# Genetic variability of bovine herpesvirus type 4 (BoHV-4) field strains from Turkish cattle herds

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Virus isolation.

**Summary**
Bovine herpesvirus type 4 (BoHV-4) is a common virus in the world that is detected in clinically ill or in apparently healthy cattle. This study provides a molecular characterization of BoHV-4 strains from 24 cattle some showing respiratory and/or reproductive problems and some without any apparent clinical sign. This study also reported the growth properties of five BoHV-4 field isolates. The 24 sampled cattle came from 13 different herds in 10 provinces collected between 2007 and 2018. Phylogenetic analysis using partially amplified nucleotide sequences of ORF8 genes coding glycoprotein B (n = 24) and ORF3 genes coding thymidine kinase (n = 9), demonstrated genetic variability among the BoHV-4 strains analysed. The partial gB gene sequences clustered in three different genotypes (genotype I, II and III) were located within the genotype I cluster, such as Movar strain. The analysis of the five BoHV-4 strains isolated from vaginal swabs (n = 2), nasal swab (n = 1), and brain samples (n = 2) revealed no significant differences in their growth properties in MDBK cell culture.

## Introduction

Bovine herpesvirus type 4 (BoHV-4), a member of the *Herpesviridae* family, subfamily *Gammaherpesvirinae* in the *Rhadinovirus* genus, is found worldwide among cattle populations (Roizman *et al.* 1992, Zimmerman *et al.* 2001). The BoHV-4 genome is a double-stranded DNA of 144 ± 6kb and a long unique coding region (LUR) or light DNA (L-DNA) with a size of approximately 108 kb. Based on restriction endonuclease patterns, almost all American strains belong to the DN-599 group whereas European strains belong to the Movar 33/63 group (Bublot *et al.* 1990). However, the restriction patterns of some strains do not completely match with those belonging to the DN-599 or Movar 33/63 groups. These are the LVR 140 strain, known as the Belgian reference strain (Thiry *et al.* 1992, Wellemans *et al.* 1986), and the Taiwan strain isolated from bovine vascular endothelial cells (Lin *et al.* 1997).

Several BoHV-4 genes have been examined in detail by various researchers (Egyed *et al.* 1996, Goltz *et al.* 1994). BoHV-4 open reading frame 8 (ORF8) codes for glycoprotein B (gB), which is a major component of the virion, essential for viral infectivity (Little *et al.* 1981, Lomonte *et al.* 1997). It is one of the most studied genes because its region is one of the most highly conserved among *Herpesviridae* family members (Goltz *et al.* 1994, Wellenberg *et al.* 2001). Tissue tropism instead is most likely associated with variations in virion surface glycoproteins (Frazier *et al.* 2002, Wellenberg *et al.* 2001). Another frequently studied gene is ORF3, which codes for thymidine kinase (TK). It helps in understanding the precise regulation of specific genes, their promoter regions, and transactivation mechanisms (Kit *et al.* 1986, Lomonte *et al.* 1992).

In Turkey, previous studies have shown that BoHV-4 infection is prevalent in dairy cattle herds (Bilge Dağalp *et al.* 2007, Bilge Dağalp *et al.* 2010, Bilge Dağalp *et al.* 2012, Gur and Dogan 2010, Kale *et al.* 2011), with seropositivity levels reaching 56.8% and 44.9% in sampled herds with reproductive disorders and healthy appearance, respectively (Bilge Dağalp *et al.* 2007). While several reports have detected BoHV-4 from cattle with or without clinical symptoms, the genetic characterization of local viruses is not yet well documented. The two

purposes of this study are therefore i) analysis of the gene regions coding gB and TK in local BoHV-4 strains from cattle suffering reproductive and respiratory disorders; ii) investigation of their growth characteristics in cell cultures.

## Materials and methods

### Samples

For this study, 24 cattle samples in which BoHV-4 was identified were selected previously (Bilge Dağalp *et al.* 2010, Bilge Dağalp *et al.* 2012). Those samples that were still positive when re-tested in this study were used for analysis of the genes coding gB. The field isolates (n = 5) and the samples that were delivered to our laboratory in the last years were also used.

The sequences used in this study were obtained from the samples that were targeted for the amplification of the gB gene region or were found positive against gB in previous two projects (Bilge Dagalp *et al.* 2010, Bilge Dağalp *et al.* 2012). Considering that the arrival dates of these samples are old, the specimens did not propagate in cell cultures and were discarded. As a result of these elimination only isolates and BoHV-4 suspected samples that were delivered to our laboratory in recent years were amplified and sequenced.

They were selected as representative of different herds, clinical disorders, and sampling years. They included diagnostic samples originated from nasal and vaginal swabs, milk, and necropsy samples collected between 2007 and 2018 from cattle with severe reproductive disorders, abortions, and/or respiratory system disorders from 13 different dairy cattle herds of 10 provinces (Table I).

### PCR

DNA extraction was carried out according to Sambrook and colleagues (Sambrook *et al.* 1989). The detection of BoHV-4 DNA was performed using oligonucleotide primers specific for BoHV-4 ORF8 gene

**Table I.** *Details of the Bovine herpesvirus type 4 positive samples .*

| Herd No/ Location | Virus code | Clinical signs | Year | Samples | Sequences of gB genes | | Sequences of TK genes[2] |
|---|---|---|---|---|---|---|---|
| | | | | | GenBank Acc.No | Genotype | GenBank Acc.No. |
| I - Bursa | K339 | Metritis | 2007 | Vaginal swab[1] | EU055543 | II | JX644990 |
| II - Aksaray | KCVS12 | Metritis, Upper respiratory disease | 2007 | Vaginal swab[1] | GQ246864 | | JX644991 |
| | KCORG | Abortus | 2011 | Organ | MK543547 | | ND |
| | KCB12 | Neonatal death | 2009 | Brain[1] | JX644988 | | JX644992 |
| | KCVS2 | Repeat breeder, upper respiratory disease | 2009 | Vaginal swab | MK543548 | | ND |
| III - Kars | KBSB9 | Abortus | 2007 | Brain | GQ246865 | | ND |
| IV - Kirklareli | TG1 | Metritis | 2007 | Leukocyte | GQ246866 | | ND |
| | TGVS550 | Repeat breeder | 2011 | Vaginal swab | MK543551 | | ND |
| | TGVS549 | Repeat breeder | 2011 | Vaginal swab | MK543550 | | ND |
| | TGVS29 | Abortus | 2011 | Vaginal swab | MK543549 | | ND |
| | TG4 | Metritis | 2007 | Vaginal swab | GQ246863 | | ND |
| V - Balikesir | T6 | Repeat breeder | 2007 | Leukocyte | GQ246867 | | ND |
| VI - Yozgat | YL | Abortus | 2007 | Leukocyte | GQ375280 | | ND |
| VII - Sivas | U-NS66 | Upper respiratory disease | 2009 | Nasal swab[1] | JX644989 | | JX644993 |
| VIII - İzmir | IZ1LK136 | Abortus | 2010 | Leukocyte | MK543546 | | ND |
| IX - Ankara | ANK.NS | Repeat breeder, upper respiratory disease | 2014 | Nasal swab | KX192364 | | ND |
| | ANK.Br. | Neonatal death | 2015 | Brain[1] | KX192363 | | KX352279 |
| X - Ankara | ANK.Teat. | Mammary lesions, mastitis | 2014 | Teat lesion | KX192365 | | KX352280 |
| XI - Kirklareli | B1AC4 | Upper respiratory disease, pneumonia | 2017 | Lung | MH318579 | | MH614620 |
| | B2AC4 | Upper respiratory disease, pneumonia | 2017 | Lung | MH318580 | | MH614621 |
| XII - Kastamonu | KAS21 | Repeat breeder | 2017 | Leucocyte | MG181944 | I | Negative |
| | KAS24 | Repeat breeder | 2017 | Leucocyte | MG181945 | | Negative |
| | KAS25 | Repeat breeder | 2017 | Leucocyte | MG181946 | | MG242137 |
| XIII - Ankara | 9870MILK | Mastitis | 2018 | Milk | MH605274 | III | Negative |

[1] BoHV-4 field strains isolated from these samples; [2] All clustered in genotype I; ND = Not done

codifying gB (5'-CCCTTCTTTACCACCACCTACA-3' and 5'-TGCCATAGCAGAGAAACAATGA-3') (Goltz *et al.* 1994), and for BoHV-4 ORF3 gene codifying TK (5'-GTTGGGCGTCCTGTATGGTAGC-3' and 5'-ATGTATGCCCAAAACTTATAATATGACCAG-3') (Egyed *et al.* 1996) PCR was conducted as described by Wellenberg and colleagues (Wellenberg *et al.* 2001) with some minor modifications. Briefly, 3 µl DNA was subjected to thermocycling in a 30 µl reaction mixture containing 2.5 U Taq DNA Polymerase (Fermentas, Lithuania), 3.5 mM dNTP mix (Fermentas, Lithuania), 10 pmol of each primer, 1.5 mM $MgCl_2$, 1 X PCR buffer, and 6% DMSO. Thermal cycling conditions were 6 min at 96 °C, followed by 40 cycles of 56 °C for 45 sec, 72 °C for 2 min, and 95 °C for 1 min. The reaction tubes were kept for a further 10 min at 72 °C for final extensions. PCR products were visualized in a transilluminator after electrophoresis in 1% agarose gel containing ethidium bromide. The North American (DN-599) reference strain of BoHV-4 was used as positive control. The reference virus strain was kindly provided by Dr. G.J. Wellenberg (Lelystad, Netherlands).

## Sequencing and phylogenetic analysis

PCR amplicons with the expected sizes (615 bp and 567 bp for genes coding gB and TK, respectively) were subjected to sequence analysis. The amplicons were firstly gel purified using a commercial kit (GeneMark, Taiwan) and sequenced by a commercial enterprise. The gB and TK gene sequence assembly and editing were performed using Bioedit (Version 7.0.5.3) and Clustral W (Hall 1999). The sequences were then compared with those of the GenBank and sequenced database for similarities using the Basic Length Alignment Search Tool (BLAST) software of the National Center for Biotechnology Information (NCBI) (Althsul *et al.* 1997).

The obtained analysis data were compared with different BoHV-4 strains by using neighbor joining method (1,000 replication) and they were phylogenetically analyzed in MEGA 6.0 software program under Kimura 2 parameter (Tamura *et al.* 2011).

## Virus isolation and determination of the replication kinetics of isolates in cell culture

All samples (n = 24) were inoculated (100 µl) onto MDBK cell cultures for virus isolation. The cells were washed 3 times with PBS-M before adding medium. The monolayers were incubated at 37 °C in a 5% $CO_2$ atmosphere and checked daily for the appearance of cytopathic effects (CPE). The monolayers were passaged at weekly intervals for a total of three passages. The supernatants of all cell cultures were tested for BoHV-4 by direct immunofluorescence (IFA) technique, using a commercial conjugate (Bio028, BioX, Belqium) as described by Nettleton and colleagues (Nettleton *et al.* 1982) and/or PCR as described in the material and method section in this study. Samples showed cytopathic effects in MDBK cell cultures, 5-7 days after 3rd passage. After isolation and identification procedure, the supernatants from third passages of isolates (n = 5) were used for titration. The virus titer was calculated using the method of Reed and Muench (Reed and Muench 1938).

To investigate the replication kinetics, five BoHV-4 strains isolated in this study were used. These BoHV-4 isolates (n = 5) were inoculated into MDBK cells grown in 24-well plates (Greiner Bio-one, Frickenhausen, Germany) at a multiplicity of infection (MOI) of 0.1. Cell cultures were incubated at 37 °C with 5% $CO_2$ atmosphere and observed daily for the presence of CPE. The supernatants were harvested at 24, 48, 72, 96, and 120 h post-infection (hpi) and frozen at - 80 °C for further viral quantitation, as described by Verna and colleagues (Verna *et al.* 2016). The percentage of CPE was recorded at the time of supernatant collection. Five replicates were performed to establish the kinetics of viral replication for each experiment (virus strain and harvest time points). Negative controls were included in each experiment. To determine the replication kinetics of these local isolates (n = 5), the infectivity titers were estimated using viral suspensions collected at 24, 48, 72, 96, and 120 hpi from every MDBK cell culture (Reed and Muench 1938).

# *Results*

## PCR, sequencing, and phylogenetic analysis

After PCR, gB gene region of 24 samples and TK region of 9 of 24 samples were also amplified. In PCR, the local viruses produced the expected size of products (615 bp) for the gene coding gB and produced the expected size of products (567 bp) for the gene coding TK (Table I). All amplicons from the local strains along with the reference strain (DN-599) were commercially sequenced and their genome sequences were deposited in GenBank under the accession numbers in Table I.

Phylogenetic analysis, performed using the gB coding gene sequences of the local viruses (n = 24) and BoHV-4 strains with different genotypes in GenBank, revealed that genotype II was prevalent in the local isolates (20/24, 83.3%) and sampled herds (11/13, 84.6%). The other two genotypes (genotype I and III ) were identified in other two

different herds (Figure 1 and Table I). The grouping in the phylogenetic tree is described by Verna and colleagues (Verna *et al.* 2012) and Areda and colleagues (Areda *et al.* 2018).

The phylogenetic analysis of BoHV-4 based on sequences of the ORF3 gene coding TK revealed three distinct genotypes: genotype I, II, and III (Figure 2). As shown in Figure 2, phylogenetic comparisons with the previously described BoHV-4 TK gene sequences indicated that all of our BoHV-4 field viruses belonged to genotype 1, which includes the European (Movar-like) reference strains, along with the Italian, Japanese, and Argentinian BoHV-4 strains.

We also compared the nucleic acid (na) sequences for the genes coding gB and TK of the local BoHV-4 samples both among themselves (Figures 3 and 4) and with the sequences deposited in GenBank from other countries (Tables II and III).

Figure 5 shows the amino acid (aa) similarity rates for genes coding TK. For genotype I, the similarity rates for genes coding TK were 100% among the BoHV-4 strains detected in this study and 98.5-100% with the other viruses. The similarities with local BoHV-4 identified as genotype II and genotype III were 98% and 96.5%, respectively (Figure 5 and Table III). Considering the sequences for the two genes at aa and na level, the differences in ORF8 sequences were greater than the differences for the ORF3 gene sequences between our viruses and BoHV-4 from other countries, and also between our local viruses. Figure 6 shows the amino acid sequence alignments of ORF8 genes coding gB of our BoHV-4 samples and other strains, including the reference viruses (Movar and DN -599).

## Virus isolation and replication kinetics

Out of 24 samples previously confirmed as positive for BoHV-4 by PCR on ORF8 gene amplification, five showed cytopathic effects in MDBK cell cultures, 5-7 days after the 3rd passages. These BoHV-4 strains were then successfully isolated and identified by immunofluorescence and/or PCR. The origin of these samples was as follows: two vaginal swabs (K339 and KC-VS12), one nasal swab (U-NS66-TR2009), and two brain samples (KC-B12 and ANK.Br.TR2015) from animals in herds I, II, VII, and IX (Table I). Figure 7 shows the infectivity titers of 5 isolates which grew in cell culture (n = 5).

# *Discussion*

BoHV-4 appears capable of significant genome drift, as demonstrated by the variability in the

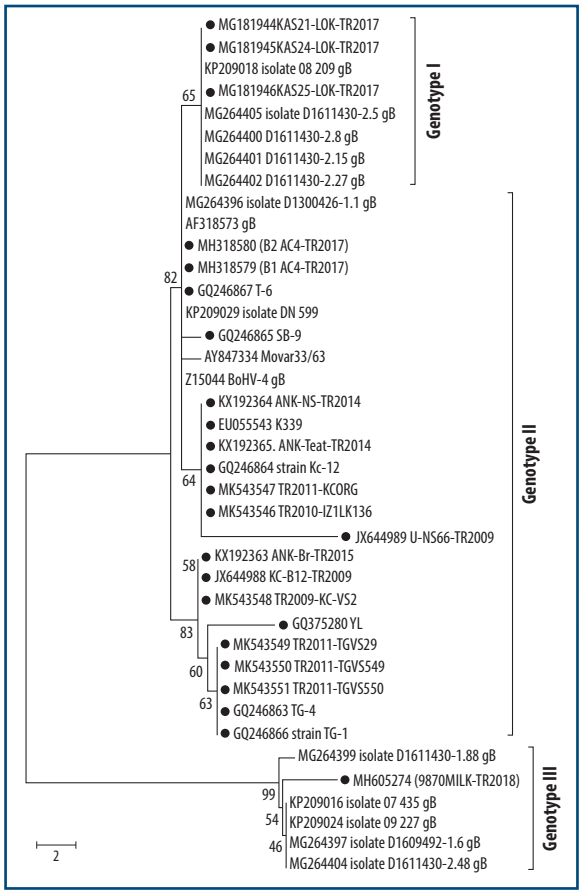


**Figure 1.** *Phylogenetic tree based on nucleotide sequences of the gB gene of Turkish BoHV-4 strains shown in round (●) shape and BoHV-4 strains from various countries.*

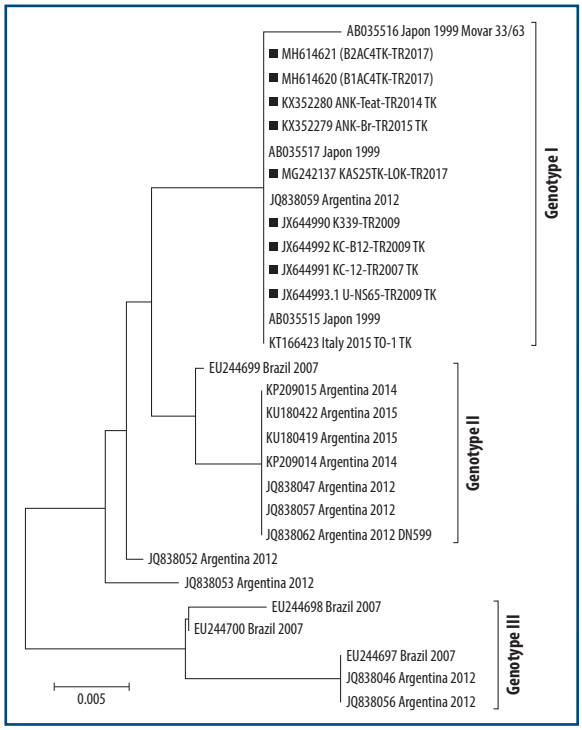


**Figure 2.** *Phylogenetic tree based on the nucleotide sequences of the TK gene of Turkish BoHV-4 strains showed in squared (■) shape and BoHV-4 strains from various countries.*

sequences of several gene regions coding gB and TK in the BoHV-4 field isolates from Turkish cattle with different clinical signs (Frazier *et al.* 2002, Areda *et al.* 2018, Verna *et al.* 2016, Gagnon *et al.* 2017).

Our molecular analysis of the partial gB gene sequences obtained from 24 local BoHV-4 strains revealed that Turkish BoHV-4 isolates clustered into three genotypes, with the separation of several viruses in different branches in genotype II (Figure 1). The majority of the local viruses were classified in genotype II, together with several European and North American BoHV-4 strains. Turkish BoHV-4 strains in genotype II (n = 20) were from various clinical samples, specifically nasal swabs (KX192364, JX644989), vaginal swabs (GQ246863, GQ246864, MK543548- MK543551, EU055543), aborted foetus tissue samples, and dead calves (MK543547, JX644988, GQ246865, KX192363), and tissue and leucocyte samples from cattle with respiratory and/or fertility system disorders (MH31879-MH31880, KX192365, MK543546, GQ375280, GQ246866-GQ246867) (Table I). Our field viruses that clustered in genotype I (MG181944, MG181945, MG181946) came from the leucocyte samples of repeat breeder cows in one specific herd (herd no: XI), which aligned with Argentinian and American BoHV-4 strains. For genotype III, only one field virus (MH605274) was detected in the milk sample from a cow with mastitis, which aligned with several North American BoHV-4 strains in the phylogenetic tree (Figure 1). Thus, the phylogenetic analysis shows that our local viruses

| | | Genotype I | | | Genotype II | | | | | | | | | | | | | | | | | | | | Genotype III |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 |
| Genotype I | 1. MG181946 KAS25-LOK-TR2017 | | 100.0 | 100.0 | 97.0 | 97.7 | 99.0 | 99.0 | 98.7 | 98.7 | 99.3 | 99.3 | 99.3 | 99.3 | 99.3 | 99.7 | 99.7 | 99.7 | 99.3 | 99.3 | 99.0 | 98.7 | 98.7 | 98.7 | 91.7 |
| | 2. MG181944 KAS21-LOK-TR2017 | 100.0 | | 100.0 | 97.0 | 97.7 | 99.0 | 99.0 | 98.7 | 98.7 | 99.3 | 99.3 | 99.3 | 99.3 | 99.3 | 99.7 | 99.7 | 99.7 | 99.3 | 99.3 | 99.0 | 98.7 | 98.7 | 98.7 | 91.7 |
| | 3. MG181945 KAS24-LOK-TR2017 | 100.0 | 100.0 | | 97.0 | 97.7 | 99.0 | 99.0 | 98.7 | 98.7 | 99.3 | 99.3 | 99.3 | 99.3 | 99.3 | 99.7 | 99.7 | 99.7 | 99.3 | 99.3 | 99.0 | 98.7 | 98.7 | 98.7 | 91.7 |
| Genotype II | 4. JX644989 U-NS66-TR2009 | 97.0 | 97.0 | 97.0 | | 95.3 | 96.7 | 96.7 | 96.3 | 96.3 | 97.7 | 97.7 | 97.7 | 97.7 | 97.0 | 97.3 | 97.3 | 97.3 | 97.7 | 97.7 | 96.7 | 96.3 | 96.3 | 96.3 | 89.3 |
| | 5. GQ375280 YL | 97.7 | 97.7 | 97.7 | 95.3 | | 98.7 | 98.7 | 98.7 | 98.7 | 97.7 | 97.7 | 97.7 | 97.7 | 97.7 | 98.0 | 98.0 | 98.0 | 97.7 | 97.7 | 98.7 | 98.7 | 98.7 | 98.7 | 90.3 |
| | 6. KX192363 ANK-Br-TR2015 | 99.0 | 99.0 | 99.0 | 96.7 | 98.7 | | 100.0 | 99.7 | 99.7 | 99.0 | 99.0 | 99.0 | 99.0 | 99.0 | 99.3 | 99.3 | 99.3 | 99.0 | 99.0 | 100.0 | 99.7 | 99.7 | 99.7 | 91.3 |
| | 7. JX644988 KC-B12-TR2009 | 99.0 | 99.0 | 99.0 | 96.7 | 98.7 | 100.0 | | 99.7 | 99.7 | 99.0 | 99.0 | 99.0 | 99.0 | 99.0 | 99.3 | 99.3 | 99.3 | 99.0 | 99.0 | 100.0 | 99.7 | 99.7 | 99.7 | 91.3 |
| | 8. GQ246863 TG-4 | 98.7 | 98.7 | 98.7 | 96.3 | 98.7 | 99.7 | 99.7 | | 100.0 | 98.7 | 98.7 | 98.7 | 98.7 | 98.7 | 99.0 | 99.0 | 99.0 | 98.7 | 98.7 | 99.7 | 100.0 | 100.0 | 100.0 | 91.7 |
| | 9. GQ246866 strain TG-1 | 98.7 | 98.7 | 98.7 | 96.3 | 98.7 | 99.7 | 99.7 | 100.0 | | 98.7 | 98.7 | 98.7 | 98.7 | 98.7 | 99.0 | 99.0 | 99.0 | 98.7 | 98.7 | 99.7 | 100.0 | 100.0 | 100.0 | 91.7 |
| | 10. GQ246864 strain Kc-12 | 99.3 | 99.3 | 99.3 | 97.7 | 97.7 | 99.0 | 99.0 | 98.7 | 98.7 | | 100.0 | 100.0 | 100.0 | 99.3 | 99.7 | 99.7 | 99.7 | 100.0 | 100.0 | 99.0 | 98.7 | 98.7 | 98.7 | 91.7 |
| | 11. KX192365. ANK-Teat-TR2014 | 99.3 | 99.3 | 99.3 | 97.7 | 97.7 | 99.0 | 99.0 | 98.7 | 98.7 | 100.0 | | 100.0 | 100.0 | 99.3 | 99.7 | 99.7 | 99.7 | 100.0 | 100.0 | 99.0 | 98.7 | 98.7 | 98.7 | 91.7 |
| | 12. EU055543 K339 | 99.3 | 99.3 | 99.3 | 97.7 | 97.7 | 99.0 | 99.0 | 98.7 | 98.7 | 100.0 | 100.0 | | 100.0 | 99.3 | 99.7 | 99.7 | 99.7 | 100.0 | 100.0 | 99.0 | 98.7 | 98.7 | 98.7 | 91.7 |
| | 13. KX192364 ANK-NS-TR2014 | 99.3 | 99.3 | 99.3 | 97.7 | 97.7 | 99.0 | 99.0 | 98.7 | 98.7 | 100.0 | 100.0 | 100.0 | | 99.3 | 99.7 | 99.7 | 99.7 | 100.0 | 100.0 | 99.0 | 98.7 | 98.7 | 98.7 | 91.7 |
| | 14. GQ246865 SB-9 | 99.3 | 99.3 | 99.3 | 97.0 | 97.7 | 99.0 | 99.0 | 98.7 | 98.7 | 99.3 | 99.3 | 99.3 | 99.3 | | 99.7 | 99.7 | 99.7 | 99.3 | 99.3 | 99.0 | 98.7 | 98.7 | 98.7 | 91.7 |
| | 15. GQ246867 T-6 | 99.7 | 99.7 | 99.7 | 97.3 | 98.0 | 99.3 | 99.3 | 99.0 | 99.0 | 99.7 | 99.7 | 99.7 | 99.7 | 99.7 | | 100.0 | 100.0 | 99.7 | 99.7 | 99.3 | 99.0 | 99.0 | 99.0 | 92.0 |
| | 16. MH318579 (B1 AC4-TR2017) | 99.7 | 99.7 | 99.7 | 97.3 | 98.0 | 99.3 | 99.3 | 99.0 | 99.0 | 99.7 | 99.7 | 99.7 | 99.7 | 99.7 | 100.0 | | 100.0 | 99.7 | 99.7 | 99.3 | 99.0 | 99.0 | 99.0 | 92.0 |
| | 17. MH318580 (B2 AC4-TR2017) | 99.7 | 99.7 | 99.7 | 97.3 | 98.0 | 99.3 | 99.3 | 99.0 | 99.0 | 99.7 | 99.7 | 99.7 | 99.7 | 99.7 | 100.0 | 100.0 | | 99.7 | 99.7 | 99.3 | 99.0 | 99.0 | 99.0 | 92.0 |
| | 18. TR2010IZ1-LK136 (MK543546) | 99.3 | 99.3 | 99.3 | 97.7 | 97.7 | 99.0 | 99.0 | 98.7 | 98.7 | 100.0 | 100.0 | 100.0 | 100.0 | 99.3 | 99.7 | 99.7 | 99.7 | | 100.0 | 99.0 | 98.7 | 98.7 | 98.7 | 91.7 |
| | 19. TR2011-KC-ORG (MK543547) | 99.3 | 99.3 | 99.3 | 97.7 | 97.7 | 99.0 | 99.0 | 98.7 | 98.7 | 100.0 | 100.0 | 100.0 | 100.0 | 99.3 | 99.7 | 99.7 | 99.7 | 100.0 | | 99.0 | 98.7 | 98.7 | 98.7 | 91.7 |
| | 20. TR2009-KC-VS2 (MK543548) | 99.0 | 99.0 | 99.0 | 96.7 | 98.7 | 100.0 | 100.0 | 99.7 | 99.7 | 99.0 | 99.0 | 99.0 | 99.0 | 99.0 | 99.3 | 99.3 | 99.3 | 99.0 | 99.0 | | 99.7 | 99.7 | 99.7 | 91.3 |
| | 21. TR2011-TG-VS29 (MK543549) | 98.7 | 98.7 | 98.7 | 96.3 | 98.7 | 99.7 | 99.7 | 100.0 | 100.0 | 98.7 | 98.7 | 98.7 | 98.7 | 98.7 | 99.0 | 99.0 | 99.0 | 98.7 | 98.7 | 99.7 | | 100.0 | 100.0 | 91.7 |
| | 22. TR2011-TG-VS549 (MK543550) | 98.7 | 98.7 | 98.7 | 96.3 | 98.7 | 99.7 | 99.7 | 100.0 | 100.0 | 98.7 | 98.7 | 98.7 | 98.7 | 98.7 | 99.0 | 99.0 | 99.0 | 98.7 | 98.7 | 99.7 | 100.0 | | 100.0 | 91.7 |
| | 23. TR2011-TG-VS550 (MK543551) | 98.7 | 98.7 | 98.7 | 96.3 | 98.7 | 99.7 | 99.7 | 100.0 | 100.0 | 98.7 | 98.7 | 98.7 | 98.7 | 98.7 | 99.0 | 99.0 | 99.0 | 98.7 | 98.7 | 99.7 | 100.0 | 100.0 | | 91.7 |
| Genotype III | 24. MH605274 (9870MILK-TR2018) | 91.7 | 91.7 | 91.7 | 89.3 | 90.3 | 91.3 | 91.3 | 91.7 | 91.7 | 91.7 | 91.7 | 91.7 | 91.7 | 91.7 | 92.0 | 92.0 | 92.0 | 91.7 | 91.7 | 91.3 | 91.7 | 91.7 | 91.7 | |

**Figure 3.** *Nucleotid identity (%) of gB gene sequences of some Turkish BoHV-4.*

| | | Genotype I | | | | | | | | | | | Genotype II | | | Genotype III | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 * | 2 * | 3 * | 4 * | 5 * | 6 * | 7 * | 8 * | 9 * | 10 | 11 | 12 | 13 | 14 | 15 | 16 |
| Genotype I | * 1. MH614621 (B2AC4TK-TR2017) | | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 99.5 | 100.0 | 98.0 | 98.0 | 98.0 | 96.5 | 96.5 |
| | * 2. MH614620 (B1AC4TK-TR2017) | 100.0 | | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 99.5 | 100.0 | 98.0 | 98.0 | 98.0 | 96.5 | 96.5 |
| | * 3. KX352280 ANK-Teat-TR2014 TK | 100.0 | 100.0 | | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 99.5 | 100.0 | 98.0 | 98.0 | 98.0 | 96.5 | 96.5 |
| | * 4. KX352279 ANK-Br-TR2015 TK | 100.0 | 100.0 | 100.0 | | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 99.5 | 100.0 | 98.0 | 98.0 | 98.0 | 96.5 | 96.5 |
| | * 5. JX644993.1 U-NS66-TR2009 TK | 100.0 | 100.0 | 100.0 | 100.0 | | 100.0 | 100.0 | 100.0 | 100.0 | 99.5 | 100.0 | 98.0 | 98.0 | 98.0 | 96.5 | 96.5 |
| | * 6. JX644991 KC-12-TR2007 TK | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | | 100.0 | 100.0 | 100.0 | 99.5 | 100.0 | 98.0 | 98.0 | 98.0 | 96.5 | 96.5 |
| | * 7. JX644992 KC-B12-TR2009 TK | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | | 100.0 | 100.0 | 99.5 | 100.0 | 98.0 | 98.0 | 98.0 | 96.5 | 96.5 |
| | * 8. JX644990 K339-TR2009 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | | 100.0 | 99.5 | 100.0 | 98.0 | 98.0 | 98.0 | 96.5 | 96.5 |
| | * 9. MG242137 KAS25TK-LOK-TR2017 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | | 99.5 | 100.0 | 98.0 | 98.0 | 98.0 | 96.5 | 96.5 |
| | 10. AB035516 Japon 1999 Movar 33/63 | 99.5 | 99.5 | 99.5 | 99.5 | 99.5 | 99.5 | 99.5 | 99.5 | 99.5 | | 99.5 | 97.5 | 97.5 | 97.5 | 96.0 | 96.0 |
| | 11. AB035517 Japon 1999 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 99.5 | | 98.0 | 98.0 | 98.0 | 96.5 | 96.5 |
| Genotype II | 12. JQ838062 Argentina 2012 DN599 | 98.0 | 98.0 | 98.0 | 98.0 | 98.0 | 98.0 | 98.0 | 98.0 | 98.0 | 97.5 | 98.0 | | 100.0 | 100.0 | 96.0 | 96.0 |
| | 13. JQ838047 Argentina 2012 | 98.0 | 98.0 | 98.0 | 98.0 | 98.0 | 98.0 | 98.0 | 98.0 | 98.0 | 97.5 | 98.0 | 100.0 | | 100.0 | 96.0 | 96.0 |
| | 14. JQ838057 Argentina 2012 | 98.0 | 98.0 | 98.0 | 98.0 | 98.0 | 98.0 | 98.0 | 98.0 | 98.0 | 97.5 | 98.0 | 100.0 | 100.0 | | 96.0 | 96.0 |
| Genotype III | 15. JQ838046 Argentina 2012 | 96.5 | 96.5 | 96.5 | 96.5 | 96.5 | 96.5 | 96.5 | 96.5 | 96.5 | 96.0 | 96.5 | 96.0 | 96.0 | 96.0 | | 100.0 |
| | 16. JQ838056 Argentina 2012 | 96.5 | 96.5 | 96.5 | 96.5 | 96.5 | 96.5 | 96.5 | 96.5 | 96.5 | 96.0 | 96.5 | 96.0 | 96.0 | 96.0 | 100.0 | |

*: Sequences obtained in this study

**Figure 4.** *Nucleotide identity (%) of partial TK gene sequences of some Turkish BoHV-4 strains.*

have high genetic similarities with BoHV-4 reported from different geographical regions worldwide, specifically Europe, and South and North America. Most of the local BoHV-4 strains with different clinical disorders were located in genotype II while BoHV-4 identified as genotype I and III were only detected in cows with repeat breeder and a cow with mastitis, respectively (Table I). Despite these findings, we cannot yet claim a definite association between the genotypes and clinical cases due to the differences in the numbers of BoHV-4 detected with different genotypes and in different clinical findings.

The phylogenetic tree for the gene coding gB revealed three main clusters, which included European (Movar-like, genotype I) and American strains isolated from cattle, the African strain isolated from buffalo (DN599-like, genotype II), and Argentinean and Brazilian strains (genotype III) from cattle.

The majority of samples (20/24) and herds (11/13) in this study were identified as genotype II. These herds were either small family herds of 1-10 cattle (III, VI, VIII, XII, XIII) or state farms (I, II, IV, V, VII, IX-XI) of about 1,000 cattle, which had been restocking from internal animal sources, transfering animals between herds, or occasionally introducing imported

**Table II.** *Nucleic acid identity (%) of gB gene sequences of Turkish BoHV-4 strains and BoHV-4 strains selected as representative for different genotypes from GenBank database.*

| Genotype | Virus Code | Acc number | Genotypes | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | I | | II | | | III | |
| | | | MG264402 | MG264401 | KP209029 DN_599 | AY847334 Movar33/63 | MG264396 | MG264404 | MG264399 |
| I | KAS25 | MG181946 | 100 | 100 | 99.7 | 99.3 | 99.7 | 92.7 | 92.3 |
| | KAS21 | MG181944 | 100 | 100 | 99.7 | 99.3 | 99.7 | 92.7 | 92.3 |
| | KAS24 | MG181945 | 100 | 100 | 99.7 | 99.3 | 99.7 | 92.7 | 92.3 |
| | | MG264402 | | 100 | 99.7 | 99.3 | 99.7 | 92.7 | 92.3 |
| | | MG264401 | 100 | | 99.7 | 99.3 | 99.7 | 92.7 | 92.3 |
| II | DN_599 | KP209029 | 99.7 | 99.1 | | 99.7 | 100 | 93.0 | 92.7 |
| | Movar33/63 | AY847334 | 99.3 | 99.3 | 99.7 | | 99.7 | 92.7 | 92.3 |
| | | MG264396 | 99.7 | 99.7 | 100 | 99.7 | | 93.0 | 92.7 |
| | U-NS66 | JX644989 | 97.0 | 97.0 | 97.3 | 97.0 | 97.3 | 90.3 | 90.0 |
| | YL | GQ375280 | 97.7 | 97.7 | 98.0 | 97.7 | 98.0 | 91.0 | 91.3 |
| | ANK-Br | KX192363 | 99.0 | 99.0 | 99.3 | 99.0 | 99.3 | 92.3 | 92.7 |
| | KC-B12 | JX644988 | 99.0 | 99.0 | 99.3 | 99.0 | 99.3 | 92.3 | 92.7 |
| | TG-4 | GQ246863 | 98.7 | 98.7 | 99.0 | 98.7 | 99.0 | 92.0 | 92.3 |
| | TG-1 | GQ246866 | 98.7 | 98.7 | 99.0 | 98.7 | 99.0 | 92.0 | 92.3 |
| | KC-12 | GQ246864 | 99.3 | 99.3 | 99.7 | 99.3 | 99.7 | 92.7 | 92.3 |
| | ANK-Teat | KX192365 | 99.3 | 99.3 | 99.7 | 99.3 | 99.7 | 92.7 | 92.3 |
| | K339 | EU055543 | 99.3 | 99[3] | 99.7 | 99.3 | 99.7 | 92.7 | 92.3 |
| | ANK-NS | KX192364 | 99.3 | 99.3 | 99.7 | 99.3 | 99.7 | 92.7 | 92.3 |
| | SB-9 | GQ246865 | 99.3 | 99.3 | 99.7 | 99.3 | 99.7 | 92.7 | 92.3 |
| | T-6 | GQ246867 | 99.7 | 99.7 | 100 | 99.7 | 100 | 93.0 | 92.7 |
| | B1 AC4 | MH318579 | 99.7 | 99.7 | 100 | 99.7 | 100 | 93.0 | 92.7 |
| | B2 AC4 | MH318580 | 99.7 | 99.7 | 100 | 99.7 | 100 | 93.0 | 92.7 |
| | IZ1LK136 | MK543546 | 99.3 | 99.3 | 99.7 | 99.3 | 99.7 | 92.7 | 92.3 |
| | KCORG | MK543547 | 99.3 | 99.3 | 99.7 | 99.3 | 99.7 | 92.7 | 92.3 |
| | KCVS2 | MK543548 | 99.0 | 99.0 | 99.3 | 99.0 | 99.3 | 92.3 | 92.7 |
| | TGVS29 | MK543549 | 98.7 | 98.7 | 99.0 | 98.7 | 99.0 | 92.0 | 92.3 |
| | TGVS549 | MK543550 | 98.7 | 98.7 | 99.0 | 98.7 | 99.0 | 92.0 | 92.3 |
| | TGVS550 | (MK543551) | 98.7 | 98.7 | 99.0 | 98.7 | 99.0 | 92.0 | 92.3 |
| III | 9870MILK | MH605274 | 91.7 | 91.7 | 92.0 | 91.7 | 92.0 | 99.0 | 98.7 |
| | | MG264404 | 92.7 | 92.7 | 93.0 | 92.7 | 93.0 | | 99.7 |
| | | MG264399 | 92.3 | 92.3 | 92.7 | 92.3 | | 99.7 | |

**Table III.** *Nucleic acid identity (%) of TK gene sequences of Turkish BoHV-4 strains and BoHV-4 strains selected as representative for different genotypes from GenBank database.*

| Virus Code | Acc number | Genotypes | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | I | | | | II | | III | |
| | | AB035516 Movar33/66 | AB035517 | JQ838059 | JQ838062 DN-599 | JQ838047 | JQ838057 | JQ838046 | JQ838056 |
| B1AC4 | MH614621 | 99.5 | 100 | 100 | 98.0 | 98.0 | 98.0 | 96.5 | 96.5 |
| B2AC4 | MH614620 | 99.5 | 100 | 100 | 98.0 | 98.0 | 98.0 | 96.5 | 96.5 |
| ANK-Teat | KX352280 | 99.5 | 100 | 100 | 98.0 | 98.0 | 98.0 | 96.5 | 96.5 |
| ANK-Br | KX352279 | 99.5 | 100 | 100 | 98.0 | 98.0 | 98.0 | 96.5 | 96.5 |
| U-NS66 | JX644993 | 99.5 | 100 | 100 | 98.0 | 98.0 | 98.0 | 96.5 | 96.5 |
| KC-12 | JX644991 | 99.5 | 100 | 100 | 98.0 | 98.0 | 98.0 | 96.5 | 96.5 |
| KC-B12 | JX644992 | 99.5 | 100 | 100 | 98.0 | 98.0 | 98.0 | 96.5 | 96.5 |
| K339 | JX644990 | 99.5 | 100 | 100 | 98.0 | 98.0 | 98.0 | 96.5 | 96.5 |
| KAS25 | MG242137 | 99.5 | 100 | 100 | 98.0 | 98.0 | 98.0 | 96.5 | 96.5 |

| | | Genotype I | | | | | | | | | | | Genotype II | | | Genotype III | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 |
| Genotype I | * 1. MH614621 (B2AC4TK-TR2017) | | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 98.5 | 100.0 | 97.0 | 97.0 | 97.0 | 92.4 | 92.4 |
| | * 2. MH614620 (B1AC4TK-TR2017) | 100.0 | | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 98.5 | 100.0 | 97.0 | 97.0 | 97.0 | 92.4 | 92.4 |
| | * 3. KX352280 ANK-Teat-TR2014 TK | 100.0 | 100.0 | | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 98.5 | 100.0 | 97.0 | 97.0 | 97.0 | 92.4 | 92.4 |
| | * 4. KX352279 ANK-Br-TR2015 TK | 100.0 | 100.0 | 100.0 | | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 98.5 | 100.0 | 97.0 | 97.0 | 97.0 | 92.4 | 92.4 |
| | * 5. JX644993. 1 U-NS66-TR2009 TK | 100.0 | 100.0 | 100.0 | 100.0 | | 100.0 | 100.0 | 100.0 | 100.0 | 98.5 | 100.0 | 97.0 | 97.0 | 97.0 | 92.4 | 92.4 |
| | * 6. JX644991 KC-12-TR2007 TK | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | | 100.0 | 100.0 | 100.0 | 98.5 | 100.0 | 97.0 | 97.0 | 97.0 | 92.4 | 92.4 |
| | * 7. JX644992 KC-B12-TR2009 TK | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | | 100.0 | 100.0 | 98.5 | 100.0 | 97.0 | 97.0 | 97.0 | 92.4 | 92.4 |
| | * 8. JX644990 K339-TR2009 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | | 100.0 | 98.5 | 100.0 | 97.0 | 97.0 | 97.0 | 92.4 | 92.4 |
| | * 9. MG242137 KAS25TK-LOK-TR2017 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | | 98.5 | 100.0 | 97.0 | 97.0 | 97.0 | 92.4 | 92.4 |
| | 10. AB035516 Japon 1999 Movar 33/63 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | | 98.5 | 95.5 | 95.5 | 95.5 | 90.9 | 90.9 |
| | 11. AB035517 Japon 1999 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | | 97.0 | 97.0 | 97.0 | 92.4 | 92.4 |
| Genotype II | 12. JQ838062 Argentina 2012 DN599 | 98.5 | 98.5 | 98.5 | 98.5 | 98.5 | 98.5 | 98.5 | 98.5 | 98.5 | 98.5 | 98.5 | | 100.0 | 100.0 | 93.9 | 93.9 |
| | 13. JQ838047 Argentina 2012 | 98.5 | 98.5 | 98.5 | 98.5 | 98.5 | 98.5 | 98.5 | 98.5 | 98.5 | 98.5 | 98.5 | 100.0 | | 100.0 | 93.9 | 93.9 |
| | 14. JQ838057 Argentina 2012 | 98.5 | 98.5 | 98.5 | 98.5 | 98.5 | 98.5 | 98.5 | 98.5 | 98.5 | 98.5 | 98.5 | 100.0 | 100.0 | | 93.9 | 93.9 |
| Genotype III | 15. JQ838046 Argentina 2012 | 95.5 | 95.5 | 95.5 | 95.5 | 95.5 | 95.5 | 95.5 | 95.5 | 95.5 | 95.5 | 95.5 | 95.5 | 95.5 | 95.5 | | 100.0 |
| | 16. JQ838056 Argentina 2012 | 95.5 | 95.5 | 95.5 | 95.5 | 95.5 | 95.5 | 95.5 | 95.5 | 95.5 | 95.5 | 95.5 | 95.5 | 95.5 | 95.5 | 100.0 | |

*: Sequences obtained in this study

**Figure 5.** *Amino acid identity of TK gene sequences of Turkish BoHV-4 and BoHV-4 strains selected as representative for different genotypes from GenBank database.*

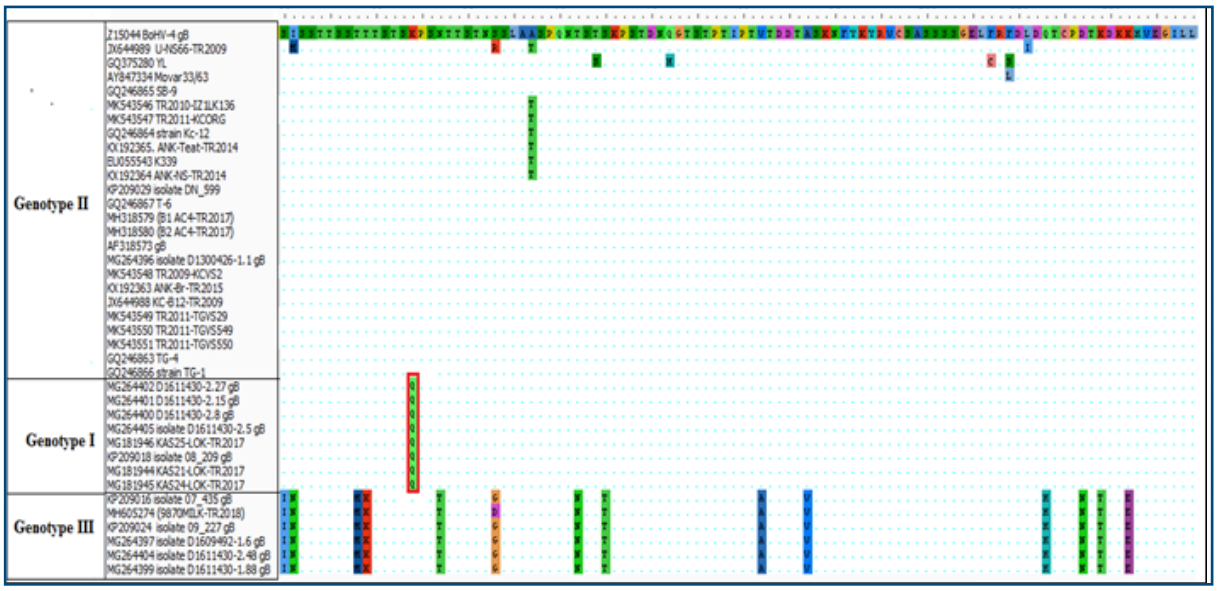


**Figure 6.** *Amino acid differences of gB gene sequences of Turkish BoHV-4 and BoHV-4 strains selected as representative for different genotypes from GenBank database.*

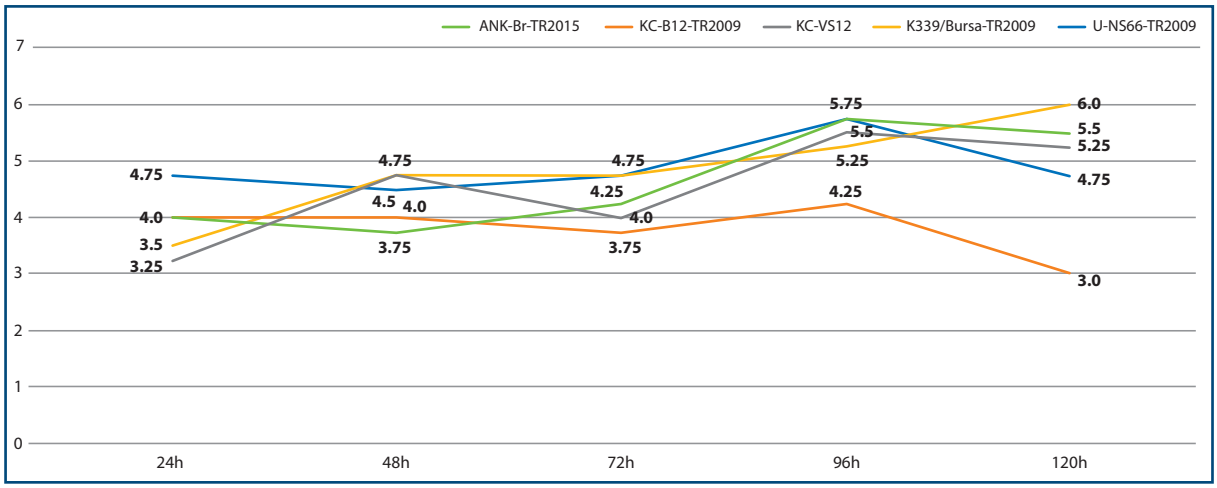


**Figure 7.** *Replication kinetics of cythopathic Bovine herpesvirus type 4 isolates in MDBK cell culture.*

animals. There is clear animal trade between either small family herds and/or industrial herds. We can speculate that animal movements between different geographical regions in Turkey and also from other countries, such as Argentina or Uruguay, especially since 2010, may have influenced the distribution of the different genotype BoHV-4 strains and their genetic resemblances. It is noteworthy that all Turkish BoHV-4 samples classified in genotype I and III, which were detected in diagnostic materials sampled in 2017 and 2018, showed close similarities with Argentinian and American BoHV-4 strains. In contrast, genotype II has been detected in cattle since at least 2007 (Table I). Given these results, the introduction into Turkey of live animals from other countries may be an important risk factor, as previously reported for BoHV-4 and several other infections (Fichtelova and Kovarcik 2010, Houe 1995). It should also be remembered that the gene region coding for gB is quite highly conserved among BoHV-4 strains (Campos *et al.* 2014, Bellino *et al.* 2015). It is therefore not useful for providing clues about the strains' geographical origins. Thus, the gB gene sequences (GQ246866, MK543551, MK543550, MK543549, GQ246863) of BoHV-4 strains from cattle with reproductive disorders, sampled at two different time points (2007 and 2011) in herd IV were placed in the same branch in the genotype II cluster (Figure 1), with the nucleotide similarities of 100%. This also supports claims that the gene coding gB is quite highly conserved, with similarities rates of (91.7-100%) among all the BoHV-4 strains analyzed in this study, despite being classified in three different genotypes (Figure 3). Additionally, the location on the different branches and/or clusters of the sequences of the samples in the phylogenetic tree from cattle with respiratory system and reproductive system disorders from different herds and years also supports claims that these genetic differences are more related to the herds sampled then clinical cases (Figure 1 and Table I). We believe that further epidemiological studies of BoHV-4 will reveal the relationship between the genotypes and geographical regions, and the association between molecular characterization and pathogenesis of infection.

This study was able to characterize nine BoHV-4 field strains for the sequences coding TK. Based on phylogenetic analysis of the TK gene sequences, these isolates (n = 9) were identified as genotype I, which includes the European (Movar-like) reference strains, together with the Italian, Japanese and Argentinian BoHV-4 strains (Figure 2). These field strains were detected in vaginal swabs (JX644990, JX644990), nasal swab (JX644993), teat lesion samples (KX352280), lungs (MH614620, MH614621), leucocytes from cows with respiratory and/or reproductive system disorders (MG242137), and brain tissue (KX352279 and JX644992) from calves that died after birth (Table I). Regarding the classification of the TK gene, the local strains were identical to each other (100%) and highly similar to other virus strains in genotype I (99.5-100%), genotype II (98%), and genotype III (96.5%) (Tables I and III, Figure 4). Consequently, these genotypes cannot imply a possible association with clinical signs. However, some researchers (Verna *et al.* 2016, Gagnon *et al.* 2017) have argued that the existence of different genotypes suggests some association between genetic variations and specific pathogenic potential. In our study, all BoHV-4 strains in genotype II and III, based on sequences for the ORF3 gene, came from Argentina or Brazil (Figure 2). This origin suggests an association between genotypes and geographic regions similar to that suggested for the gB gene sequences. This may be important for pursuing the traces of domestic or international trade.

We found nine BoHV-4 strains with both the

sequences for genes coding gB and TK. Of these, eight were identified as genotype II and one as genotype I for gB sequences while all were genotype I for TK genes (Table I). We hope that further molecular characterization studies using larger number of samples and targetting some other genes along with TK and gB genes will be able to evaluate the role of these gene(s) in the pathogenesis of BoHV-4 infection.

In our study, the nucleotide and amino acid differences were more pronounced across the gB gene than the TK gene (Figures 3-6). All the local BoHV-4 samples located in genotype I had a change in the amino acid (aa) sequences of the gB gene at positions 44 (K→Q), similar to other genotype I BoHV-4 strains. The sequences (MH605274) of one field BoHV-4 sample from milk, which was the only strain clustered in genotype III, had changes at position 53.aa. (S→D). Turkish BoHV-4 in genotype II had one aa change at 57.aa (A→T). It was detected in seven strains (JX644989, MK543546, MK543547, GQ246864, KX192365, EU055543, KX192364). These formed a branch within genotype II in the phylogenetic tree. This is important given the variety of specimen types and their origin from seven different herds (Figure 6, Table I). The greatest aa change was detected in the gB sequences of BoHV-4 strains from a nasal swab (JX644989) and a leucocyte sample (GQ375280) from cattle in two different herds (Figure 6). These results regarding aa change, especially in the seven genotype II BoHV-4 samples, reveal traces of domestic and/or international trade, although the latter change (A57T) did not match the sequences of any BoHV-4 deposited in Genbank.

The main conclusion from our results is that there are significant differences between the BoHV-4 strains circulating in Turkey, although it is not yet clear how clinical cases are related to their molecular genetic characteristics. Verna and colleagues (Verna *et al.* 2012) and Altamiranda and colleagues (Altamiranda *et al.* 2015) reported similar high variability among BoHV-4 strains in Argentina.

Of the 24 samples used in this study, BoHV-4 was isolated in five of them. These were then included in a virus isolation study to investigate their biological behavior using MDBK cell cultures. Several factors may explain our failure to isolate the virus from 19 PCR-positive samples, including the poor quality of the samples or the inherent difficulty of isolating BoHV-4 from samples (Lin *et al.* 1997, Wellenberg *et al.* 2002). We were lucky that our five positive samples, including one nasal swab (U-NS66-TR2009) and two vaginal swab samples (K339 and KC-VS12) from cattle with different clinical disorders attributed to respiratory and reproductive system disorders, and two brain tissue samples from calves (KC-B12 and ANK.Br.TR2015) were representative of the 24 different herds (herd no: I, II, VII, IX) and different sampling years (Table I).

The replication kinetics of the BoHV-4 isolates showed some differences across the five sampling points. Of BoHV-4 isolates, four (KC-VS12, ANK-Br-TR2015, KC-B12 and U-NS66-TR2009) had the highest titers at 96 hpi whereas isolate K339's titer increased constantly till 120 hpi. The highest virus titers were obtained with K339. Verna and colleagues (Verna *et al.* 2016) reported clear evidence that *in vitro* characterization (biological and molecular) of viral replication in cell culture correlates with *in vivo* virulence. Perez and colleagues (Perez *et al.* 2011) concluded that the viral strain is only weakly correlated with clinical manifestations. We therefore believe that the significant genetic variability in gB gene region sequences and the results on the *in vitro* biological behavior of our BoHV-4 field isolates provides an important resource for further studies into the relationship between pathogenesis and the molecular characterization of BoHV-4 from animals with different clinical signs or from apparently healthy cattle. Unfortunately, all the isolates that we used to investigate biological behavior in MDBK cells were genotype I for gB gene sequences, although we also identified BoHV-4 in genotypes II and III.

## Conclusions

In conclusion, we provided a precise biological and molecular characterization of the ORF8 and ORF3 gene regions coding gB and TK of BoHV-4 field strains from Turkish cattle with different health status. We hope that these findings may help future researchers to understand more clearly the molecular epidemiology of BoHV-4 infection in cattle and the possible association between the virus and pathogenesis of infection.

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