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NEW MOLECULAR DATA ON EPIDEMIOLOGICAL STUDY OF BOVINE HERPESVIRUS 1 STRAINS

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Abstract

Isolated in Bulgaria from animals with different clinical entity, bovine herpes virus 1 (BHV 1) strains are investigatied by molecular biological methods. Based on genome bank data, PCR proving gB, gD, gE and gC genes are developed and adapted and the PCR sensitivity and specificity are determined.

The developed PCR is specific for BHV 1 strains and heterologous viruses cannot be multiplied by approved primers. It is determined that PCR technique offers a high resolution capacity compared to the virus isolation test.

After investigation by restrictase fragment pattern (RFP) analysis with HPA I and HIND III restriction enzymes (REs), it is found that NIND III RE allows for the distinction of the respiratory from genital and abortogenic BHV 1 strains.

The results demonstrate that the proved PCRs identify the BHV 1 strains with a high specificity and resolution and the RFP analysis by HIND III RE allows for the differentiation among investigated BHV 1 virus strains.

Key words: bovine herpes virus 1 (BHV 1), polymerase chain reaction (PCR), gB, gD, gC, gE genes, restrictase fragment pattern (RFP) analysis, genome differentiation

Introduction. Bovine herpesvirus 1 (BHV 1) belongs to Herpesviridae family, Alphaherpesvirinae subfamily, which contains viruses characterized by a short replication cycle, relatively large host range and the ability to induce a latent infection mainly in neurons. Based on antigenic and genome analysis, three BHV 1 subtypes, BHV 1.1, BHV 1.2 a and BHV 1.2 b, have been identified [1]. The BHV 1 genome contains double-stranded linear DNA molecule arranged as a class D genome [2]. The total size is 135.3 kilo base pairs (kbp). The BHV 1 genome consists of ten genes encoding glycoproteins. Among them, six are in the unique long (UL) region – gK (UL53), gC (UL44), gB (UL27), gH (UL22), gM (UL10), gL (UL1) and the remaining four are in the unique short (US) region – gG (UL4), gD (UL6), gI (US7) and gE (US8). For the detection of BHV 1 genomes in different biological and clinical specimens, various polymerase chain reactions (PCR) have been described. Classical and nested PCR with various primers are used to amplify parts of BHV 1 genome – glycoprotein B (gB) gene [3–5], gC gene [5–7], gD gene [7, 8], gE gene [5] and the thymidine kinase (TK) gene [9, 10]. The PCR is important for rapid diagnosis but because of common clinical features sometimes a recognition of different types of BHV 1 as respiratory, genital or abortogenic is necessary for assessment of the epizootological situation. It is well known that the DNA from BHV 1 isolates, associated with respiratory infections (IBR virus), has different restriction enzyme patterns compared to the DNA isolated from viruses associated with genital infections (IPV virus) [11, 12].

The aim of the present research is to prove the amplified by PCR genomes of isolated Bulgarian BHV 1 strains as respiratory, genital and abortogenic isolates by restrictase fragment pattern (RFP) analysis.

Materials and methods. Viral strains from clinically-affected ruminants with respiratory, genital signs and abortion from different regions of Bulgaria were isolated. The sources and clinical history of BHV 1 strains included in the investigation are shown in Table 1.

Table 1

History, type and source of viral Bulgarian strains investigated by RFP analysis

Strains	Year of isolation	Clinical symptoms	Isolated from	Originated from/age
BHV1 (Ozet)	1969	respiratory	nasal swabs	calf/4 months
BHV1 (Sartze)	1973	respiratory genital	hard muscle	calf/3 months
BHV1 (Podgumer)	1974	respiratory	nasal swabs	calf/7 months
BHV1 (Tch. voda)	1974	respiratory abortion	nasal swabs abortion	pregnant heifers/ 19 months
BHV1 (Slivnitza)	1990	respiratory	lung lavage	calf/4 months
Reference "Oxford"		genital		

For virus isolation and propagation, primary bovine kidney (BK) and Madin Darby bovine kidney (MDBK) were used. Cell monolayer was cultured in Eagle's minimal essential medium (EMEM), supplemented with 10% fetal calf serum (FCS), 0.075% sodium bicarbonate, 0.2 M L-glutamine and antibiotics – penicillin 100 UI/mL and streptomycin 100 µg/mL. As maintenance the same media with 2% FCS was used. The infectious titre of viruses was calculated by the method of Reed and Muench [13].

Viral DNA from cell cultures infected with BHV 1 was extracted by GiaAmp DNA mini kit Giagen Pvt LTD according to the firm recommendation. The elution of DNA from the columns was performed in 100 µL AE buffer, or sterile double-distilled water and stored at −20 °C.

Primers coding glycoprotein gB ($M_2$1474), gE (NU06934) and gC ($M_2$7491) genes, published in genome bank, were used for the performance of PCR. Hot Start –IT^{TM} Fideli Tag^{TM}PCR Master Mix (2X) according to the firm recommendation was used. Master mix was with 12.5 µL volume, forward primer – 10 µM in quantity from 0.5 to 2 µL, reverse primer – 10 µM at quantity from 0.5 to 2 µL and DNA 1–2 µL template in quantity between 100 and 300 ng. DNA-se RNA-se free distilled water was supplemented with 7.5 µL to the micro tubes. The total volume of the reaction mix was 25 µL. Thermocycler QB – 96 (LKB) and programmes for each pair of primers were used. The thermal condition for amplification of gB, gC and gE genes was accomplished as described by Fuchs et al. [5] and for gD gene by Ros et al. [14]. For detection of gB, gC and gE genes, the samples were preliminary heated at 96 °C for 5 min. Thirty five cycles – 10 cycle for 60 s at 96 °C ; 45 s at 65 °C and 45 s at 72 °C, 15 cycles for 60 s at 96 °C, 45 s at 54 °C, 45 s at 72 °C, 10 cycles for 60 s at 96 °C, 45 s at 60 °C and 45 s at 72 °C were accomplished. After the last cycle final extension of product at 72 °C for 10 min was carried out. For proving of gD gene, the probes were heated at 95 °C for 9 min and 35 cycles, each composed of denaturation at 95 °C for 1 min, annealing 60 °C for 1 min and elongation at 72 °C for 1 min followed by final elongation at 72 °C for 7 min. The verification of reaction was completed by using the respective internal and external controls. Six µL of the PCR product from each microtube were mixed with 3 µL of gel loading buffer and electrophoresed on 1.5% agarose gel containing ethidium bromide (10 mg/mL in distilled water) together with the DNA mecular weight marker (Amersham – 100 bp Ladder, GE Healthcare UK Limited) at constant voltage of 120 V for 40 min in 0.5X TBE buffer. The amplified products were visualized as single compact band with expected size under UV light and documented by gel documentation system (Vilber Lourmat DNA photo documentation system, France). The specificity of PCR for gB gene was determined after investigation of homologous and heterologous DNA viruses. For that purpose, the bovine herpes virus 4 (BHV 4) strain "Movar 33/66", swine herpes virus (SHV 1) strain "A2", caprine herpes virus (CHV 1) strain "E/CH", buffalo herpes virus 1 (Buff HV 1) strain

"Metzler" and adenovirus strain "Chelopechene" were included. The same parameters and primers for BHV 1 amplification were used. DNA sample obtained from uninfected MDBK cells with BHV 1 and BHV 1 reference strain "Oxford" were used as negative and positive controls respectively. Analytical sensitivity of reaction was determined by using the BHV 1 strain "Ozet" with a titre $10^{-7.66}$ $TCID_{50}$/mL. Serial ten-fold dilutions of virus up 10^{-1} to 10^{-8} were performed and the MDBK cell culture was infected with the diluted strain. The viral DNA was obtained for all strain dilutions after using lysis buffer, phenol, chloroform and isopropanol treatment [15]. The DNA quantity and quality were measured with Jenway (Genova) spectrophotometer and visualized by gel electrophoresis in 2% agarose gel. The diluted samples were amplified by the same primers and conditions as the starting strains. The gB PCR product was serially ten-fold diluted and electrophoresed for additional determination of sensitivity. For purification and preparation of herpes virus DNA, the method described by Christensen et al. [16] was used.

Restriction enzymes (RE) (Anglian Biotechnology LTD) HPA I and Hind III were applied. Electrophoreses were performed in 0.6% agarose gel with TBE buffer (89 mM Tris base, 89 mM boric acid, 2.5 mM EDTA) for 18 h at 3 V/sm. The DNA was visualized by an UV Transilluminator at 302 nm and photographed with Polaroid camera.

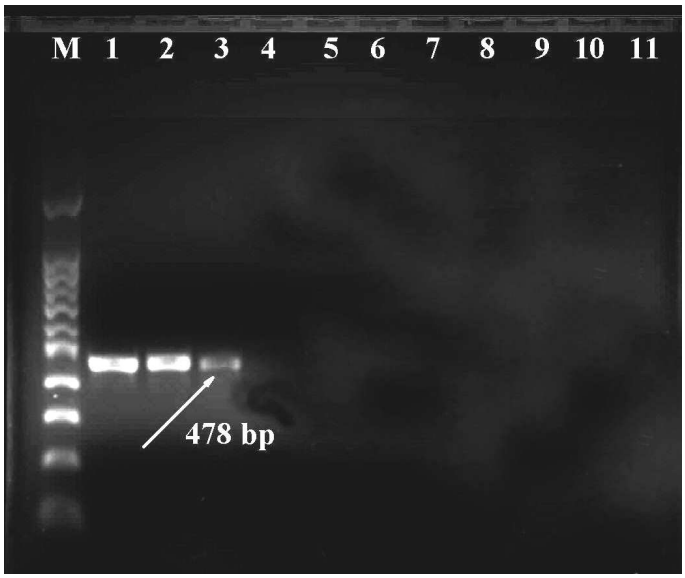

Fig. 1. Specificity of PCR with primers for gB gene amplification of 478 bp fragment (arrow). Lane M: size marker, 1 kb ladder; lane 1: BHV-1 "Ozet"; lane 2: BHV 1 "Oxford"; lane 3: Buff HV 1 "Metzler"; lane 4: SHV 1"A" ; lane 5: BHV-4 "Movar 33/63"; lane 6: goat herpes virus "E/CH"; lane 7: Bovine adenovirus "Chelopechene"; lane 8: distilled water; lane 9: uninfected MDBK cells (negative control)

Results. After using GiaAmp DNA mini kit for DNA isolation, the quality measured by the spectrophotometer was 0.870 (correlation between factor 1 and 2 at λ 260 and λ 280). The quantity of DNA for different strains varied between 100 and 300 ng/µL.

After PCR performance using primers for gB gene, the amplified fragments with 478 bp size were obtained for BHV 1 "Ozet", "Oxford" and Buff HV 1 "Metzler" strains, whereas no amplified fragments were obtained for the other investigated BHV 4, SHV 1, CHV 1 and adenovirus "Chelopechene" strains (Fig. 1). The specificity of PCR was confirmed by these findings. Serial ten-fold dilution of obtained viral DNA was amplified by the same primers and conditions as it was described for gB gene for the determination of analytical PCR sensitivity. The bands with size of 478 bp were visible till strain dilution 10^{-6}. At dilution 10^{-7} and 10^{-8}, gB fragment amplification was not observed as well as from control uninfected cell culture (Fig. 2). At the same ten-fold dilutions tested by virus isolation in cell cultures, the cytopatic effect after 10^{-5} dilutions was not observed. The measured gB PCR DNA was 789 ng/mL. In the ten-fold diluted gB PCR products, the band with DNA quantity of 0.79 ng/mL was visible (Fig. 2, lane 7). After this DNA dilution, the visible band was not observed. Specific

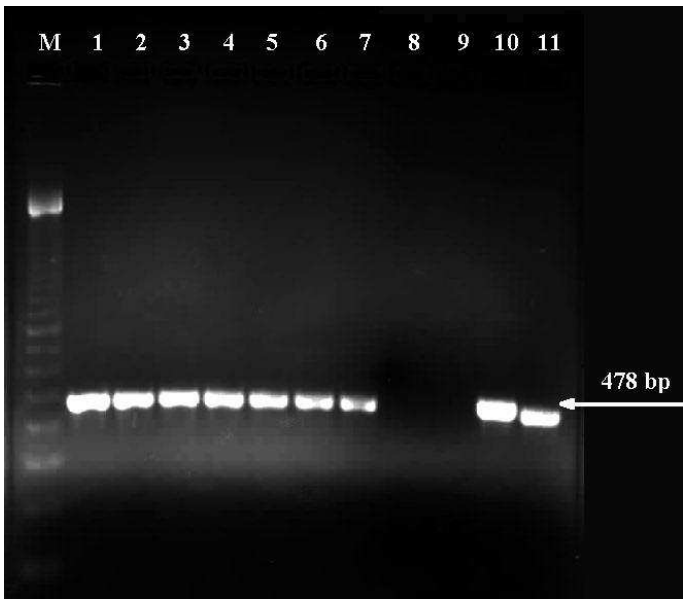

Fig. 2. Analytical sensitivity of PCR minimal detection of BHV 1 strains by amplification of the gB gene fragment (478 bp). Different dilutions of the BHV 1 "Ozet" strain were amplified by specific primers. Lane M: size marker, 1 kb ladder; lane 1: undiluted; lane 2: diluted 10^{-1}; lane 3: diluted 10^{-2}; lane 4: diluted 10^{-3}; lane 5: diluted 10^{-4} ; lane 6: diluted 10^{-5}; lane 7: diluted 10^{-6}; lane 8: diluted 10^{-7}; lane 9: diluted 10^{-8} ; lane 10: referent "Oxford" and lane 11: BHV 1 "Tch. voda" strains

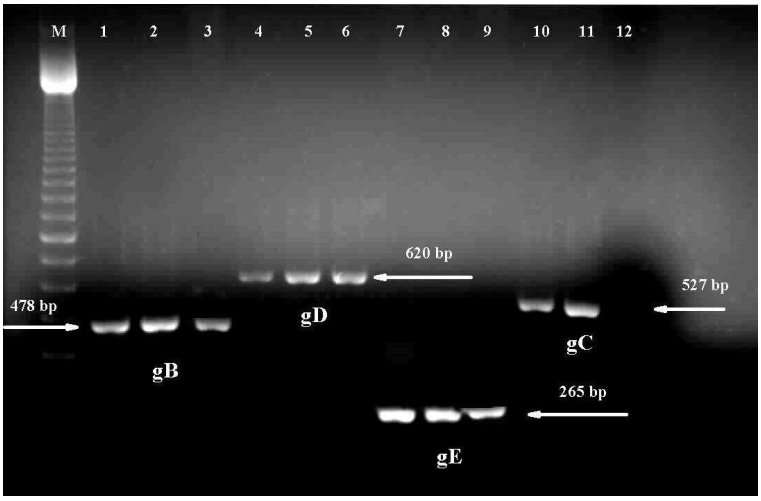

Fig. 3. Specific PCR amplification obtained with primers for gB (478 bp), gD (620 bp), gE (265 bp), and gC (527 bp) genes of BHV 1. Lane M: size marker, 1 kb ladder; lane 1: gB "Ozet"; lane 2: gB "Oxford"; lane 3: gB Buff HV 1 "Metzler"; lane 4: gD "Ozet"; lane 5: gD "Oxford"; lane 6: gD Buff HV 1 "Metzler"; lane 7: gE "Ozet"; lane 8: gE "Oxford"; lane 9: gE Buff HV 1 "Metzler"; lane 10: gC "Ozet"; lane 11: gC"Oxford"; lane 12: gC Buff HV 1 "Metzler"

amplification after PCR using primers for gB, gD, gE and gC genes fragment of expected size (478 bp for gB, 620 bp for gD, 265 bp for gE, and 527 bp for gC genes) was observed for BHV 1 – "Ozet", "Oxford" and Buff HV 1 "Metzler" strains except for the Buff HV 1 "Metzler" strain at gC gen (Fig. 3).

After application of HPA I RE, two clearly different profiles were observed. The DNA pattern with 5 fragments was typical of strains isolated from the genital tract and those with 7 fragments – from the respiratory system. The migration profile of strain "Tch. voda" originating from abortion was with 7 fragments, similarly to respiratory strains. After using Hind III RE the DNA profile of investigated virus, strains can be divided into three groups with respiratory, genital and abortogenic patterns (Fig. 4).

Discussion. The PCR is highly sensitive and rapidly provides a copy of viral genome in clinical and biological samples. By using Giagen DNA mini kit, we obtained enough quantity and quality of DNA suitable for next PCRs. For successful PCR, of great importance is the determination of correct sequence of DNA to be amplified. Nucleotide sequences in target DNA need to be conservative and to be present in all investigated strains. In the present investigation, genes coding glycoproteins gB, gD, gC and gE were selected because they are conserved in all herpes viruses and their nucleotide sequences can be found in the genome bank. The obtained products with 478 bp sizes for BHV 1 "Ozet", "Oxford" and the very closely-related Buff HV 1 "Metzler" strains and the lack of those products for heterologous investigated strains BHV 4, SHV 1, CHV 1 and

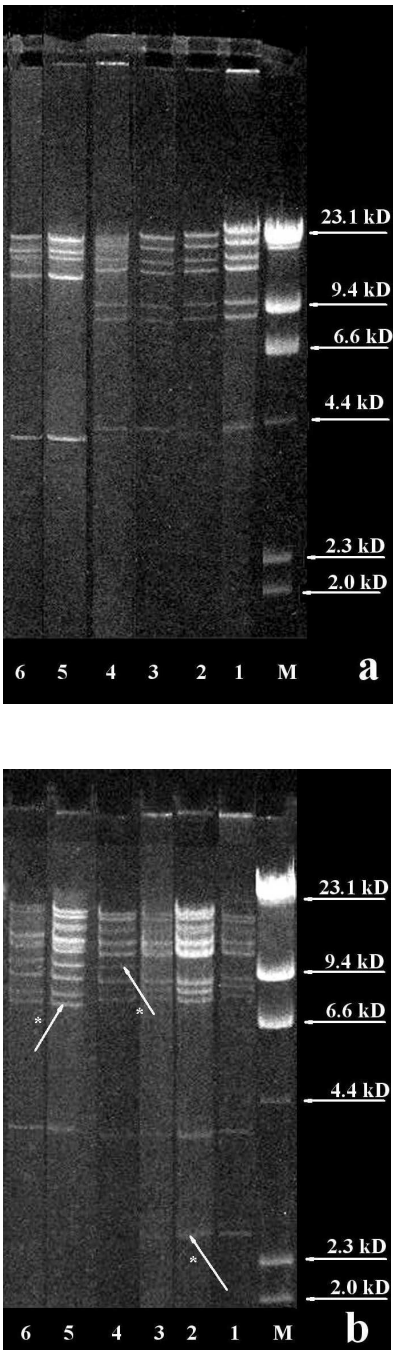

Fig. 4. Restriction enzyme analysis (REA) of different BHV-1 strains using Hpa I (**a**) and HIND III (**b**). Lane M: λDNA digested with Hind III, size marker; lane 1: "Ozet", lane 2: "Podgumer"; lane 3: "Slivnitza"; lane 4: "Tchervena voda"; lane 5: "Oxford", lane 6: "Sartze". Differences between RE patterns of respiratory, abortogenic and genital strains are noted with arrows and asterisks

adenovirus "Chelopechene" for gB gene confirm the specificity of PCR. The obtained results for PCR specificity and sensitivity prove our statement that these amplicons are exact BHV 1 genes and the investigated BHV 1 strains can be used for RFP analysis. It is known that the gE gene is missing in gE deleted BHV 1 strains. That permits us to choose the primers amplifying gE gene in this modification of reaction for a differentiation of gene deleted and virulent viruses. In this way, it is possible to determine the kind of viruses circulating in the herds [5]. During our investigation, viruses with peculiarities for gE deleted strain were not proved. That is evidence that circulating BHV 1 viruses in Bulgarian herds are not genetically deleted. Their genomes were similar to reference "Oxford" strain used as the positive control. In the current investigation, the amplification of goat gB gene with the used primers specific for BHV 1 was without success. On the contrary, Lyaku et al. [4] reported that the goat herpes virus can be proved after using primers multiplying BHV 1 gB gene and they obtained PCR product with size of 468 bp. Most probably the reason for that discrepancy is the used different type of primers with different nucleotide sequences specifically amplifying gB gene of BHV 1. That was confirmed by the fact that after amplification the obtained product for BHV 1 was with larger size (478 bp). The present investigation cannot confirm the statement that primers amplifying gC gene of BHV 1 viruses can amplify DNA obtained from the goat gC gene [5]. In our previous investigation [17] using two different sets of specific gC primers (527 and 320 bp) the gC gene from the CHV 1 was not successfully amplified as well as in the current investigation using again gC primers multiplying fragment with 527 bp size. We did not succeed to amplify the gC gene from Buff HV 1 strain "Metzler" with the primers specific for BHV 1. Most probably the reason for this is the different nucleotide sequence of used primer, amplifying specifically different nucleotide sequences of BHV 1 gC gene. According to Masri et al. [18], PCR can be proved up 0.25 to 2.5 $TCID_{50}$ BHV 1, while by viral isolation test it can be proved 250 $TCID_{50}$. Van Engelenburg et al. [19] by PCR based on gC gene multiplication determined in semen 3 to 5 molecules of BHV 1 DNA. After performed study on the analytical sensitivity of PCR, we found $1\log_{10}$ higher sensitivity of PCR in comparison to the virus isolation test. At ten-fold dilutions of gB PCR product, we obtained visible amplicons at dilution of $10^{-4}\log_{10}$. Most probably at the ten-fold virus dilution and its following amplification, the quantity of starting DNA for PCR amplification is higher and that is the reason for obtaining a visible product at dilution of 10^{-6} $TCID_{50}$. That confirms the higher sensitivity of PCR than the virus isolation test. Moreover, for the virus isolation and identification two to three blind passages are necessary which require minimum 14 days in comparison to PCR, where this time is reduced to 2–3 days.

Restriction DNA analyses have been used for subtype differentiation of BHV 1 strains but clear difference has not been determined [11]. The cause is used HPA I and HIND III REs by authors. After application of HPA I RE, we also did

not find a clear difference between respiratory, genital and abortogenic strains. Strain "Tch. voda" causing abortion was with the same migration profile as respiratory isolates [20]. However, after using Hind III RE, clear differences between respiratory, abortogenic and genital strains were existing.

Respiratory, genital and abortogenic BHV 1 strains approved by RFP analyses were with the same size after the amplification by PCR. That permits us to conclude that RFP analyses can be used for more detailed molecular epidemyological investigation, while the PCR can be used for rapid diagnostics of herpes viruses.

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