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# DEVELOPMENT AND APPLICATION OF RAPID XTREME CHAIN REACTION AND LOOP-MEDIATED ISOTHERMAL AMPLIFICATION ASSAYS FOR THE DETECTION OF LEUKAEMIA AND BRUCELLOSIS OF CATTLE

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**ABSTRACT**

**Infection of cattle with bovine leukaemia virus (BLV) and *Brucella* spp. result in economic losses due to reduced productivity, reduced milk production, and early culling. Furthermore, BLV and *Brucella* spp. can potentially infect humans. This study was conducted to develop Xtreme chain reaction (XCR) and loop-mediated isothermal amplification (LAMP) assays for the rapid and efficient detection of leukaemia and *Brucella* spp. pathogen infections in cattle. *Brucella* spp. XCR and LAMP assays targeted the host specific antigen gene and IS711 repeats from the transposase gene. To detect BLV proviral DNA, the BLV long terminal repeat region and the *Env* gene were used to develop XCR and LAMP assays. The results of LAMP assays applied to field samples were compared with those of XCR/LAMP and serological tests for BLV and *Brucella* spp. The results of the XCR and LAMP assays showed a high level of agreement with those of serological methods, and accurately detected the target sequences with no cross-reactions observed. Therefore, the XCR and LAMP assays described here are highly sensitive and specific tests to detect and differentiate between BLV and *Brucella* spp. and could help with the detection of infection in the early stages.**

**Keywords: leukaemia, brucellosis, polymerase, LAMP, XCR, genome, diagnosis.**

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## INTRODUCTION

Leukaemia of cattle is a chronic infectious disease of agricultural animals with viral etiology. The virus of leukaemia of cattle (BLV) is an infectious agent classified according to a number of signs into the genus *Deltaretrovirus* of the family *Retroviridae*. Bovine leukaemia virus is the etiological agent of enzootic bovine leukosis, a disease characterized by a highly extended course that often involves persistent lymphocytosis and culminates in B-cell lymphomas. The greatest danger is represented by animals infected with the virus and which is asymptomatic is its carrier. In 30% of cases, the virus circulates persistently and subsequently causes leukocytosis. Uncontrolled development of the virus leads to the spread of infection among livestock, which causes great economic damage to cattle breeding. Recent was detected of BLV in human breast tissue, and determination of its significant association with breast cancer in a US population [1]. World statistics show the geographical correlation between breast cancer incidence and milk consumption[2].

*Brucellae* are Gram-negative, facultative intracellular bacteria whose infections and the transmission patterns at the wildlife/livestock/human interface is of paramount importance before implementing any brucellosis control or eradication program in animals[3, 4]. Main species are recognized within the genus *Brucella*: *B.abortus*, *B.melitensis*, *B.suis*, *B.ovis*, *B.canis* and *B.neotomae* is based on differences in pathogenicity and host preference. Human brucellosis remains the commonest zoonotic disease worldwide and the infection acquire through direct contact with infected animals, by eating or drinking contaminated animal products. For preventing human brucellosis is the control and elimination of the infection in animals and the pasteurization of milk[1].

There are various techniques for detecting bovine leukaemia virus and *Brucella* infections. The polymerase chain reaction (PCR) have been used for detection of the pathogen, agar gel immunodiffusion (AGID), indirect hemagglutination (IHA), enzyme-linked immunosorbent assay (ELISA), radioimmunoassay, the indirect fluorescent antibody test and syncytium-inhibition test for antibody detection[5]. By these serological tests are not sufficient to identify cattle during the early stages of infection, especially BLV

infection that is chronic and persistent. Proviral BLV DNA is found in peripheral blood leukocytes and it can be detected by PCR.

XCR is more efficient than traditional PCR methods due to the way in which assay design and thermal amplification profile are approached. XCR uses explicitly designed denature temperatures to minimize the possibility of amplification primers binding to non-target regions, that makes the amplification more efficient from a time perspective, compared to PCR's thermal cycling need to traverse the full traditional range of ~40°C. The efficiency of the XCR reaction itself is enhanced by reducing the formation of non-specific product to focus on the formation of the desired target amplicons. Amplifying the target sequence within a 15°C temperature range that is defined by the area contained within the overlap of an annealing temperature of the pair of oligonucleotide primers and the denaturation of the target nucleic acid sequence, wherein each forward and reverse oligonucleotide primer has a melting temperature ($T_m$) within 15°C of the Tm of the target nucleic acid sequence.

Loop-mediated isothermal amplification (LAMP) is nucleic acid amplification method that relies on autocycling strand displacement DNA synthesis performed by the *Bst* DNA polymerase large fragment and a set of 4 or 6 especially designed nested primers that recognize a total of 6 or 8 distinct sequences of the target. LAMP was first described by Notomi et al. in 2000[6] is technique for rapid amplification target nucleic acids with high sensitivity, specificity, efficiency under isothermal conditions. In recent years, LAMP has become the most widely explored nucleic acid detection strategy, and is characterized by high specificity, efficiency, and speed. It has been used for the identification of various organisms such as viruses and bacteria [5; 7; 8]. A characteristic feature of LAMP is the design of 6 nested primers using 8 target DNA/RNA sequences, followed by a substitution reaction and recycling chain reaction at anisothermal temperature. The reaction begins immediately after mixing the templates, primers, *Bst* DNA polymerase, and matrix at isothermal temperature (60-65°C). The LAMP amplification efficiency is very high, and $10^9$-$10^{10}$-fold amplification can be achieved in 20 to 45 min.

Compared to conventional PCR, XCR and LAMP assays are more specific and sensitive and are less labor intensive.

The aim of this study was to develop specific XCR and LAMP assays for rapid detection and evaluate the virus of leukaemia of cattle and *Brucella* spp. infections based on specificity and sensitivity.

## METHODS AND MATERIALS

### Samples and DNA extraction

DNA was extracted from EDTA-treated blood from cattle using the QIAmp DNA mini-prep kit (Qiagen, Valencia, CA) according to the manufacturer's instructions or genomic DNA was extracted from samples of these genotypes using a modification of the CTAB extraction protocol (http://primerdigital.com/dna.html) with RNAse A treatment. The DNA samples were diluted in 1×TE buffer and the DNA quality was checked electrophoretically and spectrophotometrically with a Nanodrop apparatus (Thermo Fisher Scientific Inc.) and stored at -20°C.

### Primer design

Primers targeting conservative regions of the of LTR and *Env* gene are used to generate short amplicons that are universal in all virus of leukaemia of cattle (AB987702).All sequences of the leukaemia virus of cattle from the NCBI genebank (https://www.ncbi.nlm.nih.gov/) were first collected, then the LTR and Env gene sequences were aligned using the MUSCLE program for multiple alignment (http://www.ebi.ac.uk/Tools/msa/muscle/).

To the conservative sequence regions of the leukaemia virus of cattle, universal primers were designed, which should universally bind to any sequence of the bovine leukaemia virus.

An analysis of the whole genomes of *Brucella abortus* bacteria was carried out and the following sequences were selected: *host specificity protein* (CP008774, AE008917, CP016985, CP019679, CP008750, CP007789, CP017012, LT605585, CP019390, CP019347, LT599047, LN998033) and multicopy (up to 6 copies per chromosome) IS711repeat for *transposase* gene (CP001578,CP002079, CP003174, CP006896, CP007663, CP007681, CP007700, CP007707, CP007709, CP007763, CP008774, CP009625, CP013963, CP016973, CP016977, CP016981), representing a fairly conserved regions of the chromosomal DNA found in all bacteria of the genus *Brucella*.

For each target sequences, the sequence accessions were aligned, and the conservation was assessed with the multiple alignment procedure of MUSCLE.

The conserved segments of the target sequences were used for the design of oligonucleotide primers, which was carried out with the program FastPCR (http://primerdigital.com/fastpcr.html)[9]. The sequences of the primers are shown in Table 1,2. None of the primer chosen form dimers, and all showed high

amplification efficiency and very effective for XCR and LAMP applications. The chosen primers match motifs sufficiently conserved in the target sequences to allow amplification of almost all pathogens targets.

Using this program, compatibility primers were analysed, the main parameters of the primers were calculated, and compatible pairs for multiplex DNA amplification were selected. The conserved primers are shown in table 1,2.

**Xtreme Chain Reaction**

XCR primer sets (table 1) were designed according to the instructions (https://fluoresentric.com/principal-of-xcr/) with FastPCR software (http://primerdigital.com/fastpcr.html)[9].

The XCR assays were performed in 25 μl reactions containing 20 ng DNA template, 1x DreamTaq buffer, 200 mM dNTP, 400 nM each primer and 1 U DreamTaq polymerase (Thermo Fisher Scientific Inc.).

PCR reactions were performed on a PCR system (T100 Thermal Cycler, Bio-Rad). The PCR protocol consisted of an initial denaturation at 80-85°C for 1 minutes followed by 40 cycles of 85°C for 10 seconds and 70-75°C for 10 seconds.

Each XCR primer combinations was tested singularly in PCR reactions using DNA mixture composed of non- and infected samples. The DNA amplification products were separated by electrophoresis at 70 V for 1 hours in a 1.5% agarose gel with 1× TBE electrophoresis buffer. Gels were stained with EtBr and scanned using a Molecular Imager PharosFX™ Plus System (Bio-Rad) with a resolution of 50 μm.

**LAMP primer set design**

The primers for LAMP assays were designed according to the instructions (http://loopamp.eiken.co.jp/e/lamp/primer.html) with software PrimerExplorer V5 (http://primerexplorer.jp/lampv5e/) and FastPCR (http://primerdigital.com/fastpcr.html), in terms of the distance between primers, Tm value for primer regions, GC% contents, Linguistic Complexity (%) and the stability of primer end and secondary structure of primers. Primer sequences are listed in table 2.

LAMP assays were performed in a total of 25 μl mixture containing 20 ng DNA template, 1.6 μM (each) of the primers FIP and BIP, 0.2 μM (each) of the primers F3 and B3, 0.8 μM (each) of the primers LF and LB, 1.2 mM deoxynucleotide triphosphates, 6mM $MgSO_4$, 1 M betaine, 1x ThermoPol buffer, 8 U *Bst*DNA polymerase (New England BioLabs).

The reaction was amplified for 30-45 minutes at 65°C and was terminated by heating at 80°C for 2 minutes.

For a visual inspection of the LAMP assay products, fluorescence reagents (25 mMcalcein with 0.5 mM $MnCl_2$) were added to the reaction mixture before amplification and a color change of the reaction mixture was noted upon successful amplification. Samples that turned green were considered positive, while samples that remained orange were considered negative. The presence of a multiple bands pattern with different molecular weights indicated a positive result.

**Table 1.**The sequences of XCR primers designed in this study

| ID | Primer/Probe Sequence (5'→3') | Type | Location | nt | Tm (°C) | CG (%) | LC (%) | PQ(%) |
|---|---|---|---|---|---|---|---|---|
| *Brucella* spp. Host specificity protein<br>5224-5226: 649 bp<br>5225-5226: 202 bp | | | | | | | | |
| 5224 | ACTGATCTGGGCGACACGCTTTGAAGAGCGCA | F | 234→265 | 32 | 75.4 | 56.3 | 86 | 75 |
| 5225 | ACCCGTACCCGGCGAAAAACGATGGATCAACC | F | 681→712 | 32 | 74.8 | 56.3 | 76 | 76 |
| 5226 | CGGCTTGCGGACAGAGAGGCTCGTAACACC | R | 853←882 | 30 | 75.6 | 63.3 | 85 | 85 |
| *Brucella* spp. IS711transposase gene<br>5231-5233: 127 bp<br>5231-5234: 149 bp<br>5232-5233: 437 bp<br>5232-5234: 459 bp | | | | | | | | |
| 5231 | CAACACCCGGCCATTATGGTGACTGTCCGCAA | F | 496→527 | 32 | 74.9 | 56.3 | 94 | 88 |
| 5232 | CATCTTGTGGATGGCTGCCAATGCAGCGCACT | F | 186→217 | 32 | 75.7 | 56.3 | 90 | 80 |
| 5233 | TYGCTGGCAATGAAGGCCCTTAAGTGATCGGCAT | R | 589←622 | 34 | 74.3 | 51.5 | 87 | 82 |
| 5234 | GATCTGAGCCGTTGCCTTGAGATYGCTGGCAA | R | 613←644 | 32 | 74.1 | 54.7 | 82 | 77 |
| BLV LTR region (AB987702)<br>5239-5240: 113bp | | | | | | | | |
| 5239 | GCCCTGTCGAGTTAGCGGCACCAGAAGCGTT | F | 189→219,<br>8378→8408 | 31 | 76.4 | 61.3 | 92 | 87 |
| 5240 | CCGCTGCCGGATAGCCGACCAGAAGGTCT | R | 273←301,<br>8462←8490 | 29 | 76.1 | 65.5 | 91 | 79 |
| BLV envelope gene (AB987702) | | | | | | | | |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 5245-5247: 275 bp | | | | | | | | |
| 5245 | TCTGTCRGCCCATCCAGACTTGGAGATGCTCC | F | 4905→4936 | 32 | 74.2 | 57.8 | 90 | 84 |
| 5246 | CCCCACATAAGGGCATCGGGGCTCGCAATCA | R | 5149←5179 | 31 | 76.3 | 61.3 | 76 | 76 |
| LC – Linguistic Complexity (%); PQ – Primer Quality (%); Tm – melting temperature calculated for 1 mM $Mg^{2+}$ and 0.25 µM primer concentration. | | | | | | | | |

**Table 2.**The sequences of primers designed for LAMP assays

| ID | Primer/Probe Sequence (5'-3') | Type | Location | nt | Tm (°C) | CG (%) | LC (%) | PQ (%) |
|---|---|---|---|---|---|---|---|---|
| *Brucella* spp. Host specificity protein 5227-5228: 174 bp | | | | | | | | |
| 5227 | CACTTCCCTTCATTTATGGCAC | F3 | 194→215 | 22 | 60.2 | 45.5 | 80 | 75 |
| 5228 | TGCCAGCAATCTCGCCTT | B3 | 350←367 | 18 | 61.8 | 55.6 | 83 | 83 |
| 5229 | TCTTCAAAGCGTGTCGCCGGTTTCCGGCACACTGAT | FIP | 204→239 | 36 | 77.3 | 55.6 | 91 | 87 |
| 5230 | TGGCCCCAAAGTCACCAACCGCATTGCCGAAATAGCT | BIP | 316←352 | 37 | 77.4 | 54.1 | 87 | 87 |
| *Brucella* spp. IS711transposase gene 5235-5236: 182 bp | | | | | | | | |
| 5235 | ACGAAGCCTTACAGATGAGCAA | F3 | 81→102 | 22 | 61.8 | 45.5 | 90 | 80 |
| 5236 | CATGAACCGCTGTCCATTTGC | B3 | 242←262 | 21 | 63.5 | 52.4 | 90 | 84 |
| 5237 | ATCGGTTATCRACGCCACTGGGAACCGGATCGAAGCATATCT | FIP | 84→125 | 42 | 76.4 | 51.2 | 83 | 77 |
| 5238 | TGTCGACGCCATCTTGTGGCGAAGGTCGCAGGCAGAT | BIP | 224←260 | 37 | 79.0 | 59.5 | 82 | 75 |
| BLV LTR region (AB987702) 5241-5242: 157 bp | | | | | | | | |
| 5241 | CGGCCCTGTCGAGTTAGC | F3 | 187→204, 8376→8393 | 18 | 63.3 | 66.7 | 89 | 79 |
| 5242 | CAGCCGAGAACCACCCTC | B3 | 326←343, 8515←8532 | 18 | 62.9 | 66.7 | 81 | 80 |
| 5243 | AGAGCTCAGRACCGAGAGC-AGAAGCGTTCTTCTCCTGAGAC | FIP | 192→232, 8381→8421 | 41 | 76.7 | 54.9 | 75 | 54 |
| 5244 | AGACCTTCTGGTCGGCTATCC-GTTTGCCTTACCTGACCGCT | BIP | 297←337, 8486←8526 | 41 | 78.2 | 56.1 | 81 | 80 |
| BLV envelope gene (AB987702) 5248-5251: 205 bp | | | | | | | | |
| 5248 | TCAGTGGGGCTCACTGGAA | F3 | 5765→5783 | 19 | 62.5 | 57.9 | 78 | 58 |
| 5249 | TTTGAAAACCCAGCCGGATGTA | B3 | 5948←5969 | 22 | 62.3 | 45.5 | 92 | 87 |
| 5250 | TGATCTTGCTCCAGAACGTGGGTGTCTGCCCTTAGCCATCA | FIP | 5774→5814 | 41 | 77.3 | 53.7 | 88 | 76 |
| 5251 | ATGTGGCCTCTGTGGTYGCAACCAATCWAGCCCCCGTC | BIP | 5928←5965 | 38 | 79.0 | 59.2 | 79 | 79 |
| BLV envelope gene(AB987702) 5252-5255: 180 bp | | | | | | | | |
| 5252 | CAGTGGGGCTCACTGGAA | F3 | 5766→5783 | 18 | 61.5 | 61.1 | 81 | 61 |
| 5253 | ACCAATCWAGCCCCCGTC | B3 | 5928←5945 | 18 | 61.6 | 61.1 | 75 | 75 |
| 5254 | AGAACGTGGATCAGGGAGGTGTCTGCCCTTAGCCATCA | FIP | 5777→5814 | 38 | 76.5 | 55.3 | 89 | 76 |
| 5255 | AGATCAGCAACGCTTGATCACCACAGAGGCCACATTAAGCA | BIP | 5895←5935 | 41 | 75.7 | 48.8 | 80 | 72 |
| LC – Linguistic Complexity (%); PQ– Primer Quality (%); Tm – melting temperature calculated for 1 mM $Mg^{2+}$ and 0.25 µM primer concentration. | | | | | | | | |

**Specificity of the XCR assays**

Several sets of XCR primers were produced by FastPCR software (table 1) to optimize the reaction condition and to select most efficient set (fig. 1). XCR method indicated that the set 1 amplified the target sequences in the shortest time and was thus chosen for subsequent analyses (table 1).

Single-copy (host specificity protein) and multicopy (IS711 transposase gene) targets for the *Brucella* spp. genome were chosen. The *Brucella* spp. genome contains an insertion sequence (IS) element called IS711. The copy number of IS711 varies in the genome of the different *Brucella* spp., ranging from 7 in *B. abortus, B. melitensis* and *B. suis* to more than 30 in *B. ovis* and in *Brucella* strains isolated from marine mammals.

Specificity is an important index to evaluate a detection method. To avoid nonspecific amplification, different DNA samples from related bacteria were tested.The specificity of the XCR methods using standard strains from Family *Brucellaceae* (*Paenochrobactrum sp*., *Crabtreella saccharophila*) in laboratory collection. As shown in Fig 1, *Brucella* spp.was significantly amplified, while the other *Brucellaceae* and the blank control remained below the detection threshold for the entire experiment.

**Limit of detection of the XCR assays**

Template DNA extracted from blood samples of each DNA target was 10-fold serially diluted and quantified by using the XCR high-sensitivity assay.

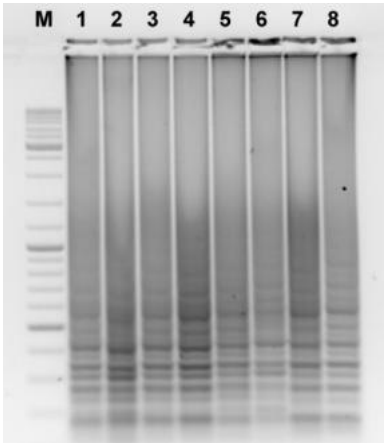
**Specificity and limit of detection of the LAMP assays**

For each LAMP assays specificity was assessed by testing the same panel of DNA targetspositive and negative control DNA that were previously used for the XCR assays. The LAMP assays limit of detection was determined by template DNA extracted from treponeme cultures of each of three phylogroup strains was 10-fold serially diluted and quantified by using. The detection limit was examined by analyzing the products yield from the 10-fold serial dilutions in duplicate. The positive reactions were visualized as a fluorescence color change under an UV lamp. The limit of detection was determined to be the last duplicate positive reaction observed in the series.

## RESULTS

**Optimization of LAMP assays**

The LAMP method is a rapid and sensitive method for target DNA detection. Also, LAMP DNA amplification methods is related to several DNA amplification factors, including temperature and time, and reaction conditions. As shown in fig.1, LAMP assays were set up at different temperatures from 60 to 68°C.

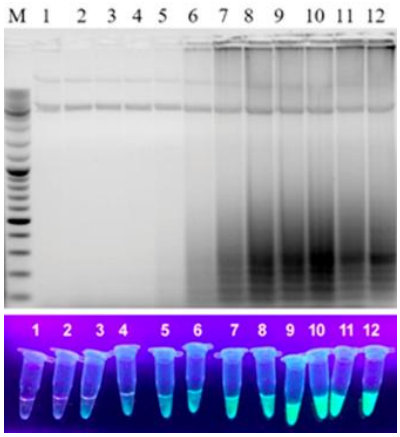



1 – 70°C; 2 –69.3°C; 3 – 68°C; 4 – 66.1°C; 5 – 63.8°C; 6 – 62°C; 7 – 60.7°C; 8 – 60°C

**Fig.1.** Results of optimization of the reaction temperature using primers for the transposase gene

As shown in Fig 1, it was found that positive reactions are demonstrated in a wide temperature range from 60°C to 70°C.

Then the LAMP reaction was lasted for different times from 15 min to 60 min. The LAMP reaction needed at least 30 min, and the product reached maximum when lasted for 60 min (fig.2).

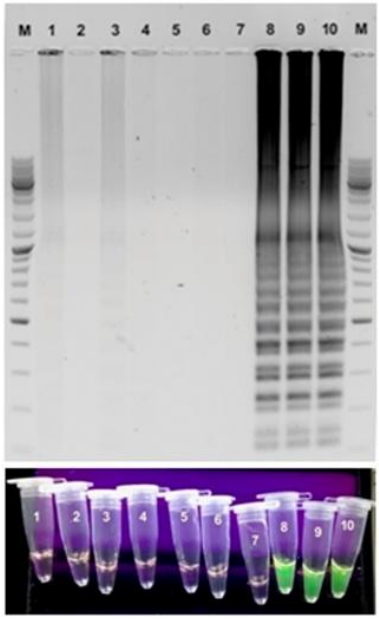



1 – 5 min.; 2 – 10 min.; 3 – 15 min.; 4 – 20 min.; 5 – 25 min.; 6 – 30 min.; 7 – 35 min.; 8 – 40 min.; 9 – 45 min.; 10 – 50 min.; 11 – 55 min.; 12 – 60 min.

**Fig. 2.** Results of optimization of the reaction time using primers on the transposase gene

As shown in fig. 2, the reaction time for optimal amplification must be at least 30min.

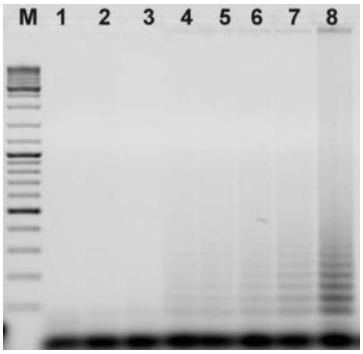
Concentration of $Mg^{2+}$ions in the reaction buffer could enhance the efficiency of the *Bst* DNA polymerase, but our result shown that lower $Mg^{2+}$ concentration (less 4mM including buffer)no amplification was detected and the optimal concentration of $Mg^{2+}$ was 6.0mM (fig.3).




1 – 0 mM $MgSO_4$; 2 – 1 mM $MgSO_4$; 3 – 2 mM $MgSO_4$; 4 – 3 mM $MgSO_4$; 5 – 4 mM $MgSO_4$; 6 – 5 mM $MgSO_4$; 7 – 6 mM $MgSO_4$; 8 – 6 mM $MgSO_4$ (sample 1); 9 – 6 mM $MgSO_4$ (Sample 2); 10 – 6 mM $MgSO_4$ (Sample 3)

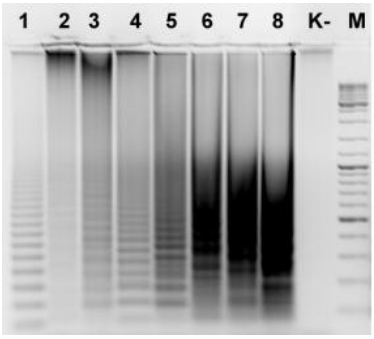
**Fig.3.**Fluorescent detection of magnesium concentration optimization results using primers on a host specific antigen

Finally, the optimal concentration of betaine was analyzed. With the betaine concentration over 0.5 M, the target fragment got well amplified, sobetaine could enhance the stability of DNA amplification and the specificity of LAMP reaction (fig. 4 and 5).




1 – 1.5 M betaine; 2 – 1 M betaine; 3 – 0.8 M betaine; 4 – 0.6 M betaine; 5 – 0.5 M betaine; 6 – 0.4 M betaine; 7– 0.2 M betaine; 8– 0 M betaine

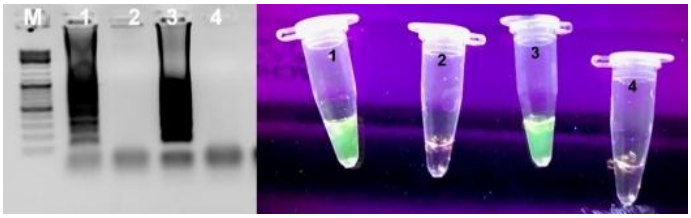
**Fig. 4.** Results of optimizing the betaine concentration using primers on the IS711 transposase gene




1 – 1.5 M betaine; 2 – 1 M betaine; 3 – 0.8 M betaine; 4 – 0.6 M betaine; 5 – 0.5 M betaine; 6 – 0.4 M betaine; 7 – 0.2 M betaine; 8– 0 M betaine; 9 – negative control

**Fig. 5.** Results of optimization of betaine concentration using primers on a host specific antigen

Taking into account all optimized parameters of isothermal amplification, specific detection of target DNA in the presence of negative control was carried out. The optimization parameters included parameters of the composition of the reaction mixture - betaine concentration, as well as the time of isothermal incubation (fig. 6).




1 –positive control of the host specific antigen gene; 2 – negative control for the gene host specific antigen; 3 – positive control of the gene transposase; 4 –Negative control of transposase

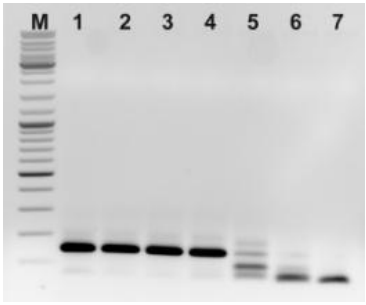
**Fig. 6.**Results of LAMP analysis using primers on the host specific antigen and transposase genes

**Optimization of XCR assays**

The XCR method is a quick and highly sensitive PCR variant method for target DNA amplification. The peculiarity of the XCR method from the standard PCR is that the reaction proceeds without the complete denaturation of the genomic DNA, and only target DNA in which melting temperature (Tm) corresponds to denaturation temperature, for example at 85°C, will participate in the XCR reaction. Melting temperature of the target XCR product calculated and used for denaturation step in the XCR reaction thermal cycles. The

primers Tm should approach the Tm of the target product. Therefore, both temperature in XCR steps should be close to each other and not exceeding 15°C. Therefore, the specificity of the XCR reaction is relied by a narrow step between the denaturation of the target DNA and primer annealing with simultaneous DNA synthesis.

XCR DNA amplification methods is related to several DNA amplification factors, including temperature for annealing and denaturation, time for each temperature steps. As shown in fig 7, XCR assays were set up at the gradient for different denaturation temperatures from 80 to 90°C and with t the gradient for different annealing temperatures from 68 to 75°C.




1 – 89.4°C; 2– 88.2°C; 3 – 88.2°C; 4 – 85.9°C; 5 – 82.1°C; 6 – 80.88°C; 7 – 80°C

**Fig. 7.** Results of optimization of the temperature gradient of denaturation of the XCR assay using primers on a host specific antigen

The maximal efficiency with minimal denaturation Tm and specificity was obtained from 80 to 85°C, with optimal annealing Tm at 70°C. The optimal time for amplification detection was 10 seconds for each temperature steps. The reaction cycles for optimal XCR DNA amplification must be at least 30. For the XCR reaction takes a total from 15 to 30 minutes.

**Specificity of *Brucella* spp.and BLV XCR assays**

All of the assays were specific to the target sequence. The assays for correctly detected *Brucella* spp. the host specificity protein andIS711 target sequences of all control samples examined, and no cross-reactions were observed with negative controls, representing 100% specificity. In general, PCR efficiency for positive samples were about 20 cycles, and no amplification signals were observed for the negative control strains. The limit of detection for the *Brucella* spp. and BLVXCR assays was about 50 fg/µl. The detection limit for the *Brucella* spp. and BLV XCR assays ranged from 50 fg/µl to 80 fg/µl depending on primers assays and DNA target.

**LAMP assays specificity and limit of detection**

The sensitivity of all LAMP assayswas analyzed by detecting the DNA amplification in conditions containing different copes of target DNA. Among of alltarget samples used to determine the specificity of the LAMP assays, false positives and false negative results were not observed.

The LAMP assays correctly identified all control samples.To estimate the detection limits of the LAMP assays in this study, serial DNA dilutions were tested. The limit of detection for the*Brucella* spp. and BLVLAMP assays wasabout 100fg/µl. The detection limit for the *Brucella* spp. and BLV LAMP assays ranged from 100fg/µl to 300 fg/µl depending on primers assays and DNA target.

**Comparison between LAMP and XCR**

A total of 10 cattle infected samples with BLV and 5 infected samples with *Brucella* spp. were subjected to the XCR and LAMP assays. After analyzing via the XCR assay, all samples were judged to be positive. Then both BLV and *Brucella* spp. positive samples were analyzed by LAMP assay with different primers assays (table 2). Among the 5 negative samples analyzed by the XCR assays were proved to be negative. However, using the LAMP assay with negative samples sometimes the nonspecific amplification was detection. For LAMP assays, only few samples out of 5 negative samples were detected to be positive, as unspecific amplification. So, the detection rate using LAMP primers was lower than the XCR.

## DISCUSSION

In this study, we designed and compare XCR and LAMP assays for the detection of cattle infections: *Brucella* spp. and BLV.The XCR methods are designed temperature profile that denature temperatures to

minimize the possibility of amplification primers binding to non-target regions, that makes the amplification more efficient from a time perspective, compared to PCR's thermal cycling. The efficiency of the XCR reaction itself is enhanced by reducing the formation of non-specific product to focus on the formation of the desired target amplicons. Amplifying the target sequence within a 15°C temperature range that is defined by the area contained within the overlap of an annealing temperature of the pair of oligonucleotide primers and the denaturation of the target nucleic acid sequence, wherein each forward and reverse oligonucleotide primer has a melting temperature ($T_m$) within 15°C of the Tm of the target nucleic acid sequence.

XCR and LAMP assays are more precise, sensitive, and specific than conventional PCR methods. Therefore, the development of XCR and LAMP assays was warranted.

The specificity of the developed XCR and LAMP assays were evaluated with a cattle infected samples. The combination of the XCR primers and the LAMP primers designed in this study correctly detected all of the 15 with no cross-reactions and no amplification in any of the negative controls strains. The detection limit for the *Brucella* spp. and BLV LAMP assays ranged from 100 fg/µl to 300 fg/µl depending on primers assays. The *Brucella* spp. and BLVLAMP assays efficiency are dependent on primers assays and DNA target due to the primer design to encompass target DNA sequence and it copy number. The *Brucella* spp. and BLVXCR assays were more specific and sensitive compare to LAMP assays. To our knowledge, there is no reported detection limits for the XCR for comparison with LAMP method. However, detection limits for *Brucella* spp. and BLV with XCR and LAMP assays were reported to be50 fg/µl and 100fg/µl, respectively. These facts imply that the developed XCR and LAMP assays are useful tools for the detection of cattle *Brucella* spp. and BLV infection.

XCR assays have many advantages over conventional PCR including higher sensitivity and specificity, lower contamination rate and they are less time consuming. Therefore, developing XCR assays for detecting cattle pathogens is very useful and easy. LAMP assays are capable of quantification with instrumentation such as bioluminescence or fluorescence readers. We chose to visualize the LAMP assays results with the naked eye for a qualitative interpretation keeping the assays practical and simple to use and cost effective. LAMP assays can be used in mobile laboratories, veterinary practices, and diagnostic laboratories. The use of the LAMP assay for identifying cattle pathogens in a sample is advantageous.In this study, we developed XCR assays that were correctly detect only positive for all cattle samples, both *Brucella* spp. and BLV. This is further validation of the specificity of the developed *Brucella* spp. and BLV specific XCR and LAMP assays. LAMP assays to screen samples for *Brucella* spp. and BLV followed by either the XCR assays to identify the cattle pathogens. Therefore, the developed XCR and LAMP assays are capable of detecting and quantifying cattle *Brucella* spp. and BLV pathogens without limitations for future studies.

## CONCLUSION

In conclusion, the XCR and LAMP assays were developed in this study can accelerate an ability to quick detect and identify the cattle *Brucella* spp. and BLV pathogens from any sample with excellent diagnostic accuracy. The difference between the XCR and LAMP assays is that the LAMP assays are sensitive to unspecific amplification and LAMP assaysrequiring more quantitatively and number of primers in reaction and single heat block to perform. These XCR and LAMP assays may facilitate detection and quantification of the cattle pathogens in relation to human transmission and can be applied to study the pathogenesis of human.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# ІРІ ҚАРА МАЛДА ЛЕЙКЕМИЯ ЖӘНЕ БРУЦЕЛЛЕЗДЫ АНЫҚТАУ ҮШІН ЖЕДЕЛ ТІЗБЕКТІ РЕАКЦИЯ ЖӘНЕ ІЛМЕКТІ ИЗОТЕРМИЯЛЫҚ АМПЛИФИКАЦИЯ ӘДІСТЕРІН ӘЗІРЛЕУ МЕН ҚОЛДАНУ

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**ТҮЙІН**

**Ірі қара малдың лейкоз вирусын (bovine leukaemia virus, BLV) және бруцеллаларды (*Brucella*spp.) жұқтыруы экономикалық шығындарға, өнімділіктің төмендеуіне, әсіресе сүт өндірісінің төмендеуі және жарамсыздығы, және адамдарға жұғу мүмкіндігіне әкелуі мүмкін. Берілген жұмыста ірі қара малдың лейкоз вирусы және *Brucella* түрінің бактериялары сияқты патогенді жұқпаларды тез және тиімді анықтау үшін Xtreme Chain Reaction (XCR) мен ілмекті изотермиялық амплификация (LAMP) әдістерінің әзірленуі жүргізілді. LAMP реакциясының нышандары ретінде *Brucella* түрінің host specific antigen гені және транспозаза генінің IS711 қайталанымдары таңдалды. Провирусты BLV ДНҚ-ы анықтау мақсатында, XCR және LAMP әдістері үшін BLV Long Terminal Repeat (LTR) аймағы мен Env гені қолданды. Сынамаларға қолданылған LAMP реакциясы XCR/LAMP және BLV мен *Brucella* spp. серологиялық талдануларымен салыстырылды. XCR және LAMP серологиялық әдістеріне сәйкестіктің жоғары дәрежесін көрсетіп, нысана тізбектерді анықтады, еш айқас реакциялар байқалмады. Алынған нәтижелер әзірленген әдістер BLV және *Brucella* spp. патогендерін анықтап саралау үшін сезімталдығы және арнайылығы жоғары тест екендігін көрсетіп, жұқпаны ерте кезеңде анықтауға көмектеседі.**

**Негізгі сөздер: лейкемия, бруцеллез, полимераза, LAMP, XCR, геном, диагностика.**