

First Isolation And Characterization Of Bovine Herpesvirus 1.2b (BoHV-1.2b) Strain From Upper Respiratory Tract Of Cattle In Türkiye

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Abstract

Analyzing fingerprints by Restriction Length Polymorphism (RFLP) analysis of complete virus genome which is laborious and time-consuming classifies Bovine Herpesvirus-1 (BoHV-1) strains into three subtypes, BoHV-1.1, 1.2a, 1.2b. These subtypes can also be referred according to clinical features, however, no clear relation was shown. Mostly BoHV-1.2b is associated with genital disease conditions. In this study, BoHV-1 isolate was obtained in a nasal swab sample taken from respiratory tract disease. In this study also, phylogenetic analysis which was targeting UL44 (Glycoprotein C) region of the genome, subtyping was carried out by a recently developed multiplex PCR targeting UL39 and US3 region followed by a RFLP analysis using Hind III enzyme. Also, success of isolation was compared in two continuous cell lines. SFT-R cell line found more susceptible for BoHV-1 field sample isolation than MDBK. The obtained isolate (ID:8640) was serologically undistinguishable from Cooper strain while molecular analysis classified as BoHV-1.2b. Current study reports the first isolation of BoHV-1.2b in Turkey as well as infrequent BoHV-1.2b isolation from clinical case of respiratory illness. Results also highlight the efficiency of PCR based RFLP analysis for easy and quick subtyping but demonstrates the requirement of more investigation to reveal differences based on genetic diversity of BoHV-1 field isolates.

Keywords: BoHV-1 subtyping, molecular characterization, restriction endonuclease analysis, virus isolation.

Introduction

Bovine herpesvirus type 1 (BoHV-1) is associated with various clinical disorders in cattle comprising respiratory and reproductive systems. Clinical signs of the infection involves nasal discharge, conjunctivitis, depression, fever, milk yield reduction, genital disorders like abortion and vulvovaginitis in female cattle and balanoposthitis in bulls. Although several European countries have successfully eradicated BoHV-1¹, introduction of the virus to all the continents had been reported² and has been found to be the cause of mass economical losses in cattle industry³.

BoHV-1, classifies in the *Varicellovirus* genus of the subfamily *Alphaherpesvirinae* of *Herpesviridae*. Virus has an enveloped icosahedral symmetry containing double stranded viral DNA which is about 135 kb in length and codes for 33 structural proteins. At least 13 of mentioned

are associated with the viral envelope⁴. According to composition and restriction site analysis of the genome, BoHV-1 strains are classified into three subtypes. BoHV-1.1 is generally responsible for infections in respiratory tissues as well as genital tract. Thus, the respiratory disease called infectious bovine rhinotracheitis (IBR) is generally caused by this subtype⁵. Subtype BoHV-1.2, generally accepted to be responsible for genital infections known as “infectious pustular vulvovaginitis (IPV) and infectious pustular balanoposthitis (IPB)”. BoHV-1.2 subtype also further divided into two groups of variants (genotype) namely BoHV-1.2a and BoHV-1.2b, based on genetic and antigenic differences. BoHV-1.2a strains can cause abortion in cattle while BoHV-1.2b is not assumed to be abortifacient⁶ despite causing common genital infections. Possibility of respiratory infections caused by BoHV-1.2b strains that is less virulent compared to BoHV-1.1, is also suggested^{5,7}. A former

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subtype (BoHV-1.3) causing encephalitis in cattle is currently classified as a distinct virus species of BoHV-5.⁸ Despite genetic variations, BoHV-1 subtypes are closely related to each other in serological testing. Since BoHV-5, BoHV-1 and its subtypes share antigenically very similar epitopes, determination of virus species and subtypes by serological differentiation is laborious. Differentiation of BoHV-1 subtypes was entirely performed by restriction site analysis using different DNA digestion enzymes⁹. Though this analysis are still employed for the same purposes, so far, there are many sequences from different parts of BoHV-1 genome deposited in the GenBank for various strains of the virus¹⁰. Hence, phylogenetic analysis on the basis of selected genes is a reliable method for subtyping BoHV-1 isolates. One of the most common part of viral DNA used for characterization and differentiation of BoHV-1 isolates is the gene coding for glycoprotein C (gC, UL44) encode for envelope glycoprotein¹¹ which is responsible for attachment of the virions onto cells.

The agent, BoHV-1, leads to a latent infection localized in sensory ganglia after primary infection that persists for lifelong. Clinical symptoms and virus shedding are induced in latently infected animals affected by various stressors or subjected to corticosteroid application². Laboratory diagnosis for virus detection can be achieved using different methods including PCR, antigen ELISA and virus isolation in cell culture. Cell lines from different tissue origin are susceptible for BoHV-1 propagation with visual cytopathogenic effect (CPE)².

The aim of this study was to present the first isolation of BoHV-1.2b from natural case of bovine respiratory infection, and its molecular characterization consisting of both phylogenetic analysis and PCR-RFLP method which is less preferred in BoHV isolates. At the same time, the success of the BoHV-1 isolation from field samples in two continuous cell lines, MDBK and SFT-R was compared.

Material and Methods

Samples: In September 2014, non-clothed blood samples from 37 beef cattle with lumpy skin disease (LSD)-like findings were submitted for laboratory diagnosis. There was no history of BoHV-1 vaccination in the herd. Skin samples from 2 of these animals and additional nasal swab sample (sample ID: 8640) from one animal exhibiting nasal discharge was also submitted. Thus, the total number of samples handled in this study was 39. All the samples were subjected to nucleic acid isolation for testing LSD virus by PCR. In the meantime, inocula prepared from samples for the purpose of virus isolation in cell culture were centri-

fuged at +4 °C, 3000 rpm for 10 minutes and the supernatants obtained were filtered via 0,2 µm and treated with antibiotics (penicillin 100 IU /ml and streptomycin 1 µg /ml) and antifungals (amphotericine B 2,5 µg /ml) solutions. All the samples and inoculum were either directly included in the test protocol or stored at -80 °C up to testing.

Cell line and the test virus: Madin Darby bovine kidney (MDBK) and Sheep fetal thymus (SFT-R) cell lines were used for virus inoculation and serum neutralization studies. Both of the cell line were grown in Dulbecco's modified minimal essential medium (DMEM) supplemented with 10% fetal calf sera. Cell lines and calf sera were tested by PCR and virus isolation protocols to be free of *Pestivirus* contamination before use. BoHV-1 strain Cooper propagated in MDBK cell line was used as positive control virus in PCR and neutralization tests.

Virus isolation: All the samples taken from total of 36 animals were inoculated onto MDBK and SFT-R cells prepared in 24 well plates. Cells cultivated in 75 cm² flasks were suspended as containing 1x10⁵ cells /mL and 1 mL of this suspension was distributed to each well of 24 well plates for using in virus isolation procedure. Culture media were changed after 24 h and samples were inoculated. All the wells were daily screened under inverted light microscope during 3 blind passages each for 7 days. The culture media from the wells in which cytopathogenic effect (CPE) was observed was individually collected and stored at -80 °C. At the end of each blind passage (day 7 pi), inoculated cell cultures in 24 well plates were freeze-thawed for 3 times and the media from each well was subjected to following test step.

ELISA: The sample (8640) producing CPE in cell culture was subjected to commercial antigen ELISA protocols detecting BoHV-1 (Pulmotest, Bio-X diagnostics, Belgium) and bovine viral diarrhoea virus (Herdcheck, IDEXX, Switzerland). While applying the commercial BVDV ELISA method originally validated for detection of persistently infected carriers, for confirmation of the validity of applied method, BVDV NADL strain produced in cell culture in the laboratory was also used as an external control, apart from the positive control included in the kit and it was found to comply with the validation criteria.

Both of ELISA protocols were applied as described by the manufacturers. The test plates were read on the ELISA reader (Thermo-Multiskan EX, Finland) at a wavelength of 450 nm.

Polymerase chain reaction: Because the sample 8640 produced positive result in BoHV-1 antigen ELISA it was further confirmed in PCR. For that purpose both the original swab sample directly taken from the animal and cell culture supernatant from 4th passage level were subjected to testing. Viral nucleic acid was isolated using a commercial kit (NucleoSpin Virus, Macharey-Nagel, Germany). The primer pair P1-forward (5'-CACGGACCTGGTG-GACAAGAAG-3') and P2-revers (5'-CTACCGTCAC-GTGCTGTGTACG-3') targeting the glycoprotein B (gB, UL26) gene of BoHV-1¹² was employed. The 50 µL of reaction mixture was consisted of nuclease free water (39,8 µL), MgCl₂ (25mM, 2 µL), 10 x PCR buffer (5 µL), forward and revers primers (50pmol, 0,5 µL each), dNTPs (10mM each, 1 µL), Taq DNA polymerase (0,2 U) and sample DNA (1 µL). Applied thermal profile was as follows: 94°C for 5 min; 35 cycles of 94°C for 1 min, 61°C for 1 min, 72 °C for 1 min and final extension at 72 °C for 10 min. The 468 bp PCR products were visualized in 2% agarose gel electrophoresis by Red-Safe™ (ABC Scientific, CA, USA) staining.

Serum neutralization assay: For serological identification, viral isolate from sample 8640 was tested in serum neutralization assay using polyclonal serum yielded against BoHV-1 Cooper strain (BoHV-1.1). Animal experiments to obtain hyperimmune sera were conducted according to rules by national and local ethical committee (HADYEK). Briefly, 2 series of 2 fold diluted hiperimmunised sera were prepared in DMEM as duplicates of 96 well plates in a volume of 50 µL. BoHV-1 Cooper strain and viral isolate from sample 8640 were prepared in the titer of 100TCID₅₀ and 50 µl volume of virus suspension was added onto each step of the serum dilutions in different series. After incubation at 37°C in a 5% CO₂ atmosphere for 2 hours, 50 µl of MDBK cell suspension including 3x10⁵ cells /ml was added to each well and incubated in the same conditions for 5 days by daily evaluation. Inhibition of the cytopathogenic virus growth was recorded as positive reaction for neutralization and the antibody titer was evaluated as the highest dilution where the last positive result was observed.

Sequencing and phylogenetic analysis: For genetic characterization of the isolate, a different PCR protocol targeting gC region of the genome was used. Test protocol including the primer pairs PF (5'-CGGCCACGACGCT-GACGA-3') and PR (5'-CGCCGCCGAGTACTACCC-3') were applied as described elsewhere¹¹. Reaction conditions were applied as given above. The 572-575 bp PCR products were visualized in 1% agarose gel electrophoresis by Red-Safe™ (ABC Scientific, CA, USA) staining. PCR products were submitted for sequencing (Genmar, Turkey) and obtained sequence data were aligned by ClustalW Multiple alignment tool using BioEdit software¹³. Phylogeny analysis

was applied on the sequences truncated as to be coding region. Neighbor-Joining method and bootstrap analysis with 1000 replicates were chosen in Mega 7 software. For phylogeny tree, reference BoHV-1 and BoHV-5 sequences were obtained from GenBank. Accession numbers for the reference strains are shown on Fig.1.

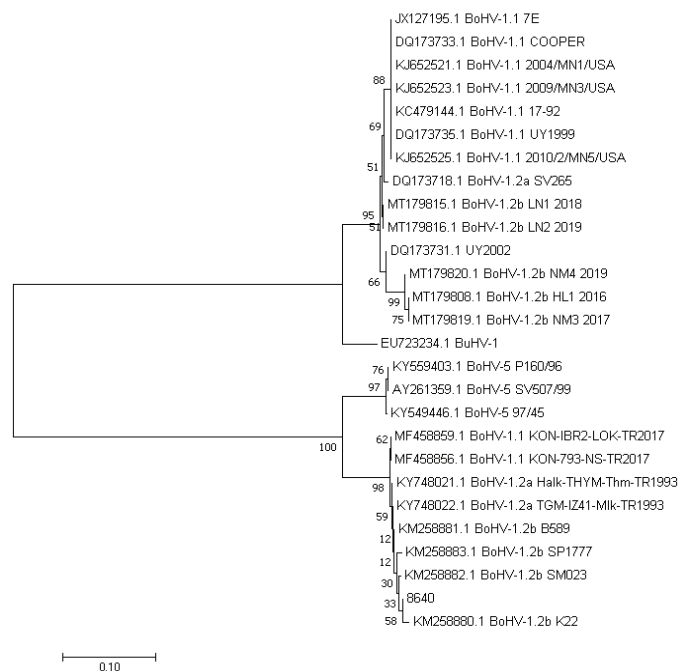


Fig. 1 Phylogenetic tree based on maximum likelihood method for glycoprotein C gene of BHV-1 field strain of 8640

The tree was displayed using Mega10 software. The numbers at branches indicates the frequency after 1000 bootstrap evaluation. Other sequences compared with 8640 isolate were obtained from the NCBI Pubmed website.

Restriction Fragment Length Polymorphism (RFLP):

Restriction site analysis were performed as described by Maidana et al.¹⁴. Multiplex PCR was carried out by using two different primer sets targeting UL39 open reading frame and the US3 upstream intergenic regions. Primers set RS1 (US3; F: 5'-TCGTCGAAGAGCGTCCACACA-3', R: 5'-ACCGCGCTGTACCGGCAGCT-3') and RS2, (US3; F: 5'-TACAAATCGGCGGCCAAA-3', R: 5'-TTGTT-GACGGCCAAGTATAA-3') amplified the fragments 493 bp and 700 bp, respectively. The multiplex PCR amplification was performed in a final volume of 50 µL, containing; 25 µL Maxima Hot Start 2x Green PCR Master Mix (Thermo Scientific™, K1061), 2,5 µL RNase/DNase free water, 5 µL of 10 pmol each sense and antisense primers, 1 µL extracted DNA and 1,5 µL DMSO. PCR products were directly cleaved with Hind III enzyme according to recommended conditions (FastDigest HindIII, Thermo Scientific™) without a purification step. The digested products were separated in 1,5 % agarose gel using 1xTAE buffer. BoHV-

1.1 reference strain Cooper was used as control virus both for multiplex PCR and digestion.

Results

Virus isolation and ELISA: Although samples refer to the laboratory with LSD-like clinical findings, characteristic CPE form encountered in cell cultures and number of days for CPE detection suggested bovine herpesvirus infection. By virus isolation studies conducted on 39 samples, the nasal swab sample (ID: 8640) and one blood sample (data not shown) produced positive result as indicated by visual CPE characterized by rounding, aggregation and lysis of the infected cells in the culture. Those of effects were observed both in MDBK and SFT-R cell cultures at 5th day pi in the first round of blind passages, while an obvious virus growth was recorded starting from 24th hours post infection by the second passage (Fig.2). Further 5 passages were achieved to confirm cytopathogenic growth of the isolates. The isolate produced a higher viral titer ($DKID_{50}$ $10^{5.75}$) in the culture of SFT-R cells comparing to MDBK

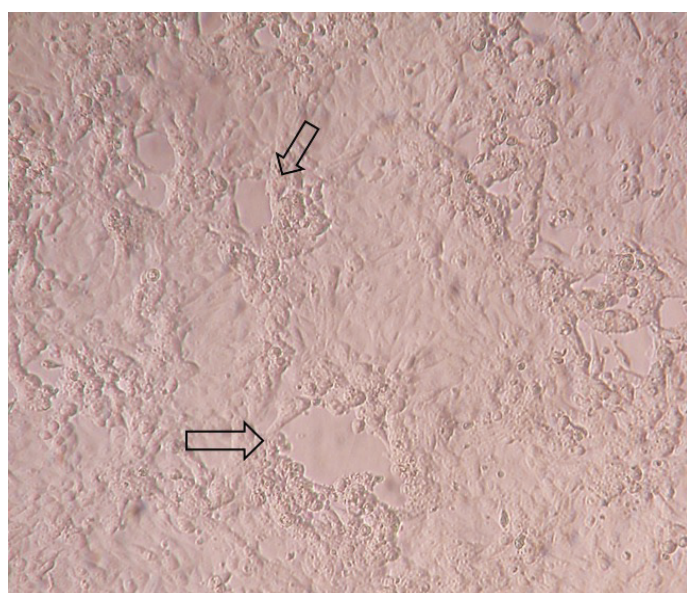


Fig. 2 Virus propagation in SFT cells
Arrows: Foci from virus growth areas (cytopathogenic effect, isolate 8640, 2nd passage, d.1 pi)

cells ($DKID_{50}$ $10^{5.25}$). All the final confirmatory tests were performed on cell culture supernatant from 4th passage of the original sample and positive results obtained in BoHV-1 Ag ELISA (OD:0.248). Beside sample was found negative by BVDV ELISA in which BVDV NADL strain produced in cell culture in the laboratory was used as an external control.

PCR and Phylogenetic analysis: BVDV negativ status of the sample was further confirmed by RT-PCR. LSD PCR test performed on the samples was determined negative. However, 486 bp amplicons were yielded by PCR target-

ing gB region of BoHV-1 genome. Nucleotide sequence analyses of gC region conducted to identify the genetic similarities between 8640 isolate (Access no. MW207646) and with subtype classification realized BoHV-1 viruses. BoHV-5 and Bubaline alphaherpesvirus-1 (BuHV-1) sequences were also included in phylogenetic analyses. Aligned nucleotide sequences revealed high degrees of identity in herpesviruses included in the assessment, but not for the BoHV-1 subtype characterization. The phylogenetic analysis showed the BoHV-1, BoHV-5 and BuHV-1 isolates grouped in distinct branches (Fig.1). The strains of BoHV-1.1 and BoHV-1.2 grouped together in two different branches. The sequences analyzed for the BoHV-1.2a and BoHV-1.2b strains are also intertwined in two different branches. Isolate 8640 revealed between BoHV-1.2 isolates but mostly similar with K22 strain (BoHV-1.2b) subtype and SM023 (BoHV-1.2b) subtype. Genomes alignment was also verified using BLAST database (NCBI, nucleotide sequence blast). BLAST analysis revealed that this sequence homologies in the context of query cover was mostly seen between strain 8640, strain B589 (BHV-1.2b), and strain K22 (BHV-1.2b), respectively. While nucleotide sequence of strain 8640 was compared, glycoprotein C region was mostly conserved according to sequence of BoHV-1.2b K22 reference strain (Fig.3). Multiple sequence alignments showed single substitutions at nucleotide position 17589 which differs from other aligned sequences of BoHV-1.2. Also another substitution revealed at 17360 position identified only in K22 strain sequence.



Fig. 3 Multiple nucleotide sequence alignment of the isolate 8640 to strains from BoHV-1 subtypes

Multiple alignment was conducted between isolates 8640 detected in this study and 6 other strains retrieved from GeneBank [BoHV-1.2b K22 reference strain (KM258880.1), BHV-1.2b B589 strain (KM258881.1), BHV-1.2b SM023 (KM258882.1), BHV-1.2b SP1777 (KM258883.1), BHV-1.2a Halk-THYM-Thm-TR1993 (KY748021.1), BoHV-1.1 Cooper (DQ173733)]. Numbers on the sequence indicate nucleotide positions in the glycoprotein C gene for each virus. Dots indicate that nucleotides in that line are identical in all sequences in the alignment.

Restriction Fragment Length Polimorphism (RFLP): For RFLP; multiplex PCR products were identical for BoHV-1.1 cooper strain. Both the UL39 and the US3 fragments were amplified as it was expected, and the isolate 8640 was detected positive for UL39 region. However, several repeats were conducted in different reaction conditions, no amplification was observed for 8640 isolate by using RS2 primer set. The restriction patterns obtained by Hind III digestions are shown in Fig.4. The fingerprints identical to BoHV-1.1 Cooper strain showed the RS1 and RS2 fragments without cleavage. For the isolate 8640, RS1 amplicon was cleaved into 2 sub-fragments. According to recommended RS1 sub-fragment patterns the field isolate 8640 was determined as BoHV-1.2b.

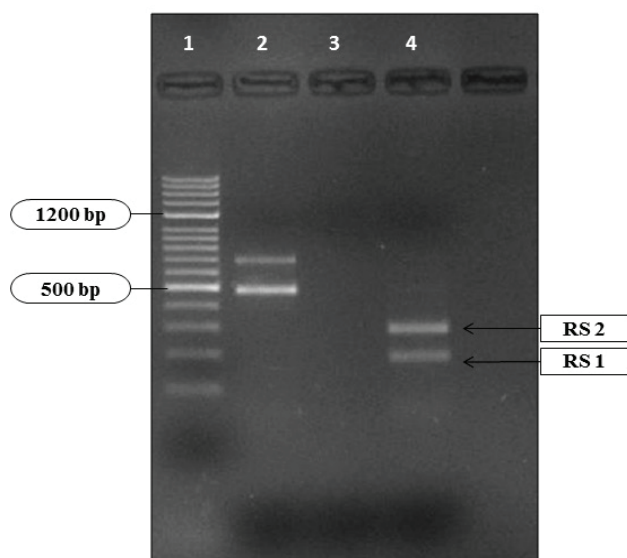


Fig. 4 Genomic fingerprints of the strain 8640 obtained after RFLP analysis of PCR products from UL39

Line 1: ladder; Line 2: BHV-1.1 strain Cooper undigested PCR amplicons of both primer sets (RS1 = amplicon of 493 bp; RS2 = amplicon of 700 bp) Line 3: strain 8640, digested amplicons of RS1; Line 4: negative control (DNase, RNase free water).

Virus neutralization assay: The viral isolate taken from nasal swab sample (BoHV-1 8640/Tur/2014) was also identified as to be BoHV-1 using serum-virus neutralization assay beside BoHV-1 antigen ELISA and PCR. Serum neutralization assay resulted with a neutralization reaction up to 1:32 serum dilution similar to BoHV-1 strain Cooper used as control virus.

Discussion and Conclusion

BoHV-1 was first recognized in Germany in early 19th century, characterized by clinical signs associated with genital tissues, known as “infectious pustular vulvovaginitis” (IPV) in cows and “infectious pustular balanoposthitis” (IPB) in bulls¹⁵. Strain K22 was the first isolate¹⁶ and today subtyped as BoHV-1.2. After half of the 19th

century, respiratory form has recognized in North America. Acute respiratory disease form, known as Infectious bovine rhinotracheitis (IBR) quickly spread to Europe¹⁵. Causative agents of those two different clinical conditions were not antigenically separated from each other by cross neutralization assays¹⁷. Analyzing fingerprints by restriction endonuclease of viral DNA allowed subtype classification. Due to the mostly isolation of BoHV-1.1 from respiratory tract disease or abortion and BoHV-1.2 in genital lesions, these subtypes can also being referred according to clinical futures⁸. However, no clear relation was shown between tropism of respiratory or genital tract infections and subtypes^{18,19}. Although BoHV-1.2b mostly associated with genital diseases in accordance with some of previous reports^{5,10,20}, the present study also reveals the molecular characterization of BoHV-1.2b isolate obtained from nasal swab sample at respiratory disease.

In this study, the phylogenetic analysis based on gC gene region clearly grouped the BoHV-1, BoHV-5 and BuHV-1 isolates into different clusters. Unlike the results of some researches which also targets gC region^{11,21,22} the phylogeny tree constructed based on nucleotide sequences in this study did not show consistent branches between BoHV-1 subtypes. The isolate 8640 sequence identity was found mostly close to BoHV-1.2b reference strain K22 and strain SM023 (BoHV-1.2b). In a study²³ some of the Chinese BoHV-1.2b isolates have been shown to be grouped separately in phylogenetic investigations and suspected as “atypical” BoHV-1.2 strains with UY2002 strain²³. The closest sequence of those three samples (MT179808, MT179819, MT179820) was also identified as UY2002. But mentioned Chinese sequences which was referred as BHV-1.2b, has fallen into a different branch separated from 8640.

In subtyping BoHV, it is accepted as the gold standard to examine the complete genome of the virus using the Hind III enzyme by RFLP method. BoHV-1, BoHV-5 differentiation and even subtype restriction endonuclease analysis profiles has distinct fingerprints²⁴⁻²⁶. But this technique needs large quantity of purified viral DNA to display patterns after virus isolation which is laborious and time consuming. Besides this classic application, alternative methods which enables restriction of BoHV PCR products have been developed for alphaherpesviruses^{14,27-29}. PCR-RFLP method is promising for rapid and easy implementation of detection and differentiation of BoHV-1 subtypes. In this study, in addition to PCR sequencing assay, one of those proposed techniques was used for subtyping. PCR-RFLP performed successfully for BoHV-1.1 strain Cooper. But isolate 8640 showed amplification only for the RS1 primer

pair. Failure of amplification using RS2 primers was proposed for BoHV-5 and BuHV-1 strains by the authors¹⁴. However RS1 cleavage was only proposed for BoHV-1.2b subtypes, not for the BoHV-1.1, BoHV-1.2a, BoHV-5 and BuHV. Comparing with the phylogenetic analysis, results was not surprising. Although it is not considered a valid method for subtype characterization, the results of phylogenetic analyses show that the isolate 8640 is not BoHV-5 or BuHV-1. And partial RFLP results obtained in this study supports digestion of RS1 amplicons, which classifies isolate 8640 as subtype BoHV-1.2b. It could be assumed that possible mutations can be responsible for the failure of RS2 amplification. Though restriction patterns for BoHV-1 by PCR-RFLP or selected amplification regions further needs to be investigated in order to enable the evaluation of possible mutations between field isolates.

BoHV infections are common in Turkey where the studies mainly involve serological investigations and limited number of molecular detections^{30,31}, and only one covers detailed characterization²¹. Beside the predominant prevalence of BoHV-1.1 or few BoHV-1.2a detection, according to our knowledge BoHV-1.2b strain has not been reported in Turkey so far. BoHV-1.2b has been isolated in Europe, America, Australia and lately in China^{10,23}. Hence the isolate 8640 is the first BoHV-1.2b isolate reported from Turkey. Our results do not provide to determine the origin of the current isolate, however, it should be noted that live animal importation can be an effective route to introduce such new viral subtypes in to country.

We also compared the cross neutralization antibody reactions between hyperimmune sera for Cooper strain and isolate 8640 (BoHV-1.2b). There was no difference between the neutralization capacities of hyperimmune serum against both the viruses tested. It was also previously reported to recognize BoHV1.2b isolate by BoHV-1.1 hyperimmune sera²¹ and due to this cross reactivity, antibody titers do not allow to subtype differentiation³².

MDBK is the commonly preferred cell line for BoHV isolations and propagation in practice. Besides, adaptation of BoHV-1 to propagate in embryoned chicken eggs³³ and susceptibility of a sheep kidney derived cell line (FLK-N3)³⁴ were established as an alternative to primary and secondary cell cultures. We obtained BoHV-1 field isolates and propagated not only in MDBK cells but also in SFT-R cell culture at first inoculation. Following 5 passages in each cell line, viral titers obtained in both cell lines were compared. Surprisingly, the virus titer of the field isolate reached higher levels in the SFT-R cells. In a previous study

which reports genomic characterization of 13 field strains only 4 could be adapted to the MDBK cell line as intended quantity (3). Regarding the data from the current study SFT-R cell lines can be realized as satisfied alternative to MDBK cell line for BoHV-1 isolation.

In the present study, the isolation and molecular characterization of BoHV-1 strain 8640 were performed from nasal swab sample of a cattle. These findings are valuable for ensuring interior or cross-country epidemiological tracking. But further research are needed to determine the prevalence of BoHV-1.2b subtype in the field. Though standardization of laborless molecular techniques are also needed to provide opportunity for extensive research in this direction. Increased complete genome sequence investments may reflect the genetic diversity and contribute diagnostic improvements.

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Ethical Statement

This study was approved by the Bursa University Animal Experiments Local Ethics Committee (2020 – 09 /10).

Conflict of Interest

The authors declare that they have no conflicts of interest.

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