Molecular detection of bovine herpesvirus 1 (BoHV-1) in cattle in Serbia

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SUMMARY
The presence of bovine herpesvirus 1 (BoHV-1) was examined in 110 samples of bovine nasal swabs collected between October 2007 and November 2015. BoHV-1 was detected in four samples by virus isolation and polymerase chain reaction (PCR). The phylogenetic analysis of glycoprotein B (gB) gene nucleotide sequences of all four BoHV-1 strains showed that they were 100% similar to each other and branched with BoHV-1 strains isolated in Egypt and USA. BoHV-1 strains isolated in Israel, India, Brazil and USA belonging to the second branch were 100% similar to each other and 98% to 99% similar to the strains from Serbia. The phylogenetic analysis of the tk gene nucleotide sequences of all four BoHV-1 strains showed that they were 100% similar and grouped together in one branch. BoHV-1 strains from USA and Australia clustered with Serbian BoHV-1 strains sharing similarity of 99%. BoHV-1 strains from India and USA clustered separately. The presented work is one of the first reports of the phylogenetic analysis of BoHV-1 isolates from Serbia.

Keywords: BoHV-1, phylogenetic analysis

RESUME
La présence de l’herpèsvirus bovin 1 (BoHV-1) a été examinée dans 110 échantillons de prélèvements nasaux bovins collectés entre octobre 2007 et novembre 2015. BoHV-1 a été détecté dans quatre spécimens par l’isolation du virus et la réaction de polymérisation en chaîne (PCR). L’analyse phylogénétique des séquences nucléotidiques du gène de la glycoprotéine B (gB) des quatre souches de BoHV-1 a montré 100% de similitude avec des souches de BoHV-1 isolées en Égypte et aux États-Unis. Les souches de BoHV-1 isolées en Israël, en Inde, au Brésil et aux États-Unis appartenant à la seconde branche étaient 100% similaires entre elles et 98% à 99% similaires aux souches de Serbie. L’analyse phylogénétique des séquences nucléotidiques du gène de la thymidine kinase (tk) des quatre souches de BoHV-1 isolées en Égypte et aux États-Unis a montré 100% de similitude à la seconde branche. Les souches de BoHV-1 des États-Unis et d’Australie se sont regroupées avec des souches de BoHV-1 isolées en Egypte et aux États-Unis partageant une similarité de 99% tandis que les souches de l’Inde et des États-Unis se sont regroupées séparément. Le résultat présenté est l’un des premiers rapports de l’analyse phylogénétique des isolats de BoHV-1 en Serbie.

Mots-clés : BoHV-1, analyse phylogénétique

Introduction

Bovine herpesvirus-1 (BoHV-1) is a member of the Herpesviridae family and Alphaherpesvirinae subfamily and an important pathogen of cattle causing respiratory and reproductive disease, abortion and neonatal infections [11]. Bovine herpesvirus-1 genome consists of a linear double-stranded DNA molecule encoding 73 proteins. The genome of the virus is subdivided into unique long (UL) and unique short (US) segments. Alphaherpesviruses, including bovine herpesvirus 1, encode several glycoproteins of the viral envelope that play crucial roles in viral pathogenicity and regulate virus attachment, entry, egress and cell to cell spread [12]. The virus is distributed worldwide with significant difference in disease prevalence in different geographical areas. Bovine herpesvirus 1, bovine viral diarrhea virus (BVDV) and bovine parainfluenza virus 3 (BPIV-3) are viruses commonly associated with bovine respiratory disease complex (BRDC) [13]. Stressors such as temperature, stocking density or inadequate transportation that lead to an impaired immune response cause the reactivation of the latently present virus with the appearance of clinical symptoms and spread of the disease in the population [13]. Bovine herpesvirus-1 infection causes significant economic losses to the livestock industry [15]. It is well known that cattle infected with BoHV-1 usually clinically recover, but remain infected for life as the virus establishes latency in neural tissue following primary infection of the conjunctiva, oral and/or nasal epithelium or reproductive organs [23]. Clinical manifestations of BoHV-1 infection are diverse and may be presented as infectious bovine rhinotracheitis (IBR), infectious pustular vulvovaginitis (IPV), infectious pustular balanoposthitis (IPB), abortion as well as generalized systemic infection of neonates [5, 17]. Vaccination against BoHV-1 does not uniformly prevent the establishment of latency. Many countries surrounding Serbia like Hungary, Slovenia, Slovakia or Austria, established national programmes for BoHV-1 eradication. In Serbia there is no such programme, however more and more farmers regularly vaccinate cattle, many of them possessing BoHV-1 free herds [14].

The laboratory diagnostics of BoHV-1 infection in cattle is based on different virological methods such as virus isolation (VI) and virus neutralization test (VN - test), [7], enzyme-linked immunosorbent assay – ELISA [2], polymerase chain reaction – PCR [24], multiplex PCR and real-time PCR [7, 8]. Molecular characterization of BoHV-1 strains from different parts of the world in order to reveal
their similarity and/or differences was performed by many authors [9, 16, 21, 22].

The objective of this examination was to provide information on the isolation, molecular detection and subsequent characterization of BoHV-1 in cattle in Serbia. The nucleotide sequences of identified BoHV-1 strains were compared with corresponding nucleotide sequences available in GenBank database (http://www.ncbi.nlm.nih.gov/BLAST/) in order to reveal the similarity or differences among them.

Materials and methods

CLINICAL SAMPLES

A total of 110 nasal swabs from non-vaccinated animals raised in different regions of Serbia (Northern and Central Serbia - surroundings of the municipalities of Bečej, Kovačica, Stara Pazova and cities of Užice and Belgrade) were examined. Most of the animals expressed non-specific clinical signs such as fever, dyspnea and nasal discharge. The samples of nasal swabs were collected between October 2007 and November 2015. Here it must be emphasized that the samples of nasal swabs were taken only from animals from extensive livestock farming systems. The number of these animals the Republic of Serbia rapidly decreased over the past ten years, especially in the Northern and Central parts of the country.

REFERENCE BOHV-1 STRAIN

Reference TN41 strain of BoHV-1, lot 971119 (American BioResearch, Sevierville, Tennessee, USA), titer $10^{8.1}\text{ TCID}_{50}$/mL, was used as a positive control for virus isolation and PCR [4].

CELL CULTURE

Vero cell line (ATCC CCL-81, IZSBS, Brescia, Italy) was used for virus isolation, VN-test and PCR sample preparation.

VIRUS ISOLATION

All nasal swab samples were centrifuged at 2000 rpm at 4°C for 15 min and the supernatants were sterilized using sterile 0.22 µm Millex syringe filter units (Merck, USA). Subsequently, 0.5 mL of the supernatant was inoculated into 24 hours old and 80% confluent monolayer of Vero cell line in 25cm² flasks. After the incubation at 37°C for 60 min, 9.5 mL of minimum essential medium (MEM, Capricorn Scientific, Germany) supplemented with 2% foetal calf serum (FBS-12A, Capricorn Scientific, Germany) was added and the cultures were further incubated at 37°C for 5 days with daily microscopic examination for the appearance of characteristic cytopathic effects (CPE). If CPE was not observed after 5 days, the flasks with the inoculated cell cultures were frozen and thawed three times and passaged for two more times at five day intervals. In case that no CPE was visible after the third passage, the sample was considered negative. Uninoculated cell monolayers were used as cell controls. If CPE appeared in the monolayer of Vero cells the virus isolates were further identified by virus neutralization test (VN-test) according to standard procedure indicated in the OIE Manual [6].

PCR

Vero cell lines with CPE of reference strain and isolated strains were centrifuged at 1500 rpm at 4°C for 15 min. The obtained cell debris was used for DNA extraction using QIAmp DNA mini kit (QIAGEN Inc., Valencia, CA, USA) according to the manufacturer's protocol. For PCR, thymidine-kinase (tk) (TK1 5’-AGA CCC CAG TTG TGA TGA ATG C-3’ and TK2 5’-ACA CGT CCA GCA CGA ACA CC-3’) and glycoprotein B (gB) (gB1 5’-TAC GAC TCG TTC TTC GCG CTC TC-3’ and gB2 5’-GGT ACG TCT CTC TC-3’) primers were used. The PCR conditions for the amplification of BoHV-1 tk and gB genes were previously described by Yason et al. [26] and Fuchs et al. [10], respectively. The presence of 183 bp band for BoHV-1 tk gene and 478 bp band for gB gene was considered as a positive identification of BoHV-1.

BOHV-1 GB AND TK DIRECT SEQUENCING

PCR products were purified with QIA quick Purification Kit (Qiagen, Valencia, CA, USA) and were subsequently sequenced with Big Dye Terminator Cycle Sequencing Kit (PE Applied Biosystems, Foster City, CA, USA) using PCR primers as sequencing primers. Direct sequencing of BoHV-1 tk and gB genes was performed according to Sanger sequencing method. Cycle sequencing protocol comprised of an initial denaturation at 96°C for 2 minutes, followed by 40 repeated cycles of denaturation at 96 °C for 10 sec, annealing at 50 °C for 5 sec and elongation at 60 °C for 4 minutes. The products were purified by isopropanol and denatured at 95°C for 2 minutes. The sequencing reactions were analyzed on the ABI Prism 310 Genetic Analyzer (Applied Biosystems) and the obtained nucleotide sequences were aligned and compared with virus sequences available in the GenBank database using BLAST software (http://www.ncbi.nlm.nih.gov/BLAST/). Multiple nucleotide sequence alignments were created using CLUSTAL W program, as implemented in MEGA 6 software. Phylogenetic analyses, construction of trees and nucleotide distance calculation were performed by both Neighbor Joining and Maximum-likelihood approach. Bootstrap support for the tree nodes of the reconstructed phylogenetic trees was calculated with 1000 replicates.

Results

VIRUS ISOLATION AND PCR ANALYSIS

Out of 110 samples of bovine nasal swabs inoculated in Vero cells, the characteristic CPE appeared in four samples (3.6%) after 48h and 72h incubation. Identification of all
isolated BoHV-1 strains was done by virus-neutralization test and PCR analysis. The presence of BoHV-1 was detected by the appearance of 478 bp bands for BoHV-1 gB gene and 183 bp bands for BoHV-1 tk gene in four samples (3.6%). All positive samples originated from animals from the same herd.

THE PHYLOGENETIC ANALYSIS OF BOHV-1 ISOLATES

The phylogenetic analysis of the gB gene nucleotide sequences of all four BoHV-1 strains showed that they were 100% similar to each other and branched with BoHV-1 strains isolated in Egypt and USA. The gB gene nucleotide sequences of 478 bp of all four BoHV-1 isolates were submitted to NCBI GenBank (Accession Nos.: MG321244; MG321245; MG321246 and MG321247). BoHV-1 strains isolated in Israel, India, Brasil and USA belonging to the second branch were 100% similar to each other and 98% to 99% similar to the strains from Serbia (fig. 1). The phylogenetic analysis of the tk gene nucleotide sequences of all four BoHV-1 strains showed that they were 100% similar and grouped together in one branch.

The tk gene nucleotide sequences of 183 bp of all four BoHV-1 isolates were submitted to NCBI GenBank (Accession Nos.: MG321248; MG321249; MG321250 and MG321251). BoHV-1 strains from USA and Australia clustered together with Serbian BoHV-1 strains sharing similarity of 99%, whilst BoHV-1 strains from India and USA clustered separately (fig. 2).

**Figure 1:** Evolutionary relationships of BoHV-1 gB gene

**Figure 2:** Evolutionary relationships of BoHV-1 tk gene

**Discussion**

The presence of BoHV-1 in Serbia is known for many years. In this study the presence of BoHV-1 was confirmed by both virus isolation and PCR in 4 out of 110 tested bovine nasal swabs. It was mentioned previously that all nasal swab samples were collected only from extensively reared animals with non-specific clinical symptoms. The appearance of nasal discharge, cough and elevated temperature are clinical symptoms related not only to BoHV-1 infection [3]. Mahajan et al. [15] compared different diagnostic tests for the detection of BoHV-1 in the cases of bovine abortion and determined that PCR is a more sensitive and specific technique for the diagnosis of IBR, i.e. virus isolation (VI) was successful in five cases and PCR detected 18 positive cases. Differently, there was a complete correlation between the mentioned methods in our study, probably due to the presence of viable BoHV-1 in all positive samples.

Wernicki et al. [25] examined the prevalence of BoHV-1 in small herds of young beef cattle in Poland and confirmed the presence of the virus by PCR using specific primers for glycoprotein D (gD) in 42 (36.5%) out of 115 examined samples of nasal swabs. Similarly to Serbia, in Poland BoHV-1 is only monitored but there is no eradication program. Bugarski et al. [5] demonstrated a low significance of BoHV-1 in the occurrence of respiratory disease in the feedlots of Vojvodina, Northern Serbia. Possible reason for such findings is that small numbers of animals are latently infected, however, it is more probable that most animals are subclinically infected. The latter is supported by the fact that BoHV-1 enzootics occur in farms after mixing animals from different herds [3]. Austria was one of the first EU member states to begin implementing the eradication program for BoHV-1. Other countries such as France, Italy, Germany, Hungary, the Czech Republic, Slovakia etc., all with different seroprevalence of BoHV-1 depending on the region and cattle rearing system, also have eradication programs based on obligatory disease reporting, vaccination, removal of infected animals, as well as regular annual control measures [1, 14].

The results of the study conducted in Bulgaria demonstrate that PCR assays using primers for gB, glycoprotein E (gE) and glycoprotein C (gC) genes identify the BoHV-1 strains with a high specificity [20]. It is well known that the envelope glycoprotein B has a significant role in virus attachment to host cells. For this reason, primers for the mentioned glycoprotein were used in PCR, as well as for phylogenetic analysis. Moore et al. [18] have shown that the use of BoHV-1 tk gene for PCR diagnostics is suitable since thymidine-kinase (tk) plays an important role in viral pathogenicity. The results of phylogenetic analysis of the gB gene nucleotide sequences showed that all four Serbian isolates obtained in this study branched together with BoHV-1 strains isolated in Egypt and USA, whilst the phylogenetic analysis of the tk gene nucleotide sequences of Serbian BoHV-1 strains
demonstrated that they branched together and clustered with BoHV-1 strains from USA and Australia sharing 99% similarity. Here It should be noted that the GenBank database does not contain sufficient data on European BoHV-1 gB and tk nucleotide sequences. Surendra et al. [21] performed the direct sequencing of gC and gB genes and phylogenetic analysis of 23 Indian BoHV-1 isolates. The results showed that these isolates clustered along with reference Cooper BoHV-1 strain and few BoHV-1 strains isolated in USA, similarly as in our investigation. Majumder et al. [16] performed gB gene sequence and phylogenetic analysis of Indian BoHV-1 isolate and showed that it shared a high level of identity (99%) with nucleotide sequences of other BoHV-1 strains from different parts of the world e.g. Brasil, China and USA. Partial BoHV-1 gB gene sequence analysis of Indian isolates revealed that they were 99% to 100% homologous, whilst the homology with Cooper strain of BoHV-1 was between 99.2% to 99.8% [19].

The results of our study confirmed the presence of BoHV-1 in extensively reared cattle in Serbia. The lack of significant genetic diversity between Serbian BoHV-1 strains and BoHV-1 strains distributed worldwide was demonstrated. In the same time, our data is one of the first reports of the phylogenetic analysis of BoHV-1 isolates from the Republic of Serbia representing a contribution to the limited data available in the GenBank database.

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Conflict of interest

The authors declare no conflict of interest.

References


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