Acta Scientiae Veterinariae, 2024. 52: 1954.

RESEARCH ARTICLE
Pub. 1954

ISSN 1679-9216

Detection of BoHV-1 - Comparative Evaluation of Real Time PCR, Real Time LAMP and Subtyping

Hatice Pelin Aslim & Oya Bulut

ABSTRACT

***Background*:** Bovine Herpesvirus 1 (BoHV-1) is an important bovine pathogen found throughout the world. It causes a subclinical, mild, or severe disease. It has different types of clinical manifestations in respiratory, genital, ocular, and encephalomyelitis forms. The aim of this study was to perform comparative diagnosis of BoHV-1 by Real Time PCR and Real Time LAMP and molecular characterization of BoHV-1.

***Materials, Methods & Results*:** In this study, BoHV-1 was investigated in nasal swab samples from cattle with clinical signs of respiratory problems using Real-Time PCR (Polymerase Chain Reaction) methods and the Real-Time Loop-Mediated Isothermal Amplification (LAMP) method, which is a cheap, fast, and reliable technique. BoHV-1 positive samples in both tests were cultured in the Madin-Darby Bovine Kidney (MDBK) cell culture. Conventional PCR was performed for the molecular characterization of the isolated samples. Eight (5.33%) nasal swab samples used in the study were found to be positive by the Real-Time PCR test, while 13 (8.66%) samples were found to be positive by the LAMP test. The results of the McNemar test showed that the difference between Real-Time PCR and Real-Time LAMP tests was not significant ($P > 0.05$). BoHV-1 was isolated from 3 of 8 samples cultured in cell culture. In the sequence analysis of these three samples after PCR, molecular characterization of the isolates was performed, and they were found to belong to the BoHV-1.1 subtype. These findings indicated that the LAMP method can be used in the rapid diagnosis of BoHV-1 infection, and the BoHV-1.1 subtype should be considered in the fight against infection.

***Discussion*:** Many studies have shown that PCR tests are more sensitive than virus isolation (VI). Similarly, in this study, only 3 of the 8 samples found to be positive by Real-Time PCR could be isolated in cell culture. The results of both tests differed in that PCR can detect any viral RNA (infectious whole virus, unpackaged viral genome, even genome strand or subgenomic fragments) whereas VI detects properly packaged and live virus particles. Although no statistical difference was recorded between Real-Time PCR and Real-Time LAMP in this study, a higher rate of positive samples was detected with LAMP, which matched the findings of other reports. These results showed that LAMP is a more resistant to PCR inhibitors and more sensitive test and due to the use of 6 different primers. BoHV-1 continues to cause significant economic losses in the cattle industry around the world. Therefore, a practical, fast, and cost-effective diagnostic tool needs to be used to identify the infection in the affected areas and control it at the earliest. In this context, the LAMP test is a promising method at the field level for the diagnosis and control of BoHV-1 infection and other diseases, as mentioned in other studies. Phylogenetic analysis was performed on 3 swab samples isolated in cell culture, after they were tested by conventional PCR with primers specific to the BoHV-1 gC region, in parallel with other reports. The isolate sequence was aligned using MUSCLE, with the sequences of other isolates obtained by a comparative analysis and multiple sequence alignment was performed in the AliView software. This analysis included the nucleotide sequences of 54 different isolates. All ambiguous locations were removed for each sequence. There were 312 nucleotides in the final dataset. Evolutionary analysis was performed using the Tamura 3-parameter model with the Neighbor-Joining algorithm in MEGA 11; in total, 1,000 iterations (bootstrap) were performed. Similar to the findings of other studies conducted in Turkey, it was determined in this study that the more common BoHV-1 strain in circulation was the BoHV-1.1 strain.

Keywords: BoHV-1, molecular characterization, Real-Time LAMP, Real-Time PCR.

DOI: 10.22456/1679-9216.139938

Received: 17 May 2024 Accepted: 29 June 2024 Published: 23 July 2024

Deparment of Virology, Faculty of Veterinary Medicine, Selcuk University-Konya, Turkey. CORRESPONDENCE: H.P. ASLIM [hpelinucan@gmail.com]. Department of Virology, Faculty of Veterinary Medicine, Selçuk University. 42003 Selçuklu, Konya, Turkey.

INTRODUCTION

Infectious Bovine Rhinotracheitis (IBR) is a viral disease of cattle caused by Bovine Herpesvirus type 1 (BoHV-1) [6,16]. BoHV-1 belongs to the genus *Varicellovirus* of the Herpesviridae family. [20]. BoHV-1 is divided into 3 subtypes: 1.1, 1.2a, and 1.2b [47]. The main clinical signs of BoHV-1 infection include high body temperature, harsh and persistent cough, and mild anorexia [18,27,30].

BoHV-1 has been eradicated from a few regions in Europe, such as Austria, Denmark, Finland, Sweden, Switzerland, and Norway. However, infection caused by BoHV-1 is widely distributed in beef and dairy cattle herds throughout the world [2,7,20,21,26,40]. Real-time PCR is used as a standard diagnostic procedure for the diagnosis of acute BoHV-1 infections. However, it is a time-consuming and expensive technique and requires specialized equipment. Therefore, simpler, faster, and cheaper applications, such as loop-mediated isothermal amplification (LAMP), have been developed for a wide range of pathogens [24,28]. It is a more sensitive and specific technique compared to PCR, given that it can amplify more than 10^9 copies of DNA in 1 hour [13,29].

This study aimed to the presence of BoHV-1 in nasal swab samples of cattle showing clinical signs of respiratory infection was comparatively assessed by Real-Time Polymerase Chain Reaction (Real-Time PCR) methods and Real-Time Loop-Mediated Isothermal Amplification (Real-Time LAMP) method. Additionally, the molecular characterization of the isolated samples was performed based on the gC gene sequences by culturing the samples that were positive for BoHV-1 in both tests in the Madin-Darby Bovine Kidney (MDBK) cell culture.

MATERIALS AND METHODS

Virus isolation materials

Nasal swab samples were collected from 150 cattle brought to the private slaughterhouse. The samples were transferred to 4 mL of DMEM containing concentrated antibiotic and centrifuged at 380-860 *g* at 4°C for 20 min.

Virus isolation

Madin Darby Bovine Kidney (MDBK) permanent cell culture and Colorado reference strain (as positive control) were used to conduct experiments in the Department of Virology, Faculty of Veterinary Medicine, Selcuk University. Nasal swab samples, which were positive in both Real-Time PCR and Real-Time LAMP tests, and the Colorado reference strain were inoculated by the adsorption inoculation technique. When BoHV-1 specific cytopathogenic effect (CPE)s was detected, the flasks were frozen thrice at -20°C and thawed at 37°C. The supernatant was centrifuged at 860 *g* at 4°C for 30 min. After ensuring that the supernatant was sterile, it was divided into 2 mL portions and stored at -20°C. Blind passages of the samples were made 3 times each. In tissue culture microscopy controls, the supernatant collected from cells with CPE was separated for sequencing by conventional PCR techniques. Virus control and cell control were performed together.

Preparation of samples for Real-Time PCR and Real-Time LAMP

-DNA extraction from nasal swab

To extract DNA, the tissue DNA Kit[1] was used. The DNA obtained was stored at -20°C until further use.

-Real-Time PCR

The presence of BoHV-1 nucleic acid in 150 nasal swab samples was determined by Real-Time PCR analysis using the LightCycler Probes Master Mix[2]. Primers, probe, reaction mix, and thermal cycling program used in the reaction were as follows. Genomic DNA extracts were analyzed with F (5-TGT GGA CCT AAA CCT CAC GGT-3') and R (5-GTA GTC GAG CAG ACC CGT GTC-3') primers [1], and the gB region of BoHV-1 was amplified. All reactions were adjusted to a final volume of 25 µL. For the reaction, 12.5 µL of 2X iGreen Master Mix, 0.5 µL of each specific primer (0.24 µM), 0.5 µL of probe, 5 µL of DNA product, and 6 µL of nuclease-free water were used. The Real-Time PCR program included initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 10 s, annealing at 60°C for 30 s, and extension at 72°C for 1 s.

-Real-Time LAMP

The presence of BoHV-1 was determined in 150 nasal swab samples by Real-Time LAMP analysis using the Isothermal Master Mix[3] Kit. The primers Pawar [31] used in this analysis were specific for the gC region of BoHV-1, F3 (5'-GTTCGACGGAAACAGCGG -3'), B3 (5'-CT-

GTTCGTAGCCCAGAACG-3'), FIP (5'-ACCGA GGGCGTGTGTACACTTGG+CGTGA CGGT-CACGACCTTG-3'), BIP (5'-GGTCAGGGGCAA GTTTGCGGG+CGATCG CGTACCGTAGCG-3'), LF (5'-GCGACATGGGCACCAA-3'), and LB (5'-CGGGCGAAGGGAAAATATATAGTTGTC -3'). After adding nuclease-free water to all lyophilized primers as indicated, 10 µL of 100 µM stocks were taken, and 90 µL of nuclease-free water was added to prepare 10 pmol portions. From these portions, 14 µL of primer mix was prepared for each sample as 4 µL each of FIP and BIP primers, 2 µL each of F3 and B3 primers, 1 µL each of LOOP F and LOOP B. Next, 46 µL of water was added to the mix. Then, 5 µL of primer for each sample was taken from this mixture, for a total volume of 60 µL (this mix was sufficient for 12 samples). These ingredients were melted in an ice bath at room temperature, mixed using a vortex, and centrifuged. The reaction mix was prepared as follows. This mixture was placed in the Genie® III instrument and analyzed at 60°C for 60 min. The reaction was stopped after 1 min of inactivation annealing at 98°C. Finally, the annealing temperature were determined.

Detection of BoHV-1 DNA by PCR method

The samples that were found to be positive in Real-Time PCR and Real-Time LAMP assays were cultured in MDBK cell culture. Among them, cells with CPEs were extracted, and PCR assays were performed using the commercially prepared Solis BioDyne[4] PCR kit. The samples that could be isolated from the cell culture underwent PCR testing with primers specific to the gC gene region. The primer pair PF 5′ CGCCGC-CGAGTACTACCC 3′) and PR 5′ CGGCCACGAC-GCTGACGA 3′ utilized by Esteves [17] was used in the analysis. The reaction mixture was prepared as 6 µL 5x HOT FIREPol Blend Master Mix[4], 1.5 µL Forward Primer, 1.5 µL Reverse Primer, 1.5 µL DMSO, 16.5 µL Nuclease-free H_2O, and 3 µL DNA. A thermal cycle program was applied to the master mix mixture following the test procedure, including initial denaturation at 95°C for 15 min, 33 cycles of 95°C for 20 s, 56°C for 30 s, 33 cycles of 72°C for 40 s, and a final extension at 72°C for 7 min.

Nucleotide sequencing and phylogenetic analysis

Bidirectional sequencing analysis of the PCR products was performed by Humanizing Genomics Macrogen Company. The chromatogram file was edited using AliView [22]. Additionally, in silico analysis of the sequences was performed in the GenBank database using the Basic Length Alignment Search Tool (BLAST) of the National Center for Biotechnology Information (NCBI) [4]. These sequences were recorded in the GenBank database. The sequences obtained were compared with the BoHV-1 reference sequences in the GenBank database and aligned using MUSCLE [14] in the AliView [22] software. A phylogenetic tree was constructed using the Tamura 3-parameter model [37] with the Neighbor-Joining algorithm in MEGA 11 [38], and 1,000 bootstraps were performed.

Statistical analysis

In order to compare the 2 methods used in the study, the McNemar test was performed using the SPSS 25 (IBM SPSS)[5] statistical program. All differences were considered to be statistically significant at $P < 0.05$. The data were reported as percentages and frequencies.

RESULTS

Virus isolation results in cell culture

The CPE characterized by shrinkage and rounding of the cells, was observed in 3 of the 8 samples analyzed 48 h after cell culture.

Real-Time PCR and Real-Time LAMP

Eight (5.33%) of the nasal swab samples obtained from the cattle were found to be positive by the Real-Time PCR test, while 13 (8.66%) samples were found to be positive by the Real-Time LAMP test (Figure 1).

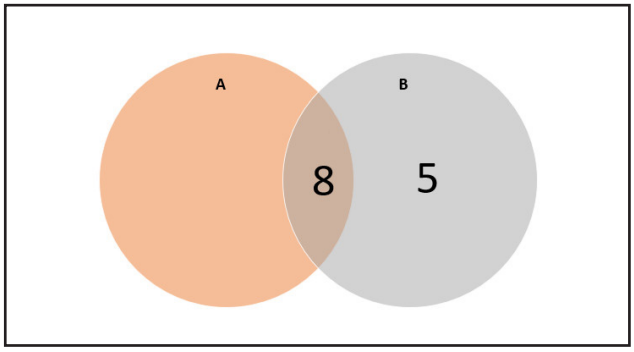

Figure 1. Number of samples that tested positive with Real-Time PCR and Real Time LAMP. Cluster A: Samples that tested positive with Real Time PCR. Cluster B: Samples that tested positive with Real Time LAMP.

PCR results of isolates obtained from cell culture

The presence of BoHV-1 nucleic acid was confirmed by observing the 575/572 bp (BoHV-1/BoHV-5) DNA band in 3 samples isolated from cell culture by the PCR method. Verification and alignment of sequences and construction of a phylogenetic tree with sequences of other isolates were performed using MUSCLE [14] in the AliView [22] software and compared with BoHV-1 reference sequences in the GenBank database. After obtaining the consensus of the aligned sequences, they were compared to other sequences in Genbank using the BLAST program. The sequences of three 575 bp BoHV-1 isolates were submitted to the GenBank database [ON350848, ON350849, and ON350850] (Figure 2).

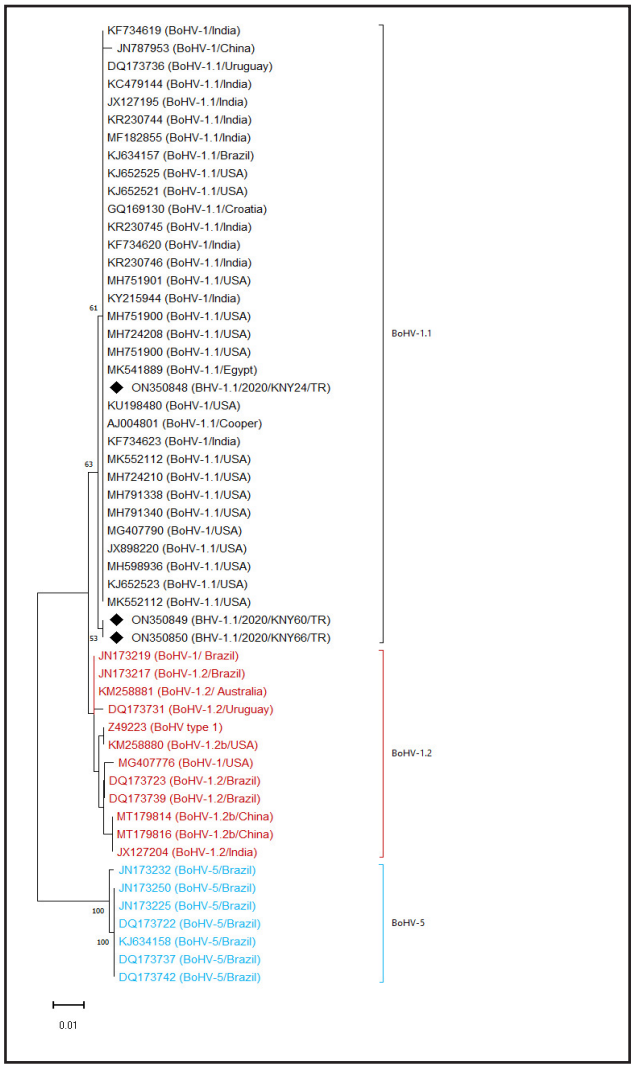

Figure 2. Phylogenetic analysis based on the nucleotide sequence of the gC gene of BoHV-1 isolates obtained from the Konya region. The phylogenetic tree containing the 312-nucleotide partial sequence was created with the Neighbor Joining algorithm in MEGA 11 software using the Tamura 3-parameter model and 1,000 repeats (bootstrap) [37,38]. Branches marked in black are BoHV-1.1, those marked in red are BoHV-1.2, and those in blue are BoHV-5. Field strains obtained from this study are marked with a black square (♦). [Scale bar indicates 0.01 nucleotide substitution].

Next, pairwise similarity and homology of the sequences were calculated. The gC region nucleotide sequence alignment of the isolates obtained from this study and previously published isolates showed variable homology percentages [95 to 100%] (Figure 3). The isolates obtained in this study [ON350848, ON350849, and ON350850] were 100% compatible with each other. At the nucleotide level, the ON350848 isolate showed the highest (100%) similarity to the AJ004801 (BoHV-1.1/Cooper) and KU198480 (BoHV-1/USA) isolates. The similarity was the lowest among JN173232 (BoHV-5/Brazil), JN173250 (BoHV-5/Brazil), JN173225 (BoHV-5/Brazil), DQ173722 (BoHV-5/Brazil), KJ634158 (BoHV-5/Brazil), DQ173737 (BoHV-5/Brazil), and DQ173742 (BoHV-5/Brazil) strains (95%). Our ON350849 and ON350850 isolates were quite similar to (99%) Z49223 (BoHV-1), KM258880 (BoHV-1.2b/USA), DQ173731 (BoHV-1.2/Uruguay), KM258881 (BoHV-1.2/Australia), JN173219 (BoHV- 1/ Brazil) and JN173217 (BoHV-1.2/Brazil), while it had the least similarity (96%) to JN173232 (BoHV-5/Brazil), JN173250 (BoHV-5/Brazil), and JN173225 (BoHV-5/Brazil), DQ173722 (BoHV-5/Brazil), KJ634158 (BoHV-5/Brazil), DQ173737 (BoHV-5/Brazil), and DQ173742 (BoHV-5/Brazil) [Figure 4].

Statistical analysis

The results of the McNemar test showed that the difference between the Real-Time PCR and Real-Time LAMP tests was not significant ($P = 0.063$) [Table 1].

Table 1. Real-Time PCR and LAMP analysis frequency and percentage values.

	Real-Time PCR		LAMP	
	n	%	n	%
Negative	142	94.7	137	91,3
Positive	8	5,3	13	8,7
Total	150	100	150	100

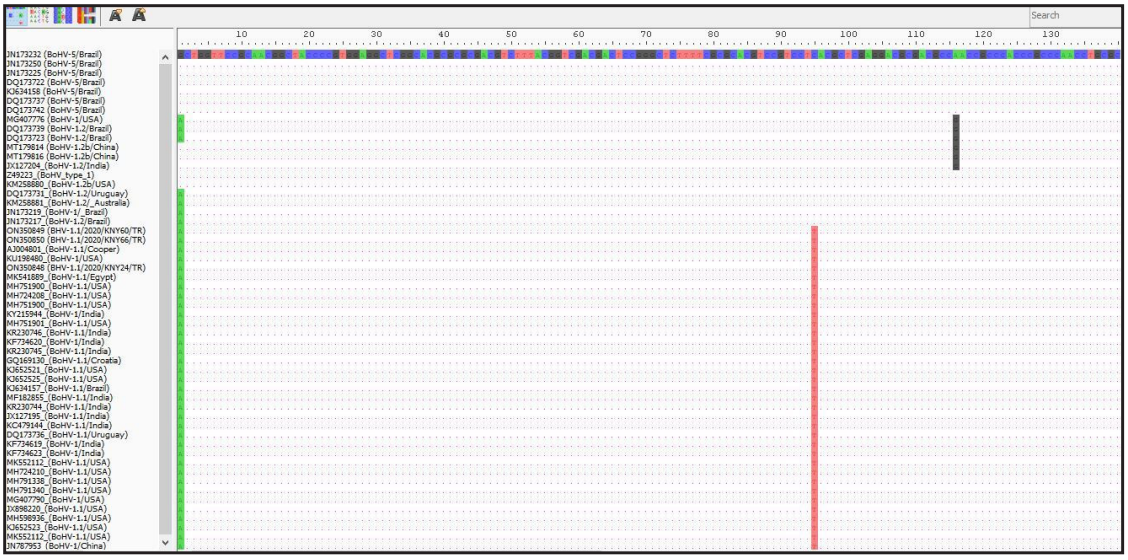

Figure 3. Nucleotide differences of BoHV-1 reference viruses and field isolates according to partial gC gene sequences.

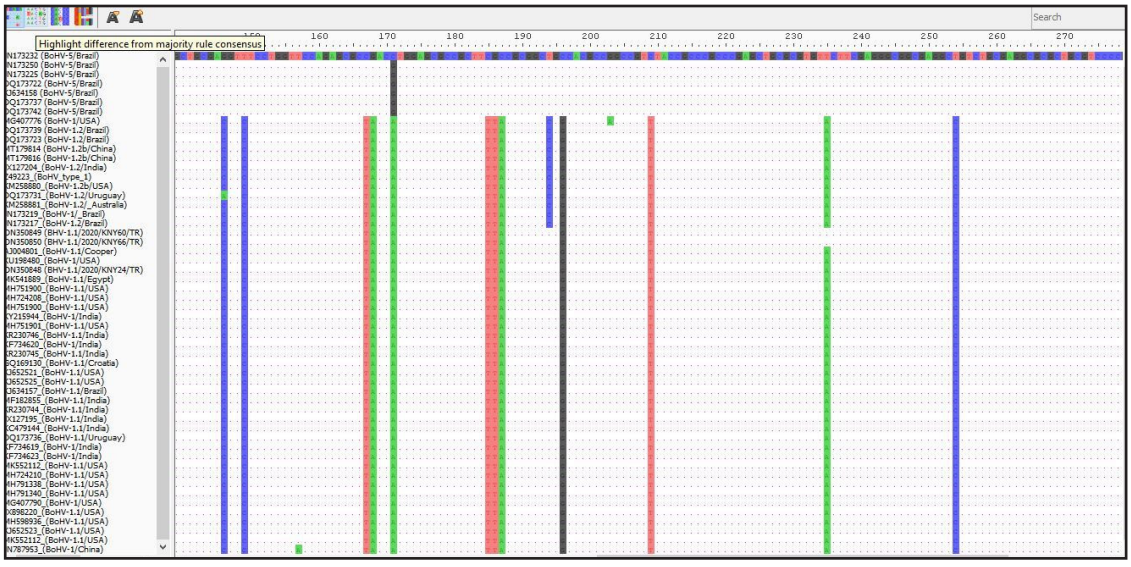

Figure 4. Amino acid differences of BoHV-1 reference viruses and field isolates according to partial gC gene sequences.

DISCUSSION

Real-time PCR is widely used for detecting microorganisms because it is fast, provides quantitative results, and has a high sample yield [35]. Several studies have used Real-Time PCR to detect various BoHV-1 strains/isolates, including genotypes 1.1 and 1.2, from different geographical regions [33,34]. The primers used in the Real-Time PCR assay in this study may cross-react with several herpesviruses closely related to BoHV-1. The primers and probe used in the Real-Time PCR assays were specific to the gB gene, a highly conserved herpesvirus gene, as this gene is highly homologous to the genes of other genetically closely related ruminant herpesviruses, including those found in deer and goats [44,46]. As stated by Chandranaik [8], the specificity and sensitivity of the Real-Time PCR test should be sufficient to detect BoHV-1 from nasal swabs. Many studies have shown that PCR tests are more sensitive than virus isolation (VI) [7,15,23,32]. Similarly, in this study, only 3 of the 8 samples found to be positive by Real-Time PCR could be isolated in cell culture. While Real-Time PCR can detect various viral components such as viral genomic DNA, viral

messenger RNA (mRNA) [45], viral glycoproteins [46], diagnosis by VI requires a fully matured virus particle [42]. The results of both tests differed in that PCR can detect any viral RNA (infectious whole virus, unpackaged viral genome, even genome strand or subgenomic fragments, and fragments formed by the destruction of viral infected cells by host immune cells), whereas VI detects properly packaged and live virus particles [5].

Although no statistical difference was recorded between Real-Time PCR and Real-Time LAMP in this study (Table 1), a higher rate of positive samples was detected with LAMP [19, 43] (Figure 1). These results showed that LAMP is a more resistant to PCR inhibitors and more sensitive test and due to the use of 6 different primers. BoHV-1 continues to cause significant economic losses in the cattle industry around the world. Therefore, a practical, fast, and cost-effective diagnostic tool needs to be used to identify the infection in the affected areas and control it at the earliest. In this context, the LAMP test is a promising method at the field level for the diagnosis and control of BoHV-1 infection and other diseases, as mentioned in other studies. The key advantages of LAMP are that it can be performed with a portable device and does not require expensive laboratory equipment or any qualified tests and personnel [36].

The gC gene plays an important role in the adhesion of virions to target cells by binding to heparin surface receptors, and therefore may contribute to cell tropism [9]. The 3' region of the gC gene (corresponding to the COOH-t region of the protein) is moderately conserved, and is therefore suitable for phylogenetic analysis in type and subtype classification of BoHV [41]. Phylogenetic analysis was performed on 3 swab samples isolated in cell culture, after they were tested by conventional PCR with primers specific to the BoHV-1 gC region, in accordance with Traesel *et al*. [41] and Chowdhury [9]. The isolate sequence was aligned using MUSCLE [14], with the sequences of other isolates obtained by a comparative analysis and multiple sequence alignment was performed in the AliView [22] software. This analysis included the nucleotide sequences of 54 different isolates. All ambiguous locations were removed for each sequence. There were 312 nucleotides in the final dataset. Evolutionary analysis was performed using the Tamura 3-parameter model [37] with the Neighbor-Joining algorithm in MEGA 11 [38]; in total, 1,000 iterations (bootstrap) were performed. The results of the phylogenetic analyses confirmed that these isolates were BoHV-1.1. It was found to be included in the subtype (Figure 2). Similar to the findings of other studies conducted in Turkey [3,11,40], it was determined in this study that the more common BoHV-1 strain in circulation was the BoHV-1.1 strain. Bovine herpesviruses were divided into 3 groups: BoHV-1.1, BoHV-1.2, and BoHV-5, with high bootstrap values (> 50%) used in phylogenetic analysis (Figure 2). The data obtained for these groups using the gC COOH-t region were similar to the phylogenetic data [17]. Strains ON350848, ON350849, and ON350850 were in the same cluster as the strains from India, China, Uruguay, Brazil, America, Croatia, and Egypt. Nucleotide sequence alignments of these three isolates were found to be 100% compatible with each other. This showed that all 3 isolates were in the same subgroup. In a study, different gene regions of BoHV-1 and BoHV-5 were analyzed, and the results showed that they shared 69-98% antigenic similarity [12]. The isolates of this study showed 94-95% similarity to BoHV-5 strains (JN173232, JN173250, JN173225, DQ173722, KJ634158, DQ173737, and DQ173742), as determined by analyzing the gC gene region, and 98-99% genetic and antigenic similarity to the BoHV-1.2 strains (DQ173739, DQ173723, MT179814, MT179816, JX127204, KM258880, DQ173731, and KM258881) [Figures 3 and 4]. Such a high degree of similarity of these isolates to the BoHV-1.2 strains suggested that the genomic differences between the 2 subtypes were very small. The results of the nucleotide sequence analysis of the gC gene region showed that the strain was BoHV-5 or BoHV-1.2 when the C nucleotide was present at the 95th position, BoHV-1.1 when the T nucleotide was present, BoHV-1.1 when the A nucleotide was present at the 116th position, and BoHV-1.2 subtype when the G nucleotide was present. The strain was BoHV-5 when there was a G at position 171, and BoHV-1.1 or -1.2 when there was an A (Figure 3). In this case, a close genetic relationship between the BoHV-1.1 and BoHV-1.2 types was expected. Although BoHV-1.1, BoHV-1.2a, and BoHV-1.2b subtypes can be distinguished by the Restriction Endonuclease Analysis (REA), the differences between nucleotide cut off points are quite small [10,25,26]. Additionally, cross-neutralization studies using hyperimmune serum cannot distinguish BoHV-1.1 from

BoHV-1.2 [25]. Thus, the 2 subtypes have a high degree of structural/antigenic similarity. The ON350848, ON350849 and ON350850 isolates identified in this study are not similar to previously reported isolates in Turkey; BoHV-1 ET-121 [39], MK919465 and MK919466 [3], KY748020, KY748021, KY748022, KY748023, KY748024, KY748025, KY748026, KY748027, MF458855, MF458856, MF458857, MF458858, MF458858, MF458859 [11], which were previously reported in Turkey, were interpreted as too many animals entering the country. Considering that the countries from which animals are imported in Turkey are also affected by IBR, the possibility of an endemic risk should be considered as these countries do not have eradication or monitoring programs for BoHV-1. Phylogenetic analysis based on gC gene nucleotide sequences revealed that the isolates obtained in this study clustered within subtype 1.1 of BoHV.1 together with the reference Cooper and some field strains. Subtype 1.1 generally contains strains responsible for respiratory diseases [41]. Certain isolates, including ON350848, ON350849, and ON350850, caused clinical symptoms such as fever, cough, loss of appetite, runny nose, and conjunctivitis in the animals in which they were detected. These results revealed that BoHV-1.1 is generally found in animals with respiratory diseases.

CONCLUSION

The findings of this study showed that using only virus isolation in the diagnosis of BoHV may lead to false-negative results. Besides reducing the effectiveness of diagnosis, false-negative results may also cause medical practitioners to not take control and prophylaxis measures. LAMP test is a fast, reliable, and affordable diagnostic tool and is recommended for areas affected by infection, especially where resources are limited. Additionally, future researchers may investigate whether the test can be applied to samples other than nasal swabs, such as semen, fetal tissue (heart, brain, lung, kidney, liver, spleen, intestinal, thymus). Studies need to perform phylogenetic analyses to determine the type of circulating virus; such information may assist in the development of vaccines to provide appropriate protection to susceptible animals. As the sequence analysis performed in this study was based only on a short piece of viral DNA and did not fully reflect the genetic diversity of the isolates, future studies may detect new circulating BoHV-1 strains by performing full-genome sequencing or REA.

MANUFACTURERS

[1]Omega Bio-tek Inc. Norcross, GA, USA.

[2]Pharma R&D. Basel, Switzerland.

[3]OptiGene. Horsham, United Kingdom.

[4]Solis BioDyne. Tartu, Estonia.

[5]International Business Machines Corporation (IBM). Armonk, NY, USA.

***Funding*.** The authors would like to thank Selcuk University for the financial support (Scientific Research Projects (BAP) (Project No. 20112002).

***Acknowledgments*.** This article was summarized from the PhD thesis of the Dr. Hatice Pelin Aslim. We thank SCI-edit.com for English editing of the article.

***Ethical approval*.** The ethics committee decision number (2023/159) of the Faculty of Veterinary Medicine Experimental Animal Production and Research Centre and the approval of the ethics committee were obtained.

***Declaration of interest*.** The authors declare no conflicts of interest. Only the authors are responsible for the content and writing of this article.

REFERENCES

1 **Abril C., Engels M., Liman A., Hilbe M., Albini S., Franchini M. & Ackermann M. 2004.** Both viral and host factors contribute to neurovirulence of bovine herpesviruses 1 and 5 in interferon receptor-deficient mice. *Virology Journal*. 78(7): 3644-3653. DOI: 10.1128/JVI.78.7.3644–3653.2004.

2 **Ackermann M. & Engels M. 2006.** Pro and contra IBR-eradication. *Veterinary Microbiology*. 113(3-4): 293-302. DOI:10.1016/j.vetmic.2005.11.043.

3 **Albayrak H., Tamer C., Ozan E., Müftüoğlu B., Kadi H., Doğan F., Cağırgan A., Elhag A., Akman A. & Kuruçay H.N. 2020.** Molecular identification and phylogeny of bovine herpesvirus-1 (BoHV-1) from cattle associated with respiratory disorders and death in Turkey. *Medycyna Weterynaryjna*. 76(6): 358-361. DOI: dx.doi.org/10.21521/mw.6393.

4 **Altschul S.F., Madden T.L., Schäffer A.A., Zhang J., Zhang Z., Miller W. & Lipman D.J. 1997.** Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* 25(17): 3389-3902. DOI: 10.1093/nar/25.17.3389.

5 **Bhat V., Chavan P., Khattry N. & Gupta S. 2021.** Dynamics of viral RNA load, virus culture, seroconversion & infectivity in COVID-19 patients: Implications on isolation policy. *Indian Journal of Medical Research*. 153(5): 585. DOI: 10.4103/ijmr.IJMR_3564_20.

6 **Biswas S., Bandyopadhyay S., Dimri U. & Patra P. 2013.** Bovine herpesvirus-1 (BHV-1)–a re-emerging concern in livestock: a revisit to its biology, epidemiology, diagnosis, and prophylaxis. *The Veterinary Quarterly*. 33(2): 68-81. DOI: 10.1080/01652176.2013.799301.

7 **Bulut O. & Yavru S. 2004.** Seroepidemiology and Comparative Diagnosis of Bovine Herpesvirus Type-1 (BHV-1) in Bulls using Enzyme-Linked Immunosorbent Assay (ELISA), Polymerase Chain Reaction (PCR) and Virus Isolation (VI). *Eurasian Journal of Veterinary Sciences*. 20(4): 61-70.

8 **Chandranaik B.M., Shivaraj C., Sanjeev K. & Renukaprasad C. 2010.** Isolation of BHV-1 from bovine semen and application of real time isolation of BHV-1 from bovine semen and application of real time PCR for diagnosis of IBR/IPV from clinical samples. *Veterinarski Arhiv*. 80(4): 467-475.

9 **Chowdhury S. 1997.** Fine mapping of bovine herpesvirus 1 (BHV-1) glycoprotein C neutralizing epitopes by type-specific monoclonal antibodies and synthetic peptides. *Veterinary Microbiology*. 58(2-4): 309-314. DOI: 10.1016/S0378-1135(97)00146-6.

10 **d'Offay J.M., Eberle R., Fulton R.W. & Kirkland P.D. 2016.** Complete genomic sequence and comparative analysis of four genital and respiratory isolates of bovine herpesvirus subtype 1.2 b (BoHV-1.2 b), including the prototype virus strain K22. *Archives of Virology*. 161: 3269-3274. DOI 10.1007/s00705-016-3026-1.

11 **Dağalp S.B., Farzani T.A., Doğan F., Alkan F. & Özkul A. 2020.** Molecular and antigenic characterization of bovine herpesvirus type 1 (BoHV-1) strains from cattle with diverse clinical cases in Turkey. *Tropical Animal Health and Production*. 52: 555-564. DOI: /10.1007/s11250-019-02042-6.

12 **Delhon G., Moraes M., Lu Z., Afonso C., Flores E., Weiblen R., Kutish G. & Rock D. 2003.** Genome of bovine herpesvirus 5. *Virology Journal*. 77(19): 10339-10347. DOI: 10.1128/JVI.77.19.10339–10347.2003.

13 **Dhama K., Karthik K., Chakraborty S., Tiwari R., Kapoor S., Kumar A. & Thomas P. 2014.** Loop-mediated isothermal amplification of DNA (LAMP): a new diagnostic tool lights the world of diagnosis of animal and human pathogens: a review. *Pakistan Journal of Biological Sciences*. 17(2): 151-166. DOI: 10.3923/pjbs.2014.151.166.

14 **Edgar R.C. 2004.** MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research*. 32(5): 1792-1797. DOI: 10.1093/nar/gkh340.

15 **Elhassan A., Fadol M., Salih M. & El Hussein A. 2015.** Bovine herpes virus-1 (BoHV-1) detection in dairy cattle with reproductive problems in Sudan. *Journal of Advanced Veterinary and Animal Research*. 2(2): 185-189. DOI: 10.5455/javar.2015.b81.

16 **Engdawork A. & Aklilu H. 2024.** Infectious bovine rhinotracheitis: Epidemiology, control, and impacts on livestock production and genetic resources. *Veterinary Research Notes*. 4(1): 1-9. DOI: 10.5455/vrn.2024.d35.

17 **Esteves P., Dellagostin O., Pinto L., Silva A., Spilki F., Ciacci-Zanella J., Hübner S., Puentes R., Maisonnave J. & Franco A. 2008.** Phylogenetic comparison of the carboxy-terminal region of glycoprotein C (gC) of bovine herpesviruses (BoHV) 1.1, 1.2 and 5 from South America (SA). *Virus Research*. 131(1): 16-22. DOI: 10.1016/j.virusres.2007.08.004.

18 **Guo J., Li Q. & Jones C. 2019.** The bovine herpesvirus 1 regulatory proteins, bICP4 and bICP22, are expressed during the escape from latency. *Journal of NeuroVirology*. 25: 42-49. DOI: 10.1007/s13365-018-0684-7.

19 **Hu S., Li M., Zhong L., Lu S., Liu Z., Pu J., Wen J. & Huang X. 2015.** Development of reverse-transcription loop-mediated isothermal amplification assay for rapid detection and differentiation of dengue virus serotypes 1–4. *BMC Microbiology*. 15: 1-15. DOI: 10.1186/s12866-015-0595-1.

20 **Irgashev A., Nurgaziev R., Nurmanov C., Asanova E. & Ishenbaeva S. 2023.** Respiratory form of infectious rhinotracheitis: Analysis of immunomorphological reactions. *Scientific Horizons*. 26(10): 32-43. DOI: 10.48077/scihor10.2023.32.

21 **Iscaro C., Cambiotti V., Petrini S. & Feliziani F. 2021.** Control programs for infectious bovine rhinotracheitis (IBR) in European countries: An overview. Animal Health Research Reviews. 22: 136-146. *Health Research Reviews*. 22: 136-146. DOI: 10.1017/S1466252321000116.

22 **Larsson A. 2014.** AliView: a fast and lightweight alignment viewer and editor for large datasets. *Bioinformation*. 30(22): 3276-3278. DOI: 10.1093/bioinformatics/btu531.

23 **Mahajan V., Banga H., Deka D., Filia G. & Gupta A. 2013.** Comparison of diagnostic tests for diagnosis of infectious bovine rhinotracheitis in natural cases of bovine abortion. *Journal of Comparative Pathology*. 149(4): 391-301.

24 **Mahdavi F., Mirjalali H., Niyyati M., Tabaei S.J.S., Shamloo A. & Aghdaei H.A. 2024.** Design and evaluation of loop-mediated isothermal amplification for rapid detection of Enterocytozoon bieneusi. *Food and Waterborne Parasitology*. 35: 1-7. DOI: 10.1016/j.fawpar.2024.e00225.

25 **Metzler A., Matile H., Gassmann U., Engels M. & Wyler R. 1985.** European isolates of bovine herpesvirus 1: a comparison of restriction endonuclease sites, polypeptides, and reactivity with monoclonal antibodies. *Archives of Virology*. 85: 57-69.

26 **Misra V., Babiuk L. & Darcel C. 1983.** Analysis of bovine herpes virus-type 1 isolates by restriction endonuclease fingerprinting. *Archives of Virology*. 76: 341-354.

27 **Moeller J.R., Adaska R.B., Reynolds J. & Blanchard P.C. 2013.** Systemic bovine herpesvirus 1 infections in neonatal dairy calves. *Journal of Veterinary Diagnostic Investigation*. 25(1): 136-141. DOI: 10.1177/1040638712470448.

28 **Nguyen T., Vinayaka A.C., Linh Q.T., Andreasen S.Z., Golabi M., Bang D.D. &Wolff A. 2023.** PATHPOD–A loop-mediated isothermal amplification (LAMP)-based point-of-care system for rapid clinical detection of SARS-CoV-2 in hospitals in Denmark. *Sensors and Actuators B: Chemical*. 392: 1-13. DOI: https://doi.org/10.1016/j.snb.2023.134085.

29 **Notomi T., Taguchi F., Kanda H., Minekawa H., Itamura S., Odagiri T. & Tashiro M. 2004.** RT-LAMP method provides a simple, rapid and specific detection system for SARS-CoV RNA. In: *Proceedings of the International Conference on SARS - One Year after the (First) Outbreak* (Lübeck, Germany). pp.8-11.

30 **O'Donoghue S., Earley B., Johnston D., McCabe M.S., Kim J.W., Taylor J.F. & Waters S.M. 2023.** Whole blood transcriptome analysis in dairy calves experimentally challenged with bovine herpesvirus 1 (BoHV-1) and comparison to a bovine respiratory syncytial virus (BRSV) challenge. *Frontiers in Genetics.* 14: 1-10. DOI: 10.3389/fgene.2023.1092877.

31 **Pawar S., Meshram C., Singh N., Sonwane A., Saini M., Rautmare S., Muglikar D., Mishra B. & Gupta P. 2014.** Rapid detection of bovine herpesvirus 1 in bovine semen by loop-mediated isothermal amplification (LAMP) assay. *Archives of Virology*. 159: 641-648. DOI: 10.1007/s00705-013-1869-2.

32 **Rana S.K., Kota S., Samayam P., Rajan S. & Srinivasan V. 2011.** Use of real-time polymerase chain reaction to detect bovine herpesvirus 1 in frozen cattle and buffalo semen in India. *Veterinaria Italiana*. 47(3): 313-322.

33 **Reddy R., Putla B., Sarangi L., Rana S., Surendra K., Ponnanna N. & Sharma G. 2020.** Shedding of bovine alphaherpesvirus-1 in bovine extended frozen semen in Indian semen stations: a longitudinal analysis. *Theriogenology*. 157: 467-471. DOI: 10.1016/j.theriogenology.2020.08.021.

34 **Sarangi L., Polapally S., Rana S., Bahekar V., Surendra K., Reddy R., Raichur A., Ponnanna N. & Sharma G. 2020.** Development and validation of duplex real-time PCR for simultaneous detection of *Brucella* and bovine alphaherpesvirus from clinical specimens. *Veterinaria Italiana*. 56(1): 35-41. DOI: 10.12834/VetIt.1728.9123.2.

35 **Satbige A.S., Kuotsu K., Patil N., Kasaralikar V., Bijurkar R. & Sandeep H. 2018.** Molecular detection of bovine herpes virus-1 (BHV-1) infection in cattle in organized and unorganized farm in Bidar, Karnataka. *Journal of Entomology and Zoology Studies*. 6(4): 1258-1260.

36 **Socha W., Rola J., Urban-Chmiel R. & Żmudziński J.F. 2017.** Application of loop-mediated isothermal amplification (LAMP) assays for the detection of bovine herpesvirus 1. *Polish Journal of Veterinary Sciences*. 20(3): 619-622. DOI: 10.1515/pjvs-2017-0078

37 **Tamura K. 1992.** Estimation of the number of nucleotide substitutions when there are strong transition-transversion and G+ C-content biases. *Molecular Biology and Evolution*. 9(4): 678-687.

38 **Tamura K., Stecher G. & Kumar S. 2021.** MEGA11: molecular evolutionary genetics analysis version 11. *Molecular Biology and Evolution*. 38(7): 3022-3027. DOI: 10.1093/molbev/msab120.

39 **Toker E.B. & Yeşilbağ K. 2021.** Molecular characterization and comparison of diagnostic methods for bovine respiratory viruses (BPIV-3, BRSV, BVDV, and BoHV-1) in field samples in northwestern Turkey. *Tropical Animal Health and Production*. 53(79): 1-11. /10.1007/s11250-020-02489-y.

40 **Toker E.B., Yeşilbağ K., Özer A., Kadiroğlu B. & Aytoğu G. 2022.** High mortality rate of shipping fever cases in cattle caused by bovine herpesvirus type 1 (BoHV-1). *Ankara Üniversitesi Veteriner Fakültesi*. 69: 69-75. DOI: 10.33988/auvfd.834671

41 Traesel C.K., Silva M.S., Spilki F.R., Weiblen R. & Flores E.F. 2013. Nucleotide sequencing and phylogenetic analysis of the 3′ region of glycoprotein C gene of South American bovine herpesviruses 1 and 5. *Research in Veterinary Science*. 94(1): 178-185.

42 Tuppurainen E.S., Venter E.H. & Coetzer J.A.W. 2005. The detection of lumpy skin disease virus in samples of experimentally infected cattle using different diagnostic techniques. *Onderstepoort Journal of Veterinary Research*. 72: 153-164.

43 Wang H., Liu X., Zeng F., Zhang T., Lian Y., Wu M., Xiao L., Zhu Y., Zhang Y. & Chen M. 2019. Development of a real-time loop-mediated isothermal amplification assay for detection of porcine circovirus 3. *BMC Veterinary Research*. 15: 305. DOI: 10.1186/s12917-019-2037-z.

44 Wang J., O'Keefe J., Orr D., Loth L., Banks M., Wakeley P., West D., Card R., Ibata G. & Van Maanen K. 2007. Validation of a real-time PCR assay for the detection of bovine herpesvirus 1 in bovine semen. *Journal of Virological Methods*. 144(1-2): 103-108. DOI: 10.1016/j.jviromet.2007.04.002.

45 Watzinger F., Ebner K. & Lion T. 2006. Detection and monitoring of virus infections by real-time PCR. *Molecular Aspects of Medicine.* 27(2-3): 254-298. DOI: 10.1016/j.mam.2005.12.001.

46 Wernike K., Hoffmann B., Kalthoff D., König P. & Beer M. 2011. Development and validation of a triplex real-time PCR assay for the rapid detection and differentiation of wild-type and glycoprotein E-deleted vaccine strains of Bovine herpesvirus type 1. *Journal of Virological Methods*. 174(1-2): 77-84. DOI: 10.1016/j.jviromet.2011.03.028

47 World Organization for Animal Health. 2024. https://www.woah.org/fileadmin/Home/fr/Health_standards/tahm/3.04.11_IBR_IPV.pdf [accessed on March 11, 2024].