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Molecular Biological Characteristics of Bovine Gamma Herpesvirus 4 Originated from Different Clinical Sources

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Abstract

Bovine herpesvirus 4 (BoHV 4) is a member of the *Herpesviridae* family in the *Gammaherpesvirinae* subfamily, *Radinovirus* genus. The purpose of this study was to isolate BoHV 4 from calves, heifers and cows presenting various clinical entities – respiratory, conjunctivitis, pneumonia, skin damage, genital lesions and abortion, and to characterize its molecular biological features.

Primary and permanent bovine cell cultures were used for virus isolation and culturing. Polymerase chain reaction (PCR) was used to confirm the gB and TK genes and the PCR products were sequenced.

After infecting the primary bovine and permanent cell lines, a cytopathic effect typical of BoHV 4 was observed and 10 cytopathic viral agents were isolated. Amplification products with molecular weights of 567 and 615 bp for the Tk and gB genes, respectively, were found after PCR.

After the BoHV 4 isolates had been sequenced, 473 and 476 nucleotide sequences were analyzed for the Tk and gB genes, respectively. The phylogenetic trees constructed based on the Tk and gB genes of Bulgarian BoHV 4 strains fall within the same branch with the reference BoHV 4 European strains deposited in the genomic bank and their genomic features were similar to European sequenced genomes of other *Radinoviruses*.

Keywords: BoHV 4, PCR, sequencing

Резюме

Говеждият херпесвирус 4 (BoHV 4) е член на семейство *Herpesviridae* в подсемейство *Gammaherpesvirinae*, род *Radinovirus*. Целта на това изследване е да се изолират BoHV 4 от телета, юници и крави с различни клинични симптоми - респираторни, конюнктивити, пневмонии, увреждания на кожата, генитални лезии и аборти, и да се охарактеризират неговите молекулярно-биологични особености.

За изолиране и култивиране на вируса са използвани първични и постоянни култури от говежди клетки. За потвърждаване на gB и TK гените е използвана полимеразна верижна реакция (PCR) и продуктите от PCR са секвенирани.

След инфектиране на първичната говежди и постоянни клетъчни линии е наблюдаван цитопатичен ефект, типичен за BoHV 4 и са изолирани 10 цитопатични вирусни агенти. След PCR са получени амплификационни продукти с молекулно тегло 567 и 615 bp за Tk и gB гените съответно.

След секвениране на BoHV 4 изолатите са анализирани 473 и 476 нуклеотидни последователности за Tk и gB гени. Конструираните филогенетични дървета на базата на Tk и gB гена на български щамове BoHV 4 попадат в един клон с референтните европейските BoHV 4 щамове, депозирани в геномната банка и техните геномни характеристики са подобни на геномите на секвенираните европейски радиновируси.

Introduction

Bovine herpesvirus 4 (BoHV 4) now named as Bovine gammaherpesvirus 4 is a member of the *Herpesviridae* family in the *Gammaherpesvirinae* subfamily, *Radinovirus* genus (Davison *et al*., 2009).

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The BoHV 4 genome is small with type B structure characterizing the subfamily *Gammaherpesvirinae* (Roizman and Pellet, 2001). It has a collinear arrangement of double-stranded nucleic acid (DNA) with a size of 144 ± 6 kb. BoHV 4 genome is organized into a long, unique segment (long unique region - LUR) with a size of 108 ± 2 kb and two repetitive double tandem sections non-coding sequences rich in guanine and cytosine (G + C), which make up the polyrepetitive (pr) DNA (pr DNA), with a size of 1450 to 3050 bp (Bublot *et al*., 1991; Broll *et al*., 1999). BoHV 4 contains a gene encoding thymidine kinase (TK) (Ehlers *et al*., 1985; Kit *et al*., 1986; Thiry *et al*., 1989; Bublot *et al*., 1992; Lomonte *et al*., 1996; Zimmermann *et al*., 2001).

Based on the restrictase fragment analysis (RFA), BoHV 4 isolates are classified into two genome types - 1) similar to the European strain Movar 33/63 and 2) similar to the American strain DN 599 (Thiry *et al*., 1989; Bublot *et al*., 1991). Another classification divides the BHV 4 strains into 3 categories: 1) isolates similar to strain DN 599, 2) similar to strain Movar 33/63 and 3) unclassified strains having a specific restrictase fragment pattern (Thiry *et al*., 1989; 1990; Bublot *et al*., 1991).

BoHV 4 was isolated from calves and heifers with different clinical entities - respiratory symptoms and keratoconjunctivitis (Bartha *et al*., 1966; Mohanty *et al*., 1971), bovine pneumonia (Smith *et al*., 1972) endometritis, abortion, enteritis, skin damage, skin nodules, pustulous mammilitis, breast ulcers, interdigital dermatitis (Reed *et al*., 1977; Cavirani *et al*., 1990, 1996; Donofrio *et al*., 2000) and animals with nodular dermatitis (House *et al*., 1990).

The purpose of this study was to isolate BoHV 4 from ruminants with different clinical symptoms and to characterize its molecular biological features.

Materials and Methods

BHV 4 virus isolation and cultivation

Parts of internal organs (lungs, liver, spleen, kidneys, lymph nodes, uterus, mucous membrane, intestines, tongue epithelium , brain, spleen, kidneys from aborted fetuses, placental cotyledons, and buffy coat) from bovine animals with various clinical signs (respiratory, genital and abortion) were used for virus isolation. The organ tissues were homogenized to 10% (w/v) suspension in cell culture medium, and then were centrifuged at 1500 x g for 10 min. The supernatants were filtered through 0.45 μm-pore-size millipore filters and used to infect the cell cultures. Nasal and ocular swabs were placed in cell culture medium, centrifuged at 1500 x g for 10 min and clear supernatants were used to inoculate the cell monolayers.

Primary bovine kidney, testes and permanent cell cultures from embryonal bovine trachea (EBTR) were used for virus isolation and culturing. As growing medium, Eagle's minimal essential medium (E MEM) was used with 10% fetal calf serum (FCS), 0.075% sodium bicarbonate, 20 mM HEPES buffer, 0.2M-L glutamine, and antibiotics, penicillin 100 UI/mL, and streptomycin 100 μg/mL. The same medium with 3% FCS was used as maintenance medium. Infectious titers were calculated using the Reed and Muench method (1938).

Molecular biological investigation

Polymerase chain reaction (PCR) for demonstration of BoHV 4 Tk and gB genes

Amplification of the Tk gene was performed by conventional and nested Tk-PCR, (Egyed *et al*., 1996) with some modifications (the DNA matrix in the classical reaction was amplified by PCR mix Fideli Tag PCR Master Mix (2X), England).

Primers selected from the sequences of the BoHV 4 Tk gene were used for amplification (Lomonte *et al*., 1992). Sequences were selected from the National Center for Biotechnology Information (NCBI Gene Bank) database (AC 49773) (Table 1). Outer primers Tk 1 and Tk 2 were used for the construction of 567-base pair fragment. The internal or nested set of primers Tk 3 and Tk 4 were used for the formation of 260 bp product. Amplification of the Tk gene was performed in a QB-96 (UK) thermal cycler in a volume of 25 μL, with amount of DNA between 0.2 and 0.4 μg/μL, 10 pmol of each primer, Fideli Tag PCR Master Mix (2X), and distilled water to 25 μL. In the first PCR round, amplification started with pre-denaturation at 95° C for 10 min, followed by 30 cycles at 94° C for 60 sec, at 60° C for 60 sec and at 72° C for 90 sec and a final extension of 7 min at 72° C. A 5 μL volume of the PCR product from the first PCR round was further amplified by a nested PCR round using the same PCR mix as described for the first round but with the nested set of primers Tk 3 and Tk 4 (Table 1).

Analysis of PCR products was carried out by horizontal agarose (1%) DNA gel electrophoresis in 0.5X Tris, Boric acid, EDTA buffer (TBE) buffer for 1 h at 120 V. Gels were stained with 1μg/mL ethidium bromide and photographed under ultraviolet light.

Table 1. Data for used primers and their nucleotide position for amplification of BHV 4 DNA

Oligo nucleotides	DNA sequencing	Nucleotide position of the primers	PCR products [bp]
Tk 1	5'-GTTGGGCGTCCTGTATGGTAGC-3'	132 - 153	567
Tk 2	5'ATGTATGCCCAAAACTTATAATATGACCAG-3'	669 - 698	567
Tk 3	5'-TTGATAGTGCGTTGTTGGGATGTGG-3'	339 - 363	260
Tk 4	5'-CACTGCCCGGTGGGAAATAGCA-3'	577 - 598	260
gB-1	5'-CCCTTCTTTACCACCACCTACA-3'	38 - 17	615
gB-2	5'-TGCCATAGCAGAGAAACAATGA-3'	555 - 576	615

PCR described by Wellenberg *et al*. (2001) with modifications (Fideli Tag DNA PCR Master Mix (2X), England, used for the conventional reaction) was used for the amplification of the gB gene.

Primers gB1 and gB2 exhibiting 100% homology to the sense chain at nucleotide positions 38 to 17 (gB 1) and the opposite nucleotide chain at positions 555 to 576 (gB 2) of the gB gene sequence of BHV 4 were used (Goltz *et al*., 1994) (Table 1).

Amplification was performed in QB 96 Thermal cycler (UK). The program began with pre-denaturation at 95° C for 10 min, followed by 45 cycles with parameters: 94° C for 10 sec, 58° C for 60 sec and 72° C for 90 sec and a final extension of 72° C for 7 min.

DNA derived from other herpes viruses - bovine herpes virus 1 (BoHV 1) Pchelarovo, goat herpes virus 1 (GHV 1) Troyan, swine herpes virus 1 (SHV 1) A 2, and EBTR cell culture uninfected with BoHV 4 were used to determine the specificity of the reactions. European strains Movar 33/63, M52 BP 90, M52 BP 10, M51 MZ 80 and the American strain DN 599 were used as reference BoHV 4 strains.

Sequencing of BHV 4 Tk and gB genes

For the sequencing of the Tk and gB genes, Bulgarian BoHV 4 strains Levski, Nikolovo, Svoboda, Kazichene, Abomasum heifer 1 Godech, Brain cow 1 Godech, Brain cow 4, Brain cow 3053 and the Hungarian strains Movar 33/63, M52 BP 90, M51 MZ, and M51 MZ 80 were used. Sequencing was performed after multiplication of portions of Tk and gB genes using specific primers (Table 1) giving the product of 567 bp for the Tk and 615 bp for gB genes, respectively. For amplification of the Tk and gB genes, the PCR programs described above were used. The amplicons obtained were purified through S-400 columns and a DNA gel purification kit (GE Healthcare, UK). Purity and amounts of the products were determined spectrophotometrically (Gene Quant II, Pharmacia LKB, UK) or after gel electrophoresis in 1% agarose gel. The amplified products were sequenced twice with each of the specific primers mentioned above.

Sequencing was performed in a MegaBACE Sequencer 1000 (Amersham, Bioscience, USA) with the DYEnamic ET Dye Terminator Cycle Sequencing Kit (GE Healthcare, UK) according to the manufacturer's instructions.

The data obtained from the nucleotide and amino acid sequences were used to construct the phylogenetic trees. The multiple alignments of the amino-acid and nucleotide sequences were performed with MUSCLE software (Edgar, 2004). The phylogenetic trees were constructed with JModelTest 0.1.1 (Posada, 2008) and ProtTest 2.4 (Abascal, 2005). For the building of the phylogenetic trees and their graphical representations, MEGA 7 software (Kumar *et al*., 2016) and 1000 bootstrap replications were used.

The data obtained after sequencing were compared to the sequences of BoHV 4 gB gene from strains AJ 617688.1, AJ 617687.1 , Movar 33/63, Z 15044.1 All Genes, EU 055543.1, JX 644989.1, MG 264399.1 USA Calif., MG 264397.1 USA Calif., MG 264404.1 USA Calif., KP 209025.1 Aregn.PLOS, MH 605274.1 Turkey, JX 644988.1, KP 209016.1 Argentina, KP 209021.1 Argentina, KU 180390.1 Argentina, GQ 375280.1, GQ 246866.1 and GQ 246863.1 deposited in NCBI. The sequences of BHV 4 Tk gene of deposited strains AB035515.1, AB035517, AB035516, AF 318573, JN 133502, KC 999113, KX 355279 and S 49773 were used for comparison to the Bulgarian BoHV 4 strains.

Results

BoHV 4 virus isolation and cultivation

After the primary bovine and EBTR permanent cell lines had been infected with 10% suspension from the internal organs of animals with various clinical manifestations (respiratory,

genital and abortion) of the BoHV 4 infection, 10 cytopathic viral agents were isolated (Levski, Nikolovo, Svoboda, Kasichene, Momchilgrad, Lungs cow Godech, Brain cow 1 Godech, Brain cow 4 Godech, Brain cow 3053 Godech and Abomasum heifer 1 Godech).

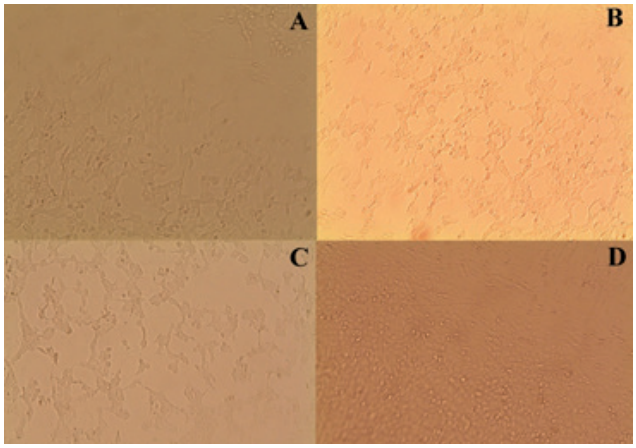

Fig. 1. Cytopathic effect at 48th hours (A), 72nd hours (B), and 120th hours (C) on EBTR cells incubated with 10% organ suspensions from the heifer Godech with characteristic for BoHV 4 clinical symptoms. Control uninfected EBTR cell culture (D). Magnification X 100.

Cytopathic changes characterized by rounding and tearing of the cell monolayer were observed at 24 – 48th h after inoculation. The changes were amplified at 72nd h and after 120th h the cell monolayers were disrupted completely (Fig. 1 B and C). The cytopathic changes of the cell monolayer were not observed in control, non-infected cell cultures (Fig. 1 D).

Molecular biological investigation
Polymerase chain reaction (PCR) for demonstration of the BoHV 4 Tk and gB genes

Amplified products of the Tk gene were with molecular weight of 567 bp after the first PCR (Fig. 2, line 1 to 9). Amplicons of the other used isolates - BoHV 1 Pchelarovo (Fig. 2, line 10), GHV 1 Troyan and SHV 1 A 2 (data not shown) were not observed. Amplification products with a size of 260 bp were obtained after the nested PCR for all BHV 4 isolates investigated (data not shown).

Amplicons with a molecular weight of 615 bp corresponding to the size of the gB gene of BoHV 4 were obtained for all Bulgarian and reference isolates of BHV 4 after the multiplication of gB gene (Fig. 3, line 1 to 8). Amplified products of the gB gene were not observed in the other isolates used - BoHV 1 Pchelarovo (Fig. 3, line 9), GHV 1 Troyan and SHV 1 A 2 (data not shown).

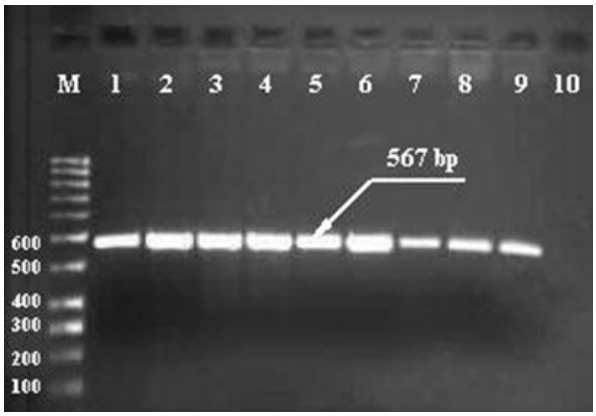

Fig. 2. Classical PCR for multiplication of Tk gene of isolated BoHV 4 strains after first PCR. Line M – molecular weight marker 100 bp, line 1 - "Nikolovo", line 2 - "Kazichene", line 3 - "Svoboda", line 4 - "Момchilgrad", line 5 - "Levski", line 6 - "Lungs cow Godech", line 7 - "DN 599", line 8 - "Movar 33/63", line 9 -"MZ 80", line 10 - BoHV 1 "Pchelarovo".

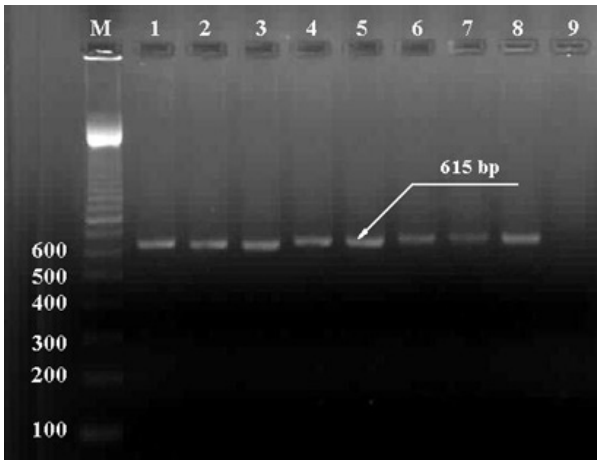

Fig. 3. Classical PCR for gB gene amplification of investigated BoHV 4 isolates. Line - M - Marker of molecular weights 100 bp, line 1 - "Nikolovo", line 2 - "Kazichene", line 3 - "Svoboda", line 4 - "Momchilgrad", , line 5 - "Lungs cow Godech", line 6 -"DN 599", line 7 - "Movar 33/63", line 8 - "MZ 80", line 9 - BoHV 1 "Pchelarovo".

Sequencing of BHV 4 strains.

After the sequencing of the Tk gene of BoHV 4 isolates, 473 nucleotide sequences were analyzed.

Bulgarian isolates Levski, Nikolovo the reference AB 035517, AF035517 and KX355279-Turkey strains formed one branch with 54% homology of the phylogenetic tree based on sequencing of the Tk gene. Referent strains KC 999113 and AB 035515-B 11 formed separate clusters of this branch (with 54% homology). On a separate branch of the phylogenetic tree, with 43% homology were locat-

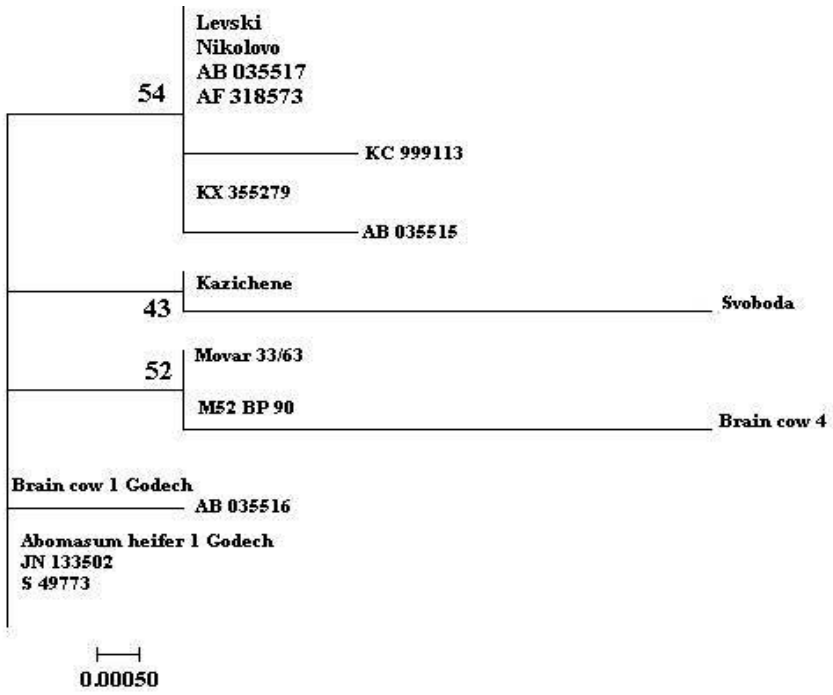

Fig. 4. Phylogenetic tree based on nucleotide sequences comparison from Tk gene of investigated Bulgarian and deposited in gene bank reference BoHV 4 strains.

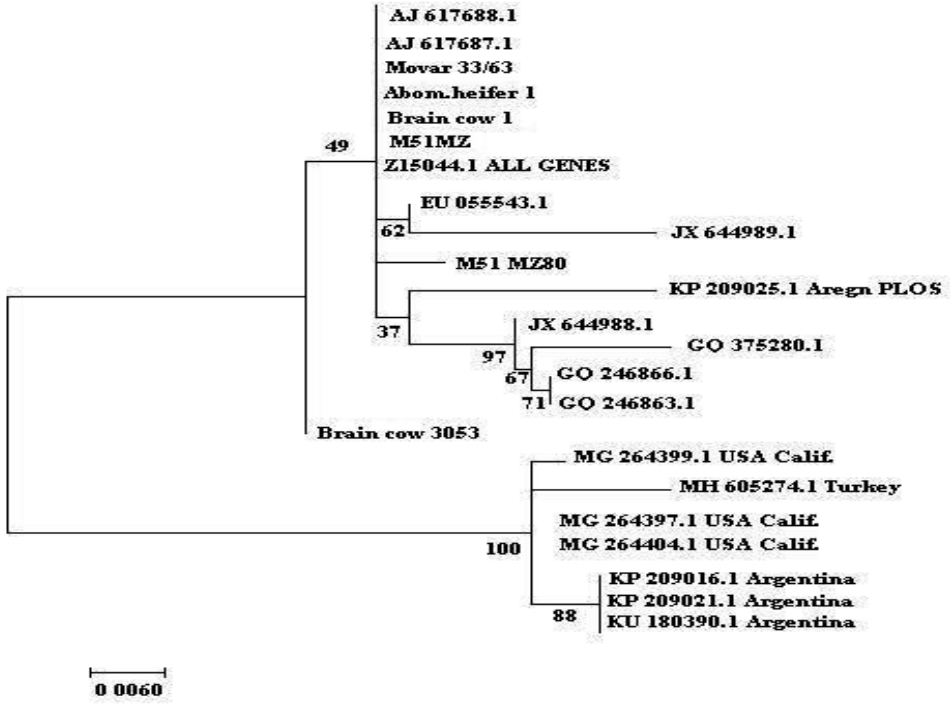

Fig. 5. Phylogenetic tree based on nucleotide sequences comparison from gB gene of investigated Bulgarian BoHV 4 strains and deposited in gene bank reference BoHV 4 strains.

ed strains Kazichene and Svoboda. Movar 33/63, M52 BP 90 and Brain cow 4 Godech were found to reside on the same branch with 52% homology. Strains Brain cow 1 Godech, Abomasum heifer 1 Godech, JN 133502-Belgium and S 49773 were located on the main branch of the phylogenetic tree. The reference strain AB 035516 formed a different clone of the tree (Fig. 4).

The analysis of 476 nucleotide sequences obtained from the gB gene was performed from base 518 to base 994, corresponding to the reference sequence.

After the construction of the phylogenetic tree based on sequencing of the gB gene, Bulgarian isolates Abomasum heifer 1 Godech, Brain cow 1 Godech as well as the European reference strain AJ 617688.1, AJ 617687.1, Movar 33/63, M51 Mz, and Z 15044.1 All Gene were shown to belong to the same branch of the phylogenetic tree with 49 % homology. Referent strains EU 055543.1 and

JX 644989.1 formed a separate cluster with 62% homology of this branch (with 49 % homology). Strains JX 644988.1, GQ 375280.1, GQ 246866.1 and GQ 246863.1 formed different branches of the phylogenetic tree with 97% homology. The Bulgarian strain Brain cattle 3053 did not belong to any branch of the tree. In different clones of the phylogenetic tree with 100% homology are the American, California MG 264399.1, MG 264397.1, MG 264404.1, Turkey MH605274.1, and the Argentinian strains KP 209016.1, KP 209021.1 KU 180390.1 with 88% homology (Fig. 5).

Discussion

After inoculation of primary and permanent EBTR cell cultures, ten cytopathic agents with typical culture characteristics for BoHV 4 were isolated. In the control uninfected cell culture cytopathic agents were not observed. The isolated viral agents were confirmed as BoHV 4 by PCR with specific primers multiplying BoHV 4 Tk and gB genes. It is known that replication and morphogenesis of BoHV 4 is different than that of the other bovine herpesviruses, which is confirmed by our investigation. The primary replication of BoHV 4 is performed in respiratory epithelial cells (Yang *et al*., 2019). The visible cytopathic changes of newly isolated strains were slow and discrete and were observed after 48 h on EBTR cell culture. Subsequently,the propagation of BoHV 4 is increased, which is evidence of the typical slow accumulation of BoHV 4.

The isolated on primary and permanent cell cultures strains from brain, spleen, buffy coat, abomasum and lungs originating form cattle and calves from different country regions were confirmed by PCR for the gB and Tk of BoHV 4 genes.

The classical and nested PCR with specific primers for Tk gene (Lomonte *et al*., 1992; Egyed *et al*., 1996) allowed us to perform more accurate studies of the in vivo prevalence of BoHV 4 in the organs and the peripheral blood leukocytes of the diseased animals. The absence of DNA multiplication obtained from the heterologous herpesvirus strains confirms the specificity of PCR. Evidence that both reactions, classical and nested PCR, are highly sensitive is the lack of bands in the other isolates used - BoHV 1 Pchelarovo (Fig. 2, line 10 and Fig. 3, line 9), GHV 1 Troyan and SHV 1 A 2 (data not shown).

The gene encoding glycoprotein B is one of the most conservative genes among? the Herpesviridae family members. Because of the fact that the primer's nucleotide sequences must be in the conserved region of the gB gene in order to prove all BoHV 4 isolates, the choice of primers for performing the gB PCR is very important. The selected gB primers based on the oligonucleotide sequences of the gB gene (Goltz *et al*., 1994) were specific as they have amplified DNA from all tested BoHV 4 strains and specific amplification for the other investigated animal herpesviruses was not observed.

The structure of the genotype of BoHV 4 is type B, similar to other Gamma herpesviruses (Roizman and Pellet, 2001). After the sequencing of the Tk gene of the Bulgarian BoHV 4 isolates, it was found that the isolates Levski and Nikolovo were grouped in the same branch with the reference BoHV 4 strains AB035517 and AB035515.1 deposited in the gene bank. BHV 4 strain isolated from Brain cow 4 Godech belonged to the same group together with the European reference strain Movar 33/63. Strains Kasichene and Svoboda are more distant in comparison with other Bulgarian strains and formed a different branch of the tree. The remaining sequenced Bulgarian strains Cow lungs Godech and Abomasum heifer No1 Godech did not fall into any of these groups. Most probably those isolates are phylogenetically more distant from the European reference strain or are closer to the American BoHV 4 strain. These results are evidence that the studied Bulgarian viral isolates are also BoHV 4. The genomic features of the sequenced Bulgarian isolates were similar to the sequenced genomes of other Radinoviruses (Broll *et al*., 1999; Zimmermann *et al*., 2001).

Conclusions

1. Bulgarian viral isolates originating from different clinical sources have molecular-biological characteristics typical of BoHV 4

2. The phylogenetic trees constructed based on the gB and Tk genes confirm that the Bulgarian BHV 4 strains have similar characteristics to the European referent BoHV 4 strains deposited in the genomic bank.

3. The amplification products obtained for the gB and Tk genes of Bulgarian BoHV 4 isolates after using specific primers allow more accurate in vivo studies of the presence of BoHV 4 in organs and peripheral blood leukocytes in animals.

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