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RESEARCH ARTICLE

SEQUENCING AND ANALYSIS OF RFLP VARIATIONS IN BULGARIAN *CAPRINE HERPESVIRUS 1* ISOLATES AND DIFFERENTIATION FROM *BOVINE HERPESVIRUS* 1 IN GOATS

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ABSTRACT

Caprineherpesvirus 1 (CapHV1) is related to *Bovine herpesvirus* 1 (BoHV1) and may cause respiratory and reproductive symptoms. CapHV1 disease pathogenesis is poorly understood and there are few studies on Bulgarian CapHV1 isolates. The aim of this study was to investigate the central region of the glycoprotein C (gC) gene of CapHV1 in fiveBulgarian isolates and compare it with other European and American CapHV1 and a BoHV1 strains. PCR amplifications yielded 414 bp products for the CapHV1 strains and BoHV1. *HpaII*and*MspI* gave different restriction patterns for the gC gene from Bulgarian and American "McK" isolates in comparison to the Western European isolates, but did not cleave the BoHV1gCgene.The sequence analysis identified differences at positions 887, 960, 1000 and 1110 in the CapHV1 gC gene of the Bulgarian isolates. Short sequence repeats were also observed in the studied region. Evolutionary relationships of CapHV1 isolates were identified. The obtained results indicate that the analyzed region of the gC gene could reveal some epizootiological aspects and is suitable for comparing different CapHV1 isolates. PCR and restrictase analysis of the gC gene could be used to distinguish between BoHV1 and CapHV1 infections in goats.

INTRODUCTION

Caprineherpesvirus 1 (CapHV1) is a viral pathogen which may cause respiratory symptoms, balanoposthitis, vulvovaginitis, necrotic lesions, vesicles, erythema with shallow ulcers on the vulvae and vagina, infertility and abortions in goats (Tempesta *et al*., 1999; Uzal *et al*., 2004). In 10–14-day-old kids the main clinical symptoms are enteritis and severe generalized disease, most often with a lethal outcome (Saito *et al*., 1974). The virus could remain latent in the nuclei of the sacral ganglia and could be reactivated under stress or immunosuppression (Plebani *et al*., 1983; Bounavoglia *et al*., 1996). CapHV1 has the typical morphology of a herpes virus: 80–100 nm indiameter, icosahedral nucleocapsid and a tegument enclosed by a lipoprotein envelope with numerous small glycoprotein peplomers containing from 4 to 15 glycoproteins (Roperto *et al*., 2000). The nucleic acid is a single linear, double-stranded DNA molecule with a size of135.7±2.7 kb and molecular weight of (90.1±1.8) × 10^6D (Engels *et al*., 1983). The genes are divided into three categories: encoding proteins connected to the viral replication, structural proteins and a heterologous set of optional genes, unessential for the viral replication (Engels and Ackermann,1996). The genes which encode the glycoprotein synthesis are with a different degree of conservativity and determine the genetic relationship between the different types of herpes viruses (Ros and Belak, 1999). The glycoprotein C (gC) gene is responsible for the viral pathogenesis, the adsorption to the targeted host cells by its heparan-like receptors (Okazaki *et al*., 1994; Byrne *et al*., 1995) and determines the virus cell tropism (Rijsewijk*et al*., 1999). The conducted analysis of different CapHV 1 isolates was able to identify the presence of short sequence repeats (SSR) in a region of 400 to 464 bp size products (Tarsitano *et al*., 2010). In order to clarify the role of these SSRs and different substitutions in the genome for the viral pathogenesis and cell tropism, further phylogenetic investigations should be performed. *Bovine herpesvirus 1*(BoHV1) and CapHV1 are genetically and serologicallyrelated (Ros and Belak, 1999;

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Thiry *et al*., 2006). Moreover, goats can serve as a reservoir of BoHV1 (Six *et al*., 2001).The aim of the present study was to investigate the CapHV1 gC gene region with a size of 414 bp in five Bulgarian isolates and compare it with four European and American CapHV1 strainsand a BoHV1 strain. The possibility to differentiate between CapHV1and BoHV1 in goats was also explored. An attempt to identify correlations between the characteristics of the isolates, the clinical presentation, the epizootiological data from the affected farms and experimental infection of young goats was made.

MATERIALS AND METHODS

Virus strains,cells and cultivation

Five Bulgarian viral strains isolated from clinically affected goats from the Bulgarian White Milk Goat (BWM) breed were used in this study (Table 1). European strains "E/CH", "Sp1" and "Sp2", the American "McK" CapHV1 strain and the heterologous BoHV1 strains "Oxford" and "Tchervenavoda", were used as positive controls(Table 1). All CapHV1 and BoHV1 strains were cultivated in MDBK cell line (CCLV 1992) as previously described (Sirakov *et al*., 2011). Infected cells were collected when 90% cytopathic effect was observed.

Table 1. CapHV1 and BoHV1 strains used in this study

Herpes virus strains and isolates	Origin	Symptoms	Source
CapHV1			
McK	USA	Abortions, enteritis and severe generalized disease in kids	Reference strain, used as control
E/CH	Switzerland	Ulcerative enteritis	Reference strain, used as control
Sp1	Spain	Latent infection	Reference strain, used as control
Sp2	Spain	Latent infection	Reference strain, used as control
Ba1	Italy	Latent infection	Reference strain, used as control
Suhindol	Bulgaria	Abortions and infertility	Peshev R. *et al*., 2008
Troyan	Bulgaria	Abortionsand infertility	Peshev R. *et al*., 2008
Biser	Bulgaria	Abortions	Peshev R. *et al*., 2008
PavelBanya	Bulgaria	Enteritis and severe generalized disease in kids	Peshev R. *et al*., 2008
Kyustendil	Bulgaria	Infertility, balanoposthitis, vulvovaginitis	Peshev R. *et al*., 2008
BoHV 1			
Oxford	UK	Genital infection	Reference strain, used as control

Nucleic acid extraction and amplification

Viral DNA from cell cultures inoculated with CapHV1 and BoHV1 strains was extracted using Virus DNA/RNA extraction kit (GeneShun Biotech, China), according to the manufacturer's recommendations. DNA was resuspended in 70 µl of nuclease-free H_2O and kept at -20 °C until use.PCR amplification was carried out with Usb MasterMix (USB Corporation, Ohio, USA), according to the method described by Tempesta *et al*., (1999) and Fuchs *et al.*, 1999). The following primers were used for amplification of the CapHV1 gC gene between positions 759 and 1172 bp (Hecht *et al*., 1995): forward primer 5'- AGGGCGCCGGTGGATGCTCTG -3', reverse primer 5'- GGCGGGCGGTGCGTCGTGA-3'. The primers used for amplification of the BoHV1 gC gene between positions 563 bp and 1087 bp were: forward 5'-AGGAGCGCAAGTGGATGCTCTG-3', and reverse 5'-GTAGCCGTTGCGGAACCAGTGC-3'.The CapHV1 reference strain "E/CH", BoHV1 strains "Oxford" and "Tchervenavoda", and cell supernatant from an uninfected cell culture were included in the test procedure as a homologous positive control, a heterologous positive control and a negative control, respectively. The amplified products were purified through S400 columns (GE Healthcare, Giles, UK), according to the manufacturer's recommendations.Nucleic acid concentrations of all investigated isolates were measured by a GeneQuant II spectrophotometer (Pharmacia LKB, Biochrom, UK) for control of their quantity and quality.The quality of the PCR amplification and the purification were tested by 2% agarose gel electrophoresis (GE Healthcare, Giles, UK) with a 100 bp DNA Ladder (GE Healthcare, Giles, UK). The electrophoresis conditions were: 120 V, 45 mA, 45 min.

Restriction enzyme analysis (REA)

The PCR products obtained from the gC gene of the CapHV1 strains and the heterologous BoHV1 strain "Oxford" were analysed by the restriction enzymes *HpaII* and *MspI* for detection of the methylation sites, according to themanufacturer's instruction (Fermentas, Lithuania). Both enzymes recognize CCGG sites, but *Hpa II* cleavage is blocked by CpG methylation of the target DNA. Mega5 version 5 software (Tamura *et al*., 2011) was used to determine the size of the specific restriction bands. Gel electrophoresis was performed with 4% ready-to-use gels (Reliant Gel System, Lonza, USA) and low DNA Leader (GeneShun Biotech, China) at 5 V/cm^2 for 4 h at 4^{o}C.

Sequencing and data processing

The PCR products were sequenced two times using the DYEnamic ET Dye Terminator Cycle Sequencing Kit(GE Healthcare, Giles, UK), according to the instructions of the producer, with each of theprimers specific for CpHV1 described above. Sequence determinationwas performed with a MegaBACE1000 automatic sequencer (Amersham Biosciences). An M13mp18DNA controlof reaction and a MegaBACE 4 ColourStandart (control of reading) were included in the sequencing procedure. The obtained sequences were analyzed for close homology by the Basic Local Alignment Search Tool (BLAST) available at the National Center for Biotechnology Information (NCBI; Bethesda, MD) (http//:www.nbi.nlm.nih.gov/BLAST). The amino acid and nucleotide sequences were compared with the gC gene sequences deposited in NCBI GenBank for CapHV1 strains "E/CH" (Z49225.1) and "Ba1" (AY821804.1). The BoHV1 strain (Z49223) was used as an out-group control in the phylogenetic analysis.

The multiple alignments of the amino-acid and nucleotide sequences were performed with MUSCLE (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, PhyML 3.0 (Guindon and Gascuel, 2003) by Phylemon 2 (Sanchez *et al*., 2011), 1000 bootstrap replications and the Fig Tree1.4.0software (http://tree.bio.ed.ac.uk/) were used.

RESULTS

After the amplification step, a 414 bp PCR product was observed in all of the studied CapHV1 strains (Figure 1, upper panel). Specific amplicons were not found in any of the CapHV1 strains when the PCR was performed with primers targeting the BoHV1 gC gene (data not shown). The amplification of the BoHV1 strain "Oxford" gave specific products with a size of 527 bp as well as amplicons 414 bp in size (Figure 1, lower panel). The analysis performed with the software Mega 5 (Tamura *et al*., 2011) showed that the primers targeting the CapHV1 gC gene could recognize and attach to the BoHV1 gC gene between nucleotide positions 768 and 1175. The CapHV1 forward primer had mismatches with the sequence of the referent BoHV1 (Z49223) strain in primer positions 1 (**A→G**), 3 (**G→A**), 8 (**C→A**) and 9 (**G→A**). A mismatch in position 5 (**G→C**) was detected for the CapHV1 reverse primer. The detached fragment of BoHV1 between both primers was 408 bp in size, shorter than that in the amplification of CapHV1. In the 857–862 bp fragment of BoHV1 (Z49223) there was a deletion with a size of 6 bp as compared to CapHV1 (Z49225.1).

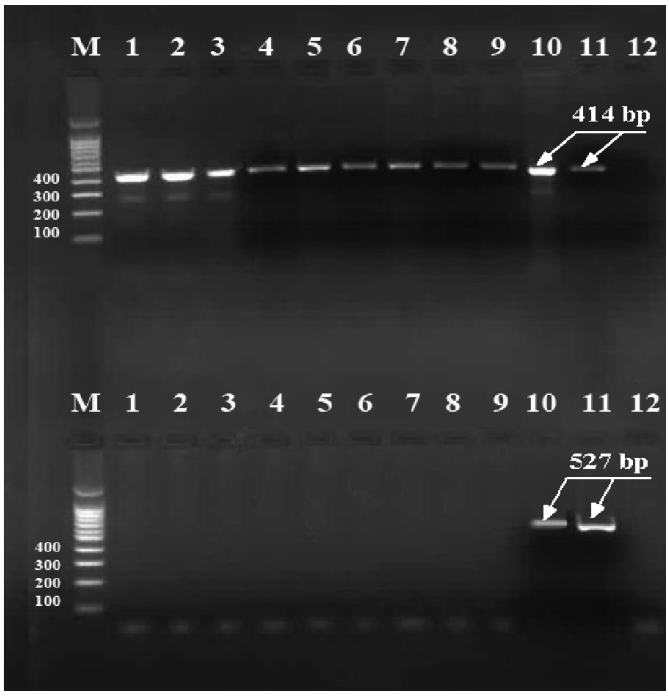


Fig. 1. PCR amplification results

Comparison of the gC gene restriction profiles obtained by Hpa II digest or by Msp I digest showed no difference in the fragments size and number. Different restriction profiles of the gC gene for the Bulgarian CapHV1 isolates and the American strain "McK" in comparison to the Western European isolates were found (Figure 2). Using theMEGA 5 software, the following restriction enzyme cleavage patterns were identified: Western European isolates– 8 bp, 14 bp, 44 bp, 46 bp, 92 bp, 96 bp and 114 bp; Bulgarian isolates (except "Biser") and the American strain "McK" – 8bp, 14bp, 28bp, 44bp, 46bp, 68bp, 92 bp and 114 bp; isolate "Biser" 8 bp, 14 bp, 28 bp, 44 bp, 68 bp, 92 bp, 160 bp.After the gel electrophoresis we observed the following results: three bands, 44 bp, 92 bp and 114 bp in size, in the Western European isolates (Figure 2, lanes 2, 3 and 4) and five bands with a size of 28 bp, 44 bp, 68 bp, 92 bp and 114 bp in Bulgarian isolates"Troyan", "Suhindol", "PavelBanya" and "Kyustendil" and in the American "McK" strain (Figure 2, lanes 1,5,6,7 and 9).

In the CapHV1 isolate "Biser" there were also five bands but the band with the largest size was 160 bp, in contrast to the 114 bp fragment observed in the other isolates included in this study (Figure 2, lane 8). The specific bands for the Bulgarian and the American CapHV1 strains were 28 bp and 68 bp, except for the "Biser" isolate, where the specific bands were 28 bp and 160 bp. The REA with *HpaII* and *Msp*Ienzymes was unsuccessful forthe gC gene of the BoHV1 strain "Oxford"(Figure2, lane 10).

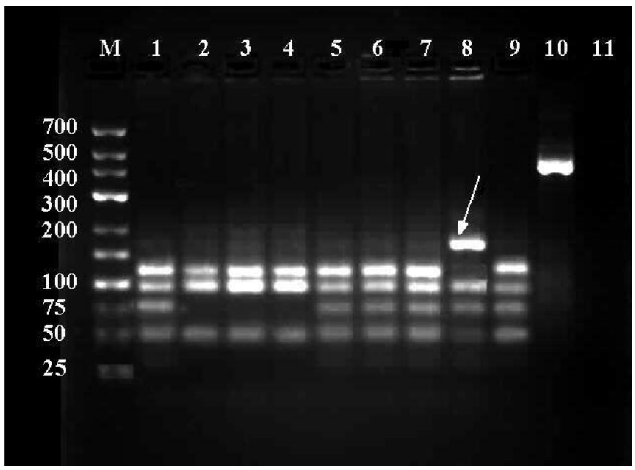
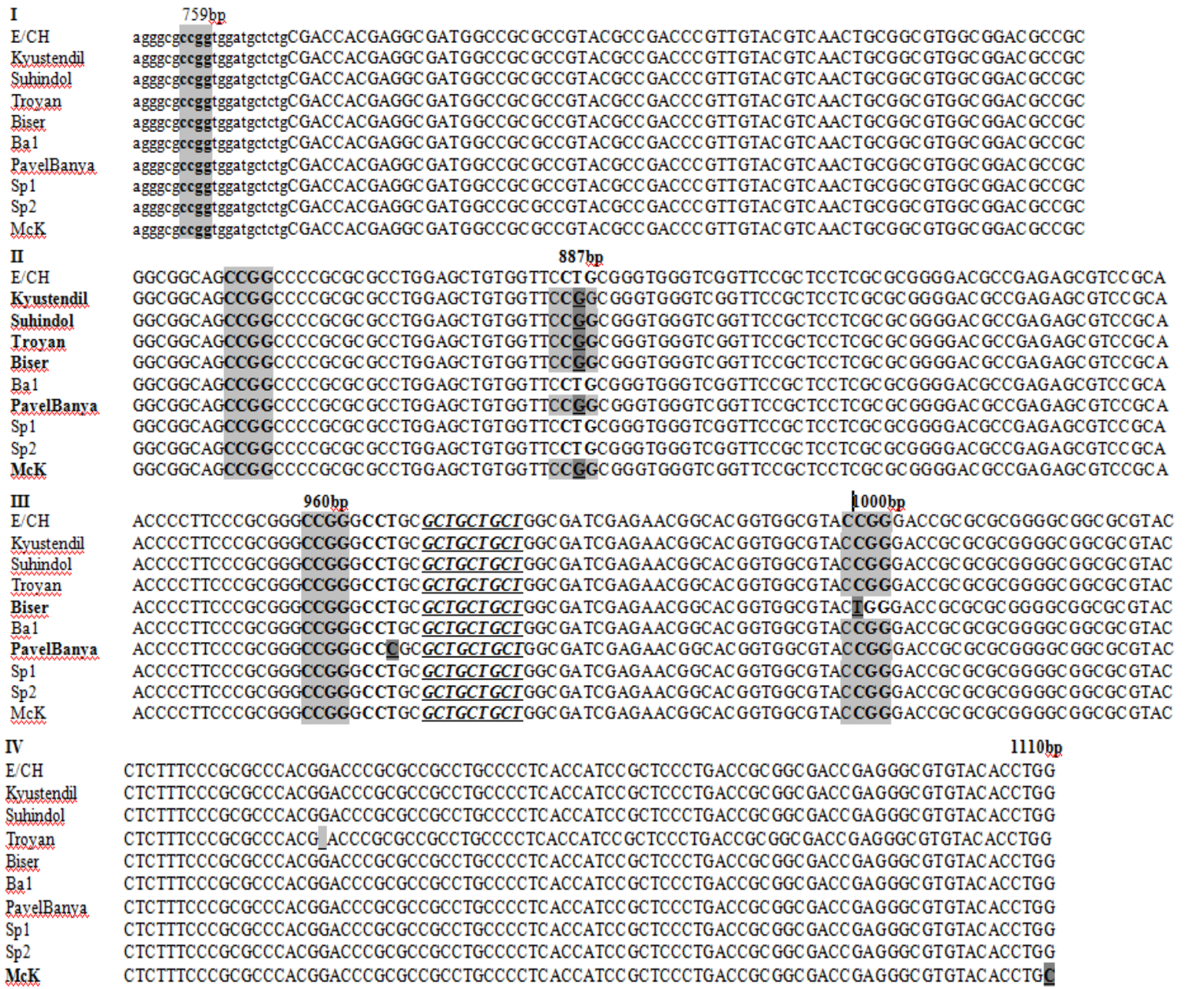


Fig. 2. Restriction analysis of the PCR products

Two additional bands with a size of 8 bp and 14 bp were observed in all CapHV1 isolates when the data were analyzed with the MEGA 5 software. Specific bands with a size of 96 bp were found for the Western European isolates and 46 bp, for all CapHV1 isolates except "Biser". In order to investigate the relationship between the observed different restriction pattern of the studied CapHV1 isolates and the virus origin, sequencing analysis was performed.All PCR products of the CapHV1 gC gene were sequenced and after multiple alignments by the MUSCLE method, the gC gene products with a size of 351bp were used in the subsequent nucleotide sequence analysis. The identity for all studied CapHV1 isolates and the BoHV1 "Oxford" strain was 99% after comparison by BLAST analysis with the gC gene sequences deposited in NCBI GenBank for CapHV1 and BoHV1, respectively.

Figure 3.**Sequencing of the PCR products.** Sequencing results and detected point mutations (marked in bold, underlined and dark grey colour) in the gC gene. Short sequence repeats (SSR) consisting of three nucleotide units – GCT are underlined andin italic. Restriction sites of Hpa II / Msp I enzymes are in bold and light grey colour. Forward primer alignment is shown with lowercase letters.



Differences at positions 887, 960, 1000 and 1110 of the studied CapHV1 gC gene for all Bulgarian isolates were found after multiple alignment (Figure 3). The mutations in position 887, 1000 and 1110 were nonsynonymous, leading to amino acid changes, respectively: leucine → arginine;arginine → tryptophan and tryptophan → cysteine (Tables 2 and 3). In the 351 bp PCR fragment of the central part of the gC gene in all of the investigated CapHV1 isolates (759 bp and 1172 bp) there were short sequence repeats (SSR) consisting of three nucleotide units, GCT, with three repetitions at position 963–971 bp (Figure 3).

Table 2. Detected point mutations and their position in the nucleotide sequence of the CapHV1 gC gene, positions 759 – 1172 bp

CapHV1isolates/ strains	Gen Bankaccession No.	Position of point mutations in the gC gene nucleotides				
		887	960	1000	1045	1110
E/CH	Z49225.1	T	T	C	T	G
Sp1	not submitted	T	T	C	T	G
Sp2	not submitted	T	T	C	T	G
Ba1	AY821804.1	T	T	C	T	G
Biser	JX993260	G	T	T	T	G
Kyustendil	JX993261	G	T	C	T	G
PavelBanya	JX993262	G	C	C	T	G
Suhindol	JX993263	G	T	C	T	G
Troyan	JX993264	G	T	C	Δ	G
McK	not submitted	G	T	C	T	C

Legend: T- Thymine; C- Cysteine; G- Guanine.Bold nucleotides represent detected point mutations; "**Δ**" – deletion

Table 3. Amino acid changes in the CapHV1 gC protein region 253 – 391amino acids

CapHV 1isolates	Position of nonsynonymous changes in the gC gene		
	296	334	370
E/CH	L	R	W
Sp1	L	R	W
Sp2	L	R	W
Ba1	L	R	W
McK	R	R	C
Kyustendil	R	R	W
Suhindol	R	R	W
Troyan	R	R	W
Biser	R	W	W
PavelBanya	R	R	W

Legend: L – Leucine; R – Arginine; W – tryptophan; C – cysteine;

A TMI3 model of a phylogenetic tree based on the nucleotide sequences is presented on Figure 4A. Two main branches were formed. The first branch grouped all of the investigated CapHV1 isolates and the second one included only the "PavelBanya".Based on the differences at position 887 of the gC gene, the first branch was divided into two subgroups. The first subgroup included all the Western European strains: "E/CH", "Sp1", "Sp2" and "Ba1", and the second subgroup, the Bulgarian strains "Troyan", "Kyustendil" and "Suhindol" and two branches also as a result of the changes at position 1000 (C→T) for the "Biser" isolate and at position 1110 (G→C) for the "McK" strain. When a JTT phylogenetic tree based on the amino-acid sequences was build, all Bulgarian isolates and the American isolate "McK" were grouped together as a subgroup, and the Western European isolates formed another subgroup (Figure 4B).

All the sequences of the Bulgarian CapHV1 isolates have been deposited in NCBI GenBank under the following accession numbers: "Biser" (JX993260), "Kyustendil" (JX993261), "PavelBanya" (JX993262), "Suhindol" (JX993263) and "Troyan" (JX993264).

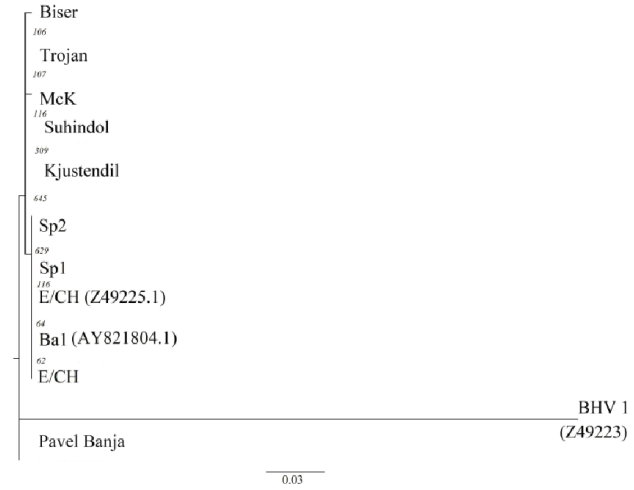


Fig. 4a

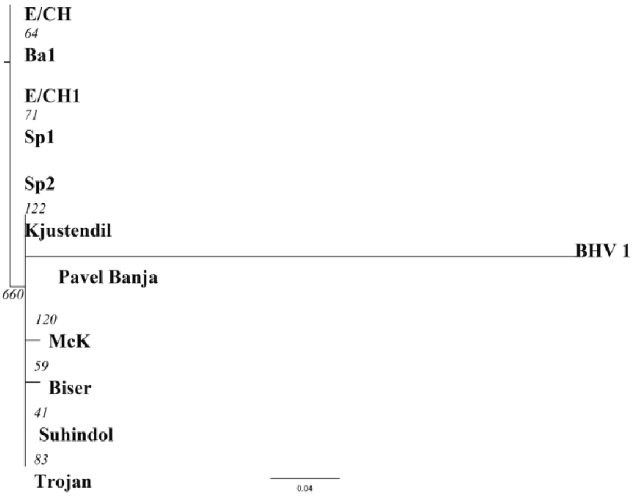


Fig. 4b

Fig. 4. Phylogenetic analysis

DISCUSSION

In many countries, goats andsheep are reared in close contact with cattle, sharingpastures, water, food and facilities. With the developmentof the agricultural reform in Bulgaria the number of ruminants has decreased dramatically and they are reared in small private farms of 2 to 5 animals or big farms with 50–200. Moreover, a single farm is often used for rearing different types of animals. The ability of different alphaherpesviruses to circulatein the farms and to cross the species barrier increases the possibility for infections in heterologous animal species. Thus, it is possible a recombination to occur with a related ruminant alphaherpesvirus and new viruses to be generated or the presence of another virus to be undetected, which would complicate the eradication programs.

BoHV1 infection is a serious problem for livestock breeding and it is under EU regulations 64/432/EEC and 88/407/EEC. CapHV1is closely related toBoHV1 (Ros and Belak, 1999,

Thiry *et al*., 2006), and the clinical symptoms and pathogenesis of CapHV 1 and BoHV1are similar (Engels*et al*., 1992). These two viruses show serologic cross-reactivity(Nixon *et al*., 1988) and sequence identity on the nucleotide level for the gB gene (Ros and Belak, 2002).Goats can be a reservoir for BoHV1 (Six *et al*., 2001) and have been experimentally proved to be sensitive to BoHV1 (Wafula *et al*.,1985; Six *et al*., 2001). Moreover, BoHV1 causes latent infection in trigeminal ganglia of goats and can be reactivated after dexametazone treatment for several days (Engels *et al*., 1992; Six *et al*., 2001). BoHV1 has also been isolated from naturally infected goats (Fulton*et al*., 1982,Whetstone*et al*., 1988, Kalman and Egyed, 2005).This, together with the fact that the CapHV1 pathogenesis is still not fully understood, highlights the significance of studies focused on identification of mutations in the CapHV1 genome and their differential role in disease pathogenesis. It is also important to develop an array of methods for differentiation between CapHV1 and BoHV1 and, respectively, for screening of goats and other non-bovine species for infection with BoHV1.

Closely related alpha herpesviruses can be differentiated and identified by PCR and REA. The obtained PCR products demonstrate that the used sets of primers for the CapHV1 gC gene (Hecht *et al*., 1995) could specifically amplify DNA sequences of the BoHV1 gC gene, similar to what has been established for the gB and gD gene in other herpes viruses (Lyaku *et al*., 1996; Ros and Belak, 1999). In the investigated part of the gC gene, the shorter amplified product, which was scored by the software but was otherwise unclear in the 2% gel, was due to the shorter DNA sequence of BoHV1 in comparison to CapHV1 as a result of a deletion or a frame-shift mutation in the investigated region. Consequently, in our experiments, the CapHV1-specific primers could amplify BoHV1 but not vice versa (the primersets specific for the BoHV1 gCgene did not amplify products from the CapHV1 gCgene).

The REA results are evidence for the lack of methylation sites in the investigated region of the gC gene – enzymes *Hpa II* and *Msp I* cut equal the target DNA. This finding showed that the strains included in our study could not be differentiated based on DNA methylation(Waalwijk and Flavell 1978). Other restriction enzymes (*Pst I*, *BstE II, Xho I* and *BamHI*) have been used to identify differences of restriction patterns in the whole CapHV1 genomewithbetween New Zeland ("3338") and Australian ("Glenfield") isolates (Tisdall, *et al*., 1984), European ("E/CH", "BA1", "BA 6") and American isolates "McK" (Engels *et al*., 1987;Pratelli*et al*., 2000) and referent strains "1/W", "BA1" and "BA 2" (Buonavoglia *et al*., 1996), but the authors did not connect those findings with clinical signs of animals from which the CapHV1 had been isolated.

In the present study we also found differences in the restriction patterns between investigated CapHV1 isolates, despite the small length of the gC region. The differences in the number of bands visible after the restriction endonuclease analysis and those determined by MEGA 5 could be attributed to the small number of base pairs as fragment lengths (8 bp and 14 bp) as well as the undetectable differences between the size of bands (44 bp vs. 46 bp and 92 bp vs. 96 bp), which, in turn, affects the restriction pattern determined for different isolates and their differentiation.

The mutations found in the gC gene of the investigated CapHV1 isolate sconfirm that genome differences among the CapHV1 strains could exist. Other researchers have observed similar findings when the gC gene of BoHV 1.1 and BoHV 1.2 strains were examined (Rijsewijk *et al*., 1999). These results are in agreement with the hypothesis of Keuser *et al*., (2004) for differences in the gC amino-acid sequences and their correlation with differences in the tropism *in vivo*. Non-synonymous mutations in the gC gene are probably connected with virus adaptation to heparansulphate proteoglycan cell receptors and aiding in the second stage of virus penetration in cells, namely, the connection of glycoprotein D with second cell receptors from HVEM/TNF/NGFfamily, virus penetration with the participation of glycoprotein Band virus replication (Wagner, 2003). The observed gC gene mutations in the "Pavel Banya" and "Biser" isolates at positions 960 and 1000 most probably are a part of the complex changes affecting both the central and variable N-terminal parts of the gC gene, as observed in different her pesviruses (BoHV1, BoHV5, CapHV1, CerHV1, RanHV1). Mutations in the gC and gD gene are responsible for the specific tropism and pathology (Ros *et al*., 1999). In our previous report (Peshev *et al*., 2008), a latent infection without clinical symptoms was found in the flock in the Troyan region (with sporadic abortions and mating difficulties) by antibody seroconversion against CapHV1. In the regions Biser, Kyustendil, Suhindol and Pavel Banya, typical clinical symptoms for CapHV1 (sterility, abortions and kidsdead) were observed and the mortality rateofkids was highest (90%) in the Pavel Banyafarm. This could probably be connected with the specific pathogens is as arersul to fg C genemutations:a synonymous mutation atposition 960 in the "PavelBanya" isolate and a nonsynonymous one at position 1000 for the "Biser" isolate.

The glycoprotein C gene of herpes viruses is less conservative and is virus- and strain- specific (Engels *et al*., 1992). In related herpes viruses the central part of the gC gene is conservative, while the N-terminal part is very variable (Ros *et al*., 1999). Based on the changes in the gC gene carboxy-terminal region Esteves *et al*. (2008) determined exactly the investigated bovine isolates as separate groups (BoHV1.1, BoHV1.2. and BoHV5). Differences between the molecular weight of gC for CapHV1 and BoHV1 found by Keuser *et al*. (2004) indirectly confirmed the variability in the N-terminal part of the gC gene. The authors explained these variations by differences of the ORF length, type and number of glycosylation sites. Determined subgroups and branches for investigated CapHV1 isolates after phylogenetic analysis in current research are also evidence for variability in the central conservative region of the gC gene.

The results obtained by PhyML 3.0 and the bootstrap test for 11 nucleotide sequences suggest that the investigated Bulgarian isolates ("Biser", "Troyan", "Kyustendil", "Suhindol") and the American "McK" one originate from a common ancestor strain and form a separate subgroup different from that of the Western European isolates ("E/CH", "Sp1", "Sp2" and "Ba1").

This differentiation could most likely be a result of isolation of the both goat populations (the Bulgarian White Milk Goat and the Western European goats) as an independent event of antigen drift. The similarity between the American and Bulgarian isolates at position 887 bp is most probably due to independent evolution resulting in replacement of the amino acid leucine by arginine at that position (codon 296) of the gC gene. Unexpectedly, the "Pavel Banya" isolate was separately positioned in the nucleotide phylogenetic analysis due to a CCT→ CCC substitution at position 960, corresponding to an identical nucleotide sequence in BoHV1. Thus, the "PavelBanya" isolate was defined as more closely related to BoHV1than the other studied isolates. The substitution is synonymous and does not change the amino acid coded by the codon. Hence, the amino acid phylogenetic analysis determined the "Pavel Banya" isolate in the same subgroup together with the other Bulgarian isolates. The "PavelBanya" isolate was previously shown to have better adaptability to bovine cells as compared to the other Bulgarian isolates (Peshev *et al*., 2008). The "PavelBanya" isolate also gave a higher titer than the other isolates in a hemagglutination reaction after cultivation on bovine cells (Sirakov and Peshev, 2011).

In the "PavelBanya" farm goats are reared together with cattle and upon disease outbreak the newborns were affected with 90% lethality. Therefore, it is possible that the mutations might have originated as a result of adaptation of the virus to young animals' cells and/or as a result of multiple virus passages and virulentization of the virus. Although the mutation in position 960 does not lead to changes in the encoded amino acid, it might still affect the protein function, since, according to Mullard, 2007, synonym mutations can affect protein folding and function. Simple sequence repeats (SSRs) or microsatellites are short repeats with a sizeof 1–6 bp (the smallest repeated DNA motif) situated in protein coding and non-coding regions of DNA sequences (vanBelkum *et al*., 1998). SSRs modulate gene activity to provide an important source ofgenetic variation (Li *et al*., 2004). SSRs directly affect the correspondinggene products and even cause phenotypicchanges. SSR alterations are the main cause for evolution and sequencediversity that drives adaptation. Due to insertion or deletion mutations of one or more types of repeats, SSRs have a high level of length polymorphism. Theseloci mutate by insertions or deletions of one or a fewrepeat units. In our study we identified SSRs consisting of three nucleotide units (GCT) with three repetitions at position 963–971 bp in the central part of the gC gene for all investigated CapHV1 isolates. SSRs containing 15 nucleotides with 4repeats in a nasal strain, andtwo SSRs in seven vaginal and nasal strains and an SSR in three strains (two vaginal and a nasal one) was found in the gC gene by Tarsitano *et al*. (2010). SSRs facilitate genome rearrangement through recombination and impact on protein structure and possibly protein–protein interactions. The presence of SSRs next to position 960 most probably facilitates the mutation at this site.

The epizootological data revealed that the farms in the regions of Kyustendil, Suhindol and PavelBanya had imported bucks and goats from the town of Troyan, but the origin of the animals in the Biser farm remains unknown. Based on the results obtained in the present study, it could be speculated that in all of the investigated Bulgarian herds, except for the Biser farm, there is circulation of one and the same or several closely related CapHV1 strains.

The differences observed in the migration profile of the CapHV1 isolates could be related to the clinical signs in goats, kids and bucks, similar to what has been observed in bovine species (Engels *et al*., 1981; Metzler *et al*., 1985; Peshev and Christova, 2010). This suggestion is supported by the mutations identified in the CapHV1 isolates, but additional examinations are necessary to further elucidate this hypothesis.

Conclusion

The observed changes in the central conservative part of the gC gene suggest that they are stable and could be used in identification of strains and in molecular epizootiological studies. Our data that CapHV1-specific primers could amplify BoHV1 warn against a risk of compromising BoHV1 eradication programs in mixed-species flocks.

The obtained results suggest that the analyzed region of the gC gene could be considered suitable for comparison of different CapHV1 and BoHV1 isolates and could be used as a method for differentiation of CapHV1 strains in future epidemiologic studies. BoHV1 and CapHV1 infections could be distinguished by PCR and restriction endonuclease analysis. Clarification of the significance of point mutations observed could improve the understanding of the disease pathogenesis in different groups of animals. Our results also demonstrated that both PCR and subsequent restriction enzyme analysis of the amplified product are needed to diagnose CapHV1 with precision and differentiate it from BoHV1 in goats.

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