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ABSTRACT

As the causative agent of Infectious Bovine Rhinotracheitis (IBR) and Infectious Pustular Vulvovaginitis/Balanoposthitis (IPV/IPB), Bovine alphaherpesvirus 1 (BoHV-1) is responsible for high economic losses in the cattle industry worldwide. This study aimed to establish a fast, colorimetric loop-mediated isothermal amplification (LAMP) assay for the detection of viral DNA. Phenol red is used as pH-sensitive readout, relying on a distinct color change from pink to yellow in case of a positive reaction. LAMP reactions with different primers were compared and a newly designed set targeting the gene encoding the tegument protein V67 provided best results, enabling readout within 8–30 min. LAMP showed less cross-reactions with other ruminant alphaherpesviruses than qPCR but was 10-fold less sensitive. However, LAMP still detected down to 14 copies. The test performance was evaluated using 26 well-characterized nasal swabs from cattle with respiratory disease. All samples were correctly identified when using column-extracted DNA. Using a simple DNA precipitation method, only two weak-positive samples turned indeterminate. Combining this DNA precipitation with a makeshift water bath heated by a gastronomic immersion heater allowed successful application of the colorimetric LAMP assay under resource-limited conditions. This technique can therefore help in managing IBR/IPV outbreaks where sophisticated laboratory equipment is unavailable.

1. Introduction

Bovine alphaherpesvirus 1 (BoHV-1) is a highly contagious pathogen responsible for considerable economic losses in the cattle industry worldwide due to a variety of clinical manifestations and imposed trade restrictions. It is the causative agent of Infectious Bovine Rhinotracheitis (IBR), and Infectious Pustular Vulvovaginitis (IPV) / Infectious Pustular Balanoposthitis (IPB).

BoHV-1 is a member of the genus *Varicellovirus* within the *Herpesviridae* family. Three subtypes of BoHV-1 are distinguished. Subtype 1.1 is associated with respiratory disease, while subtypes 1.2a and 1.2b are related to IPV/IPB (Nandi et al., 2009). The viral genome consists of linear double-stranded DNA with a total length of 136 kilo base pairs (kbp) (Schwyzer and Ackermann, 1996).

Upon primary respiratory infection, animals can develop nasal discharge, cough, pyrexia, apathy and anorexia, often accompanied by a notable milk drop. Disease severity can vary greatly from asymptomatic to severe clinical manifestations, depending on host, viral and environmental factors (Nettleton and Russell, 2017). Abortion can be a sequela of respiratory infection of seronegative cows (Muylkens et al., 2007). During genital infection, animals display frequent micturition and hyperemic genital organ mucosa covered with pustules. Reduced fertility and poor semen quality are possible consequences (Nandi et al., 2009).

Following primary infection, the virus establishes latency in the sensory neurons of regional ganglia, e.g. the trigeminal ganglion in the case of IBR, from where it can reactivate under stressful conditions or corticosteroid treatment (Nandi et al., 2009). Latently infected carriers therefore constitute an epidemiologically important reservoir. They pose a constant risk for re-excreting BoHV-1 and infecting other animals, particularly given the fact that re-excretion often occurs asymptomatically (Muylkens et al., 2007). The above-mentioned symptoms and characteristics of the virus have an extensive economic impact on the cattle industry – directly by decreasing the fitness of the animals, as well as indirectly through trade restrictions imposed by the listing of IBR/IPV as notifiable disease in the Terrestrial Animal Health Code (OIE). To date, only a few countries have been able to eradicate BoHV-1, namely Switzerland, Sweden, Norway, Finland, Denmark and Austria

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(Ackermann and Engels, 2006). In many countries, especially in Europe, national eradication campaigns are ongoing. Otherwise, the virus is still widespread around the globe with varying seroprevalence (Biswas et al., 2013).

Eradication programs and control measures against the spread of IBR mostly rely on antibody detection because it also enables the identification of latently infected animals. Virus isolation and propagation in cell culture and PCR, on the other hand, are common methods to detect infectious virus and viral DNA, respectively, in acute clinical outbreaks. Although qPCR secured its place as a standard diagnostic procedure for the diagnosis of acute BoHV-1 infections, the duration, the costs and need for special equipment can be hindering factors, particularly under resource-limited circumstances. Simpler, faster and cheaper applications such as loop-mediated isothermal amplification (LAMP) have already been established for an extensive variety of pathogens. The method was developed by Notomi (2000) and was steadily improved (Notomi et al., 2015). LAMP uses a polymerase with an intrinsic strand displacement activity, which allows for incubation at a constant temperature and no thermocycler is needed to run the assay. Six primers are used to amplify the target region, making LAMP highly specific. Furthermore, the mechanism of target amplification is extremely efficient, leading to an accumulation of up to 10^9 copies in less than an hour (Notomi, 2000). This massive amplification enables optical readout options such as color change or turbidimetric measurement. Thus, LAMP is an attractive alternative tool in resource-limited areas and in situations where time plays a crucial role in controlling the spread of a disease. Several LAMP assays for the detection of BoHV-1 have already been developed (El-Kholy et al., 2014; Fan et al., 2018; Pawar et al., 2014; Socha et al., 2017). However, these assays mostly rely on fluorescence measurements and gel electrophoresis, which both require further special equipment. In this study we therefore aim to establish a BoHV-1 LAMP assay with a simpler readout, using phenol red as a visual indicator dye. In addition, a fast and easy DNA precipitation method was evaluated, and new primers were designed and tested. The goal was to shorten and simplify the overall procedure - not only of the LAMP reaction itself, but also of the preceding sample preparation - to enable early disease management during outbreaks while also saving resources.

2. Material & methods

2.1. Samples

2.1.1. Cells and viruses

BoHV-1.1 strain Jura served as positive control for the establishment of this LAMP assay. Strain Jura was originally isolated from an IBR outbreak in the canton of Jura in Switzerland in 1972 (Metzler et al., 1986). Furthermore, two other BoHV-1 strains (BoHV-1.1 strain Cooper (Mayfield et al., 1983) and BoHV-1.2 strain K22 (Kendrick et al., 1958)) and the following bovine and other ruminant alphaherpesviruses were included in the study: Bovine alphaherpesvirus 5 (BoHV-5, strain N569 (French, 1962)), Bovine alphaherpesvirus 2 (BoHV-2 strain V766), Cervid alphaherpesvirus 1 and 2 (CvHV-1 (Inglis et al., 1983), CvHV-2 (Ek-Kommonen et al., 1986)) and Bubaline alphaherpesvirus 1 (BuHV-1 (St George and Philpott, 1972)). While BoHV-1 strains Jura, Cooper, and K22, and BoHV-2 and BoHV-5 were specifically passaged for this work, CvHV-1, CvHV-2, and BuHV-1 cell culture supernatant was provided by the diagnostics unit of the Institute of Virology (University of Zurich, Switzerland). All herpesvirus species used in the study underwent a limited number of *in vitro* passages on Madin-Darby bovine kidney (MDBK) cell culture. Cultivation and harvest were carried out according to an in-house protocol, which is based on the guidelines of the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (OIE, 2012).

2.1.2. BoHV-1 BAC

A BoHV-1.1 BAC (bacterial artificial chromosome) (strain Jura), previously described by Gabev et al. (2009), was used to compare the analytical sensitivity of the qPCR and the LAMP assay. The DNA concentration of the starting material was measured to be 204 ng/µl using a Qubit™ 2.0 Fluorometer (Life Technologies™, Carlsbad, CA, USA) and the Qubit™ dsDNA BR Assay Kit (Thermo Fisher Scientific,Waltham, MA, USA) according to the manufacturer's instructions. Copy numbers were calculated to be $1{,}4 \times 10^9$ copies/µl and were then serially 10-fold diluted down to $1{,}4 \times 10^{-1}$ copies/µl in UTM® nasal swab medium (Copan Italia S.p.A, Brescia, Italy).

2.1.3. Clinical samples

Supernatant from 26 nasal swabs (in Virus Transport Medium) collected from cattle with respiratory disease was kindly provided by the Virus Surveillance Unit (VSU) of the Moredun Research Institute (MRI) in Scotland (Penicuik, UK). The swabs were sent to MRI by different external veterinarians from 2017 to 2019. Twelve nasal swabs tested positive for BoHV-1 by qPCR at the MRI and therefore served as a positive control group. Fourteen nasal swabs that tested negative for BoHV-1 were used as negative controls. As proven by diagnostic evaluation at the MRI (Multiplex-Tandem Bovine Respiratory Diagnostic PCR, Ausdiagnostics Pty Ltd, Australia), all 26 samples showed co-infection with one or more pathogens involved in the development of bovine respiratory disease (BRD) such as Parainfluenza virus type 3 (PI-3), Bovine Respiratory Syncytial Virus (BRSV), *Pasteurella multocida,* and *Mannheimia haemolytica*. More detailed information on the samples can be found in the supplementary material section (Tab. A). Upon arrival, supernatants were aliquoted and stored at −20 °C until further use.

2.2. DNA isolation

2.2.1. QIAamp® DNA mini kit

DNA was extracted using the QIAamp® DNA Mini Kit from Qiagen (Düsseldorf, Germany) according to the protocol supplied by the manufacturer. An eluate negative control was included in every extraction series in order to detect contamination during the extraction process. The extracted DNA was eluted in 60 µl of diethylpyrocarbonate (DEPC) treated water and stored at −20 °C until further use. BoHV-1.1 strain Jura DNA extracted by this protocol served as positive control.

2.2.2. Extraction after Vingataramin and Frost (2015)

In the course of adapting the assay to the usage in limitedly equipped laboratories, DNA was also extracted according to a protocol published by Vingataramin and Frost (V&F) in 2015 with minor modifications.

Briefly, 100 µl of nasal swab transport medium were added to 455 µl of extraction solution in a 1.5 ml Eppendorf tube. The mixture was incubated at 80 °C for 10 min and then centrifuged at 2000 x *g* with a portable LLG-uniCFUGE 2 bench-top mini-centrifuge (LLG Labware, Wilmington, DE, USA) for 3 min. The supernatant was thoroughly discarded and the DNA pellet at the bottom of the tube retained. This pellet was re-suspended in 60 µl of DEPC treated water and stored at −20 °C until further use. Prior to LAMP incubation, the eluted DNA was diluted 1:5 in DEPC treated water.

2.3. BoHV-1 and 12S qPCR

A qPCR targeting the gB coding region of BoHV-1 was carried out as reference method. The assay for BoHV-1 was performed according to Abril et al. (2004) with minor modifications in the template volume. The qPCR was run at a final volume of 25 µl per reaction, consisting of 12.5 µl TaqMan™ Universal Master Mix (Applied Biosystems, Waltham, MA, USA), 240 nM of forward and reverse primer, 160 nM of the probe, 8.9 µl DEPC treated water and 2 µl template.

As an internal control to verify successful DNA extraction, a qPCR amplifying a part of the reference gene encoding for 12S mitochondrial rRNA was used (Stahel et al., 2013). Primer and probe design were based on a consensus sequence between previously published 12S rDNA

sequences of various bovid taxa (Gatesy et al., 1997; Stahel et al., 2013). The final reaction mixture of 25 µl for the 12S qPCR contained 12.5 µl TaqMan™ Universal Master Mix (Applied Biosystems, Waltham, MA, USA), 600 nM of forward and reverse primer, 160 nM of the probe, 8.9 µl DEPC treated water and 2 µl template.

The PCR reactions were run on a QuantStudio™ 3 Real-Time PCR System (Applied Biosystems, Waltham, MA, USA) and the cycling conditions for both qPCR assays corresponded to those published by Abril et al. (2004). The threshold value was set manually at 0.1 for BoHV-1 and at 0.05 for 12S. The result was considered positive if the fluorescence signal exceeded the threshold value and exponential amplification was observed.

2.4. LAMP primer design

Seven primer sets were compared at the beginning of this study. The sets targeting the genes encoding for gB (Fan et al., 2018), gC (Pawar et al., 2014), gD, and gE (Socha et al., 2017) were already published (Suppl. Tab. B). Additionally, three sets targeting the sequences of the catalytic subunit of the Polymerase (Pol), the Helicase (Hel), and the tegument protein V67 (V67) (Table 1) were designed using the LAMP designer 1.16 software from Premier Biosoft (Palo Alto, CA, USA). These three regions were chosen because of their relatively low GC content (60.2 %, 61.8 % and 61.8 % respectively). Regions of low GC-content were identified in the NCBI reference strain sequence of BoHV-1 (GenBank accession number NC_001847.1) with the GeneQuest application of the DNASTAR Lasergene V.17 software (DNASTAR, Madison, WI, USA). This reference strain was also used for primer design, with primers designed within the above regions.

2.5. LAMP assay and detection of LAMP products

In a first stage, the real-time WarmStart® LAMP Kit (New England Biolabs, Ipswich, MA, USA), which includes a master mix and a separate tube of fluorescent dye for readout in a qPCR machine, was used for the optimization of the LAMP assay. The real-time measurement allowed for a precise representation and evaluation of amplification and melt curve data during the establishment of the assay. The Colorimetric WarmStart® LAMP 2x Master Mix (New England Biolabs, Ipswich, MA, USA) was used as a visual assay. The Colorimetric WarmStart® LAMP 2x Master Mix contains the same components (except for a low-Tris reaction buffer and additional phenol red) as the master mix of the real-time WarmStart® LAMP Kit. Because the Colorimetric Master Mix does not come with a fluorescent dye, but a precise representation of amplification and melt curve to control the visual readout during the second stage of the assay establishment was still desired, Syto™ 9 Green Fluorescent Nucleic Acid Stain (Invitrogen, Carlsbad, CA, USA) was added to the Colorimetric Master Mix. This allowed the concurrent evaluation of two readouts (real-time fluorescence and end-point visual color change). The performance of Syto™ 9 and the fluorescent dye of the real-time WarmStart® LAMP Kit was compared beforehand in a separate experiment and was identical for both dyes (data not shown).

The final reaction volume of 12.5 µl for the real-time WarmStart® LAMP Kit consisted of 6.25 µl WarmStart® LAMP 2x Master Mix, 0.25 µl LAMP Fluorescent Dye (50x), 1.25 µl 10x primer mix, 3.75 µl DEPC treated water and 1 µl of template.

The final reaction volume of 12.5 µl for the Colorimetric WarmStart® LAMP Master Mix consisted of 6.25 µl Colorimetric WarmStart® LAMP 2x Master Mix, 0.5 µl 25x Syto™ 9 Green Fluorescent Nucleic Acid Stain (Invitrogen, Carlsbad, CA, USA), 1.25 µl 10x primer mix, 3.5 µl DEPC treated water and 1 µl of template. Syto™ 9 Green Fluorescent Nucleic Acid Stain was prepared as a 25x stock solution according to Tanner and Evans, 2014. The template was denatured at 95 °C for 5 min and immediately placed on ice before being added to the reaction mix.

The reaction mix was then incubated at 65 °C for 60 min. Afterwards, a melt curve consisting of the following steps was added: 1.6 °C/sec up to 95 °C, 15 s at 95 °C, 1.6 °C/sec down to 60 °C, 1 min at 60 °C, 0.1 °C/sec up to 95 °C and finally 15 s at 95 °C. A QuantStudio™ 3 Real-Time PCR System (Applied Biosystems, Waltham, MA, USA) was used to run the assay.

Two readout methods were used in this work. First, a real-time fluorescence measurement was used throughout the entire study. Second, a visual readout based on phenol red was employed in a second stage, once the assay was optimized.

Regarding the real-time readout, fluorescence emission of the fluorescent dye from the real-time WarmStart® LAMP Kit and of the Syto™ 9 was measured in the SYBR channel. A reaction was considered positive when meeting the following criteria: the signal exceeded the manually set fixed threshold value (beginning of stable exponential amplification) of 16,480, it amplified exponentially, and the melt curve analysis showed a peak at around 89 °C. The time point where the fluorescence signal crosses the threshold is called time to reaction (TTR) or reaction time. Reactions meeting the following criteria were considered negative: no measurement of an exponential amplification curve, and appearance of a flat line in the melt curve analysis. Reactions with an amplification curve exceeding the threshold, but with a melt curve peak at a lower temperature than 89 °C or with multiple melt curve peaks, were considered false positive due to non-specific amplification.

Phenol red is included in the Colorimetric WarmStart® LAMP 2x Master Mix as a visual indicator dye. Due to a drop in pH caused by an accumulation of protons during a positive LAMP reaction, the dye changes color from pink to yellow (Tanner et al., 2015). In the case of a negative reaction, the color remains pink.

2.6. Optimization of LAMP conditions

The effect of different concentrations of Betaine (0 M – 1.6 M), DMSO (0 %–7.5 %), additional $MgSO_4$ (0 mM – 2 mM), or additional dNTPs (0 mM – 0.4 mM) in the reaction mix was analyzed. Also, the effect of doubling the concentration of FIP/BIP primer and doubling the concentration of all primers was evaluated. Further, the effect of using different volumes of template (1 – 5 µl) was analyzed. Lastly, a

Table 1
Sequences and final concentrations of the newly designed LAMP primers used for the final assay.

Target gene Genome position[1]	Primer	Sequence	Final Concentrations
V67 [U_S 1.67] 114′483 – 114′731	F3	5′-GTACTGGCTCATGTTTCCC-3′	0.2 µM
	B3	5′-CTGCTGGTGAAAGTTCCC-3′	0.2 µM
	FIP	5‘-TCGTTAAGCTTCCGCACAACTGTTGAGGTAGAAGCGGTC-3‘	1.6 µM
	BIP	5‘-TGTTCCGTCGTAAAGCTGACGGTTCACTTTGAATGTGTTCCC-3‘	1.6 µM
	LF	5′-AGACGAGTGCTACGAGGA-3′	0.4 µM
	LB	5′-AAAGAACTGCAGCGGTCG-3′	0.4 µM

[1]The genome position refers to the position of the LAMP amplicon and is relative to the reference sequence for BoHV-1 (GenBank accession number NC_001847.1). F3 = outer forward primer; B3 = outer backward primer; FIP = forward inner primer; BIP = backward inner primer; LF = forward loop primer; LB = backward loop primer; V67 = tegument protein V67; U_S = unique short sequence. The sequences of the Pol and Hel primer sets are only provided in Suppl. Tab. B as they proved inferior to V67.

temperature gradient (61 °C – 70 °C) was carried out to determine the optimal incubation temperature.

2.7. Analytical specificity and sensitivity

Extracted DNA of the ruminant alphaherpesviruses was first diluted to approximately the same Cq value (ΔCq 24,4 ± 0.8). In a next step, the diluted samples were analyzed by LAMP by means of real-time fluorescence measurement and concurrent visual detection at the end of incubation. Results were then compared to those obtained by qPCR.

To evaluate the analytical sensitivity, the minimum detectable copy number was determined by simultaneously testing the serially diluted BoHV-1 BAC (see chapter 2.1.2) by BoHV-1 qPCR, and concurrent visual and qLAMP.

2.8. Testing of clinical samples

A total of 26 nasal swabs supernatants kindly provided by the Moredun Research Institute (MRI) (Penicuik, UK) were used to test the performance of the newly established LAMP assay with field samples. The samples had been tested for 9 bacterial and viral agents causing bovine respiratory tract diseases, among them BoHV-1, in a multiplexed-tandem PCR (AusDiagnostics Pty Ltd, Australia). For the LAMP assay, the samples were first used untreated, i.e. no DNA extraction step was included, only the denaturation of the nasal swab supernatant at 95 °C for 5 min was performed. Alternatively, DNA was extracted using the QIAmp® DNA Mini Kit (Qiagen, Düsseldorf, Germany) (see chapter 2.2.1) and applying the method described by Vingataramin and Frost (see chapter 2.2.2). Finally, the untreated nasal swabs, as well as the extracted DNA, were tested by LAMP and qPCR. Where sufficient nasal swab supernatant was available, extractions were performed in duplicate to ensure biological replicability. Each extraction series was tested twice by qPCR and twice by LAMP to ensure technical replicability.

2.9. Adaptation to resource-limited settings

The following set up was assessed in order to make the test applicable in situations where only limited laboratory facilities are available. A commercial gastronomic immersion heater for low-temperature long-time cooking of vacuumed goods, a so-called sous-vide stick (Aicok Sous Vide SV-8001), was placed in an insulating rubber ice bucket containing water. The sous-vide stick has ability to heat water up to 99 °C and maintain the temperature with an accuracy of ±0.5 °C. This heated water bath was used for DNA precipitation, DNA denaturation and LAMP incubation. To ensure consistency of temperature everywhere in the water bath, temperatures (65, 80 and 95 °C) were controlled at different spatial points with a TFN 520 thermometer (ebro GmbH, Urdorf, Switzerland). A bench-top mini-centrifuge was used for the preparation of the LAMP reaction mix and the DNA extraction. Apart from these two tools, pipets and pipet tips, 0.1 ml PCR tubes, 1.5 ml Eppendorf tubes, racks that fit and fix the different tubes for incubation in the water bath, a rack to prepare the DNA precipitation and the LAMP reaction mix on the bench, a frozen cold pack, and a weight to submerge the rack for the LAMP incubation were used. With this equipment, all the necessary steps could be performed, which included the DNA precipitation after V&F, denaturation of the DNA and the LAMP assay. For the detailed and illustrated protocol see Suppl. Fig. A.

2.10. Statistical analyses

To compare the TTR results of different primer sets and of TTR between different incubation temperatures, the NCSS 10 statistical software (NCSS, LLC; Utah, USA) package was used. For overall comparison, the Kruskal-Wallis One-Way ANOVA on Ranks and for pair-wise comparison the paired *t*-test were applied. P values below 0.05 were regarded as statistically significant.

3. Results

3.1. LAMP primer

Seven primer sets were tested for their reaction time to determine the most efficient set (Fig. 1) using DNA extracted from BoHV-1 (strain Jura) positive cell culture supernatant (~ Cq value 18). The newly designed primers targeting the gene for the tegument protein V67 showed a significantly faster TTR (~ 8.3 min; $p < 0.000001$) compared to the second best primer set tested (Pol, ~ 13.5 min) and were therefore used for all following experiments. All other sets had far longer TTRs and the primers targeting the gene encoding gB showed the longest TTR (~ 44 min).

3.2. Optimization of LAMP conditions

Different conditions were evaluated to optimize the qLAMP assay using the real-time WarmStart® LAMP Kit. None of them had a positive impact on the TTR and the master mixes of the WarmStart® kits were therefore used as recommended by the manufacturer. The temperature gradient revealed the optimal incubation temperature to be 65 °C.

3.3. Analytical specificity

The analytical specificity was determined by testing various bovine and other ruminant alphaherpesviruses by qPCR, and by visual and concurrent qLAMP using the Colorimetric WarmStart® LAMP master mix with an added fluorescent dye (Fig. 2). All DNA samples were diluted to approximately the same Cq values of 24-25. While the BoHV-1 qPCR cross-reacted with all examined viruses except for BoHV-2, the LAMP assay only amplified the different BoHV-1 strains as well as CvHV-2 and BuHV-1. However, the TTR for BoHV-1 strains was much lower, ranging from 8.6–16.9 min and leading to a clear color change from pink to yellow. CvHV-2 and BuHV-1 only gave a positive result in qLAMP readout after 42 and 56.4 min respectively, but the colorimetric readout remained indeterminate. Only when using undiluted BuHV-1 (~Cq value of 18.2) a cross-reaction occurred after 27.7 min resulting also in a color change to yellow (data not shown). BoHV-5 did only cross-react at ~ 33.8 min resulting in color change when used at the original concentration (~ Cq value of 18.2) instead of the diluted template (~ Cq value of 25), as did CvHV-1 (~ Cq value of 18.2 equaled a TTR of ~ 50.9 min, but no color change) (data not shown). Long reaction times are usually suspicious for non-specific amplification (e.g. primer dimer) but melt curve analysis confirmed the specificity of the reactions.

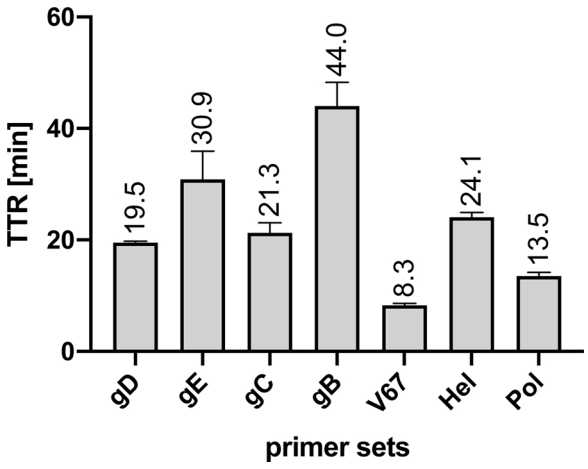

Fig. 1. Comparison of different primer sets. The experiment was run twice and in triplicate for each primer set. Data are shown as mean ± SD. V67: tegument protein V67; Hel: Helicase; Pol: Polymerase.

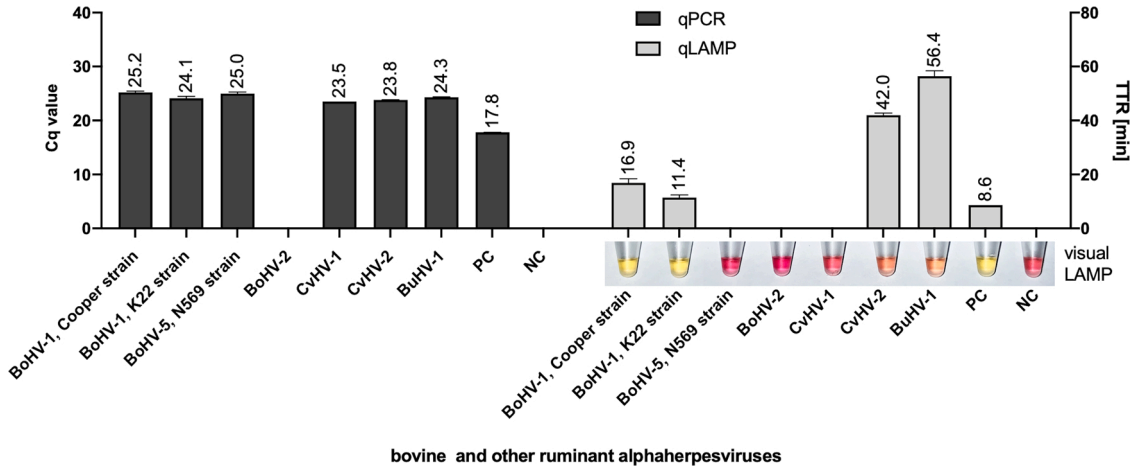

Fig. 2. Cross-reactivity of the BoHV-1 qPCR and the qLAMP assay with different bovine and ruminant alphaherpesviruses. Below the graph on the right side, the corresponding results of the visual readout are added. The experiment was run twice and in duplicates. Data are shown as mean ± SD. PC: positive control (BoHV-1.1 strain Jura); NC: negative control (DEPC treated water).

3.4. Analytical sensitivity

The sensitivity of the assay was evaluated using a serially tenfold diluted BoHV-1.1 BAC (strain Jura) ranging from 1.4×10^5 to 1.4×10^{-1} copies/µl. The dilution series was tested by qPCR, and by visual and concurrent qLAMP (Fig. 3). While the qPCR detected as few as $1.4 \times 10^{\circ}$ DNA copies, the detection limit of the LAMP assay was 1.4×10^1 DNA copies. Cq values of the qPCR ranged from 21 for the highest, to 37.5 for the lowest detectable copy number. TTRs ranged from 12.6–26.9 min respectively. Thus, the LAMP assay is slightly less sensitive than the qPCR, detecting ten times less copies.

3.5. Testing of clinical samples

Twenty-six nasal swabs supernatant from clinical cases of bovine respiratory disease were tested for the presence of BoHV-1 DNA by qPCR and by visual and qLAMP (Fig. 4). Initial attempts of using the nasal swab supernatant directly without DNA extraction did not yield satisfactory results due to many false negative and false positive results (data not shown).

When extracting DNA using the QIAmp® DNA Mini Kit, the results of the qPCR and of the visual and concurrent qLAMP were identical (Fig. 4, uppermost row of three). Samples 16, 17 and 26 originally tested positive for BoHV-1 at the MRI with Cq values of around 40 but always tested negative in qPCR at our lab. Since this may be due to DNA degradation by freeze-thawing or shipment, we used our qPCR results to determine the status of the samples. With DNA precipitated by the method of V&F on the other hand, an inconsistency of results could be observed (Fig. 4, middle row of three). The qPCR gave false-negative results in some replicates of 5 positive samples. Interestingly, the qLAMP was less affected, as all positive samples were detected, also when using DNA extracted by columns. The visual LAMP however, delivered variable results and color change could not be clearly judged at times.

When diluting V&F DNA 1:5 in DEPC treated water prior to denaturation and addition to the reaction mix, results became more consistent (Fig. 4, bottom row of three). All samples apart from number 18 and 19 gave the same result as samples extracted with the QIAmp® DNA Mini Kit. Sample 18 had a mean Cq value of 33.4 when extracted with the QIAmp® DNA Mini Kit, and a Cq value of 36.7 when extracted after V&F and diluted 1:5 in DEPC treated water; sample 19 had Cq values of 33.2 and 38.2 respectively. The detection limit of the LAMP assay was estimated to equal approximately a Cq value of 37 (Fig. 3) and samples 18 and 19 had Cq values exactly below or above the detection limit, depending on the DNA extraction method. When comparing all samples, Cq values of the column-based extractions were on average 2.8 cycles

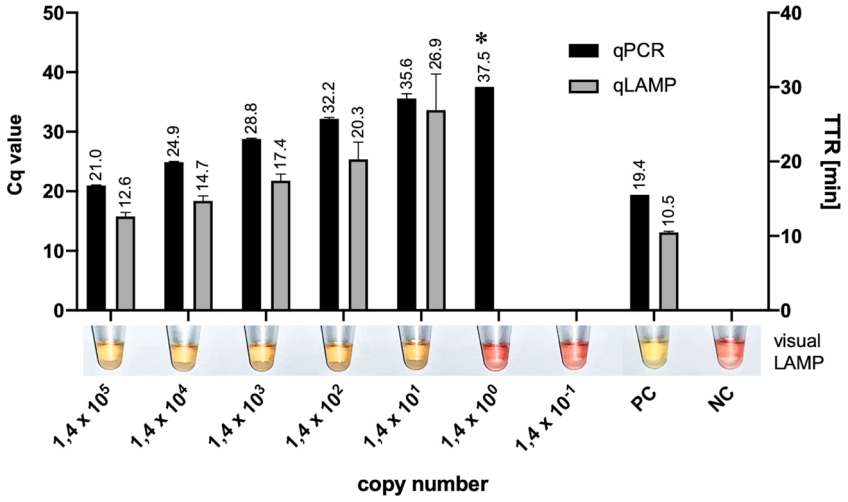

Fig. 3. Data are shown as mean ± SD. qPCR as "gold standard" was run once in duplicates, the LAMP assay was performed twice and in duplicates. ✱ represents a single value because one reaction of the duplicate remained negative. PC: positive control (BoHV-1.1 strain Jura); NC: negative control (DEPC treated water).

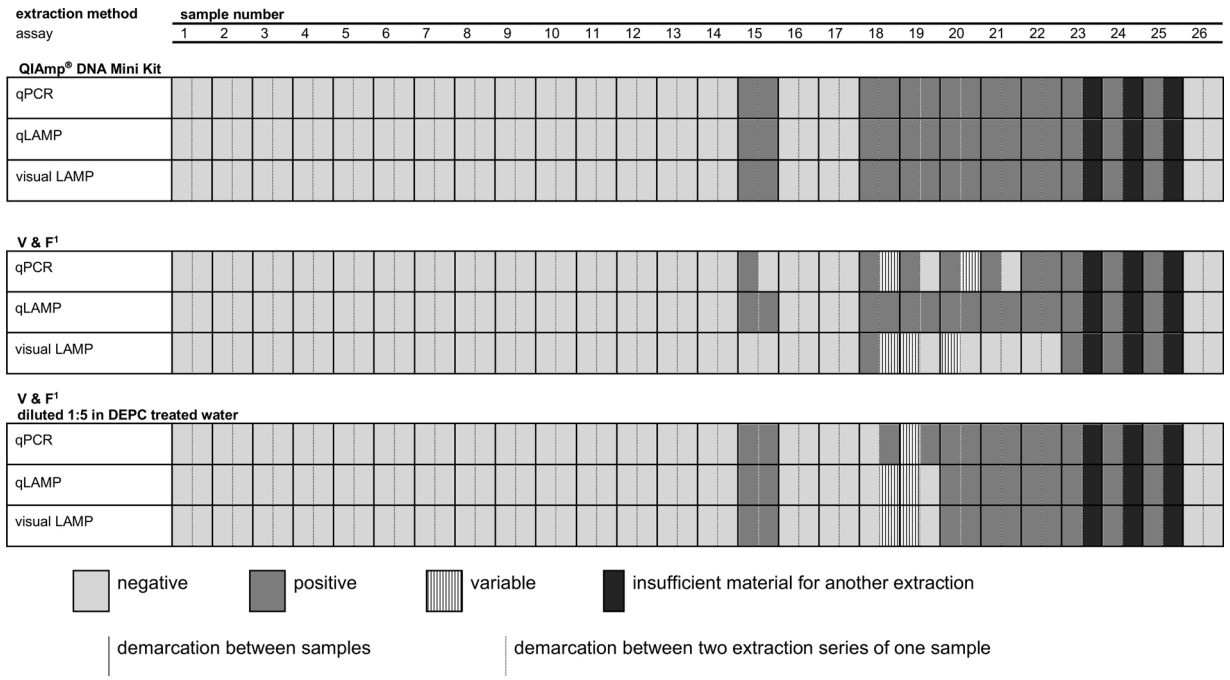

Fig. 4. Overview of the results of the clinical samples tested (n = 26). Samples 16, 17 and 26 originally tested positive for BoHV-1 at the MRI with Cq values of around 40, 40 and 32, respectively, but always tested negative in qPCR at our lab. Since this may be due to DNA degradation by freeze-thawing or shipment, we used our qPCR results to determine the status of the samples. DNA of the samples was extracted and processed in three different manners: (i) with the standardized DNA extraction method using the QIAmp® DNA Mini Kit (uppermost row of three); (ii) according to the protocol of Vingataramin and Frost (middle row of three); (iii) as in (ii) but diluting the eluted DNA 1:5 in DEPC treated water (bottom row of three). For clarity of presentation, a ternary depiction of the results was chosen. With each of the two extraction series, two independent experiments were carried out in duplicates (LAMP) or single assay (PCR). variable: Within the total of four replicates, replicates were not always negative or positive.
[1]Extraction method after Vingataramin and Frost, 2015.

lower than Cq values of the precipitation after V&F (data not shown), indicating an inferior extraction efficiency of the precipitation after V&F compared to the commercial column-based extraction. While the number of tested samples is too small for reliable calculation of test performance, no false positive reactions were observed, indicating a high diagnostic specificity. The diagnostic sensitivity seems to be the same for the qPCR, and visual and qLAMP when using column-based extracted DNA. When using 1:5 diluted DNA after V&F precipitation, samples with high Cq values were not always recognized, resulting in a lower diagnostic sensitivity compared to column-based extraction. However, more samples need to be tested to allow for reliable determination of sensitivity and specificity of the LAMP test.

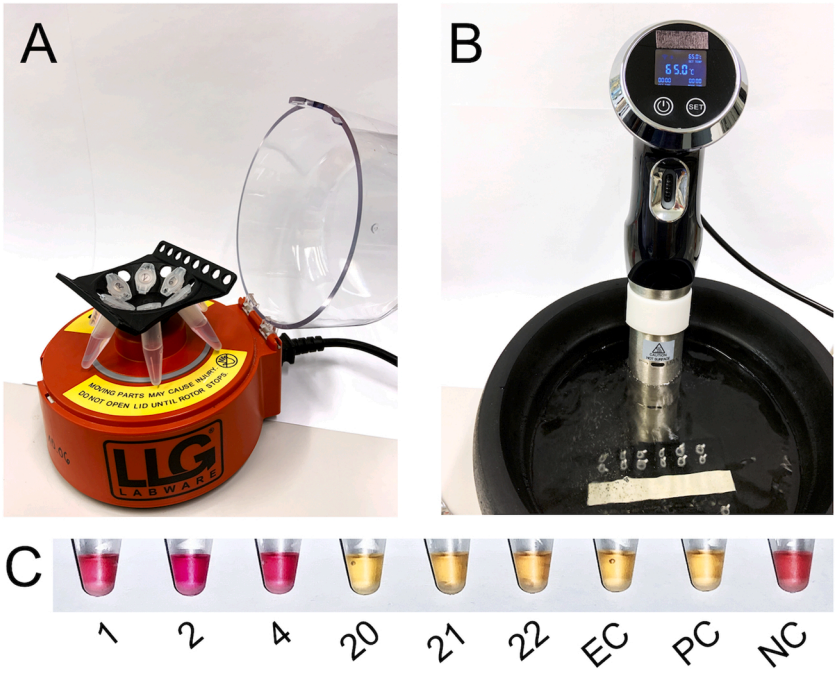

Fig. 5. Adaption to limited equipment. A: mini-centrifuge used for DNA precipitation and preparation of the LAMP reaction mix; B: Sous-vide stick heating water to different temperatures in a rubber ice bucket; C: Results of 6 samples to test this simple set up and only relying on visual readout. Sample numbers match numbers in figures 1 and 6. Samples 1, 2 and 4 were negative for BoHV-1, samples 20, 21 and 22 positive. EC: positive extraction control; PC: positive control (BoHV-1 strain Jura; NC: negative control (DEPC treated water).

3.6. Adaptation to limited equipment

DNA was extracted from six of the clinical samples with sufficient material left and tested by visual LAMP using only the equipment listed in chapter 2.10 to imitate the use of the assay under limited equipment conditions. The core of the experimental set up consisted of a portable mini-centrifuge (Fig. 5A) and a water bath, which could be heated to different temperatures with a sous-vide stick (Fig. 5B). Measuring the temperature in the water bath with an independent thermometer proved consistency and correctness of temperature (±0.3 °C), also at points that were the farthest away from the heat source. An extraction control consisting of BoHV-1 (strain Jura) cell culture supernatant was added to prove successful DNA extraction. After the centrifugation step, a pellet was not clearly visible in two of the six samples. In these cases, the extraction solution was decanted carefully, and possible visible traces of extracted DNA eluted in DEPC treated water. A color change became apparent in some of the samples already after 15 min of LAMP incubation. After 30 min however, a color change was clearly visible in all positive samples and results were identical to those obtained by qLAMP and qPCR. We therefore recommend assessing the result after 30 min of incubation, avoiding thereby also potential cross-reactions with closely related herpesviruses (Fig. 2). For better visibility of the color change, a double volume of reaction mix (23 µl) and template (2 µl) were used for the LAMP assay in this experiment. For illustration of the exact set up, and single steps of DNA precipitation and LAMP incubation, a picture series with descriptions is provided in Suppl. Fig. A.

4. Discussion

Due to its simplicity, speed and the dispensability of sophisticated laboratory equipment, LAMP has become a popular tool for point-of-care diagnostics and has proven particularly useful in situations where no well-equipped lab is at hand such as for diagnosis of leishmaniosis in rural Brazil (Celeste et al., 2019).

BoHV-1 is still widely spread causing significant economic losses to cattle industries worldwide. Affected regions, especially resource-limited areas and regions lacking functioning infrastructure, would profit from an easy, fast, and cost-efficient diagnostic tool.

Here, we report the design of new LAMP primers for the detection of BoHV-1, showing significantly faster reaction times than previously published primer sets (Fan et al., 2018; Pawar et al., 2014; Socha et al., 2017) (Fig. 1). The gB primer set showed the longest reaction time (44 min). This relatively weak performance may be explained by the absence of loop primers in the gB primer set, while the other sets include loop primers. Even though not essential for LAMP, loop primers have been shown to accelerate the LAMP reaction greatly (Nagamine et al., 2002). Since the BoHV-1 genome has an exceptionally high GC content of 70 % (Thiry et al., 2006), choosing the V67, Hel and Pol regions was thought to facilitate strand displacement and therefore enhance amplification and reduce reaction time. However, melting temperature, primer secondary structures and stability at the end of the primers can also influence the efficacy of a primer set. While amplicons obtained with our primers show a lower percentage of GC compared to previously published assays, there is no difference in GC content of amplicons between V67, Hel and Pol primers that could explain the significantly better performance of V67 primers (Suppl. Tab. B). Thus, the GC content seems not to be the only influencing factor even though high GC content is known to hinder efficiency of amplification assays (Kool, 2001).

To our best knowledge, this is the first LAMP assay for the detection of BoHV-1 which uses phenol red as a readout. This indicator dye is already included in the Colorimetric WarmStart® LAMP master mix. Previous studies mainly focused on the usage of hydroxynaphthol blue (HNB) (Goto et al., 2009; Pawar et al., 2014) and SYBR Green I stain or gel electrophoresis (El-Kholy et al., 2014; Pawar et al., 2014; Socha et al., 2017) as visual readout methods. These methods, however, require additional time and consumables. Furthermore, the color change of HNB from purple to sky blue is sometimes difficult to discern by eye and adding SYBR Green stain I post incubation poses a considerable risk to contaminate surfaces or equipment when opening the tubes containing massively amplified LAMP products. Moreover, the color change of phenol red from pink to yellow can be clearly distinguished. A further advantage of this master mix is the possibility of a set up at room temperature – a helpful feature for field applications. Yet a disadvantage lies within the pH-sensitivity of this indicator dye. When using nasal swab supernatant directly or eluting DNA in anything else than water, the pH of the buffer can have an effect on the starting pH and therefore the color of the reaction mix. This can complicate or even render a visual readout impossible. The manufacturer of the Colorimetric Kit therefore advises not to use more than 10 % template volume compared to the final reaction volume to reduce the impact of the pH on the color change. Thus, only 1 µl of template per 12.5 µl reaction was used.

The assay successfully detected all three tested BoHV-1 strains. Cross-reactivity with BuHV-1 and CvHV-2 was observed at very late stages of incubation (Fig. 2) and with BoHV-5 and CvHV-1 only if copy numbers were relatively high (~ Cq values of 18, data not shown). One could therefore argue that the risk of cross-reactions rises with increased titers of shed virus. However, using a LAMP incubation time of only 30 min reduces this risk. Furthermore, inter-species transmissions of related alphaherpesviruses to cattle do not seem to play an important role in BoHV-1 epidemiology (Thiry et al., 2006) and false positive results due to infection with other ruminant herpesviruses may therefore be rather unlikely. However, if such an infection is suspected, additional, confirmative analyses are recommended. Previous reports of naturally occurring homologous recombination between BoHV-1 / BoHV-5 (Maidana et al., 2017), and vaccine / wild-type BoHV-1 (d'Offay et al., 2019) may represent a more significant challenge. Therefore, in regions where vaccines are administered frequently and/or where BoHV-1 and BoHV-5 occur simultaneously, a secondary test to identify the virus, sequencing of the LAMP product or probably restriction enzyme analysis (El-Kholy et al., 2014) may be necessary.

The analytical sensitivity of the colorimetric LAMP assay seems to be slightly lower than that of the qPCR, however LAMP still detected 14 copies/µl equaling to a Cq value of 35.6 (Fig. 3). In case of clinically overt IBR outbreaks, animals are typically shedding high quantities of viral particles (Muylkens et al., 2007; Nettleton and Russell, 2017), therefore, a slightly decreased sensitivity may be acceptable for tests deployed in this scenario. However, for surveillance programs where an eradication of IBR is required, the suitability of the LAMP assay should first be thoroughly evaluated.

When testing column-extracted DNA from 26 clinical nasal swabs, our LAMP assay performed equally well as the qPCR (Fig. 4). The unsatisfactory results obtained by testing supernatant without DNA extraction, might be explained by inhibition due to contaminants, such as humic acid or complex polysaccharids originating e.g. from animal feed or bedding, or unsuitable swab medium. Using DNA extracted after V&F without further dilution also proved unsuitable due to remaining traces of NaOH from the extraction solution inhibiting the qPCR and influencing the pH of the LAMP reaction, thereby disabling visual read out – even if amplification occurred (Fig. 4). A 1:5 dilution of the DNA in water abolished the NaOH-related problems but diagnostic sensitivity decreased due to two weak positive samples falling below the detection limit of the assay. This may be due to the V&F precipitation being generally somewhat less efficient than the column-based extraction and/or a stronger influence of impurities of the nasal swabs (e.g. amount of nasal mucus, purulent material, abraded skin particles) on precipitation. In short, detection of DNA precipitated after V&F and diluted 1:5 in DEPC water is somewhat less sensitive than DNA extracted with the QIAmp® DNA Mini Kit. However, the speed, the costs and the limited equipment needed may compensate for the decreased sensitivity in specific circumstances such as field settings or the lack of a well-equipped laboratory. Therefore, we tested the applicability of our LAMP assay under "field conditions". A simple set up using an insulated

rubber ice bucket and a commercial gastronomical immersion heater to form a makeshift water bath and a portable mini-centrifuge to precipitate the DNA proved sufficient for the DNA precipitation after V&F and the visual LAMP assay (Figs. 5, Suppl. A), offering a cost saving alternative to qPCR and column-based DNA extraction.

Due to the absence of IBR in Switzerland, obtaining positive clinical samples was difficult. Twenty-six supernatants from nasal swabs, kindly provided by the VSU of the MRI (Penicuik, UK), where tested to this end. Based only on these few samples we cannot completely and reliably assess the diagnostic sensitivity and specificity of the assay. However, the samples were known to be also positive for several other pathogens of the bovine respiratory tract such as paramyxoviruses and *Pasteurella multocida* and it was important to proof absence of cross-reaction or non-specific reaction in cases of such mixed infections.

In conclusion, a fast, colorimetric LAMP assay for the detection of BoHV-1 was developed. Analytical specificity appears to be higher than that of the qPCR used as a reference method in this study, but the LAMP assay is around tenfold less sensitive. Compared to other qPCRs however, analytical sensitivity and specificity may differ.

The LAMP assay may be flexibly applied in different settings. Using the real-time readout option and extracted DNA it represents a fast (30 min run time) and cost-efficient alternative to qPCR in routine veterinary diagnostic labs. On the other hand, while sensitivity is slightly reduced, the use of a simple and fast DNA precipitation (Vingataramin and Frost, 2015), portable equipment and the visual readout option (Figs. 5, and Suppl. A) enables application of the assay as an affordable and practicable diagnostic tool in resource-limited settings.

To reliably assess the diagnostic sensitivity and specificity of the test as well as intra- and inter-assay variation, a larger number of clinical samples needs to be tested which is ideally done in a country where, unlike Switzerland, BoHV-1 is still circulating. Also, further studies could focus on whether the assay is applicable to other specimen than nasal swabs, e.g. semen, abortion material or fetal tissue.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jviromet.2020.114041.

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