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Original article

STUDIES ON THE MOLECULAR BIOLOGICAL PECULIARITIES OF BOVINE HERPESVIRUS 4

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Summary

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Studies on the molecular biological features of bovine herpesvirus 4 (BHV 4) strains isolated in Bulgaria have been conducted. Two types of polymerase chain reaction have been developed and applied to confirm the *gB* and *TK* genes. A restrictase fragment analysis was performed using various types of restrictase enzymes. The tested Bulgarian strains differed in their restrictase genomic profile from the reference European strain Movar 33/63 and from the American strain DN 599, and were clearly different each from the other. No clear relationship has been established between the restrictase enzyme profiles and the tropism of the isolated viruses. Sequencing of isolated BHV 4 strains showed homology with the reference European strain Movar 33/63. After construction of the phylogenetic tree, three BHV 4 strains were at one branch of the phylogenetic tree, while two other strains were at the branch of reference Movar 33/63 strain. Applied molecular biology methods can be successfully used for differentiation and detailed genetic characterisation of the isolated BHV 4 strains.

Key words: BHV 4, PCR, restrictase fragment analysis (RFA), sequencing

INTRODUCTION

Bovine herpesvirus 4 (BHV 4) was first isolated and characterised from calves and a heifer with respiratory symptoms and keratoconjunctivitis (Bartha *et al*., 1966; Mohanty *et al*., 1971), bovine pneumonia (Smith *et al*., 1972), endometritis, abortion, enteritis, skin damages, 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).

BHV 4 is classified as a member of the Herpesviridae family in the *Gammaherpesvirinae* subfamily, *Radinovirus* genus, and is distinct from the *Betaherpesvirinae* subfamily with its smaller genome and the typical for subfamily *Gammaherpesvirinae* type B genomic structure (Roizman & Pellet, 2001). It contains a gene encoding thymidine kinase (TK) which is absent in the members of the *Betaherpesvirinae* subfamily and has a collinear nucleic acid arrangement (Ehlers *et al*., 1985; Kit *et*

al., 1986; Thiry *et al*., 1989; Bublot *et al*., 1992; Lomonte *et al*., 1996; Zimmermann *et al*., 2001). The genome is composed by two-stranded DNA with a size 144±6 kb, organised into a long, unique segment (long unique region – LUR or light DNA L-DNA) with a size of 108±2 kb and two repetitive and non-coding sequences of guanine-rich and cytosine-rich (G+C) double tandem section, forming the so-called polyrepetitive DNA (pr DNA), or heavy H-DNA with a size of 1450 to 3050 bp (Bublot *et al*., 1990; Broll *et al*., 1999).

Based on the restrictase fragment analysis (RFA) and the variations in three restrictase sites (two located in the unique central part and one located in the polyrepetitive region of the DNA), the BHV 4 isolates are classified into two types – similar to the European strain Movar 33/63 and similar to the American strain DN 599 (Thiry *et al*., 1989; Bublot *et al*., 1990). Another classification divides the BHV 4 isolates into 3 categories: 1) strains with RFA similar to strain DN 599, 2) RFA similar to the strain Movar 33/63 and 3) unclassified strains having specific RFA (Thiry *et al*., 1989; Bublot *et al*., 1990; Thiry *et al*., 1990).

Sequencing of the genome of BHV 4 revealed that its genomic features were similar to sequenced genomes of other Radionoviruses (Broll *et al*., 1999; Zimmermann *et al*., 2001).

The purpose of this study was to characterise the molecular biological features of Bulgarian BHV 4 isolates.

MATERIALS AND METHODS

The research included nine BHV 4 viral strains (Levski, Nikolovo, Svoboda, Kasichene, Momchilgrad, Lungs cow Godech, Brain cow No 4 Godech, Abomasum heifer No 1 Godech, and Omurtag) isolated from bovines with various clinical signs (respiratory, genital and abortion) and identified in the Viral Diseases of the Ruminants laboratory of NDRMVI. BHV 4 strains Movar 33/63, M-52 BP 90, BP 10, MZ 80, AB035515.1, AB035517, AB035518, and DN 599 and heterologous BHV 1 strain Pchelarovo were used as reference strains.

PCR for demonstration of BHV 4 gB gene

PCR used for investigation of the Bulgarian BHV 4 isolates was as described by Wellenberg *et al*. (2001) with modifications (the DNA master mix in the conventional reaction was amplified by PCR mix Fideli Tag PCR Master Mix (2×), England).

Primers *gB1* and *gB2* exhibiting 100% homology to the sense chain at nucleotide positions 38 to 17 (*gB1*) and the opposite nucleotide chain at positions 555 to 576 (*gB2*) of the gB gene sequence of BHV 4 were used (Goltz *et al*., 1994) (Table 1). Amplification was performed in QB 96 Termal cycler (UK). The programme began with a 95 °C denaturation for 10 min, followed by 45 cycles with parameters: 94 °C for 10 s, 58 °C for 60 s and 72 °C for 90 s and a final extension of 72 °C for 7 min.

Nested PCR for demonstration of BHV 4 TK gene

Amplification of TK DNA target of BHV 4 was based on conventional and nested TK-PCR (Egyed *et al*., 1996) with some modifications (the DNA matrix in the classical reaction amplified by PCR mix Fideli Tag PCR Master Mix (2×), England.

Primers selected from the sequences of the BHV 4 *TK* gene were used for the amplification of the *TK* gene (Lomonte *et*

Table 1. Type of olygonucleotides for PCR

Olygonucleotide type	Position in DNA sequencing	Primers nucleotide position	PCR products (bp)
gB-1	5’-CCCTTCTTTACCACCACCTACA-3’	38–17	615
gB-2	5’-TGCCATAGCAGAGAAACAATGA-3’	555–576	615
TK-1	5’-GTTGGGCGTCCTGTATGGTAGC-3’	132–153	567
TK-2	5’-ATGTATGCCCAAAACTTATAATATGACCAG-3’	669–698	567
nested TK 3	5’-TTGATAGTGCGTTGTTGGGATGTGG-3’	339–363	260
nested TK 4	5’-CACTGCCCGGTGGGAAATAGCA-3’	577–598	260

al., 1992). Sequences were selected from the genomic Genebank database (AC 49773) (Table 1). Outer primers *TK* 1 and *TK* 2 were used for the construction of the 567 base pair fragment, and internal or nested set of primers *TK* 3 and *TK* 4 were used for the formation of a 260 bp product. Denaturation at 95 °C for 10 min, followed by 30 cycles at 94 °C for 60 s, 60 °C for 60 s and 72 °C for 90 s and a final extension of 7 min at 72 °C was performed in the first PCR circle. Five µL volume of the PCR product from the first PCR circle was further amplified in a nested PCR circle using the same PCR mix as described for the first round but with the nested primers 3 and 4 (Table 1).

Amplification of the *TK* gene was performed in a QB-96 (UK) thermocycler in a volume of 25 µL, with the amount of DNA between 0.2 and 0.4 µg/µL, 10 pmol of each primer, and Fideli Tag PCR Master Mix (2X), and distilled water to 25 µL. The first 5 PCR amplification cycles consisted of denaturation at 94° C for 45 s, annealing of the primers at 56° C for 1 min and synthesis at 72° C for 1.5 min. After that 25 cycles of denaturation at 94 °C for 45 s, attachment of the primers at 51 °C for 1 min and elongation at 72 °C for 1.5 min and final extension at 72 °C for 7 min were performed. Analysis of PCR products was carried out by horizontal agarose (1%) DNA gel electrophoresis in 0.5× Tris, Boric acid, EDTA buffer (TBE) buffer for 1 h at 120 V. Gels were stained with 1 µg/mL ethidium bromide and photographed under ultraviolet light.

DNA derived from relative herpes viruses – bovine herpes virus 1 (BHV 1), goat herpes virus 1 (GHV 1), swine herpes virus 1 (SHV 1), and uninfected with BHV 4 cell culture were used for determination of the reaction specificity. Ten-fold dilutions of DNA of BHV 4 strains with initial titres: Momchilgrad ($10^{6.3}$ TCID50/mL), Movar 33/63 ($10^{5.3}$ TCID50/mL) and DN 599 ($10^{7.0}$ TCID50/mL) were used for determination of the PCR sensitivity of *gB* and *TK* genes. Reference strains DN 599 and Movar 33/63, and uninfected cell culture MDBK and distilled water were used as positive and negative control in the reactions, respectively.

Restriction fragment analysis of BHV 4

DNAs of the BHV 4 Bulgarian strains were investigated by using the RFA. DNA was obtained by the methods described by Christensen & Norman (1992). Restriction enzymes (RE) Hpa I, Bam H I, Hind III and Eco R I (Anglian Biotechnology LTD, UK) were used according to the manufacturer’s instructions for the RFA of

DNA. The RE-treated DNAs were examined by electrophoresis in 0.8% agarose gel in 0.5× Tris, Boric acid, EDTA buffer (TBE buffer) for 18 h at 3 V/cm and was photographed with photo-documentation system Wilber Lourmat (France). The BHV 1 strain Pchelarovo was used as a heterologous virus strain.

Sequencing of Bulgarian BHV 4 strains

Sequencing of *gB* and *TK* genes of BHV 4 was performed after multiplication of portions of them using specific primers giving the product of 615 and 567 bp for the *gB* and TK genes respectively and 260 bp for nested *TK* gene. For amplification of the *gB* and *TK* genes were used the described PCR programmes above. The obtained PCR products were purified through S-400 columns and DNA gel purification kit (GE Healthcare, UK). The purity and amounts of the products were determined spectrophotometrically (Gene Quant II, Pharmacia LKB, UK) and after gel electrophoresis in 1% agarose gel. The sequencing was performed by using the DYEnamic ET Dye Terminator Cycle Sequencing Kit (GE Healthcare, UK) according to the manufacturer's instructions. The amplified products were sequenced twice with each of the mentioned above specific primers. Sequencing was performed using a Mega Bace Sequencer 1000 (Amersham, Bioscience, USA). The deposited in GenBank sequences of *gB* and *TK* genes from Movar 33/63 and DN 599 BHV 4 strains (NCBI GenBank) were used as a positive controls.

Multiple clustering of the obtained nucleotide sequences was performed with Clustal W or Muscle. The obtained sequences were analysed for narrow homology through the Basic Local Alignment Search Tool (BLAST), NCBI (http: www.ncbi.nlm.nih.gov/BLAST). Data analysis was done with MEGA 5 Softwere. The evolutionary deviations between the nucleotide sequences of the isolates were determined by the Jukes-Santor, Tamura-Nei, p-distance and substitution of purine with purine or pyrimidine with pyrimidine (transition) and purine substitution with pyrimidine bases and vice versa (transversion).

The obtained data from the nucleotide and amino acid sequences were used to construct phylogenetic trees by the Neighbour-joining method, grouping the viruses according to their different types and subtypes (Saitou & Nei, 1987), bootstrap test-2000 (Felsenstein, 1985) and p-distance model and programme Jmodel Test 0.1.1 (Guindon & Gascuel, 2003) and ProTest 2.4 (Abascal *et al*., 2005), PhyML 3.0 (Guindon & Gascuel, 2003, Posada, 2008) and Phylemon 2 (Sanchez *et al*., 2011), 1000 bootstrap iterations and Fig Tree 1.4.0 software (http://tree.bio.ed.ac.uk/), MEGA 4 (Tamura *et al*., 2007) and MEGA 5 (Tamura *et al*., 2011). The data obtained after sequencing were compared to the BHV 4 sequences deposited in GenBank. Sequences of *gB* and *TK* genes from BHV 4 strains (AB035517, AB035515.1 and AB035518) were used as positive controls and BHV 4 strains, "Movar 33/63" and "DN 599"cultivated in our laboratory – as additional positive controls. Cytomegalovirus sequences were used as an outside group.

RESULTS

PCR for the demonstration of BHV 4

Products with a molecular weight of 615 bp corresponding to the size of the *gB* gene of BHV 4 were obtained after PCR amplification of the *gB* gene for Bulgarian and reference BHV 4 isolates – evidence for the presence of the virus (Fig. 1).

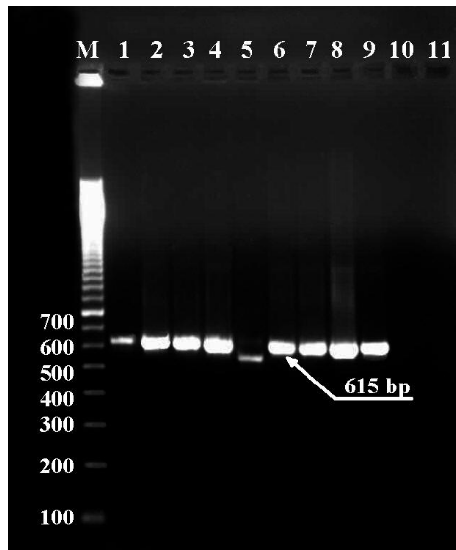

Fig. 1. PCR for *gB* gene amplification of investigated BHV 4 isolates. Lane M: molecular weight marker 100 bp, lane 1: BHV 4 Nikolovo: lane 2: BHV 4 Kazichene: lane 3: BHV 4 Svoboda, lane 4: Lungs cow Godech; lane 5: DN 599; lane 6: BHV 4 Momchilgrad; lane 7: reference strain BP 10; lane 8 - reference strain MZ 80; lane 9 - reference strain Movar 33/66: lane 10: negative control (uninfected cell culture MDBK); lane 11: negative control (distilled water).

The amplified products of the *TK* gene after the first and the nested PCR were with molecular weights of 567 and 260 bp respectively for all viral isolates (Fig. 2 and 3).

RFA of the genome of BHV 4 strains

The three studied isolates Kazichene, Levski and Momchilgrad showed a unique restriction profile after RFA with RE Bam H I. The number of fragments in the unique central region were between 6 and 8 bands with molecular weights from 21568 to 4.4 kDa and varied in the individual strains. The isolates were with the different position of the bands in the central unique region. Fragment five at all three isolates was two-molar, and the sixth fragment of lesser intensity was immediately next to it. In the Levski strain, the fifth fragment was greatest compared to the same fragments in the other two isolates studied. The isolates were different in the polyrepetitive region of the genome and they contained between two and four bands. Two fragments with a molecular weight of 1933 and 1229 kDa were visible in the Kazichene strain. Only one band with a molecular weight of 1913 kDa was found in the Momchilgrad strain. Four bands with molecular weights between 2333 and 1513 kDa were observed in the Levski isolate (Fig. 4B, lanes 2, 3, 4). Pr DNA in BHV 1 Pchelarovo strain was not observed.

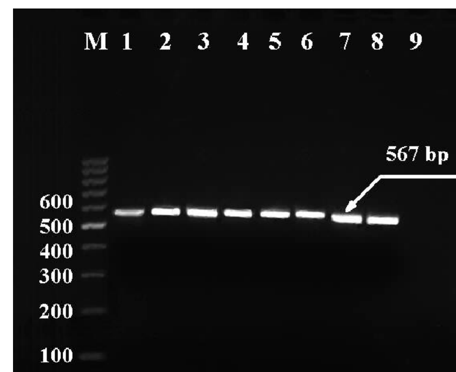

Fig. 2. Multiplication of *TK* gene of isolated BHV 4 strains after first PCR. Lane M: molecular weight marker 100 bp: lane 1: BHV 4 Nikolovo: lane 2: BHV 4 Kazichene: lane 3: BHV 4 Svoboda, lane 4: BHV 4 Moмchilgrad; lane 5: BHV 4 Levski; lane 6: BHV 4 Lungs cow Godech; lane 7: DN 599; lane 8: Movar 33/63, lane 9: negative control (distilled water).

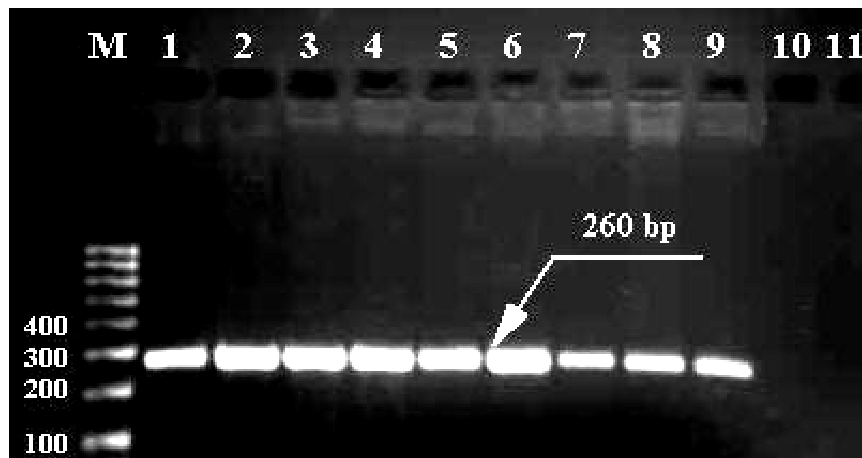

Fig. 3. Multiplication of *TK* gene of isolated BHV 4 strains after nested PCR. Lane M: – molecular weight marker 100 bp; lane 1: BHV 4 Nikolovo; lane 2: BHV 4 Kazichene; lane 3: BHV 4 Svoboda; lane 4: BHV 4 Moмchilgrad; lane 5: BHV 4 Levski; lane 6: BHV 4 Lungs cow Godech; lane 7: BHV 4 Omurtag; lane 8: reference strain Movar 33/63, lane 9: reference strain DN 599, Lane 10: negative control (distilled water); lane 11: uninfected cell culture MDBK.

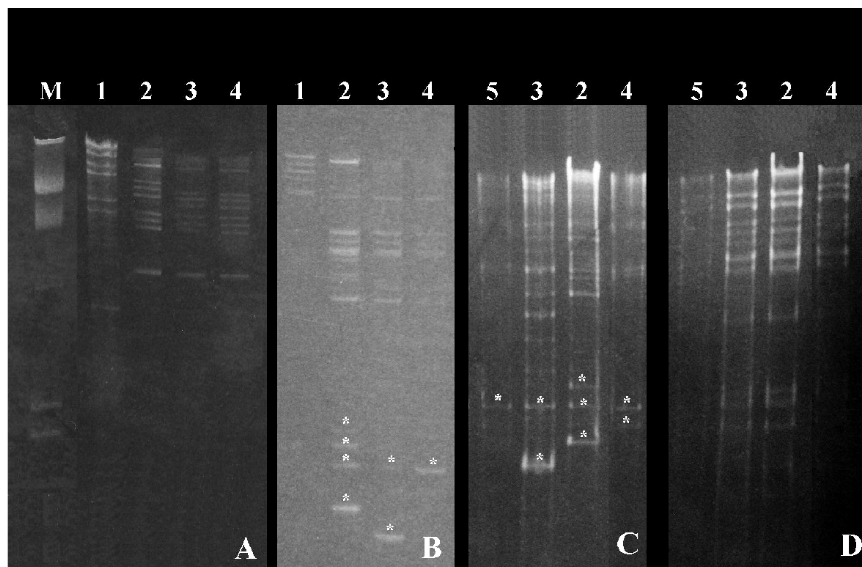

Fig. 4. Restrictase fragment analysis of entire BHV 4 and BHV 1 strains by restrictase enzymes Hpa I (A), Bam H I (B), Eco R I (C) and Hind III (D). Lane M: marker λ DNA, treated with Hind III RE; lane 1: BHV 1 Pchelarovo; lane 2: BHV 4 Levski; lane 3: BHV 4 Momchilgrad; lane 4: BHV 4 Kazichane; lane 5: BHV 4 Movar 33/63. Fragments from pr DNA are marked with an asterix.

Table 2. Data from *gB* gene sequencing of investigated isolates

Isolate	Position in sequencing						
	535	637	928	962	963	964	992
Levski	G	A	T	T	A	A	T
Nikolovo	G	A	T	T	A	A	T
Kazichene	G	G	C	T	A	A	T
Svoboda	G	A	C	T	A	C	C
Lungs cow Godech	G	G	T	T	A	A	T
Brain cow No 4 Godech	C	G	T	C	T	A	C
Abomasum heifer No 1 Godech	G	G	T	T	A	A	T
Movar 33/63	C	G	T	T	A	A	T
M-52 BP 90	C	G	T	T	A	A	T
AB035515.1	G	A	T	T	A	A	T
AB035518	G	G	T	T	A	A	T
AB035517	G	A	T	T	A	A	T

Table 3. Data of TK sequencing of investigated isolates

Isolate	Position in sequence										
	522	529	535	546	637	839	880	928	962	963	964
Levski	–	C	G	A	A	A	T	T	T	A	A
Nikolovo	–	C	G	A	A	A	T	T	T	A	A
Kazichene	–	C	G	A	G	A	T	C	T	A	A
Svoboda	–	C	G	A	A	A	T	C	T	A	C
Lungs cow Godech	–	C	G	A	G	A	T	T	T	A	A
Brain cow No 4 Godech	–	C	C	A	G	A	T	T	C	T	A
Abomasum heifer No 1 Godech	–	–	G	–	G	A	T	T	T	A	A
Movar 33/63	–	C	C	A	G	A	T	T	T	A	A
M-52 BP 90	–	C	C	A	G	A	T	T	T	A	A
AB035515.1	T	C	G	A	A	A	A	T	T	A	A
AB035518	T	C	G	A	G	C	T	T	T	A	A
AB035517	T	C	G	A	A	A	T	T	T	A	A

Nine bands for all investigated isolates were found in the unique central region after treatment with Hpa I RE, but the pr DNA fragments characteristic for the BHV 4 isolates were not detected (Fig. 4A, lanes 2, 3, 4).

Hypermolar fragments in the unique central part of the isolates were not detected after using EcoR I RE. In this re-

gion small fragments with molecular weight of 21.3 to 6.4 kDa whose number varied between 6 and 8 were visible. Two to three fragments with a molecular weight between 1.2 and 2.3 kDa were found in the pr region of DNA in the studied Bulgarian strains and in the reference Movar 33/63 strain (Fig. 4C, lanes 2 –5).

Different number of bands with a molecular weight of 21.3 kDa to 1.5 kDa were visible after using Hind III RE in all investigated BHV 4 isolates, but DNA fragments of low molecular weight (below 2.3 kDa) were not observed (Fig. 4D, lanes 2–5).

The restriction profile of BHV 1 isolates was different compared to that of BHV 4 strains, in both the location of the bands and the absence of pr DNA.

Sequencing of Bulgarian BHV 4 strains

The *gB* gene from the Bulgarian BHV 4 strains Levski, Nikolovo, Kazichene, Svoboda, Abomasum heifer No 1 Godech, Lung cow Godech, Brain cow No 4 Godech and Hungarian strains Movar 33/63, M52 BP 10, MZ 90 and M52 BP 90 were sequenced. The BHV 4 strains sequences AB035515.1, AB035517 and AB035518 deposited in the genome bank were used as reference.

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

The isolate Brain cow No 4 Godech and the reference strains Movar 33/63 and M-52 BP 90 had cytosine (*C*) at position 535 while all the other tested strains had guanine (*G*). The reference strains Movar 33/63, M-52 BP 90, AB035518 and the Bulgarian isolates Kazichene, Lung cow Godech, Brain cow No 4 Godech and Abomasum heifer No 1 Godech at position 637 had guanine (*G*) while the strains Levski, Nikolovo, Svoboda, AB 035515.1 and AB 035517 had adenine (*A*). The strains Kazichene and Svoboda at position 928 had *C* while all the other strains – thymine (*T*). The isolate Brain cow No 4 Godech had *C* and *T* at the 962 and 963 positions, while all the other strains – *T* and *A* respectively. The Svoboda strain had *C* at the 964 position while the re-

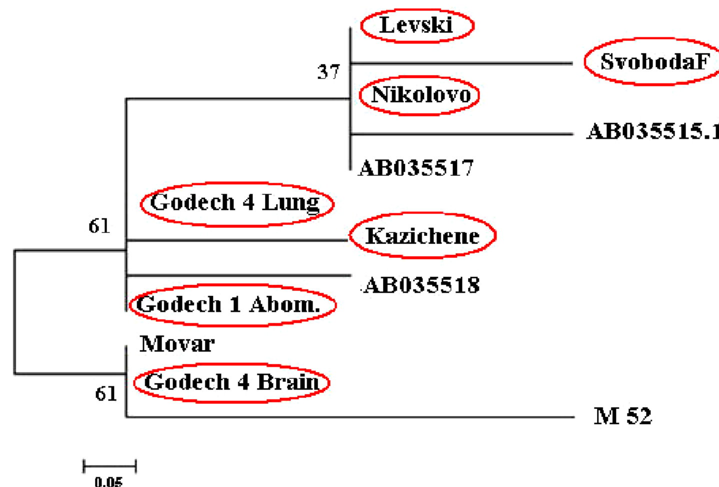

Fig. 5. Phylogenetic tree based on nucleotide sequences comparison from *TK* gene of investigated Bulgarian BHV 4 strains and BHV 4 strains deposited in GenBank.

maining isolates *A*. The isolates Brain cow No 4 Godech and Svoboda had *C* at position 992 while all the other strains *T* (Table 2).

After sequencing of the thymidine kinase gene of BHV 4 isolates, 473 nucleotide sequences were analysed. At position 522 there was a deletion (–) in the Bulgarian sequenced isolates (Levski, Nikolovo, Svoboda, Kazichene, Lung cow Godech, Brain cow No 4 Godech, Abomasum heifer No 1 Godech) as well as in the reference strains Movar 33/63 and M-52 BP 90. In the other reference strains at that position there was *T* (Table 3). The isolate Abomasum from heifer No 1 Godech had a deletion (–) at positions 529 and 546 while the other strains had *C* and *A*, respectively. Strains Movar 33/63, M-52 BP 90 and Brain cow No 4 Godech had *C* at position 535 while the other tested strains – *G*. The isolates Levski, Nikolovo, Svoboda, AB035515.1 and AB035517 had *A* at position 637 while the remaining tested strains – *G*. The reference strain AB035518 had *C* at position 839 while all the others had *A*. Strain AB035515.1 had *A* at position 880, while all the others strains – *T*. At position 928 Kazichene and Svoboda isolates had *C*, while the others strains – *T*. The strain Brain cow No 4 Godech had *C* and *T* at positions 962 and 963 respectively, while the rest had *T* and *A*. At position 964 the isolate Svoboda had *C* while the others strains had *A* (Table 3).

Investigated Bulgarian BHV 4 strains Levski, Nikolovo and Svoboda were in one group with the reference strains AB 035517 and AB 035515.1 after construction of the phylogenetic tree based on the nucleotide sequences of the *TK* gene of BHV 4. The M52 and the Brain cow No 4 Godech strains fell into one group with the European reference strain Movar 33/63. The remaining sequenced strains Kazichene, Cow lungs Godech and Abomasum heifer No 1 Godech did not fall into any of these groups (Fig. 5).

DISCUSSION

The gene encoding glycoprotein B is one of the most conservative genes in 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 BHV 4 isolates, the choice of primers for performing the *gB* PCR is very important. The *gB* primers used by us based on the oligonucleotide sequences of the gB gene (Goltz *et al*., 1994) were specific as they have amplified DNA from all tested BHV 4 strains and specific amplification for other animal herpesviruses was not observed.

The classical and nested PCR with specific primers for *TK* gene (Lomonte *et al*., 1992; Egyed *et al*., 1996) allowed performing more accurate studies of the *in vivo* prevalence of BHV 4 in the organs and the peripheral blood leukocytes of the diseased animals. The absence of DNA multiplication obtained from heterologous herpesvirus strains confirms the specificity of PCR.

The restriction profile of BHV 4 DNA completely differs from that of the other herpesviruses (Ludwig, 1983). The BHV 4 isolates differ by their RE profile, as the differences are in both the unique long segment and the monomeric pr DNA (Thiry *et al*., 1986). In the unique segment, the differences were in the large molar fragments obtained after digestion with Eco RI and BamHI RE endonucleases. Different numbers and molecular weights of the DNA fragments were found in the Kazichene, Momchilgrad and

Levski strains after using RE Bam H I. That is the proof for the heterogeneity of the isolates. Cutting of DNA by the EcoR I RE permits differentiation of all isolates. After using Hind III RE, slicing of pr DNA was not observed, and the presence of hypermolar fragments only was found (Bublot *et al.,* 1991). After analysis of the genomic structure of BHV 4, Ehlers *et al*. (1985) found that the linear double stranded DNA had a size of 144±6 kb and contained approximately 15 monomeric pr DNA segments for the genome, similarly to the other cytomegaloviruses, and based on this features the strains can be divided in 3 classes: Class I and Ia (1950 bp), Class II (2350 bp) and Class III (2750 bp). The authors found differences of 400 bp between the three classes. After monitoring the pr DNA fragments from the studied BHV 4 isolates, the Bulgarian strains Kazichene and Momchilgrad can be classified as Class I and Ia (size of monomeric pr DNA 1913, 1229–1933, respectively) and the Levski strain can be referred to class II with a size of 2333 bp.

REF analysis of BHV 4 strains with different types of RE demonstrated that the Bulgarian strains belonged to the strains with a genomic REF profile similar to the European strain (Movar 33/63). Despite the high heterogeneity of the investigated BHV 4 strains, we were not able to connect the viral tropism to the REF profile of the strains. Similar conclusions are made by Bublot *et al*. (1991) and Naeem *et al*. (1991). A larger number of BHV 4 strains isolated from the animals with different clinical symptoms need to be studied by REF analysis for determination of the viral tropism.

The structure of the genotype of BHV 4 is type B, similar to other gamma herpesviruses (Roizman & Pellet, 2001). After the sequencing of the *TK* gene of the Bulgarian BHV 4 isolates, the Bulgarian isolates Levski, Nikolovo and Svoboda were grouped in one group with the reference BHV 4 strains AB035517 and AB035515.1 deposited in the GenBank. This provides evidence that the studied Bulgarian viral isolates are BHV 4. The BHV 4 "Brain cow № 4 Godech" strain fell into one group with the European reference strain Movar 33/63. The remaining sequenced strains Kazichene, Cow lungs Godech and Abomasum heifer No 1 Godech did not fall into any of these groups. Most probably those isolates are phylogenetically more distant from the European reference strain or closer to the American BHV 4 strain.

CONCLUSIONS

The specific products obtained for the *gB* and *TK* genes after using specific amplification primers allow for more accurate *in vivo* studies of the incidence of BHV 4 in organs and peripheral blood leukocytes in animals.

The REF profile of the Bulgarian BHV 4 isolates obtained after the REF analysis was similar to that of the European prototype strain Movar 33/63.

The Bulgarian isolates had different REF profiles, and the most suitable RE for investigation of DNA were Eco RI and Bam HI.

After construction of the phylogenetic tree based on the TK gene, the Bulgarian BHV 4 strains Levski, Nikolovo and Svoboda fell into one branch with the reference BHV 4 strains deposited in the genomic bank.

Bulgarian BHV 4 strains Brain cow No 4 Godech fell into one group with the reference BHV 4 Movar 33/63. The BHV 4 strains Abomasum heifer No 1 Godech

and Cow lungs Godech configured a separate group.

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