Univerza v Ljubljani
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HERPESVIRUSI PRI IZBRANIH SKUPINAH PROSTOŽIVEČIH PTIC

Doktorska disertacija

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HERPESVIRUSES IN SELECTED GROUPS OF FREE-LIVING BIRDS

Doctoral dissertation

Ljubljana, 2022

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Herpesvirusi pri izbranih skupinah prostoživečih ptic

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IZVLEČEK

Ključne besede: bolezni ptic – virologija; *Alphaherpesvirinae*; herpesvirusne infekcije – diagnostika – genetika – klasifikacija; verižna reakcija s polimerazo – metoda; analiza zaporedja DNA; filogeneza; sove

Ptice so gostitelji številnih herpesvirusov (HV). Okužba pri njih lahko ostane neopažena ali povzroči (letalno) bolezen. Za razliko od HV pri perutnini so podatki o okužbi, prisotnosti in vplivu HV na prostoživeče ptice skopi. Celotni seznam naravnih gostiteljev HV pri pticah ni poznan. Vzorce organov, orofaringealnih in/ali kloakalnih brisov 1212 živih in mrtvih prostoživečih ptic iz 15 redov smo pregledali na prisotnost odseka gena DNA polimeraze HV. HV smo dokazali v vzorcih organov osmih od 55 (14,5 %) pregledanih poginjenih sov, v brisih kloake štirih od 525 (0,7 %) pregledanih prostoživečih pevk, odlovljenih med jesensko selitvijo, v orofaringealnih in/ali kloakalnih brisih 34 od 447 (7,5 %) prostoživečih bolnih, poškodovanih ali oslabelih ptic, ki so potrebovale nujno veterinarsko pomoč in so bile sprejete na Kliniko za ptice, male sesalce in plazilce Veterinarske fakultete, in v orofaringealnih brisih 16 od 170 (9,4 %) živih prostoživečih sov, vzorčenih na območju Krima in Jelovice. HV smo odkrili tako pri poginjenih kot tudi pri živih, klinično zdravih prostoživečih pticah. V gnezdeči populaciji sov nismo ugotovili vpliva HV na produktivnost sov, saj se število jajc in mladičev med okuženimi in neokuženimi gnezdi ni razlikovalo. Filogenetska analiza nukleotidnih zaporedij odseka gena DNA polimeraze HV je pokazala, da se HV, dokazani pri prostoživečih pticah, uvrščajo v poddružino *Alphaherpesvirinae* in da v populaciji prostoživečih ptic v Sloveniji krožijo različni HV, ki se v večini primerov razlikujejo od že znanih HV, objavljenih v genski banki. Nekateri HV so specifični za gostiteljsko vrsto ptic, medtem ko smo v nekaterih primerih v različnih redovih ptic odkrili zelo podobna oziroma identična nukleotidna zaporedja, kar pomeni, da HV niso vedno omejeni na vrsto ptice. Do prenosa HV bi lahko prišlo s plenjenjem ptičjega plena ali celo s superplenilstvom. Ugotovili smo tudi, da mali glodavci kot plen ne predstavljajo vira okužbe s HV za ptice iz reda sov, sokolov in ujed. Z opravljenimi študijami smo močno razširili seznam dovzetnih gostiteljskih vrst prostoživečih ptic in ugotovili, da je za dokaz HV pri prostoživečih pticah z neznanim statusom okužbe treba odvzeti tako brise orofarinksa kot brise kloake.

ABSTRACT

Key words: bird diseases – virology; *Alphaherpesvirinae*; herpesvirus infections – diagnosis – genetics – classification; polymerase chain reaction – methods; sequence analysis, DNA; phylogeny; *Strigiformes*

Birds are hosts of many herpesviruses (HVs), in which the infection can go unnoticed or cause (lethal) disease. In contrast to HV in poultry, data on infection with and the presence and impact of HV in wild birds are limited. In birds, the full list of natural hosts of HV is unknown. Samples of organs and oropharyngeal and/or cloacal swabs of 1,212 live and dead free-living birds from 15 orders were examined for the presence of a fragment of the HV DNA polymerase gene. HV was detected in organ samples in eight out of 55 (14.5%) dead owls, in cloacal swabs in four out of 525 (0.7%) free-living songbirds caught during the autumn migration, in oropharyngeal and/or cloacal swabs in 34 out of 447 (7.5%) free-living birds admitted as wildlife casualties to the Clinic for Birds, Small Mammals, and Reptiles at the Faculty of Veterinary Medicine, and in oropharyngeal swabs in 16 out of 170 (9.4%) live free-living owls sampled on Mount Krim and the Jelovica Plateau. HVs were detected in individuals found dead as well as in live individuals with no clinical signs of illness. Furthermore, no productivity deviances (i.e., in clutch and brood size) were recorded in a breeding population of clinical healthy owls. Phylogenetic analysis of nucleotide sequences of the partial DNA polymerase gene of HVs showed that HVs detected in free-living birds belong to the subfamily *Alphaherpesvirinae* and that different HVs have been circulating in the population of free-living birds in Slovenia, which in most cases are different from known HV nucleotide sequences published in GenBank. Some HVs are specific to the host bird species, whereas in some cases HVs very similar to identical nucleotide sequences were found in different bird orders, meaning that HVs are not always restricted to bird host species. HV transmission could occur through bird predation or even superpredation. It was also found that small rodents are not a source of HV infections in owls, falcons, and birds of prey. The results of the research greatly expand the list of HV-susceptible host species of free-living birds and show that free-living birds with unknown infection status should be tested with a combination of oropharyngeal and cloacal swabs, which would maximize the probability of HV detection.

KAZALO VSEBINE

SEZNAM KRATIC IN OKRAJŠAV

kbp	kilobazni par
DNA	deoksiribonukleinska kislina (angl. *deoxyribonucleic acid*)
HV	herpesvirus
NCBI	Nacionalni center za biotehnološke informacije (angl. *National Center for Biotechnology Information*)
PCR	verižna reakcija s polimerazo (angl. *polymerase chain reaction*)
ILT	infekciozni laringotraheitis (angl. *infectious laryngotracheitis*)
CoHV	herpesvirus golobov (angl. *columbid herpesvirus*)
StHV	herpesvirus sov (angl. *strigid herpesvirus*)
FaHV	herpesvirus sokolov (angl. *falconid herpesvirus*)
PaHV	herpesvirus pevk (angl. *passerine herpesvirus*)
PsHV	herpesvirus papig (angl. *psittacid herpesvirus*)
GaHV	herpesvirus kokoši (angl. *gallid herpesvirus*)
MeHV	herpesvirus puranov (angl. *meleagrid herpesvirus*)
MD	Marekova bolezen (angl. *Marek's disease*)
PD	bolezen Pacheco (angl. *Pacheco's disease*)
SuHV	herpesvirus prašičev (angl. *Suid herpesvirus*)
CMV	citomegalovirus (angl. *cytomegalovirus*)
MHV	mišji gama herpesvirus (angl. *murine gammaherpesvirus*)

1 UVOD

1.1 Predstavitev problematike

Herpesvirusi (HV) so dokazani pri gostiteljih v skupinah vseh vretenčarjev: ribah, dvoživkah, plazilcih, pticah in sesalcih (1). HV so zunaj organizma razmeroma neobstojni in hitro propadejo, zato je najpogostejši neposreden prenos HV med osebki gostitelja. Virusna DNA polimeraza je ključen encim v litični fazi HV infekcije, ki omogoča replikacijo virusnega genoma (2). Genom predstavlja ena linearna dvojnovijačna molekula DNA v velikosti od 125 do 290 kbp (3) in vsebuje od 32 do 74 % gvanina in citozina (4). Genom kodira več kot 80 različnih strukturnih in nestrukturnih proteinov. Razlike med posameznimi vrstami HV so v organizaciji (zaporedju in orientaciji), velikosti (125–290 kbp) in sestavi (predvsem variacija deleža gvanina in citozina) genoma (3). Skupna lastnost HV je zmožnost prikrite (latentne) in trajne okužbe, kar pomeni, da je okužena žival lahko dosmrtni prikriti nosilec virusa, ki se občasno reaktivira in predstavlja potencialni vir okužbe za druge živali (2). Družina *Herpesviridae* je razdeljena v tri poddružine: *Alphaherpesvirinae*, *Betaherpesvirinae* in *Gammaherpesvirinae* (5). Vsi HV, dokazani pri pticah, so trenutno klasificirani v poddružino *Alphaherpesvirinae*, vendar številni od njih niso popolnoma raziskani in so uvrščeni med neklasificirane viruse znotraj družine *Herpesviridae* (2). Za viruse iz poddružine *Alphaherpesvirinae* so značilni širok krog gostiteljev, kratki replikacijski cikli virusa, hitra destrukcija inficiranih celic, hitro širjenje virusa po gostitelju in velikokrat akuten potek bolezni. Poleg tega imajo sposobnost vzpostavitve vseživljenjske latentne okužbe v senzornih in tudi preostalih ganglijih (5). HV iz poddružine *Betaherpesvirinae* imajo ožji krog gostiteljev, dolg replikacijski cikel in značilno počasno širjenje okužbe po telesu. Latentna ali persistentna okužba se vzpostavi v limforetikularnih celicah, sekretorne žlezah in ledvicah. Okužba največkrat povzroči povečanje določenih celic oziroma citomegalijo. Okužbe ponavadi ne povzročajo kliničnih znakov, edino v primeru, ko se virus zanese v neokuženo populacijo. HV, ki spadajo v poddružino *Gammaherpesvirinae*, so ponavadi omejeni na gostiteljsko družino ali red. Virusi te poddružine okužijo limfocite B ali limfocite T in lahko povzročijo limfoproliferativne bolezni ter vzpostavijo latentno stanje v limfoidnih tkivih. Pri glavnem gostitelju okužba pogosto povzroči pojav bolezenskih znakov, niso pa izključeni pogini tudi pri drugih gostiteljih (5). HV pri perutnini povzročajo zelo dobro raziskane bolezni, kot sta Marekova bolezen (angl. *Marek's disease* – MD) in infekciozni laringotraheitis (angl. *infectious*

laryngotracheitis – ILT), med okrasnimi in sobnimi pticami pri papigah povzročajo bolezen Pacheco (angl. *Pacheco's disease* – PD) (6, 7). Pri prostoživečih pticah so podatki o okužbi s HV skopi in pri pticah celotnega seznama naravnih gostiteljev HV ni. HV imajo bolj kot kateri koli drugi virusi nagnjenost k prikriti in trajni okužbi. Prehod v produktivno okužbo in pojav bolezni se lahko pojavita ob različnih stresnih dejavnikih, kot so odlov, rokovanje, sprememba okolja, dodajanje novih ptic v jato, poškodbe/travme ter sočasne okužbe z drugimi bolezenski povzročitelji (8). Pri prostoživečih pticah so za bolezni, ki jih povzročajo HV, značilni nespecifični klinični znaki, kot so oslabelost, anoreksija, poliurija, konjuktivitis, rinitis in difteroidni traheitis, v nekaterih primerih tudi nenadni ali povišani pogini (9). Večina opravljenih študij temelji na dokazu HV pri poginjenih pticah (8). Prisotnost HV pri prostoživečih pticah je trenutno najbolj raziskana pri domačih golobih (*Columba livia domestica*). Pri njih HV najpogosteje povzroči latentno okužbo, ki ima malo ali nič vpliva na zdravje golobje populacije. Ob pojavu kliničnih znakov imajo golobi znake respiratornega obolenja. Pri poginjenih golobih so opisane fokalne nekroze jeter, vranice, kostnega mozga, požiralnika in nekroze ob področju sapišča (10). Kot je opisano v nekaterih študijah, so okuženi golobi kot plen vir okužbe za prostoživeče plenilske ptice iz reda ujed (*Accipitriformes*), sokolov (*Falconiformes*) in sov (*Strigiformes*). Pri njih so opisani podobni klinični znaki in patološke spremembe kot pri golobih, vendar z višjo intenziteto bolezni in višjo stopnjo smrtnosti (11, 12, 13, 14). Čeprav približno kar polovica vseh vrst ptic spada med ptice pevke (*Passeriformes*), je izolacija in karakterizacija HV pri omenjenem redu ptic redko opisana (15, 16) in trenutno ni objavljenih študij o okuženih prostoživečih pticah pevkah v Evropi. Iz literature je razvidno, da večina objavljenih podatkov o HV pri prostoživečih pticah temelji na dokazu prisotnosti virusa pri poginulih pticah, redkejši so opisi detekcije HV pri živih pticah. Poleg tega je število pregledanih osebkov, predvsem znotraj posameznih družin in vrst, skromno.

1.2 Namen dela in hipoteze

Namen doktorske disertacije je bil dokazati prisotnost HV pri prostoživečih pticah v Sloveniji in razširiti seznam dovzetnih gostiteljskih vrst prostoživečih ptic. Dokazanim HV smo določili tudi nukleotidna zaporedja DNA polimeraze ter jih primerjali med seboj in z virusnimi sevi iz drugih držav, ki smo jih dobili iz podatkovne zbirke Nacionalnega centra za biotehnološke informacije (angl. *National Center for Biotechnology Information* – NCBI GenBank). Zanimal

nas je tudi način prenosa HV, vpliv HV na določeno populacijo prostoživečih ptic ter povezava z ekološkimi lastnostmi obravnavanih vrst ptic s HV.

Hipoteze

1) Pri prostoživečih pticah v Sloveniji so prisotne okužbe s HV. Ti so genetsko podobni, kakor tudi različni od HV, ki so trenutno objavljeni v genski banki. Z molekularnimi metodami bomo HV dokazali pri novih vrstah ptic, pri katerih do zdaj še niso bili dokazani.

2) Prenos HV poteka tako na horizontalni ravni med plenilci kot tudi s plena na plenilca.

2 OBJAVLJENA ZNANSTVENA DELA

2.1 Detekcija in filogenetska analiza herpesvirusov pri prostoživečih vrstah sov v Sloveniji

Detection and phylogenetic analysis of herpesviruses detected in wild owls in Slovenia

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Izvleček

HV smo z metodo verižne reakcije s polimerazo (angl. *polymerase chain reaction* – PCR) dokazali v vzorcih organov pri 14,5 % (8/55) pregledanih prostoživečih sov, ki so bile najdene poginule na različnih lokacijah v Sloveniji med letoma 1995 in 2015. HV smo dokazali pri veliki uharici (*Bubo bubo*), kozači (*Strix uralensis*) in mali uharici (*Asio otus*). Filogenetska analiza nukleotidnega zaporedja odseka gena DNA polimeraze HV je pokazala, da so dokazani HV podobni drugim HV ptic in sesalcev iz poddružine *Alphaherpesvirinae*. Nukleotidni zaporedji dveh HV sov sta bili zelo podobni oziroma identični znanim nukleotidnim zaporedjem HV ptic. Eno nukleotidno zaporedje HV, dokazano pri veliki uharici, je bilo identično nukleotidnemu zaporedju HV golobov 1 (angl. *Columbid herpesvirus 1* – CoHV1), drugo nukleotidno zaporedje HV, dokazano pri kozači, pa je bilo zelo podobno nukleotidnemu zaporedju HV kokoši 2 (angl. *Gallid herpesvirus 2* – GaHV2). Preostala nukleotidna zaporedja HV, dokazana pri eni veliki uharici, dveh malih uharicah in treh kozačah, so pokazala manjšo homologijo z nukleotidnimi zaporedji HV ptic in sesalcev ter so se v filogenetskem drevesu razvrstila v ločeno skupino znotraj poddružine *Alphaherpesvirinae*. Gre za prvo študijo, ki poroča o prisotnosti različnih HV pri prostoživečih sovah.

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Detection and Phylogenetic Analysis of Herpesviruses Detected in Wild Owls in Slovenia

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SUMMARY. Herpesvirus (HV) was detected using PCR in the organs of eight of 55 wild owls (14.5%) from seven species that were found dead in various locations in Slovenia between 1995 and 2015. HV was detected in three species: the Eurasian eagle owl (*Bubo bubo*), Ural owl (*Strix uralensis*), and long-eared owl (*Asio otus*). Phylogenetic analysis of partial DNA polymerase gene nucleotide sequences showed that the detected HVs are similar to the avian and mammal alphaherpesviruses. Two sequences were very similar to known bird HV sequences. One sequence was identical to the columbid herpesvirus 1 (CoHV1) sequence, and the other was very similar to the gallid herpesvirus 2 (GaHV2) sequence. The phylogenetic tree revealed a lower similarity of the other six analyzed Slovenian sequences with the sequences of alphaherpesviruses of birds and mammals. This is the first study to report the detection of different HVs in owls.

RESUMEN. Detección y análisis filogenético de herpesvirus detectados en búhos silvestres en Eslovenia
Se detectaron virus herpes mediante PCR en los órganos de ocho de 55 búhos silvestres (14.5%) pertenecientes a siete especies y que se encontraron muertos en varios lugares de Eslovenia entre los años 1995 y 2015. Se detectaron virus herpes en tres especies: el búho real (*Bubo bubo*), cárabo uralense (*Strix uralensis*) y el búho chico (*Asio otus*). El análisis filogenético de las secuencias de nucleótidos parciales del gene de la polimerasa de ADN mostró que los virus herpes detectados son similares a los alphaherpesvirus aviares y de mamíferos. Dos secuencias fueron muy similares a las secuencias de virus herpes de aves conocidas. Una secuencia fue idéntica a la secuencia del herpesvirus 1 de palomas (CoHV1) y otra fue muy similar a la secuencia del herpesvirus 2 del pollo (GaHV2). El árbol filogenético reveló menores similitudes entre las otras seis secuencias analizadas de Eslovenia con las secuencias de alphaherpesvirus de aves y de mamíferos. Este es el primer estudio que informa la detección de diferentes virus herpes en búhos.

Key words: herpesvirus, avian, owls, phylogenetic, PCR, Slovenia

Abbreviations: aa = amino acid; AcHV1 = accipitrid herpesvirus 1; BLAST = Basic Local Alignment Search Tool; bp = base pair; CoHV1 = columbid herpesvirus 1; FaHV1 = falconoid herpesvirus 1; GaHV = gallid herpesvirus; HV = herpesvirus; MDV = Marek's disease virus; MeHV1 = meleagrid herpesvirus 1; NCBI = National Center for Biotechnology Information; nt = nucleotide; PCR = polymerase chain reaction; StHV = strigid herpesvirus 1

INTRODUCTION

Herpesviruses (HVs) are DNA viruses that affect a wide range of mammalian and avian hosts (19). HVs of mammals, birds, and reptiles are classified in the family Herpesviridae, which is further subdivided into three subfamilies: Alpha-, Beta-, and Gammaherpesvirinae (50). The subfamily Alphaherpesvirinae is divided into five genera: *Iltovirus, Mardivirus, Scutavirus, Simplexvirus,* and *Varicellovirus* (7). Columbid herpesvirus 1 (CoHV1) from the genus *Mardivirus*, falconoid herpesvirus 1 (FaHV1), strigid herpesvirus 1 (StHV1), and accipitrid herpesvirus 1 (AcHV1), which are currently unassigned (7), cause a fatal disease known as inclusion body disease or herpesvirus hepatitis in pigeons (Columbiformes), falcons (Falconiformes), owls (Strigiformes), and birds of prey (Accipitriformes), respectively (13,36,49).

The disease was first described in the great horned owl (*Bubo virginianus*) (15) in the United States in 1935, and later in the Eurasian eagle owl (*Bubo bubo*), long-eared owl (*Asio otus*), and snowy owl (*Bubo scandiacus*) in Europe (4). Since then, StHV1 infections have been reported in captive and wild owls in Asia, Europe, and North America (38). The disease has been reported in European owl species: the Eurasian eagle owl, long-eared owl, snowy owl, little owl (*Athene noctua*), and boreal owl (*Aegolius funereus*) (5).

Generally, affected owls are depressed and anorectic for 2 to 5 days before death occurs (38). Leukopenia may be noted during the acute phase of infection (12). Necrosis of the liver, spleen, and bone marrow with intranuclear inclusion bodies as well as pharyngeal ulceration and necrosis and gastrointestinal necrosis are characteristic of StHV1 infections (22,39). Unilateral keratitis and conjunctivitis may develop in surviving owls (22).

HV DNA polymerase gene contains a highly conserved region suitable for targeting with degenerate primers (47) that has been widely used for detection and phylogenetic analysis of mammalian and avian HVs (8,9,26,32,51). Sequencing of PCR products generated with degenerative primers that targeted the partial HV DNA polymerase gene lead to the hypothesis that the disease in pigeons, falcons, and owls is caused by the same virus (9). However, more recent investigations, including complete genome sequences of CoHV1 and FaHV1, have suggested that closely related viruses rather than the same virus cause the disease (16,45).

In order to investigate HV infections in wild owls in Slovenia, the detection of HV DNA polymerase gene region by PCR was performed in 55 dead owls from seven species collected between 1995 and 2015. The detected HV DNA was characterized by direct sequence analysis of the PCR products to provide greater insight into the phylogenetic relationship and epidemiology of HVs detected in wild owls in Slovenia.

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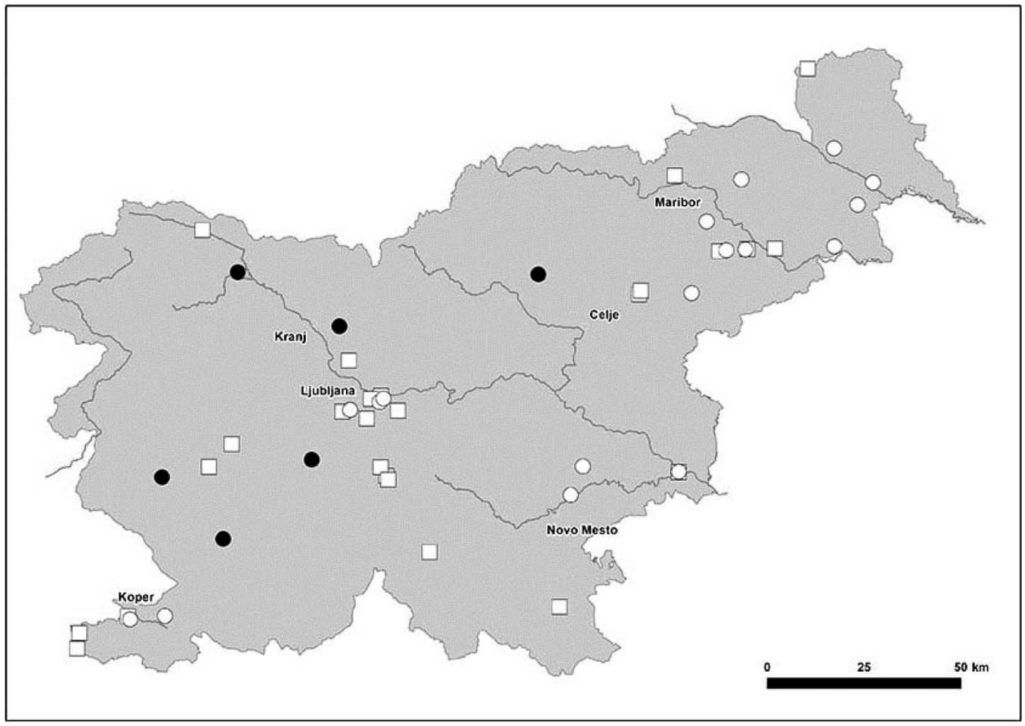

Fig. 1. Overview of collection sites of dead owls in Slovenia used in the study. White squares indicate sites of owl species found in the study as non-susceptible to the herpesvirus: tawny owl (*Strix aluco*), Eurasian scops owl (*Otus scops*), western barn owl (*Tyto alba*), and short-eared owl (*Asio flammeus*); white circles indicate sites of uninfected specimens of susceptible owl species: Eurasian eagle owl (*Bubo bubo*), Ural owl (*Strix uralensis*), and long-eared owl (*Asio otus*); and black circles indicate infected owl specimens.

MATERIALS AND METHODS

Sample collection. Altogether, 55 dead wild owls were collected by the Slovenian Museum of Natural History in Ljubljana at various locations in Slovenia between 1995 and 2015 as a part of the museum collection program for bird specimens (Fig. 1). The owls collected belonged to seven species: the tawny owl (*Strix aluco*, 19 individuals), long-eared owl (15 individuals), Ural owl (*Strix uralensis*, 11 individuals), western barn owl (*Tyto alba*, three individuals), Eurasian scops owl (*Otus scops*, three individuals), Eurasian eagle owl (three individuals), and short-eared owl (*Asio flammeus*, 1 individual; Table 1). Sex was determined for 48 carcasses. The shortest and longest air distances between two collected carcasses were 3 and 234 km, respectively (Fig. 1). The time distribution of owls collected was as follows: one in 1995, two in 2002, two in 2003, two in 2005, five in 2006, two in 2008, three in 2009, two in 2010, four in 2011, four in 2012, 11 in 2013, 10 in 2014, and seven in 2015. The carcasses were kept individually in plastic bags at −20 C until they were submitted to the Institute for Poultry, Birds, Small Mammals, and Reptiles at the University of Ljubljana's Veterinary Faculty, where partial necropsy was performed. During the necropsy, lung, kidney, liver, spleen, and brain samples were taken from each carcass using cleaned and autoclaved instruments. A different set of instruments was used for each necropsy to prevent DNA contamination. Tissues were either processed immediately or stored at −70 C until use.

DNA extraction and PCR of a DNA polymerase gene region using HV consensus primers. Tissue samples from individual birds were pooled and homogenized in phosphate-buffered saline. The homogenates were clarified by centrifugation at 3500 RPM for 10 min. Total DNA and RNA were extracted from 140 µl of the supernatant by the QIAamp Viral RNA Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. Viral DNA was detected by nested PCR using a set of primers that target the HV DNA polymerase gene region as described by VanDevanter *et al.* (47). The PCR volume was 20 µl, and it contained 10 µl of DreamTag Green PCR Master Mix (2×) (Thermo Scientific, Europe), 1 µM of each PCR primer, 2 µl of isolated DNA, and deionized water up to 20 µl. The parameters for prime and nested PCR were denaturation at 95 C for 5 min, followed by 45 cycles of denaturation at 94 C for 30 sec, annealing at 46 C for 1 min, extension at 72 C for 1 min, and final extension at 70 C for 7 min.

Detection, sequencing, and phylogenetic analysis of PCR products. Amplified products were separated by electrophoresis on a 1.8% agarose gel (Sigma-Aldrich, USA) containing ethidium bromide. PCR products of 215 to 315 bp were excised and purified with a FastGene Gel/PCR extraction kit (Nippon Genetics, Europe) and sent for sequencing to the Macrogen Laboratory (Macrogen Inc., the Netherlands). The nucleotide (nt) sequences obtained were first analyzed by BLAST (2) to identify sequences relevant for further analyses within the NCBI database. Nucleotide alignments were constructed with ClustalW implemented in MEGA 7.0 (25).

Table 1. Detection of the herpesvirus DNA polymerase gene in dead wild owls in Slovenia.

Species	Owls tested (*n*)	Positive (*n*)	Positive (%)
Tawny owl (*Strix aluco*)	19	0	0.0
Long-eared owl (*Asio otus*)	15	2	13.3
Ural owl (*Strix uralensis*)	11	4	36.4
Western barn owl (*Tyto alba*)	3	0	0.0
Eurasian scops owl (*Otus scops*)	3	0	0.0
Eurasian eagle owl (*Bubo bubo*)	3	2	66.7
Short-eared owl (*Asio flammeus*)	1	0	0.0
Total	55	8	14.5

Phylogenetic analysis of the 153 nt-long sequences was performed by the neighbor-joining method with the Kimura two-parameter model and 2000 bootstrap replicates by MEGA 7.0 (25). The accession numbers of HV sequences used for phylogenetic analysis are included in Fig. 2.

RESULTS

Detection of HV by PCR. The HVs were detected by nested PCR using a set of primers that target a region of the HV DNA polymerase gene in eight out of 55 owls (14.5%). HVs were detected in Eurasian eagle owls (2/3), Ural owls (4/11), and long-eared owls (2/15), but not in tawny owls, western barn owls, Eurasian scops owls, and short-eared owls (Table 1). HV DNA was detected in one long-eared owl in 1995 (AO/AO2/95), in one Eurasian eagle owl in

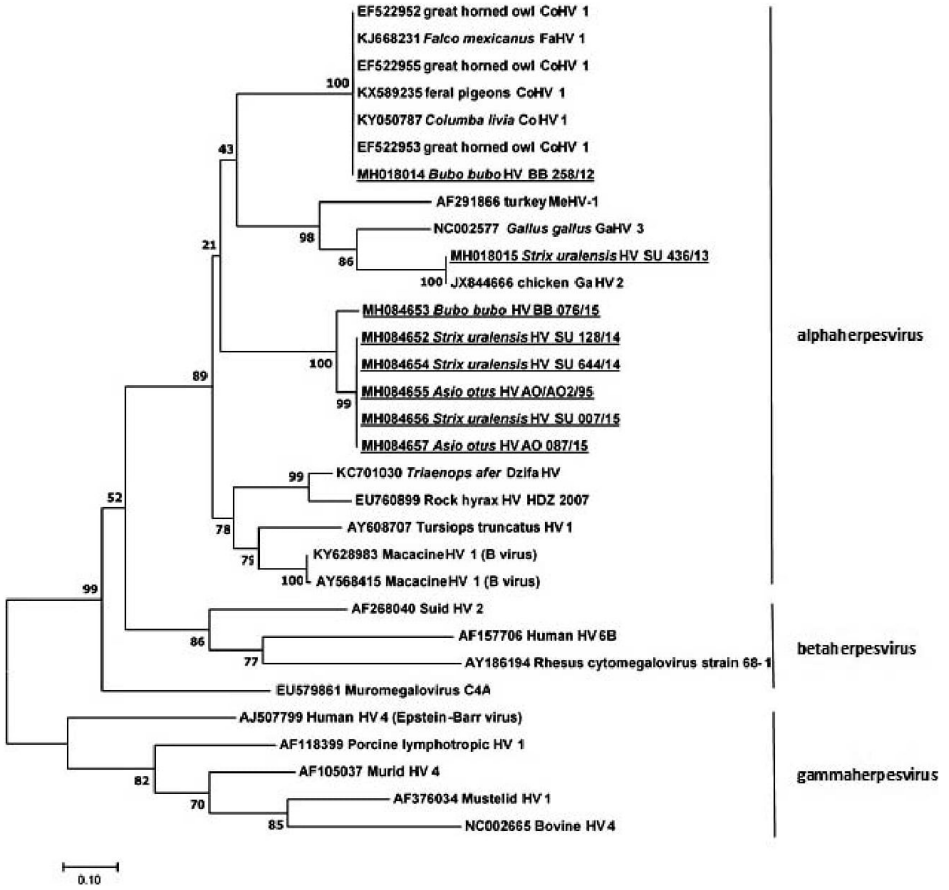

Fig. 2. Phylogenetic relationship based on partial DNA polymerase gene nucleotide sequences of HVs from wild owls in Slovenia and herpesviruses derived from the GenBank database. The tree was generated by the neighbor-joining method with the Kimura-2 parameter substitution model and 2000 bootstrap replicates to assign confidence level to branches. Scale bar indicates substitutions per site. GenBank accession numbers for sequences are given before the host and herpesvirus names. Nucleotide sequences obtained in the current study are additionally underlined.

Table 2. Details of HV sequences obtained in the current study.

Host species	HV sequence	GenBank accession number	PCR product size (bp)
Eurasian eagle owl (*Bubo bubo*)	BB 258/12	MH018014	183
	BB 076/15	MH084653	243
Long-eared owl (*Asio otus*)	AO/AO2/95	MH084655	237
	AO 087/15	MH084657	246
Ural owl (*Strix uralensis*)	SU 436/13	MH018015	231
	SU 128/14	MH084652	192
	SU 644/14	MH084654	246
	SU 007/15	MH084656	246

2012 (BB/258/12), in one Ural owl in 2013 (SU/436/13), in two Ural owls in 2014 (SU/128/14 and SU/644/14), and in one Eurasian eagle owl (BB/076/15), Ural owl (SU/007/15), and long-eared owl (AO/087/15) each in 2015. Four out of 26 (15.38%) male owls and four out of 22 (18.18%) female owls were positive for HV. The shortest and longest distances between the collection sites of HV-positive owls were 40 and 140 kilometers, respectively (Fig. 1).

Phylogenetic and sequence analysis. Phylogenetic analysis of the partial DNA polymerase gene nt sequences (Table 2) of eight Slovenian owl HV detected between 1995 and 2015 and other avian and mammal alpha-, beta-, and gammaherpesviruses showed that Slovenian owl HVs were grouped into three distinct, highly supported genetic clusters that are most closely related to the avian and mammal alphaherpesviruses (Fig. 2). An owl HV sequence, BB/258/12, detected in 2012 in a Eurasian eagle owl was grouped together with sequences of CoHV1 detected in pigeons, FaHV1 detected in a falcon, and CoHV1 detected in owls (Fig. 2), with nt identities ranging up to 100% with sequences of CoHV1 detected in pigeons and owls as well as with sequence of FaHV1, and 96.7% nt identities with the sequence of CoHV1 detected in a rock pigeon (*Columba livia*). A sequence SU/436/13, detected in 2013 in a Ural owl, was grouped together with sequences of *Gallid herpesvirus* (GaHV) 2, GaHV3, and *Meleagrid herpesvirus* 1 (MeHV1) (Fig. 2), and showed 95% nt identity with GaHV2. Lower nt identities, 73.3% and 67.5%, were observed with GaHV3 and MeHV1, respectively. The phylogenetic tree showed that partial DNA polymerase gene sequences of six Slovenian owl HVs detected in Ural owls, long-eared owls, and a Eurasian eagle owl formed a highly supported separated genetic cluster (Fig. 2). High nt identities (93% to 100%) were observed between the sequences from this cluster. Five sequences (SU/644/14, AO/AO/02/95, SU/007/15, SU/128/14, and AO/087/15) were identical and shared 93% nt identities with the BB/076/15 sequence. The phylogenetic tree revealed a distant relationship of the aforementioned sequences with other known sequences of alphaherpesviruses of birds and mammals (Fig. 2). The highest nt identities, ranging from 58% to 63.8%, were observed with alphaherpesviruses from the *Macacine herpesvirus* 1 cluster. Similar nt identities, ranging from 60.2% to 61.2%, were observed with alphaherpesviruses from the CoHV1 and FaHV1 clusters.

The alignment of predicted amino acid sequences used in the study showed similar clustering of HV sequences to that obtained from phylogenetic analyzes of nt sequences. Sequence BB/258/12 was identical to aa sequence of CoHV1 and sequence SU/436/13 was identical to aa sequence of GaHV2. There was 92% aa identity between sequence BB/076/15 and sequences SU/644/14, AO/AO/02/95, SU/007/15, SU/128/14, and AO/087/15. Those sequences shared 70% to 72% aa identity with CoHV1 and 65% aa identity with GaHV2 (Fig. 3).

DISCUSSION

The results of our study showed that HVs with different partial DNA polymerase gene sequences have been circulating in the population of wild owls in Slovenia, and that HV sequences different from CoHV1 sequences can be detected in owls. HVs were detected by the PCR method in eight out of 55 (14.5%) owls among seven different species, with the Eurasian eagle owl (2/3), Ural owl (4/11), and long-eared owl (2/15) being positive (Table 1). A recent study of CoHV1 detection with real-time PCR method in rock pigeons, birds of prey, and nonraptors in Poland showed that one out of seven (14.2%) of the wild owls examined was positive for CoHV1 (51). Although both studies were carried on birds found dead and the incidence of HV detections are similar, caution should be applied in assessing the results of both studies because a different methodology was used. The major differences were in the number of owls investigated, the tissue samples tested, and the specificity of PCR assays.

A CoHV1 specific set of primers was used to detect HV in brain tissues in a study performed in Poland (51), whereas HV consensus primers, which also detect HVs different from CoHV1 (47), were used for viral detection in pools of various tissues, including the brain, in this study. In our study, only one (1.8%) sequence identical or very similar to known partial DNA polymerase sequences of

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KX589235:CoHV1       832 NGLLPCLNVAATVTTIGRNMLLAVRDYIHRRWASWDALIKEFPQLDGHAK 881
MH018014:BB 258/12     1 NGLLPCLNVAATVTTIGRNMLLAVRDYIHRRWASWDALIKEFPQLDGHAK  50

JX844666:GaHV2       806 NGLLPCIDVAATVTTIGRNMLLTVRDYIHKQWGTRDALLREFPNLSNFMR 855
MH018015:SU 436/13     1 NGLLPCIDVAATVTTIGRNMLLTVRDYIHKQWGTRDALLREFPNLSNFMR  50

MH084653:BB 076/15     1 NGLLPCLQVAATVTTVGRDMLLATRDYIHSKWATLDDLTTAFPDLETTNL  50

MH084652:SU 128/14     1 NGLLPCLQVAATVTTVGRDMLLATRDYIHSRWATLDDLTAAFPGLETPNA  50
MH084654:SU 644/14     1 NGLLPCLQVAATVTTVGRDMLLATRDYIHSRWATLDDLTAAFPGLETPNA  50
MH084655:AOAO2/95      1 NGLLPCLQVAATVTTVGRDMLLATRDYIHSRWATLDDLTAAFPGLETPNA  50
MH084656:SU 007/15     1 NGLLPCLQVAATVTTVGRDMLLATRDYIHSRWATLDDLTAAFPGLETPNA  50
MH084657:AO 087/15     1 NGLLPCLQVAATVTTVGRDMLLATRDYIHSRWATLDDLTAAFPGLETPNA  50
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Fig. 3. Alignment of partial DNA polymerase amino acid sequences. Numbers at the beginning and at the end of sequence indicate aa positions in complete protein sequence of CoHV1 and GaHV2 and the length of sequences obtained in the current study. Identical aa in all aligned sequences are in bold.

CoHV1 was detected in addition to seven HVs that have sequences different from CoHV1. The observed diversity between the detected sequences might lead to the assumption that HV consensus primers are more suitable for monitoring owls for HVs by PCR methods.

A seroprevalence study of FaHV1 and StHV1 antibodies in wild and captive raptors showed that seroprevalance for StHV1 in wild owls was 18.6%, and that seroprevalance for FaHV1 in wild owls ranged from 8.2% to 30% depending on the FaHV1 strain used as an antigen in the sero-neutralization test (52). The results of our study showed a similar incidence of HV infections in owls as seroprevalence using the StHV1 strain as an antigen (52). Although virus isolation and serotypization of HV detected by PCR in Slovenia were not carried out, it could be assumed that the genetic differences detected could result in antigenically different viruses. The genetic differences observed are in agreement with the suggested existence of more than one serovar of FaHV1 (52) and the observed existence of different serovars of psittacine herpesvirus (20). Possibly genetically different HVs, latent infection with HVs (12,38), and the possible existence of different serovars (52) could be a significant challenge to selecting the most suitable methods for studying the prevalence of HV infections in owls.

CoHV1 has been associated with fatal infections in hawks, falcons, and owls in North America and Europe (9,35). In the domestic pigeon (*Columba livia domestica*) population, HV is prevalent and has little to no effect on the health of this species (9). HVs are not always restricted to a specific host or tissue, and crossing host or tissue barriers can considerably alter the pathogenicity of the virus (12). Many previous studies indicated that CoHV1-infected pigeons are a source of the infection for birds of prey (1,13,37) and that the genome of the virus detected in owls is indistinguishable from that detected in pigeons and falcons (9,34). Moreover, all recently reported HVs in owls are genetically very similar to CoHV1 (9,33,34,39,51). Interestingly, in our study, only one partial DNA polymerase sequence (BB/258/12) detected in a Eurasian eagle owl in 2012 out of eight identified HVs was identical to partial CoHV1 sequences detected in pigeons, owls, and falcons (Fig. 2). Among the owls, CoHV1 has been detected in the great horned owl (9,39), barking owl (*Ninox connivens*), powerful owl (*Ninox strenua*) (33,34), and tawny owl (51); therefore this is the first report of detection of partial DNA polymerase sequence that is identical or very similar to known CoHV1 sequences in the Eurasian eagle owl. Although our results confirmed previous findings (9,33,34,51) that partial DNA polymerase gene sequences of CoHV1 detected in owls could be identical to sequences detected in pigeons and falcons, the results obtained from a comparison of partial DNA polymerase gene sequences should be interpreted with care. The latest studies (16,45) on the complete genome of a CoHV1 and a FaHV1 showed that at least one gene differs markedly between FaHV1 and CoHV1 and that the viruses are closely related rather than the same (45). In the future, full-length genome characterization of HVs detected in owls should be performed to obtain a clearer picture of HV diversity in birds of prey. The source of HV infection of the Eurasian eagle owl (BB258/12) is unknown, but it might be, as often hypothesized (1,9,13,33,34,37), due to the ingestion of infected birds, which could present up to 50% of the species' diet (28), including known infected species such as pigeons and falcons (29).

A partial sequence of HV SU/436/13 detected in a Ural owl in 2013 was grouped together with GaHV2, GaHV3, and MeHV1 (Fig. 2). A detected sequence SU/436/13 was very similar (95%) to the GaHV2 sequence. Lower nt identities were observed with GaHV3 and MeHV1, respectively. GaHV2 is also known as Marek's disease virus (MDV), which causes a neoplastic lymphoproliferative disease of gallinaceous birds that produces visceral and/or cutaneous lymphoma, immunosuppression, paralysis, and/or death (42). Natural hosts of MDV infections are chickens and game fowl, quails, turkeys, and pheasants, and some species of ducks and geese are also susceptible to infection and disease. Other avian species including sparrows, partridges, pigeons, and peafowls are probably refractory to the infection (42). Gross pathological and histopathological lesions consistent with MD, but without demonstration of the virus, have been described in the Eurasian eagle owl, little owl, and great horned owl (17,21).

The phylogenetic tree (Fig. 2) showed that six Slovenian HV sequences detected between 1995 and 2015 in owls were grouped together and had a distant relationship with other known partial DNA polymerase gene sequences of alphaherpesviruses of birds and mammals (Fig. 2). Over the last decade, novel alphaherpesviruses have been described in various animal species, such as the Indian gyps vulture (*Gyps indicus*) (6), the mountain peacock pheasant (*Polyplectron inopinatum*), the Malayan peacock pheasant (*Polyplectron malacense*), the Congo peafowl (*Afropavo congensis*) (43), the domestic rabbit *(Oryctolagus cuniculus*) (18), the Indonesian pteropodid bat (*Cynopterus titthaecheilus)* (41), the rock hyrax (*Procavia capensis*) (10), and the common bottlenose dolphin (*Tursiops truncatus*) (44). Our testing of multiple owl species has shown that susceptibility of different owl species to HVs appears to differ. Although we can rule out the western barn owl, Eurasian scops owl, and short-eared owl due to our low sample size (only one to three individuals per species), assuming that HV could be underdetected in populations studied, we could not rule out the HV-infection-free tawny owl given the relatively large sample of owls tested ($n = 19$). All of the owl species studied, except the predominantly insectivorous Eurasian scops owl, are small mammal predators, with voles and mice being their main prey (30), and thus have a relatively similar diet. However, our results indicate that larger owl species (e.g., the Eurasian eagle owl and the Ural owl) seems to be more infected than smaller species, which could be related to their diet, that is, superpredation by taking larger birds and mammals, and even predators (27,31). On the other hand, this does not explain the HV-infected long-eared owl that was found, which is a specialized vole-eating bird (3,46). Even more, the infection also seems to be unrelated to predominant habitat use (e.g., the tawny owl as forest species *vs.* the long-eared owl as an open land species) (30) and to the owls' phylogenetic relationship (e.g., closely related *Strix* species, tawny owl *vs.* Ural owl) (24). Given the sample size acquired, the only uninfected species with a reliable sample size was the tawny owl. The tawny owl is a highly polymorphic species, which is reflected not only in different color morphs, but also in high variability in the physiology and survival capability of tawny owls in variable environmental conditions (11,14,40). In contrast, the infected owl species are not, or are much less, polymorphic than the tawny owl. Polymorphism was to some extent found only in the Ural owl (48), but to a much lower extent compared to the tawny owl. We speculate that greater polymorphism in the population might generally lead to increased resistance to certain diseases, which might not be able to develop epidemic expansion within certain species in the wild (23). Thus, the more polymorphic tawny owl could be a species less susceptible to HV infections compared to the less polymorphic Eurasian eagle owl, Ural owl, and long-eared owl, but this should be explored more in future studies.

To conclude, our research on HVs in wild owls showed that owls could be infected with HVs that are different, at least on the analyzed region of DNA polymerase gene, from CoHV1.

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2.2 Detekcija herpesvirusov pri pticah pevkah, odlovljenih v obdobju jesenske selitve v Sloveniji

Detection of herpesviruses in passerine birds captured during autumn migration in Slovenia

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Izvleček

HV smo z metodo PCR dokazali v brisih kloake pri 0,76 % (4/525) pregledanih klinično zdravih prostoživečih ptic pevk pri 32 različnih vrstah. Ptice pevke so bile odlovljene z ornitološkimi mrežami v obdobju jesenske selitve med letoma 2014 in 2017. HV smo dokazali pri črnoglavki (*Sylvia atricapilla*), kosu (*Turdus merula*) in plavčku (*Cyanistes caeruleus*). Filogenetska analiza nukleotidnih zaporedij odseka gena DNA polimeraze HV je pokazala oddaljeno sorodstvo z drugimi alfaherpesvirusi ptic. V filogenetskem drevesu so bili dokazani HV uvrščeni skupaj in najbolj sorodni HV, odkritimi pri rumenočopastem kakaduju (*Cacatua galerita*) in olivnem kormoranu (*Phalacrocorax brasilianus*), pa tudi z bolj znanimi HV, kot so HV kokoši 1 (angl. *Gallid HV 1* – GaHV1), HV papig 1 (angl. *Psittacid HV 1* – PsHV1) in HV papig 2 (angl. *Psittacid HV 2* – PsHV2). Z opravljeno študijo smo dokazali prisotnost različnih nukleotidnih zaporedij HV z relativno nizko homologijo, kar kaže na to, da v vzorčenih pticah pevkah v jesenski selitvi v Sloveniji krožijo različni HV, ki nimajo zaznavnega vpliva na zdravje teh populacij ptic.

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DETECTION OF HERPESVIRUSES IN PASSERINE BIRDS CAPTURED DURING AUTUMN MIGRATION IN SLOVENIA

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ABSTRACT: Herpesviruses (HVs) were detected by PCR in the cloacal swabs of 0.76% (4/525) clinically healthy free-living passerine birds from 32 different species captured in mist nets in Slovenia during the 2014 and 2017 autumn migrations. Herpesviruses were detected in the Eurasian Blackcap (*Sylvia atricapilla*), the Common Blackbird (*Turdus merula*), and the Eurasian Blue Tit (*Cyanistes caeruleus*). Phylogenetic analysis of partial DNA polymerase gene nucleotide sequences of the HV strains showed a distant relationship with other alphaherpesviruses of birds. In the phylogenetic tree, the HVs detected were clustered together with HV detected in Sulphur-crested Cockatoo and Neotropic Cormorants, as well as with known HVs such as gallid HV1, psittacid HV1 and HV2, and passerine HV1. Different sequences of HVs with relatively low identity were detected in our study, suggesting that different HVs were circulating in passerines sampled during the autumn migration in Slovenia.

Key words: Autumn migration, avian, herpesvirus, passerine birds, PCR, Slovenia.

INTRODUCTION

Some avian herpesviruses (HVs) are well researched and known to cause diseases such as Marek's disease and infectious laryngotracheitis in gallinaceous birds (Galliformes; Kaleta and Redmann 1997; Voelckel et al. 1999; Pennycott et al. 2003), duck virus enteritis in Anseriformes (Jansen and Wemmenhove 1965; Keymer and Gough 1986; Spieker et al. 1996; Campagnolo et al. 2001), Pacheco's disease in parrots (Psittaciformes; Randall et al. 1979), and inclusion body disease or herpesvirus hepatitis in pigeons (Columbiformes), owls (Strigiformes), and birds of prey (Falconiformes; Saik et al. 1986; Gailbreath and Oaks 2008). In passerines, HVs have been detected in sick or dead captive-bred Canaries (*Serinus canaria* f. *domestica*; Widen et al. 2012), Gouldian Finches (*Erythrura gouldiae*; Wellehan et al. 2003; Paulman et al. 2006; Widen et al. 2012), and caged Superb Starlings (*Lamprotornis superbus*; Tomaszewski et al. 2004). All avian HVs are members of the genera *Iltovirus* and *Mardivirus* of the subfamily *Alphaherpesvirinae*; however, many viruses detected in wild birds have not been completely characterized and therefore remain unassigned in the family *Herpesviridae* (Kaleta and Docherty 2007). Clinical manifestations from HV infection in passerines are poorly understood; however, in captive-bred finches, clinical signs such as reduced activity; weight loss; labored breathing; respiratory snicks; unilateral or bilateral conjunctivitis with red, swollen, crusty, adherent eyelids; and high morbidity and mortality were reported (Paulman et al. 2006). In addition to dead or sick birds, HVs have also been detected in healthy captive-bred White-rumped Munias (*Lonchura striata*), Bronze Mannikins (*Lonchura cucullata*), Northern Cardinals (*Cardinalis cardinalis*), and Zebra Finches (*Taeniopygia guttata*; Widen et al. 2012). Recently, two partial DNA polymerase gene and UL16 gene sequences of HV were published: passerine HV1 (PaHV1), detected in captive-bred caged finches in Canada and the US, and psittacid HV1 (PsHV1), detected in caged Superb Starlings (Wellehan et al. 2003; Tomaszewski et al. 2004; Paulman et al. 2006).

The isolation and molecular characterization of HVs from free-living passerine birds is

rarely described (Widen et al. 2012), and only one report appears to be published about the detection and partial characterization of HV in free-living passerine birds; namely, the Hooded Crow (*Corvus cornix*) and the Song Thrush (*Turdus philomelos*; Woźniakowski et al. 2013). The viruses detected were classified as columbid HV1 through analysis of partial DNA polymerase gene sequences (Woźniakowski et al. 2013). Because the current understanding of HV in free-living passerine birds is quite limited, we sought to expand upon the current knowledge of HV infections in free-living passerine birds. Our aims were to investigate the occurrence of HV in free-living passerine birds and to characterize and compare the virus sequences with previously known HV of birds. We used PCR to detect HV DNA in free-living passerine birds captured and sampled during the autumn migration in Slovenia. The HV DNA that we detected was characterized by direct sequence analysis of the PCR product to provide greater insight into the phylogenetic relationships and epidemiology of the HVs detected.

MATERIALS AND METHODS

Birds and samples

Free-living passerine birds were captured with mist nets during the autumn migration, from the end of September to early October 2014 and 2017, at the bird ringing station near Vrhnika in central Slovenia as part of a bird migration study (Vrezec and Fekonja 2018). Each bird was clinically examined by a veterinarian, and 525 cloacal swabs from 32 different passerine species were collected (Table 1). A total of 292 cloacal swabs from 30 species and 233 cloacal swabs from 16 species was collected in 2014 and 2017, respectively. All birds were sampled with special sterile microswabs (Deltalab, Barcelona, Spain) to avoid cloacal damage. All sampled birds were clinically healthy, and no birds were harmed during the sampling procedure. All birds were released after ringing at the site of capture soon thereafter. Dry swabs were placed in the refrigerator at 4 C stationed at the ringing station facility. At the end of each day, samples were transported in an isolated cooling bag and finally stored at –20 C for up to 10 d until analyzed.

DNA extraction and PCR of a DNA polymerase gene region with HV consensus primers

Cloacal swabs were individually vortexed in 2 mL of phosphate-buffered saline for 2 min, and 100-µL aliquots of each swab in phosphate-buffered saline were pooled to produce 500-µL samples for genomic nucleic acid extraction. Samples were pooled by the date of sampling.

Total DNA and RNA were extracted from 140 µL of pooled samples by the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Viral DNA was detected by nested PCR with a set of primers that target the HV DNA polymerase gene region as described by VanDevanter et al. (1996). The PCR volume was 20 µL, and it contained 10 µL of 2X DreamTaq Green PCR Master Mix (Thermo Scientific, Dreieich, Germany), 1 µM of each PCR primer, 2 µL of isolated DNA, and deionized water up to 20 µL. The parameters for prime and nested PCR were denaturation at 95 C for 5 min, followed by 45 cycles of denaturation at 94 C for 30 s, annealing at 46 C for 1 min, extension at 72 C for 1 min, and final extension at 70 C for 7 min. Individual samples from positive pools of samples were tested individually, as previously described.

Detection, sequencing, and phylogenetic analysis of PCR products

Amplified products from individual samples were separated by electrophoresis on a 1.8% agarose gel (Sigma-Aldrich, St. Louis, Missouri, USA) containing ethidium bromide. The PCR products of 215–315 base pairs were excised and purified with a FastGene Gel/PCR Extraction Kit (Nippon Genetics, Duren, Germany) and sent for sequencing to the Macrogen Laboratory (Macrogen Inc., Amsterdam, the Netherlands). The nucleotide sequences obtained were first analyzed by BLAST (Altschul et al. 1990) to identify sequences relevant for further analyses (National Center for Biotechnology Information 2019). Nucleotide alignments were constructed in Geneious Prime 2019 software suite version 1.3 (Biomatters Ltd., Auckland, New Zealand) with MAFFT translation alignment (Katoh and Standley 2013). Phylogenetic analysis was performed using the maximum likelihood method with the Tamura 3-parameter model and 1,000 bootstrap replicates by MEGA 7.0 (Kumar et al. 2016). The percentage of similarity among sequences was calculated by the *p*-distance model (pairwise distance) in MEGA 7.0. Mustelid HV1 (accession AF376034) was selected as the outgroup sequence. The accession numbers of HV sequences obtained in this study are MN274972–MN274975.

TABLE 1. Free-living passerine birds captured in Slovenia during autumn migration in 2014 and 2017 and tested on partial DNA polymerase gene nucleotide sequences of herpesviruses. Positive passerine species are marked in bold type.

Species	Common name	No. positive/no. tested		
		2014	2017	Total
Sylvia atricapilla	**Eurasian Blackcap**	**0/30**	**2/101**	**2/131**
Erithacus rubecula	European Robin	0/30	0/41	0/71
Turdus merula	**Common Blackbird**	**1/30**	**0/7**	**1/37**
Sylvia borin	Garden Warbler	0/8	0/25	0/33
Phylloscopus collybita	Common Chiffchaff	0/30	0	0/30
Prunella modularis	Dunnock	0/30	0	0/30
Remiz pendulinus	Eurasian Penduline	0/30	0	0/30
Turdus philomelos	Song Thrush	0/27	0/1	0/28
Parus major	Great Tit	0/11	0/16	0/27
Acrocephalus scirpaceus	Eurasian Reed Warbler	0/14	0/5	0/19
Acrocephalus schoenobaenus	Sedge Warbler	0/7	0/10	0/17
Passer montanus	Eurasian Tree Sparrow	0/12	0/1	0/13
Ficedula hypoleuca	European Pied Flycatcher	0	0/12	0/12
Cyanistes caeruleus	**Eurasian Blue Tit**	**0/7**	**1/1**	**1/8**
Sitta europea	Eurasian Nuthatch	0/2	0/2	0/4
Acrocephalus arundinaceus	Great Reed Warbler	0	0/4	0/4
Emberiza schoeniclus	Common Reed Bunting	0/3	0	0/3
Troglodytes troglodytes	Eurasian Wren	0/3	0	0/3
Coccothraustes coccothraustes	Hawfinch	0/3	0	0/3
Fringilla coelebs	Common Chaffinch	0/3	0	0/3
Carduelis carduelis	European Goldfinch	0/3	0	0/3
Phoenicurus phoenicurus	Common Redstart	0/1	0/1	0/2
Sylvia curruca	Lesser Whitethroat	0/2	0	0/2
Sturnus vulgaris	Common Starling	0/2	0	0/2
Locustella luscinioides	Savi's Warbler	0	0/2	0/2
Lanius collurio	Red-backed Shrike	0	0/2	0/2
Motocilla alba	White Wagtail	0/1	0	0/1
Acrocephalus melanopogon	Moustached Warbler	0/1	0	0/1
Certhia brachydactyla	Short-toed Treecreeper	0/1	0	0/1
Acrocephalus palustris	Marsh Warbler	0/1	0	0/1
Muscicapa striata	Spotted Flycatcher	0	0/1	0/1
Spinus spinus	Eurasian Siskin	0	0/1	0/1
Total		1/292	3/233	4/525

RESULTS

Detection of HV by PCR

The HVs were detected by nested PCR with a set of primers that target a region of the HV DNA polymerase gene in four out of 525 (0.76%) free-living passerine birds. Herpesviruses were detected in three passerine species: a Eurasian Blue Tit (*Cyanistes caeruleus*; 1/8; 12.60%) and two Eurasian Blackcaps (*Sylvia atricapilla*; 2/131; 1.53%) in 2017 and a Common Blackbird (*Turdus merula*; 1/37; 2.70%) in 2014. Herpesvirus was not detected in 29 other passerine species (Table 1).

Phylogenetic and sequence analysis

The partial nucleotide HV sequences (174 nt) detected in four Slovenian free-living passerine birds in 2014 and 2017 were compared with the sequences of DNA polymerase gene of other avian and mammal HVs to determine their phylogenetic relationship.

The accession numbers of other HV sequences used for phylogenetic analysis are included in Figure 1. The phylogenetic analyses showed that these four HV sequences are most closely related to the alphaherpesvirus sequences detected in other bird species (Fig. 1). More precisely, the sequences of detected HVs clustered together with novel HV sequences detected in the Sulphur-crested Cockatoo (*Cacatua galerita*) and Neotropic Cormorant (*Phalacrocorax brasilianus*), as well as with known HVs such as gallid HV1 (GaHV1), PaHV1, PsHV1, and PsHV2 (Fig. 1). Known HV sequences detected in pigeons and birds of prey, such as columbid HV1, falconoid HV1, and GaHV2 and meleagrid HV1, as well as novel HV sequences detected in owls, were grouped together in the other cluster (Fig. 1). The sequences detected in 2017 in Eurasian Blackcaps were identical and were most similar (73.1%) to the sequence detected in the Common Blackbird in Slovenia in 2014. When compared with known HV sequences, the highest identity (72.5%) was observed with the currently unclassified PsHV2 sequence detected in the African Grey Parrot (*Psittacus erithacus*). Lower similarity (70.8% and 67.8%) was observed between the HV sequence detected in the Eurasian Blue Tit in 2017 and the other three HV sequences detected in Eurasian Blackcaps and the Common Blackbird in Slovenia, respectively. When compared with known HV sequences, the HV sequence detected in the Eurasian Blue Tit shared the highest identity (66.1%) with the HV sequence detected in the Gouldian Finch, whereas the HV sequence detected in the Common Blackbird shared the highest identity (72.5%) with the sequence of PsHV1.

DISCUSSION

This study detected HVs in free-living passerine birds caught and sampled during the autumn migration in Slovenia. The occurrence of HVs in free-living passerines in our study was low (0.76%), with only four birds out of 525 testing positive. Generally, only a few studies have been published on the prevalence of HVs in free-living birds, which is apparently low but present, as this study also shows. However, it appears that the prevalence of HV-associated infections differs among free-living bird species. In wild raptor species, detections of HV infections range from 14.5% (8/55; Žlabravec et al. 2018) to 17.8% (8/45; Woźniakowski et al. 2013) for owls in Slovenia and for selected birds of prey in Poland, respectively. A slightly lower HV prevalence ranging from 3.85% (4/104; Verdugo et al. 2019) to 5.6% (14/250; Niemeyer et al. 2017) was detected in seabirds. The reasons for these differences in HV prevalence detected among different bird species could be complex, involving factors such as the biology and ecology of the bird species tested, intermittent shedding of HVs by the cloaca, trachea, or both, and the immune status of infected birds (Kaleta and Lierz 2016). The differences may also arise from varying study approaches; in previous studies, HVs were mostly detected in dead birds (Woźniakowski et al. 2013; Žlabravec et al. 2018), in which the prevalence might be higher than in living and clinically healthy birds, and our findings were based on detection of HVs only in cloacal swabs. Hence, the results should be treated with considerable caution because a higher occurrence could be expected if oropharyngeal swabs had also been investigated (Phalen et al. 2017). Moreover, the low detection rate could be linked to some technical reasons. Collection, storage, and transport conditions of samples are important factors for an accurate and reproducible diagnosis (Borsanyiova et al. 2018). Although swabs with viral transport media are better for virus recovery and detection than dry swabs (Spackman et al. 2013), some studies have reported successful usage of dry swabs in the diagnosis of viral infection such as in the families *Orthomyxoviridae* (Druce et al. 2012) and *Paramyxoviridae* (Moore et al. 2008), and in the family *Herpesviridae* (Boppana et al. 2011). Because the samples from passerine birds were taken and transported similarly to procedures described in these studies, we believe that only

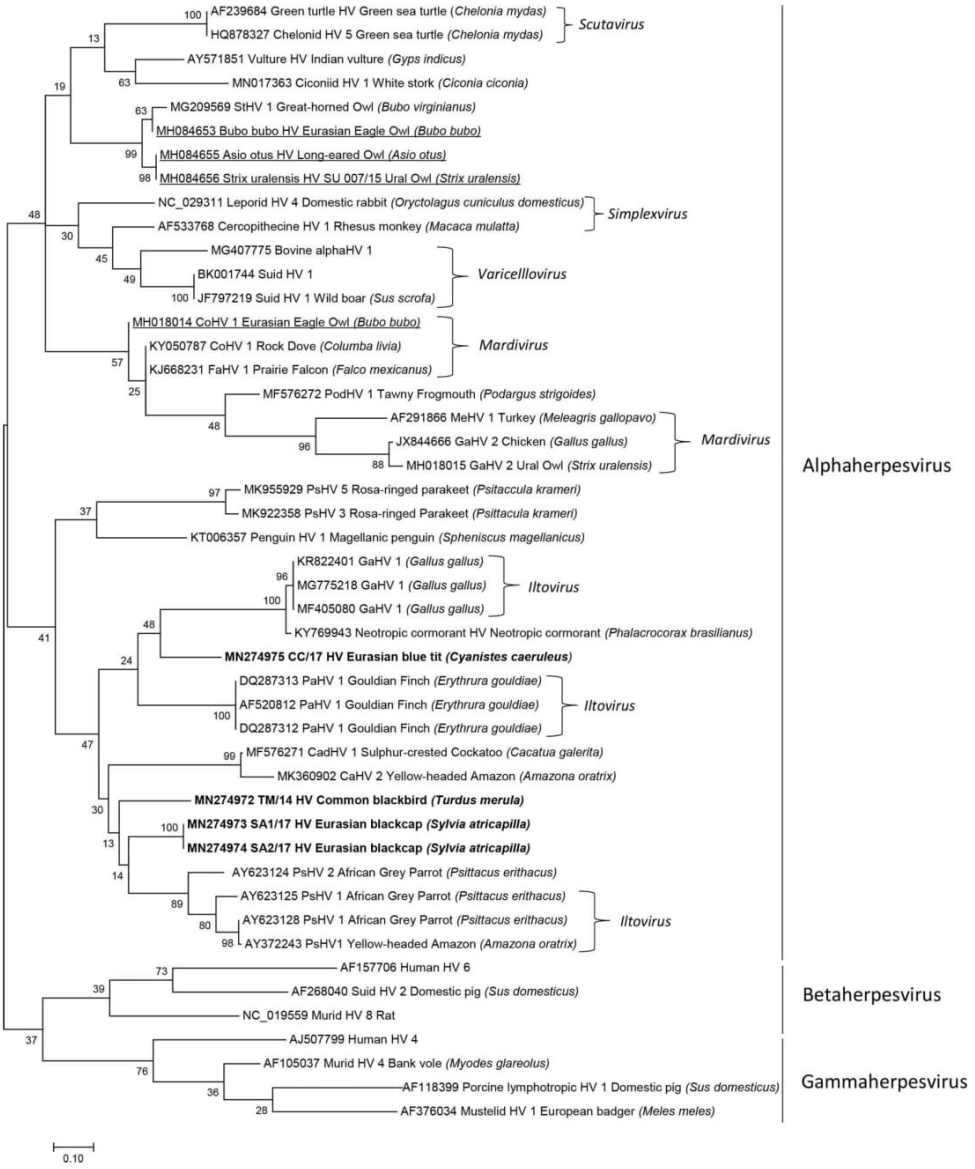

FIGURE 1. Phylogenetic relationship from partial DNA polymerase gene nucleotide sequences of herpesviruses from free-living passerine birds captured in Slovenia during autumn migration in 2014 and 2017 and herpesviruses derived from the GenBank database. The tree was generated by the maximum likelihood with the Tamura 3-parameter model and 1,000 bootstrap replicates to assign confidence levels to branches. The scale bar indicates substitutions per site. GenBank accession numbers for sequences are given before herpesvirus names and the host. Nucleotide sequences obtained in this study are marked in bold, and nucleotide sequences of herpesviruses detected from wild owls in Slovenia are underlined.

storage of the samples at –20 C for up to 10 d could have some influence, but not significant, on the low detection rate of HV.

Although HVs were mostly detected in sick or dead passerines in past studies, some reports describe the detection of HV in apparently healthy captive-bred caged passerine birds (Widen et al. 2012). As in these cases, all HV-positive passerines in our study lacked clinical signs of disease. It is assumed that most natural HV infections are acquired during early life, and many healthy birds could remain viremic or persistently infected for prolonged periods of time. Some internal or environmental stress factors could probably alter the host–viral balance and lead to later development of the disease (Ritchie 1995; Ramis et al. 1996; Phalen 1997).

On the other hand, a potential benefit of HV as part of a symbiotic relationship with the host needs to be considered. Numerous studies have shown that asymptomatic HV infection has a protective effect on hosts against viral and bacterial infection (Barton et al. 2007). Mice infected with murine gammaherpesviruses were more resistance to infection with *Listeria monocytogenes* and *Yersinia pestis* and had a higher survival rate for influenza A virus infection (Barton et al. 2007; Saito et al. 2013; Furman et al. 2015). Under certain circumstances, chronic HV infection was related to increased resistance to tumors in mice (White et al. 2010; Raffegerst et al. 2015). Therefore, detection of HVs in cloacal swabs of healthy animals suggests that the relationship between host and virus likely includes both symbiosis and infection that could result in disease.

The use of a relatively short fragment of the DNA polymerase gene of mammalian and avian HVs for phylogenetic analyses is well supported by numerous studies (Ehlers et al. 1999; Li et al. 2000; Pagamjav et al. 2005; Gailbreath and Oaks 2008; Woźniakowski et al. 2013). The phylogenetic analyses showed that the HV sequences detected in four free-living passerine birds in our study are the most closely related to the alphaherpesvirus sequences detected in different bird species (Fig. 1). More precisely, the HV sequences detected clustered together with HV sequences detected in Sulphur-crested Cockatoo and Neotropic Cormorants, as well as with better-known HVs such as GaHV1, PaHV1, PsHV1, and PsHV2. Despite the limited sequence data on HVs detected in passerine birds, it could be that different sequences of HVs with relatively low identity were detected (Fig. 1). This finding points to the probability that different HVs were circulating in the passerine population in our study. Our detection of identical HV sequences in two Eurasian Blackcaps is consistent with the detection of identical HV sequences in two separate outbreaks of the disease in captive-bred Gouldian Finches (Wellehan et al. 2003; Paulman et al. 2006; Fig. 1). These findings suggest that HVs detected in passerine birds might be species specific; however, their ability to cross the tissue or host barriers described for HVs remains unknown (Gerlach 1994).

All infected species in our study are short-distance migrants or sedentary species, with the highest occurrence being detected in the Eurasian Blue Tit (12.6%), but this could be a consequence of the small sample size of this species (n=8). Surprisingly, no infected long-distant migrants were found, although they were included in the study in reasonably large numbers, including Reed Warblers (*Acrocephalus* spp.), Garden Warblers (*Sylvia borin*), and Common Chiffchaffs (*Phylloscopus collybita*). Migration has extremely selective power on migrating birds (Newton 2003), selecting against less fit birds (i.e., infected specimens). Selective pressure is high, even in short-distance migrants, as indicated by the very low HV infection occurrence we found. Susceptibility to HV infection should be studied in the future by combining sampling on living birds as well as dead birds, in which the occurrence is expected to be higher if the species is susceptible. Our phylogenetic analysis also showed that HV sequences detected in free-living passerine birds in Slovenia are grouped together with HV sequences detected in exotic parrots, passerines, and poultry. Future studies should reveal whether the HVs found in passerines are of indigenous Euro-

pean origin or if they were transferred from imported exotic birds to wild avifauna, which is already a known transmission pathway for various diseases (Lockwood et al. 2013).

Our findings indicate that HVs with different partial DNA polymerase gene sequences have been circulating in the population of free-living songbirds caught during the autumn migration in Slovenia. Further work needs to be done to establish whether the partial viral sequences detected are novel HVs circulating in the passerine population and to study the epidemiology of HVs and their potential effects on the health of free-living songbirds.

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2.3 Okužba s herpesvirusom v gnezdečih populacijah dveh sobivajočih sov rodu *Strix*

Herpesvirus infection in a breeding population of two coexisting *Strix* owls

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Izvleček

Pri pticah, ki so gostiteljice številnih HV, lahko okužba ostane neopažena ali povzroči bolezen (letalno). Podatkov o prisotnosti, vplivu in možnem prenosu HV v populaciji prostoživečih sov je zelo malo. Z metodo PCR smo odsek gena DNA polimeraze HV dokazali v brisih orofarinksa pri 9,4 % (16/170) pregledanih sov, ki so bile ujete v gnezdilnicah ali blizu njih. HV smo odkrili pri kozači, in sicer tako pri odraslih kot pri mladičih. HV nismo dokazali pri lesni sovi (*Strix aluco*). Pri rumenogrlih miših (*Apodemus flavicollis*), ki so glavni plen lesnih sov in kozač na območju raziskave, je bil HV odkrit v organih 5 % (2/40) miši, ujetih na istih lokacijah kot sove. Filogenetska analiza je pokazala, da se nukleotidna zaporedja HV, odkrita pri kozačah, razlikujejo od nukleotidnih zaporedij HV, odkritih pri rumenogrlih miših. Izsledki raziskave kažejo, da je v populaciji prostoživečih kozač prisotna okužba s HV, vendar okužene sove niso pokazale nobenih kliničnih ali produktivnih odstopanj. Na podlagi filogenetske primerjave dokazanih nukleotidnih zaporedij HV in nukleotidnih zaporedij HV, dobljenih iz baze podatkov NCBI GenBank, se zdi, da rumenogrla miš in drugi glodavci niso vir HV okužbe sov. Najverjetnejša pot prenosa je intraspecifična, zlasti z odraslih osebkov na njihove mladiče.

animals

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Article

Herpesvirus Infection in a Breeding Population of Two Coexisting *Strix* Owls

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Simple Summary: Although infection with herpesvirus in owls has commonly been described as a highly lethal disease, there is very little information about the presence of herpesvirus and its potential impact in living owls in wild populations. Our study detected herpesvirus in a breeding population of Ural owls, which showed no clinical signs of illness nor productivity deviances (i.e., in clutch and brood size). Herpesvirus was detected in Ural owl adults and chicks, but not in a young tawny owl (despite the fact that they were in same nest and in persistent contact). Furthermore, herpesviruses were also detected in yellow-necked mice as both owls' main prey. However, comparison of the herpesviruses detected showed that different herpesviruses are present in the owls and mice. The results of this study show that herpesvirus may be present in a Ural owl breeding population without any consequences on health and breeding performance. However, in the case of tawny owls, it seems that they are not susceptible to infection, which could be related to their polymorphism. It seems that small rodents are not a source of herpesvirus infection in owls and that the probable herpesvirus transmission pathway takes place intraspecifically, mostly from adults to young.

Abstract: Birds are a frequent host of a large variety of herpesviruses, and infections in them may go unnoticed or may result in fatal disease. In wild breeding populations of owls, there is very limited information about the presence, impact, and potential transmission of herpesvirus. The herpesvirus partial DNA polymerase gene was detected using polymerase chain reaction in oropharyngeal swabs of 16 out of 170 owls examined that were captured in or near nest boxes. Herpesvirus was detected in Ural owls (*Strix uralensis*), in both adults and young, but not in tawny owls (*Strix aluco*). In yellow-necked mice (*Apodemus flavicollis*), as the main prey of tawny owls and Ural owls in the area, herpesvirus was detected in the organs of 2 out of 40 mice captured at the same locations as the owls. Phylogenetic analysis showed that the herpesvirus sequences detected in the Ural owls differed from the herpesvirus sequences detected in the yellow-necked mice. The results indicate that herpesvirus infection exists in the breeding wild Ural owl population. However, herpesvirus-infected owls did not show any clinical or productivity deviances and, based on a phylogenetic comparison of detected herpesvirus sequences and sequences obtained from Genbank database, it seems that mice and other rodents are not the source of owl infections. The most probable transmission pathway is intraspecific, especially from adults to their chicks, but the origin of herpesvirus in owls remains to be investigated.

Keywords: wildlife; predator-prey interaction; disease transmission; Ural owl; tawny owl; yellow-necked mouse; polymerase chain reaction; Slovenia

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1. Introduction

Some pathogens pose significant natural hazards for wild bird populations [1], and they even have potentials for outbreaks in humans, especially when ecosystems and thus regulatory ecosystem services are depleted [2]. Raptors, as predators at the top of the food chain, are particularly good environmental sentinels for detection of wildlife zoonosis [3]. However, the prevalence, transmission, and impacts of viruses in free-ranging raptors are still a poorly understood phenomenon, and this is probably reflected in cumulative effects in raptor mortality and fecundity combined with other environmental impacts [4,5], as well as individual variation [6].

Diverse herpesviruses have frequently been found in different free-living bird species [7–11]. In owls, herpesvirus was discovered in the 1970s [12], later known as Strigid herpesvirus (StHV 1), and it has been reported in captive and free-ranging owls in Asia, Europe, North America, and Australia [10]. General, all avian herpesviruses are members of the genera *Iltovirus* and *Mardivirus* of the subfamily Alphaherpesvirinae. However, many viruses detected in wild birds have not been completely characterized, including StHV 1, and therefore has not been approved as species and are marked as other related viruses which may be member of the family Herpesviridae [13,14].

The clinical signs of herpesviruses infections in owls, known as inclusion body disease or herpesvirus hepatitis, appear as general depression and anorexia before death or sudden death [15]. In addition, more specific clinical signs such as ulcerative superficial keratitis, proliferative conjunctivitis, and iris pigmentary changes have been described [16]. Hepatitis and disseminated focal necrosis in the liver, spleen, and bone marrow are most commonly seen in owls dying of herpesvirus infections [13]. The actual impact of herpesvirus on the wild owl population became apparent in cases of repopulation of eagle owls (*Bubo bubo*) where serologically herpesvirus-negative birds released back to the wild led to the establishment and expansion of the population [17]. Earlier studies of owl herpesvirus in Europe reported that, on the one hand, the virus can be lethal to some owl species but non-infective to some others, especially species with dark eyes (i.e., the tawny owl (*Strix aluco*) and barn owl (*Tyto alba*) [18,19]), while the third European owl species with dark eyes, the Ural owl (*Strix uralensis*), was not tested. A later study, which included Ural owls as well, did not confirm this hypothesis but indicated that polymorphic species are seemingly more resistant than the species showing lower variability in overall plumage color [11]. However, all of these studies were conducted on dead or injured birds from wildlife rehabilitation centers or birds in laboratory experiments rather than in free-living wild populations.

The viral DNA polymerase is a key enzyme in the lytic phase of the infection by herpesviruses [20]. PCR assays [21,22] with degenerate primers for amplification of the herpesvirus DNA polymerase gene sequence have shown tremendous effectiveness in detecting previously unknown herpesvirus [23]. Based on a partial herpesvirus DNA polymerase sequence phylogenetic study showed that herpesvirus in owls and herpesvirus endemic in the pigeon population—namely, *Columbid herpesvirus 1* (CoHV 1)—are the same virus, and that the pigeons are responsible for transmission of the virus to the owls [7,24]. However, a recent study of phylogenetic analysis of herpesvirus DNA polymerase partial nucleotide sequences detected in dead owls showed that owls were also infected with herpesviruses that are divergent from CoHV 1 [11]. In general, inhalation of virus-containing dust derived from feathers, nasal excretion, saliva, nasal discharge, urine, feces, and crop milk is the predominant means of herpesvirus transmission in birds, and no vertical transmission has been proven [25].

To the best of our knowledge, this study is the first on herpesvirus in owls conducted in the wild breeding owl population. We studied two closely related, ecological similar, and coexisting owl species, the Ural owl and tawny owl [26], which were found to have different susceptibility to herpesvirus infection in previous study on dead birds [11]. In Europe, the Ural and tawny owl largely overlap in range, habitat, prey, and nest site selection [26–28], indicating close contact between the species and thus high exposure to interspecific virus transmission. The larger Ural owl can also outcompete the smaller

tawny owl through direct interspecific territoriality and even predation [27,29]. Thus, our aim was to ascertain whether herpesvirus is present in free-living owls, to determine its potential impact on clinical health and breeding parameters taking into account adult breeding birds and young of both species, and to characterize herpesviruses in line with previously detected herpesviruses. We predicted two possible means of virus transmission, namely via prey (the main prey of both species are mice and voles [30,31]) or via intra- and interspecific direct contact between interacting owls.

2. Materials and Methods

2.1. Field Sampling of Strix Owls

Field sampling was conducted between 2017 and 2019 on Mount Krim (45°58′ N, 14°25′ E; central Slovenia) and the Jelovica Plateau (46°18′ N, 14°8′ E; northern Slovenia), which are large mixed forest areas dominated by beech (*Fagus*), fir (*Abies*), and spruce (*Picea*) at elevations between 300 and 1600 m [32]. Tawny and Ural owls coexist in relatively high breeding densities [33] in the area, and they were found to compete for space, nest sites, and food [26]. In both areas, a network of nestboxes for large owls was established to allow detailed study of both species' biology. We trapped adult breeding birds and their young in or near the nestboxes during annual nest inspections and ringing under license no. 3561-40/2017-4, issued by the Slovenian Environment Agency. Each bird was clinically examined, and oropharyngeal and cloacal swabs were collected from 43 breeding adults and 127 young of the tawny owl and Ural owl. No birds were harmed during the sampling procedure, and all 170 owls were released at the site of capture soon thereafter. Dry FloqSwabs (Copan Italia SpA, Brescia, Italy) were immediately placed in sealed plastic at 4 °C, transported to the laboratory, and stored at −20 °C until analyzed. In each sampled nest we also recorded clutch (no. eggs per nest) and brood size (no. of young per nest) as a measure of productivity.

2.2. Field Sampling of Mice

The main prey of tawny and Ural owls in the area are forest-dwelling rodents, especially the yellow-necked mouse (*Apodemus flavicollis*) [32,34], which is one of the dominant small mammal species in temperate forests [35]. Mice were sampled in 2019 with snap traps baited with a mixture of canned sardines and rolled oats in both areas, two sampling locations at low and high elevations, in the forest at the end of the owl breeding season in June, comprising on average 122 ± 43 trap-nights each year.

Forty mouse carcasses were kept individually in plastic bags and stored at −20 °C at the Institute for Poultry, Birds, Small Mammals, and Reptiles at the University of Ljubljana's Veterinary Faculty, where partial necropsy was performed. During the necropsy, oropharyngeal and rectal swabs, and lung, kidney, liver, spleen, and brain samples were taken from each carcass using cleaned and autoclaved instruments. A different set of instruments was used for each necropsy to prevent DNA contamination. Tissues were either processed immediately or stored at −70 °C until use.

2.3. DNA Extraction and PCR of a DNA Polymerase Gene Region Using Herpesvirus Consensus Primers

Oropharyngeal and cloacal swabs collected from free-living owls and oropharyngeal and rectal swabs from free-living rodents were individually vortexed in 2 mL phosphate-buffered saline for 2 min. Oropharyngeal and cloacal/rectal swabs were separately pooled per five samples together; 100 µL aliquots of each swab in PBS were pooled to produce 500 µL samples for genomic nucleic acid extraction. Tissue samples from individual free-living rodents were pooled and homogenized in phosphate-buffered saline in ratio 1:10 volume/volume. The homogenates were clarified by centrifugation at 1000× *g* for 10 min.

Total DNA and RNA were extracted from 140 µL from pooled samples by the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's

instructions. Viral DNA was detected by nested PCR using a set of degenerate primers that target the herpesvirus DNA polymerase gene region as described by VanDevanter et al. [21]. The PCR volume was 20 µL, and it contained 10 µL of DreamTag Green PCR Master Mix (2×; Thermo Scientific, Dreieich, Germany), 1 µM of each PCR primer, 2 µL of isolated DNA, and deionized water up to 20 µL. Cycling parameters for both methods of PCR used an initial denaturation at 95 °C for 5 min, followed by 45 cycles of 94 °C for 30 s, 46 °C for 60 s, 72 °C for 60 s, and final extension at 70 °C for 7 min. Individual samples from positive pools of samples were tested individually, as previously described.

2.4. Detection, Sequencing, and Phylogenetic Analysis of PCR Products

PCR products were analyzed in 1.8% agarose gel (Sigma-Aldrich, St. Louis, MO, USA) containing ethidium bromide by electrophoresis, purified using a FastGene Gel/PCR extraction kit (Nippon Genetics, Duren, Germany) and sent for sequencing to the Macrogen Laboratory (Macrogen Inc., Amsterdam, The Netherlands). The nucleotide sequences obtained were first analyzed by BLAST [36] to identify sequences relevant for further analyses within the NCBI database. Nucleotide sequences were aligned in Geneious with MAFFT translation alignments [37]. Phylogenetic analysis was performed using the maximum likelihood method with the Tamura 3-parameter plus gamma distribution plus invariable site and 1000 bootstrap replicates by MEGA 7.0 [38]. The percentage of similarity among sequences was calculated by the p-distance model (pairwise distance) in MEGA 7.0. The accession numbers of the herpesvirus sequences obtained in this study are MW315868–MW315883 and MW345631–MW345632. The accession numbers of other herpesvirus sequences used for phylogenetic analysis are included in Figure 1.

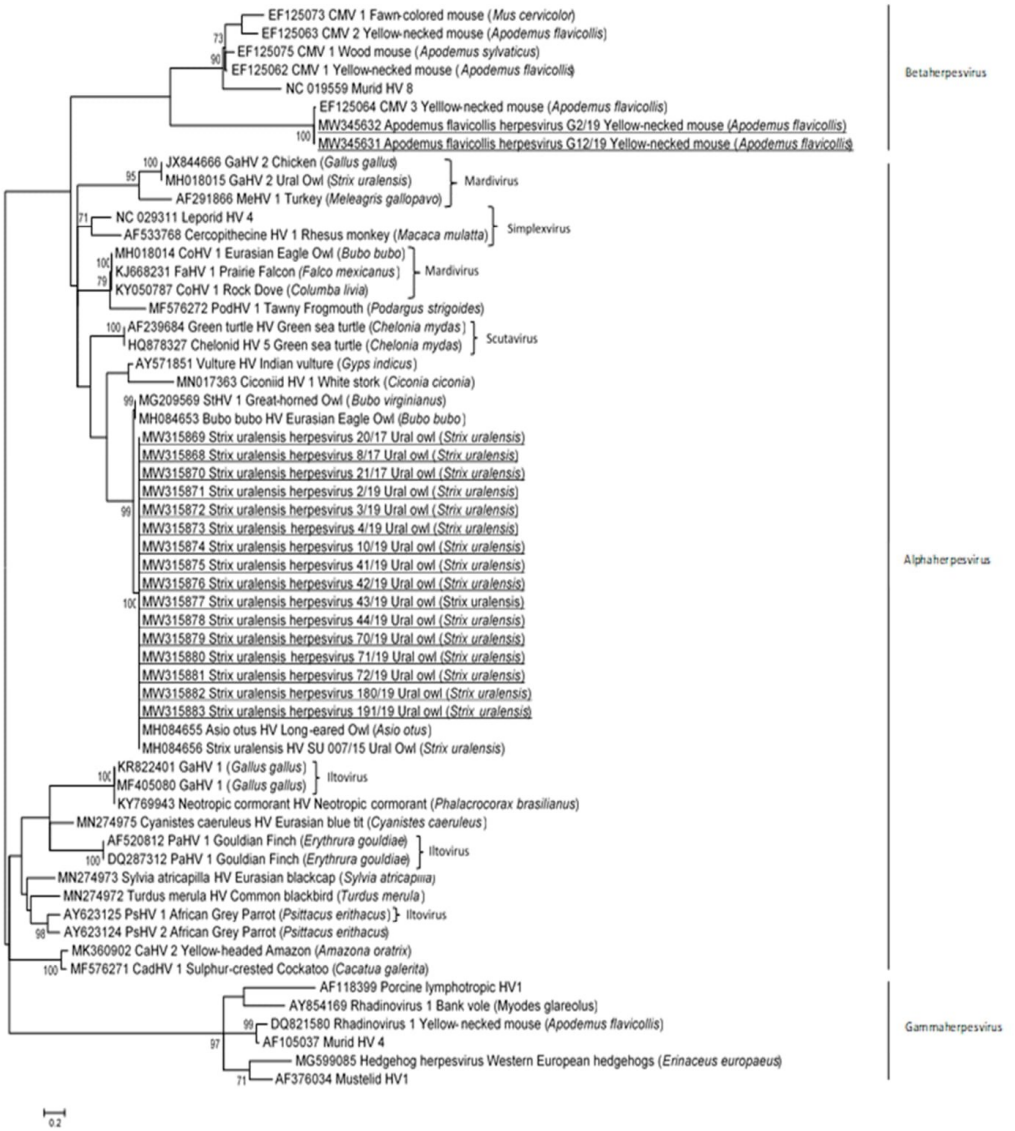

Figure 1. Phylogenetic relationship based on partial DNA polymerase gene nucleotide sequences of herpesviruses from Ural owls and yellow-necked mice captured in Slovenia and other herpesviruses derived from the GenBank database. The scale bar indicates substitutions per site. Nucleotide sequences obtained in the current study are additionally underlined.

3. Results

3.1. Herpesvirus Detection in Owls and Yellow-Necked Mouse Populations

The partial sequence of herpesvirus DNA polymerase gene was detected only in oropharyngeal swabs in 16 out of 170 owls examined (9.4%). However, the herpesvirus was detected only in Ural owls, in adults and young, but not in tawny owls (Table 1). Out of

45 Ural owls from Mount Krim, 14 were positive (31.1%), and out of 10 Ural owls from the Jelovica Plateau, 2 were positive (20.0%), but the infection prevalence was not significantly different between the areas ($\chi^2 = 0.11$, $p = 0.74$). The infection prevalence was found to be higher in young than adult birds, although the difference was not significant ($\chi^2 = 0.59$, $p = 0.44$). We found that about half of the Ural owl nests were infected (Table 1), but we did not necessarily confirm infection in all birds from each infected nest. The herpesvirus prevalence was found to be similar in adult females (22.2%, $n = 9$) and males (16.7%, $n = 6$). In the nests with more than one young, of which at least one was positive ($n = 5$ nests), the median herpesvirus prevalence in the young was 50.0% (range 20.0 to 100.0%). In the nests with tested breeding female and chicks, all chicks were infected when female was positive ($n = 2$ nests), while positive chicks were found also in the nests with females found negative for herpesvirus infection ($n = 4$ nests). In one nest in 2019 on Mount Krim, the Ural owl pair successfully raised one Ural and one tawny owl young, which was a consequence of competitive expulsion of the tawny owl by the larger Ural owl from the nestbox. Both Ural owl parents were herpesvirus positive as well as the Ural owl young, but not the tawny owl young raised in the same nest. All the owls sampled were clinically healthy, and the productivity and clutch and brood size between infected (median 4.0/3.5) and uninfected nests (median 3.5/3.0) was similar (Mann–Whitney $U = 20–23$, $p = 0.6–1$).

Table 1. Herpesvirus prevalence (% of infected among all tested birds/nests) in wild coexisting populations of tawny owls *(Strix aluco)* and Ural owls *(Strix uralensis)* in Slovenia in 2017 and 2019. The numbers of birds or nests examined are given in parentheses.

	Tawny Owl	**Ural Owl**
Infected birds	0.0% (115)	29.1% (55)
Infected adults	0.0% (27)	18.7% (16)
Infected young	0.0% (88)	33.3% (39)
Infected nests	0.0% (30)	53.0% (17)

In 2019, 40 yellow-necked mice captured at Mount Krim and the Jelovica Plateau were examined for the presence of herpesvirus. Pharyngeal and rectal swabs and tissue samples were analyzed with panherpesvirus PCR targeting the DNA polymerase gene. Of 40 free-living mice examined, 2 were herpesvirus positive (5%).

3.2. Phylogenetic and Sequence Analysis

The partial nucleotide herpesvirus sequences (205 nt) detected in wild Ural owls and yellow-necked mice were compared to the sequences of DNA polymerase gene of other avian and mammal herpesviruses to determine their phylogenetic relationship.

The phylogenetic analyses showed that the herpesvirus sequences detected in Ural owls are most closely related to the alphaherpesvirus sequences detected in other owls, whereas the herpesvirus sequences detected in small rodents formed a separate group among betaherpesviruses (Figure 1). More precisely, the sequences of herpesviruses detected in owls clustered together with novel herpesvirus sequences detected in the Eurasian eagle owl (Bubo bubo HV), Ural owl (Strix uralensis HV), long-eared owl (Asio otus HV), and great horned owl (StHV 1). The phylogenetic tree (Figure 1) showed that partial DNA polymerase gene sequences of wild Ural owls from Slovenia were identical and showed 100% nt identities with the herpesvirus sequence detected in the long-eared owl and Ural owl, and 90.6% and 90.0% nt identities with the Eurasian eagle owl and great horned owl, respectively. Known herpesvirus sequences detected in pigeons (CoHV 1) and birds of prey (CoHV 1, FaHV 1, GaHV 2) were grouped in the other clusters, and they shared 60.3% to 62.2% nt identities with novel herpesvirus sequences detected in Ural owls.

The DNA polymerase sequences detected in yellow-necked mice were identical and shared 98.7% nt identity with the most closely related betaherpesvirus sequence detected in yellow-necked mice (Apodemus flavicollis cytomegalovirus 3) from Germany. Lower nt identities, 47.2 to 48.4%, were detected with other betaherpesvirus sequences in the yellow-

necked mouse (Apodemus flavicollis cytomegalovirus 2 and 1), fawn-colored mouse (Mus cervicolor cytomegalovirus 1), and wood mouse (Apodemus sylvaticus cytomegalovirus 1).

There was low nt identity (51.1%) between the herpesvirus sequence detected in wild mice and owls in Slovenia.

4. Discussion

Based on the herpesvirus DNA polymerase gene detected in wild owls, the results of our study showed that herpesvirus infection exists in breeding wild owl populations, but only the Ural owl appeared to be a herpesvirus reservoir. However, the fact that no herpesviruses were detected in tested tawny owls should be taken with caution, as several factors may influence the detection of herpesvirus, such as the small number of birds tested, viral shedding at the time of sampling, pooling of samples, and the sensitivity and specificity of the PCR method. All of these factors can influence on negative results, especially when virus levels in the sample are low or in the case of novel viruses. Infected birds did not show any clinical or productivity deviances, despite the fact that infection with herpesvirus is known as a fatal disease in owls [15,25], but the effects might be evident in long-term productivity and survival, especially in low-prey seasons [4,39]. Based on the herpesvirus DNA polymerase gene detected in oropharyngeal swabs in clinical healthy wild owls, this could imply that asymptomatic shedding of the virus was detected. Herpesviruses have the ability to establish lifelong latency within the host and to periodically reactivate [25], and it is not unusual that an individual might be carrying the pathogen without any visible manifestation of the disease until exposure to an environmental stressor triggers activation of viral replication and spread to a new host organism. Shedding of herpesvirus from the oropharyngeal cavity of birds is an important factor from the point of view of potential virus transmission from infected to uninfected individuals. In owls, the male commonly offers food to the female during the courtship ritual and, furthermore, the male and female both feed their offspring during the reproduction period [27]. The results showed that oropharyngeal swabs are more suitable for detection of herpesvirus in owls; however, although herpesvirus was not detected in cloacal swabs, which could also be due to the presence of inhibitors in the swabs, the importance of cloacal swabs in the detection of herpesvirus should not be neglected, as previous reports have shown [23,40]. Our study was conducted during peak prey years [41], and thus with low nutritional stress for owls, but even here transmissions within the nests were detected. We speculate that during low-prey seasons herpesvirus infections might have more detrimental effects on Ural owls. Such infections are known to completely suppress breeding in low-prey seasons, which is not the case in tawny owls [41].

In general, herpesviruses are widespread, with various clinical manifestations in several avian species throughout the world [42]. However, neither transmission nor pathogenesis are fully understood in free-living birds. Although that some studies have suggested that raptors may contract the infection and consequently disease through the oral route by ingesting CoHV 1 infected pigeons [7], the detection of the partial sequence of herpesvirus DNA polymerase gene in healthy owls raises the question whether all herpesvirus infections in this species [7,24] cause illness with a fatal outcome in some cases. GaHV 2, also known as Marek's disease virus [11], CoHV 1 [7,10,11], StHV 1 [16], and the herpesviruses detected in our previous [11] and present study are currently known herpesviruses in owls. They are genetically different, and it could be possible that some of them, such as CoHV 1, are a more fatal threat to owls than others, and also that they could differ from each other in transmission and pathogenesis. For more complete information on the potential pathogenicity of herpesvirus in owls, a virus isolation, serotyping, and full genome characterization of these different herpesviruses should be performed in the future.

The herpesvirus sequences detected in Ural owls were grouped together with herpesvirus sequences detected in a dead free-living Eurasian eagle owl, Ural owl, and long eared owl (*Asio otus*) from our previous study [11], and in live free-living great horned owl (*Bubo virginianus*) from the United States [16], suggesting that this herpesvirus is spread

transcontinental. Although, it seems that this herpesvirus infects only species within group Striginae, which is a monophyletic clade [43] with owls from the genus *Bubo* and *Strix* being most closely related [44], what additionally supports our and previous findings. However, our study revealed that even within Striginae herpesvirus susceptibility is not equal. Within the genus *Strix,* tawny and Ural owls were found to be closely related species [43], but our study showed that species differ significantly in herpesvirus susceptibility, however a possibility of other herpesviruses presence should be emphasized, which could be missed out by used PCR method. We believe that a simple correlation between herpesvirus and the owl molecular phylogeny is insufficient to explain the host-parasite relationship. Possible reasons in apparently greater immunity of the tawny owl against herpesvirus infection may lay in owl metabolic traits, as suggested in a previous study [11] or in recent co-evolutionary processes that differed between different owl hosts and herpesvirus. Both owl species are highly diverged in life traits. In our study area, the Ural owl is regarded as a glacial relict species with a major part of its population found in boreal climate zone of Eurasia, while the tawny owl is a temperate species that has recently spread to the Northern Europe [45–47]. Our study was confined to a small geographic scale within temperate climate zone, where both species coexist and where Ural owl reach its southern limit of distribution [32]. Future studies should take into account larger geographical scale of herpesvirus sampling in tawny and Ural owl wild populations across Europe to study climate and trait related impacts of herpesvirus infection prevalence in both *Strix* species, suggesting that populations at the limit of their distribution may be more susceptible to infections.

Although host-virus co-evolution is thought to be the primary mode of herpesvirus evolution, cross-species transmission events have been known to occur [7,48,49]. Phylogenetic analysis of the partial sequence of herpesvirus DNA polymerase gene showed identical sequences within both positive animal species, in Ural owls and yellow-necked mice. The herpesviruses detected in mice were different from the herpesvirus sequences detected in owls. The complete natural host range of most herpesviruses is not known, and it appears that the range of natural hosts is very narrow for some herpesviruses, but others affect many different bird species (Figure 1). A herpesvirus that infects both mammals and birds has been rarely described. To our knowledge, Suid herpesvirus 1, causative agent of pseudorabies also named as Aujeszky's disease which belongs to alfaherpesviruses is the only herpesvirus that causes generally mild disease in swine and severe disease including mortality in some other mammals, and also in chickens or pigeons; however, herpesvirus transmission from mammals to birds was reported only under experimental conditions [50]. The herpesvirus DNA polymerase sequences detected in mice in this study were clustered with cytomegalovirus 3 (CMV 3) (subfamily betaherpesviruses) detected in yellow-necked mice in Germany. Other herpesviruses were also detected in the same host; namely, CMV 1, CMV 2, *Apodemus flavicollis rhadinovirus 1* (AflaRHV1), and *Murine gammaherpesvirus 68*, which were clustered in other groups among betaherpesviruses and gammaherpesviruses [51]. In this large-scale study, with over 1100 samples of blood and tissue samples, many known and novel beta- and gamma-herpesviruses were also detected that are found in other species of free-living rodents, such as the bank vole (*Clethrionomys glareolus*), field vole (*Microtus agrestis*), common vole (*Microtus arvalis*), long-tailed field mouse (*Apodemus sylvaticus*), house mouse (*Mus musculus*), Norway rat (*Rattus norvegicus*), and black rat (*Rattus rattus*). Similar to our study, no evidence of alphaherpesvirus was obtained. Low prevalence, unknown loci of latency, low viral loads of samples, or the hypothesis that alphaherpesvirus in rodents never developed or became extinct during herpesvirus evolution were some of the reasons that were proposed [51].

On the other hand, no detected rodent herpesviruses were found in owls, despite the fact that they represent a large part of their diet. The herpesviruses detected in owls are currently unassigned in the family Herpesviridae. However, phylogenetic analysis of DNA polymerase sequences detected in owls in Slovenia and North America shows that they are most closely related to subfamily alphaherpesvirus. Betaherpesviruses and gamma-

herpesviruses are known for their biological characteristic of restricted host range [52], which could be one of the reasons why no rodent herpesviruses (betaherpesviruses and gammaherpesviruses) were detected in owls. Nevertheless, because of the large proportion of rodent diet in owls and relatively sensitive PCR method, the detection of rodent herpesvirus in the oropharyngeal region and cloaca would be expected, at least as part of contamination with herpesvirus. To conclude, based on our results and the results presented in the herpesvirus study by Ehlers et al. [51], it seems we can rule out the prey–predator herpesvirus transmission pathway since there was very low similarity between the herpesvirus sequences detected in owls and mice and also other rodents. However, due to the high level of diversity among herpesviruses it should be emphasized that used consensus PCR method could miss a novel herpesvirus occurring [23]. The strongest and probably the most important transmission pathway is intraspecific, especially from adults to their chicks. However, within the nest, transmission among the chicks seemed to be lower.

We confirmed the conclusions of previous studies on dead and experimental birds [11,18] that tawny owls are not susceptible to herpesvirus, not even when in close contact with infected Ural owls. However, there is still a pending question about lack of susceptibility of tawny owls to herpesvirus, although tawny and Ural owls are closely related species [43]. Žlabravec et al. [11] suggested polymorphism and greater genetic and physiological variability as a possible reason for tawny owl resistance to infection, but there might be other historical reasons, with the tawny owl being an ancient and coevolved herpesvirus host, whereas infections in Ural owls might be relatively recent, which should be explored further in future studies.

5. Conclusions

In conclusion, this study has provided greater insight into the herpesvirus presence in free-living owls, their epizootiology, and the wildlife impact on the breeding population of owls in temperate forests in Europe. Herpesvirus was detected in live free-living breeding population of Ural owls, but not in tawny owls. The herpesviruses detected in owls were phylogenetically identical and were most similar to herpesvirus sequences detected in organs of dead free-living owls from our previous study. However, comparison with the herpesvirus detected in yellow-necked mice and other rodents ruled out small rodents as a possible source of herpesvirus infection in owls. It seems that most important herpesvirus transmission occurs from adults to young, but the origin of the herpesvirus detected in owls remains to be further investigated. Nevertheless, despite the fact that herpesvirus in owls was commonly described as a fatal disease, no clinical signs or productivity deviances were recorded in breeding population of herpesvirus-positive owls.

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2.4 Detekcija herpesvirusov pri bolnih, oslabelih ali poškodovanih prostoživečih pticah v Sloveniji

Detection of herpesviruses in wild bird casualties in Slovenia

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Izvleček

Celotnega gostiteljskega seznama HV pri prostoživečih pticah ni, informacije o nukleotidnih zaporedjih HV so na voljo le v nekaterih primerih. Namen te študije je bil ugotoviti prisotnost HV pri različnih prostoživečih pticah in dobiti več informacij o njihovem filogenetskem razmerju. V brisih orofarinksa in kloake 447 prostoživečih bolnih, poškodovanih ali oslabelih ptic iz 15 različnih vrst, ki so bile sprejete na Kliniko za ptice, male sesalce in plazilce Veterinarske fakultete, smo z metodo PCR dokazovali prisotnost odseka gena DNA polimeraze HV. HV so bili odkriti v brisu orofarinksa in/ali kloake pri 34 pticah (7,5 %), ki pripadajo 11 vrstam iz šestih različnih ptičjih redov: *Accipitriformes*, *Charadriiformes*, *Columbiformes*, *Falconiformes*, *Passeriformes* in *Strigiformes*. Filogenetska analiza je pokazala, da so v populaciji prostoživečih ptic prisotna različna nukleotidna zaporedja HV. Nekateri HV so specifični za gostiteljsko vrsto ptic, medtem ko smo v nekaterih primerih v različnih redovih ptic odkrili zelo podobna nukleotidna zaporedja, kar potrjuje ugotovitve, da HV niso vedno omejeni na vrsto ptic. Zdi se, da bi lahko do prenosa HV prišlo s plenjenjem iz ptičjega plena in celo s superplenitvijo – na primer velike sove, kot sta velika uharica ali kozača, ki plenijo manjše plenilske ptice. To lahko povzroči večjo izpostavljenost okužbam in je v skladu z dejstvom, da so bile plenilske ptice najbolj okužene skupine vrst. Dokaz HV samo v enem od obeh odvzetih brisov kaže na to, da je za dokaz HV pri prostoživečih pticah treba odvzeti tako orofaringealni kot kloakalni bris.

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Detection of Herpesviruses in Wild Bird Casualties in Slovenia

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The complete host range of avian herpesviruses in wild birds is unknown, and information about nucleotide sequences is available only in limited cases. The aim of this study was to detect the presence of herpesviruses in wild birds and to gain more information about their phylogenetic relationship. Oropharyngeal and cloacal swabs from 447 wild birds from 15 different orders presented as wildlife casualties were examined for herpesvirus presence with PCR targeting a fragment of the DNA polymerase gene. Herpesviruses were detected in oropharyngeal and/or cloacal swabs in 34 (7.5%) birds belonging to 11 species from six different avian orders: Accipitriformes, Charadriiformes, Columbiformes, Falconiformes, Passeriformes, and Strigiformes. The results of phylogenetic analysis showed that various herpesviruses sequences are present in the wild bird population. Some herpesviruses are host species–specific, whereas in some cases very similar sequences were detected through different avian orders, which confirms findings that herpesviruses are not always restricted to bird species. It seems that herpesvirus transmission could occur by predation from avian prey, and even by superpredation—for example, large owls, such as the Eurasian eagle owl (*Bubo bubo*) or Ural owl (*Strix uralensis*), preying on smaller raptors. This can lead to greater infection exposure and is in line with the fact that raptors were the most infected species group. Nevertheless, the individual or simultaneous detection of herpesviruses in oropharyngeal and cloacal swabs shows that both swab samples should be used for herpesvirus detection in wild birds.

Keywords: wildlife, herpesvirus, avian, polymerase chain reaction, Slovenia

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INTRODUCTION

Avian herpesviruses cause some of the more familiar diseases of birds, such as Marek disease (1, 2), infectious laryngotracheitis (3), duck plague in poultry (4), and Pacheco disease in parrots (5), as well as diseases in free-living birds that are equally important. However, because of several disadvantages regarding disease surveillance in free-living birds, including obtaining biological samples, virus infections in these animals are less studied. Generally, many herpesviruses replicate in healthy birds with little or no apparent signs of infection, but under certain environmental conditions various forms of disease associated with high rates of mortality may occur. Clinical signs of herpesvirus infections in wild birds may comprise a broad spectrum of non-specific signs ranging from respiratory to enteric problems, such as depression, reduced/absent appetite, regurgitation, biliverdinuria, diarrhea, conjunctivitis, or sudden death (6).

Pigeons are the natural hosts of pigeon herpesvirus 1, in the new nomenclature also known as columbid herpesvirus 1 (CoHV-1) (7). In infected pigeon flocks, mature birds are asymptomatic carriers and some of them may intermittently shed virus (8). CoHV-1 causes the disease known as Smadel's disease or ingluvinitis of pigeons, a contagious disease of predominantly young pigeons of racing and fancy breeds or immunocompromised adult pigeons (6). Numerous field studies and detection of CoHV-1 in raptors suggest that consumption of infected prey species, in particular pigeons, is most likely the source of herpesvirus infections in hawks, eagles, and owls (9–11). Furthermore, CoHV-1 was also detected in other non-raptorial birds (12), and recent studies have shown that many additional herpesviruses, which differ from CoHV-1, have been identified in various live-free living birds such as owls and songbirds (13–15). In birds of prey, the disease caused by CoHV-1 is known as herpesvirus hepatitis or inclusion body disease, and in some cases it is described as fatal, with mortality approaching 100% (11). Disease outbreaks and also mortality due to herpesvirus infections have been reported among free-ranging aquatic birds, marine birds, and waterfowl (16–19). Fatal cases of animals infected with passerid herpesviruses have also been reported in songbirds from aviary enclosures in America and Canada (20, 21). In general, severe and fatal disease caused by herpesvirus infections in birds are mostly described in interspecies infections, whereas in intraspecies infections clinical signs are mild and followed by a period of latency (22).

Based on biological properties and genomic attributes, the Herpesviridae have been divided into three subfamilies: Alphaherpesvirinae, Betaherpesvirinae, and Gammaherpesvirinae (23). The Alphaherpesvirinae subfamily is divided into five genera; namely, *Iltovirus*, *Mardivirus*, *Scutavirus*, *Simplexvirus*, and *Varicellovirus* (24). For universal detection of unknown herpesviruses in birds and mammals, a PCR with pan-herpes degenerate primers for detection of the highly conserved herpesvirus DNA polymerase gene showed good results in previous studies (10, 12–14, 25). All avian herpesvirus analyzed to date are phylogenetically most closely related to the members of the Alphaherpesvirinae subfamily; however, it should be emphasized that, although herpesvirus infections have been described in various species of free-living birds, only limited herpesvirus nucleotide (nt) sequence data are available in free-living birds.

This study further extends insight into the spread of herpesviruses in wild avian hosts from various orders, studies their phylogenetic relationship, and presents more information about the ubiquitous features of herpesviruses in the free-living bird population.

MATERIALS AND METHODS

Bird Species and Samples

Cloacal and oropharyngeal swabs were collected from 447 wild birds treated at the Clinic for Birds, Small Mammals, and Reptiles, Faculty of Veterinary Medicine, University of Ljubljana as wildlife casualties for veterinary diagnosis, treatment, and care between October 2017 and December 2019 (**Table 1**). A clinical examination was performed by a veterinarian after admission. Samples were collected from live avian patients during the clinical procedure or were taken while birds were anesthetized using inhalational isoflurane and oxygen delivered via mask to facilitate clinical examination and diagnostics. All efforts were made to minimize animal stress and discomfort. Sterile dry swabs (Copan, Italy) were used to separately swab the oropharynx and cloaca of live birds. Swabs were stored in a refrigerator at 4°C up to 48 h until analyzed.

The free-living birds belonged to 15 different orders; bird taxonomy follows Gill et al. (26): Accipitriformes (n = 44 individuals), Anseriformes (n = 7), Apodiformes (n = 2), Bucerotiformes (n = 1), Charadriiformes (n = 11), Ciconiiformes (n = 1), Columbiformes (n = 109), Falconiformes (n = 34), Galliformes (n = 1), Gruiformes (n = 3), Passeriformes (n = 183), Pelecaniformes (n = 5), Piciformes (n = 7), Podicipediformes (n = 2), and Strigiformes (n = 42). More detailed information is shown in **Table 1**.

DNA Extraction and PCR

Cloacal and oropharyngeal swabs were individually vortexed in 2 ml phosphate-buffered saline for 1 min and supernatant was stored for nucleic acid extraction. In each assay, a DNA of a alphaherpesvirus-positive sample was included as a positive control. As negative control extracted phosphate-buffered saline was used.

Total DNA and RNA were extracted from 140 µl of samples using the QIAamp Viral RNA Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. PCR was used to detect diverse herpesvirus as previously described by VanDevanter et al. (27). The degenerated primers DFA (5′-GAYTTYGCNAGYYTNTAYCC-3′), ILK (5′-TCCTG GACAAGCAGCARNYSGCNMTNAA-3′) and KG1 (5′-GTCTTGCTCACCAGNTCNACNCCYTT-3′) targeted the DNA polymerase protein. This procedure was followed by a nested-PCR with the primers TGV (5′-TGTAACTCGGTGTAYG GNTTYACNGGNGT-3′) and IYG (5′-CACAGAGTCCGTRTC NCCRTADAT-3′). The PCR volume was 20 µl, and it contained 10 µl of 2× DreamTag Green PCR Master Mix (Thermo Scientific, Europe), 1 µM of each PCR primer, 2 µl of isolated DNA, and deionized water up to 20 µl. The parameters for primer and nested PCR were denaturation at 95°C for 5 min, followed by 45 cycles of denaturation at 94°C for 30 s, annealing at 46°C for 1 min, extension at 72°C for 1 min, and final extension at 70°C for 7 min.

Detection and Sequencing of PCR Products

Amplified products were separated by electrophoresis on 1.8% agarose gel (Sigma-Aldrich, USA) containing ethidium bromide. PCR products of 215 to 315 bp were excised and purified with a FastGene Gel/PCR extraction kit (Nippon Genetics, Europe) and sent for sequencing to the Macrogen Laboratory (Macrogen Inc., Netherlands).

TABLE 1 | Detection of herpesvirus by avian order, family, and species.

Order	Family	Genus and species	Common name	% HV positive	*n*
Accipitriformes *n* = 44	Accipitridae *n* = 44	*Aquila chrysaetos*	Golden eagle	100	1
		Buteo buteo	Common buzzard	16	32
		Accipiter gentilis	Northern goshawk	0	1
		Accipiter nisus	Eurasian sparrowhawk	0	7
		Circaetus gallicus	Short-toed snake eagle	0	1
		Pernis apivorus	European honey buzzard	0	2
Anseriformes *n* = 7	Anatidae *n* = 7	*Anas platyrhynchos*	Mallard	0	2
		Cygnus olor	Mute swan	0	5
Apodiformes *n* = 2	Apodidae *n* = 2	*Apus apus*	Common swift	0	2
Charadriiformes *n* = 6	Laridae *n* = 2	*Larus michahellis*	Yellow-legged gull	50	2
	Scolopacidae *n* = 4	*Scolopax rusticola*	Eurasian woodcock	0	4
Ciconiiformes *n* = 1	Ciconiidae *n* = 1	*Ciconia ciconia*	White stork	0	1
Columbiformes *n* = 109	Columbidae *n* = 109	*Columba livia domestica*	Domestic pigeon	18	89
		Columba palumbus	Common wood pigeon	20	5
		Streptopelia decaocto	Eurasian collared dove	0	1
		Streptopelia turtur	European turtle dove	0	14
Bucerotiformes *n* = 1	Upupidae *n* = 1	*Upupa epops*	Eurasian hoopoe	0	1
Falconiformes *n* = 34	Falconidae *n* = 34	*Falco peregrinus*	Peregrine falcon	0	1
		Falco subbuteo	Eurasian hobby	0	1
		Falco tinnunculus	Common kestrel	3	32
Galliformes *n* = 1	Phasianidae *n* = 1	*Phasianus colchicus*	Common pheasant	0	1
Gruiformes *n* = 3	Rallidae *n* = 3	*Fulica atra*	Eurasian coot	0	1
		Rallus aquaticus	Water rail	0	2
Passeriformes *n* = 183	Corvidae *n* = 52	*Corvus corax*	Common raven	0	2
		Corvus cornix	Hooded crow	7	40
		Coloeus monedula	Western jackdaw	0	3
		Garrulus glandarius	Eurasian jay	0	2
		Pica pica	Eurasian magpie	0	5
	Emberizidae *n* = 1	*Emberiza calandra*	Corn bunting	0	1
	Fringillidae *n* = 17	*Carduelis carduelis*	European goldfinch	0	6
		Spinus spinus	Eurasian siskin	0	1
		Chloris chloris	European greenfinch	33	3
		Coccothraustes coccothraustes	Hawfinch	0	5
		Fringilla coelebs	Common chaffinch	0	2
	Hirundinidae *n* = 7	*Delichon urbicum*	Common house martin	0	1
		Hirundo rustica	Barn swallow	0	6
	Motacillidae *n* = 1	*Motacilla alba*	White wagtail	0	1
	Muscicapidae *n* = 6	*Erithacus rubecula*	European robin	0	5
		Muscicapa striata	Spotted flycatcher	0	1
	Paridae *n* = 10	*Periparus ater*	Coal tit	0	1
		Parus major	Great tit	0	8
		Poecile palustris	Marsh tit	0	1
	Passeridae *n* = 33	*Passer domesticus*	House sparrow	0	33
	Sittidae *n* = 2	*Sitta europaea*	Eurasian nuthatch	0	2
	Sturnidae *n* = 8	*Sturnus vulgaris*	Common starling	0	8
	Sylviidae *n* = 1	*Sylvia atricapilla*	Eurasian blackcap	0	1
	Turdidae *n* = 45	*Turdus merula*	Common blackbird	0	40
		Turdus philomelos	Song thrush	0	5
Pelecaniformes *n* = 5	Ardeidae *n* = 5	*Ardea cinerea*	Grey heron	0	4
		Botaurus stellaris	Eurasian bittern	0	1

(Continued)

TABLE 1 | Continued

Order	Family	Genus and species	Common name	% HV positive	*n*
Piciformes *n* = 7	Picidae *n* = 7	*Dendrocopos major*	Great spotted woodpecker	0	3
		Drycopus martius	Black woodpecker	0	1
		Picus canus	Grey-headed woodpecker	0	2
		Picus viridis	European green woodpecker	0	1
Podicipediformes *n* = 2	Podicipedidae *n* = 2	*Podiceps cristatus*	Great crested grebe	0	1
		Podiceps nigricollis	Black-necked grebe	0	1
Strigiformes *n* = 42	Strigidae *n* = 42	*Asio otus*	Long-eared owl	43	7
		Athene noctua	Little owl	0	2
		Bubo bubo	Eurasian eagle owl	25	4
		Otus scops	Eurasian scops owl	0	4
		Strix aluco	Tawny owl	0	17
		Strix uralensis	Ural owl	12	8
Total				34	447

Phylogenetic Analysis

The nucleotide sequences obtained were first analyzed by BLAST (28) to identify sequences relevant for further analyses within the NCBI database. Nucleotide alignments were constructed in Geneious Prime 2019 v1.3 software suite (Biomatters Ltd., Auckland, New Zealand) with MAFFT translation alignment (29). Phylogenetic analysis was performed using the maximum likelihood method with the Tamura 3-parameter model and 1,000 bootstrap replicates by MEGA 7.0 (30). The genetic distances among sequences were calculated using the *p*-distance model (pairwise distance) in MEGA 7.0.

RESULTS

PCR and Sequencing

The presence of herpesviruses in clinical samples was investigated by PCR. A PCR product was detected in swabs of 34 out of 447 live free-living birds. In four birds, herpesvirus was detected in oropharyngeal and cloacal swabs, in three birds herpesvirus was detected only in cloacal swabs, and in 27 birds herpesvirus was detected only in oropharyngeal swabs. Herpesvirus was found in 11 species from six different avian orders: Accipitriformes, Charadriiformes, Columbiformes, Falconiformes, Passeriformes, and Strigiformes (**Tables 1**, **2**).

Phylogenetic Analysis

Nucleotide sequences of the partial DNA polymerase gene of herpesviruses were obtained from 34 herpesvirus-positive samples and used for phylogenetic analysis. Phylogenetic comparison of herpesvirus nt sequences from Slovenian free-living birds from this study and other avian and mammal alpha-, beta-, and gammaherpesvirus revealed high phylogenetic diversity among alphaherpesvirus (**Table 2**, **Figure 1**). All herpesvirus nt sequences investigated in this study clustered with alphaherpesviruses, with nt identity ranging from 49.8 to 100% among them.

Herpesvirus sequences detected in domestic pigeons (*Columba livia domestica*) (238/17, 31/18, 229/18, 293/18, 294/18, 314/18, 392/18, 1,665/18, 11/19, 136/19, 183/19, 231/19, 255/19, 633/19, 903/19, 904/19) shared 99.51 to 100% nt identity and clustered together with CoHV-1 detected in an Eurasian eagle owl (*Bubo bubo*) (353/19), and with FaHV-1 detected in a prairie falcon (*Falco mexicanus*) with 97.53 to 99.02% and 97.55 to 98.04% nt identity, respectively.

The herpesvirus sequence detected in a common wood pigeon (*Columba palumbus*) (360/19) was distinct from the herpesvirus sequences detected in domestic pigeons, with 60.78 to 61.27% nt identity and clustered together with a herpesvirus sequence detected in a yellow-legged gull (*Larus michahellis*) (831/19), with 99.51% identity between them.

In owls, the herpesvirus nucleotide sequences belonged to two genetically distinct groups sharing 56.8% nt identity. The herpesvirus sequence (353/19) detected in a Eurasian eagle owl (*Bubo bubo)* was very similar to the CoHV-1 group of sequences in domestic pigeons in this study (97.53 to 99.02% identity), whereas the herpesviruses detected in a Ural owl (*Strix uralensis*) (1,544/19) and in long-eared owls (*Asio otus*) (690/19, 750/19/, 969/19) were identical and grouped together with the herpesviruses detected in a Eurasian eagle owl (MH084653) and in long-eared owls (MH084655), sharing from 90.74 to 100% nt identity.

In common buzzards (*Buteo buteo*), identical herpesvirus sequences (594/19, 245/17, 241/17, 298/18, 1,062/18) were detected and were most similar to herpesviruses detected in an Indian vulture (*Gyps indicus*), with 83.33% identity, and golden eagle (*Aquila chrysaetos*), with 75.98% identity.

Among falcons, the herpesvirus nt sequence detected in a common kestrel (*Falco tinnunculus*) (338/18) clustered together with the PsHV-1/PsHV-2 group with 81.59 to 86.07% nt identity and was most closely related to PSHV-2 (AY623124) detected in an African gray parrot (*Psittacus erithacus*), with 86.07% identity.

FIGURE 1 | Phylogenetic relationship based on partial DNA polymerase gene nucleotide sequences of herpesviruses from wild birds in Slovenia and other herpesviruses derived from the GenBank database. The scale bar indicates substitutions per site. Nucleotide sequences obtained in the current study are additionally underlined.

TABLE 2 | Details of HV sequences obtained in this study.

Host species	ID number	Sample tested positive	Sequence acc. number	Sequence length (bp)
Common buzzard (*Buteo buteo*)	241/17	Oropharynx	MW533123	231
Common buzzard (*Buteo buteo*)	245/17	Oropharynx	MW533124	235
Common buzzard (*Buteo buteo*)	298/18	Oropharynx	MW533125	216
Common buzzard (*Buteo buteo*)	1062/18	Oropharynx, cloaca	MW533126	222
Common buzzard (*Buteo buteo*)	594/19	Oropharynx	MW533127	228
Golden eagle (*Aquila chrysaetos*)	367/19	Oropharynx	MW533128	228
Common kestrel (*Falco tinnunculus*)	338/18	Cloaca	MW533129	210
Long-eared owl (*Asio otus*)	690/19	Oropharynx	MW533132	231
Long-eared owl (*Asio otus*)	750/19	Oropharynx	MW533133	228
Long-eared owl (*Asio otus*)	969/19	Oropharynx	MW533134	228
Eurasian eagle owl (*Bubo bubo*)	353/19	Oropharynx	MW533135	210
Ural owl (*Strix uralensis*)	1544/19	Oropharynx	MW533137	228
Yellow-legged gull (*Larus michahellis*)	831/19	Oropharynx	MW533138	219
Hooded crow (*Corvus cornix*)	416/18	Oropharynx	MW533139	231
Hooded crow (*Corvus cornix*)	417/18	Oropharynx, cloaca	MW533140	201
Hooded crow (*Corvus cornix*)	115/19	Cloaca	MW533141	228
European greenfinch (*Chloris chloris*)	19/19	Oropharynx	MW533143	204
Domestic pigeon (*Columba livia domestica*)	238/17	Oropharynx	MW625922	225
Domestic pigeon (*Columba livia domestica*)	31/18	Oropharynx	MW625923	234
Domestic pigeon (*Columba livia domestica*)	229/18	Oropharynx	MW625925	225
Domestic pigeon (*Columba livia domestica*)	293/18	Oropharynx	MW625926	234
Domestic pigeon (*Columba livia domestica*)	294/18	Oropharynx, cloaca	MW625927	225
Domestic pigeon (*Columba livia domestica*)	314/18	Oropharynx	MW625928	231
Domestic pigeon (*Columba livia domestica*)	392/18	Oropharynx	MW625929	225
Domestic pigeon (*Columba livia domestica*)	1665/18	Oropharynx	MW625930	234
Domestic pigeon (*Columba livia domestica*)	11/19	Oropharynx	MW625931	219
Domestic pigeon (*Columba livia domestica*)	136/19	Oropharynx	MW625934	207
Domestic pigeon (*Columba livia domestica*)	183/19	Oropharynx	MW625935	234
Domestic pigeon (*Columba livia domestica*)	231/19	Oropharynx	MW625936	225
Domestic pigeon (*Columba livia domestica*)	255/19	Cloaca	MW625937	225
Domestic pigeon (*Columba livia domestica*)	633/19	Oropharynx	MW625938	225
Domestic pigeon (*Columba livia domestica*)	903/19	Oropharynx, cloaca	MW625939	234
Domestic pigeon (*Columba livia domestica*)	904/19	Oropharynx	MW625940	210
Common wood pigeon (*Columba palumbus*)	360/19	Oropharynx	MW626272	222

In songbirds, herpesvirus nt sequences detected in hooded crows (*Corvus cornix*) (416/18, 115/19, 417/19) were 100% identical and shared 76.12% nt identity with the closest relative; namely, the herpesvirus nt sequence of the common kestrel (338/18) detected in our study. The nt sequence of herpesvirus detected in a European greenfinch (*Chloris chloris*) (19/19) shared 68.16% nt identity with nt sequences of herpesviruses detected in hooded crows in this study (416/18, 115/19, 417/19).

DISCUSSION

This study describes the detection of novel and known herpesviruses in free-living avian species that are most similar to bird alphaherpesviruses according to a phylogenetic analysis based on partial DNA polymerase sequences. The occurrence of herpesviruses in free-living bird species in our study was 7.6%, with 34 out of 447 birds testing positive. Generally, different studies have shown different levels of occurrence of herpesvirus in free-living bird species. In owls, herpesvirus was detected at levels from 9.41% (16/170) (15) to 14.5% (8/55) (13), and in seabirds from 3.8% (4/104) (31) to 5.6% (14/250) (32), whereas a lower presence of herpesvirus was detected in passerine birds, at only 0.8% of positive samples (4/525) (14). In studies that examined free-living birds from different orders, the highest occurrence of herpesvirus (20.4%; 18/88) was seen in a study from Poland, where the presence of herpesvirus was detected in domestic pigeons, birds of prey, and non-raptorial birds (12). A lower prevalence was detected in a study from Australia, where 403 birds from 13 different genera were investigated and herpesvirus was detected in only three out of 409 birds (0.7%) (33). The differences in herpesvirus occurrence detected among different bird species may arise from varying study approaches. The major reasons could be the detection of herpesvirus in dead

birds, which was performed in most studies (12, 13, 31, 33), and the use of different PCR assay tests. However, other reasons should also be considered, such as the biology and ecology of the bird species tested, intermittent shedding of herpesviruses via the cloaca and/or trachea, and the immune status of infected birds (34).

Although sampling of birds presented to a veterinary clinic as wildlife casualties is a useful method for studying viruses in free-living birds, it should be considered that the inherently biased population of birds (e.g., injured birds, particular locations, and common species in an area) (33) presented to a veterinary facility could have impact on virus detection. Herpesviruses are known for their subclinical or latent infections, in which under certain conditions—particularly conditions which we may have encountered, such as collision/trauma and/or concurrent infections—can trigger reactivation, causing recurrent infection or asymptomatic shedding and consequently detection of herpesvirus in clinical samples (swabs). However, detection of herpesvirus in oropharyngeal and/or cloacal swabs in this study could indicate the detection of herpesvirus in an initial or reactivated phase of infection.

In raptorial birds, herpesvirus DNA was detected in 12 out of 121 birds (Accipitriformes 13.6%, 6/44; Falconiformes 2.9%, 1/34; and Strigiformes 11.9%, 5/42). Herpesviruses were detected in golden eagle (1/1), common buzzards (5/32), common kestrel (1/32), Eurasian eagle owl (1/4), long-eared owls (3/7), and Ural owl (1/8). The prevalence in susceptible species ranged from 16 to 100%.

Herpesvirus is the etiological agent responsible for inclusion body disease or herpesvirus hepatitis in hawks, falcons, and owls (9, 10). The disease is fatal, with mortality approaching to 100% (35). In the eagle and falcon population, individual cases of herpesvirus infection have been reported in different species (12, 36–39); however, only limited sequence data are available. Previous reports showed that herpesvirus sequences detected in eagles, owls, and falcons are very similar if not identical to the pigeon herpesvirus (CoHV-1) and that the consumption of infected pigeons or infected birds of prey is the most likely mode of transmission in raptors. The detection of CoHV-1 in a Eurasian eagle owl with 98.53 to 99.02% nt identity to CoHV-1 detected in domestic pigeons in this study could support the theory of prey-related herpesvirus transmission because the diet of the Eurasian eagle owl also includes potentially infected birds, especially in temperate regions and at low elevations (40). In Slovenia, for example, this may include known herpesvirus-infected bird species among birds of prey, pigeons, and corvids (41). However, detection and phylogenetic analysis of other herpesvirus sequences detected in three long-eared owls and one Ural owl in this study showed that these herpesvirus sequences have a distant relationship with known CoHV-1 and other alphaherpesvirus partial DNA polymerase sequences. These findings confirm the presence of different herpesvirus in owls (13), in which virus transmission between conspecifics was found to be more likely than transmissions from small mammals consumed as prey (15). However, interspecific disease transmission can take place at least occasionally due to intraguild superpredation; for example, when the Ural owl preys upon the long-eared owl (42). Herpesvirus sequences different from previously published ones were obtained also in falcons, eagles, and other bird orders.

In general, very different herpesvirus nt sequences of the partial DNA polymerase gene were detected among orders of raptors in this study. In our study, 51.7 to 54.7%, 60.29 to 63.3%, and 55.2 to 56.2% nt identities were shown between Acciptriformes and Falconiformes, Acciptriformes and Strigiformes, and Falconiformes and Strigiformes, respectively, and only one out of 12 herpesvirus nt sequences detected in raptors was similar to CoHV-1. The common kestrel herpesvirus DNA polymerase sequence was clustered together with known PsHV1 and PsHV2 detected in the African gray parrot and yellow-headed amazon, and it was different from previously detected CoHV-1 in various species of falcons, including the common kestrel (10, 12, 43). In raptors we have found clear herpesvirus clade divergence by grouping Accipitriformes and Strigifomes in one, and Falconiformes and Psittaciformes together with Passeriformes in other clade. This distinction is in line with avian phylogenetic relationships with Falconiformes being more related to Psittaciformes and Passeriformes than to other raptor groups (44). Apparently, herpesvirus evolution followed evolution history of their hosts rather than ecological convergences. Within Accipitriformes, herpesvirus sequences detected in five common buzzards were identical and were clustered together with the herpesvirus sequence detected in a golden eagle in our study and with the vulture herpesvirus detected in an Indian vulture (*Gyps indicus*). Even though raptors, Accipitriformes and Strigiformes, were the most infected group of species, even in this group we found herpesvirus-resistant species despite the higher number of individuals being examined—for example, sparrowhawks (*Accipiter nisus*) and especially tawny owls (*Strix aluco*)—which also agrees with previous studies (13, 15, 45).

It seems that herpesviruses in raptors are more or less species- or order-specific, and that they differ from herpesviruses detected in pigeons, which provide opposite results from previous reports, in which the hypothesis was that identical herpesviruses are detected in raptor and pigeon populations (10, 12, 33). Furthermore, they differ from herpesviruses detected in songbirds, showing 49.7 to 76.1% nt identity to herpesviruses detected in songbirds in this and previous reports (14), which excludes the possibility of songbirds as the source of herpesvirus infection in birds of prey and owls. There appear to be only two published reports on the detection and partial characterization of herpesvirus in wild passerine birds. In Poland, the viruses that were studied and detected in brain samples of the hooded crow and song thrush (*Turdus philomelos*) were classified as CoHV-1 through analysis of partial DNA polymerase gene sequences (12). A recent study of 525 herpesvirus-tested free-living passerine birds showed the presence of herpesvirus in two Eurasian blackcaps (*Sylvia atricapilla*), a common blackbird (*Turdus merula*), and a Eurasian blue tit (*Cyanistes caeruleus*) with relatively low nt identity between host species; however, within the same species (e.g., Eurasian blackcaps), identical herpesvirus sequences were detected (14). Similar results, with the detection of identical herpesvirus in three hooded crows and a diverse sequence detected in a European greenfinch, obtained in this study, could implicate the species-specific

feature of herpesviruses in songbirds as well. Furthermore, both studies showed that herpesviruses different from CoHV-1 are present in passerine birds. The natural host range of avian herpesviruses is highly restricted, and most herpesviruses are thought to have evolved in association with a single host species (23). Although previous studies have detected different partial DNA polymerase sequences in owls and their prey (14, 15), some studies describe the detection of identical partial DNA polymerase sequences in different bird species, also suggesting the possible transmission of herpesvirus between species; for example, in domestic pigeons and raptors (10–13). Some studies also show that some species of large gulls could predate on other bird species, including feral pigeons (46–48). A very similar DNA polymerase sequence detected in a yellow-legged gull and common wood pigeon, where the difference in sequence identity could arise from an adaptation step in the emergence of host-switching viruses (49), could suggest that pigeons could be the source of herpesvirus infection; however, based on the dietary habits of the yellow-legged gull (50), this seems very unlikely. The results clearly show that the yellow-legged gull is susceptible to herpesviruses that differ from previously described CoHV-1 detected in brain samples in a dead herring gull (*Larus argentatus*) (12). Interestingly, the DNA polymerase sequence detected in a wood pigeon showed only 60.78 to 61.30% nt identity to CoHV-1 detected in the order Columbiformes in this study. All the CoHV-1 strains detected in domestic pigeons are genetically very similar (99.51 to 100%) to each other and cluster together with the CoHV-1 detected in a Eurasian eagle owl in this study and the CoHV-1 detected in a prairie falcon. These results align with the fact that domestic pigeons are considered a natural reservoir of CoHV-1 and are a potential source of infection for any susceptible native species that might coexist with them or consume them (51). However, in tawny owls no CoHV-1 was detected in this or previous studies (13, 15) even though they occasionally prey on domestic pigeons (52); this may be related to their polymorphism (13) or coevolved lower susceptibility to herpesvirus, but this requires further study.

In this study, the herpesvirus DNA polymerase sequence was readily detected in oropharyngeal and/or cloacal swabs. Although the herpesvirus presence in oropharyngeal swabs was detected with a higher detection rate than in cloacal swabs (81.6 vs. 18.4%), one cannot not rule out the importance of cloacal swabs in the detection of herpesvirus in wild birds, especially because in three out of 34 live wild birds herpesvirus was detected only in a cloacal swab. This could point to herpesvirus replicating poorly in the intestinal tract and/or kidneys, or to the herpesvirus detected in the cloaca shedding in the oropharyngeal region and then passing to and being digested through the digestive system. Nevertheless, these results suggest that wild birds with an unknown infection status and unknown duration of infection should be tested with a combination of oropharyngeal and cloacal swabs, which would maximize the probability of herpesvirus detection. However, even with this combination of sample types, it must be emphasized that the likelihood of herpesvirus-infected birds testing negative in both samples remains a possibility because the latent non-productive stage of herpesvirus in live wild birds is difficult to detect due to challenging (in some cases inaccessible) sampling of possibly latency sites of alphaherpesvirus, such as sensory ganglia or mononuclear cells (34, 53). Furthermore, the various herpesvirus sequences detected in this study that differ from known avian herpesvirus could mean that different tissue tropism and replication sites could also be present, as previously described for herpesvirus. This remains to be investigated.

This study has contributed valuable information regarding herpesvirus present in wild birds in Slovenia, and it has shown that very different herpesvirus sequences are present in the wild bird population, at least for the region of the DNA polymerase gene analyzed. Some detected herpesviruses could be prey-related, whereas others show a tendency to be order- or species-specific. In the future, full-length genome characterization should be performed to establish whether the (previously unknown) partial viral sequences are novel herpesviruses and to gain more information regarding the diversity of herpesvirus circulating in the wild bird population.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/supplementary material.

ETHICS STATEMENT

Ethical review and approval was not required for the animal study because all the diagnostic methods were made to provide proper treatment and care of injured (clinical) birds/patients. All the birds were treated at the Clinic for Birds, Small Mammals, and Reptiles, Faculty of Veterinary Medicine, University of Ljubljana as wildlife casualties.

AUTHOR CONTRIBUTIONS

ZŽ, AV, and JR: conceptualization and writing—original draft preparation. ZŽ, AV, BS, UK, ZG, and JR: methodology. ZŽ, AV, UK, and BS: software. BS: validation. ZŽ, AV, JR, BS, and OZR: investigation. ZŽ, AV, JR, BS, ZG, and OZR: resources. ZŽ, BS, AV, UK, JR, OZR, and ZG: writing—review and editing. All authors have read and agreed to the published version of the manuscript.

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3 RAZPRAVA

Ugotavljanje in genetska karakterizacija HV pri poginjenih prostoživečih sovah v Sloveniji

Študija o prisotnosti HV pri poginjenih prostoživečih sovah je pokazala, da so v populaciji prostoživečih sov v Sloveniji prisotni HV z različnimi nukleotidnimi zaporedji odseka gena DNA polimeraze, ki se večinoma (7/8) razlikujejo od predhodno opisanih HV pri sovah in tudi od drugih nukleotidnih zaporedij HV, ki so objavljeni v genski banki. Z metodo PCR smo HV dokazali pri 8 od 55 (14,5 %) pregledanih sov. HV smo ugotovili pri veliki uharici (2/3), kozači (4/11) in mali uharici (2/15). Podobno incidenco (14,2 %) prisotnosti HV pri sovah so ugotovili tudi v poljski študiji leta 2012 (17), vendar je treba poudariti, da so v omenjeni študiji poleg manjšega števila pregledanih sov (n = 7) in detekcije HV samo v vzorcih možganov uporabili specifične začetne oligonukleotide za detekcijo CoHV1 z metodo PCR v realnem času. V naši študiji smo odkrili le eno nukleotidno zaporedje odseka gena DNA polimeraze HV, ki je bilo identično znanemu nukleotidnemu zaporedju CoHV1. Za razliko od uporabljenih specifičnih oligonukleotidnih zaporedij smo v naši raziskavi z uporabo konsenznih začetnih oligonukleotidov za detekcijo HV in metodo PCR ugotovili veliko raznolikost med odkritimi nukleotidnimi zaporedji HV pri poginjenih prostoživečih sovah v Sloveniji. Predvsem genetsko različni HV, latentna okužba s HV (18, 19) in možen obstoj različnih serovarov (20) predstavljajo izziv pri izbiri najprimernejših metod za proučevanje razširjenosti okužb s HV pri pticah.

Študiji iz Severne Amerike sta pokazali, da okužba s CoHV1 povzroča pogine pri jastrebih, sokolih in sovah (11, 12). V populaciji domačih golobov (*Columba livia*) je CoHV1 pogosto prisoten in nima skoraj nobenega vpliva na zdravje te vrste (11). Znano je, da HV niso vedno omejeni na določenega gostitelja in/ali na tarčni organ in da preskok na drugega gostitelja ali drug organski sistem lahko precej spremeni patogenost virusa (18). Različne študije so pokazale, da golobi, okuženi s CoHV1, predstavljajo vir okužbe za ptice ujede (21, 22, 23) in da se nukleotidno zaporedje HV, odkrito pri sovah, ne razlikuje od nukleotidnega zaporedja HV, odkritega pri golobih in sokolih (11, 13). Tako so vse študije genetske karakterizacije HV pri sovah pokazale, da so nekateri dokazani HV pri sovah genetsko zelo podobni ali celo identični CoHV1 (11, 13, 14, 17, 24). V naši študiji o detekciji HV pri poginjenih prostoživečih sovah (25) je bilo samo eno od osmih ugotovljenih nukleotidnih zaporedij HV pri sovah

identično CoHV1, in sicer HV sev BB/258/12, ki je bil dokazan pri veliki uharici, najdeni leta 2012. Do zdaj je bil CoHV1 ugotovljen pri virginijskem viru (*Bubo virginianus*) (11, 24), pasjem bubuku (*Ninox connivens*) in velikem bubuku (*Ninox strenua*) (13, 14). Na podlagi nam znanih podatkov in primerjave nukleotidnega zaporedja odseka gena DNA polimeraze HV nam je kot prvim uspelo dokazati prisotnost CoHV1 pri veliki uharici. Čeprav so naši rezultati potrdili prejšnje podatke iz študij o identičnosti CoHV1, ugotovljenega pri golobih, sovah in preostalih ujedah (11, 13, 14, 17), je treba poudariti, da je primerjava nukleotidnega zaporedja v omenjenih raziskavah potekala na nukleotidnem zaporedju odseka gena DNA polimeraze HV, ne pa na celotnem genomu. Študiji iz leta 2014 in 2017 (26, 27), v katerih so avtorji primerjali genom CoHV1, ki je bil dokazan pri domačem golobu, in genom sokoljega herpesvirusa 1 (angl. *Falconid herpesvirus 1* – FaHV1), ki je bil dokazan pri prerijskem sokolu (*Falco mexicanus*), sta pokazali, da se genoma razlikujeta vsaj v enem genu in da sta virusa zelo podobna, ne pa enaka. Da bi dobili jasnejšo sliko o raznolikosti HV pri sovah in pticah ujedah, bi bilo treba izvesti karakterizacijo genoma v celotni dolžini HV, kar je cilj prihodnjih študij. Vir okužbe s CoHV1, ki smo ga dokazali pri veliki uharici, ostaja neznan, vendar avtorji nekaterih študij (11, 13, 14, 22) predpostavljajo, da se virus prenese s plena na plenilca. Do 50 % prehrane velike uharice predstavljajo ptice (28), med katerimi so tudi vrste ptic, pri katerih je bil dokazan HV, kot so golobi in sokoli (29).

Nukleotidno zaporedje HV SU/436/13, dokazano pri poginjeni kozači, najdeni leta 2013, je bilo v primerjanem nukleotidnem zaporedju odseka gena DNA polimeraze HV najbolj podobno (95 %) nukleotidnemu zaporedju GaHV2. V filogenetsko drevo sta se poleg nukleotidnega zaporedja HV SU/436/13 in GaHV2 uvrstila tudi GaHV3 in puranji herpesvirus 1 (angl. *Meleagrid herpesvirus 1* – MeHV1). GaHV2 je znan tudi kot virus Marekove bolezni (angl. *Marek's disease* – MD), ki povzroča neoplastično limfoproliferativno bolezen ptic. Ta povzroča imunosupresijo z visceralnim in/ali kožnim limfomom, paralizo in/ali smrt (7). Naravne gostiteljice virusa MD so kokoši. Za okužbo in bolezen so dovzetne tudi prepelice, purani, fazani ter nekatere vrste rac in gosi. Druge vrste ptic, vključno z vrabci, jerebicami, golobi in pavi, so domnevno odporne na okužbo (7). Patološke in histopatološke lezije, ki so skladne z opisom MD, so bile opisane pri veliki uharici, čuku (*Athene noctua*) in virginijskem viru (30, 31), vendar v nobenem primeru ni bila dokazana dejanska prisotnost HV. Dokaz HV, ki je v primerjani regiji zelo podoben GaHV2, potrjuje predhodne opise o dovzetnosti sov za okužbo z virusom MD. Način okužbe kozače z virusom MD ni znan, vendar bi lahko domnevali –

podobno kot pri okužbah ptic ujed s CoHV1 (11, 13, 14, 22) – da je vir okužbe plen, okužen z virusom MD.

Preostalih šest nukleotidnih zaporedij HV, dokaznih pri poginjenih sovah, najdenih med letoma 1995 in 2015 v Sloveniji, se je v filogenetskem drevesu združilo v enotno skupino in so v oddaljenem sorodstvu z drugimi znanimi nukleotidnimi zaporedji DNA polimeraze alfaherpesvirusov ptic in sesalcev. Ti rezultati niso presenetljivi, saj se novi HV nenehno ugotavljajo pri različnih vrstah ptic, kot so indijski plešec (*Gyps indicus*) (32), gorski pavovec (*Polyplectron inopinatum*), malajski pavovec (*Polyplectron malacense*) in afriški pav (*Afropavo congensis*) (33), pa tudi pri drugih živalskih vrstah, kot so domači kunec (*Oryctolagus cuniculus*) (34), indonezijski pteropodidni netopir (*Cynopterus titthaecheilus*) (35), planinski pečinar (*Procavia capensis*) (36) in velika pliskavka (*Tursiops truncatus*) (37). Poleg tega smo z našo študijo pokazali, da so različne vrste sov različno dovzetne za okužbo s HV. Iz proučevanih populacij sov, dovzetnih za okužbo s HV, smo zaradi majhne velikosti vzorca (1–3 predstavniki posameznih vrst) izključili pegasto sovo (*Tyto alba*), velikega skovika (*Otus scops*), močvirsko uharico (*Asio flammeus*) in veliko uharico. Na podlagi razmeroma velikega števila pregledanih osebkov (n = 19) lesne sove, pri katerih HV nismo dokazali, predvidevamo, da lesna sova kot predstavnik svoje vrste ni dovzetna za okužbo s HV. Lesna sova je zelo polimorfna vrsta, kar se ne kaže le v različnih barvnih odtenkih in oblikah, temveč tudi v veliki fiziološki variabilnosti in sposobnosti preživetja v spremenljivih okoljskih razmerah (38, 39, 40, 41). Pri okuženih vrstah sov je bil polimorfizem do neke mere ugotovljen le pri kozači (42), vendar v primerjavi z lesno sovo v precej manjšem obsegu. V primeru okužbe s HV smo ugotovili, da je bolj polimorfna lesna sova manj dovzetna za okužbo v primerjavi z manj polimorfnimi vrstami sov, kot so velika uharica, kozača in mala uharica. Domnevamo, da večji polimorfizem v populaciji na splošno poveča odpornost proti nekaterim boleznim, vendar je to treba še dodatno raziskati. Vse proučevane vrste sov, razen velikega skovika, ki se pretežno hrani z žuželkami, imajo sorazmerno podobno prehrano. To so mali sesalci, med katerimi njihov glavni plen predstavljajo voluharice in miši (43). Študija je tudi pokazala, da so večje vrste sov (npr. velika uharica in kozača) bolj okužene kot manjše vrste, kar bi bilo lahko povezano z njihovim načinom prehranjevanja, t. i. superplenilstvom, z večjimi pticami in tudi sesalci (44, 45). HV smo dokazali tudi pri mali uharici, ki se za razliko od že omenjenih vrst prehranjuje pretežno z voluharicami (46, 47). Pri primerjavi sov iz različnih habitatov, npr. lesne sove kot gozdne vrste in male uharice kot vrste odprtih okolij, smo ugotovili, da okužba

ni povezana z življenjskim prostorom oziroma habitatom (43). Pri primerjavi zelo sorodnih sov iz rodu *Strix*, kozače in lesne sove, smo ugotovili, da okužba ni odvisna od filogenetska odnosa (48).

Okužba s HV v gnezdečih populacijah dveh sobivajočih sov rodu *Strix*

Naša študija, v kateri smo prisotnost HV ugotavljali pri živih prostoživečih sovah, ki so bile vzorčene na območju Krima in Jelovice med letoma 2017 in 2019, je pokazala, da je HV prisoten v populaciji kozač (29,1 %; 16/55), vendar nima zaznanega vpliva oziroma posledic na zdravje in reprodukcijo kozač. Pri živih in klinično zdravih lesnih sovah kljub razmeroma visokemu številu pregledanih sov (n = 115) nismo dokazali HV. Na detekcijo HV lahko vpliva več dejavnikov, kot so premajhno število testiranih ptic, neizločanje HV med vzorčenjem, zbiranje in shranjevanje vzorcev ter občutljivost in specifičnost metode PCR. Vsi ti dejavniki lahko vplivajo na negativne rezultate, zlasti če je koncentracija virusa v vzorcu nizka ali v primeru prisotnosti novih virusov. Okužene ptice niso pokazale nobenih kliničnih ali produktivnih odstopanj, in to kljub dejstvu, da je okužba s HV pri sovah opisana kot bolezen, ki se konča s poginom (10, 19). Obstaja tudi možnost, da so posledice okužbe vidne šele na dolgoročni produktivnosti in preživetju sov, zlasti v letih, ko v okolju primanjkuje hrane oziroma plena (49, 50). Detekcija odseka gena DNA polimeraze HV v orofaringealnih brisih klinično zdravih sov dokazuje asimptomatsko izločanje HV. HV imajo sposobnost vzpostaviti prikrito (latentno) stanje v gostitelju in se občasno reaktivirati. Prav tako je znano, da s HV okuženi posameznik lahko prenaša HV brez vidnih znakov bolezni (19). Izločanje HV v področje orofarinksa ptic je pomemben dejavnik z vidika potencialnega prenosa virusa z okuženega na neokuženega posameznika. Pri sovah samec običajno hrani samico med dvorjenjem, kasneje v obdobju starševstva pa samec in samica sodelujeta pri hranjenju svojih mladičev (43). V obeh primerih je to možen način prenosa HV. Čeprav HV v brisih kloake nismo odkrili, kar bi lahko bila tudi posledica prisotnosti inhibitorjev, ne smemo zanemariti pomena kloakalnih brisov pri dokazovanju HV, saj je bil v nekaterih študijah HV dokazan tudi v brisih kloake (8, 51). Naša študija je bila opravljena v letih, ko je bilo na voljo veliko plena (52), torej z nizkim prehranskim stresom za sove, a tudi tukaj so bili zaznani prenosi HV znotraj gnezd. V obdobju pomankanja hrane (manj plena) bi pri kozači lahko pričakovali intenzivnejše izločanje ter prenos virusa in tudi morebitni pojav različnih kliničnih znakov.

Različne študije kažejo, da se HV bolezen pri pticah ujedah lahko razvije po zaužitju CoHV1 okuženih golobov (11, 13, 14, 22). Ob dokazu odseka DNA polimeraze HV pri zdravih sovah se postavlja vprašanje, ali vse okužbe s HV pri sovah (11, 14) povzročijo bolezen, ki je v nekaterih primerih opisana celo kot bolezen s 100-odstotnim poginom. Trenutno znani HV pri sovah so GaHV2, znan tudi kot virus Marekove bolezni (25), CoHV1 (11, 25, 53), herpesvirus sov 1 (angl. *Strigid herpesvirus 1* – StHV1) (54) in HV, ugotovljeni pri poginjenih (25) in živih prostoživečih sovah v Sloveniji, ki smo jih poimenovali po gostiteljski vrsti (npr. *Strix uralensis* HV). Vsi omenjeni HV so genetsko različni in možno je, da so nekateri od njih, npr. CoHV1, bolj patogeni za populacijo sov kot drugi, prav tako bi se lahko med seboj razlikovali po načinu prenosa in patogenezi. Za popolnejše informacije o potencialni patogenosti HV pri sovah bi bilo potrebno v prihodnosti opraviti izolacijo virusa, določanje serotipov in popolno karakterizacijo genoma teh različnih HV.

Nukleotidna zaporedja HV, dokazana pri kozačah na Krimu in Jelovici, so se v filogenetsko drevo uvrstila skupaj z nukleotidnimi zaporedji HV, dokazanimi pri poginjeni veliki uharici, kozači in mali uharici iz naše predhodne študije (25), in z nukleotidnim zaporedjem StHV1, dokazanim pri živem prostoživečem virginijskem viru iz Amerike (54). Kljub dokazu HV pri tesno sorodnih sovah iz rodu *Bubo* in *Strix* (55) je naša študija pokazala, da dovzetnost za okužbo s HV znotraj monofiletske skupine Striginae (56) vendarle ni enaka. Že znotraj rodu *Strix* je ugotovljeno, da sta lesna sova in kozača tesno sorodni vrsti (56), vendar so naši rezultati pokazali, da se vrsti bistveno razlikujeta po dovzetnosti za okužbo s HV. Treba je pa poudariti možnost prisotnosti nedetektiranih HV in se zavedati, da preprosta korelacija med HV in molekularno filogenijo sove ne zadostuje za razlago odnosa med gostiteljem in parazitom. Možni razlogi za morebitno manjšo dovzetnost lesne sove za okužbo s HV so lahko v presnovnih lastnostih (metabolizmu), kot je bilo predlagano v prejšnji študiji (25), ali v nedavnih koevolucijskih procesih, ki so se razlikovali med različnimi gostitelji sove in HV. Obe vrsti sov se zelo razlikujeta v življenjskih lastnostih. Na našem proučevanem območju kozača velja za ledenodobno reliktno vrsto, katere večji del populacije najdemo v borealnem podnebnem pasu Evrazije, medtem ko je lesna sova vrsta zmernega pasu, ki se je nedavno razširila v severno Evropo (52, 57, 58, 59). Naša študija je bila omejena na manjše geografsko območje znotraj zmernotoplega podnebnega pasu, kjer vrsti sobivata in kjer kozača doseže svojo južno mejo razširjenosti (60). Za dokaz, da so vrste sov, ki živijo na meji svojega naravnega habitata, bolj dovzetne za okužbe s HV ter za natančnejšo proučitev vpliva podnebja

in življenjskih lastnosti sov na okužbo s HV bi bilo treba zajeti predvsem večje geografsko območje, na katerem bi vzorčili sove iz roda *Strix*.

Čeprav je koevolucija gostitelja in virusa primarni način evolucije HV, so v literaturi opisani tudi medvrstni prenosi (11, 61, 62). Filogenetska analiza delnega nukleotidnega zaporedja DNA polimeraze HV, ugotovljenega pri rumenogrli miši in kozači z istega območja, je pokazala, da se HV, dokazani pri rumenogrli miši, razlikujejo od HV, dokazanih pri kozači. HV, ki okuži tako sesalce kot ptice, je redko opisan. Po nam znanih podatkih je prašičji herpesvirus 1 (angl. *Suid herpesvirus 1* – SuHV1) povzročitelj bolezni Aujeszkega (znana tudi kot psevdosteklina), ki spada v poddružino *Alphaherpesvirinae*, edini HV, ki pri prašičih večinoma povzroča blago obliko bolezni. Hujšo obliko bolezni, vključno s poginom, povzroča pri drugih vrstah sesalcev, kakor tudi pri kokoših in golobih. Prenos HV je bil s sesalcev na ptice do zdaj opisan le v eksperimentalnih pogojih (63). Nukleotidni zaporedji odseka gena DNA polimeraze HV, dokazani pri rumenogrli miši v naši študiji, sta se v filogenetskem drevesu uvrstili v poddružino *Betaherpesvirinae* skupaj s citomegalovirusom 3 (angl. *Cytomegalovirus 3* – CMV 3), ki je bil ugotovljen pri rumenogrli miši v Nemčiji. Pri enakem gostitelju so bili odkriti tudi drugi HV, in sicer CMV 1, CMV 2, *Apodemus flavicollis rhadinovirus 1* (AlphaRHV1) in *Murine gammaherpesvirus 68* (MHV-68), ki so bili uvrščeni v druge skupine v poddružinah *Betaherpesvirianae* in *Gammaherpesvirinae* (64). V tej obsežni študiji iz Nemčije so bile ob več kot 1100 pregledanih vzorcih krvi in tkiva odkriti številni že znani in novi betaHV in gamaHV pri različnih vrstah prostoživečih glodavcev, kot so gozdna voluharica (*Clethrionomys glareolus*), travniška voluharica (*Microtus agrestis*), poljska voluharica (*Microtus arvalis*), belogrla miš (*Apodemus sylvaticus*), hišna miš (*Mus musculus*), siva podgana (*Rattus norvegicus*) in črna podgana (*Rattus rattus*). Tako kot mi tudi avtorji nemške študije niso dokazali prisotnosti alfaHV pri glodavcih. Možni vzroki so nizka prevalenca alfaHV pri glodavcih, neznano mesto lokacije v latentni fazi okužbe, nizka koncentracija virusa v vzorcih ali hipoteza, da se alfaHV pri glodavcih med evolucijo HV nikoli niso razvili ali so dejansko izginili (64).

Čeprav glodavci predstavljajo visok delež prehrane sov, pa vendar pri kozači in tudi pri drugih vrstah sov, kot sta velika uharica in mala uharica, nismo zaznali prisotnosti HV glodavcev. Filogenetska analiza dokazanih nukleotidnih zaporedij odseka gena DNA polimeraze HV pri prostoživečih sovah v Sloveniji in Ameriki kaže, da ugotovljeni HV spadajo v poddružino

Alphaherpesvirinae. Virusi iz poddružine *Betaherpesvirinae* in *Gammaherpesvirinae* so znani po specifičnosti oziroma omejenosti na določene naravne gostitelje (2), kar bi lahko bil eden od razlogov, da pri sovah nismo odkrili HV glodavcev. Kljub temu bi zaradi velikega deleža prehrane, ki jo predstavljajo glodavci, in relativno občutljive metode PCR pričakovali prisotnost DNA polimeraze HV glodavcev v orofaringealnem predelu in kloaki ptic, vsaj kot posledico kontaminacije oziroma zaužitja s HV okuženega glodavca. Na podlagi naših izsledkov in študije, ki so jo napisali Ehlers in sod. (64), lahko izključimo pot prenosa HV z glodavcev na sove, saj je bila ugotovljena zelo majhna podobnost med nukleotidnimi zaporedji odseka gena DNA polimeraze HV sov, miši in drugih vrst glodavcev. Zaradi visoke stopnje raznolikosti med HV velja poudariti, da bi uporabljena metoda PCR v določenih primerih lahko tudi zgrešila pojav novega HV (8). Najverjetnejši način prenosa HV pri sovah je znotraj vrste, predvsem s staršev na njihove mladiče.

V gnezdu na gori Krim smo ugotovili, da sta imela samec in samica kozače v svojem gnezdu enega mladiča kozače in enega mladiča lesne sove, kar je bila posledica tekmovalnega izgona lesne sove s strani večje kozače iz gnezda. DNA polimerazo HV smo dokazali pri samcu in samici kozače ter pri mladiču kozače, pri mladiču lesne sove pa HV nismo ugotovili. S študijo smo potrdili prejšnje ugotovitve (25, 65), da lesne sove niso dovzetne za okužbo s HV, tudi če so v tesnem stiku z okuženimi in tesno sorodnimi kozačami. Možni razlogi nedovzetnosti lesne sove za okužbo s HV so poleg že omenjenega večjega polimorfizma ter genetske in fiziološke variabilnosti (25) lahko tudi zgodovinski evolucijski razlogi. Lesna sova je zgodovinsko gledano dlje prisotna in je koevoluiran gostitelj HV, medtem ko bi se okužbe s HV pri kozačah lahko pojavile relativno nedavno, vendar bi bilo to treba v prihodnje podrobneje raziskati.

Detekcija HV pri pticah pevkah, odlovljenih v obdobju jesenske selitve v Sloveniji

Pri prostoživečih pticah pevkah, ki smo jih ujeli med jesensko selitvijo v Sloveniji, smo DNA polimerazo HV dokazali pri 0,76 % (4/525) testiranih ptic. Če to primerjamo s preostalimi študijami, opazimo, da je razširjenost HV pri testiranih pticah pevkah v Sloveniji dokaj nizka. Pri prostoživečih pticah ujedah je HV prisoten v 9,4 % do 17,8 % pregledanih ptic (17, 25, 66), pri prostoživečih morskih pticah je razširjenost HV nekolika nižja, in sicer med 3,8 % (4/104) in 5,6 % (14/250) (67, 68). Razlogi za različno razširjenost HV pri pticah so številni in vključujejo dejavnike, kot so biologija in ekologija testiranih vrst ptic, občasno/različno izločanje HV preko kloake in/ali žrela ter imunski status okuženih ptic (8). Razlike lahko

izhajajo tudi iz različnih študijskih pristopov; v nekaterih študijah so HV dokazali pri mrtvih pticah (17, 25), pri katerih bi bila razširjenost lahko višja kot pri živih in klinično zdravih pticah. Večjo pojavnost HV pri prostoživečih pticah pevkah na migraciji bi pričakovali, če bi v študijo poleg kloakalnih brisov vključili tudi orofaringealne brise (14). Poleg tega je ugotovljena nizka prisotnost HV pri pticah pevkah na migraciji lahko povezana tudi z nekaterimi tehničnimi razlogi. Postopki vzorčenje, shranjevanja in transporta vzorcev imajo pomemben vpliv na natančno in ponovljivo diagnostiko (69). Čeprav so brisi z virusnim transportnim medijem primernejši za detekcijo virusa kot suhi brisi (70), različne študije opisujejo uporabo suhih brisov kot učinkovito metodo pri diagnostiki virusnih okužb, npr. pri virusih iz družin *Orthomyxoviridae* (71) in *Paramyxoviridae* (72), pa tudi *Herpesviridae* (73). Vzorci kloak ptic pevk, odlovljenih med jesensko selitvijo, so bili odvzeti in transportirani v skladu z opisanimi postopki v omenjenih študijah, zato menimo, da ima uporaba suhih brisov manjši, vendar ne bistven vpliv na nizko stopnjo detekcije HV.

S HV okužene prostoživeče ptice pevke, ki so bile odlovljene med jesensko selitvijo, ob veterinarskem pregledu niso imele kliničnih znakov bolezni. V omenjeni študiji smo HV dokazali pri klinično zdravih pevkah, kar je v nasprotju z izsledki prejšnjih študij, v katerih so bili HV dokazani pri bolnih in poginulih pticah pevkah (5, 15, 16). Podobno kot pri drugih vrstah, se tudi pri pticah pevkah domneva, da se s HV naravno okužijo v zgodnjem obdobju življenja, v katerem nato različni stresni dejavniki v življenju spremenijo ravnovesje med gostiteljem in virusom ter povzročijo pojav bolezni (19, 74, 75). Treba je omeniti tudi potencialno korist HV kot del simbiotskega odnosa z gostiteljem. Nekatere študije so pokazale, da ima asimptomatska okužba s HV pri gostitelju zaščitni učinek pred virusno in bakterijsko okužbo (76). Miši, okužene z mišjimi gamaHV, so bile manj dovzetne za okužbo z bakterijama *Listeria monocytogenes* in *Yersinia pestis* ter so imele višjo stopnjo preživetja po okužbi z virusom influence tipa A (76, 77, 78). Prav tako je bila pri kronično okuženih miših s HV ugotovljena manjša pojavnosti tumorjev (79, 80). Dokaz HV v vzorcih zdravih živali tako poleg okužbe in možne bolezni dopušča tudi možnost simbioze, ki zaščiti gostitelja pred drugimi boleznimi.

Filogenetska analiza je pokazala, da so dokazana nukleotidna zaporedja odseka gena DNA polimeraze HV pri štirih prostoživečih pticah pevkah, odlovljenih med jesensko selitvijo, najbolj sorodna nukleotidnim zaporedjem DNA polimeraze HV, ki so bili dokazani pri velikim

rumenočopastem kakaduju in olivnem kormoranu, ter bolj znanim HV, kot so GaHV1, PsHV1 in PsHV2, ki so dokazani pri perutnini in papigah. Dokaz treh nukleotidnih zaporedij DNA polimeraze HV z relativno nizko homologijo nakazuje na verjetnost, da v proučevani populaciji prostoživečih ptic pevk na migraciji v Sloveniji krožijo različni HV. Dokaz identičnih nukleotidnih zaporedij DNA polimeraze HV pri dveh črnoglavkah in dokaz identičnih nukleotidnih zaporedij DNA polimeraze HV pevk (angl. *Passerine herpesvirus 1* – PaHV1) v dveh ločenih izbruhih bolezni pri Gouldinovi amadini v ujetništvu (15, 16) kaže, da so HV, odkriti pri pticah pevkah vrstno specifični, velja pa poudariti, da sposobnost preskoka gostitelja pri HV ostaja neznana (18). Izziv prihodnjih študij je ugotoviti, ali so dokazani HV pri pevkah avtohtonega evropskega izvora ali so bili vir prenosa pobegle uvožene eksotične ptice v prostoživečo avifavno (populacijo), kar je že znana pot prenosa različnih bolezni (81).

Vse s HV okužene vrste ptic pevk, odlovljene med jesensko selitvijo v naši raziskavi, so vrste, ki migrirajo na kratke razdalje, ali sedentarne vrste. Najvišja pojavnost HV je bila pri plavčku (13 %), vendar je to lahko tudi posledica majhne velikosti vzorca te vrste (n = 8). Nobene okužbe ni bilo ugotovljene pri pticah pevkah, ki migrirajo na dolge razdalje, čeprav so bile v študijo vključene v razmeroma velikem številu, vključno s trstnicami (*Acrocephalus* spp.), vrtno penico (*Sylvia borin*) in vrbjim kovačkom (*Phylloscopus collybita*). Selitev ima izjemen selekcijski vpliv na ptice (82), še predvsem na morebitno oslabele, obolele ali v našem primeru okužene osebke. Selekcijski pritisk je visok, tudi pri selivkah na kratke razdalje, kar kaže tudi ugotovljen nizek pojav okužb s HV. Dovzetnost za okužbo s HV pri prostoživečih pticah pevkah bi bilo treba v prihodnje proučiti s kombiniranim vzorčenjem živih in mrtvih ptic, pri čemer bi pričakovali višjo pojavnost pri dovzetnih vrstah ptic pevk.

HV pri bolnih, poškodovanih ali oslabelih prostoživečih pticah, sprejetih na Kliniko za ptice, male sesalce in plazilce

Pri prostoživečih vrstah ptic, sprejetih na Kliniko za ptice, male sesalce in plazilce Veterinarske fakultete, smo odsek gena DNA polimeraze HV dokazali pri 7,6 % (34/447) pregledanih ptic. Odvzem orofaringealnih in kloakalnih brisov pri kliničnem pregledu in za diagnostiko bolnih, poškodovanih ali oslabelih ptic se je izkazal kot uporabna metoda za proučevanje virusov pri prostoživečih pticah, saj smo se s tem izognili nepotrebnemu stresu, ki bi ga sicer povzročili z lovljenjem in fiksacijo ptic. Poudariti je treba, da je bila v tem primeru v študijo vključena pristranska populacija ptic, npr. poškodovane ptice, ptice z določenih območij, najbolj pogoste

ptice z območja itd., kar lahko vpliva na pojavnost HV v proučevani populaciji. HV so znani po svojih subkliničnih ali latentnih okužbah, ki se pod določenimi vplivi – v primeru ptic, sprejetih na kliniko, so to trki oziroma travme in/ali sočasne okužbe – reaktivirajo, kar vodi v pojav bolezni ali asimptomatsko izločanje in posledično tudi možno detekcijo HV v kliničnih vzorcih (brisih). Poleg detekcije DNA polimeraze HV v fazi reaktivacije je treba omeniti tudi možnost primarne okužbe in s tem povezan dokaz DNA polimeraze HV pri prostoživečih pticah.

Pri plenilskih pticah je bila DNA polimeraza HV dokazana pri 9,9 % (12/121) pregledanih ptic. HV smo dokazali pri 13,6 % (6/44) pregledanih ujed (*Accipitriformes*), pri 2,9 % (1/34) pregledanih sokolov (*Falconiformes*) in 11,9 % (5/42) pregledanih sov (*Strigiformes*). DNA polimerazo HV smo dokazali pri planinskem orlu (*Aquila chrysaetos*) (1/1), kanji (*Buteo buteo*) (5/32), postovki (*Falco tinnunculus*) (1/32), veliki uharici (1/4), mali uharici (3/7) in kozači (1/8).

Izsledki prejšnjih raziskav kažejo, da so nukleotidna zaporedja HV, dokazana pri ujedah, sovah in sokolih, zelo podobna in v nekaterih primerih identična CoHV1 ter da je plenjenje okuženih golobov ali okuženih plenilskih ptic najverjetnejši način prenosa pri roparskih pticah (11, 13, 14, 25, 53). Dokaz CoHV1 pri domačih golobih, sprejetih na kliniko, z 98,53- do 99,02-odstotno homologijo v nukleotidnem zaporedju s CoHV1, dokazanim pri veliki uharici (obe vrsti sta bili sprejeti na kliniko), podpira teorijo s plenom povezanega prenosa HV. Dokazano je, da prehrana velike uharice predvsem v zmernih regijah in nizki nadmorski višini (28) vključuje tudi potencialno okužene vrste ptic, kot so manjše ujede in golobi (29). Nukleotidna zaporedja HV, dokazana pri kozači in treh malih uharicah, sprejetih na kliniko, so bila med seboj identična in so se razlikovala od nukleotidnih zaporedij CoHV1. Te ugotovitve potrjujejo izsledke prejšnje raziskave o prisotnost različnih HV pri sovah (25). Dokaz identičnih nukleotidnih zaporedij HV pri različnih vrstah sov, sprejetih na kliniko, kaže na morebitni medvrstni prenos HV zaradi t. i. znotrajcehovskega plenjenja, npr., ko kozača upleni malo uharico (83).

Na splošno smo pri prostoživečih plenilskih pticah, sprejetih v veterinarsko oskrbo, dokazali različna nukleotidna zaporedja DNA polimeraze HV. Primerjava dokazanih HV pri ujedah in sokolih je pokazala od 51,7- do 54,7-odstotno homologijo v nukleotidnem zaporedju, med ujedami in sovami od 60,29- do 63,3-odstotno homologijo v nukleotidnem zaporedju in med

sokoli in sovami od 55,2- do 56,2-odstotno homologijo v nukleotidnem zaporedju. Samo ena od 12 dokazanih nukleotidnih zaporedij HV je bila podobna CoHV1, ki je bil še pred nedavnim edini opisani HV pri plenilskih pticah. Nukleotidno zaporedje DNA polimeraze HV, ugotovljeno pri postovki, je bilo v filogenetskem drevesu uvrščeno skupaj s PsHV1 in PsHV2, odkritimi pri sivem žakoju (*Psittacus erithacus*) in rumenoglavi amazonki (*Amazona oratrix*), in se je razlikovalo od predhodno dokazanega CoHV1 pri postovki in drugih vrstah sokolov (11, 17, 26). V filogenetskem drevesu so se HV, ugotovljeni pri plenilskih pticah, razvrstili v dve ločeni skupini. HV, ugotovljeni pri pticah iz redov *Accipitriformes* in *Strigiformes*, so se združili v eno klado ter HV, ugotovljeni pri pticah iz redov *Falconiformes*, *Psittaciformes* in *Passeriformes*, v drugo klado. Razlika v filogenetski razvrstitvi ugotovljenih nukleotidnih zaporedij HV je v skladu s filogenetskimi odnosi ptic, kjer je red *Falconiformes* bolj soroden z redoma *Psittaciformes* in *Passeriformes* kot z drugimi plenilskimi pticami (84). Na podlagi tega predvidevamo, da je evolucija HV pri prostoživečih pticah sledila evoluciji njihovih gostiteljev, ne pa ekološkim konvergencam. Pri ujedah *Accipitriformes* so bila nukleotidna zaporedja HV, dokazana pri petih kanjah, identična in so bila v filogenetskem drevesu združena skupaj z nukleotidnim zaporedjem HV, dokazanim pri planinskem orlu in indijskem plešcu. Čeprav sta bila reda *Accipitriformes* in *Strigiformes* med plenilskimi pticami najbolj okuženi skupini ptic, so tudi v tej skupini kljub visokemu številu pregledanih osebkov prisotne vrste ptic, ki so nedovzetne za okužbo s HV, npr. skobec (*Accipiter nisus*) in predvsem lesna sova, kar potrjujejo tudi prejšnje raziskave HV pri poginjenih in klinično zdravih odlovljenih sovah v Sloveniji (25, 65, 66).

HV, dokazani pri plenilskih pticah, se z 49,7- do 76,1-odstotno homologijo v nukleotidnem zaporedju razlikujejo tudi od HV, dokazanih pri pticah pevkah med jesensko selitvijo (51), in od HV, dokazanih pri pticah pevkah, sprejetih na kliniko, kar izključuje možnost, da so ptice pevke vir okužbe s HV pri plenilskih pticah. Pri treh sivih vranah (*Corvus cornix*), sprejetih na kliniko, smo dokazali identična nukleotidna zaporedja HV, ki so se razlikovala od nukleotidnega zaporedja HV, dokazanega pri zelencu, ki je bil prav tako sprejet na kliniko. Podobne so bili tudi izsledki raziskave o prisotnosti HV pri pevkah med jesensko selitvijo; ugotovili smo različna nukleotidna zaporedja HV pri različnih pticah pevkah, vendar identična nukleotidna zaporedja HV pri predstavnikih iste vrste. Pri pticah pevkah, sprejetih na kliniko, smo potrdili prisotnost vrstno specifičnih HV, ki se razlikujejo od predhodno dokazanega CoHV1.

Zelo podobni nukleotidni zaporedji DNA polimeraze HV (99,51 %) sta bili dokazani tudi pri rumenonogem galebu (*Larus michahellis*) in grivarju (*Columba palumbus*). Prenos HV med različnimi vrstami pticami je bil opisan v številnih študijah, predvsem med pticami ujedami in njihovim ptičjim plenom, kot je domači golob (11, 14, 17, 25). Znano je, da nekatere vrste galebov plenijo tudi druge vrste ptic, vključno z golobi (85, 86, 87), vendar je glede na prehranjevalne navade rumenonogega galeba (88) majhna verjetnost, da bi prišlo do prenosa HV z grivarja na rumenonogega galeba. Razlika v nukleotidnem zaporedju HV bi lahko nastala zaradi procesa prilagoditve virusa na novega gostitelja (89). Rumenonogi galeb je tako dovzeten za HV, ki se razlikuje od predhodno opisanega CoHV1, dokazanega v vzorcih možganov poginjenega srebrnega galeba (*Larus argentatus*) (17). Dokazana DNA polimeraza HV pri grivarju je imela le 60,8- do 61,3-odstotno homologijo v nukleotidnem zaporedju s CoHV1, ki je bil dokazan pri domačih golobih. Dokaz različnih DNA polimeraze HV pri grivarju in mestnem golobu (znotraj istega reda ptic) ni presenetljiv, saj smo podobne rezultate dobili tudi pri pticah iz drugih redov. Nukleotidna zaporedja CoHV1, dokazana pri domačih golobih, sprejetih na kliniko, so si z 99,51- do 100-odstotno homologijo v nukleotidnem zaporedju med seboj genetsko zelo podobna/identična in so se v filogenetskem drevesu razvrstila skupaj s CoHV1, dokazanim pri veliki uharici, sprejeti na kliniko, in CoHV1, dokazanim pri prerijskem sokolu. Ti rezultati so v skladu z dejstvom, da domači golobi veljajo za naravni rezervoar CoHV1 in so potencialni vir okužbe za vse za okužbo s HV dovzetne avtohtone vrste, ki bi lahko sobivale z njimi ali jih zaužile (14).

Pri prostoživečih pticah, sprejetih na kliniko, je bila DNA polimeraza HV dokazana v brisu orofarinksa in/ali brisu kloake. Čeprav je bila DNA polimeraza HV pogosteje dokazana v odvzetih brisih orofarinksa (81,6 %) kot v odvzetih brisih kloake (18,4 %), je treba poudariti pomen kloakalnih brisov pri detekciji HV pri prostoživečih pticah, saj je bil HV pri treh pticah, sprejetih na kliniko, dokazan le v brisu kloake. Nizka detekcija HV v kloakalnih brisih lahko kaže na slabo pomnoževanje HV v prebavnem traktu in/ali ledvicah. Druga možnost je, da se je HV izločal v orofaringealni regiji in širil v prebavni trakt, kjer se je DNA polimeraza HV dokazala v brisih kloake. Ti rezultati dokazujejo, da je treba prostoživečim pticam z neznanim statusom okužbe in z neznanim trajanjem okužbe za dokaz HV odvzeti tako orofaringealne kot tudi kloakalne brise. Kljub okužbi je treba poudariti možnost nedetektiranega virusa v odvzetih vzorcih, kajti latentno neproduktivno stopnjo HV pri živih prostoživečih pticah je težko odkriti zaradi zahtevnega in v nekaterih primerih nedostopnega vzorčenja morebitnih latentnih mest

alfaHV, kot so senzorični gangliji ali mononuklearne celice (8, 90). Poleg tega so se številna ugotovljena nukleotidna zaporedja HV razlikovala od že znanih alfaHV, kar pomeni, da so lahko prisotni tudi drugi tkivni tropizmi in mesta replikacije, kot je trenutno opisano in znano za HV.

4 SKLEPI (ZAKLJUČKI)

Z opravljenimi raziskavami in objavljenimi študijami smo prvo postavljeno hipotezo (»Pri prostoživečih pticah v Sloveniji so prisotne okužbe s HV. Ti so genetsko podobni HV, kakor tudi različni od HV, ki so trenutno objavljeni v genski banki. Z molekularnimi metodami bomo HV dokazali pri novih vrstah ptic, pri katerih do zdaj še niso bili dokazani.«) **potrdili.**

- Z opravljenimi raziskavami smo pri prostoživečih pticah v Sloveniji dokazali prisotne okužbe s HV. Ugotovili smo, da v populaciji prostoživečih ptic krožijo različni HV. Nekateri HV so genetsko podobni že znanim HV, večina pa se jih razlikuje od trenutno objavljenih v genski banki.
- Z opravljenimi raziskavami smo razširili seznam dovzetnih gostiteljskih vrst prostoživečih ptic za okužbo s HV.

Z opravljenimi raziskavami in objavljenimi študijami smo drugo postavljeno hipotezo (»Prenos HV poteka tako na horizontalni ravni med plenilci kot tudi s plena na plenilca.«) **delno potrdili**.

- Z opravljenimi raziskavami smo ugotovili, da se HV prenaša z okuženih staršev na njihove mladiče. HV se lahko prenaša tudi prek plena, okuženega s HV, kot so okuženi golobi, na plenilsko ptico.
- Rezultati študije so pokazali, da so večje vrste sov (npr. velika uharica in kozača) bolj okužene kot manjše vrste, kar bi bilo lahko povezano z njihovim načinom prehranjevanja, t. i. superplenilstvom, z večjimi pticami in tudi sesalci.
- Prenosa HV z malih sesalcev na prostoživečo plenilsko ptico nismo zaznali. Z opravljenimi raziskavami in objavljenimi študijami smo drugo hipotezo potrdili, vendar to velja le za prenose s ptice na ptico, ne pa tudi s sesalca na ptico, kar kaže določeno evolucijsko specifičnost HV.

Druge ugotovitve:

- Pri primerjavi sov iz različnih habitatov, npr. lesna sova kot gozdna vrsta in mala uharica kot negozdna vrsta smo ugotovili, da okužba ni povezana z življenjskim prostorom. Okužba prav tako ni odvisna od filogenetska odnosa sov.
- HV smo dokazali tudi v populaciji živih prostoživečih kozač in ugotovili, da okužba s HV nima zaznanega vpliva oziroma posledic na zdravje in reprodukcijo kozač.

- Kljub tesnim sorodstvom lesne sove in kozače so naši izsledki pokazali, da se vrsti bistveno razlikujeta po dovzetnosti za okužbo s HV.
- S študijo smo dokazali HV pri klinično zdravih pevkah, kar je v nasprotju z izsledki prejšnjih študij, v katerih so bili HV dokazani pri bolnih in poginulih pticah pevkah. HV nismo dokazali pri pticah pevkah, ki se selijo na dolge razdalje.
- Za dokaz HV pri živih prostoživečih pticah se mora odvzeti tako orofaringealne kot kloakalne brise.

5 POVZETEK

Študija o prisotnosti HV pri poginjenih prostoživečih sovah je pokazala, da so v populaciji prostoživečih sov v Sloveniji prisotni HV z različnimi nukleotidnimi zaporedji DNA polimeraze HV. Predhodne študije genetske karakterizacije HV pri sovah so pokazale, da so dokazani HV pri sovah genetsko zelo podobni oziroma identični HV, dokazanim pri golobih in poimenovanim CoHV1. Izsledki naše raziskave kažejo, da se kar sedem od osmih ugotovljenih nukleotidnih zaporedij HV razlikuje od predhodno opisanih HV pri sovah in tudi od drugih nukleotidnih zaporedij HV, ki so objavljeni v genski banki. Na podlagi razmeroma velikega števila pregledanih osebkov lesne sove, pri katerih HV nismo dokazali, predvidevamo, da lesna sova kot predstavnik svoje vrste ni dovzetna za okužbo s HV. Lesna sova je zelo polimorfna vrsta, kar se ne kaže le v različnih barvnih odtenkih in oblikah, temveč tudi v veliki fiziološki variabilnosti in sposobnosti preživetja v spremenljivih okoljskih razmerah. Dokazali smo, da so večje vrste sov (npr. velika uharica in kozača) pogosteje okužene kot manjše vrste, kar bi bilo lahko povezano z njihovim načinom prehranjevanja, t. i. superplenilstvom, z večjimi pticami in tudi sesalci. Ob pregledu literature ugotavljamo, da smo prisotnost HV prvič dokazali pri veliki uharici. Prisotnost HV smo dokazali tudi pri živih prostoživečih sovah, ki smo jih odlovili na območju Krima in Jelovice med letoma 2017 in 2019. Ugotovili smo, da HV kljub prisotnosti nima zaznanega vpliva oziroma posledic na zdravje in reprodukcijo kozač. Pri živih in klinično zdravih lesnih sovah, kljub razmeroma visokemu številu pregledanih sov, HV nismo dokazali, tudi če je bila ta vrsta v testnem stiku s kozačo, okuženo s HV. Na podlagi naših rezultatov in drugih študij lahko izključimo pot prenosa HV prek uplenjenih okuženih glodavcev na sove, saj je bila med nukleotidnimi zaporedji HV, dokazanih pri sovah, miših in drugih glodavcih, zelo majhna podobnost (51,1 %). Verjetnejši je prenos HV znotraj vrste, s staršev na mladiče, in sicer prek sline oziroma pri hranjenju mladičev. Prisotnost DNA polimeraze HV smo dokazali tudi pri prostoživečih pticah pevkah, ki smo jih ujeli med jesensko selitvijo v Sloveniji. Ob primerjavi z drugimi študijami smo ugotovili, da je razširjenost HV pri testiranih pticah pevkah v Sloveniji dokaj nizka (0,8-odstotna; 4/525). Prostoživeče ptice pevke, ki so bile odlovljene med jesensko selitvijo, ob veterinarskem pregledu niso kazale kliničnih znakov bolezni, kar pomeni, da smo v omenjeni študiji dokazali HV pri klinično zdravih osebkih, kar je v nasprotju z izsledki prejšnjih študij, v katerih so HV dokazani le pri bolnih in poginulih pticah pevkah. Dokaz treh nukleotidnih zaporedij HV z relativno nizko homologijo kaže na verjetnost, da pri proučevanih selečih se pticah pevkah v Sloveniji krožijo različni HV.

Pri prostoživečih vrstah ptic, sprejetih v veterinarsko oskrbo, smo dokazali zelo različna nukleotidna zaporedja HV. Nekateri ugotovljeni HV se prenašajo prek okuženega plena, kot so domači golobi, medtem ko so bili drugi ugotovljeni HV bolj specifični za posamezno vrsto in/ali red ptic in bi pri pticah lahko bili del koevolucijskega razvoja virusa in gostitelja. Odvzem orofaringealnih in kloakalnih brisov pri kliničnem pregledu in diagnostiko bolnih, poškodovanih ali oslabelih ptic se je izkazal kot uporabna metoda za proučevanje virusov pri prostoživečih pticah, saj smo se s tem izognili nepotrebnemu stresu, ki bi ga sicer povzročili z lovljenjem ptic. Izsledki prav tako dokazujejo, da je treba prostoživečim pticam z neznanim statusom in z neznanim trajanjem okužbe za dokaz HV odvzeti tako orofaringealne kot tudi kloakalne brise. Pri številnih vrstah prostoživečih ptic smo z opravljeno raziskavo prvič dokazali prisotnost HV.

6 SUMMARY

A study of the presence of HV in dead wild owls showed that HVs with different nucleotide sequences of HV DNA polymerase are present in the population of wild owls in Slovenia. Previous genetic characterization studies of HVs in owls have shown that HVs in owls are genetically very similar to or identical with the HVs detected in pigeons, named CoHV-1. The research showed that seven out of eight HV nucleotide sequences identified differ from previously described HVs in owls and also from other HV nucleotide sequences published in GenBank. Based on the relatively high number of tawny owls examined, in which HVs were not detected, it is assumed that the tawny owl as a representative of its species is not susceptible to HV infection. The tawny owl is a very polymorphic species, which is reflected not only in different color morphs, but also in high variability in its physiology and survival capability in variable environmental conditions. The results indicate that larger owl species (e.g., the Eurasian eagle owl and Ural owl) are more often infected than smaller species, which could be related to their diet; that is, superpredation by taking larger birds and mammals. This is believed to be the first report of HV in the Eurasian eagle owl. HV was also detected in live free-living owls captured on Mount Krim and the Jelovica Plateau between 2017 and 2019. However, HV-infected owls did not show any clinical or productivity deviances. No HVs were detected in live free-living tawny owls, despite the relatively high number of owls tested and even if they were in close contact with HV-infected Ural owls. Based on these results and the results presented in other HV studies, it is possible to rule out a HV transmission route via infected small rodents to owls because there was very little similarity between the HV nucleotide sequences detected in owls, mice, and other rodents. It is more likely that HV is transmitted in an intraspecific manner, especially from adults to their chicks. This study also detected the HV DNA polymerase gene in free-living songbirds that were caught during the autumn migration in Slovenia. The occurrence of HV in free-living songbirds was quite low (0.8%; 4/525). During veterinary examination, free-living songbirds that were caught during the autumn migration showed no clinical signs of the disease, which means that HV was detected in clinically healthy individuals, which contrasts with previous studies, in which HV was detected only in sick and dead songbirds. The detection of HV with different partial DNA polymerase gene sequences indicates the probability that different HVs have been circulating in the population of free-living songbirds in Slovenia. Very different HV nucleotide sequences were detected in wild birds presented to the Clinic for Birds, Small Mammals, and Reptiles at the Faculty of Veterinary

Medicine as wildlife casualties. Some HVs detected are transmitted through infected prey, such as domestic pigeons, whereas other HVs detected were more specific to bird species and/or orders and could be part of the coevolutionary process of their bird host. Oropharyngeal and cloacal swab sampling as part of the clinical examination and diagnostics of sick, injured, or weakened birds has proven to be a useful method for detecting viruses in wild birds because this avoids unnecessary stress that would otherwise be caused by hunting birds. The results of this study suggest that wild birds with an unknown infection status and unknown duration of infection should be tested with a combination of oropharyngeal and cloacal swabs to maximize the probability of herpesvirus detection. HVs were detected for the first time in many wild bird species.

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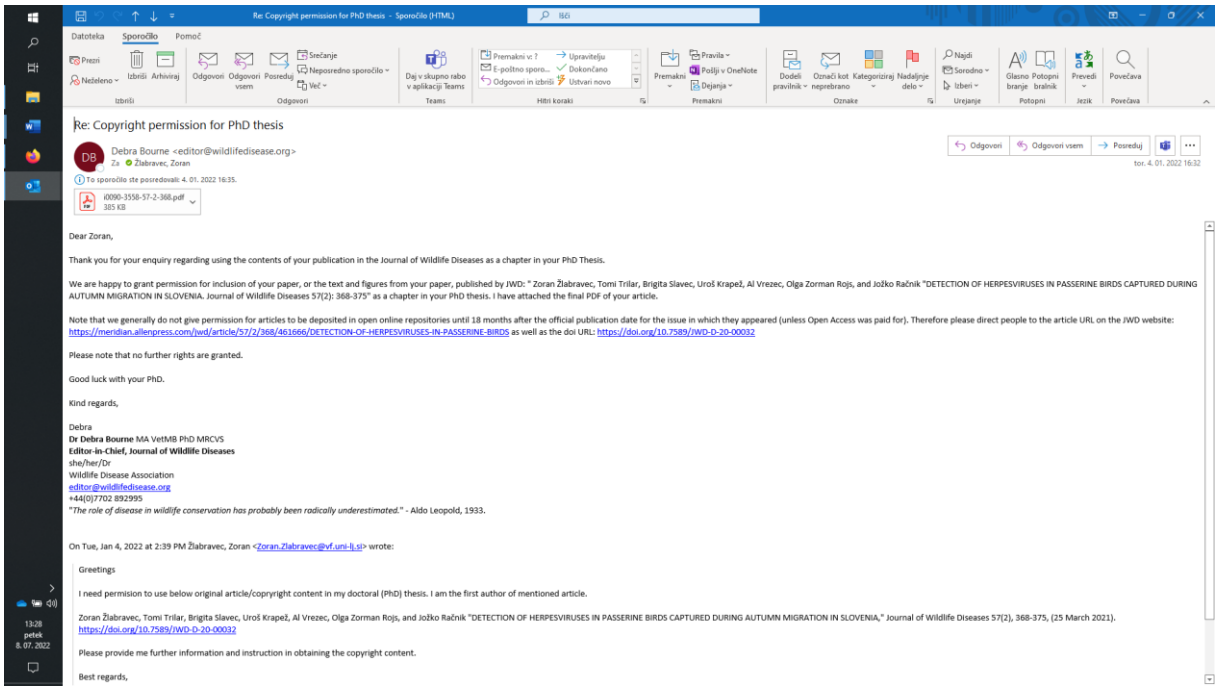

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