymerase is not hindered by inhibitors, which is an additional credit to the technique of LAMP [33]. Isolation and distinguishing proof of the species was endeavored in the underlying stages of the examination without productive outcomes, as entire blood is not a good for the isolation of *Brucella*. Traditional PCR is ordinarily utilized as molecular device for recognition of brucellosis and henceforth efforts were made to contrast LAMP and ordinary PCR, as opposed to the more refined real time PCR. The clinical affectability, specificity and sensitivity of the indicative test were altogether observed to be right around 100% and thus the LAMP test can be used as a helpful demonstrative apparatus for screening of brucellosis in field conditions, with more extensive appropriateness around the world. The LAMP measure demonstrated promising outcomes when utilized for epidemiological screening of brucellosis in cows and this investigation additionally uncovered the pervasiveness of cow-like brucellosis in various parts of Pakistan. In the LAMP assay blend is lyophilized, it might be utilized as a field test to distinguish *Brucella spp*. outwardly inside 30 min, which would examine the sub-atomic the study of disease transmission of this essential zoonotic pathogen.


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DOI: https://doi.org/10.54393/fbt.v4i02.119

# CONCLUSIONS

This is principal report of use of loop-mediated isothermal amplification for recognition of *Brucella* species. Our study will be helpful in prevention and control of animal and human brucellosis in Pakistan.

## Authors Contribution

Conceptualization: MJ
Methodology: MJ, SA, AH[1], MAR, AA
Formal analysis: YA
Writing, review and editing: MJ, AH[2]

All authors have read and agreed to the published version of the manuscript.

## Conflicts of Interest

The authors declare no conflict of interest.

## Source of Funding

The authors received no financial support for the research, authorship and/or publication of this article.

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