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PREVALENCE OF COLUMBID HERPESVIRUS INFECTION IN FERAL PIGEONS FROM NEW SOUTH WALES AND VICTORIA, AUSTRALIA, WITH SPILLOVER INTO A WILD POWERFUL OWL (*NINOX STRUENA*)

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ABSTRACT: Columbid herpesvirus-1 (CoHV-1) is widespread in feral pigeons in North America and Europe. We used a PCR assay to detect CoHV-1 DNA in oral and cloacal tissues and oral swabs from naturally infected pigeons. Fifty-three feral pigeons from five flocks in Australia (n=3 from south-central Victoria and n=2 from Sydney) were examined for CoHV-1 DNA. We detected CoHV-1 DNA in oral mucosa and cloacal mucosa, with higher concentrations in the oral mucosa. The sensitivity of testing oral swabs was the same as testing the tissue, indicating that testing of oral swabs from live birds is an effective means of screening flocks for CoHV-1 infection. Infection was found in all five of the flocks examined and the prevalence of infection ranged from 70% to100%. Most positive birds could be detected with a single-amplification PCR, but a nested amplification was required to detect others. Oral swabs from Australian native doves and pigeons (n=18) and the introduced Collared Dove (*Streptopelia chinensis*; n=2) were also tested by the nested PCR and all were negative for CoHV-1 DNA. We describe a fatal infection of CoHV-1 in a wild Powerful Owl (*Ninox strenua*) that was observed feeding on feral pigeons. This is the first known case of CoHV-1 causing death in a wild bird of prey in Australia. Our data suggest that CoHV-1 is widespread in feral pigeon flocks in Australia but we did not find it in native doves and pigeons. Spillover into native avian predator species may be occurring.

Key words: Australia, *Columba livia*, columbid herpesvirus-1, *Ninox strenua*, pigeon, polymerase chain reaction, Powerful Owl, Rock Dove.

INTRODUCTION

Emerging viral diseases have had significant impacts on both domestic and wild animals ranging from amphibians (ranavirus; Robert 2010) and fish (iridovirus; Whittington et al. 2010), to mammals (canine distemper virus, West Nile virus; Packer et al. 1999; Hofmeister 2011) and birds (West Nile virus; Hofmeister 2011). One such virus is the columbid herpesvirus-1 (CoHV-1). This virus was first described in the domestic pigeon or Rock Dove (*Columba livia*) lofts in the US in 1945 (Smadel et al. 1945) and has subsequently been documented in pigeon lofts from around the world (Phalen et al. 2011; Stenzel et al. 2012). When introduced into a naïve or immunosuppressed population of pigeons, CoHV-1 causes a multisystemic disease with high mortality (Raue et al. 2005; Marlier and Vindevogel 2006). Birds that survive infection remain subclinically infected. Horizontal infection between parent and young occurs but the young do not develop disease, possibly because of passive transfer of antibody and, as a result, there has been the establishment of large populations of subclinical carrier birds (Vindevogel and Pastoret 1980; Marlier and Vindevogel 2006). The movement of subclinical carrier birds between lofts locally and internationally is the likely source of CoHV-1 dissemination. The virus also appears to be widespread in feral pigeons and whether this is the result of spread from domestic birds to feral birds, or whether this virus has always been present in feral pigeons is not known (Marlier and Vindevogel 2006). Genetic studies using the DNA polymerase gene show that

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this virus contains very limited genetic variation no matter the species or geographic origin (Rose et al. 2012; Woźniakowski et al. 2013).

The CoHV-1 has a remarkably broad host range and infects and has caused mortality in a number of captive (Kunkle and Duhamel 1991; Phalen et al. 2011) and wild species of birds (Gailbreath and Oaks 2008; Woźniakowski et al. 2013). Historically, systemic herpesvirus infections have been seen in some captive hawks and owls that have been fed pigeons (Pinkerton et al. 2008; Phalen et al. 2011). While originally thought to be separate viruses (Maré and Graham 1973; Kaleta 1990), it is now known that most of these infections are caused by CoHV-1. Because CoHV-1 causes disease in wild raptors, it has the potential to have significant ecological impacts and in Canada has been shown to cause persistent mortality in urban and periurban Great Horned Owls (*Bubo virginianus*; Rose et al. 2012). A recent study in Poland found that CoHV-1 DNA could be found in the brain tissue of a Herring Gull, (*Larus argentatus*), Gray Heron (*Ardea cinerea*), and two passerine species, the Hooded Crow (*Corvus cornix*) and Song Thrush (*Turdus philomelos*), expanding the host range of CoHV-1 (Woźniakowski et al. 2013). These birds died in care, but it was not known what species they may have been exposed to while in care or if CoHV-1 infection was the cause of death or an incidental finding.

Given the potential impact of CoHV-1 on domestic pigeons, captive breeding programs of raptors, and wild raptors, and our currently limited understanding of its host range, there is a need for a diagnostic assay that can be used to detect clinical and subclinical infections in the live bird. The ability to detect birds subclinically infected with CoHV-1 will have many benefits, including the ability to screen birds for infection before entry into a collection and to screen wild birds captured for banding or other purposes to determine the host range and epizootiology of this virus.

Two potentially useful screening tools for CoHV-1 infection include virus neutralization assays to detect antibody-positive birds and PCR assays to detect virus shedding in infected birds. Virus neutralization has been used to screen infected domestic and wild pigeons, but this assay requires that a laboratory have CoHV-1 in culture and is not widely available (Marlier and Vindevogel 2006). Studies using PCR of infections of parrots with psittacid herpesvirus-1 (PsHV-1) showed that PsHV-1 viral DNA is present on the mucosal surfaces of the oral cavity and cloaca in subclinically infected parrots and that this virus can be found in these birds for more than a year after infection and possibly for their entire lifetime (Tomaszewski et al. 2006). This finding suggests that a PCR assay of samples from oral and cloacal mucosa of birds infected with CoHV-1 may allow their infection status to be identified. Stenzel et al. (2012), using cloacal swabs and a single-amplification PCR, showed that CoHV-1 can be detected in the cloacal swabs of captive pigeons. In their survey of racing and fancy pigeons, they found a prevalence of infection that ranged from 0% to 20%. Given the prevalence of seropositive pigeons in other studies (Thompson et al. 1977), it is likely that the actual prevalence of infected birds is higher than this and that more sensitive assays such as a nested or real-time PCR would detect a higher prevalence of infection.

Our study aimed to determine the detectability and prevalence CoHV-1 in feral pigeons in parts of Australia. Specifically, we sought to determine whether CoHV-1 could be found in the oral cavity and cloaca of infected pigeons, the comparative sensitivities of a single or nested PCR, and if there is a difference in sensitivity of PCR of DNA obtained from oral swabs as compared to tissue samples or oral mucosa. Then, using the PCR assay, we screened a small subset of native doves for evidence of CoHV-1 infection. Lastly, we documented an apparent spillover of CoHV-1 from pigeons into a wild Powerful Owl (*Ninox strenua*) resulting in its death, thus demonstrating the potential risk of CoHV-1 to native Australian birds of prey.

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MATERIALS AND METHODS

Sources of pigeons and sampling

Fifty-three feral pigeons were included in our study. Six originated from Werribee Open Range Zoo (37°55′22″S, 144°40′2″E), three from Healesville Sanctuary (37°40′56″S, 145°31′54″E), 10 from Melbourne Zoo (37°78′40″S, 144°95′16″E), nine from Fairfield (33°87′43″S, 150°95′81″E), and 25 from Merrylands (33°83′74″S, 150°99′17″E) in Victoria and New South Wales. All of the pigeons were killed as part of pest control programs. The pigeons from Werribee Open Range Zoo, Melbourne Zoo, and Merrylands were frozen immediately after euthanasia and kept frozen (–80 C) until examined. Pigeons collected in Fairfield were chilled immediately after collection and sampled on the same day. Oral swabs were obtained immediately after euthanasia from the pigeons collected at Melbourne Zoo and were frozen until they were processed.

The initial phase of the study was carried out on the six pigeons from Werribee Open Range Zoo. All six pigeons underwent a full necropsy and small samples from oral mucosa and cloacal mucosa were collected by sharp dissection with instruments that had been washed and then disinfected by soaking them first in 5% sodium hypochlorite and then in 7% formaldehyde. Each tissue was collected with a separate set of instruments.

Only oral mucosal tissue samples were collected from the three pigeons from Healesville Sanctuary and the 25 pigeons from Merrylands. Two samples were taken from the pigeons from Fairfield, an oral swab (Copan, Bruescia, Italy) and an oral mucosal tissue sample. Only swabs were taken from the oral cavities of the pigeons from Melbourne Zoo. All samples were stored at –80 C until they were processed.

After the initial phase of the study, oral swabs were collected from 20 wild native or feral pigeons and doves that presented to the veterinary service at Healesville Sanctuary (n=19) or the University of Sydney's Avian Reptile and Exotic Pet Hospital, Camden, New South Wales (34°03′40″S, 150°65′67″E; n=1). All birds were euthanized because of their condition at admission and swabs were collected after their death. Species swabbed included the native species, the Crested Pigeon (*Ocyphaps lophotes*; n=2), White-headed Pigeon (*Columba leucomela*; n=9), Common Bronze-wing Pigeon (*Phaps chalcoptera*; n=1), Brush Bronze-wing Pigeon (*Phaps elegans*; n=2), Bronze-wing (species not reported; n=1) and Wonga Pigeon (*Leucosarcia melanoleuca* n=1), and feral Spotted Dove (*Streptopelia chinensis*; n=2).

DNA extraction, PCR amplification of CoHV-1 DNA, sequencing of amplicons and sensitivity

We extracted DNA from all tissues and swabs using a commercial DNA extraction kit (DNeasy® Blood & Tissue Kit, Qiagen Group, Doncaster, Victoria, Australia) following the manufacturer's protocol. Amplification of CoHV-1 was done using a nested PCR (Phalen et al. 2011). Primer set A (5′-TCTGTACCCCAGCATCATCC-3′ and 5′-AGTAGTCCTCCCCCGCCTCC-3′) was used in the initial amplification reaction to produce a 480-base pair amplicon. Primer set B (5′-TCTTGCTGGACAAACAGCAG-3′ and 5′-CTCGCCCACCGCCGGTGTATGTA-3′) was used in the second (nested) PCR reaction to produce an amplicon of 170 base pair. Both primer sets were selected from the published sequence of the CoHV-1 DNA polymerase (GenBank, National Center for Biotechnology, Bethesda Maryland, USA: accession no. AF141890) using MacVector 10.0 (MacVector, Cary, North Carolina, USA). Initial amplifications were done in a programmable heating block (Corbett Research, Montlake, New South Wales, Australia). Each 20-μL reaction contained 1 μL of extracted DNA, 20 pmol of each primer, 0.1 mmol of each of four dexoynucleotide triphosphates, 2.5 mM magnesium chloride, 0.15 unit Go Taq Flexi, and 1× buffer (all reagents Promega, Madison, Wisconsin, USA). We amplified DNA according to the following protocol: A single cycle of 94 C for 4 min was followed by 40 cycles of an annealing temperature of 62 C for 30 s, an extension temperature of 72 C for 30 s, and a melting temperature of 94 C for 30 s; a single 5-min extension cycle of 72 C was done after the 40 cycles were completed. Reactions were immediately chilled to 5 C after the extension cycle. Sterile water, DNA from a budgerigar (*Melopsittacus undulates*) that was euthanized because it was infected with psittacine beak and feather disease virus, tissue DNA containing PsHV-1 (genotypes 1, 2, 3, and 4), and psittacid herpesvirus-3 were used as negative controls. Positive-control DNA was from the liver of a captive Powerful Owl with a confirmed infection of CoHV-1 (Phalen et al. 2011). Nested amplification was done using the same reaction conditions with the new reaction mix containing 2 μL of the original PCR reaction.

Amplification products were separated by electrophoresis on a 2% agarose gel containing ethidium bromide and visualized with ultraviolet light. To verify that the amplification products were in fact CoHV-1 DNA, amplification products for one bird from Healesville were submitted for sequencing. Unused primers were removed from final PCR reaction using ExoSAP-IT (USB Corp., Cleveland, Ohio, USA). Amplicons were se-

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quenced directly from both ends (Australian Genome Research Facility, Sydney, New South Wales, Australia). Sequences were aligned and compared to the original CoHV-1 sequence using CLC Main Workbench (Qiagen, Redwood City, California, USA).

To determine the sensitivity of the PCR assay, amplification products were isolated and, beginning with 1,000,000 copies/μL of template, they were 10-fold serially diluted. The dilutions were then amplified to estimate the level of sensitivity of the single- and double-amplification PCR assays.

Powerful Owl infection

A moribund Powerful Owl originating from St. Kilda, Victoria, Australia (37°86′75″S, 144°97′90″E) was presented to the Australian Wildlife Health Centre, at Healesville Sanctuary. The bird was euthanized because of its poor condition; tissues were formalin-fixed, paraffin-embedded, sectioned at 5 μm and routinely stained with H&E. Fresh liver was frozen at –80 C until it could be processed and DNA from the liver was tested for the presence of CoHV-1 as described earlier. Amplification products were also sequenced as recently described.

RESULTS

Identification of pigeon tissues containing CoHV-1 DNA and viral prevalence

Six pigeons from Werribee Zoo were necropsied and determined to be healthy, based on pectoral muscle mass, presence of body fat, and the absence of gross lesions. We extracted DNA from both oral and cloacal tissues taken from each pigeon and amplified in a single PCR reaction. Two of six combined samples tested positive for CoHV-1. Following a second nested amplification, using a dilution of the reaction mix from the initial amplification, all six samples tested positive for CoHV-1. To determine which site was more sensitive for the detection of the virus, the oral and cloacal samples were tested separately. After the initial amplification, five of six oral samples tested positive for CoHV-1, whereas only one out of six cloacal samples returned a positive result (Fig. 1a). Following the second amplification, all six oral samples produced positive responses for CoHV-1, as did all six cloacal samples (Fig. 1b). Based on these results, further testing for CoHV-1 was restricted to using DNA from oral mucosal samples.

Nonspecific amplification products of various sizes and faint to moderate intensity were seen in the negative samples, but were not seen in the positive samples. None of the amplification products were the same size as the target amplicon.

CoHV-1 flock infection was detected in pigeons from all five geographic locations (flock prevalence of infection=100%). Combining the results for individual birds from all sites the prevalence of infection was 81%. The prevalence between flocks ranged from 70% at Melbourne Zoo, 76% in Merrylands, and 89% in Fairfield, to 100% in Werribee and Healesville (Table 1). Using a binomial distribution probability and the minimum prevalence (70%), it would only be necessary to sample three birds to have a 95% confidence of finding at least one positive bird. Columbid herpesvirus-1 PCR was not detected in any of the samples from the native and feral pigeons and doves presented to wildlife hospitals.

Sensitivity of two PCR assays and sample types

Test results from all oral samples were pooled to determine sensitivity of the single-amplification PCR, as compared to the single-amplification PCR followed by nested PCR. Thirty-four samples tested positive on a single-amplification PCR and 43 were positive when a nested PCR was added (Table 1). Therefore, the nested PCR resulted in an increased sensitivity of 26%.

To determine if a live bird could be tested for CoHV-1, a comparison was made between the test results from swabs and oral tissues taken from pigeons collected in Fairfield (n=9). Both oral swabs and tissue DNA samples from the same seven birds were positive for CoHV-1 using single-amplification PCR. Using a nested PCR for the swab and tissue samples, an additional eighth bird was identified as positive (Fig. 1b, c). These results show an identical sensitivity of PCR analysis of swab and tissue samples from the oral cavity

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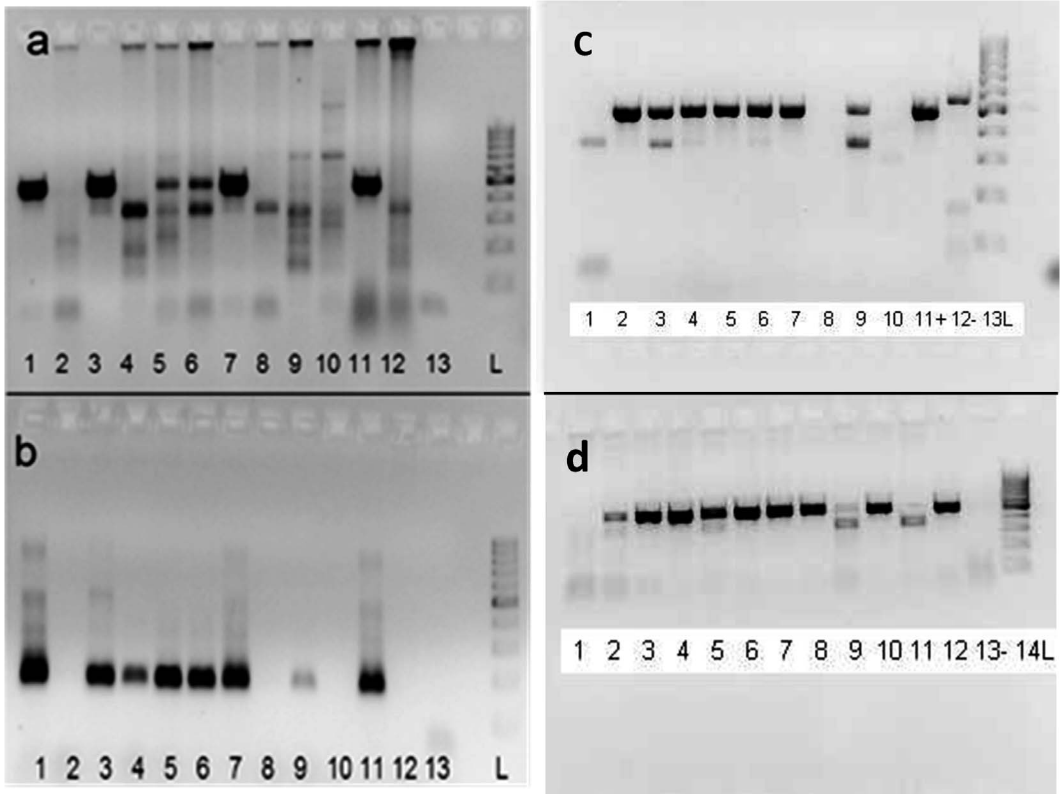

FIGURE 1. (a, b) Agarose gel electrophoresis PCR products from six feral pigeons (*Columba livia*) from Werribee Zoo. From the top: (a) First and (b) nested amplifications. Lane 1: Oral mucosa (OM) sample from pigeon 380 (P380); Lane 2: Cloacal mucosa (CM) sample from P380; Lane 3: OM-P381; Lane 4: CM-P381; Lane 5: OM-P382; Lane 6: CM-P382; Lane 7: OM-P383; Lane 8: CM-P383; Lane 9: OM-P384; Lane 10: CM-P384; Lane 11: OM-P385; Lane 12: CM-P385; Lane 13: columbid herpesvirus-1 negative control. The lanes marked L contain a 100 base pair (bp) ladder, the most intense band in this ladder is 500 bp. (c,d) Images of agarose gel electrophoresis of single-amplification PCR products obtained from nine pigeons (Lanes 1–9) in Fairfield comparing the sensitivity of PCR from DNA extracted from (c) oral tissue and (d) DNA extracted from swabs from the oral cavity. (c) Lane 10: water negative control; Lane 11+: positive control; Lane 12-: tissue negative control. (d) Lanes 10 and 12: positive controls; Lane 11: tissue DNA used as a negative control; Lane 13-: water as a negative control. Lanes 13L and 14L contain a 100 bp ladder, the most intense band in this ladder is 500 bp.

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TABLE 1. Presence of columbid herpesvirus-1 DNA detected by PCR in feral pigeons (*Columba livia*) from five flocks across Sydney and Melbourne.

Source of pigeons	No. of pigeons	Single-amplification PCR oositive (%)	Nested PCR positive (%)
Werribee (Melbourne)	6	5 (83)	6 (100)
Healesville (Melbourne)	3	3 (100)	3 (100)
Melbourne Zoo (Melbourne)	10	3 (30)	7 (70)
Fairfield (Sydney)	9	8 (89)	8 (89)
Merrylands (Sydney)	25	15 (60)	19 (76)
Total	53	34 (64)	43 (81)

and suggest that swabs could be substituted for tissue in this assay. Using single-amplification PCR, 10,000 was the lowest number of copies of CoHV-1 that could be detected. Using nested PCR, 100 copies could be detected.

To verify that the amplification products observed were indeed CoHV-1, DNA amplicons from one bird from Healesville produced by two primer sets in the initial and nested PCR reactions were submitted for sequencing by both the forward and reverse primers. The sequences of these amplicons (GenBank accession no. KY050787) were identical to that of the original CoHV-1 DNA sequence and to those reported from a captive Powerful Owl, two Southern Boobook Owls (*Ninox novaeseelandiae*), and an Australian Hobby (*Falco longipennis*) that died with CoHV-1 infection at Healesville Sanctuary (Phalen et al. 2011). None of the negative control samples produced amplicons of the expected sizes (Fig. 1).

Powerful Owl

On gross postmortem the owl was found to be a female with a moderate degree of pectoral muscle atrophy. It was judged to be approximately 8 mo old based on its juvenile plumage and time of the year. The bird had been seen with ring-tailed possum (*Pseudocheirus peregrinus*) and pigeon carcasses in the weeks leading up to her death.

Microscopic lesions included severe multifocal to coalescing areas of acute necrosis throughout the spleen (Fig. 2a), liver (Fig. 2b), pancreas, adrenal gland, and mucosa of the intestine. Deeply staining eosinophilic intranuclear inclusion bodies were seen in hepatocytes (Fig. 2c), pancreatic acinar cells, and adrenal cortical cells. A mixed bacterial population was found in the necrotic intestinal mucosa. There was a multifocal, mild, acute tubular necrosis with intraluminal crystal formation. The PCR of DNA extracted from this bird's liver was strongly positive for CoHV-1. The specificity of this reaction was confirmed by sequencing the amplification products from both directions. The sequence (GenBank accession no. KY050786) was then confirmed to be CoHV-1by comparing its sequence to that of CoHV-1 found in GenBank using BLAST (Madden 2002).

DISCUSSION

Columbid herpesvirus-1 latently infects domestic and feral pigeons and likely has a worldwide distribution (Smadel et al. 1945; Marlier and Vindevogel 2006; Stenzel et al. 2012). It is documented in Australia and may be widespread in Australia in domestic pigeons (Boyle and Bingington 1973; Phalen and Walker 2008). Prior to this study there were no data on its prevalence in feral pigeons in Australia.

We showed that CoHV-1 DNA can be detected in both oral and cloacal mucosal samples from feral pigeons. However, oral samples contained a higher concentration of CoHV-1 than did cloacal samples and thus the oral cavity should be considered to be the preferred site for sample collection. Virus concentrations at this site, in most of the infected birds, were sufficiently high that they could be detected with a single PCR amplification; however, the sensitivity of the PCR reaction was improved with a second nested amplification. The sensitivity of the nested PCR was similar to a real-time PCR developed by Woźniakowski et al. (2013) and therefore these two assays are assumed to be interchangeable.

Only five sources of pigeons were examined in this study and nothing was known about the history of the flocks and in some cases the age and sex of the bird was also not known. Therefore, our results should be considered preliminary, as virus concentrations in infected birds may be affected by the duration of infection and the age of the bird and its reproductive and immune status. Previous research showed that parrots infected with PsHV-1 shed virus for many years after infection and may shed virus for life. The amount of virus detected on the mucous membranes of these parrots, however, is at its highest concentration following a recent

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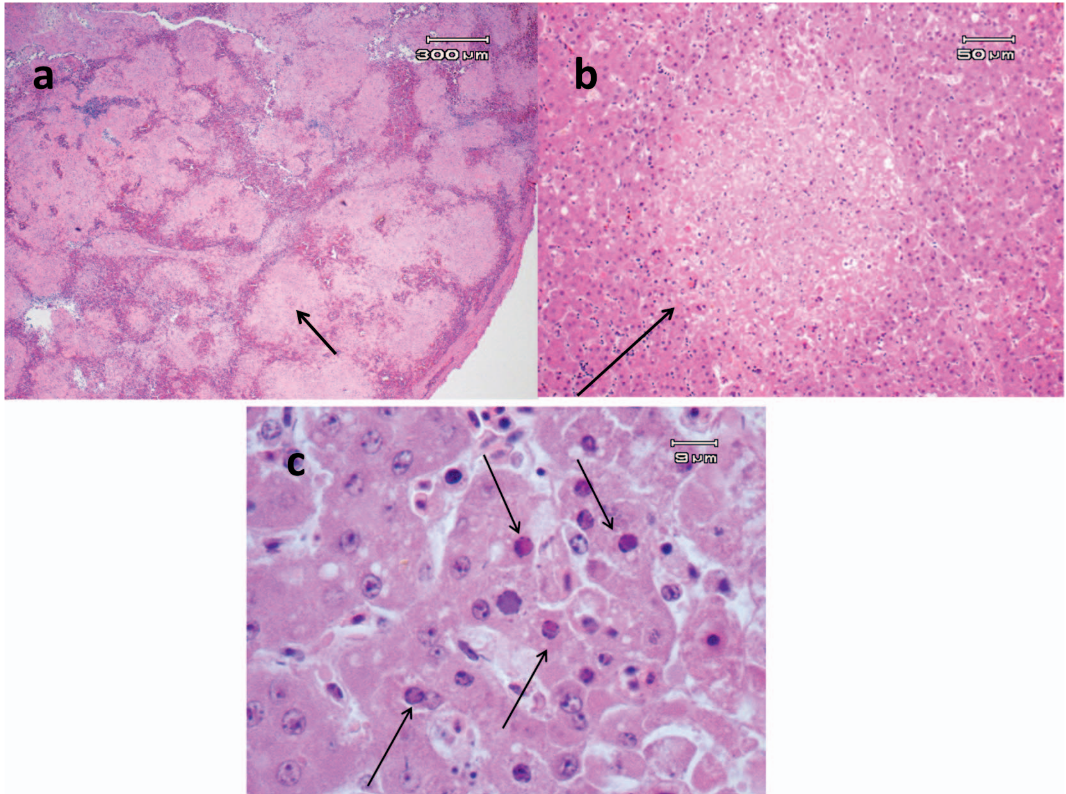

FIGURE 2. Three H&E-stained sections from a Powerful Owl (*Ninox strenua*) with columbid herpesvirus-1 infection. (a) Spleen demonstrating mutifocal areas of necrosis. A representative focus is indicated by the arrow. (b) Liver demonstrating focus of necrosis (arrow). (c) Liver demonstrating viral inclusion bodies (arrows).

infection and dwindles in the months and years that follow (Tomaszewski et al. 2006). Therefore, the sensitivity of the PCR assay used in this study for CoHV-1 may diminish in birds that have been infected for long periods of time.

Columbid herpesvirus-1 is known to cause death in a wide range of birds of prey including hawks, falcons, and owls in captivity and in the wild as the result of being fed or eating pigeons (Phalen et al. 2011; Rose et al. 2012). Infection resulting in death has been documented in three captive species of Australian raptors including a Powerful Owl (Phalen et al. 2011). This is the first documented case of lethal infection in a wild Australian bird of prey.

Columbid herpesvirus-1 infections in raptors are characterized by necrosis of the spleen, liver, pancreas, and intestinal mucosa. Eosinophilic and, less commonly, basophilic intranuclear inclusion bodies are most commonly seen in the liver, but can be found in any affected tissue (Zsivanovits et al. 2004; Gailbreath and Oak 2008). The lesions seen in our Powerful Owl are consistent with those reported in other raptor species. The cause of the intestinal lesion is unclear. The necrotizing lesion of the mucosa is consistent with a viral etiology, but inclusion bodies were not seen. The bacterial colonization of this lesion could potentially be primary or it could have been secondary to virus-induced necrosis.

Powerful Owls are widespread in southeastern Australia and they can inhabit both natural and highly urbanized environments (Isaac et al. 2014). They primarily consume arboreal marsupials, but when these are limited they will take avian prey (Bilney 2013). The home range of the owl in this

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study was an urban environment and it was observed eating pigeons prior to its death. This suggests that the ability of Powerful Owls to utilize urban environments may be constrained by availability of mammal prey and their choice of prey. Precedence for this occurs in urban and peri-urban Great Horned Owls in Canada, where significant numbers of deaths have been caused by CoHV-1 as the result of these owls consuming pigeons (Rose et al. 2012).

Despite the preliminary nature of these findings, some important conclusions can be drawn from them. Clearly CoHV-1 is widespread in feral pigeons in and around Melbourne and Sydney and it is very likely that CoHV-1 is well established in feral pigeon flocks wherever they are found in Australia. This means that captive or wild Australian raptors that are fed, hunt, or are exposed to feral pigeons are at risk of infection with CoHV-1. The extent of CoHV-1 infection in captive pigeon flocks in Australia is unknown, but based on a survey of postmortem examinations of captive pigeons in Australia (Phalen and Walker 2008) and similar studies in New Zealand (Thompson et al. 1977) and Europe (Marlier and Vindevogel 2006; Stenzel et al. 2012), it is likely that this virus is also widespread in these flocks as well. Based on these findings, pigeons should not be used as a food source for raptors in Australian collections and feral pigeon control in these facilities is indicated to reduce exposure to CoHV-1.

Columbid herpesvirus-1 can infect a range of species other than the pigeon (Rose et al. 2012; Woźniakowski et al. 2013). This means that feral and domestic pigeons infected with CoHV-1 are a potential source for infection to any susceptible native species that might cohabitate with them or consume them. The initial preliminary results of this study failed to demonstrate transmission of CoHV-1 to native doves and pigeons and to the introduced Collared Dove. The numbers of birds that we tested so far is small and additional studies with larger numbers of birds are needed.

In conclusion, CoHV-1 is readily detected in the oral mucosal tissue and oral mucosal swabs in many feral pigeons infected with CoHV-1 using a single-amplification and nested PCR. Screening of feral pigeons with this assay strongly suggests that CoHV-1 is enzootic in feral pigeon flocks across Australia and that these birds should not be used as food for raptors. Our work also documents, at least in one instance, a CoHV-1 transfer from feral pigeons to a wild Powerful Owl and suggests that CoHV-1 may prove a limiting factor in the distribution of Powerful Owls in urban environments.

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