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Original Study

Detection of Herpesviruses in Cockatoos (*Cacatuidae*) in Europe

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Abstract: Avian herpesviruses, including psittacid herpesvirus 1 (PsHV1; *Iltovirus psittacidalpha1*), are of particular concern in avian collections because they can lead to severe disease with high mortality. In recent years, distinct novel avian alphaherpesviruses were detected in wild cockatoos originating from Victoria, Australia. These were further characterized as cacatuid herpesvirus 1 (CaHV1) and cacatuid herpesvirus 2 (CaHV2). The cockatoos affected by these viruses exhibited severe signs of disease attributed to concurrent infections with other pathogenic agents. The clinical significance of these novel herpesviruses remains unknown, and no information on the pathogenicity and prevalence of these viruses is available. During routine diagnostic testing, 2 clinically healthy pet sulphur-crested cockatoos (*Cacatua galerita*) from Vienna, Austria, tested positive for a CaHV. To gain more information on the prevalence of this virus in cockatoos kept in Europe, a retrospective evaluation of samples from cockatoos submitted to a European diagnostic laboratory between 2016 and 2023 was initiated. In total, 468 samples from cockatoos were evaluated. Herpesviruses were detected in 16 (3.4%, 95% confidence interval: 1.8-5.1) samples. Fourteen of the positive samples were further screened, 13 were most closely related to the previously described CaHVs, and 1 was psittacid herpesvirus 1. Phylogenetic analysis of amino acid sequences from 11 of the detected herpesviruses showed that 5 were identical to CaHV1, 2 were distinct but closely related to CaHV1, and 3 were identical to one another and clustered with CaHV1 and CaHV2 but on a separate branch. Due to the lack of further information from these positive tested cockatoos, the clinical importance of these viruses remains unknown.

Key words: Herpesvirus, polymerase chain reaction, avian, cockatoo, Cacatuidae, psittacine, sulphur-crested cockatoos, *Cacatua galerita*

INTRODUCTION

Several herpesviruses have been identified in birds, many associated with various, often severe, disease manifestations.[1–7] In psittacine species, psittacid herpesviruses belonging to the subfamily *Alphaherpesvirinae* and the genus *Iltovirus* are of particular concern. Psittacid herpesvirus 1 (PsHV1, *Iltovirus psittacidalpha1*) is the cause of Pacheco disease, an acute, rapidly fatal disease of parrots.[8–11] Infections with PsHV1 genotypes 1, 2, and 3 have also been associated with mucosal papillomas, and genotype 3 has been linked to biliary and pancreatic duct carcinomas.[10,12–14] Birds that are subclinically infected or have survived herpesviral disease remain persistently infected for life, posing a risk to other birds in contact with them.[15]

There are limited reports available on herpesviruses in cockatoos (family Cacatuidae). Recently, novel avian alphaherpesviruses were detected in wild cockatoos originating from Victoria, Australia. A sulphur-crested cockatoo (*Cacatua galerita*) first tested positive for an alphaherpesvirus in 2018.[16] The virus was named cacatuid herpesvirus 1 (CaHV1). A related but genetically distinct herpesvirus was isolated from a little corella (*Cacatua sanguinea*) and named cacatuid herpesvirus 2 (CaHV2).[17] This second virus has since been classified in the species *Iltovirus cacatuidalpha2*.[18] Phylogenetic analyses found both CaHVs to cluster most closely with PsHV1. Both birds with these CaHVs exhibited severe signs of illness, likely related to concurrent infection with other pathogenic agents. As

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a result, the pathogenicity of these novel herpesviruses remains uncertain.

To the authors' knowledge, these 2 are the only descriptions of CaHV1 and CaHV2 in the literature, and no occurrence has been previously described in Europe. The occasion for this study was the detection of a CaHV in 2 cockatoos in a veterinary clinic in Austria. Due to the lack of available information on the prevalence of these viruses in cockatoo species outside of Australia, a retrospective study was undertaken to evaluate the prevalence of CaHVs in cockatoos in a routine diagnostic laboratory in Europe over a period of 7 years and to evaluate the relationships of the detected herpesviruses to one another and to the previously described CaHV1 and CaHV2 based on available data. We also aimed to determine whether there were any indications of changes in infection prevalence over the study period. We hypothesized that the prevalence of CaHV-like viruses in cockatoos in Europe would be $>$ 0%, and that the majority of herpesviruses detected in cockatoos in Europe would be most closely related to these viruses.

MATERIALS AND METHODS

A pair of sulphur-crested cockatoos from a mixed collection was presented in 2023 for preventive clinical examination. These included tests for the presence of psittacine beak and feather disease virus (PBFDV), polyomaviruses, herpesviruses, avian bornaviruses, and *Chlamydia*. The samples were analyzed by an accredited diagnostic laboratory (LABOKLIN GmbH & Co KG, Bad Kissingen, Germany). The cockatoos were part of a collection of 31 psittacine birds, consisting of 26 grey parrots (*Psittacus erithacus*) and 3 red-crowned parakeets (*Cyanoramphus novaezelandiae*). Although the species were housed in separate aviaries, they were kept in the same room. Each bird underwent a physical examination by a qualified avian specialist. In addition to the physical examination, sterile rayon-tipped dry transport swabs were collected from the crop and the cloaca, feather samples were obtained, and whole blood samples were collected from the right jugular vein and placed into EDTA tubes. To minimize the risk of contamination during sampling, the individuals collecting the samples used powder-free sterile gloves during the exams and changed gloves between handling each bird. After collection, the swabs were placed individually into sterile tubes without transport medium. Due to financial constraints, the samples were pooled according to species groups for polymerase chain reaction (PCR) testing rather than testing each sample individually. The physical examination and sampling of the 2 sulphur-crested cockatoos were repeated after 91 days. The birds did not show signs of illness during this period. During this second examination, sterile dry crop and cloacal swabs were again collected from each bird to repeat the testing for herpesviruses; this time, the swabs were tested individually. Only 22 days later, the female bird had to be euthanized due to an unrelated cause. The owners declined a complete pathological examination. However, postmortem swabs were taken from the crop and cloaca along with biopsy samples from the liver and central nervous system.

Testing for infectious agents was carried out using previously described methods for PBFDV,[19] bornaviruses,[20] and *Chlamydia*.[21] Detection of polyomaviruses was carried out using a proprietary PCR targeting the VP1 gene. Detection of herpesviruses was carried out using a pan-herpesvirus PCR targeting a portion of the DNA-dependent DNA polymerase gene,[22] followed by Sanger sequencing in 1 direction using primer IYG from the second round of the PCR reaction to confirm the specificity of the reaction. Internal extraction controls were used during DNA preparation for all samples. Positive and negative controls for all reactions consisted of samples with confirmed presence of the target pathogen and PCR-grade water, respectively.

The pooled sample from the 2 sulphur-crested cockatoos tested positive for CaHV. Due to the lack of information available on herpesviruses in cockatoos and possible consequences for an affected collection, a retrospective study of samples from cockatoos submitted to the diagnostic laboratory (LABOKLIN) was carried out. For the study, all samples from birds submitted to the laboratory for herpesvirus PCR testing between January 1, 2016, and December 31, 2023, were searched, and all samples for which the species was clearly identified as a cockatoo were included. All samples had been tested using the same protocol listed above. Sample types included swabs, feathers, whole blood in EDTA, and tissues. Multiple sample types were frequently submitted; however, only 1 positive result for the animal was documented. Exact identification of sample origin was often not possible. Data on herpesvirus-positive samples were collected, and if available, sequences from the PCR products were analyzed by BLAST.[23] For samples for which sequence quality was sufficient, the amino acid sequences were trimmed to equal lengths and aligned with corresponding sequences from CaHV1 and CaHV2; PsHV1, PsHV2, and PsHV3; and a gallid alphaherpesvirus 1 using Multiple Sequence Comparison by Log-Expectation[24] and a maximum likelihood distance tree constructed using Molecular

Table 1. Retrospective evaluation of samples from cockatoos tested for herpesviruses by polymerase chain reaction testing.

Species	Number tested	Number positive (% positive)
Galah (*Eolophus roseicapilla*)	117	1 (0.85)
White cockatoo (*Cacatua alba*)	39	1 (2.56)
Sulphur-crested cockatoo (*Cacatua galerita*)	37	3 (8.11)
Yellow-crested cockatoo (*Cacatua sulphurea*)	35	1 (2.86)
Tanimbar corella (*Cacatua goffiniana*)	28	2 (7.14)
Salmon-crested cockatoo (*Cacatua moluccensis*)	28	3 (10.71)
Palm cockatoo (*Probosciger aterimus*)	15	1 (6.67)
Solomons cockatoo (*Cacatua ducorpsii*)	14	1 (7.14)
Pink cockatoo (*Laphochroa leadbeateri*)	13	0
Red-tailed black cockatoo (*Calyptorhynchus banksia*)	11	0
Gang-gang cockatoo (*Callocephalon fimbriatum*)	4	0
Little corella (*Cacatua sanguinea*)	3	0
Red-vented cockatoo (*Cacatua haematuropygia*)	1	0
Blue-eyed cockatoo (*Cacatua ophthalmica*)	1	0
Long-billed corella (*Cacatua tenuirostris*)	1	0
Unknown	121	3 (2.48)
Total	468	16 (3.42)

Evolutionary Genetics Analysis, version 11,[25] to evaluate possible relationships between the individual herpesviruses detected and previously described avian herpesviruses. Calculations were carried out using the Jones-Taylor-Thornton substitution model, uniform substitution rates, and the nearest neighbor interchange inference option. Bootstrapping was carried out with 500 replications.

The influence of species and year on herpesvirus detection in cockatoos was evaluated by chi-squared test (SAS OnDemand for Academics, SAS Institute Inc, Cary, NC, USA).[26] The cutoff for significance was set at $P < 0.05$. No additional factors (age, sex, clinical signs) were evaluated due to the lack of data on these factors in most cases.

RESULTS

Neither of the 2 sulphur-crested cockatoos originally examined nor any of the 29 psittacine birds from the same collection showed any signs of disease upon initial presentation. Negative results were obtained for PBFD-virus, bornavirus, polyomavirus, and *Chlamydia* in all birds. Herpesvirus testing was also negative in all birds except the pooled sample from the 2 sulphur-crested cockatoos. The BLAST analysis indicated that the herpesvirus was most closely related to CaHV1. The repeated testing of individual swabs from the 2 sulphur-crested cockatoos after 91 days and the euthanized female 113 days after initial testing resulted in negative PCR results for herpesviruses.

The retrospective exploration of the data for samples originating from cockatoos tested at the diagnostic laboratory identified 468 samples that had been tested for herpesviruses between 2016 and 2023, of which 16 (3.4%, 95% confidence interval: 1.8-5.1) were positive (Table 1). There was no statistically significant difference in detection rates based on cockatoo species ($P = 0.76$) or year of detection ($P = 0.06$).

Sequence data was available for 14 of the 16 herpesvirus PCR products (Table 2). Available sequence data was generally short and of varying quality. The BLAST analysis indicated that 13/14 (92.9%) detected herpesviruses were most closely related to either CaHV1 or CaHV2. The sequence from 1 (7.1%) of the herpesviruses detected from a Solomons cockatoo (*Cacatua ducorpsii*) in 2022 was 100% identical to the corresponding sequence of PsHV1 (GenBank accession #AY623127.1).

Three of the available sequences were not included in the further phylogenetic analysis due to issues with the quality of the sequences (Table 2). The sequences from the remaining 11 herpesviruses detected in this study were used for the phylogenetic analysis. Amino acid sequences from these viruses were trimmed to a length of 38 amino acids. The sequences from 5 of the detected viruses, including 1 from the sulphur-crested cockatoos described here (sample 2305A43567; Table 2) as well as from an additional sulphur-crested cockatoo, a Goffin's cockatoo (*Cacatua goffiniana*), a Palm cockatoo (*Probosciger aterrimus*), and a yellow-crested cockatoo (*Cacatua sulphurea*), were identical to the corresponding sequence from CaHV1 (Fig 1). Two additional sequences from an umbrella cockatoo (*Cacatua alba*) and a cockatoo of unknown species were also most closely related to

Table 2. Herpesvirus-positive cockatoo samples and their country of origin. Confirmation of herpesviruses was done using polymerase chain reaction testing, sequence length, and results of the Basic Local Alignment Search Tool analysis.

							BLAST results			
Lab number	Year	Country	Sex	Species	Age, years	Sequence length, bp	Closest matching sequence(s)	Virus name	Query covery	Identity
1809S42349	2018	UK	u	Cockatoo	u	137	MF576271.1	CaHV1	98%	88.15%
							NC_076966.1	CaHV2	96%	87.88%
1903M04807	2019	Sp	u	Goffin's cockatoo (*Cacatua goffiniana*)	u	140	MF576271.1	CaHV1	100%	91.43%
							NC_076966.1	CaHV2	99%	91.37%
2002R13435[a]	2020	Ger	m	Goffin's cockatoo (*Cacatua goffiniana*)	26	126	NC_076966.1	CaHV2	90%	92.5%
2002R21627	2020	Hun	f	Palm cockatoo (*Probosciger aterrimus*)	u	139	MF576271.1	CaHV1	100%	96.43%
2002R37391	2020	Pol	u	Umbrella cockatoo (*Cacatua alba*)	u	126	MF576271.1	CaHV1	100%	92.06%
							NC_076966.1	CaHV2	100%	91.27%
2008B10795[a]	2020	Slv	u	Salmon-crested cockatoo (*Cacatua moluccensis*)	6	n.a.	n.a.	n.a.	n.a.	n.a.
2009S03769[a]	2020	Ger	u	Sulphur-crested cockatoo (*Cacatua galerita*)	u	144	MF576271.1	CaHV1	100%	93.2%
2201S02367	2022	Ger	u	Salmon-crested cockatoo (*Cacatua moluccensis*)	u	144	MF576271.1	CaHV1	95%	87.68%
							NC_076966.1	CaHV2	93%	87.41%
2211S14928	2022	Ger	u	Yellow-crested cockatoo (*Cacatua sulphurea*)	u	125	MF576271.1	CaHV1	100%	92.8%
							NC_076966.1	CaHV2	100%	92%
2208R08074	2022	Pol	u	Solomons cockatoo (*Cacatua ducorpsii*)	u	126	AY623127.1	PsHV1	100%	100%
2211M23298[a]	2022	Sp	u	Cockatoo	u	130	MF576271.1	CaHV1	100%	86.92%
							NC_076966.1	CaHV2	99%	86.05%
2210R04055[a]	2022	CZ	u	Galah (*Eolophus roseicapilla*)	7	n.a.	n.a.	n.a.	n.a.	n.a.
2305A43567	2023	Aus	u	Sulphur-crested cockatoo (*Cacatua galerita*)	u	127	MF576271.1	CaHV1	100%	92.91%
							NC_076966.1	CaHV2	100%	92.13%
2307S19689	2023	Ger	u	Salmon-crested cockatoo (*Cacatua moluccensis*)	38	125	MF576271.1	CaHV1	100%	88.8%
							NC_076966.1	CaHV2	100%	88.8%
2308R10394	2023	Ger	f	Sulphur-crested cockatoo (*Cacatua galerita*)	u	128	MF576271.1	CaHV1	100%	98.44%
							NC_076966.1	CaHV2	100%	92.97%
2310R60036	2023	Ger	u	Cockatoo	u	126	MF576271.1	CaHV1	100%	91.27%
							NC_076966.1	CaHV2	100%	91.27%

Abbreviations: BLAST, Basic Local Alignment Search Tool; bp, base pairs; UK, United Kingdom; Sp, Spain; Ger, Germany; Hun, Hungary; Pol, Poland; Slv, Slovakia; CZ, Czech Republic; Aus, Austria; m, male; f, female; u, unknown; n.a., no sequence available; CaHV, cacatuid herpesvirus; PsHV, psittacid herpesvirus.

[a] Marked samples were not used for the phylogenetic analysis either due to lack of availability of sequence data or poor quality of the available sequence.

CaHV1 although the sequences from these 2 viruses differed in individual amino acid positions from that of CaHV1 and from one another. The sequences from 3 of the herpesviruses (2 salmon-crested cockatoos [*Cacatua moluccensis*] and a cockatoo of unknown species) were identical to one another but differed from those of previously described avian herpesviruses. These viruses clustered with CaHV1 and CaHV2 but on a separate branch (Fig 1).

DISCUSSION

This study suggests a low prevalence (3.4%, 95% confidence interval: 1.8-5.1) of herpesvirus infection in cockatoos in Europe, which has not changed significantly over recent years. The viruses detected were from a variety of cockatoo species, all kept in human care, and originated from a wide range of European countries (Table 2). Whereas the detected prevalence was low, this study does indicate that herpesviruses are relatively widespread in captive psittacine collections in Europe. However, the use of samples submitted to a diagnostic laboratory by veterinarians also means that the sample population was biased toward animals either with clinical disease or that were presented to veterinary practices for other reasons. It is, therefore, not possible based on these results to evaluate the true prevalence of herpesvirus infections in cockatoos in human care in Europe. Gaining more understanding of the prevalence of CaHV in captive populations and the species most commonly infected is important for future investigations and for developing routine screening recommendations.

Most of the herpesviruses analyzed in this study were closely related to CaHV1 and CaHV2, and these viruses were first discovered in a wild sulphur-crested cockatoo[16] and a wild little corella,[17] respectively. The phylogenetic

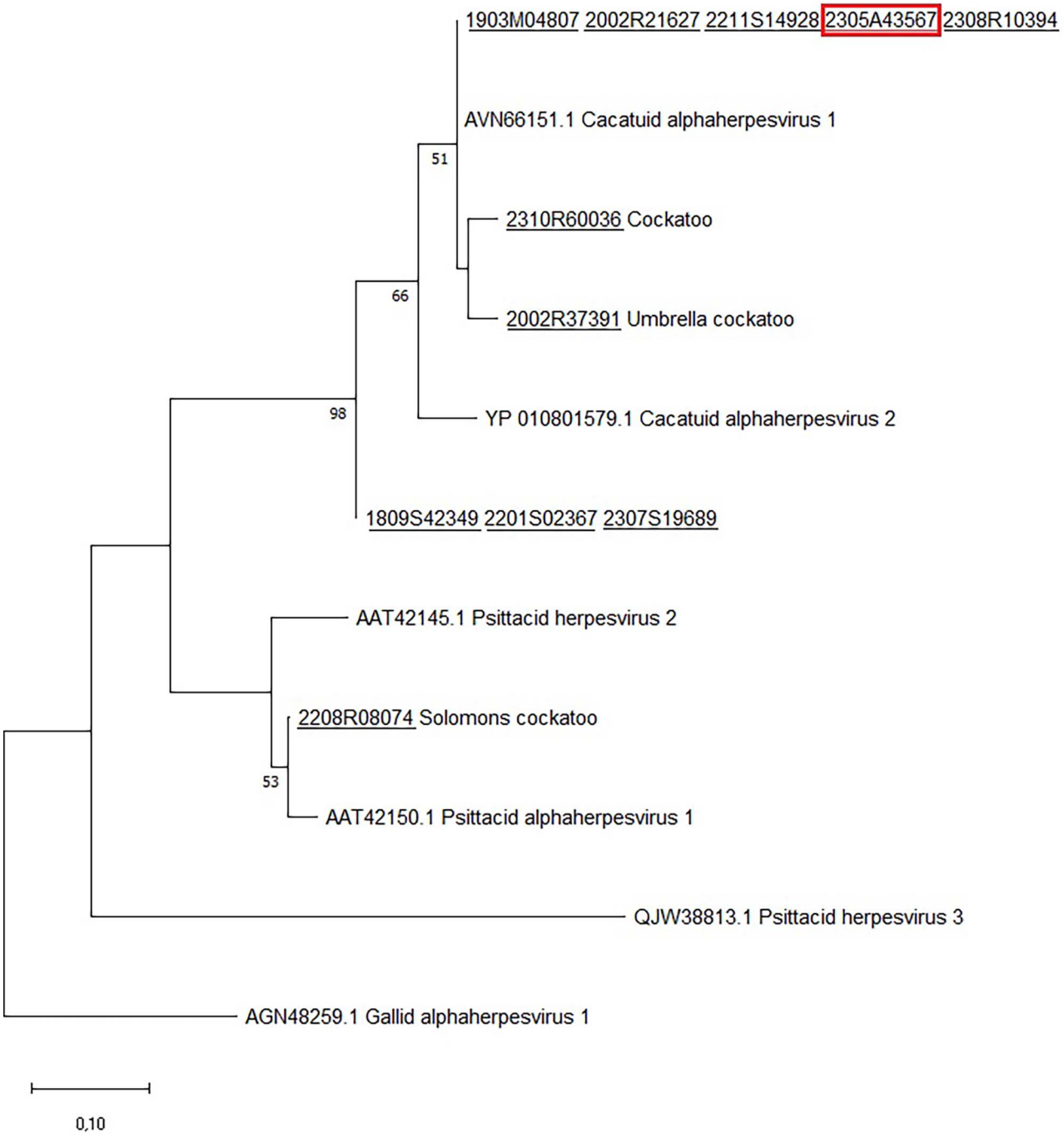

Figure 1. Maximum likelihood phylogenetic tree based on 38 amino acid sequences from 11 detected cockatoo herpesviruses with cacatuid alphaherpesvirus 1 and 2; psittacid alphaherpesvirus 1, 2, and 3; and gallid alphaherpesvirus 1. Gallis alphaherpesvirus 1 was used as the root. GenBank accession numbers of the previously described avian herpesviruses are included in the labels. Sequences from the present study are designated by their laboratory numbers and underlined. The herpesvirus detected in the sulphur-crested cockatoos (*Cacatua galerita*) described here is indicated by a red box. Branchings with >50% support of 500 bootstrap replicas are shown on the individual nodes. The scale bar indicates the number of amino acid substitutions per site.

analysis of the detected herpesviruses indicates the presence of related but previously undescribed viruses as well. While these conclusions are based on short viral sequences, they indicate that additional study of the herpesviruses found in cockatoos is needed. In addition to these diverse CaHVs, PsHV1 was detected in 1 cockatoo. Given that PsHV1 is known to infect a wide range of psittacine birds,[10] this finding was not surprising. The

finding of genetically diverse herpesviruses in the cockatoos sampled here was possible due to the use of degenerate primers using a PCR designed to detect a wide range of members of the family *Orthoherpesviridae*.[22] The primers used here have repeatedly been used for diagnostic testing of a wide variety of species.[16,17,27,28] The PCR products obtained with this assay should always be sequenced to both confirm the specificity of results and better understand what herpesvirus has been detected. Given the diversity of herpesviruses detected in this study, the use of methods capable of detecting a range of herpesviruses in cockatoos should be considered when screening these animals for herpesviruses.

The clinical relevance of CaHVs in cockatoos is not yet understood. Both CaHV1 and CaHV2 were originally described in cockatoos that were suffering from disease possibly caused by concurrent infections, and the role of the herpesviruses was not clear.[16,17] No clinical information was available for the cockatoos included in the present retrospective study. The 2 sulphur-crested cockatoos originally examined in this study were both considered clinically healthy and remained so over a 4-month monitoring period. However, as is common with herpesviruses, infection does not always result in overt disease.[29] Further studies are necessary to better understand the biology and potential pathogenicity of herpesviruses in cockatoos.

Interestingly, the 2 sulphur-crested cockatoos from the known collection only shed the virus during the initial testing. At that time, the birds experienced the stress of confiscation and relocation. Herpesviruses are known for their capacity to cause subclinical infections, which can reactivate under stressful conditions.[8,30,31] Viral shedding may depend on multiple factors, and long-term shedding of herpesviruses has been recorded in birds.[13,15]

Based on current data, it is clear that genetically diverse herpesviruses can be found in cockatoos in human care in Europe. More work is needed to fully understand this diversity and to understand the relationships between these viruses, their host ranges, and their pathogenic potential.

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