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Ecology driven patterns of herpesvirus spread in cervids in Poland

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Abstract

Cervids are considered as a potential hosts for various human and animal pathogens but studies on herpesvirus infections among these species remain limited. We have used a panherpesvirus PCR to investigate herpesvirus diversity and prevalence and a BoHV-4-specific ELISA to assess exposure to BoHV-4." Among 1257 cervids tested for the herpesvirus DNA, 389 (30.9%) were positive. The prevalence was highest among fallow deer (63.9%), followed by red deer (29.8%) and roe deer (19.4%); among captive animals (42.1%) and in forest districts with low (31.2%) or medium (32.9%) forest coverage. No positive cases were found in moose. BoHV-4 specific antibodies were found in 107 out of 1179 (9.1%) tested cervids. Seroprevalence was highest among roe deer (18.7%) and in regions with the highest forest coverage (13.9%). Two gammaherpesvirus species: Fallow deer lymphotropic herpesvirus, Elk gammaherpesvirus 1; and one betaherpesvirus – Capreolus herpesvirus 1 were for the first time identified in Polish deer. Although, none of these viruses is currently considered pathogenic for cervids, the study revealed distinct ecological patterns of herpesvirus circulation among cervid species. These findings expand current knowledge of herpesvirus diversity in Polish cervids and demonstrate the

value of herpesvirus surveillance for understanding pathogen transmission and wildlife–livestock interactions.

Keywords: cervids, gammaherpesviruses, betaherpesviruses, deer, Poland

Introduction

Wildlife species, including cervids are considered a potential hosts for various pathogens of man and animals [1]. In Poland, the role of deer species as a source of viral infections has been studied in regard to Bovine herpesvirus 1 (BoHV-1), Hepatitis E Virus (HEV), Small Ruminant Lentivirus (SRLVs), Bluetongue virus (BTV), Schmallenberg virus (SBV) and in smaller degree for SARS-CoV-2. For all of these viruses, except BTV and SBV, their prevalence was low, suggesting a marginal role in the health of cervids and as reservoirs of disease [2–5]. However, gammaherpesvirus and betaherpesvirus infections of deer have gone largely unresearched. From the perspective of animal health the most significant gammaherpesvirus are those associated with malignant catarrhal fever (MCF). Malignant catarrhal fever is an infectious disease with several different patterns of symptoms although most often it is characterised by sudden death in acute form, and fever, diarrhoea, inappetence, eye and nasal discharges, head oedema in milder form of the disease. In deer, range of presentations has been observed including acute, with potentially haemorrhagic diarrhoea; a head and eye form similar to cattle and also a cutaneous form, with the presence of skin lesions [6]. Depending on the infected host, animals may function either as asymptomatic carriers of the virus or be susceptible to the disease. In susceptible species, mortality can be very high (over 90%) and, in deer, death can occur within 48 hours after first signs of infection [7,8]. In less susceptible species, the course of infection could be longer, lasting 2 to 4 weeks. Contrary to symptomatic infections in cattle that may lead to recovery, disease results in death of all individuals [6]. Cases of MCF have been described in at least 13 species of deer such as: Pere David's deer (*Elaphurus davidianus)*, white-tailed deer (*Odocoileus virginianus)*, sika deer *(Cervus nippon)*, sambar deer (*Rusa unicolor)*, red deer (*Cervus elaphus)*, fallow deer *(Dama dama)*, roe

deer (*Capreolus capreolus)* or elk/wapiti (*Cervus elaphus canadensis)* [9–11]. Susceptibility among deer species varies, with Père David's deer being the most susceptible and red deer, elk and fallow deer usually less affected when infected [10]. Among gammaherpesviruses infecting ruminants we can distinguish members of at least two genera: macavirus and rhadinovirus as well as multiple unclassified viral species. Although most of the MCF viruses belong to macavirus genus, there are some MCFV species that are not assigned to this genus. At the same time, macavirus genus also includes lymphotropic viruses, that could infect ruminants without causing MCF [12,13]. Currently there are at least 4 gammaherpesviruses known to cause MCF in various deer species; 3 macaviruses: Ovine Herpesvirus 2 (OvHV-2), Caprine Herpesvirus 2 (CpHV-2), Alcelaphine gammaherpesvirus 2 (AlHV-2), and Caprine Herpesvirus 3/MCF-white-tailed-deer gammaherpesvirus (CpHV-2/MCF-WTD) not classified to any gammaherpesvirus genus [13]. Among gammaherpesviruses infecting ruminants, there are also non-MCF related while still possessing pathogenic potential such as Bovine Herpesvirus 4 (BoHV-4) – member of the rhadinovirus genus, known as associated with respiratory diseases and abortions in cattle [14–17]. This virus was previously identified in cervids in Hungary, suggesting that although its prevalence was lower in wild ruminants in comparison to local cattle, deer might still play some role in spread of BoHV-4 [18]. Additionally multiple other gammaherpesviruses have been isolated in recent years, such as Fallow deer lymphotropic herpesvirus (Fallow deer LHV), Elk gammaherpesvirus (Elk GHV) or sambar gammaherpesvirus (Sambar GHV) [19,20] but these have uncertain influence on the health status of deer.

There are four main species of cervids in Poland. Three of them - roe deer (*Capreolus capreolus*), red deer (*Cervus elaphus*) and moose (*Alces alces*) are native, whereas fallow deer (*Dama dama*), is an introduced but well-established species [21]. Free-ranging sika deer (Cervus nippon), another non-native species, occurs only locally in Poland following historical

escapes and introductions from captive populations [22,23]. The population size of most cervid species has nearly doubled in the last twenty years, with the most abundant being roe deer and red deer [21]. This growth occurred despite a downward trend in the area of their natural habitats and an increase in the number of their natural predators. The change may be the result of, among others, changes in forest management, hunting restrictions, milder winters, and urbanisation, which reduces the access for predators (e.g. wolves and lynxes) but does not necessarily interfere with deer ecology, which are able to adapt to mosaic agricultural and suburban landscapes [24]. This trend is particularly evident in roe deer forming separate forest and field subpopulations with the latter one migrating towards agricultural crops, closer to human and livestock habitats [25]. Another species increasingly appearing in the human-wildlife interface is the moose (*Alces alces*), the population of which has been increasing since its hunting was suspended in 2001 [21]. The migratory strategy of the moose in response to environmental changes makes this species a potentially important vector of pathogens [26,27]. The population and distribution of the fallow deer is also growing in Poland. Fallow deer had appeared in the south of Poland by the 13th century and began to be bred in parks and game reserves in the 17th century. The fallow deer population increased in the following centuries due to subsequent introductions as game or escapes from breeding farms. Currently, this species is widespread throughout the country, with the largest concentrations in western Poland [21]. Fallow deer are commonly bred for venison in Poland, while in the wild, due to their size and food preferences, they may compete with roe deer for suitable habitats, especially where resources are limited and both species cohabit [28]. Free-ranging sika deer, currently occurs in wild in four locations in Poland and is kept at various deer farms as livestock [22,23].

Understanding the ecological patterns of virus transmission between different deer species is important for insight into pathogen-environment-host interactions, including within the One Health concept.

In order to assess the drivers and risks for herpesvirus transmission among cervids, we analysed the frequency of infections in relation to ecological, biological and environmental factors.

Material and methods

Sample collection

Minimum representative sample size was calculated for each cervid species using population data based on hunting bags provided by the Polish Hunting Society using Epitools calculators (https://epitools.ausvet.com.au/samplesize) assuming 95% confidence level [29–31]. The population sizes of native cervids in the country include 236,000 red deer; 810,000 roe deer and 42,000 moose (*Alces alces*) [21]. The fallow deer free-living population is estimated at around 24,000 individuals. The free ranging invasive alien species sika deer population is estimated at approximately 200 individuals, which occur in four locations in Poland [22,23].The vast majority of this species is kept at various deer farms. Given the lack of reference data on gammaherpesviruses of deer in Europe, for estimating the overall prevalences of GHV and BoHV-4 infections, 10-20% was adopted on the basis of values obtained in similar studies [19,32,33]. The required sample sizes were 138–246 animals for red deer, roe deer and moose, 138–243 for fallow deer, and 82–111 for sika deer, depending on the assumed prevalence.

A total of 1319 cervids were sampled for the purpose of this study, with some previously collected samples retrieved from biobank storage [33]. The sampled animals represented five species of Cervidae of Poland, including 731 red deer, 270 roe deer, 189 fallow deer, 21 moose and one farmed sika deer. Therefore, the target sample size was achieved for red deer, roe deer and fallow deer but not for moose and sika deer because of the protected status and very limited availability, respectively. For 107 animals, no data was available on the species. The samples came from collective hunts and were collected either by a veterinarian or a trained hunter according to Polish

hunting regulations [34]. Sampling kits were prepared in advance, and sampling was carried out under the supervision of the State Forests. Whole and clotted blood and spleen samples were collected post mortem from cervids from a total of 68 locations during three hunting seasons: 2013/2014; 2023/2024; and 2024/2025. Additionally, 149 captive cervids, including 82 fallow deer, 37 red deer, 1 sika deer, 14 moose and 15 red deer, were sampled at four farms, two wildlife reserves, two mini zoos and a rehabilitation centre, mostly ante mortem, that included additional nose swab samples. Clotted blood from the heart or from body cavities was collected into the sterile 9 ml tubes. After separation of 1-2 ml of whole blood or clot, it was centrifuged within 24 hours and the obtained serum was archived frozen at -70°C with the rest of the samples retained. In cases where there was insufficient whole blood for PCR, a fragment of the spleen or a nasal swab from a live animal was used. Spleen tissue was homogenised in PBS to obtain a 10% suspension using Lysing Matrix D tubes (1.4 mm Zirconium-Silicate spheres; MP Biomedicals, France) on the Fastprep-24 5G system (MP Biomedicals, USA). Nasal swabs were collected using Virocult swabs with liquid transport medium (MWE, Wiltshire, England).

Panherpesvirus PCR

DNA was extracted from 200 µL of samples described above using an IndiMag Pathogen Kit (INDICAL Bioscience, Leipzig, Germany) according to the manufacturer's guidelines. The nucleic acid was eluted in 90 µL of elution buffer and stored at -65°C or used directly for polymerase chain reaction (PCR). Nested PCR for pan-herpesvirus detection was run using degenerate primers described by Van Devanter et al., 1996 [35] (Table S1). A two-step reaction was run using Allin^{TM} HS Red Taq Mastermix 2x (highQu, Kraichtal, Germany). The first step was run in a 28 µL reaction mix consisting of 10 µL of water, 13 µL of Allin^{TM} HS Red Taq Mastermix 2x (highQu, Kraichtal, Germany), 1 µL of each panherpes forward (DFA, ILK) and reverse primer (KG1) (20 µM) and 2 µL of DNA sample. After 5-minute incubation at 94°C, 45 cycles of amplification were run each consisting of 30 s of denaturation at

94°C, 60 s of annealing at 46°C, 60 s of elongation at 72°C. The reaction was finished with a 7-minute incubation at 72 °C. The second step reaction was run under the same conditions with 1 µL of each nested pan-herpes forward (TGV) and reverse primer (IYG) (20 µM) and 3 µL of PCR product (from the first reaction). The PCR cycling conditions and cycle numbers were the same as the first step. For analysis, 6 µl of each PCR product was visualised by 3% agarose gel electrophoresis, with positive samples visible at 227 bp. As a positive control AlHV-1 strain C500, provided by Moredun Research Institute, was used.

Sanger sequencing and phylogenetic analysis

Samples positive in the pan-herpesvirus nested PCR were sequenced using the Sanger method with previously described primers TGV and IYG (Table S1) by Genomed S.A. (Warsaw, Poland)[35]. Partial, 174 bp long sequences of the DNA polymerase gene were aligned, using MEGA software (Version 12.1.1; www.megasoftware.net), with sequences of known herpesviruses of artiodactyls, available from GenBank, in order to identify the species of herpesvirus involved in infection [36]. Pairwise sequence similarity was calculated between each identified herpesvirus sequence and its closest GenBank reference sequence.

A neighbour-joining (NJ) tree based on all identified herpesvirus sequences and selected references was constructed using the p-distance model with 1,000 bootstrap replicates and pairwise deletion of ambiguous positions [37,38]. Branches supported by less than 5% bootstrap replicates were collapsed. Additionally, a Maximum Likelihood (ML) tree was reconstructed using a representative subset of 20 viral sequences selected to represent the observed genetic diversity and all analysed host species. The ML analysis was performed using the Tamura–Nei substitution model with 1,000 bootstrap replicates [39]. For cophylogenetic analyses, representative viral sequences from the Fallow deer lymphotropic herpesvirus-related, Elk gammaherpesvirus-related and Capreolus herpesvirus 1 related groups were

selected. Host phylogeny was reconstructed from cytochrome b sequences of Cervus elaphus, Cervus nippon, Dama dama, Capreolus capreolus and Alces alces retrieved from GenBank using the Maximum Likelihood method and the Tamura–Nei substitution model [39]. Host-virus cophylogenetic relationships were evaluated in R version 4.5.3 using the ape, vegan and ade4 packages. Congruence between host and virus phylogenies was assessed using the ParaFit test with 9,999 permutations [40].

ELISA test

To complement molecular detection of active herpesvirus infections, sera were additionally screened for antibodies against BoHV-4 using a commercial ELISA. BoHV-4 was selected because it is the only ruminant gammaherpesvirus for which a validated commercial serological assay is currently available and because previous studies have suggested its circulation in both cattle and cervids, making it relevant for investigating the wildlife–livestock interface. For the purpose the monoscreen AbELISA BoHV-4/indirect, monowell ELISA kit (Bio-X Diagnostics, Rochefort, Belgium) was used following the manufacturer's instructions.

Statistics

The effects of animal-level (species; age group; sex) and population-level variables (population type: captive or free-ranging; cervid density; forest cover) on the binomial results for HV detection or BoHV-4 seropositivity were estimated using univariable logistic regression reporting odds ratio (OR). The data at the forest district level including overall cervid density and densities of individual species such as red deer (mean 1.72; range: 0.04-7.18/km^2), roe deer (mean; 3.50; range: 0.54-7.0/km^2) and fallow deer (mean: 0.32; range: 0.01-1.52/km^2) as well as forest cover (mean: 39.80; range: 10.3-88.66) were retrieved from Forest Data Bank (https://www.bdl.lasy.gov.pl/portal/en). Since these values did not meet the conditions for normal distribution, the densities (expressed as the number of individuals per square kilometer) and

forest cover (expressed as percentage of forested area) to the total district were categorized by the 25th and 75th centiles into three groups (low, medium, high). Deer were also grouped by age into calves (< one year old), juveniles (1-2 years old) and adults (≥3 years old). The correlations between all the variables were assessed by logistic regression or Spearman rank test. The generalized linear mixed models (GLMMs) with logit transformation were developed by backward elimination of insignificant (with $P>0.05$) predictor variables one-by-one. The collinearity (Cramér's V above 0.3; Spearman $\rho>|0.5|$; $P<0.05$) between the predictors was considered when building up the multivariable model. To account for clustering, models including random intercept were assessed by checking the variance of the component and other covariates. The model with lowest Akaike Information Criterion (AIC) and highest Bayesian Information Criterion (BIC) was considered better fitting. All the analyses were performed using STATA v.13.0 software (StataCorp LP, Texas, USA). The P value ≤ 0.05 was considered significant in all analyses.

Results

Herpesvirus (HV) prevalence

In total, herpesvirus DNA was detected in 389 of 1257 tested cervids. Prevalence varied from 0 to 66.7% between forest districts, without any distinctive geographical pattern (Figure 1a).

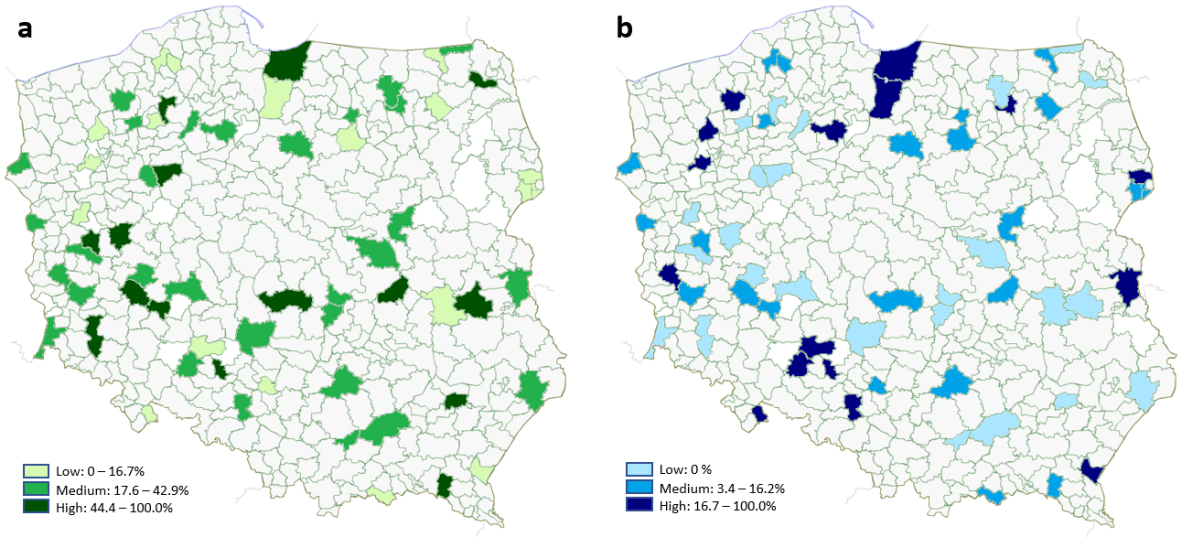

Figure 1. The occurrence of herpesvirus infections in cervids at the forest district level. The forest districts were marked with different shades of green corresponding to HV prevalence (A) and blue for BoHV-4 seroprevalence (B). Values graded by colour intensity were graded based on limits set by the 25th and 75th quartiles at the forest district-level.

Significant differences in herpesvirus infection were observed between species with prevalence of infection highest among fallow deer, followed by red deer and roe deer (Table 1). The high HV prevalence in fallow deer was found in both captive (65.4%) and free-ranging (61.7%) animals. The single sika deer tested was positive for herpesvirus, while no positive cases were found among the 20 moose tested. No impact of sex or age on HV detection was observed (Table 1). Significantly higher HV prevalence was observed in captive deer, while this was not related to the overall cervid density in free-ranging populations. Nevertheless, the association of HV infection with forest cover was significant, with the lowest HV prevalence in the most highly forested districts (with over 53% coverage).

Table 1. Descriptive statistics of herpesvirus infections in cervids based on the presence of genetic material (PCR) and specific antibodies to bovine herpesvirus type 4 (BoHV-4) and the results of univariate logistic analysis of the effects of animal and population-level risk factors for the infection.

Variables	HV prevalence					BoHV-4 seroprevalence				
	n/N[a]	%	95% CI[b]	OR[c]	P	n/N[a]	%	95% CI[b]	OR[c]	P

Animal-level										
Species				0.36	**<0.001**				2.85	**<0.001**
fallow deer	120/188	63.9	56.5-70.7			2/169	1.2	0.1-4.2		
red deer	207/694	29.8	26.4-33.4			40/649	6.1	4.4-8.3		
roe deer	52/268	19.4	14.8-24.6			44/235	18.7	13.9-24.3		
moose	0/20	0	0-16.8			2/20	10.0	1.2-11.7		
sika deer	1/1	100	2.5-100			-	-			
unspecified	9/86	10.5	4.9-18.9			19/106	18.0	11.1-26.6		
Sex				0.68	0.49				0.94	0.81
female	212/668	31.7	28.2-35.4			49/609	8.0	6.0-10.5		
male	144/427	33.7	29.2-38.4			29/388	7.4	5.0-10.5		
missing data	33/162	20.4	14.4-27.4			29/182	15.9	10.9-22.0		
Age group				1.18	0.07				1.09	0.62
calves (<1 year)	48/165	29.1	22.1-36.1			10/152	6.6	2.6-10.6		
juveniles (1-2 years)	29/93	31.2	21.6-40.8			6/90	6.7	1.4-11.9		
adults (≥3 years)	213/587	36.3	32.4-40.2			41/555	7.4	5.2-16.5		
missing data	99/412	24.0	19.9-28.1			50/382	13.1	9.7-16.5		
TOTAL	389/1257	30.9	28.4-33.6			107/1179	9.1	7.5-10.9		
Population-level										
Population type				1.73	**0.003**				0.81	0.52
free-ranging	330/1117	29.5	26.9-32.1			96/962	10.0	8.1-12.0		
captive	59/140	42.1	33.9-50.7			11/134	8.2	4.2-14.2		
Cervid density (individual/km^2)[d]				1.07	0.73				1.07	0.6
low (1.9-4.1/km^2)	72/227	31.7	25.7-38.2			17/197	8.6	5.1-13.4		
medium (4.2-6.5/km^2)	161/582	27.7	24.1-31.5			44/476	9.2	6.8-12.2		
high (6.6-12.6/km^2)	89/259	34.4	28.6-40.5			22/222	9.9	6.3-14.6		
missing data	8/49	16.3	7.3-29.6			13/67	19.4	10.8-30.9		
Forest cover (%)[d]				0.80	**0.02**				1.74	**0.001**
low (10.3-28.6%)	78/250	31.2	25.5-37.3			12/199	5.7	2.8-5.6		
medium (29.5-51.4%)	193/586	32.9	29.1-36.9			43/501	8.1	5.7-10.5		
high (53.5-88.7%)	52/242	21.5	16.5-27.2			35/229	13.9	9.6-18.2		
missing data	7/39	18.0	7.5-35.0			6/33	18.2	7.0-34.5		

[a]number of HV PCR positive or BoHV-4 seropositive deer (excluding inconclusive)/all tested; [b]binomial exact 95% confidence interval or 97.5% one sided confidence interval; [c]odds ratio. OR >1 indicates higher odds of the outcome relative to the reference category, OR <1 indicates lower odds, and OR =1 indicates no association. ORs were interpreted as measures of association and not as evidence of causal or protective effects. Significant differences (P<0.05) are shown in bold; [d]applicable only to free-ranging cervids.

The effect of cervid species on the association between explanatory variables and HV prevalence was studied using the Spearman rank correlation test (Table S2-S4). In the case of animal-level variables, only roe deer age was negatively associated with the percentage of HV infected animals, with the highest proportion of infected calves (30.3%) in contrast to yearlings (17.4%) and adults (15.3%) (Table S4). Again, no association was found between HV prevalence and density of individual cervid species. A significant negative association between HV prevalence and forest cover was observed in red deer and fallow deer (Figure 2a). In contrast, no association was found in roe deer, likely reflecting their broader habitat use and occurrence in both forested and non-forested landscapes(Figure 2a).

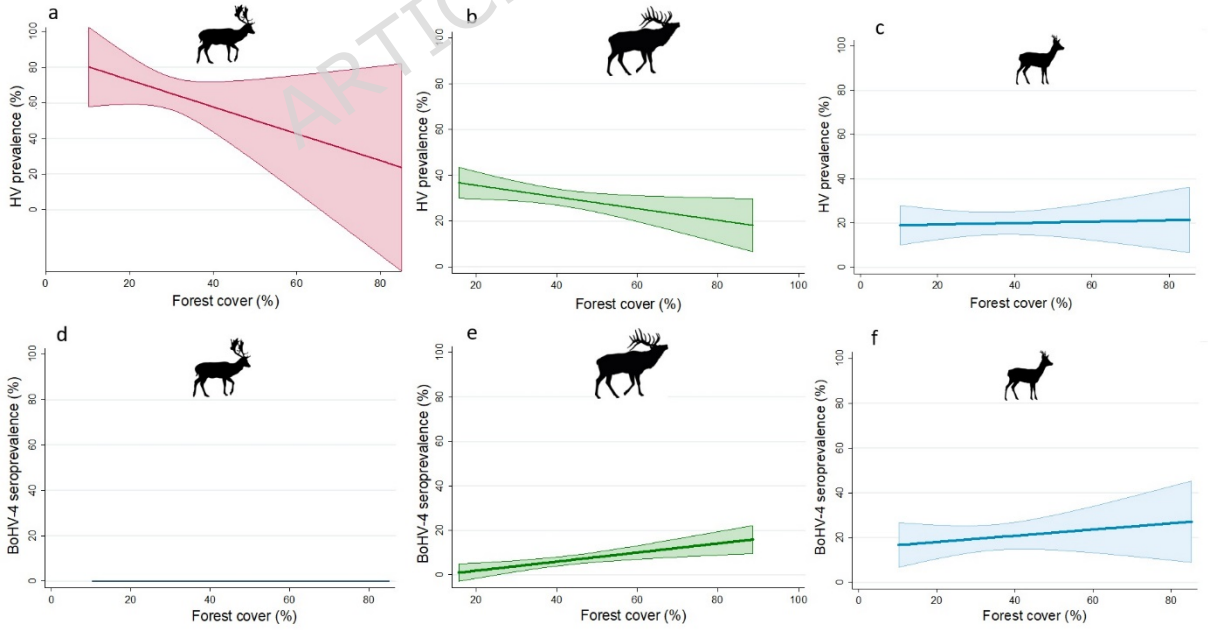

Figure 2. Predicted effects of forest cover on herpesvirus (HV) prevalence and BoHV-4 seroprevalence with 95% confidence intervals in the free-

ranging populations of three most represented cervid species: fallow deer (a, d), red deer (b, e) and roe deer (d, f). In the case of BoHV-4, the absence of a graph for fallow deer (d) is due to the fact that all animals were seronegative.

BoHV-4 seroprevalence

The seroprevalence to BoHV-4, expressed as a percentage of cervids in which serum virus-specific antibodies were detected, was affected by two variables: cervid species and forest cover (Table 1). Overall, only 107 cervids among 1179 tested were seropositive against BoHV-4, while in 83 cases the ELISA result was interpreted as inconclusive because the OD value was elevated, but did not exceed the assay cut-off. These inconclusive samples were excluded from further analysis. Seroprevalence varied between forest districts, but no geographical or endemic patterns were observed (Figure 1b). Interestingly, unlike the detection of HV genetic material, BoHV-4 seroprevalence was highest in roe deer, and the lowest in fallow deer (Table 1). Only in roe deer was a correlation with age observed. However, the percentage of animals with antibodies increased with age, rather than decreasing, as was observed for detection of HV genetic material (Table S4). Moreover, BoHV-4 seroprevalence increased with forest cover in free-ranging cervids. This trend was particularly noticeable in the case of red deer and roe deer (Figure 2b), although the changes were relatively low. Furthermore, the presence of BoHV-4 antibodies was inversely correlated to the presence of HV genetic material in red deer and roe deer (Table S3 and S4) which was most likely attributed to the relatively high prevalence of BoHV-4 in these two species (Table 1).

Herpesvirus genetic characterization

The sequence analysis of PCR products allowed three lineages of viruses to be distinguished in the deer species tested: Fallow deer LHV related, Elk GHV related and Capreolus HV-1 related (Table S5, Figures 3, 4).

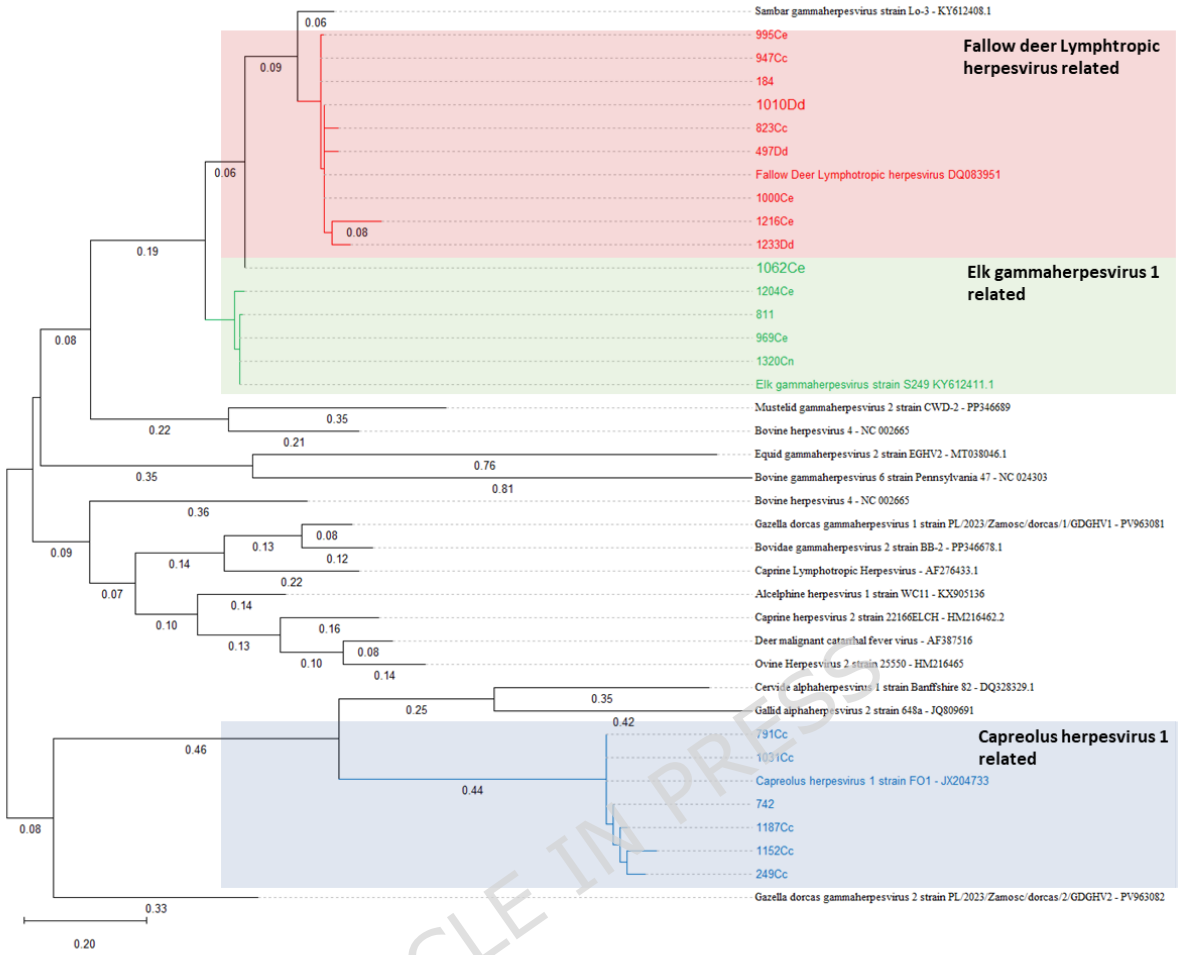

Figure 3. Maximum likelihood phylogenetic tree constructed with adaptive bootstrap basing on the set of selected sequences acquired from different species of cervids and sequences of selected herpesvirus available in GenBank. Viral isolates identified in the study were marked by the consecutive sample number and letter signifying host species: Ce – red deer (*Cervus elaphus*), Dd – fallow deer (*Dama dama*), Cc – roe deer (*Capreolus capreolus)* and Cn – sika deer (*Cervus nippon*).

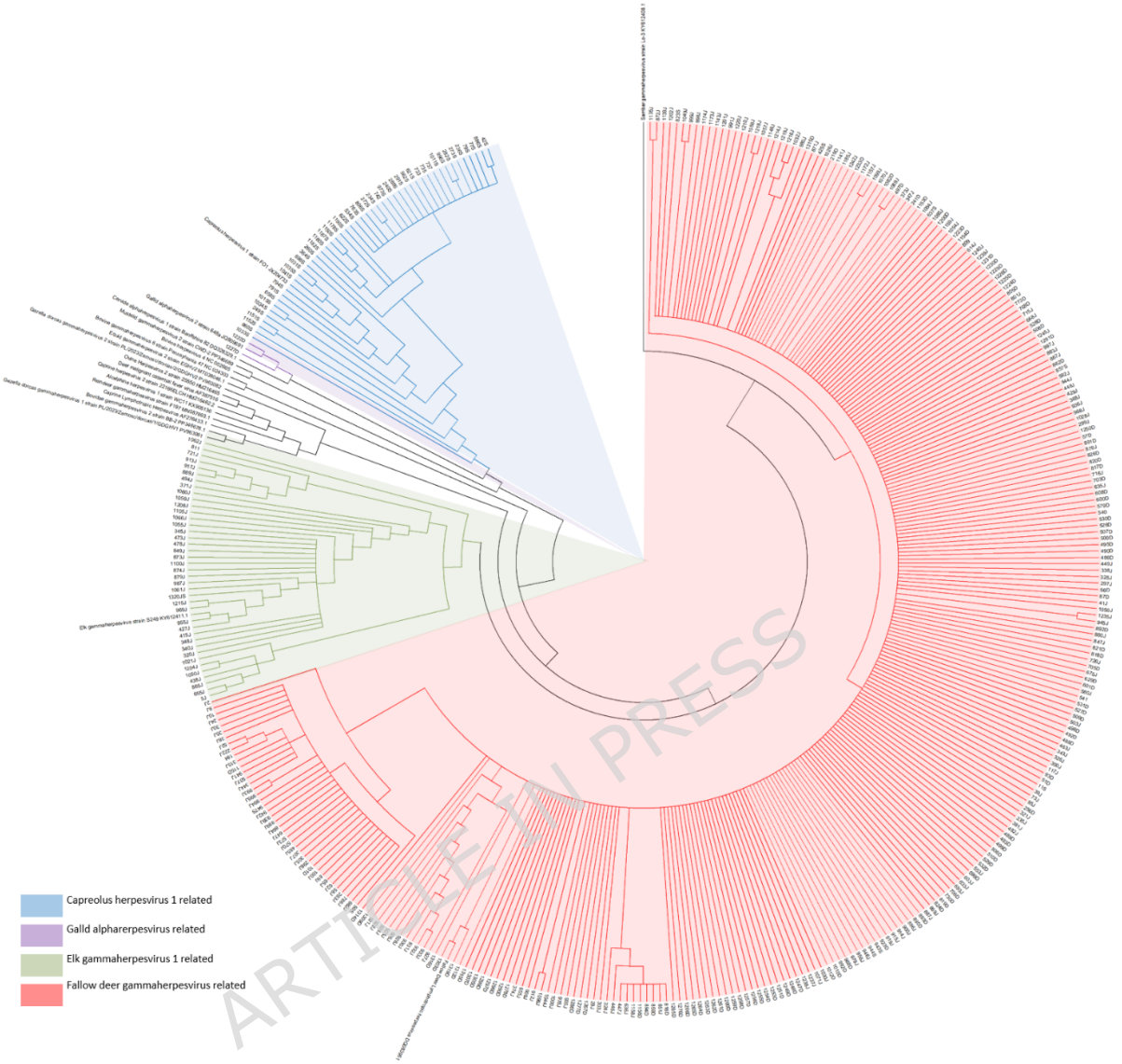

Figure 4. Neighbour-joining phylogenetic tree constructed based on 174 bp long fragment of gene encoding DNA polymerases of herpesviruses identified in the study and 19 strains selected from sequences available in GenBank. Tree was constructed in MEGA12 v.12.1.1, and interior branches condensed with each branch of less than 5% cut-off bootstrap value collapsed. Viral isolates identified in the study were marked by the consecutive sample number and letter signifying host species: Ce – red deer (*Cervus elaphus*), Dd – fallow deer (*Dama dama*), Cc – roe deer (*Capreolus capreolus)* and Cn – sika deer (*Cervus nippon*). Based on the closest related herpesvirus sequence identified; viral strains were divided into 4 groups marked by different colour: Caprelous herpesvirus 1 related (92.55-100% homology, 46 isolates, blue), Fallow deer lymphotropic virus related (91.5-100% homology, 303 isolates, red), Elk gammaherpesvirus related (89.35-100%, 40 isolates, green) and Gallid alphaherpesvirus related (91.95-99.43% homology, 2 isolates, violet).

The most widespread herpesvirus showed a high level of identity (91.5-100%) to Fallow deer lymphotropic herpesvirus was found in 303 animals

(24.1%) (Table 2). This virus was mostly present in red deer (169/694; 24.4% of all animals of this species), fallow deer (120/188; 63.8%) and was also identified in 3.7% (10/268) of roe deer. The next most frequent sequence species, assigned as Capreolus HV-1 (92.55-100% identity), clustered with betaherpesviruses and was detected exclusively in 42 roe deer and in 4 samples from unspecified species. The third lineage which showed the highest identity to Elk gammaherpesvirus (ElkGHV) (89.35-100%) was detected in red deer (38/694; 5.5%) and the single sika deer tested. Interestingly, a Gallid alphaherpesvirus 2 was identified in two nose swab samples from farmed fallow deer where ornamental chicken birds were kept nearby. To better visualize sequencing results, two phylogenetic trees were constructed. First, Maximum likelihood phylogenetic tree based on the selected sequences The acquired viral sequences were submitted to GenBank under accession numbers: PX691557 - PX691856 for Fallow deer lymphotropic herpesvirus related sequences; PX691517 - PX691556 for Elk GHV related and PX691857 - PX691901 for Capreolus HV-1. Additional Maximum Likelihood phylogenetic trees, including a tree based on representative herpesvirus strains and a host phylogeny reconstructed from cytochrome b sequences, are provided. These trees were used for cophylogenetic analyses to evaluate potential host–virus coevolution. The global ParaFit test revealed significant congruence between host and virus phylogenies (ParaFitGlobal = 381.64, p = 0.0004), supporting a non-random association between herpesvirus lineages and their cervid hosts (Figure 5). Distinct differences in host distribution were observed among the identified herpesvirus groups. Capreolus herpesvirus 1 was detected exclusively in roe deer, whereas Elk gammaherpesvirus was detected predominantly in red deer, with a single detection in sika deer. In contrast, Fallow deer lymphotropic herpesvirus exhibited the broadest host range and was identified in red deer, fallow deer and roe deer.

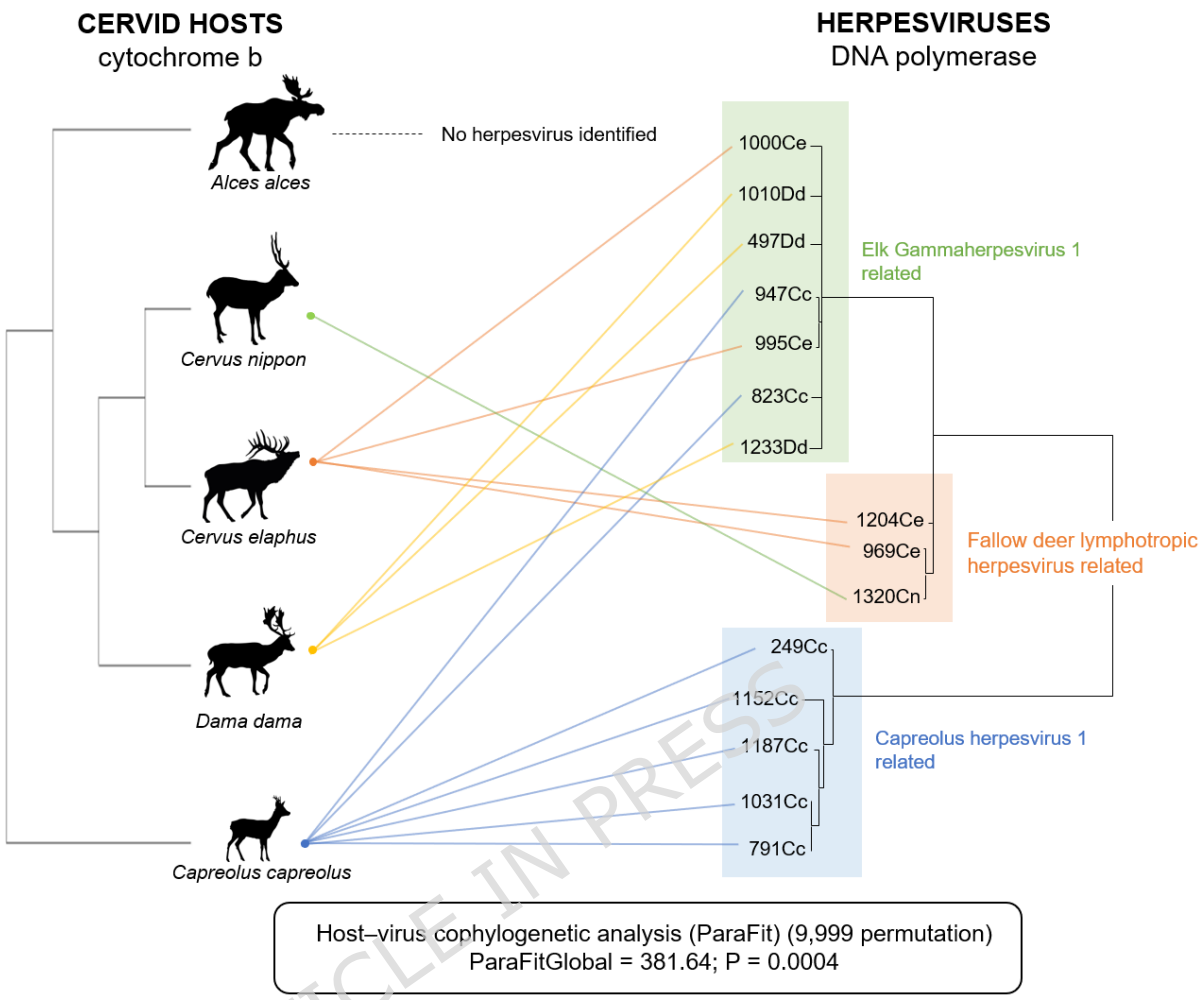

Figure 5. Host-virus cophylogenetic relationships between cervid hosts and selected, representative herpersvirus strains identified in this study. Both, hosts and herepesviral trees were constructed using Maximum Likelihood methods in MEGA12 v.12.1.1.

No correlation was observed between the origin and occurrence of Fallow deer LHV and Elk GHV homologous sequences in red deer suggesting simultaneous circulation of both viruses in the environment. Both strains were detected in twenty out of 63 forest districts, although the prevalence of Fallow deer LHV-related gammaherpesvirus sequences was higher (Figure 1).

Table 2. Distribution of specific gammaherpesvirus lineages among five native cervid species.

Species	Fallow deer LHV			Elk GHV			Capreolus herpesvirus 1		
	n/N[a]	%	95% CI[b]	n/N[a]	%	95% CI[b]	n/N[a]	%	95% CI[b]
red deer	169/694	24.4	21.2-27.6	38/694	5.5	3.8-7.2	0/694	0	0-0.4
fallow deer	120/188	63.8	56.9-70.7	0/188	0	0-1.6	0/188	0	0-1.6
roe deer	10/268	3.7	1.4-6.0	0/268	0	0-1.1	42/268	15.7	11.4-20.0
moose	0/20	0	0-13.9	0/20	0	0-13.9	0/20	0	0-13.9
sika deer	0/1	0	0-95.0	1/1	100	5.0-95.0	0/1	0	0-95.0
unspecified	4/86	4.6	0,1-9,1	1/86	1.2	0.0-3.5	4/86	4.6	0,1-9,1

[a]number of positive/all tested, [b]binomial exact 95% confidence interval

Moreover, the effect of herpesvirus genotype on the associations between explanatory variables and HV prevalence was examined by analogy with cervid species impact (Table 5S). The prevalence of different virus strains was associated only with population type which was probably due to the fact that the dominant Fallow deer LHV occurred in farmed cervids, which consisted only of red, fallow and one sika deer. While, putative to roe deer Capreolus HV-1 was present only in the reservoir which is not a farmed species. The fifteen captive roe deer included in the study originated from wildlife reserves or a rehabilitation centre. Moreover, it was also found that Fallow deer LHV infections in roe deer were significantly more common in forest districts with higher densities of fallow deer (OR = 0.18; P=0.026) but not red deer (OR = -0.46; P = 0.34). In fact, the infection rate with this virus species was also associated to the fallow deer population density in red deer (OR = 1.5; P = 0.01) with the HV prevalence over 2 times higher in the districts with the highest fallow deer density of ≥ 0.07 fallow deer/km^2.

Risk factor analysis

Generalized linear mixed-effect models were developed to assess the effect of different animal-level and population variables on gammaherpesvirus infection and BoHV-4 seroprevalence in all cervids and in relation to each of

the individual deer species. After the backward elimination procedure several models were developed (Table 3). The random effect of forest district was accounted for in both models. The only significant other risk factor associated with HV infection was the species. The two native cervid species: red and roe deer were significantly less likely to be infected with HV than the invasive fallow deer (Table 2). The forest district accounted for significant variability in the model, especially since the fallow deer came from only a few farms and 19 out of 69 forest districts, where cervids were sampled.

Table 3. The final generalized linear mixed model (GLMM) presenting species as a single risk factor for GHV infection in cervids in Poland with the forest district as a random effect (number of observations = 1156).

Variable	Category	OR[a]	β (SE)[b]	z[c]	P > |z|	95%CI[d]
Species	fallow deer	reference				
	red deer	0.24	0.06	-6.14	<0.001	0.15-0.38
	roe deer	0.13	0.04	-7.54	<0.001	0.08-0.22
	moose	-	-	-	-	-
	sika deer	-	-	-	-	-
Random effect						
	Forest district	0.36	0.13	-	-	0.18-0.72

[a]odds ratio; [b]standard error for the estimate of the variance reported in the previous column; [c]Wald *z* statistic; [d]95% confidence interval.

Table 4. The final generalized linear mixed model (GLMM) presenting species and forest cover as risk factors for BoHV-4 seropositivity in cervids in Poland with the forest district as a random effect (number of observations = 847).

Variable	Category	OR[a]	β (SE)[b]	z[c]	P > |z|	95%CI[d]
Species	fallow deer	reference				
	red deer	4.60	3.58	1.96	0.05	0.10-21.12
	roe deer	19.81	15.33	3.86	<0.001	4.34-90.31

		moose	5.83	6.56	1.57	0.12	0.64-52.84
		sika deer	-	-	-	-	-
Forest cover (%)		low (10.3-28.6%)	reference				
		medium (29.5-51.4%)	1.40	0.55	0.84	0.40	0.64-3.02
		high (53.5-88.7%)	2.60	1.09	2.28	0.02	1.14-5.62
Random effect							
		Forest district	0.41	031	-	-	0.10-1.76

[a]odds ratio; [b]standard error for the estimate of the variance reported in the previous column; [c]Wald *z* statistic; [d]95% confidence interval.

Discussion

While alphaherpesvirus infection of deer in Poland has been previously described, this study for the first time demonstrated infections with beta- and gammaherpesviruses in Polish cervids [33]. Furthermore, data on the spread of gammaherpesviruses in native herbivorous fauna was limited to studies on reindeer in Norway and cervids in China and Chile [19,32,41]. Serological tests for detecting antibodies against gammaherpesviruses tend to be almost species-specific due to their genetic and antigenic variability [42], so that the pan-herpesvirus nested PCR coupled with Sanger sequencing, has become the test of choice [35] for detection and identification of herpesviruses of 3 herpesvirus subfamilies: alpha-, beta- and gammaherpesvirus. The results of sequencing of 389 samples demonstrated the presence of three different groups of viruses among cervid populations related to: Capreolus HV-1, Fallow deer LHV and Elk GHV. Similarity of each of the newly identified strains to respective reference sequences ranged between 89.5% to 100% which suggests that we have detected strains of previously known viruses rather than new viruses that have not been described yet. However to unequivocally prove it, sequencing of larger fragments or full genomes of those strains would be necessary. Additionally, and surprisingly, Gallid alphaherpesvirus type 1 (GaHV-1) sample was identified in nasal swabs collected from two farmed fallow deer. Gallid alphaherpesvirus is responsible

for Marek's disease and is associated with significant economic losses in the poultry industry, with a host range limited to avian species [43]. In this case, the positive samples were collected from nasal swabs of fallow deer living in close proximity to poultry with a history of herpesvirus infections. The virus was probably inhaled from the environment and, considering the limited host range of GaHV-1, it is highly unlikely that the virus was propagated in the fallow deer. Although, both cervid herpesvirus 1 (CvHV-1) and BoHV-1 are known to infect cervids, in our study neither of this viruses was detected in tested samples. In contrast, almost 30% overall alphaherpesvirus seroprevalence was observed in Polish deer in 2017 [33]. While panherpesvirus PCR was confirmed to be able to detect BoHV-1, detection of CvHV-1 by this method has not been described yet [44]. The most probable explanation of almost complete lack of alphaherpesvirus detection is different cell and tissue tropism of this subfamily in comparison to beta- and gamma-herpesviruses. As this study was originally designed for detection of gammaherpesviruses, blood and spleen samples were the main source of genetic material chosen for diagnostic tests, because those viruses establish systemic infection of lymphoid cells including B lymphocytes [45,46]. In contrast, alphaherpesvirus infection may be detected in blood lymphocytes, virus is typically found in epithelial cells and neurons [46,47].

The prevalence of the gamma- and beta- herpesviruses detected was characterized by species specificity. This was especially clear for Capreolus HV-1 related sequences which were detected only in roe deer. While our study is the first large scale research that describes this virus in cervids and there is little information on betaherpesviruses of wildlife, these results were in accord with previous observations that betaherpesviruses showed the strictest host specificity among herpesviruses [48]. Furthermore, overlaying the phylogenetic trees of cervid herpesviruses and their reservoir species reveals a close relationship, suggestive of co-evolution (Figure 6). In primates, the most prominent betaherpesviruses are human cytomegalovirus

(HCMV) and rhesus cytomegalovirus (RhCMV) known to cause congenital birth defects and act as opportunistic pathogens in immunosuppressed individuals [49]. However, because Capreolus herpesvirus 1 showed the highest nucleotide sequence homology (74%) to the bat betaherpesviruses YB34_L (of unknown pathogenic potential), it is hard to draw conclusions about the importance of this virus for roe deer health [50]. One of the previous studies showed that BoHV-4 infections could be common among various deer species [18]. However, in our study, while over 9% samples were seropositive to BoHV-4, the sequencing failed to confirm the presence of this virus. Instead, two other gammaherpesviruses were detected, Fallow deer LHV and Elk GHV. A similar discrepancy was observed by de Boer et al. 2014, who found Bovine Lymphotropic herpesvirus in some BoHV-4 seropositive cattle [51]. One of the explanations suggested by the authors of that study, was possible serological cross-reactivity. However, in this study, only a small number of BoHV-4 seropositive animals had detectable gammaherpesviral infection, which makes this explanation improbable. The simpler explanation may be, the fact that in case of BoHV-4 viral shedding is short lasting (3 to 10 days) while antibodies could remain detectable for at least 1 year after infection [52,53]. Therefore observed seroprevalence may by the result of past contact with the virus. Fallow deer LHV was much more prevalent among fallow deer than in other deer species, while Elk GHV was only detected in red deer. Although those viruses were prevalent (303/1257 – 24.1% tested individuals), there is currently no data to suggest that they could negatively affect the health status of cervid hosts [19]. We have demonstrated that most deer beta- and gammaherpesviruses identified in the study were specific to their reservoir host and, as demonstrated before, may have co-evolved alongside them (Figures 6, 7) [54]. This co-evolution has resulted in a long-term, often asymptomatic, relationship where the virus establishes latent infection in lymphocyte subsets. The viral genome persists without actively producing new viruses, allowing the virus to evade the host

immune response [55] Simultaneously, the host developed ways to manage the infection without succumbing to severe illness.

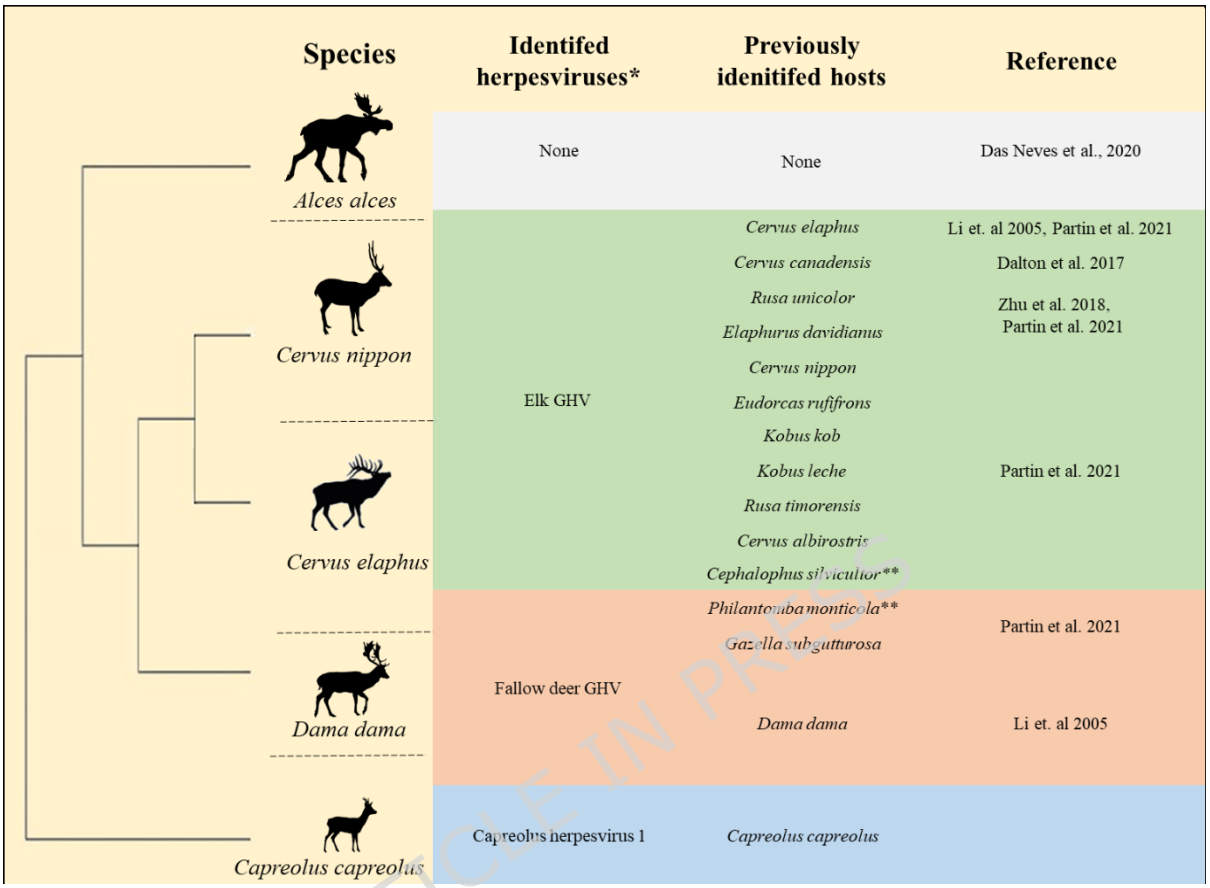

Figure 6. Identified herpesvirus and their hosts. Phylogenetic relationship between cervids is shown on maximum likelihood tree based on sequences of cytochrome b gene of the tested species. *Herpesviruses representing closest match in GenBank to identified strains;**host species in which infection with particular herpesvirus was accompanied by MCF-like disease symptoms

The epidemiological analysis also revealed some differences between cervid species which may also be related to the biology of the reservoir hosts themselves. Herpesvirus transmission in all cervids tested appeared independent of population density, however, in case of fallow deer and red deer, increasing forest cover was associated with lower HV prevalence, what suggested a protective effect against infection. Although, forest cover, particularly in the case of red deer, was positively linearly related to the density of the population of this species (Figure7b), the risk of infection

decreased. The observed association with forest cover is more likely to reflect differences in habitat use among cervid species than a direct effect of forest cover on virus transmission. Deer density is complex, variable both seasonally and locally due to the breeding cycle, agricultural use, climate, landscape and habitat characteristics, therefore, simple relationships are not straightforward to demonstrate as shown for instance for vector-borne pathogens [56,57]. Lower probability of HV infection in red deer in the increasing forest cover gradient may indicate a suitable habitat with lower pressure and a more resilient population (Figure 7b). Moreover, in forest ecosystems, unlike their non-forested areas, deer are more active in searching for food and are subject to pressure from predators such as wolves. This results in more active movement and population dispersion, which is comparable to the dilution effect [58]. High deer density can increase disease prevalence for directly transmitted diseases like Chronic Wasting Disease (CWD), it can have the opposite effect for vector-borne diseases like Lyme disease through a "dilution effect" where a higher deer population can dilute the impact of disease-carrying vectors like ticks [57,59]. We have also observed that some vector-borne infections in European bison were frequency-dependent, rather than density-dependent [60].

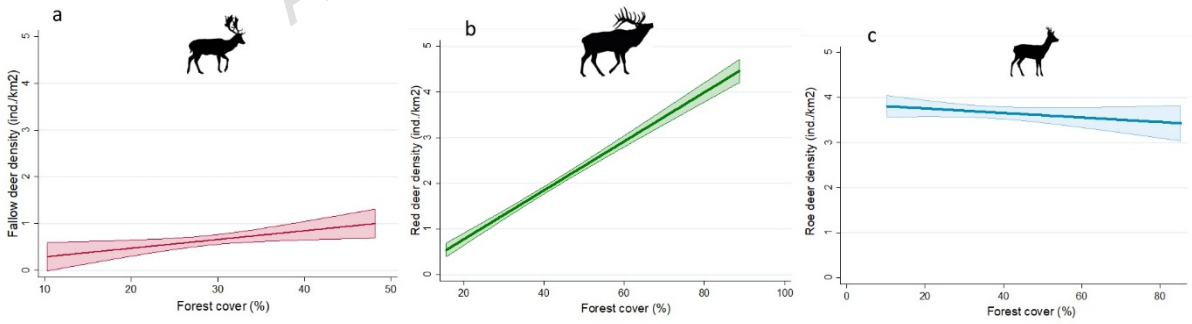

Figure 7. The density of free-ranging fallow deer (*n*=107), red deer (*n*=694) and roe deer (*n*=270) populations in the forest cover gradient based on data at the forest district level obtained for the study from the Forest Data Bank.

Indigenous species of red deer prefer woodlands, midforest grasslands and upland areas [61], while roe deer favour the edges of deciduous and mixed

woodlands, where they can access both cover and open fields. They cohabit same area, however, do not compete for the same food niche [62,63]. This is confirmed by the graph showing the relationship between density and forest cover based on our data (Figure 7b and c). Fallow deer are highly adaptable and are found in a wider variety of habitats, including deciduous and mixed woodlands, coniferous forests, and open fields (Figure 7a). Fallow deer and roe deer tend to share or compete for the same habitats, which may be the explanation for detection of Fallow deer LHV in roe deer as a potential spill-over event [64–66]. We have demonstrated that the frequency of detection of this strain in roe deer was related to the density of the free-ranging fallow deer population, and their numbers have been growing in recent years to around 24 thousand individuals in 2023 [21]. In addition, infection of red deer by Fallow deer LHV might have been a consequence of the introduction of fallow deer into the ecosystem, since the high prevalence of this virus in both free-ranging and captive fallow deer suggest its role as the reservoir host, with red deer as an overspill host. In the study, we also observed a significant correlation between the presence of Fallow deer LHV and areas with free-ranging fallow deer. However, given the long history of fallow deer in the country, this transmission could have occurred long ago, but as shown by the lower prevalence of HV in red deer, the latter species does not have the same potential to maintain and spread the virus as the reservoir species. The second virus identified in red and sika deer (Elk GHV) was previously identified in red deer and milu (*Elaphus davidianus*) from China [19]. Fallow deer have also been identified as potential or confirmed reservoirs for other viruses and bacterial pathogens, including SARS-CoV-2, Hepatitis E virus (HEV), and certain Shiga toxin-producing *Escherichia coli* (STEC) or multidrug-resistant *Yersinia enterocolitica* strains [67–70]. Interestingly, this deer species is suspected as a possible reservoir species of SARS-Cov-2 Omicron strain in Europe [67], by analogy to white-tailed deer (O*docoileus virginianus*) in North America [71].

Herpesvirus transmission in deer probably occurs through direct contact, respiratory droplets, and contact with contaminated environment. Herpesviruses can spread over long distances with air currents and dust, which was already demonstrated for malignant catarrhal fever virus [72]. During our research (unpublished data), we also found the opposite situation when we detected Fallow deer LHV in the nasal swabs taken from clinically healthy sheep living next to a fallow deer enclosure, although there is no data on propagation of Fallow deer LHV in sheep. Therefore, we know that the transmission of putative to cervids gammaherpesviruses to domestic ruminants may potentially occur, but there is no evidence in the literature that farm animals are susceptible and that this may be of any significance.

In addition to the mode of transmission of herpesviruses in cervids, the study also indicated the time of possible infection. In most species, we did not observe any effect of age on infection, except for roe deer, in which the curve was rather unexpectedly downward sloping. This may indicate infection early in life. Primary infection in immunonaive hosts leads more often to lytic infection with high level of viremia that is more likely to be detected. After primary infection, herpesviruses usually remain dormant in lymphoid cells in a latent state. The virus is most often reactivated due to stress or other immunosuppressive factors, which can lead to sporadic shedding of the virus and a risk of transmission to other animals. As a result increased detection of the virus in young roe deer could reflect the detection of active infections rather than total infected animals which, when older, remain mostly undetected as they are in latent state. There is little epidemiological data on the beta and gammaherpesviruses [41]. Most studies have been conducted using ELISA serological tests based on AlHV-1 or OvHV-2 antigens, the value of which is questionable due to antigenic differences [73,74]. Even the value of serological tests for MCF should be revised, if only because the mortality rate in susceptible species after infection with viruses capable of causing MCF before seroconversion is very high. These tests do however have other

uses, particularly in reservoir species, and in vaccine studies. Epidemiological studies of non-reservoir species should consider developing serological diagnostic tests based on putative virus antigens, which can be produced in recombinant systems [75,76]. Since only AlHV-1 may be propagated in cell cultures, most serological tests rely on this virus. The studies in both reindeer and other deer species have consistently shown that the risk of exposure to gammaherpesvirus was age-dependent, what resulted is significantly higher seroprevalence in adults compared to calves. We have also made similar observations for alphaherpesviruses [33]. An MCFV infection in a carrier host, such as a wildebeest or sheep, is typically acquired shortly after birth, either in utero or through direct contact with the mother or other infected young animals. The virus is then carried for life, with most shedding occurring in the first few months of life, what may be connected to MCF outbreaks observed in in-contact susceptible species. We have recently confirmed that infection with lymphotropic gammaherpesviruses in pigs occurs also in the first weeks of their life [77]. This may explain the lack of correlation with age and the fact that Capreolous HV-1 detection is even more common in younger roe deer. While the gammaherpesviruses and betaherpesvirus of roe deer identified in cervids were characterised by their reservoir species, the serological detection of Bovine herpesvirus 4 (BoHV-4) infection showed a different pattern. The only indicator of infection was the presence of antibodies to the virus, as we did not find any BoHV-4 DNA in the samples tested. BoHV-4 is primarily found in cattle, but the infection was reported in cervids in Hungary, indicating the virus can infect wild ruminants [18]. Earlier studies failed to demonstrate BoHV-4 infection in wild cervids in France and Belgium and in Russia [78,79]. We have previously demonstrated the presence of antibodies to BoHV-4 in approx. 6% of European bison in Poland, which is comparable to the 9% seroprevalence of the virus in deer in the country determined here [60]. While the pathogenic role of BoHV-4 in cattle is unclear and infection may be opportunistic, its presence in a new host species such as deer raises questions about its potential impact and

prevalence in wildlife populations, though no disease was reported in the deer studied. It is interesting to note that roe deer were the species with the highest seroprevalence of this virus, and it is roe deer, which are often found in transitory habitats, that seem to be the link between urban and forest environments. It can therefore be assumed that BoHV-4 infection in roe deer is the result of interspecies transmission from domestic ruminants. This is also substantiated by the age-dependence of BoHV-4 seroprevalence in roe deer. This is the most common pattern for pathogens spread by direct contact. The time factor influences the increasing risk of contact with an infected animal and the immune response. Since no association between BoHV-4 and deer density could be established, a frequency-dependent model, where infection occurs in the presence of a virus reservoir, possibly domestic cattle should be addressed. In Europe, roe deer are considered transitional species between sylvatic and synanthropic environments and therefore considered a good bioindicator in One Health context [57,69,80,81].

The present study has several limitations. First, serological analyses were restricted to BoHV-4 because validated commercial assays are not available for most cervid herpesviruses. Consequently, the ELISA results should be interpreted as evidence of exposure to BoHV-4 or antigenically related viruses rather than as a comprehensive assessment of herpesvirus seroprevalence. Second, although virus neutralization assays could provide greater specificity, they require virus isolation and validated cell culture systems, which are currently unavailable for most cervid herpesviruses detected in this study. Although data on livestock density could have been accessed, they were aggregated at the county level, whereas cervid abundance was estimated at the forest district level. The mismatch in the spatial resolution of these datasets precluded a meaningful analysis of livestock density as a risk factor. Moreover, information on grazing practices, pasture sharing and direct contact opportunities were not available for the evaluation of herpesvirus transmission at the wildlife–livestock interface.

Conclusions

Two gammaherpesviruses were found circulating among Polish native deer. The lymphotropic herpesviruses seem to be specific to their reservoir species, apart from Fallow deer LHV that crossed species barriers. The density and age-independence of those infections suggests that there are some other environmental factors involved and the exposure may occur in young age leading to a lifelong latent infection. Due to the very high prevalence of Fallow deer LHV in fallow deer, the correlation between infections in roe deer and red deer, and the occurrence and density of fallow deer in the same forest districts, it is possible that the continued expansion of fallow deer may lead to further exposure of other native deer species to the Fallow deer LHV. However, no evidence exists regarding the pathogenic potential of these viruses in wild cervids and the possibility of transmission to non-cervids. The situation was different in the case of BoHV-4, with highest seroprevalence in roe deer, which are increasingly common in farmland and meadows, suggesting that transmission from cattle could occur. In summary, monitoring of gammaherpesvirus infections and determining the biodiversity of viruses proved to be a useful model for tracking interspecies interactions among deer and their potential contact with farm animals. It was also possible to rule out the significance of the wild reservoir in strategies for protecting livestock from these threats. No BoHV-4 was detected in samples from deer species, therefore, this suggests that deer are unlikely to act as a reservoir of this virus, however, the relatively high percentage of seropositive roe deer can be considered a sentinel to possible spill-over from cattle in cases.

Ethical statement and farm permissions

Samples from wild cervids were collected during collective hunting in accordance with the Regulation on the detailed conditions for the performing hunting and marking carcasses, with amendments (Dz.U. z 2005 Nr 61 poz. 548), issued by Ministry of Climate and Environment of the Republic of Poland in 2005. Samples from live animals were collected during other

procedures performed in the farms which required animal immobilisation. Prior to sample collection, explicit permission was obtained from the directors of all participating deer farms. None of the animals were killed or immobilized for the purpose of the present study. The collection of samples, their analysis, and the laboratory testing methods used on game animals do not require approval from an ethics committee.

Data Availability

All sequences acquired in this study are available in NCBI GenBank under accession numbers PX691517- PX691901. Other data that support the findings of this study are available from the corresponding author upon reasonable request.

Competing interests

The author(s) declare no competing interests.

Authors' contributions

W.S, M.L. participated in study design, analysis of the results, figure preparation, writing up and editing of the final manuscript, A.J., M.K.K. participating in sampling and editing of the final manuscript, P.K participated in results analysis and writing up of the final manuscript, G.R. participated in writing up and editing of the final manuscript.

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