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A new method for simultaneous detection and discrimination of Bovine herpesvirus types 1 (BoHV-1) and 5 (BoHV-5) using real time PCR with high resolution melting (HRM) analysis

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Bovine herpesvirus types 1 (BoHV-1) and 5 (BoHV-5) are antigenically and genetically similar. The aim of this study was to develop a simple and reliable one-step real time PCR assay with high resolution melting (HRM) analysis for the simultaneous detection and differentiation of BoHV-1 and BoHV-5. Optimization of assay conditions was performed with DNA from reference strains. Then, DNA from field isolates, clinical samples and tissue samples of experimentally infected animals were studied by real time PCR-HRM. An efficient amplification of real time PCR products was obtained, and a clear melting curve and appropriate melting peaks for both viruses were achieved in the HRM curve analysis for BoHV type identification. BoHV was identified in all of the isolates and clinical samples, and BoHV types were properly differentiated. Furthermore, viral DNA was detected in 12/18 and 7/18 samples from BoHV-1- and BoHV-5-infected calves, respectively. Real time PCR-HRM achieved a higher sensitivity compared with virus isolation or conventional PCR. In this study, HRM was used as a novel procedure. This method provides rapid, sensitive, specific and simultaneous detection of bovine alpha-herpesviruses DNA. Thus, this technique is an excellent tool for diagnosis, research and epidemiological studies of these viruses in cattle.

1. Introduction

Bovine herpesvirus types 1 (BoHV-1) and 5 (BoHV-5) are two closely related alpha-herpesviruses that infect cattle. Bovine herpesviruses (BoHV) are responsible for a variety of clinical syndromes including respiratory disease, conjunctivitis, abortion and genital infections. Whereas BoHV-1 is neuroinvasive and can sporadically cause encephalitis (Barenfus et al., 1963; d'Offay et al., 1993; Meyer et al., 2001), BoHV-5 is the primary aetiological agent of non-suppurative necrotizing meningoencephalitis in calves (Pérez et al., 2002). BoHV-5 has occasionally been isolated from aborted bovine foetuses (Schudel et al., 1986) as well as from the genital and respiratory tracts of cattle (Schudel et al., 1986; Esteves et al., 2003; Kirkland et al., 2009). BoHV-1 and BoHV-5 display a remarkable similarity in genetic, antigenic and biological aspects. Differentiation between BoHV-1 and BoHV-5 can be achieved by antigenic or molecular analysis (Ashbaugh et al., 1997; Claus et al., 2005; Silva et al., 2007). However, there is a need for highly sensitive and specific tests that are suitable for virus detection and differentiation, particularly in tissue samples. Although several strategies have been developed for rapid virus detection, classic cell culture is still "the gold standard" method (Bleotu et al., 2006) for isolation of bovine alpha-herpesviruses. However, due to the difficulties in differentiating both BoHV-1 and BoHV-5 in cell culture and by serological methods, classical virological techniques have limited value to differentiate both viral types. Additionally, virus isolation is less sensitive compared with other methods

Abbreviations: BoHV-1, Bovine herpesvirus type 1; BoHV-5, Bovine herpesvirus type 5; HRM, high resolution melting; T_m, melting temperature; MDBK, Madin–Darby Bovine Kidney cells; dpi, days post-infection; Ct, cycle threshold.

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(Takiuchi et al., 2005). Therefore, the technique employed for the diagnosis of these cattle alpha-herpesviruses should successfully detect and differentiate both virus types.

High resolution melting (HRM) analysis is a recently developed molecular technique. The development of HRM curve analysis as an extension to real time PCR provides a cost-efficient, close-tube system that allows for high-throughput analysis without any post-PCR processing (Wittwer et al., 2003). HRM analysis is based on the determination of fluorescence changes because of melting double-stranded PCR products in response to a temperature increase. The melting temperature (T_m) and the characteristic shape in the melting curve profile of the amplified products are highly dependent on the nucleotide sequence. A single base substitution can change the T_m of an amplified product. This change can be detected using instrumentation capable of real time fluorescence monitoring combined with variations in high-resolution temperature (Tong and Giffard, 2012).

Recently, real time PCR with HRM analysis has been effectively applied to detect and discriminate among animal pathogens (Rebelo et al., 2011; Douarre et al., 2012; Vorimore et al., 2012). Furthermore, the method was used for simultaneous detection and typing of herpes simplex virus in human clinical samples (Dames et al., 2007). However, there are no reports on the use real time PCR with HRM for identification of bovine alpha-herpesviruses. The aim of this study was to develop a simple and reliable one-step real time PCR assay with HRM analysis for the simultaneous detection and differentiation of BoHV-1 and BoHV-5. Furthermore, viral distribution in the respiratory tract in experimentally BoHV-1- and -5-infected calves was analyzed and discussed. The findings of this study might contribute to the knowledge and understanding of the tissue distribution of both viruses during primary replication and dissemination of bovine alpha-herpesviruses in the respiratory system.

2. Materials and methods

2.1. Cell line and culture conditions

Madin–Darby Bovine Kidney (MDBK) cells from American Type Culture Collection (ATCC, Rockville, MD, USA) were used in this study. MDBK cells were propagated in Minimum Essential Medium (Eagle), with Earle salts (MEM-E) (Sigma–Aldrich, Saint Louis, MO, USA) supplemented with 10% foetal bovine serum (Bioser, Buenos Aires, Argentina), which was free from viruses and antibodies and contained antibiotic–antimycotic solution (Gibco, Langley, OK, USA) including 100 U/ml penicillin G, 100 μg/ml streptomycin sulphate and 0.025 μg/ml amphotericin B. Cells were incubated at 37 °C in a 5% CO_2 atmosphere.

2.2. Viruses and samples

Strains used for this study included: reference strains Los Angeles (LA) (BoHV-1.1), Cooper (BoHV-1.1), N569 (BoHV-5a) and A663 (BoHV-5b), 10 Argentinean BoHV-1 field isolates as well as the Argentinean BoHV-5 field strain identified as 97/613, which had been previously described (Pérez et al., 2002; Marin et al., 2012), characterized and provided by the Specialized Veterinary Diagnostic Service, INTA Balcarce (Argentina). The Argentinean field strains were isolated from cattle with clinical–pathological signs that were compatible with BoHV infection. Virus identification was confirmed by virus isolation in cell culture followed by direct immunofluorescence using a polyclonal antibody against BoHV (American BioResearch, Sevierville, TN, USA) and two conventional PCR methods to differentiate BoHV-1 and BoHV-5 (Claus et al., 2005; Campos et al., 2009). Viral stocks were propagated in MDBK cells using T-175 flasks (Greiner Bio-one, Frickenhausen, Germany) for 24 h. Supernatants were harvested and stored at −80 °C until use. Virus titres were determined by the endpoint titration method and expressed as tissue culture infective doses ($TCID_{50}$), according to Reed and Müench (1938).

In total, five clinical samples in which BoHV DNA was detected by conventional PCR according to Claus et al. (2005) and Campos et al. (2009) were also included in this study. These samples were 1 spleen from an aborted foetus that was positive for BoHV-1 and -4 foetal brains that were positive to BoHV-5, which had been previously characterized by Marin et al. (2013). Furthermore, 45 samples from the respiratory tract of experimentally infected animals were studied as detailed below.

2.3. Experimental design and BoHV challenge

Five BoHV-1- and BoHV-5-free, seronegative crossbred, one year-old calves were used for this study. Calves were randomly assigned to the following experimental groups: Group 1 (BoHV-1, primary acute infection) composed of 2 calves that were intranasally inoculated with 10 ml $10^{6.3}$ $TCID_{50}$ BoHV-1 Cooper strain (Calf Nos. 1–2); Group 2 (BoHV-5, primary acute infection) composed of 2 calves that were intranasally inoculated with 10 ml $10^{6.3}$ $TCID_{50}$ of the BoHV-5 field strain 97/613 (Calf Nos. 3–4); Group 3 (mock-infected) included 1 calf that was intranasally inoculated with MEM-E as a placebo (Calf No. 5). All calves were euthanatized 6 days post-infection (dpi). Previous studies (Narita et al., 1976; Pérez et al., 2002) have demonstrated that these viral inocula caused acute infection. In this study, success of the experimental infection was demonstrated by detection of virus shedding in nasal and/or ocular secretions until 6 dpi (time of euthanasia). Animal handling and experimentation procedures were performed according to the Animal Welfare Committee of the University of the Center of Buenos Aires Province, Argentina (Res. 087/02). At necropsy, respiratory system samples were collected from each calf including epithelium of the nasal mucosa, trachea, bronchi, the apical, middle and diaphragmatic lobes of the lungs, and retropharyngeal, bronchial and mediastinal lymph nodes.

2.4. DNA extraction

Infected cell culture supernatants and tissue sample homogenates in 1X Hank's solution (10%, w/v) were clarified by centrifugation for 10 min at 1500 × *g* at 4 °C. DNA was extracted from 200 μl clarified viral isolates or tissue samples using a commercial kit (DNeasy Blood and Tissue Kit, Qiagen Inc., Valencia, CA, USA), as recommended by the manufacturer.

DNA from respiratory samples of experimentally infected animals was also obtained by extraction with the phenol:chloroform method to compare the efficiency with respect to the commercial method. Five hundred microlitres of homogenized tissue supernatants were placed in 1 ml lysis buffer (10 mM Tris–HCl, pH 7.4, 25 mM EDTA, pH 8, 100 mM NaCl, 0.5% SDS and 100 mg proteinase K [Promega, Madison, WI, USA]) and digested overnight at 37 °C. Total DNA was extracted with phenol:chloroform:isoamyl alcohol (25:24:1) and precipitated with 0.3 M sodium acetate and cold 70% ethanol. After incubation for 1 h at −80 °C, the nucleic acid solution was centrifuged at 12,000 × *g* for 30 min. The supernatant was discarded, and the pellet was rinsed with cold 70% ethanol. The pellet was vacuum dried for 10 min and re-suspended in 50 μl sterile water.

Sample DNA concentrations were measured by absorbance at 260 nm using an Epoch Microplate Spectrophotometer (BioTeK, Winooski, VT, USA).

Table 1
Primer pairs used in this study.

ID	Primer	5′–3′sequences	Amplicon size (bp)	Target region	Reference
BoHV-1 forward	Forward	TGTGGACCTAAACCTCACGGT	97	BoHV-1 and -5 glycoprotein B gene	Abril et al. (2004)
BoHV-1 reverse	Reverse	GTAGTCGAGCAGACCCGTGTC			
PF2	Forward	CGGCCACGACGCTGACGA	575/572	BoHV-1 and -5 glycoprotein C gene	Campos et al. (2009)
PR1	Reverse	CGCCGCCGAGTACTACCC			
PF	Forward	CTAACATGGAGCGCCGCTT	161	BoHV-1 glycoprotein C gene	Campos et al. (2009)
PR	Reverse	CGGGGCGATGCCGTC			
PF	Forward	GTGGAGCGCCGCTTCGC	236	BoHV-5 glycoprotein C gene	Campos et al. (2009)
PR	Reverse	TATCGCGGAGAGCAGGCG			
BoHV-1 forward	Forward	GGTACATGTCCAGGGAAAC	189	BoHV-1 and -5 DNA polymerase gene	Diallo et al. (2011)
BoHV-1 reverse	Reverse	GGTACAACATCGTCAACTTC			
VAC 1	Forward	ATTAAGGACATCTTAGGGGCCCTCT	134	Bovine cytochrome B gene	Santaclara et al. (2007)
VAC 2	Reverse	GGGTTTGATGTGAGGGGGTGTGTTG			

2.5. Internal control for real time PCR-HRM

The success of DNA extraction and the absence of inhibitory substances in the extracted DNA that might affect amplification was assessed by real time PCR amplification in a final volume of 20 μl using EvaGreen as an intercalating fluorescent dye (KAPA HRM FAST, Biosystems, Woburn, USA) and primers that amplify a 134 bp fragment of bovine cytochrome B DNA (Santaclara et al., 2007) (Table 1). The cycling programme consisted of an initial denaturation of 2 min at 95 °C and 40 cycles of 10 s at 95 °C, 15 s at 56 °C and 20 s at 72 °C. Samples with bovine cytochrome B cycle threshold (Ct) values below 35 were considered to be suitable for further analysis. The T_m of the bovine cytochrome B specific amplification fragment was 84 °C. All of the real time PCR reactions were performed in a Rotor Gene Q thermocycler in duplicate.

2.6. Optimization

To identify the most effective primer set for detecting both alpha-herpesviruses by real time PCR and differentiating BoHV-1 and BoHV-5 by using HRM curve analysis, three primer pairs were evaluated on DNA from BoHV reference strains using real time PCR-HRM. These primers are described in Table 1. The first primer set amplifies a 97 bp fragment from glycoprotein B gene of both alpha-herpesviruses (Abril et al., 2004), the second primer set amplifies a 575/572 fragment from BoHV-1 and BoHV-5 glycoprotein C gene, respectively (Campos et al., 2009) and the third primer set amplifies a 189 bp fragment from DNA polymerase gene of both alpha-herpesviruses (Diallo et al., 2011), as tested by BLAST bioinformatics analysis at the website of the National Institutes of Health (NIH) (NCBI – National Center for Biotechnology Information, NIH, USA). The primers described by Diallo et al. (2011) (Table 1), were selected to optimize the assay and to analyze the viral isolates and the clinical and experimental samples in the present work. The BoHV-1 (accession number AJ004801.1) and BoHV-5 (accession number NC_005261.2) sequences obtained from the GenBank database at the website of the NIH (NCBI – National Center for Biotechnology Information, NIH, USA), that corresponded to the target amplified fragment with the chosen pair primers, were aligned and compared using the MAFFT multiple sequence alignment programme (Katoh and Standley, 2013). Assay conditions were optimized using varying primer concentrations, annealing temperatures and times. Artificial in vitro mixes containing BoHV-1 and -5 DNA were made and analyzed using real time PCR-HRM as detailed below to determine whether the selected primer pair could differentiate between BoHV types in case of dual field infections.

2.7. General real time PCR-HRM procedure

Real time PCR assays to detect BoHV-1 and -5 DNA were performed using primers that amplify a 189 bp fragment from the DNA polymerase gene of BoHV-1 and BoHV-5 (Diallo et al., 2011) (Table 1). This primer set was selected based on its higher efficiency and capacity for differentiating between BoHV-1 and BoHV-5 DNA, as described in Section 3.1. Real time PCR reactions were performed in a Rotor Gene Q thermocycler in a final volume of 20 μl using EvaGreen as an intercalating fluorescent dye, containing 640 nM forward and reverse primers, 1× PCR Master Mix (KAPA HRM FAST Master Mix, Biosystems, Woburn, USA) and 2 μl of DNA sample. The cycling programme consisted of an initial denaturation of 3 min at 95 °C and 50 cycles of 15 s at 95 °C, 15 s at 58 °C and 20 s at 72 °C. In all cases, the experiments were performed in duplicate. Amplification was followed by a high resolution melting curve analysis; BoHV identification was performed using Rotor Gene Q software, version 1.7.94. The genotype confidence cut off value of 93% was set up in the software genotyping module.

2.8. Real time PCR-HRM sensitivity and specificity

Analytical sensitivity of the real time PCR-HRM procedure was determined using BoHV-1 and BoHV-5 reference strains grown and titrated on MDBK cells. For this purpose, ten-fold dilutions (from 1×10^5 to 1×10^1 viral particles) were performed and DNA from each dilution was extracted. To confirm the number of virus particle present in the diluted sample, virus titres were corroborated by titration on MDBK cells, as described in Section 2.2. For analytical specificity, Bovine herpesvirus type 4 (BoHV-4) and a range of bacteria that infect cattle including *Campylobacterfetus venerealis*, *Brucella abortus* and *Leptospira interrogans* serovars canicola and pomona and the parasites *Trichomonas fetus* and *Neospora caninum* were tested using the real time PCR-HRM assay.

This technique aimed to simultaneously detect and differentiate between BoHV-1 and BoHV-5. Therefore, the criteria for a positive result by real time PCR-HRM included the analysis of the amplification plot and the shape of the melting peak, i.e., a reaction was considered positive only when the melting curve allowed for unequivocal identification of the BoHV type and when the amplification plot was positive.

2.9. Virus isolation and conventional PCR

The real time PCR-HRM was compared with virus isolation and conventional PCR techniques using tissue samples from experimentally infected animals.

2.9.1. Virus isolation

Tissue samples were homogenized in 1× Hank's solution (10%, w/v), and the suspensions were centrifuged at 1000 × *g* for 15 min at 4 °C. Fifty microlitres of supernatant were inoculated in duplicate into MDBK cells in 96-well plates (GreinerBio-one, Frickenhausen, Germany) and incubated at 37 °C. Samples were passaged 4–6 times every 3 days and monitored daily for the presence of cytopathic

effects. At the end of each passage, samples were tested by direct immunofluorescence using a polyclonal antibody against BoHV conjugated with fluorescein isothiocyanate (American BioResearch, Sevierville, TN, USA).

2.9.2. Conventional PCR

For conventional PCR, DNA was extracted from tissue samples with a commercial kit (DNeasy Blood and Tissue Kit, Qiagen Inc., Valencia, CA, USA). PCR was performed as described by Campos et al. (2009). Primers used in the first PCR round anneal to recognize both BoHV-1 and BoHV-5 and they amplify a region of 575 and 572 bp of glycoprotein C gene, respectively (Table 1). The first PCR reaction (25 ml) contained 1 mM $MgCl_2$ (Promega, Madison, WI, USA), 0.3 mM each primer, 10% dimethylsulfoxide (DMSO), 1 U Taq DNA polymerase (Promega, Madison, WI, USA), 10% PCR buffer (Promega, Madison, WI, USA) and 0.6 mM deoxynucleoside triphosphates per reaction. Cycling conditions were: 5 min at 94 °C; followed by 35 cycles of 1 min at 94 °C, 1 min at 62 °C, 1 min at 72 °C; followed by 5 min at 72 °C. To differentiate BoHV-1 and BoHV-5, two type-specific PCRs were performed using the product of the first PCR round as a template. To detect BoHV-1 DNA the primer pair which amplifies a 161 bp product (Table 1) was used. To detect BoHV-5 a primer pair which gives a product of 236 bp (Table 1) was used. The reaction conditions for type-specific PCRs were the same as for the first PCR round, with exception of the primers concentration, which was 0.2 mM. The cycling conditions were: 94 °C for 5 min, followed by 35 cycles of 1 min at 94 °C, 1 min at 61 °C and 1 min at 72 °C and a final extension time of 5 min at 72 °C. All tests were performed in duplicate.

2.10. Statistical analysis

Concordance between the three methodologies was assessed using Cohen's Kappa coefficient. The difference among coefficients was calculated using the Chi-square test. Differences were considered to be significant when the P value was <0.05. All analyses were performed with Epidat 3.0 software (Panamerican Health Organization [PHO]/World Health Organization [WHO]).

3. Results

3.1. Assay design and optimization

The primers described by Diallo et al. (2011) were selected for assay optimization because of their reaction efficiency and higher capacity for BoHV type differentiation, when compared to the other tested primers. The reaction efficiency obtained for the amplification of the genome of both viruses with the selected primers was 95% while with the other primer sets it was lower than 90%. The primer set described by Campos et al. (2009), which amplifies a larger fragment, was not able to generate an appropriate amplification plot by real time PCR. Therefore it is not considered suitable for further analysis by HRM. As is detailed below, T_m differences between both viruses was of 1.4 °C with the primers described by Diallo et al. (2011). However it was only of 1.1 °C with the primers described by Abril et al. (2004). This shows that with the selected primers a larger difference between viruses T_m is achieved. The rate of change in relative fluorescence units (RFU) with time (T) [$-d(\text{RFU})/dT$] vs temperature (°C), also was higher with the primers described by Diallo et al. (2011) than with those designed by Abril et al. (2004) (2 vs 1, respectively). The confidence percentage for differentiating both viruses was 93% with the selected primers and only 50% with the primers by Abril et al. (2004). The experimental primer set allowed the simultaneous detection of BoHV-1 and BoHV-5 by real time PCR. Furthermore, with these primers it was possible to differentiate BoHV types using HRM analysis of the PCR amplicons based on the differences in the sequence within the target region. The absence of unspecific products in agarose gels was also corroborated. Amplified fragment sequence variation and alignment for BoHV-1 and BoHV-5 are summarized in Fig. 1. A few point nucleotides differed between both viruses throughout the amplified sequences, detecting 14 different nucleotides.

Once the primer pair was selected, following subsequent optimization steps, it was possible to obtain an efficient amplification strategy by real time PCR (data not shown), a clear melting curve for differentiating BoHV-1 and BoHV-5 based on the differences in the sequence within the target region (Figs. 2 and 3), and appropriate melting peaks for both PCR amplicons (Fig. 4). Fig. 2 displays normalized melting curves for the two BoHV types, in which it is possible to observe a characteristic profile for each BoHV type. In Fig. 3, HRM data were plotted as difference curves to visually magnify differences between the melting profiles of the different viral types within the same genotype. Curve differences were magnified by subtracting each curve from a user-defined reference, which in this case was the melting profile of a BoHV-1 strain which was chosen as a horizontal baseline. Finally, melting peaks for the two BoHV types, and the corresponding melting temperatures are shown in Fig. 4. The PCR primer concentrations as well as the annealing temperature and time were critical assay parameters. The optimal concentration for both forward and reverse primers was 640 nM, and the most suitable annealing temperature was 58 °C. Using the virus reference strains as positive controls, the melting points obtained for BoHV-1 and BoHV-5 were 89.7 °C and 91.1 °C, respectively, using the optimized conditions (Fig. 4). These results demonstrated that BoHV types could be distinguished by the characteristic T_m shift in the melting curves. Moreover, from dual artificial mixes of BoHV-1 and BoHV-5 DNA, it was possible to obtain distinguishable melting peaks for each BoHV type, confirming that the selected primer pair can differentiate mixed infections (data not shown).

3.2. Detection of BoHV-1 and BoHV-5 in field isolates and clinical samples

A summary of BoHV-1 and BoHV-5 detection from isolates and clinical samples is presented in Table 2. The concentration of DNA from the different isolates ranged from 10 to 150 ng/μl and for tissues samples the DNA recovered was in the range of 300–3000 ng/μl. BoHV was identified in all isolates and clinical samples from our collection when assessed by real time PCR-HRM assay and BoHV types were properly differentiated. The HRM analysis results were consistent with viral isolation and conventional PCR data. Furthermore, viral DNA detection was successful in samples in which the virus could not be isolated.

3.3. BoHV-1 and BoHV-5 detection in tissues from experimentally infected animals

The protocol for DNA extraction from respiratory tissues with phenol:chloroform yielded inconsistent results, with DNA samples giving Ct values of 35 or higher for the internal control. Using this method, only two respiratory samples corresponding to retropharyngeal lymph nodes and tracheal epithelium (Calf No. 3) were positive for BoHV-5 using the real time PCR-HRM assay. Thus, DNA extraction from tissue samples was performed with a commercial kit, which yielded DNA suitable for further analysis.

The results of viral DNA detection in different sections of the respiratory tract of BoHV-1- and BoHV-5-infected calves as assessed using the real time PCR-HRM assay is presented in Table 2. BoHV-1 DNA was detected in 12/18 samples from BoHV-1-infected calves, including bronchial lymph nodes, epithelium of nasal mucosa, trachea and bronchi (2/2), and it was also frequently

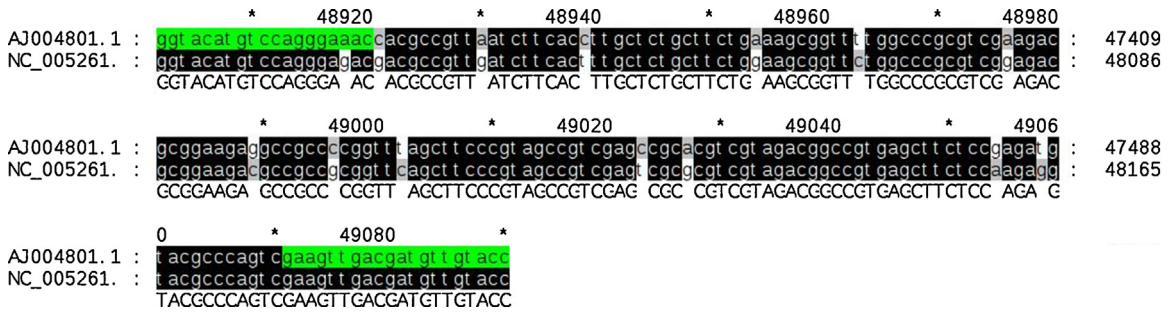

Fig. 1. Nucleotide sequence alignment of the amplified fragment of BoHV-1 (AJ004801.1) and BoHV-5 (NC_005261.2) DNA polymerase genes using MAFFT multiple sequence alignment software. The forward primer (5′-GGTACATGTCCAGGGAAAC-3′) and the complementary sequence of the reverse primer (5′-GGTACAACATCGTCAACTTC-3′) are highlighted. Different nucleotides between both sequences are indicated in grey.

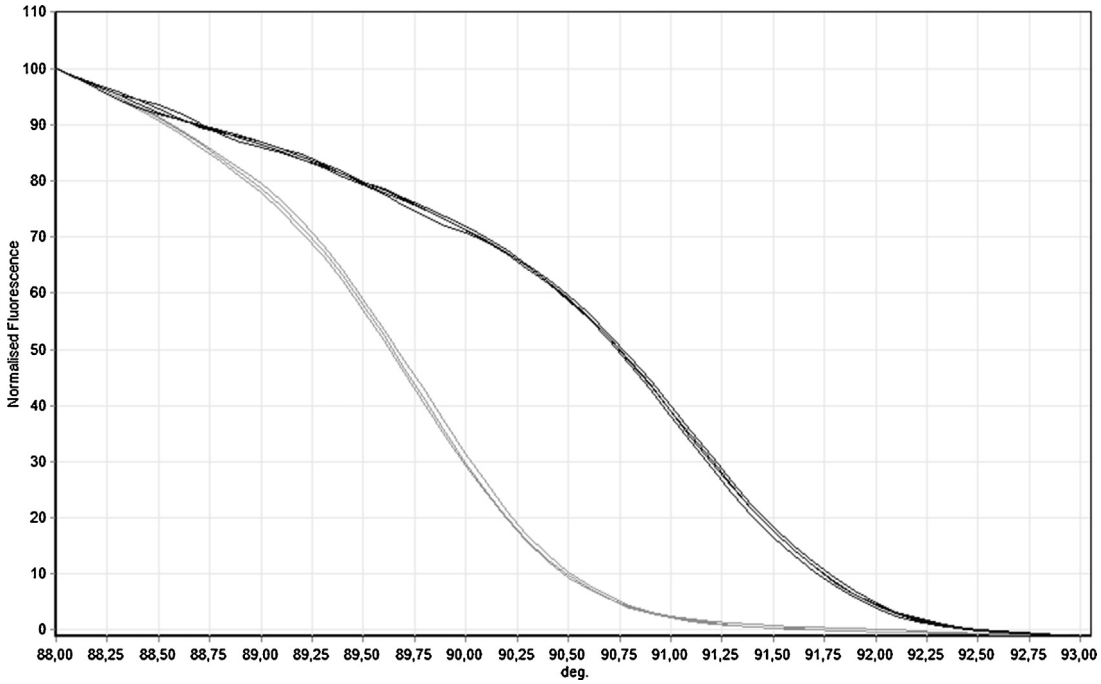

Fig. 2. Normalized graph illustrating BoHV typing using high resolution melting in a Rotor Gene Q thermocycler (Rotor Gene Q software, version 1.7.94). BoHV-1 (grey curves) and BoHV-5 (black curves) reference strains and randomly selected samples were tested. The figure displays normalized melting curves for the two BoHV types.

detected in retropharyngeal lymph nodes, as well as the apical, middle and diaphragmatic lobes of the lungs (1/2). BoHV-1 DNA was not detected in mediastinal lymph nodes. BoHV-5 DNA was detected in 7/18 samples from BoHV-5-infected calves, including retropharyngeal lymph nodes and tracheal epithelium (2/2). Furthermore, it was possible to detect virus DNA in mediastinal lymph nodes as well as the nasal mucosa and bronchial epithelium (1/2). BoHV-5 DNA was not detected in bronchial lymph nodes or lungs. As expected, viral DNA was not detected in any of the 9 samples from uninfected control animals.

The detection rate achieved with real time PCR-HRM was higher compared with virus isolation or conventional PCR. Out of 45 samples from the respiratory tract, it was possible to detect BoHV DNA in 19 samples by real time PCR-HRM. However, by conventional PCR 14 samples were positive, and infectious virus was only detected in 5 samples (Table 2). There were no discrepancies in the HRM analysis results in relation to the other techniques, i.e., all samples that were positive by any of the conventional methodologies were positive when evaluated by HRM. The concordance coefficient between real time PCR-HRM and conventional PCR was 0.76, between real time PCR-HRM and virus isolation was 0.29 and between conventional PCR and virus isolation was 0.43. Significant differences were observed when comparing the three techniques against each other ($P < 0.05$). Real time PCR-HRM had the highest sensitivity compared with the other methodologies.

3.4. Sensitivity

Analytical sensitivity of the method was assessed by amplifying ten-fold dilutions of viral DNA from BoHV-1 and BoHV-5 reference strains. The real time PCR-HRM assay allowed the detection of both BoHV-1 and BoHV-5, even when DNA from 10 viral particles was assayed. Further testing of the capacity of the real time PCR-HRM assay to detect and differentiate BoHV-1 and BoHV-5 demonstrated that all BoHV isolates including the reference and field strains as well as tissue samples were positive for the corresponding virus. Moreover, evaluation of samples containing BoHV-1 and BoHV-5 DNA demonstrated that the sensitivity of the assay was similar to that obtained with samples containing DNA from only one virus type. There was no evidence of reaction inhibition; DNA from BoHV-1 or BoHV-5 was amplified by the specific primers, and the appropriate melting curve was obtained, similar to reactions containing BoHV-1 or BoHV-5 DNA alone.

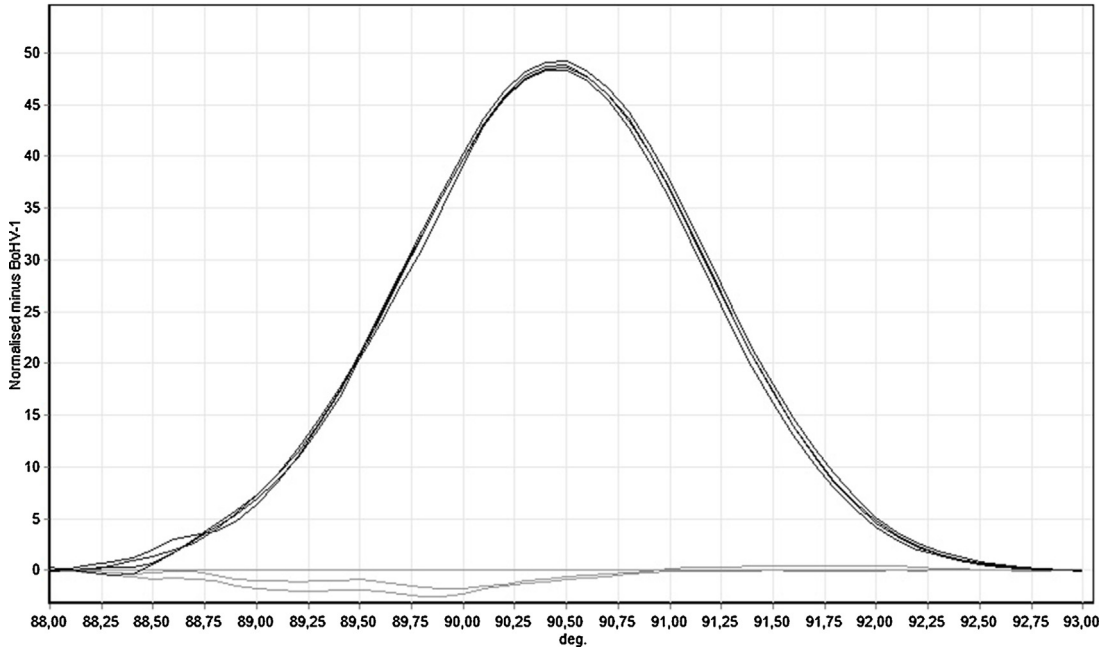

Fig. 3. Normalized temperature-shifted difference graph illustrating BoHV typing using high resolution melting in a Rotor Gene Q thermocycler (Rotor Gene Q software, version 1.7.94). The melting profile of a BoHV-1 strain was chosen as a horizontal baseline. BoHV-1 (grey curves) and BoHV-5 (black curves) reference strains and randomly selected samples were tested.

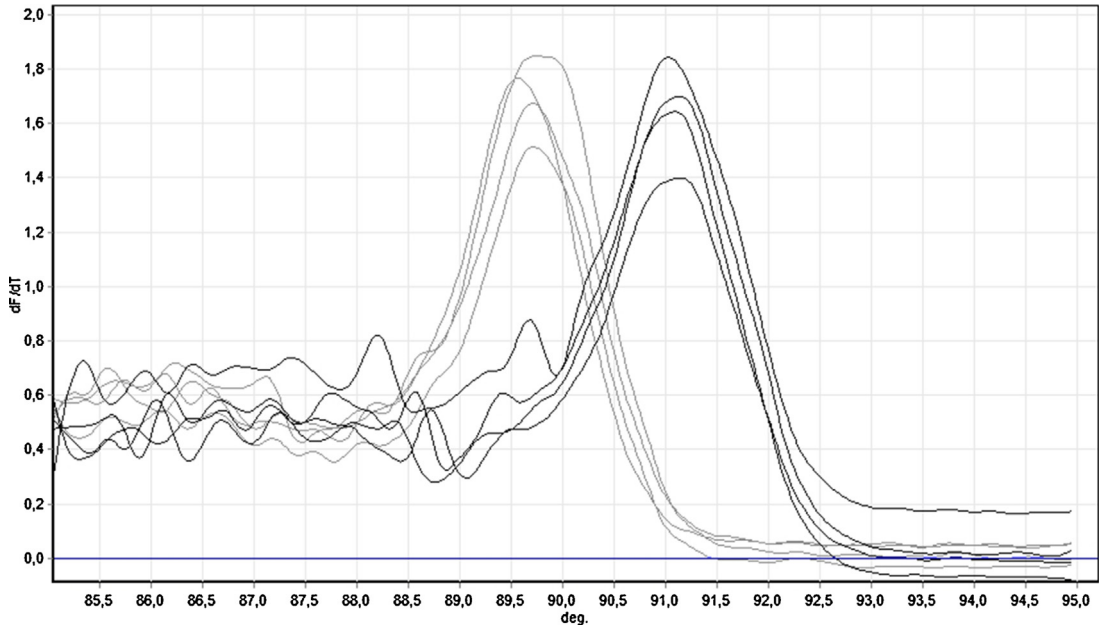

Fig. 4. Melting peaks for the two bovine alpha-herpesvirus types using high resolution melting in a Rotor Gene Q thermocycler (Rotor Gene Q software, version 1.7.94). Reference strains and randomly selected samples were tested. Melting temperatures were 89.7 °C and 91.1 °C for BoHV-1 (grey curves) and BoHV-5 (black curves), respectively.

3.5. Specificity

There was no cross-reaction between BoHV-1 and BoHV-5 DNA detection. DNA from BoHV field isolates, reference strains and tissue samples produced only their respective melting peak. To ensure that there was no cross-reaction in the detection of each virus, mixed DNA samples were tested, and they had distinguishable melting peaks, as expected. Furthermore, DNA from BoHV-4 and all bacteria and parasites analyzed were negative. Amplification was not detected when negative samples from uninfected animals were evaluated.

4. Discussion

BoHV-1 and BoHV-5 can be isolated from very similar clinical cases (Silva et al., 2007). Therefore, the association of these viruses with their respective clinical syndromes appears not to be definitive and mutually exclusive. This finding, together with the existence of mixed infections in cattle, indicates the need for correctly identifying BoHV field isolates to achieve an accurate final aetiological diagnosis and to better understand their epidemiology and pathogenesis. In this study, HRM was used as a novel procedure for

Table 2
Detection of BoHV-1 and BoHV-5 in field isolates, clinical samples and tissues from experimentally infected animals using viral isolation, conventional PCR and real time PCR-HRM.

Isolate/clinical sample/calf	Sample	BoHV type	Virus isolation	Conventional PCR		Real time PCR-HRM	
				BoHV-1	BoHV-5	BoHV-1	BoHV-5
Field isolate 98/759	Nasal and ocular swabs	1	+	+	–	+	–
Field isolate 01/156	Vaginal discharge	1	+	+	–	+	–
Field isolate 01/211	Vaginal discharge	1	+	+	–	+	–
Field isolate 02/221	Vaginal discharge	1	+	+	–	+	–
Field isolate 02/701	Preputial swabs	1	+	+	–	+	–
Field isolate 03/41	Vaginal discharge	1	+	+	–	+	–
Field isolate 03/134	Nasal and ocular swabs	1	+	+	–	+	–
Field isolate 03/404	Vaginal discharge	1	+	+	–	+	–
Field isolate 09/210	Nasal swabs	1	+	+	–	+	–
Field isolate 12/255	Ocular swabs	1	+	+	–	+	–
Field isolate 97/613	Brain	5	+	–	+	–	+
Clinical sample 04/536	Foetal spleen	1	–	+	–	+	–
Clinical sample 07/415	Foetal brain	5	–	–	+	–	+
Clinical sample 07/447	Foetal brain	5	–	–	+	–	+
Clinical sample 07/487	Foetal brain	5	–	–	+	–	+
Clinical sample 08/523	Foetal brain	5	–	–	+	–	+
Calf No. 1	Retropharyngeal lymph node	1	–	+	–	+	–
	Bronchial lymph node		–	–	–	+	–
	Mediastinal lymph node		–	–	–	–	–
	Epithelium of nasal mucosa		+	+	–	+	–
	Tracheal epithelium		–	+	–	+	–
	Bronchial epithelium		–	+	–	+	–
	Apical lung lobe		–	–	–	+	–
	Middle lung lobe		–	–	–	–	–
	Diaphragmatic lung lobe		–	–	–	–	–
Calf No. 2	Retropharyngeal lymph node	1	–	–	–	–	–
	Bronchial lymph node		–	–	–	+	–
	Mediastinal lymph node		–	–	–	–	–
	Epithelium of nasal mucosa		+	+	–	+	–
	Tracheal epithelium		+	+	–	+	–
	Bronchial epithelium		–	+	–	+	–
	Apical lung lobe		–	–	–	–	–
	Middle lung lobe		–	+	–	+	–
	Diaphragmatic lung lobe		–	–	–	+	–
Calf No. 3	Retropharyngeal lymph node	5	+	–	+	–	+
	Bronchial lymph node		–	–	–	–	–
	Mediastinal lymph node		–	–	–	–	+
	Epithelium of nasal mucosa		–	–	–	–	–
	Tracheal epithelium		+	–	+	–	+
	Bronchial epithelium		–	–	–	–	–
	Apical lung lobe		–	–	–	–	–
	Middle lung lobe		–	–	–	–	–
	Diaphragmatic lung lobe		–	–	–	–	–
Calf No. 4	Retropharyngeal lymph node	5	–	–	+	–	+
	Bronchial lymph node		–	–	–	–	–
	Mediastinal lymph node		–	–	–	–	–
	Epithelium of nasal mucosa		–	–	+	–	+
	Tracheal epithelium		–	–	+	–	+
	Bronchial epithelium		–	–	+	–	+
	Apical lung lobe		–	–	–	–	–
	Middle lung lobe		–	–	–	–	–
	Diaphragmatic lung lobe		–	–	–	–	–
Calf No. 5	Retropharyngeal lymph node	Control	–	–	–	–	–
	Bronchial lymph node		–	–	–	–	–
	Mediastinal lymph node		–	–	–	–	–
	Epithelium of nasal mucosa		–	–	–	–	–
	Tracheal epithelium		–	–	–	–	–
	Bronchial epithelium		–	–	–	–	–
	Apical lung lobe		–	–	–	–	–
	Middle lung lobe		–	–	–	–	–
	Diaphragmatic lung lobe		–	–	–	–	–

analysis and differentiation of BoHV types in cell culture as well as in tissue samples.

Several studies have been performed using conventional PCR for differentiating BoHV-1 from BoHV-5 (Alegre et al., 2001; Wang et al., 2001; Claus et al., 2005; Campos et al., 2009). Campos et al. (2009) described a nested PCR that amplified BoHV-1 DNA as well as BoHV-5 DNA in the first round followed by two type-specific PCRs to discriminate between BoHV-1 and BoHV-5 DNA (one per virus). However, the authors have demonstrated that some samples may contain concentrations of viral DNA which are below the PCR detection limit. Moreover, this technique is also a nested PCR that requires the use of agarose gels similar to all conventional PCRs, and special care to prevent cross-contamination must be taken. The primers employed in the first round of PCR amplified a fragment

of 572/575 bp for BoHV-1 and BoHV-5. This set of primers was evaluated for application by HRM analysis. However, poor reaction efficiency was obtained, probably due to the amplified fragment length.

Real time PCR greatly reduced the risk of contamination compared with conventional PCR. It is a rapid, reliable and quantitative detection method, which is more sensitive than conventional PCR. Real time PCR has been successfully applied for differential diagnosis of BoHV-1 and BoHV-5 (Abril et al., 2004; Diallo et al., 2011). However, this technique required the use of a set of primers and probe for each viral type. Therefore, in this study, the primer efficiency described for this real time PCR was evaluated with the purpose of selecting a primer pair that identifies both BoHV types, which allowed their discrimination by the application of HRM analysis. Finally, the selection of a universal primer set (Diallo et al., 2011) that generated an amplicon suitable for HRM analysis for both bovine alpha-herpesviruses was achieved. The correct primer choice is critical when increasing variant resolution because small sequence variations can dramatically affect the results (Erali and Wittwer, 2010). Due to the high nucleotide sequence identity between these viruses, the primers can detect them simultaneously, which makes the technique rapid, simple and inexpensive. Furthermore, the use of this new methodology exploits the possibility of differentiating these pathogens by their slight differences in specific nucleotides, as observed in the amplified fragment alignment. The alignment reveals differences in only 14 nucleotides, giving melting peaks that are completely distinguishable for each virus.

In this study, the viral DNA extraction and the real time PCR-HRM assay protocols were validated. This assay was highly sensitive and could detect the BoHV-1 and BoHV-5 genome even when DNA from only 10 viral particles was used. The test was also sensitive enough to detect BoHV DNA in all of the samples that had been previously characterized as positive by virus isolation and/or conventional PCR. The assay is also highly specific, as unique amplifications of BoHV-1 and BoHV-5 were obtained, i.e., cross-reactions were not detected. Furthermore, DNA amplification was not detected from herpesviruses from a different subfamily, bacteria or parasites. An additional advantage of this assay is its suitability for viral detection in tissue samples. It can also be used as a confirmatory or identification test for viruses isolated in cell culture without needing to grow the virus to high titres before performing the test. This technique also allows the identification of both BoHV types in one sample, which is relevant for detection of dual infections, which can frequently occur in nature (Campos et al., 2009). The use of an internal control is important to monitor for false-negative results due to nucleic acid extraction failure or presence of inhibitor components in the reaction. The use of such an internal control, as it was achieved in the present study, ensured that DNA extracts from “difficult” samples were PCR-competent, avoiding the reporting of false-negative results and increasing assay robustness.

Additionally, these results demonstrate the relevance of evaluating different DNA extraction protocols when a new methodology is applied. The discrepancy between the phenol:chloroform method and the commercial kit is likely due to the quality of the DNA sample obtained. During phenol:chloroform extractions, traces of chemicals may inhibit the real time PCR reaction, interfere with dye incorporation or modify the thermal properties of the mixture, which might affect the melting profile of the sample.

Little is known about the behaviour of different alpha-herpesviruses regarding host invasion at primary replication sites such as the respiratory tract. The ability of our novel approach to detect and differentiate BoHV types in samples of the respiratory tract of experimentally infected-calves was further assessed. BoHV-1 DNA exhibited a wide distribution in the bovine respiratory system. With the only exception of mediastinal lymph nodes, the virus genome was detected in all other samples evaluated. As expected, BoHV-5 distribution in the respiratory tract was more restricted. The virus only reached the upper respiratory tract, and viral DNA was not detected in bronchial lymph nodes and lungs. Alpha-herpesviruses are known for their efficient cell-to-cell transmission (Muylkens et al., 2007; Nauwynck et al., 2007). According to Steukers et al. (2012) epithelial basal cells and fibroblasts are in close proximity in the trachea. Taken together with the observed smaller reticular lamina thickness, this could explain why virus invasion of the trachea and bronchi is more efficient. These findings emphasize the involvement of these tissues in the respiratory syndrome caused mainly by BoHV-1 and also correlate with the in vivo situation. After primary infection by intranasal inoculation, BoHV-1 and BoHV-5 replicate at the portal of entry in the respiratory mucosa (Bagust and Clark, 1972; Meyer et al., 2001). These findings confirmed that BoHV-1 and BoHV-5 display similar biological features after primary infection and consequently need to be considered together in the control of BoHV infections.

Additionally, the real time PCR-HRM results were compared with those obtained by viral isolation and conventional PCR. As expected, real time PCR-HRM demonstrated the highest sensitivity compared with the other methodologies.

Real time PCR-HRM provides a rapid, sensitive and specific detection of bovine alpha-herpesviruses DNA. Furthermore, it can be useful for simultaneous detection of two bovine alpha-herpesvirus types. Thus, this technique is an excellent tool for diagnosis, research and epidemiological studies of these viruses in cattle.

Conflict of interest statement

The authors declared no potential conflicts of interest with respect to the research, authorship, and publication of this article and/or financial and personal relationships that could inappropriately influence this work.

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