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## PRELIMINARY SEROLOGICAL AND MOLECULAR INVESTIGATION OF SELECTED VIRAL PATHOGENS IN CROATIAN CERVID SPECIES

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A total of 131 blood samples and 175 spleen samples were collected from three cervid species: roe deer (*Capreolus capreolus*), red deer (*Cervus elaphus*) and fallow deer (*Dama dama*) inhabiting the continental part of Croatia. Serum samples were tested for antibodies against bovine herpesvirus 1, parainfluenza-3 virus, bluetongue virus, bovine respiratory syncytial virus, hepatitis E virus, bovine viral diarrhoea virus and enzootic bovine leukosis virus. The tested sera were negative for bovine viral diarrhoea virus, enzootic bovine leukosis virus, bluetongue virus, bovine respiratory syncytial virus and hepatitis E virus antibodies. The antibody prevalence in roe deer and red deer samples was 21.11% for bovine herpesvirus 1 and 75.55% for parainfluenza-3 virus. Sera from bovine herpesvirus 1 positive animals were subsequently tested with comparative virus neutralization test and bovine herpesvirus 1 neutralising antibodies were found in 18 (out of 19) sera. In fallow deer, no antibodies against any of the viral pathogens were detected. All spleen samples tested for bovine viral diarrhoea virus and enzootic bovine leukosis virus came back negative, except for one red deer spleen sample found to be weakly diarrhoea virus-positive. Our findings provide the first information on the exposure of Croatia-inhabiting cervid species to viral pathogens, and could serve as valuable baseline data for future investigations regarding deer exposure to various pathogens and the distribution of diseases shared between wildlife and livestock. As of now, the epidemiology of these viruses in the Croatian cervid population has been only poorly understood, so that further research is recommended.

**Key words:** Croatia, deer, livestock, serology, viral diseases

### INTRODUCTION

Over the past decades, the interest in the epidemiology of infectious diseases emerging in livestock due to the transmission from wildlife has greatly increased [1]. Many

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infectious diseases are shared between wildlife and domestic animals and may have implications on animal production industry and wildlife health [2].

Some of them have been recognized as a permanent threat to wild ruminants and domestic animals health, as well as a threat to the livestock production industry. Namely, when present in wildlife, some of these infectious diseases can be transmitted to humans and livestock. Contrary to the significant attention paid to livestock diseases, understanding of disease processes going on at the livestock-wildlife interface and their management still remains poor and insufficient [3]. Serological monitoring of livestock and wildlife is of vital importance for establishing the incidence of such infections and their possible spreading patterns.

It is well known that enzootic bovine leukosis (EBL) is responsible for economic losses in livestock farming. The highest seroprevalence of EBL virus in the first year of the national eradication programme was detected in Osijek-Baranja County followed by Sisak-Moslavina County, and Brod-Posavina County, [4]. These counties represent Croatian territories in which the livestock population reaches the highest numbers.

In the past some studies about infectious bovine rhinotracheitis (IBR) and bovine viral diarrhoea (BVD) seroprevalence have been conducted in Croatia [5-7]. These viruses have constantly been present in cattle herds and caused periodical disease outbreaks [7]. However, only bulls for breeding should be serologically tested for IBR and BVD. The first report on bovine herpesvirus 1 (BoHV-1) presence in Croatian dairy herds was submitted in 2011 [8], clustering the isolates into the BoHV-1 subtype 1.1 cluster. As for the BVD virus, its presence was also confirmed in domestic cattle, the isolates thereby belonging to the genotype-1 viruses, subgroups 1b and 1f [9], and the first reports on bluetongue virus (BTV) presence in cattle, goats and sheep in Croatia have been reported by Listeš et al. [10,11].

Several serological surveys have been carried out in Europe recently in order to investigate the health status of wild cervids. In Hungary, the prevalence of BoHV-1 in deer species was reported to be in the range from 21% to 35% [12]. In Germany, antibodies against three different  $\alpha$ -herpesviruses were detected in samples retrieved from free-ranging red, roe and fallow deer, with the following prevalence: BoHV-1, 14%; cervid herpesvirus 1, 10.8%; and caprine herpesvirus 1, 6.9% [13]. In Italy, antibodies against bovine respiratory syncytial virus (BRSV) (41%), bovine parainfluenza virus-3 (PI-3 virus) (17%) and pestiviruses (18%) were detected in roe deer [14]. An increased distribution of bluetongue virus in five wild ruminant species, with a mean seroprevalence ranging from 17% to 29% was found in Spain [15], while in another survey of the Spanish population of wild ruminants an antibody prevalence of 48.6% was found [16]. The evidence that BTV-8 infects the wild cervid population was found in Belgium, too [17]. Antibodies against BVDV (1.5%) and herpesvirus (0.2%) were detected in roe deer in Spain [18]. A BVDV prevalence of 1.7% in wild ruminant species (roe deer, red deer, Alpine chamois and Alpine ibex) was reported in Switzerland [19]. A survey carried out in Austria showed that free-ranging and farmed

red deer were BVDV-negative [20]; the same was reported for red deer, roe deer and fallow deer in Spain [21]. Cervid pestivirus infection (prevalence, 0.6%) was reported in the Czech Republic [22], while in central Italy the prevalence of this infection in fallow deer was reported to be 4.5% [23]. In the south-western Italian Alps, antibodies against pestiviruses in roe deer samples failed to be found [24]. Outside Europe, a BVDV seroprevalence of 1.2% was detected in Alabama [25], while in Mexico a high prevalence rate of 63.5% was evidenced [26]. In the USA, the presence of BTV antibodies in mule deer in prevalence from 4% to 62% was reported in Wisconsin [27].

As for hepatitis E virus (HEV) infection in the deer population, 2.6% of sika deer samples in Japan were positive for anti-HEV IgG [28]. The red deer population in Spain was tested for HEV antibodies and 10.4% of serum samples were IgG positive [29]. In the Netherlands, HEV specific antibodies were detected in 5% of red deer and none of roe deer samples [30]. In Poland, no HEV antibodies were found in any wild ruminant species including roe deer, red deer and fallow deer [31], while in Germany the HEV antibody prevalence ranged from 2% to 3.3% in red deer and from 5.4% to 6.8% in roe deer [32]. For Italian red deer, an anti-HEV seroprevalence of 13.9% was reported [33].

Wild ruminants, deer in particular, are hunting game species common for Croatia. The overall number of deer population has increased during the last decade, so that, according to the data obtained from the Croatian Bureau of Statistics, the Croatian Hunting Union and the Ministry of Agriculture, in 2014 the estimated population of red deer was around 4,382 and the number of roe deer thereby approximating to 16,800 [34]. To our knowledge, no information on the epidemiology of viral pathogens in Croatian cervid species is available.

The aim of this study was to find out whether the wild cervid population in Croatia is exposed to viral pathogens important for livestock and for human health. To that effect, the wildlife blood samples were tested for selected viral antibodies. The identification of these antibodies may help estimating the disease risk in cervid species and livestock, and allows for the prediction of the impact of such diseases on the animal production industry. Our results could be of use in establishing the baseline exposure level across the targeted wildlife groups, the infection incidence rates and its possible spreading patterns.

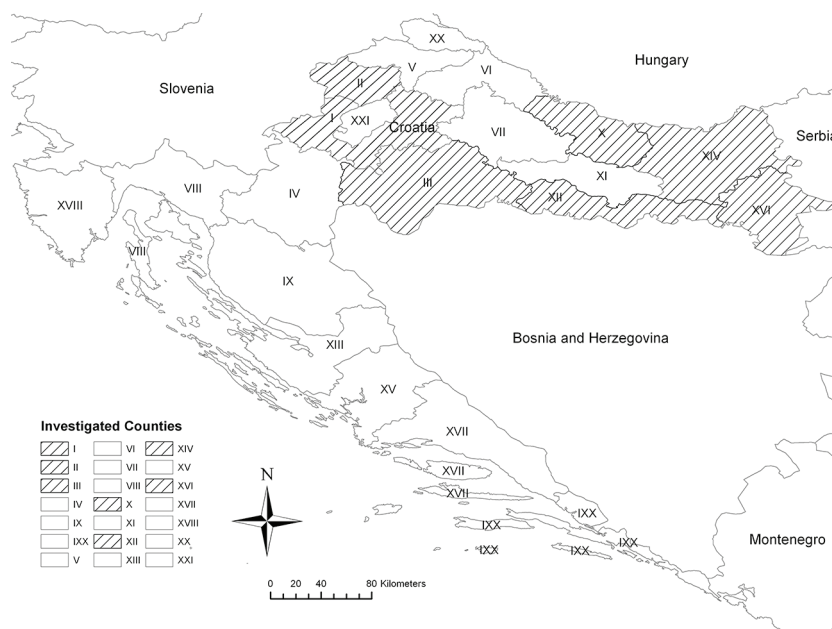
## MATERIALS AND METHODS

### Study areas

Animals belonging to three deer species: red deer (*Cervus elaphus*), fallow deer (*Dama dama*) and roe deer (*Capreolus capreolus*) had been shot by accredited hunters in the hunting grounds located in seven counties of continental Croatia (Figure 1).

The counties in question are geographically connected and situated along the borders with Slovenia (Krapina-Zagorje County), Hungary (Virovitica-Podravina and Osijek-

Baranja County), Serbia (Vukovar-Srijem and Osijek-Baranja County) and Bosnia and Herzegovina (Brod-Posavina and Sisak-Moslavina County). The field sampling lasted from 2009 to 2011, on the occasion of which sex and age of shot animals were recorded, as well. At the time, all animals appeared to be healthy from the clinical standpoint.



**Figure 1.** Map of Croatia with counties where sampling has been done. Cross-hatching identifies the counties in continental Croatia: Zagreb County (I); Krapina-Zagorje County (II); Sisak-Moslavina County (III); Virovitica-Podravina County (X); Brod-Posavina County (XII); Osijek-Baranja County (XIV) and in Vukovar-Srijem County (XVI).

### Sample collection

Blood samples were taken from 131 animals, as follows: 32 male roe deer (aged 1-7), 58 female red deer (aged 1-11), and 41 male fallow deer (aged 1-6). Samples were collected by heart puncture immediately after the animals were shot and stored into sterile test tubes. Live animals were bled-out by jugular or brachial venipuncture, their blood subsequently being stored in anticoagulant-free vacuum tubes at 4 °C. Each sample was centrifuged at 1,200 G for 15 min, decanted and stored at – 20 °C until testing and assayed for antibodies against the selected viral agents.

Spleen samples were obtained from 175 animals, as follows: 169 red deer, 5 fallow deer and 1 roe deer, and tested for BVDV and EBLV using the reverse transcription-polymerase chain reactions (RT-PCR).

County-based representation of blood and spleen samples is given in Table 1.

**Table 1.** The number of deer blood (n=131) and spleen samples (n=175) collected in different Croatian counties

| County               | Red deer |        | Roe deer |        | Fallow deer |        |
|----------------------|----------|--------|----------|--------|-------------|--------|
|                      | Blood    | Spleen | Blood    | Spleen | Blood       | Spleen |
| Krapina-Zagorje      | 0        | 0      | 12       | 0      | 0           | 0      |
| Zagreb               | 0        | 0      | 5        | 0      | 0           | 0      |
| Sisak-Moslavina      | 0        | 15     | 8        | 0      | 0           | 0      |
| Virovitica-Podravina | 0        | 0      | 0        | 0      | 21          | 0      |
| Brod-Posavina        | 0        | 14     | 0        | 0      | 0           | 0      |
| Osijek-Baranja       | 58       | 62     | 7        | 0      | 0           | 0      |
| Vukovar-Srijem       | 0        | 78     | 0        | 1      | 20          | 5      |
| Total                | 58       | 169    | 32       | 1      | 41          | 5      |

## Serological methods

The sera were tested using commercial immunoenzymatic assays (ELISA). All ELISA tests procedures and the interpretation of results were performed according to the manufacturer's instructions.

The presence of specific anti-HEV IgG antibodies was performed by MP Diagnostics HEV ELISA reagents (MP Biomedicals Suisse S.A., Switzerland) originally designed to detect HEV-specific antibodies in human sera. Peroxidase-labeled antibodies to deer IgG KPL, Guilford peroxidase-conjugated rabbit anti-deer IgG antibodies (1:500) were used instead of anti-human conjugates with few modifications because of the lack of positive deer sera. All deer sera were tested in duplicate, the dilution thereby being 1:21. The presence or absence of HEV specific IgG antibodies was determined by comparing the absorbance of the specimens against the plate cut off value. Optical density was measured using a plate reader (Microplate Autoreader TECAN Sunrise-Magellan, Austria) at 450 nm wavelength.

In order to determine the levels of anti PI-3 virus antibodies, all serum samples were tested using a haemagglutination inhibition test (HI-test) performed according to the standard protocol [35]. The SF-4 strain (ATCC VR-28) of PI-3 virus was used for the HI-test. The results were recorded once the erythrocyte sedimentation in the control wells was completed, the antibody titre thereby being defined as the reciprocal value of the last serum dilution in which the inhibition of agglutination was clearly visible. Samples in which the end-point titres of  $\geq 4$  were recorded were considered positive.

The virus neutralization test in 96-well microplates was used for the detection of anti-BoHV-1 antibodies with RVB-0073 Schönböken strain received from the Friedrich Loeffler Institute, Germany. The test was performed according to the OIE Terrestrial Manual [36]. The test results are expressed as the reciprocals of the dilution of the serum that neutralised the virus in 50% of the wells.

The tested viral agents, the tests employed and the positive titre cut off values of each test in use are summarized in Table 2.

**Table 2.** Serologic tests employed for serological assay of deer sera sampled

| Agent <sup>a</sup> | Tests <sup>b</sup>   | Antigen                              | Conjugate   | Positive threshold <sup>d</sup>     |
|--------------------|--|--------------------------------------|---|-------------------------------------|
| IBR                | IDEXX IBRgB Blocking (IDEXX, Montpellier, France)                        | glycoprotein gB                      | mAb anti-bovine IgG peroxidase                      | S/N ≤ 50%                           |
| BVDV               | PrioCHECK BVDV Ab (PRIONICS Lelystadt B.V.AG Lelystadt, the Netherlands) | protein p80 (NS-3)                   |   | PI ≥ 30%                            |
| BRSV               | PrioCHECK BVDV Ab (PRIONICS Lelystadt B.V.AG Lelystadt, the Netherlands) | BRSV antigen                         | anti-bovine polyclonal antibody peroxidase          | PP ≥ 15%                            |
| BTV                | Ingezim BTV COMPAC 1.2.BTV.K.3. (Ingenasa, Madrid, Spain)                | recombinant VP7                      |   | OD sample ≤ pos cut off (60% of NC) |
| EBL                | Svanovir BLV gp51 Ab-Screening (Svanova, Uppsala, Sweden)                | gp51                                 | mAb anti-bovine IgG peroxidase                      | PP ≥ 20%                            |
| HEV                | MP Diagnostic HEV ELISA (MP Biomedicals Suisse S.A. Switzerland)         | recombinant HEV antigen              | peroxidase-conjugated rabbit anti-deer IgG antibody | OD sample ≥ to the cut off          |
| PI-3 virus         | Haemagglutination inhibition test (HI)                                   | SF 4strain ATCC VR-281               |   | 1:4                                 |
| BoHV-1             | VNT on Au-Bek cell   | BHV-1 strain Schönböken RVB-0073 FLI |   | 1:4                                 |

<sup>a</sup>IBR=Infectious bovine rhinotracheitis; BVDV=Bovine viral diarrhoea virus; BRSV=Bovine respiratory syncytial virus; BTV=Bluetongue virus; EBLV=Enzootic bovine leukosis virus; HEV=Hepatitis E virus; PI-3 virus=Parainfluenza virus; BoHV-1=Bovine herpes virus 1

<sup>b</sup>ELISA=Enzyme-linked immunosorbent assay; HI test=haemagglutination inhibition test; VNT=virus neutralization test

<sup>c</sup>S/N=sample/negative ratio; PI=percentage inhibition; PP=Percent Positivity Values; IgG=immunoglobulin G; OD=optical density

## Molecular methods

### Sample preparation and RNA isolation

Approximately 0.1 g of each spleen sample was homogenised and diluted in 1 mL of phosphate-buffered saline (PBS; pH 7.4). The homogenised samples were then vortexed for 1 min and centrifuged for 15 min at 13,000 rpm. An aliquot (140 µL) of each sample's supernatant was used for the isolation of viral RNA using a QIAamp viral RNA extraction kit (Qiagen, Germany) according to the manufacturer's protocol. The RNA samples were stored at -80 °C until further use.

### Detection of BVDV using RT-PCR

The amplification of BVDV 5'NTR fragments was adapted from the previously published methodology [37]. Six µL of RNA extracts were used for the reverse transcription by SuperScript III reverse transcriptase using random hexamers



(Invitrogen, USA). For the purpose of amplification of the 284-nucleotide long product of 5'NTR, primers described by Vilcek *et al.* [37] were used. Fifty  $\mu\text{L}$  reaction mixtures containing 6  $\mu\text{L}$  of cDNA were prepared according to the manufacturer's instructions (PlatinumTaq DNA polymerase, Invitrogen, USA). The amplification procedure for the 5'NTR region run as follows: denaturation at 94 °C for 2 min; incubation at 94 °C for 1 min, at 60 °C for 1 min, and at 72 °C for 1 min (35 cycles); and incubation at 72 °C for 10 min. Eventually, the reaction mixtures were maintained at 4 °C until being removed from the device.

### **Detection of EBLV using nested RT-PCR**

The amplification of EBLV gp51 gene fragment was adapted from the previously published methodology [38]. Six  $\mu\text{L}$  of RNA extracts were used for the reverse transcription by SuperScript III reverse transcriptase using random hexamers (Invitrogen, USA).

Two pairs of EBLV primers were used for direct and nested PCR amplification of 341-nucleotide long gp51 product (OBLV1A/OBLV6A—direct and OBLV3/OBLV5—nested) [38]. Fifty  $\mu\text{L}$  reaction mixtures containing 6  $\mu\text{L}$  of cDNA were prepared according to the manufacturer's instructions (PlatinumTaq DNA polymerase, Invitrogen, USA). Direct and nested PCR parameters of an optimised amplification assay were mutually identical. The amplification procedure for the gp51 region run as follows: denaturation at 94 °C for 3 min; incubation at 94°C for 30 s, at 58 °C for 30 s, and at 72 °C for 45 s (30 cycles); and incubation at 72°C for 5 min. Eventually, the reaction mixtures were maintained at 4 °C until being removed from the device.

### **Gel electrophoresis**

The PCR products were separated using agarose gel electrophoresis in a 1.5%-agarose gel stained with ethidium bromide and visualised using an UV trans-illumination. All standard precautions were taken to prevent PCR contamination. As a positive control, previously characterised BVDV and EBLV strains were used.

### **Statistical analysis**

The lower and upper limits of the 95% confidence interval (CI) for a proportion were calculated. To define the differences in the occurrence of serologically positive results in roe and red deer, Fisher Exact Probability Test was used.  $P \leq 0.05$  was considered statistically significant.

### **Ethics**

No ethical approval was obtained because the blood samples were collected during the health control surveillance programme.



## RESULTS

No antibodies against bovine viral diarrhoea virus (BVDV), enzootic bovine leukosis virus (EBLV), bluetongue virus (BTV), respiratory syncytial virus (RSV) and hepatitis E virus were detected. However, the results reported herein demonstrated the presence of antibodies against BoHV-1 and PI-3 virus.

Overall seroprevalence of antibodies against the BoHV-1, recorded in red deer and roe deer, was 21.11%, while that of antibodies against the PI-3 virus amounted to 75.55%. As far as the fallow deer population is concerned, no antibodies against any of the viral pathogens were detected.

BoHV-1 antibodies were detected in two out of seven investigated counties. In Sisak-Moslavina County, 25% of roe deer were BoHV-1-positive (2 positive/8 tested), while in Osijek-Baranja County that share equalled to 28.57% (2 positive/7 tested). Based on the results of the ELISA assay, a total percentage of roe deer positive for antibodies against BoHV-1 was 12.50 (4 positive/32 tested).

The prevalence of antibodies against bovine BoHV-1 among red deer was 25.86% (15 positive/58 tested), but only in one county (Osijek-Baranja County).

To confirm the ELISA screening results and to determine the specificity of the antibodies, sera of 4 roe deer and 15 red deer were tested using comparative VNT that detects BoHV-1 neutralizing antibodies.

The VNT test confirmed the results obtained in 18 out of 19 sera considered positive (4 roe deer and 14 red deer were positive) based on the outcome of the ELISA, while 1 red deer serum formerly tagged as positive reacted negatively. Most animals had relatively low titres of antibodies against the pathogen tested, ranging from 1:8 to 1:16 in roe deer samples and 1:16 to 1:32 in red deer samples.

Moreover, the recorded seroprevalence for PI-3 virus among red and roe deer was 75.55%. Antibodies to PI-3 virus were detected in 68.75% of roe deer (22 positive/32 tested). The positive animals were found in Sisak-Moslavina County (6 positive/8 tested), Osijek-Baranja County (6 positive/7 tested), Krapina-Zagorje (7 positive/12 tested) and in Zagreb County (3 positive/5 tested). As far as red deer are concerned the antibody prevalence for PI-3 virus was 79.31% (46 positive/58 tested) that was found in the Osijek-Baranja County.

The samples contained detectable amounts of antibodies against the PI-3 virus, the titres thereby ranging from 1:4 to 1:64. Results are summarized in Table 3.

According to the results of Fisher Exact Probability Test we found no statistical significance in the serological results in roe deer when compared to the serological results of red deer regarding BoHV ( $P=0.1100$ ) and PI-3 virus ( $P=0.1941$ ).

Out of 175 tissue samples tested for BVDV using the RT-PCR and tested for EBLV using the nested RT-PCR, 174 were found to be virus-negative. Only one red deer sample coming from Sisak-Moslavina County was weakly BVDV-positive.

**Table 3.** Results of serological tests for BoHV-1 and PI-3 virus and seroprevalence in roe and red deer

|       | Roe deer<br>(number of tested/positive/negative<br>samples; seroprevalence and<br>95% CI in parenthesis) |                                   | Red deer<br>(number of tested/positive/negative<br>samples; seroprevalence and<br>95% CI- in parenthesis) |                                   |
|-------|--|-----------------------------------|---|-----------------------------------|
|       | BoHV-1   | PI-3 virus                        | BoHV-1  | PI-3 virus                        |
| ELISA | 32/4/28; 12.5%<br>(4.97-28.07)   | n.d.                              | 58/15/43; 25.86%<br>(16.34-38.38)   | n.d.                              |
| VNT   | 32/4/28; 12.5%<br>(4.97-28.07)   | n.d.                              | 58/14/44; 24.14%<br>(14.96-36.53)   | n.d.                              |
| HI    | n.d.   | 32/22/10; 68.50%<br>(51.43-82.05) | n.d.  | 58/46/12; 79.31%<br>(67.23-87.75) |

n.d.= no data

However, the weakly positive sample was not sequenced since the available template was insufficient; therefore the raw data signal was too low to allow a correct interpretation.

## DISCUSSION

This study provides evidence of exposure of Croatia-inhabiting cervid species (red, roe and fallow deer) to viral pathogens of importance for livestock and human health. The study comprised seven Croatian counties characterized by forested areas that represent a suitable wildlife habitat. Geographically speaking, three of the investigated counties border with Bosnia and Herzegovina, two with Hungary, one with Slovenia and two with Serbia, so that an uncontrolled migration of wild animals is possible. We hope that the results of our study will at least provide information of value for future research on wildlife health and risks associated with the transmission of viruses from wildlife to livestock. Serology is the first step in determining the potential significance that pathogens could play.

The results reported herein revealed the presence of antibodies in tested sera that are developed against two viral pathogens capable of affecting livestock. Seropositive outcomes were recorded in roe deer and red deer samples; exposure to bovine BoHV-1 occurred in 21.11%, and that to PI-3 virus in 75.55% of animals tested. As far as the fallow deer population is concerned, no antibodies against any of the viral pathogens under study were detected.

Detection of BoHV-1 in non-bovine species might be of the outmost importance, especially since the finalization of eradication programs in European livestock. Roe and red deer populate Europe in high numbers, many of them thereby being infected with BoHV-1 [12]. In view of the above, BoHV-1-seropositive cervids are expected to be found in Croatia, as well, as detected in our preliminary study.

The prevalence of BoHV-1 was lower in roe deer (12.5%) in comparison to red deer (25.86%), even though no statistical significance was found when positive samples in

both species were compared. These results correspond to the prevalence of BoHV-1 of 10.55% in roe deer and that of 23.3% in red deer found in Germany [13], as well as to the prevalence of 21.4% in roe deer and 28.8% in red deer established in Hungary [12].

The number of samples identified as positive based on the BoHV-1-screening ELISA test and the confirmatory VNT test are quite consistent. Namely, subsequent VNT testing of 19 samples identified as positive using ELISA showed the presence of neutralising antibodies in 18 out of 19 tested samples, the antibody titres thereby ranging from 1:8 to 1:32.

Although the sero surveys have been proven to be a fundamental tool when it comes to disease surveillance, serological testing for antibodies against bovine herpesviruses can be made difficult due to possible cross-reactions with other herpesviruses [39]. Unfortunately, as standard serological tests cannot distinguish between different types of bovine herpesvirus infections encountered in deer, we were unable to provide information regarding closely related viruses with the similar antigenic profile that might have caused the seroconversion. Just like in our study, the presence of BoHV-1 antibodies has been reported by other European countries without any mention of differentiation between BoHV-1 and cross-reacting herpesviruses [18]. Therefore, blocking ELISA commercial kits that make use of the gB antigen can be used for screening purposes, but do not offer the possibility of differentiating herpesviruses [40].

BoHV-1 disease is an important cattle disease with a long history in Croatia [5]. In general, the Croatian cattle population is BoHV-1-seropositive [7]. During the time-period in which deer samples within this study were collected, the presence of the virus was proven in cattle inhabiting eastern and the south-western parts of the country, where the density of both cattle and cervids is very high [8], making these areas the most probable infection sites.

In the present study, PI-3 virus antibody prevalence was relatively high and varied from 68.75% in roe deer to 79.31% in red deer, with titres ranging from 1:4 to 1:64. The highest number of seropositive reactors and the highest antibody titres were found among red deer.

The seroprevalence confirmed by this study was higher in comparison to other countries. A prevalence of 26% in fallow deer was reported in Italy [14, 41], while in the USA the seroprevalence in red deer equalled to 24.7% [27]. We do not know for a fact how the animals were exposed to the PI-3 virus, but exposure may depend upon local environmental factors, including deer and/or cattle density. Parainfluenza-3 virus is likely to be transmitted to deer from cattle; however, the disease can also be maintained in the deer population having no contact with cattle at all [42].

We assume that a more frequent exposure to BoHV-1 and PI-3 virus seen in north-eastern Croatia (Osijek-Baranja County) comes as a consequence of higher densities of deer species and cattle in this part of Croatia. Also, this part of Croatia is bordering

with Hungary, where positive cervids were detected, as well as with Serbia, so that the possibility of migration of wild animals cannot be ruled out. Evidence of exposure of deer populations to any other agent assayed in this study failed to be found. The results of this study suggest a very limited contact of red deer, fallow deer and roe deer populating the continental part of Croatia with these infective agents. The HEV-negative results speak in proof of the previously reported absence of such an infection in Croatian cervids [43].

Only one sample was shown to be weakly BVDV-positive by RT-PCR. Even though sequencing of the BVDV isolate from our study was not performed due to a low RNA load, the sequencing and the phylogenetic analyses results of the 5'NTR genome region within the same time frame (2009-2011) show the presence of genotypes BVDV-1b and BVDV-1f circulating in Croatia [9]. Our results are comparable to those reported by Austria [20] and in contrast to those reported by Spain, where BVDV infection allegedly affects more than 22% of red deer regardless of age [44]. Even though no antibodies against bovine viral diarrhoea virus (BVDV) were detected within this study frame, a low number of tested animals should be kept in mind and/or the dynamics of antibody development may differ in regards to different cervid species [18]. Therefore, based merely on these negative results, a possible involvement of deer in the epidemiology of this disease cannot be ruled out, especially in view of the fact that BVDV has been isolated in 9 Croatian dairy herds [9] and that the virus circulates among cattle.

Although the analysed samples were negative for most of the selected pathogens, the detected antibodies against BoHV-1 and PI-3 virus indicate that some of the cervids had been exposed to viral agents common in, and relevant for, livestock. However, in order to better clarify these findings, further studies are needed as well as the investigation of transmission routes from wild ruminants to livestock and *vice versa*, that are still poorly understood.

Even though our preliminary results do not give a complete picture of the epidemiological status of Croatian deer population but our research emphasizes the need for further epidemiological studies. Permanent surveillance programmes of livestock and wildlife in the hunting grounds can be a reliable and effective detection programme of these agents and possible patterns of their spreading.

### **Authors' contributions**

RB, TS, FT and JL participated in the design and coordination of the study, analysis and interpretation of data and performed the statistical analysis. PJ carried out the molecular genetic studies, participated in the sequence alignment and drafted the manuscript. OS and BI have been involved in sampling and drafting the manuscript. JA and KT carried out the immunoassays and conceived of the study. All authors read and approved the final manuscript.

## Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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## REZULTATI PRELIMINARNIH SEROLOŠKIH I MOLEKULARNIH ISTRAŽIVANJA ODABRANIH VIRUSNIH INFEKCIJA JELENSKE DIVLJAČI U REPUBLICI HRVATSKOJ

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U ovom istraživanju prikazani su rezultati ispitivanja uzoraka 131 seruma i 175 slezina poreklom od srna (*Capreolus capreolus*), jelena (*Cervus elaphus*) i jelena lopatara (*Dama dama*) prikupljenih u kontinentalnom delu Hrvatske. Uzorci seruma ispitani su na prisustvo specifičnih antitela za goveđi herpes virus-1, virus parainfluence-3, virus plavog jezika, goveđi respiratorni sincicijski virus, hepatitis E virus, virus virusne dijareje goveda i virus enzooske leukoze goveda. U uzorcima seruma nisu utvrđena antitela za virus enzooske leukoze goveda, virus plavog jezika, goveđi respiratorni sincicijski virus, hepatitis E virus i virus virusne dijareje goveda. Specifična antitela za goveđi herpes virus-1 u dokazana su u 21,11% ispitanih seruma srna i jelena kao i u 75,55% uzoraka za virus parainfluence-3. Serumi pozitivni na goveđi herpes virus-1 dodatno su ispitani virus neutralizujućim testom i u njih 18 (od 19 pozitivnih) utvrđena su neutralizaciona antitela. U ispitanim serumima jelena lopatara nisu dokazana antitela ni za jedan od navedenih virusa. Uzorci slezina ispitani su na prisustvo virusa za virusnu dijareju goveda i enzooske leukoze goveda bili su negativni, osim jednog uzorka slezine jelena koji je dao slabo pozitivnu reakciju na virus virusne dijareje goveda. Dobijeni rezultati predstavljaju prvo takvo istraživanje sprovedeno na jelenskoj divljači u Republici Hrvatskoj i mogu biti osnova za dalje istraživanje širenja virusa između divljih i domaćih životinja. S obzirom da je epizootička situacija u populaciji jelena slabo poznata, ovi podaci bi mogli biti korisni za dalja istraživanja.