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*Short communication*

# Application of loop-mediated isothermal amplification (LAMP) assays for the detection of bovine herpesvirus 1

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## Abstract

Bovine herpesvirus-1 (BoHV-1), a causative agent of Infectious Bovine Rhinotracheitis (IBR), is responsible for high economic losses in cattle farming industry. The use of testing methods that allow early detection of BoHV-1-infected animals is a key element of each program of IBR eradication. The aim of the study was to design and evaluate two variants of LAMP isothermal tests with SYBR Green fluorescence probes, specific to the genes encoding gD and gE glycoproteins of BoHV-1. LAMP gE BoHV-1 assay was able to distinguish between gE- and gE+ strains of the virus. Both LAMP gD and gE assays were specific to BoHV-1 and did not react with other related to BoHV-1 alphaherpesviruses. Sensitivity of LAMP gD was $2x10^4$ copies of the viral genome whereas for LAMP gE it was $2x10^5$. Diagnostic sensitivity calculated for LAMP gD was 64.7% whereas for LAMP gE it was 80%. Diagnostic specificity for LAMP gD and LAMP gE was 78.9% and 89.3%, respectively. LAMP assay can be a rapid and simple method of diagnosis of acute BoHV-1 infections and discrimination of $gE^-$ strains. However, relatively low diagnostic sensitivity of the method can limit its use in routine diagnostics.

**Key words**: BoHV-1, diagnostics, LAMP

## Introduction

Bovine herpesvirus-1 (BoHV-1) is a causative agent of Infectious Bovine Rhinotracheitis (IBR), responsible for serious economic losses in cattle. Several European countries have introduced programs to eradicate BoHV-1 and six of them are officially IBR-free (Raaperi et al. 2014). This status was achieved by elimination of all seropositive animals and ban of vaccination against IBR. However, in countries with high prevalence of BoHV-1 this approach is too expensive and vaccination programs based on gE-marker vaccines are used instead. Deletion of gE glycoprotein of BoHV-1 does not affect immunizing potential of the vaccine, allowing at the same time differentiation between vaccinated and infected animals (Belknap et al. 1999). Serological methods, most commonly used for the discrimination are not always sufficient. As an alternative BoHV-1-specific PCR (Fuchs et al. 1999) and real time PCR (Wernike et al. 2011)

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Table 1. Primers designed for the diagnostic of BoHV-1 using LAMP assay.

| Gene | Primer | Sequence |
|---|---|---|
| gD | B3 | 5'-GTCCCAGCTCGTCGTC-3' |
| | F3 | 5'-TGGGAAGCGGTACGCC-3' |
| | FIP | 5'-ATGTAGTACAGCGGCCGGGCTTTTCGTACAACGCCACGGTCAT-3' |
| | BIP | 5'-GGAGTACACCGAGTGCGAGCCTTTTGCCAGGAAGCTGTCCCAA-3 |
| | LoopF | 5'- ATGGTACAAGATCGAGAGCGGGTGC – 3' |
| | LoopR | 5'- CAGGAAGCACTTTGGGTACTGCCGCTAC – 3' |
| gE | B3 | 5'-CGTGTACAGCCGGCTGT- 3' |
| | F3 | 5'-GGGCGATTCCTTTCTGCTAT- 3' |
| | FIP | 5'-TACCAGTCGATGCTGGCCGATTTTGGTGCGTCTGCAGTCTG- 3' |
| | BIP | 5'-ACGAGACGTGCATCTTCCACCTTTTGTACGGCGACGCGAAG- 3' |
| | LoopF | 5'- AGCATCGACTGGTACTTCCTGCGGA -3' |
| | LoopB | 5'- AGGCACCGGCCTGCCTGCA-3' |

assays were developed. Although specific and rapid, those assays require well equipped laboratory and trained personnel. Recently, various isothermal amplification assays for diagnosis of infections with human and animal pathogens were designed (Zanoli et al. 2012), allowing for the rapid and specific diagnostic with little need of equipment. The aim of our study was to design and evaluate two variants of loop-mediated isothermal amplification (LAMP) assays specific for gD and gE of BoHV-1 as a simple diagnostic tool.

## Materials and Methods

The specificity of each assay was evaluated with pool of different herpesviruses including: BoHV-1.1 – strain Colorado, BoHV-1.2 – strains IPV468 and K22, BoHV-1 – gE negative vaccine strain Difivac Rispoval® (IBR marker virus), Cervid herpesvirus 1 (CvHV-1), Cervid herpesvirus 2 (CvHV-2), Caprine herpesvirus 1 (CpHV-1) and five selected Polish field. As a non-template control, redistilled water was used. DNA was extracted using QIAamp® DNA Mini Kit (Qiagen). Sensitivity of PCR and LAMP assays was tested using serial 10-fold dilutions of specified quantity of oligonucleotides representing partial sequences of genes encoding gD and gE of BoHV-1.2, the strain K22. To evaluate diagnostic sensitivity and specificity of the LAMP gD assay, 36 nasal swabs collected from animals from 3 different farms were tested. Two sets of LAMP primers specific for gD and gE of BoHV-1 were designed using PrimerExplorer V4 (Table 1).

Both BoHV-1 LAMP assays were composed of 6 µl of Isothermal Master mix (Optigen ISO-001), 50 pmol of each inner primer, 5 pmol of each outer primer and 5 pmol of each loop primer, 0.5 µl of DMSO and 2 µl of DNA sample. Total volume of reaction mix was 20 µl. Reactions were run in thermoblock for 60 min at 66°C. Results were visualized by addition of 1 µl of 1000x concentrated SYBR Green per sample. Interpretation was done in visible light, in UV and by electrophoresis on 1.5% agarose gel. Two nested PCRs specific to gD and to gE protein genes of BoHV-1 were performed as a reference method (Wiedmann et al. 1993, Fuchs et al. 1999).

## Results and Discussion

The minimal copy number of specific oligonucleotides detected by PCR gD was 20, whereas for LAMP gD it was $2x10^4$. Sensitivity of PCR gE and LAMP gE was 200 copies and $2x10^5$, respectively. Sensitivity of the LAMP assays was the same regardless of the detection method: visible light evaluation, fluorescence in UV light or electrophoresis on agarose gel. LAMP gD was able to detect all BoHV-1 strains of both subtypes of the virus. Neither CvHV-1 and CvHV-2 nor CpHV-1 were amplified (Fig. 1). Similarly PCR and LAMP assays specific to gE detected only BoHV-1 strains having gE gene (Fig. 1). Diagnostic sensitivity for LAMP gD was 64.7%, whereas for LAMP gE it was 80%. Diagnostic specificity for LAMP gD and LAMP gE was 78.9% and 89.3%, respectively.

The first LAMP BoHV-1 assay was developed by Pawar et al. (2014) and it was specific to gene encoding gC protein. It was characterized by very high analytical sensitivity, comparable to real-time PCR and 100 times higher than conventional PCR for the same glycoprotein gene. El-Kholy et al. (2014) designed LAMP assay specific to BoHV-1 gE that showed sensitivity comparable to conventional PCR. Both of the assays were specific to BoHV-1 and did not react with any other herpesviruses. Contrary to the previous results, analytical and diagnostic sensitivity of our assays was lower in comparison to the corresponding conventional PCR methods specific to gD and gE genes. Possibly this was caused by the fact that for our LAMP assays

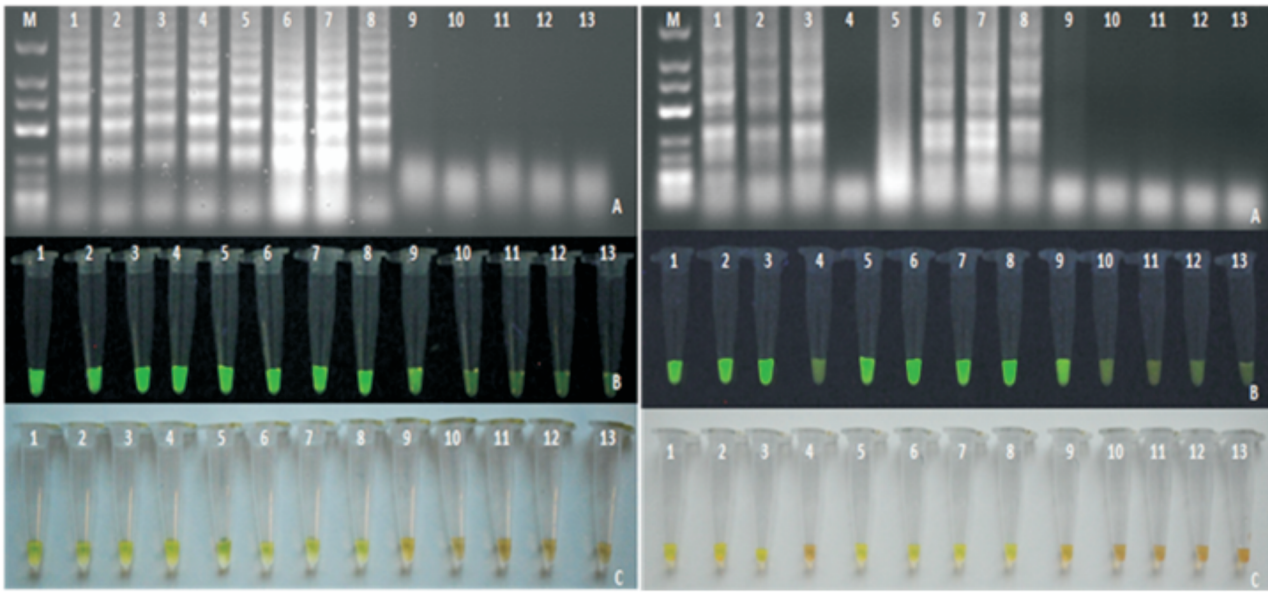


Fig 1. Specificity test of LAMP gD (left) and LAMP gE (right) . A – 1,5% agarose gel, B – UV light, C – visible light. Lines 1 – 9 BoHV-1 strains: 1 – IPV468,2 – Colorado, 3 – K22, 4 – Rispoval gE- vaccine strain, 5 – 9 – Polish field strains, Line 10 – CvHV-1, Line 11 – CvHV-2, Line 12 – CpHV-1, Line 13 – K(-) – NTC.

we have used different isothermal master mix compared to Pawar et al. (2014) and El-Kholy et al. (2014). Different primers used for amplification could also affected the efficiency of the reaction. Primers were designed using PrimerExplorer V4, commonly used tool for various LAMP applications (El Kholy et al. 2014) which should enable for obtaining optimal primer sequences. However, in order to avoid variable regions of target genes, we have included some minor changes to proposed primers.

LAMP assays designed in our study were characterized by high analytical specificity as no reaction with other, closely related to BoHV-1, herpesviruses was observed. However, diagnostic specificity was relatively low with four false positives in LAMP gD and three false positives in LAMP gE. In comparison to our study no false positive results were recorded in LAMP BoHV-1 gD assays designed by other authors. This difference could be the result of biological material used in the study, as Pawar et al. (2014) used semen samples instead of nasal swabs. Alternatively, smaller number of samples used in previous studies as in El-Kholy et al. (2014) could have precluded detection of possible false positives. False positives observed in our study, could also stem from cross-contamination between positive and negative samples in the final stage of the test run. It was shown by Karthik et al. (2014) that it is possible to contaminate working area when tubes are opened after isothermal amplification for addition of fluorescent dye. Replacing SYBR Green with calcein or hydroxyl methyl blue added before reaction (Goto et al. 2009) could eliminate this problem.

We confirmed that LAMP assay can be rapid and simple method of diagnosis of BoHV-1 infections and discrimination of gE$^-$ mutants of the virus. However, low diagnostic sensitivity of the method and presence of false positives means that method require further improvement before it could replace PCR based laboratory diagnostic tests for BoHV-1 detection.

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