*VETERINARSKI ARHIV 86* (1), 35-47, 2016

# Rapid and visual loop mediated isothermal amplification (LAMP) test for the detection of *Brucella* spp. and its applicability in epidemiology of bovine brucellosis

**Kumaragurubaran Karthik[1], Rajesh Rathore[1], Prasad Thomas[1], Konasagara N. Viswas[1], Rajesh K. Agarwal[1], Valsala Rekha[1], Ramesh V. Jagapur[1], and Kuldeep Dhama[2]***

[1]*Division of Bacteriology and Mycology, Indian Veterinary Research Institute, Izatnagar, Bareilly, Uttar Pradesh, India*

[2]*Division of Pathology, Indian Veterinary Research Institute, Izatnagar, Bareilly, Uttar Pradesh, India*

**KARTHIK, K., R. RATHORE, P. THOMAS, K. N. VISWAS, R. K. AGARWAL, V. REKHA, R. V. JAGAPUR, K. DHAMA: Rapid and visual loop mediated isothermal amplification (LAMP) test for the detection of *Brucella* spp. and its applicability in epidemiology of bovine brucellosis. Vet. arhiv 86, 35-47, 2016.**

**ABSTRACT**

Brucellosis is a devastating disease, once an abortion storm starts in a herd, and hence early diagnosis is important. Loop mediated isothermal amplification **(**LAMP) has enlightened the darker part of the diagnostic world by its simplicity and swiftness, and has the advantage of working in isothermal conditions so that it can be applied to the field. LAMP for *Brucella* spp., that already exists has not been exploited for visual detection and the time needed for completion of the reaction has not been reduced. In the present study, an attempt was made to seal these cracks by developing a fast and visually detectable LAMP for *Brucella* spp., which needs only 30 min detection time, and SYBR green aided easier result visualization. The LAMP test targeting the o*mp*25 gene was found to be highly sensitive and specific for detecting *Brucella* spp. Comparison of the LAMP test with the available PCR assay revealed that the LAMP test was more sensitive than PCR, following testing of 438 field samples of cattle origin, and it showed prevalence of Brucella of 14.7 % in different parts of India. Being highly sensitive, specific and speedy, the standardized visual LAMP test can be widely used for epidemiological surveys of this economically important and zoonotic pathogen.

**Key words:** visual loop mediated isothermal amplification test, Brucella, loop primers, *omp*25, SYBR green

*Corresponding author:

Kuldeep Dhama, Principal Scientist, Division of Pathology, Indian Veterinary Research Institute, Izatnagar, Bareilly, Uttar Pradesh 243 122, India, Phone: +91 85 3282 4316; E-mail: kdhama@rediffmail.com

ISSN 0372-5480
Printed in Croatia

**Introduction**

Brucellosis, familiarly known as "Bang's Disease "or "Contagious Abortion in cattle," is one of the world's major zoonoses of veterinary and public health importance. *Brucella* does not show any bias towards sex and age in animals (ALTON, 1975). Many aspects of *Brucella* have been explored by scientists but there is still much lacking in terms of the accurate diagnosis of the disease. The gold standard test for diagnosis of brucellosis is isolation and identification of the organism. To achieve this, 5-10 % carbon dioxide is required for its growth. Even with all the conditions conducive for the growth of *Brucella*, it will take around 3-5 days for a colony to appear. At times it may even take longer; hence culturing work is laborious and time consuming. The major setback in culturing is that Brucellosis is zoonotic and the handlers are always at the risk of infection (PIKE et al., 1965). Hence, it needs level 3 biocontainment facilities and highly skilled technical personnel for handling live culture (ALTON et al., 1988).

To overcome these problems, serological tests such as the Rose Bengal Plate Test (RBPT), Standard Tube Agglutination Test (STAT) and Enzyme Linked Immunosorbent Assay (ELISA) are commonly employed (CHAND and SHARMA, 2004). These tests are inexpensive, fast and highly sensitive, but not necessarily highly specific, as antibodies may cross react with *Yersinia enterocolitica* serotype O:9. Polymerase Chain Reaction (PCR), a DNA detection method, has revolutionized the world of diagnostics by rendering accurate diagnosis and minimizing the time needed (YU and NIELSEN, 2010; HABTAMU et al., 2013). Although DNA detection using PCR serves as a good tool for diagnosis, it needs sophisticated instruments, amplification time takes 2 - 3 hours and result visualization requires electrophoresis, so practically it does not have wide field applicability.

The loop mediated isothermal amplification (LAMP) test has revolutionized the diagnostic world by its accuracy, swiftness and ability to visualize the results without a post amplification process (NOTOMI et al., 2000; DHAMA et al., 2013). *Bst* polymerase enzyme, having strand displacing property, works at an isothermal temperature and is the vital component of LAMP. Incorporation of loop primers increases the swiftness of the reaction (NAGAMINE et al., 2002). Addition of DNA binding dyes to the post amplified reaction tube results in differences in colour production in positive and negative samples. LAMP for detecting *Brucella* has been developed against the *Brucella* cell surface protein (*bcsp*) 31 gene and the *omp*25 gene, and sensitivity of both LAMP assays has been shown to be higher than PCR (OHTSUKI et al., 2008; LIN et al., 2011). Recently, visual LAMP using a calcein metal indicator has also been developed (PAN et al., 2011), also *B. abortus* specific LAMP has been reported (KARTHIK et al., 2014a)

Though papers on the development of LAMP for *Brucella* spp. have recently been available (SOLEIMANI et al., 2013; PÉREZ-SANCHO et al., 2013), no study reports the screening of a good number of field samples from cattle, to find the potency and accuracy

of the developed LAMP in detecting *Brucella* pathogens. The present study reports the use of a rapid and visual LAMP assay for detecting *Brucella* from field samples, targeting the genus conserved *omp*25 gene. Loop primers were incorporated in our study to reduce the time taken for the reaction and SYBR green was used to visualize the results. DNA from whole blood samples was screened for *Brucella* spp. By the LAMP test and the results were compared with conventional PCR to evaluate the clinical specificity and sensitivity. The results obtained indicated that LAMP may be adopted as a promising field level diagnostic test for *Brucella* spp.

## Materials and methods

*Bacterial strains.* Two major species of *Brucella* commonly encountered in ruminants, namely *Brucella abortus* (*B. abortus* S99 - diagnostic reference strain and *B. abortus* S19 - vaccine strain), *Brucella melitensis* (field isolate and *B. melitensis* 16M- reference strain) and 11 other non-*Brucella* spp. (*Escherichia coli, Salmonella* Typhimurium, *Yersinia enterocolitica, Pasteurella multocida* B:2, *Clostridium chauvoei, Clostridium perfringens, Staphylococcus aureus* field isolate, *Listeria monocytogenes*, *Campylobacter jejuni* field isolate 1, *Campylobacter jejuni* field isolate 2 and *Shigella flexneri*) were selected to standardize the LAMP test, and to assess its specificity. The bacterial strains were obtained from repositories of different referral laboratories of the Indian Veterinary Research Institute, India.

*DNA template preparation.* Bacterial DNA of the four *Brucella* isolates was extracted using the RODRIGUEZ et al. (1997) standard protocol.

*Standardization and development of LAMP*

*LAMP primers.* LAMP primers targeting the well-conserved *Brucella* specific *omp*25 gene were designed using primer explorer online software, version 4. B3 and BIP primers were selected from LIN et al. (2011) and F3 and FIP were self-designed so as to generate

Table 1. LAMP primers used in the study

| S. No | Name | Sequence (5'-3') | Base pair length | Reference |
|---|---|---|---|---|
| 1 | F3 | ATCCAGGAACAGCCTCCG | 18 | Self- designed |
| 2 | B3 | GCATCACCTTCAACACCGTA | 20 | LIN et al. (2011) |
| 3 | FIP | TTGTTCCAGCCATAGCCAAGGTGGTTGAAGTA GCTCCCCA | 40 | Self- designed |
| 4 | BIP | CAGCACCGTTGGCAGCATCATCTGGTCCTGCT GGAAGTT | 39 | LIN et al. (2011) |
| 5 | LF | ATAGCCACCAGCCCAGCTATA | 21 | Self- designed |
| 6 | LR | AAGGCTGGCGCCTTTGCTG | 19 | Self- designed |

space for loop primers, and loop primers were also designed using the same software. The primers used in the study are highlighted in Table 1. LAMP primers were synthesized by Eurofins India Pvt Ltd.

*Optimization of LAMP reaction conditions.* LAMP conditions were adopted as those reported by LIN et al. (2011) and were standardized for time, temperature and for different chemicals. Reaction time with and without the use of loop primers was assessed so as to learn the influence of loop primers on the rapidity of LAMP.

In brief, the final reaction mixture for the LAMP contained 5 pM of each outer primer (F3 and B3), 40 pM of each inner primer (FIP and BIP) and 20 pM of each loop primer (LF and LB). Other components included of 2.5 µL of 10x Thermopol buffer [1x buffer comprised of 25 mM Tris- HCl pH 8.8, 12.5 mM KCl, 12 mM $(NH_4)_2SO_4$ and 0.125 % Tween 20 (New England Biolabs, USA)], 8 mM $MgSO_4$, 1.2 mM dNTPs, 1 M Betaine and the reaction mixture was adjusted to 22 µL with sterile nuclease free water (NFW). Then 2 µL of the standard *Brucella abortus* S99 (Reference diagnostic strain) DNA template was added, the mixture was heated to 95 °C for 5 min to denature the template, and reaction mixture was kept on ice to prevent re-annealing. *Bst* DNA polymerase (8 Units) was added to the reaction mixture, which was followed by incubation at 63 °C for 30 min. The reaction was terminated by heating at 95 °C for 5 min. Negative and positive controls were included in each run. Nuclease free water was used as the negative control, while DNA froma known standard strain (*B. abortus* S99) was used as the positive control.

*Analysis of LAMP products.* LAMP products were visualized directly for the formation of turbidity due to magnesium pyrophosphate formation during the course of the reaction. Gel electrophoresis (2 % agarose) was also carried out with 5 µL of LAMP products to visualize the ladder like pattern. The amplicons were digested with *Hha* I (Thermo Scientific, USA) using 5 µL of LAMP product at 37 °C for 3 hours. The LAMP products were added with 1 µL of SYBR green dye I (1000 X concentration, Sigma, USA) to visualize the results both in day and UV light. Hydroxy naphthol blue (HNB), at a concentration of 120 µM, was also used for some reactions.

*Analytical specificity of LAMP.* Specificity of LAMP primers was evaluated using the four *Brucella* strains and the eleven other non-*Brucella* strains mentioned earlier. Optimized reaction conditions were maintained during the course of specificity assessment.

*Analytical sensitivity of LAMP.* Analytical sensitivity of the LAMP test was examined by performing an optimized LAMP reaction with serially diluted (10 fold) *Brucella abortus* S99 DNA. The initial concentration of purified DNA, estimated using Nanodrop, was 700 ng/µL (Thermo Scientific, USA). The detection limit of LAMP with and without loop primers was observed as the positive reaction obtained at the lowest dilution of DNA.

*Applicability of LAMP*

*Field samples.* A total of 428 cattle whole blood samples were collected from three different states of India, namely: Uttar Pradesh (225 samples), Uttarakhand (103 samples) and Tamil Nadu (100 samples), where there was a history of abortion or *Brucella* infection. Simultaneously, 428 cattle serum samples were also collected from these animals for serological examination. In addition, ten aborted foetal stomach contents from cattle were collected from Uttar Pradesh. All the samples were collected from slaughter houses, organized and unorganized farms from Bareilly (125 samples), Mathura (100 samples) of Uttar Pradesh, Haridwar (65 samples) and Rishikesh (38 samples) of Uttarakhand, Salem (32 samples) and Coimbatore (68 samples) of Tamil Nadu. Out of the 438 samples, 285 samples were from adult female cattle which had calved at least once, 85 samples from heifers and 68 samples from bulls. The 438 samples comprising whole blood and aborted foetal stomach contents were subjected to the standardized LAMP testing for the presence of *Brucella* infection.

*DNA extraction.* DNA from the 428 cattle whole blood samples was extracted using modified protocol of SAMBROOK and RUSSELL (2001). DNA from the aborted foetal stomach contents (n = 10) was extracted using the RODRIGUEZ et al. (1997) method mentioned earlier.

*Screening of field samples by PCR and comparison with LAMP.* All the 438 field samples were subjected to PCR screening using *Brucella* genus specific primers (synthesized by *Eurofins India Pvt Ltd*.) targeting *bcsp*31 gene as per BAILY et al. (1992) in order to compare the PCR testing results with those of LAMP. The PCR was standardized with initial denaturation at 95 °C for 10 min, followed by 35 cycles of denaturation (94 °C for 1 min), annealing (55 °C for 1 min) and extension (72 °C for 1 min). Final extension was carried out at 72 °C for 7 min. The sensitivity limit of the PCR test was also tested using standard *B. abortus* S99 DNA and the results compared with those of LAMP.

*Serological examination.* Serum samples (n = 428) were subjected to serological testing for *Brucella* antibodies employing Rose Bengal Plate Test (RBPT) and Standard Tube Agglutination Test (STAT) according to the standard protocols described by ALTON et al. (1975).

*Statistical analysis.* DNA from whole blood samples were used for testing using LAMP and PCR. The results of LAMP, PCR, RBPT and STAT were analysed with Kappa statistics, using GraphPad prism V4 software. The clinical sensitivity and specificity of the LAMP test were determined keeping RBPT and STAT as the standard test.

**Results**

*Optimization of LAMP reaction conditions.* LAMP reaction conditions were standardized for discovering the optimum temperature, time, dNTP concentration, $MgSO_4$

and Betaine. Conditions were optimized by considering the band visibility in agarose gel electrophoresis. Reaction time with and without the use of loop primers was assessed to discover the influence of loop primers on the rapidity of LAMP. For reaction time without the loop primers, amplification was noticed at 60 min, while with loop primers it was noticed at 30 min (Fig. 1).

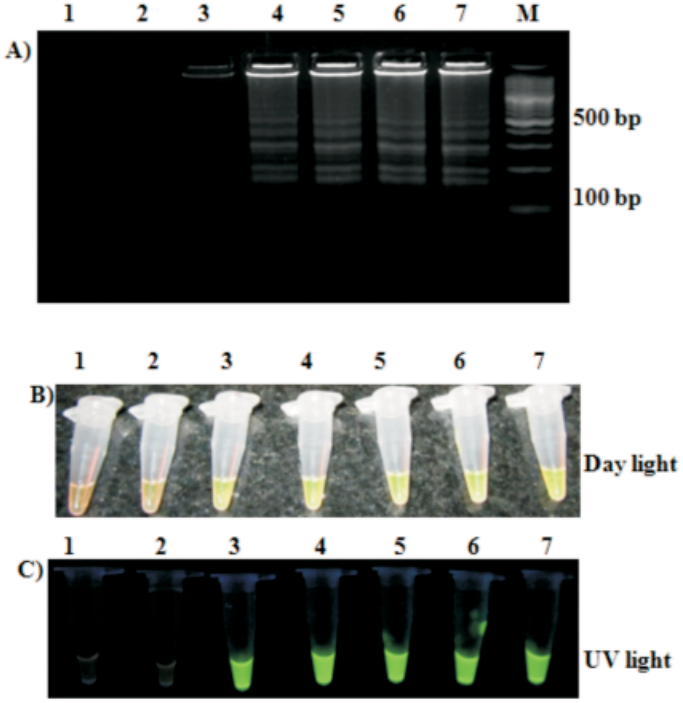


Fig. 1. Effect of time on LAMP with loop primers. A) Resolution of LAMP products in agarose gel. Lane 1 to 7- 10, 20, 30, 40, 50 and 60 min. M- 100 bp ladder. B) SYBR green based detection under day light. Lane 1- negative control, Lane 2 to 7 - 10, 20, 30, 40, 50 and 60 min. C) SYBR green based detection under UV light. Lane 1 - negative control, Lane 2 to 6 - 10, 20, 30, 40, 50 and 60 min.

*Analysis and visualization of LAMP products.* The visual LAMP assay results can be seen directly in day light (Positive- green and Negative- orange colour) and in UV light (Positive- green fluorescence and Negative- orange fluorescence). With HNB dye, a positive reaction showed a sky blue colour and a negative reaction showed a violet colour, but on some occasions there was intermediate colour production between sky blue and violet, which hindered result interpretation. Hence SYBR green was employed

for further reactions and visualization of LAMP reaction. RE digestion of the LAMP products yielded products corresponding to theoretical calculation.

Table 2. Epidemiological data of whole blood samples screened for bovine brucellosis by LAMP assay

| S. No | Origin of sample | | Number of samples | Sample positive by LAMP | Prevalence (%) | |
|---|---|---|---|---|---|---|
| | State | Place | | | Place wise | State wise |
| 1 | Uttar Pradesh | Bareilly | 125 | 21 | 16.8 | Uttar Pradesh- 14.7 |
| | | Mathura | 100 | 12 | 12 | |
| 2 | Uttarakhand | Haridwar | 65 | 9 | 13.8 | Uttarakhand- 14.56 |
| | | Rishikesh | 38 | 6 | 15.7 | |
| 3 | Tamil Nadu | Salem | 32 | 5 | 15.6 | Tamil Nadu- 11 |
| | | Coimbatore | 68 | 10 | 14.7 | |
| | | Total | 428 | 63 | 14.7 | |

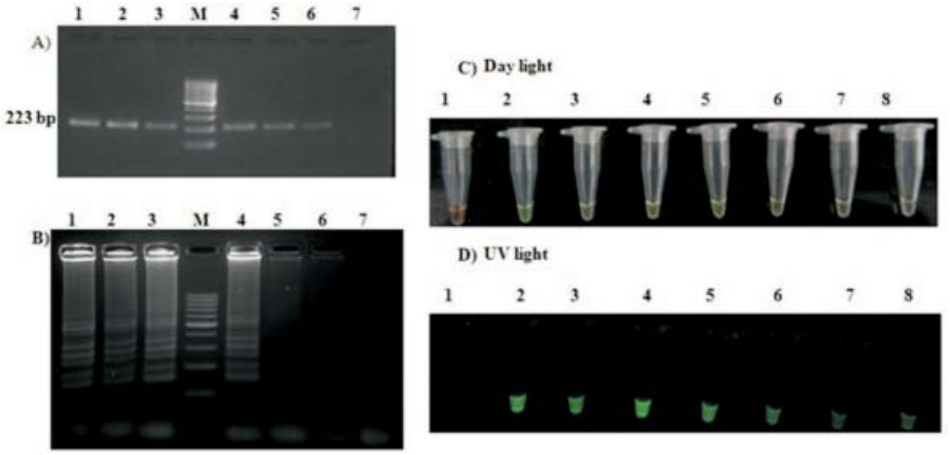


Fig. 2. Analytical sensitivity of LAMP (without loop primers) and PCR.. A) Resolution of PCR amplicons in agarose gel. Lane 1 to 7 - 700 ng/µL DNA, 70 ng, 7 ng, 700 pg, 70 pg, 7pg and negative control. B) Resolution of LAMP products (without loop primers). Lane 1 to 7 - 700 ng/µL DNA, 70 ng, 7 ng, 700 pg, 70 pg, 7 pg and negative control. M - 100 bp ladder. C) and D) SYBR green based detection under day light and UV light respectively. Lane 1- negative control, Lane 2 to 8 - 700 ng/µL DNA, 70 ng, 7 ng, 700 pg, 70 pg, 7 pg and 700 fg.

*Analytical specificity and sensitivity.* The LAMP primers only specifically amplified the four *Brucella* DNA and no amplification was observed with the other eleven bacterial DNA and negative controls. LAMP could detect up to 70 pg/µL of DNA without loop

primers (Fig. 2) and 700 fg/µL with loop primers (Fig. 3). PCR could detect only up to 7 pg/µL (Fig. 2). Hence, by employing loop primers, LAMP could detect DNA 10 fold more than PCR.

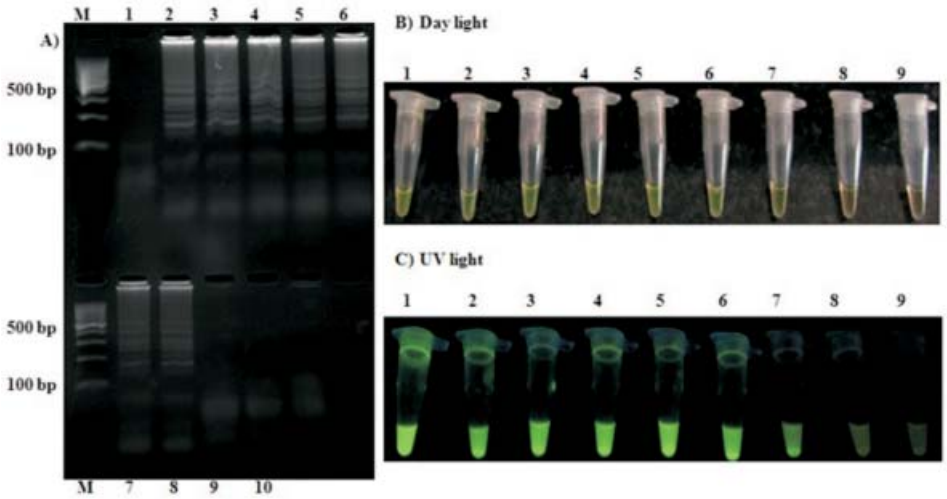


Fig. 3. Analytical sensitivity of LAMP with loop primers. A) Resolution of LAMP products (without loop primers). Lane 1 - negative control, Lane 2 to 10 - 700 ng/µL DNA, 70 ng, 7 ng, 700 pg, 70 pg, 7 pg, 700 fg, 70 fg and 7 fg. M - 100 bp ladder. B) and C) SYBR green based detection under day light and UV light respectively. Lane 1 to 8 - 700 ng/µL DNA, 70 ng, 7 ng, 700 pg, 70 pg, 7 pg, 700 fg, 70 fg and Lane 9 - negative control.

*Comparative evaluation of LAMP, PCR, RBPT and STAT for Brucella.* The results of the screening of field samples of cattle origin for the presence of *Brucella* spp., applying different diagnostic tests (LAMP, PCR, RBPT and STAT), revealed that out of the 428 cattle whole blood samples, 63 (14.7 %) samples were positive by LAMP of which only 60 (14.0 %) samples were positive by PCR. Testing of the aborted foetal stomach content samples showed 5 (50 %) samples to be positive by both PCR and LAMP. Out of the 428 serum samples, 66 (15.4 %) samples were positive by RBPT, of which 65 (15.2 %) samples were positive by STAT. There was homogeneity between the serum samples, which were serology positive and the whole blood samples, which were LAMP and PCR positive. The epidemiological data of samples screened by LAMP for Brucella is given in Table 2. In the present study, out of the 63 positive samples recorded by LAMP, 42 were from adult cattle which had calved at least once and the remaining positive samples were from heifers. Unorganized dairy farms showed more LAMP positivity, with 45 samples, when compared with organized farms. The clinical specificity of LAMP was 100 % and

sensitivity was 95.45 %, when compared to RBPT and 96.92 % when compared with STAT. Since PCR detected 3 samples fewer than LAMP, sensitivity and specificity were not calculated for LAMP in comparison with PCR. Statistical analyses of the comparative results with the different diagnostic tests are given in Table 3.

Table 3. Statistical analysis for clinical samples

| Tests | Clinical specificity | Clinical sensitivity | Accuracy | Positive predictive value | Negative predictive value | Kappa value | P value |
|---|---|---|---|---|---|---|---|
| LAMP Vs RBPT | 100 % | 95.45 % | 99.29 % | 100 % | 99.18 % | 0.973 | 0.0001 |
| LAMP Vs STAT | 100 % | 96.92 % | 99.53 % | 100 % | 99.45 % | 0.982 | 0.0001 |

LAMP- Loop mediated isothermal test; RBPT- Rose bengal plate test; STAT- Standard tube agglutination test

**Discussion**

LAMP assay, developed a decade ago by NOTOMI et al. (2000), assured that diagnosis can be made accurately as well as swiftly. At present LAMP has been designed for numerous microorganisms (PARIDA et al., 2005; GEOJITH et al., 2011; XU et al., 2012). There are reports about the development of LAMP test for *Brucella* spp. (OHTSUKI et al., 2008; LIN et al., 2011; PAN et al., 2011; SONG et al., 2012), but there are no reports on completion of the LAMP reaction in 30 min for detecting *Brucella* spp. and also there are no reports of the use of SYBR green for visual detection of *Brucella* spp.

In this study, SYBR green dye was found to be superior as the intensity and clarity of colour was much more than that of HNB. Moreover, HNB occasionally produced intermediate colours (CHEN et al., 2012). Completion of the LAMP reaction has been reported to be 60 minutes in earlier studies (LIN et al., 2011), but in the present study it could be achieved in 30 minutes, with the help of loop primers, indicating that loop primers accelerated the reaction (NAGAMINE et al., 2002). Thus, with this LAMP, Brucellosis may be detected within a span of 30 min, which is helpful in the accurate and early diagnosis of Brucellosis. The LAMP primers designed were highly specific to *Brucella* spp. and not to other bacterial spp.

In this study, the detection limit of *Brucella* DNA using LAMP was 70 pg/µL (without loop primers) and 700 fg/µL (with loop primers), and that of PCR was 7 pg/µL. Hence, loop primers improved the sensitivity of LAMP reaction 10 fold compared to PCR. Similar findings were reported by LIN et al. (2011). Designing of the primers is the critical area of LAMP to ensure good specificity and sensitivity. On rare occasions

false positives were obtained in negative controls, which may be attributed to carry over contamination (PARIDA et al., 2008; DHAMA et al., 2013; KARTHIK et al., 2014b).

The results of the samples screened showed that LAMP could detect 3 positive samples more than PCR, but RBPT could detect 3 more positive samples than LAMP. However, serological tests are not always specific and may cross react with *Yersinia enterocolitica* O:9 (GARIN-BASTUJI et al., 1999; MUÑOZ et al., 2005; KARTHIK et al., 2014c). Similarly, serological tests detect antibodies in the chronic stages also, but the organism lodges itself in lymph nodes, mammary gland, uterus etc., hence DNA could not always be recovered from whole blood (LEARY et al., 2006). β-actin primers (JOSEPH et al., 2010) were used as internal control during PCR reactions to rule out the chance of PCR inhibitors. Out of the 3 LAMP positive samples, which were PCR negative, one sample did not produce amplification with β-actin primers and hence that one sample may have had a PCR inhibitor, which led to the negative result on PCR. Cattle whole blood samples were processed by conventional methods for DNA extraction and hence PCR inhibitors may exist, but the LAMP test is a sturdy assay as the enzyme *Bst* polymerase is not inhibited by inhibitors, which is an added credit to the list of advantages of LAMP (PARIDA et al., 2008). Isolation and identification of the organism was attempted in the initial stages of the study without fruitful results, as whole blood is not an appropriate sample for isolation. Conventional PCR is commonly employed as a molecular tool for detection of brucellosis and hence attempts were made to compare LAMP with conventional PCR, rather than the more sophisticated real time PCR. The clinical sensitivity, specificity and accuracy of the diagnostic test were all found to be almost 100 % and hence the LAMP test can be exploited as a useful diagnostic tool for screening of brucellosis in field conditions, with wider applicability worldwide. Kappa values showed that there is almost perfect agreement between the different tests used in this study. The LAMP assay showed promising results when used for epidemiological screening of brucellosis in cattle and this study also revealed the prevalence of bovine brucellosis in different parts of India. If this LAMP reaction mixture is lyophilized and marketed, it may be used as a field test to detect *Brucella* spp. visually within 30 min, which would help to study the molecular epidemiology of this important zoonotic pathogen.

## References

ALTON, G., L. M. JONES, R. D. ANGUS, J. M. VERGER (1988): Techniques for the Brucellosis Laboratory. INRA, Paris.

ALTON, G. G., L. M. JONES, A. PIETZ (1975): Laboratory Techniques in Brucellosis. World Health Organization, Geneva.

BAILY, G. C., J. B. KRAAHN, B. S. DRASAR, N. G. STOKEER (1992): Detection of *Brucella melitensis* and *Brucella abortus* by DNA amplification. J. Trop. Med. Hyg. 95, 271- 275.

CHAND, P., A. K. SHARMA (2004): Situation of brucellosis in bovines at organized cattle farms belonging to three different states. J. Immunol. Immunopathol. 6, 11-15.

CHEN, X., X. WANG, N. JIN, Y. ZHOU, S. HUANG, Q. MIAO, Q. ZHU, J. XU (2012): Endpoint visual detection of three genetically modified rice events by loop-mediated isothermal amplification. Int. J. Mol. Sci. 13, 14421-14433.

DHAMA, K., K. KARTHIK, S. CHAKRABORTY, R. TIWARI, S. KAPOOR, A. KUMAR, P. THOMAS (2013): Loop-mediated isothermal amplification of DNA (LAMP) - a new diagnostic tool lights the world of diagnosis of animal and human pathogens: a review. Pak. J. Biol. Sci. 17, 151-166.

GARIN-BASTUJI, B., N. HUMMEL, G. GERBIER, C. CAU, R. POUILLOT, M. DA COSTA, J. J. FONTAINE (1999): Non specific serological reactions in the diagnosis of bovine brucellosis: experimental oral infection of cattle with repeated doses of *Yersinia enterocolitica* O:9. Vet. Microbiol. 66, 223-233.

GEOJITH, G., S. DHANASEKARAN, S. P. CHANDRAN, J. KENNETH (2011): Efficacy of Loop Mediated Isothermal Amplification (LAMP) assay for the laboratory identification of *Mycobacterium tuberculosis* isolates in a resource limited setting. J. Microbiol. Methods 84, 71-73.

HABTAMU, T. T., R. RATHORE, K. DHAMA, K. KARTHIK (2013): Isolation and molecular detection of *Brucella melitensis* from natural disease outbreak in sheep and *Brucella abortus* from organized cattle farm by *IS*711 and *omp*2a gene based PCR. Int. J. Curr. Res. 5, 1920-1925.

JOSEPH, S. J., S. L. PRATT, E. PAVAN, R. REKAYA, S. K. DUCKETT (2010): Omega-6 fat supplementation alters lipogenic gene expression in bovine subcutaneous adipose tissue. Gene Regul. Syst. Bio. 4, 91-100.

KARTHIK, K., R. RATHORE, P. THOMAS, T. R. ARUN, K. N. VISWAS, R. K. AGARWAL, H. V. MANJUNATHACHAR, K. DHAMA (2014a): Loop-mediated isothermal amplification (LAMP) test for specific and rapid detection of *Brucella abortus* in cattle. Vet. Q. 34, 174-179.

KARTHIK, K., R. RATHORE, P. THOMAS, T. R. ARUN, K. N. VISWAS, K. DHAMA, R. K. AGARWAL (2014b): New closed tube loop mediated isothermal amplification assay for prevention of product cross contamination. MethodsX. 1, e137-e143.

KARTHIK, K., R. RATHORE, P. THOMAS, A. ELAMURUGAN, T. R. ARUN, K. DHAMA (2014c): Serological and molecular detection of *Brucella abortus* from cattle by RBPT, STAT and PCR, and sample suitability of whole blood for PCR. Asian J. Anim. Vet. Adv. 9, 262-269.

LEARY, S. O., M. SHEAHAN, T. SWEENEY (2006): *Brucella abortus* detection by PCR assay in blood, milk and lymph tissue of serologically positive cows. Res. Vet. Sci. 81, 170-176.

LIN, G. Z., F. Y. ZHENG, J. Z. ZHOU, X. W. GONG, G. H. WANG, X. A. CAO, C. Q. QIU (2011): Loop-mediated isothermal amplification assay targeting the omp25 gene for rapid detection of *Brucella* spp. Mol. Cell. Probes 25, 126-129.

MUÑOZ, P. M., C. M. MARÍN, D. MONREAL, D. GONZÁLEZ, B. GARIN-BASTUJI, R. DÍAZ, R. C. MAINAR-JAIME, I. MORIYÓN, J. M. BLASCO (2005): Efficacy of several

serological tests and antigens for diagnosis of bovine brucellosis in the presence of false-positive serological results due to *Yersinia enterocolitica* O:9. Clin. Diagn. Lab. Immunol. 12, 141-151.

NAGAMINE, K., T. HASE, T. NOTOMI (2002): Accelerated reaction by loop-mediated isothermal amplification using loop primers. Mol. Cell. Probes 16, 223-229.

NOTOMI, T., H. OKAYAMA, H. MASUBUCHI, T. YONEKAWA, K. WATANABE, N. AMINO, T. HASE (2000): Loop-mediated isothermal amplification of DNA. Nucleic Acids Res. 28, e63.

OHTSUKI, R., K. KAWAMOTO, Y. KATO, M. M. SHAH, T. EZAKI, S. I. MAKINO (2008): Rapid detection of *Brucella* spp. by the loop mediated isothermal amplification method. J. Appl. Microbiol. 104, 1815-1823.

PAN, W., J. Y. WANG, H. Y. SHEN, M. Q. ZHAO, C. M. JU, X. Y. DONG, L. YI, J. D. CHEN (2011): Development and application of the novel visual loop mediated isothermal amplification of *omp25* sequence for rapid detection of *Brucella* spp. J. Anim. Vet. Adv. 10, 2120-2126.

PARIDA, M., K. HORIOKE, H. ISHIDA, P. K. DASH, P. SAXENA, A. M. JANA (2005): Rapid detection and differentiation of dengue virus serotypes by a real-time reverse transcription-loop-mediated isothermal amplification assay. J. Clin. Microbiol. 43, 2895-2903.

PARIDA, M., S. SANNARANGAIAH, P. K. DASH, P. V. L. RAO, K. MORITA (2008): Loop mediated isothermal amplification (LAMP): a new generation of innovative gene amplification technique; perspectives in clinical diagnosis of infectious diseases. Rev. Med. Virol. 18, 407-421.

PÉREZ-SANCHO, M., T. GARCÍA-SECO, L. ARROGANTE, N. GARCÍA, I. MARTÍNEZ, A. DIEZ-GUERRIER, A. PERALES, J. GOYACHE, L. DOMÍNGUEZ, J. ALVAREZ (2013): Development and evaluation of an IS711-based loop mediated isothermal amplification method (LAMP) for detection of *Brucella* spp. on clinical samples. Res. Vet. Sci. 95, 489-494.

PIKE, R. M., S. E. SULKIN, M. L. SCHULZE (1965): Continuing importance of laboratory-acquired infections. Am. J. Public Health Nations Health 5, 190-199.

RODRIGUEZ, J. C., E. FUENTES, G. ROYO (1997): Comparison of two different PCR detection methods. Application to the diagnosis of pulmonary tuberculosis. APMIS. 105, 612-616.

SAMBROOK, J., D. W. RUSSELL (2001): Molecular cloning-A laboratory manual. Cold Spring Harbour Laboratories press, Cold Spring Harbour, New York.

SOLEIMANI, M., S. SHAMS, A. K. MAJIDZADEH (2013): Developing a real-time quantitative loop-mediated isothermal amplification assay as a rapid and accurate method for detection of Brucellosis. J. Appl. Microbiol. 115, 828-834.

SONG, L., J. LI, S. HOU, X. LI, S. CHEN (2012): Establishment of loop-mediated isothermal amplification (LAMP) for rapid detection of *Brucella* spp. and application to milk and blood samples. J. Microbiol. Methods 90, 292-297.

XU, Z., L. LI, J. CHU, B. M. PETERS, M. L. HARRIS, B. LI, L. SHI, M. E. SHIRTLIFF (2012): Development and application of loop-mediated isothermal amplification assays on rapid detection of various types of *Staphylococci* strains. Food. Res. Int. 47, 166-173.

YU, W. L., K. NIELSEN (2010): Review of detection of *Brucella* spp. by polymerase chain reaction. Croat. Med. J. 51, 306-313.

Received: 17 December 2014
Accepted: 9 June 2015

**KARTHIK, K., R. RATHORE, P. THOMAS, K. N. VISWAS, R. K. AGARWAL, V. REKHA, R. V. JAGAPUR, K. DHAMA: Brzo i vidljivo petljom posredovano izotermno umnažanje za dokazivanje *Brucella* spp. i njegova primjena u epidemiologiji bruceloze goveda. Vet. arhiv 86, 35-47, 2016.**

**SAŽETAK**

Bruceloza je razorna bolest kad se u stadu pojavi u obliku pobačaja što iziskuje brzo postavljanje dijagnoze. Upotreba metode nazvane „petljom posredovano izotermno umnažanje“ (engl. loop mediated isothermal amplification, LAMP)“ omogućila je poboljšanje dijagnostike svojom jednostavnošću i brzinom, a prednost joj je da se može izvesti na postojanoj temperaturi pa se može primijeniti u terenskim uvjetima. U dosada razvijenim postupcima LAMP-a nije bio rabljen vizualni dokaz i nije se uspjelo skratiti vrijeme reakcije. U ovom istraživanju pokušalo se ukloniti te nedostatke razvitkom brzog postupka za vizualni dokaz *Brucella* spp., za što je potrebno samo 30 minuta i SYBR zeleno. LAMP test za dokaz gena *omp*25 pokazao se visoko osjetljivim i specifičnim za dokaz *Brucella* spp. Pretraga 438 terenskih uzoraka podrijetlom od goveda pokazala je da je LAMP osjetljiviji od raspoloživih testova PCR-a. Tim je testom dokazana prevalencija bruceloze od 14,7 % u različitim dijelovima Indije. Kao vrlo osjetljiv, specifičan i brz, LAMP test se može naširoko upotrijebiti u epidemiološkim istraživanjima bruceloze.

**Ključne riječi:** petljom posredovano izotermno umnažanje, brucele, početnice, *omp*25, SYBR zeleno