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Phylogenetic analysis of bovine herpesvirus 1 isolated in Croatia

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ABSTRACT

In the present study, we report the detection, isolation, molecular characterisation and phylogenetic analysis of bovine herpesvirus type 1 (BoHV-1) in nasal swabs, sera and tissue samples derived from naturally infected cattle. In the spring of 2008, clinical symptoms of the respiratory disease were observed in three Croatian dairy herds. Nasal swabs and sera were collected on the 5th and 27th days after appearance of symptoms. A tissue sample (lungs) from an eight week old calf was also collected. Specific antibodies against BoHV-1 were not found in sera samples collected after the first sampling, but all sera samples were positive after the second sampling. The cytopathic effect on the bovine embryo kidney (BEK) cells was visible after 72 hours for the nasal swab samples, and after 48 hours for the lung tissue sample. Sensitive PCR assays specific for BHV-1 glycoproteins gB and gC were also performed. All nasal swab samples and the lung sample were positive with both sets of PCR primers. The gC PCR product obtained from one sample from each farm as well as from the lung sample DNA were sequenced. Phylogenetic analysis of gC gene sequence clustered our isolates with BoHV subtype 1.1.

Key words: bovine herpesvirus-1.1, nasal swabs, phylogenetic analysis

Introduction

Infectious bovine rhinotracheitis (IBR) is a highly contagious infectious disease which infects cattle and buffaloes and is caused by bovine herpesvirus 1 (BoHV-1). Infection with BHV-1 is an important cause of losses in livestock worldwide.

BoHV-1 is a member of *Herpesviridae* family, *Alphaherpesvirinae* sub-family, *Varicellovirus* genus. According to antigenic and genomic characteristics, BHV-1 was further subdivided in two distinct but closely related subtypes: BoHV-1.1 and BoHV-1.2 (METZLER et al., 1986; MILLER et al., 1991; MINSON et al., 2000). Both subtypes are able

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to infect respiratory and genital tract of cattle, however, BoHV-1.1 is better adapted to respiratory and BoHV-1.2 to genital tract (EDWARDS et al., 1991; RIJSEWIJK et al., 1999). Like other alphaherpesviruses, BoHV-1 can establish latency in ganglion neurons after infection. Furthermore, latency and reactivation also occur within germinal centres of pharyngeal tonsils (WINKLER et al., 2000). Former BoHV-1 subtype 1.3., which attacks central nervous system (MINSON et al., 2000; WOODBINE et al., 2009), today is recognized as a distinct virus species, BoHV-5.

BoHV-1 is associated with several clinical conditions including infectious bovine rhinotracheitis (IBR), infectious pustular vulvovaginitis (IPV), balanoposthitis and conjunctivitis and generalized disease in newborn calves. Upon primary infection, BoHV-1 replicates in the mucous membranes of the respiratory or genital tract. From there, it accesses local sensory neurons of the peripheral nervous system and establishes lifelong latency in the corresponding ganglia (ACKERMANN et al., 1990; MUYLKENS et al., 2007). Furthermore, the virus may spread locally to the deeper respiratory tract, causing IBR, or systemically, may lead to abortion as a consequence of infection of the foetus. Respiratory disease caused by BoHV-1.1 is a most common in heifers older than 6 months, it is not lethal, but is usually followed by secondary bacterial pneumonia.The incubation period is 2 to 6 days, usually without clinical symptoms. With respect to respiratory disease, clinical symptoms include high fever, anorexia, coughing, excessive salivation and nasal discharge, inflamed nares, and dyspnoea. Conjunctivitis with lachrymal discharge is obligatory but sometimes the only symptom. In the absence of bacterial pneumonia, recovery typically occurs 4 to 5 days after the onset of clinical symptoms.

Specific antibodies to BoHV-1 can be detected by serum-neutralisation test or ELISA, two to four weeks after infection. Diagnostic samples for detection and isolation of BoHV-1 are nasal and lachrymal discharges, nasal and tracheal swabs and bronchial and lung tissue. Several laboratory methods are available for BHV-1 detection including virus isolation, direct fluorescent antibody examination of the infected tissue, and antigen and antibody detection by enzyme-linked immunosorbent assay (ELISA). The polymerase chain reaction (PCR) to detect the presence of the viral DNA in nasal swabs, blood, tissue and semen has advantages over above mentioned methods for its sensitivity and rapidity (VAN ENGELENBURG et al., 1993; MOORE et al., 2000; BELAK and HAKHVERDYAN, 2006).

In the present study, we report the detection, isolation, partial sequencing and phylogenetical analysis of BoHV-1 in nasal swabs samples and tissue sample derived from naturally infected cattle from Croatia.

Materials and methods

Samples. Clinical symptoms from the upper respiratory tract (conjunctivitis and serosal to mucopurulent nasal discharge), high fever and anorexia were observed in three unvaccinated dairy farms. The first farm (farm A) was located in central Croatia with 200 Holstein breed cows and 180 Simmental cows. Farm B numbered around 1100 Holstein cows and it was located in eastern Croatia. Small family farm with 20 Simmental cows (farm C) was located in south-western part of country. All three outbreaks appeared in spring in 2008.

Sera and nasal swabs from the animals were collected at 5th and 27th day after the farm veterinarians noticed the appearance of symptoms. Twenty and 40 sera and nasal swab samples were taken from the farm A and B, respectively. Tissue sample (lung) from eight weeks old calf from the farm A was also collected. Ten sera and swab samples were taken from the farm C. The nasal swabs were dipped in Eagle's MEM (Gibco, Karlsruhe, Germany) containing antibiotics (Anti-anti, Gibco), vortexed and centrifuged at 1000 g for 10 minutes. The supernatants from nasal swabs and tissue sample were used for virus isolation and for viral DNA extraction.

Serological examination. BoHV-1 specific antibodies in sera were detected using HerdCheck IBR gB Ab ELISA kit (Idexx, Liebefeld-Bern, Switzerland) specific for gB glycoprotein of BoHV-1. The test was performed according to manufacturer's instructions.

Virological examination. Virus isolation test was performed in bovine embryo kidney (BEK) cell line grown in 25 cm^2 cell culture flasks (Nunc, Roskilde, Denmark). Two to three days old monolayer of BEK cells was inoculated with 500 µL of earlier obtained supernatants from five nasal swabs per farm and from the lung sample. The plates were incubated for one hour at 37 ºC and then the inoculum was replaced with maintenance medium (Modified Eagle's MEM with addition of 2% foetal calf serum) and incubated at 37 ºC in 5% CO_2 atmosphere. Infected BEK cells were examined microscopically for the presence of cytopathic effect (CPE) on the daily basis for the next five to seven days.

DNA extraction, PCR and sequencing. Viral DNA was extracted from 200 µL of all collected nasal swabs and tissue sample supernatants with High Pure Viral Nucleic Acid Kit (Roche, Mannheim, Germany) according to the manufacturer's instructions. Oligonucleotide primers selected from the gene encoding gB (gB1: 5′TACGACTCGTTCGCGCTCTC-3′ and gB2: 5′-GGTACGTCTCCAAGCTGCCC-3′) (FUCHS et. al., 1999) and gC (PF: CGGCCACGACGCTGACGA and PR: CGCCGCCGAGTACTACCC) (ESTEVES et al., 2008) genes of BoHV-1 were used in PCR. The reaction was performed in a 50 µl mixture containing 1X PCR buffer (200 mM Tris-HCl (pH 8.4), 500 mM KCl), 0.2 mM dNTP, 1.5 mM $MgCl_2$, primers at 0.5 µM each, 2.5 units of Platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA) and 5 µL of extracted DNA. A GeneAmp PCR system

9700 (Applied Biosystems, Foster City, CA, USA) was used for the amplification. The PCR amplified products were electrophoresed on a 1.5 % agarose gel and visualized with ethidium bromide. The 575 bp PCR products corresponding to the gC gene were purified by QIAquick purification kit (Qiagen, Valencia, CA, USA). Sequencing was carried out in both directions using the Big Dye Terminator 1.1 Cycle Sequencing kit (Applied Biosystems) as recommended by the manufacturer. Sequencing analysis was performed on an automatic sequencer ABI PRISM 310 Genetic Analyzer (Applied Biosystems). The nucleotide sequence obtained from our isolate Cro08 was deposited in GenBank with accession number GQ169130.

Multiple alignment and phylogenetic analysis. The sequence data were initially aligned with the corresponding nucleotide sequences published for BoHV isolates: Cooper (DQ173733), Lam (DQ173724, La (AF135441), UY 1999 (DQ173735), UY 2004 (DQ173732), PG 1779 (DQ173723) and EVI 100 (DQ184912) using the basic BLAST search program (http://www.ncbi.nlm.nih.gov/BLAST/). Sequence analysis was performed with the Lasergene 6 package (DNAstar Inc., Madison, USA). Phylogenetic analyses of the 461-bp fragment BoHV-1 gC were conducted using MEGA version 3.1 (KUMAR et al., 2004) using the maximum parsimony method with 500 bootstrap replicates.

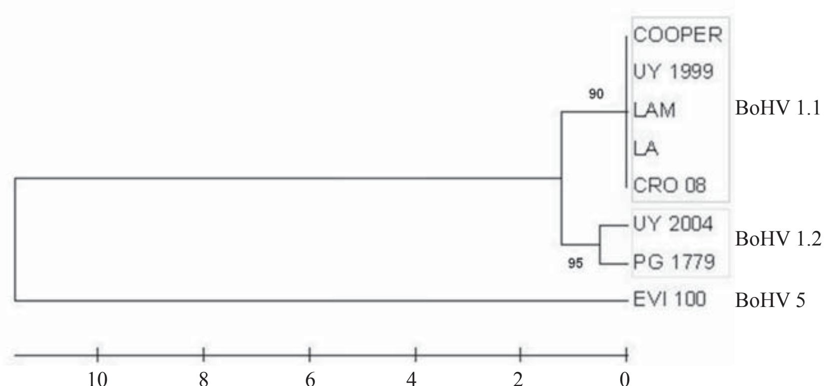
Results

All sera and nasal swab samples collected at 5th and 27th day after appearance of clinical symptoms of IBR as well as the tissue sample from eight weeks old calf were examined. Specific antibodies against BoHV-1 were not found in sera samples collected after first sampling (5th day), but all sera samples were positive after second sampling (27th day).

The supernatants from nasal swabs dipped in Eagle's MEM containing antibiotics were used for virus isolation in the bovine embryo kidney cells and for viral DNA extraction. The CPE was visible after 72 hours for the all tested nasal swabs samples, and after 48 hours for the lung sample. Additionally, glycoprotein B specific PCR confirmed infection of the cell culture with BoHV-1.

Sensitive PCR assays specific for gB and gC were performed. All nasal swab samples and a lung sample were positive with both sets of primers. Three gC PCR positive samples (first from the lung sample and second and third from the swab samples from farms B and C, respectively) were sequenced and 400-nt fragment of BoHV-1 gC encoding gene was analysed. When compared to each other, a 100% identity was found. When aligned with the corresponding nucleotide sequences published for BoHV isolates worldwide, an 100 % identity was found with the isolates UY1999, LAM, LA and COOPER. Phylogenetic

analysis of nucleotide and aminoacid gC sequence clustered our isolates with BoHV subtype 1.1. (Fig. 1).



Discussion

BoHV-1 is one of the most important pathogens of cattle and is distributed worldwide. Novel investigation showed that number of serologically positive animals has increased dramatically since the 1960s especially in old cattle and big herds, with example from Great Britain where approximately 83% of the unvaccinated herds in the South West of the country were serologically positive (WOODBINE et al., 2009). IBR was first recorded in Croatia in 1967 (CVETNIĆ et al., 1967) and since then, it is constantly present in cattle herds with periodical outbreaks (BIUK-RUDAN et al., 1999; MADIĆ et al., 1989). Despite the fact that IBR is a disease with long history in Croatia, this is the first report on the detection of BHV-1 in Croatian dairy herds, based on sensitive laboratory techniques and characterisation of BoHV-1 by phylogenetic analysis. Differentiation between BoHV-1 subtypes 1.1. and 1.2. is possible by sequence analysis of the gC amplified PCR products, and that's why we picked the primers described by ESTEVES et al. (2008). Compared to each other, a 100% sequence identity was found among our analysed sequences. For that reason only one sequence was chosen for GenBank submission and further analysis. Phylogenetic analysis of nucleotide and aminoacid gC sequence clustered our isolate Cro08 with BHV subtype 1.1. with other respiratory isolates, separated from genital strains (BoHV-1.2) and BoHV-5 (Fig. 1).

Europe has a long history of fighting against BoHV-1 infections. However, only a small number of countries have achieved the goal of IBR-eradication. The gE-deleted vaccine has been used successfully to eradicate IBR from a number of European countries. These marker vaccines, either inactivated or live attenuated, used together with a serological detection of gE-specific antibodies, allow discriminating infected from

vaccinated animals (KAASHOEK et al., 1995; VAN OIRSCHOT et al., 1997). The BoHV-1 vaccines are effective at reducing the clinical impact of BoHV-1 infection. However no vaccine is able to prevent the infection and the establishment of latency by challenge and field strains. Eradication programs are based on the repeated vaccination of infected herds or, when the prevalence of latent carriers is low, on the detection and the culling of seropositive animals (ACKERMANN and ENGELS, 2006, MUYLKENS et al., 2007).

The BoHV-1 eradication programme was not introduced in diary and beef cattle herds in Croatia. However, depending on the farm management, several farms are performing active immunisation using conventional vaccine against bovine respiratory diseases. According to the NN 159/08-Croatian Directive of control measures for protection of the animals from infectious diseases: "bulls for *natural* or artificial *insemination* should be clinically and serologically tested for IBR/IPV. All heifers and cows intended for production should be clinically and serologically tested for IBR/IPV, except in the case they were identified as having been vaccinated".

For further investigation any future outbreak should be covered with molecular characterisation of the isolated BoHV strain for possible existence of other virus subtypes.

References

ACKERMANN, M., S. BELAK, V. BITSCH, S. EDWARDS, A. MOUSSA, G. ROCKBORN, E. THIRY (1990): Round table on infectious bovine rhinotracheitis infectious pustular vulvovaginitis: virus infection, diagnosis and control. Vet. Microbiol. 23, 361-363.

ACKERMANN, M., M. ENGELS (2006): Pro and contra IBR-eradication. Vet. Microbiol. 113, 293-302.

BELAK, S., M. HAKHVERDYAN (2006): Recent achievements and trends in the molecular diagnosis of bovine viral diseases - a view from the „OIE Collaboration centre for the application of polymerase chain reaction methods for the diagnosis of viral diseases in veterinary medicine". Dtsch. Tierärztl. Wochenschr. 13, 129-133.

BIUK-RUDAN, N., S. CVETNIĆ, J. MADIĆ, D. RUDAN (1999): Prevalence of antibodies to IBR and BVD viruses in dairy cows with reproductive disorders. Theriogenology 51, 875-881.

CVETNIĆ, S., E. TOPOLNIK, M. KRALJ (1967): Aetiology of infection of respiratory and digestive system of cattle. IV. Detection of neutralisation antibodies against bovine rinotracheitis virus. Vet. arhiv 37, 53-56.

EDWARDS, S., R. H. NEWMAN, H. WHITE (1991): The virulence of British isolates of bovid herpesvirus 1 in relationship to viral genotype. Br. Vet. J. 147, 216-231.

ESTEVES, P. A., O. A. DELLAGOSTIN, L. S. PINTO, A. D. SILVA, F. R. SPILKI, J. R. CIACCI-ZANELLA, S. O. HÜBNER, R. PUENTES, J. MAISONNAVE, A. C. FRANCO, F. A. RIJSEWIJK, H. B. C. R. BATISTA, T. F. TEIXEIRA, D. DEZEN, A. P. OLIVEIRA, C. DAVID, C. W. ARNS, P. M. ROEHE (2008): Phylogenetic comparison of the carboxy-

terminal region of glycoprotein C (gC) of bovine herpesviruses (BoHV) 1.1., 1.2 and 5 from South America (SA). Virus Res. 131, 16-22.

FUCHS, M., P. HÜBERT, J. DETTERER, H.J. RZIHA (1999): Detection of bovine herpesvirus type 1 in blood from naturally infected cattle by using a sensitive PCR that discriminates between wild-type virus and virus lacking glycoprotein. J. Clin. Microbiol. 37, 2498-2507.

KAASHOEK, M. J., A. MOERMAN, J. MADIĆ, K. WEERDMEESTER, M. MARIS-VELDHUIS, F. A. RIJSEWIJK, J. T. VAN OIRSCHOT (1995): An inactivated vaccine based on a glycoprotein E-negative strain of bovine herpesvirus 1 induces protective immunity and allows serological differentiation. Vaccine 13, 342-346.

KUMAR, S., K. TAMURA, M. NEI (2004): MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. Brief Bioinform. 5, 150-163.

MADIĆ, J., S. CVETNIĆ, N. BIUK-RUDAN, B. LUGOVIĆ (1989): Antibodies to BRS, BVD, and IBR viruses in bovine sera in S.R. Croatia, S.F.R. Yugoslavia. Vet. Arhiv 59, 233-238.

METZLER, A. E., A. A. SCHUDEL, M. ENGELS (1986): Bovine herpesvirus 1: molecular and antigenic characteristics of variant viruses isolated from calves with neurological disease. Arch. Virol. 87, 205-217.

MILLER, J. M., C. A. WHETSTONE, M. J. VAN DER MAATEN (1991): Abortifacient property of bovine herpesvirus type 1 isolates that represent three subtypes determined by restriction endonuclease analysis of viral DNA. Am. J. Vet. Res. 52, 458-461.

MINSON, A. C., A. DAVISON, R. EBERLE, R. C. DESROSIERS, B. FLECKENSTEIN, D. J. MCGEOCH, P. E. PELLETT, B. ROIZMAN, M. J. STUDDERT (2000): Family *Herpesviridae*. In: Virus Taxonomy: Seventh Report of the International Committee on Taxonomy of Viruses. (van Regenmortel, M. H. V., C. M. Fauquet, D. H. L. Bishop, E. B. Carstens, M. K. Estes, S. M. Lemon, J. Maniloff, M. A. Mayo, D. J. McGeoch, C. R. Pringle and R. B. Wickner, Eds). Academic Press. San Diego. pp.203-225.

MOORE, S. J., M. GUNN, D. WALLS (2000): A rapid and sensitive PCR-based diagnostic assay to detect bovine herpesvirus 1 in routine diagnostic submissions. Vet. Microbiol. 75, 145-153.

MUYLKENS, B., J. THIRY, P. KIRTEN, F. SCHYNTS, E. THIRY (2007): Bovine herpesvirus 1 infection and infectious bovine rhinotracheitis. Vet. Res. 38, 181-209.

RIJSEWIJK, F. A., M. J. KAASHOEK, J. P. LANGEVELD, R. MELOEN, J. JUDEK, K. BIENKOWSKA-SZEWCZYK, M. A. MARIS-VELDHUIS, J. T. VAN OIRSCHOT (1999): Epitopes on glycoprotein C of bovine herpesvirus-1 (BHV-1) that allow differentiation between BHV-1.1 and BHV-1.2 strains. J. Gen. Virol. 80, 1477-1483.

VAN ENGELENBURG, F. A. C., R. K. MAES, J. T. VAN OIRSCHOT, F. A. RIJSEWIJK (1993): Rapid and sensitive detection of bovine herpesvirus type 1 in bovine semen by a polymerase chain reaction based assay. J. Clin. Microbiol. 31, 3129-3135.

VAN OIRSCHOT, J. T., M. J. KAASHOEK, M. A. MARISVELDHUIS, K. WEERDMEESTER, F. A. M. RIJSEWIJK (1997): An enzyme-linked immunosorbent assay to detect antibodies against glycoprotein gE of bovine herpesvirus 1 allows differentiation between infected and vaccinated cattle. J. Virol. Methods 67, 23-34.

WINKLER, M. T., A. DOSTER, C. JONES (2000): Persistence and reactivation of bovine herpesvirus 1 in the tonsils of latently infected calves, J. Virol. 74, 5337-5346.

WOODBINE, K. A., G. F. MEDLEY, S. J. MOORE, A. M. RAMIREZ-VILLAESCUSA, S. MASON, L. E. GREEN (2009): A four year longitudinal sero-epidemiological study of bovine herpesvirus type-1 (BHV-1) in adult cattle in 107 unvaccinated herds in South west England. Bio. Med. Central Vet. Res. 5, 5-16.

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SAŽETAK

U radu je opisano dokazivanje, izdvajanje, molekularna karakterizacija i filogenetska analiza goveđega herpesvirusa-1 (GHV-1) iz nosnih briseva i organa prirodno zaraženih goveda. U proljeće 2008., u tri domaća stada mliječnih krava pojavili su se klinički simptomi dišne bolesti. Peti i 27. dan nakon pojave simptoma sakupljeni su uzorci krvi i nosnih briseva kao i uzorak pluća uginuloga teleta u dobi od od 8 tjedana. Svi uzorci seruma prikupljeni prvim uzorkovanjem dali su negativan rezultat na prisutnost specifičnih protutijela za GHV-1, a pozitivan nakon drugoga uzorkovanja. Citopatski učinak na stanicama bubrega goveđega zametka bio je vidljiv nakon 72 sata u slučaju infekcije stanica inokulumom pripravljenim od nosnoga brisa te nakon 48 sati u slučaju suspenzije organa. Napravljena je i lančana reakcija polimerazom (PCR) upotrebom specifičnih početnica za gene gB i gC GHV-1. Svi uzorci nosnih briseva kao i pluća dali su pozitivnu reakciju s oba para početnica. Nakon PCR, odsječcima gC DNA određen je nukleotidni slijed, a filogenetska analiza GHV-gC sekvencija svrstala je naš izolat u podtip 1.1.

Ključne riječi: goveđi herpesvirus-1, nosni brisevi, filogenetska analiza

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