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Source: Journal of Wildlife Diseases, 21(3) : 193-198

Published By: Wildlife Disease Association

URL: https://doi.org/10.7589/0090-3558-21.3.193

Journal of Wildlife Diseases, 21(3), 1985, pp. 193–198

VIRAL INFECTIONS OF LITTLE BLUE PENGUINS (*EUDYPTULA MINOR*) ALONG THE SOUTHERN COAST OF AUSTRALIA

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ABSTRACT: Serum antibodies to strains of avian paramyxovirus and flavivirus were detected in little blue penguins sampled at Port Campbell and Phillip Island, Victoria, Australia. No antibody to Newcastle disease virus (NDV) was detected in 267 sera collected, although one penguin captured for experimental studies had a hemagglutination-inhibition antibody titer of 2^4 to this virus. Experimental studies showed that the avian paramyxovirus designated APMV-IM and strain V4 of NDV were non-pathogenic for penguins, although the penguins could have been previously infected with these or similar virus strains. A flavivirus designated Saumarez reef virus, and an unnamed virus isolated from ticks on Macquarie Island, Southern Ocean were pathogenic causing disease and mortality in penguins inoculated with the viruses.

INTRODUCTION

Morgan et al. (1981) isolated six strains of avian paramyxovirus (APMV) from royal penguins (*Eudyptes chrysolophus schelegili*) and king penguins (*Aptero-dytes patagonica*), as well as 23 viral strains from the tick *Ixodes uriae*, on Macquarie Island. Subsequently two further APMV strains were isolated from Adelie penguins (*Pygoscelis adeliae*) in Antarctica and serological testing showed a high prevalence of serum antibody to APMV strains in penguins on Antarctica and Macquarie Island (Morgan and Westbury, 1981; Morgan et al., 1981). Specific serum antibody to Newcastle disease virus (NDV) and avian influenza virus (hemagglutinin 7) and group specific antibody to flavivirus (group B togavirus) was also detected indicating that infection of penguins with these viruses had occurred.

These findings prompted the investigation of the occurrence of specific antibody to these virus strains in little blue penguins from the Australian coastline as Macquarie Island penguin species are occasionally observed on this coastline, and Antarctic penguin species have been recorded on Macquarie Island. Experimental infections of little blue penguins with NDV, an APMV strain and viruses isolated from bird ticks were also undertaken in an attempt to obtain knowledge of the pathogenicity of these virus strains for penguins.

Received for publication 16 June 1981.

MATERIALS AND METHODS

Little blue penguins were captured from their burrows at several sites on Phillip Island and at Port Campbell, Victoria. These birds were bled from the brachial vein and the pharynx and cloaca of each bird was swabbed with a sterile cotton wool swab stick. The swabs were individually transported to the laboratory in brain–heart infusion broth containing penicillin (1,000 IU/ml) and streptomycin (1,000 μg/ml). Virus isolation attempts from swabs were undertaken by inoculation of broth into embryonating chicken eggs (Westbury et al., 1979). Ticks were also collected from infested penguins.

Serological studies: Penguin serum was examined for the presence of hemagglutination-inhibition (HI) antibody to NDV, avian influenza virus hemagglutinin 7 (H7), flavivirus (Murray Valley encephalitis virus) and to an APMV strain designated AMPV-IM isolated from royal penguins on Macquarie Island. The procedures used in these tests were as described by Morgan et al. (1981).

Virus isolation from ticks: Ticks collected from penguins were pooled into groups of either three engorged or eight unengorged specimens. Each pool was homogenized in the cold by grinding in a mortar with 3 ml of phosphate buffered saline pH 7.2 containing 200 IU penicillin, 200 μg streptomycin and 0.2% bovine

serum albumin. Following centrifugation at 800 *g* for 5 min, the supernatant of the homogenized suspension was inoculated intracerebrally (IC) into litters of 1- to 2-day-old mice using 0.015 ml per mouse. Litters were observed daily for 14 days for evidence of clinical disease and samples of brain from apparently normal inoculated mice were then passaged into a further mouse litter. Failure to induce clinical signs or neurological disease following this passage was considered to indicate the absence of virus in the original homogenate.

Transmission studies: Nine little blue penguins were captured on Phillip Island with the permission of the Division of Fisheries and Wildlife, Department of Conservation, Forests and Land. The penguins were bled and pharyngeal and cloacal swabs taken on arrival at the laboratory. The birds were hand-fed twice daily with whole small fish, each penguin receiving about 300 g of fish each meal. In addition each penguin was given a vitamin-mineral capsule (Myadec, Parke-Davis and Co., Nth Carringbah, New South Wales, Australia), and a vitamin A and D tablet (Vitamin A and D tablet, H. W. Woods Pty. Ltd., Victoria, Australia) daily. The birds were housed on a concrete floor without litter material and had free access to a swimming pool containing fresh water. The pen was cleaned twice daily. Penguins were transferred to a high security isolation ward for virus transmission studies.

Experiment #1: Three penguins were given 10^6 50% chicken embryo-infective doses (EID_{50}) of strain V4 of NDV by the oro-nasal route. These birds were bled at 7-day intervals and pharyngeal and cloacal swabs taken daily for 21 days after administration of virus. These swabs were used in virus isolation procedures as described earlier. Another three penguins were inoculated with APMV-IM in the same way. The procedure used for the NDV infected penguins was applied. Penguins infected with NDV and APMV-IM, and uninfected birds were observed for 21 days for the development of clinical disease.

Experiment #2: Two penguins were inoculated subcutaneously (SC) with 1.0 ml of a 1% suspension of mouse brain tissue containing a flavivirus strain (Saumarez reef (SAZ)). The inoculum contained 5×10^5 50% mouse lethal doses (LD_{50}) of virus. This virus was isolated from ticks infesting birds (St. George et al., 1977) and subsequently from ticks (*Ixodes uriae*) on Macquarie Island. Penguins were observed for the development of clinical disease. The brain, liver, kidney and spleen from moribund or dead penguins were collected at post-mortem and used for viral isolation and histopathological examination.

Experiment #3: Six penguins used in experiment #1, and one other, were used to study the virulence of virus strains designated AUST-MI-411 and AUST-MI-418 isolated from *Ixodes uriae* from Macquarie Island (Morgan et al., 1981). Initially, one penguin was inoculated SC with AUST-MI–411, and one with AUST-MI-418. The same dose of virus and procedures were used as in experiment #2. Tissue suspensions of liver and spleen from the penguin inoculated with AUST-MI-411 were inoculated into three penguins, two with the liver suspension, one with the splenic suspension. The liver suspension was also inoculated IC into suckling mice in the way described earlier for isolation of tick-borne viruses. A 10% suspension of mouse brain tissue, from mice exhibiting clinical disease following inoculation of the liver suspension was inoculated into a further two penguins. Those developing clinical disease were killed and tissue samples were collected for use in virus isolation and histopathological examination.

RESULTS

Serological studies: Serum antibody to APMV was detected in six (7%) of 108 sera collected in the Port Campbell region. No antibody to this virus was detected in the 159 sera collected from Phillip Island penguins. Twenty-eight (32%) of 88 sera collected on Phillip Island had group specific antibody to flavivirus. An additional 32 (36%) showed presumptive evidence of this antibody, there being insufficient serum in these samples to permit the complete removal of non-specific inhibitors to hemagglutination. Six of 30 (20%) sera from Port Campbell had significant titers of group specific antibody to flavivirus. No serum antibody to NDV or avian influenza virus H7 was detected.

Virus isolation: No hemagglutinating viruses were isolated from pharyngeal or cloacal swabs using egg inoculation techniques. Similarly, no viruses were isolated from about 800 ticks (*Ixodes kohlsi*) following mouse inoculation.

Transmission studies: Experiment #1: Penguins became infected with NDV and APMV-IM as judged by isolation of the

TABLE 1. The number of little blue penguins from which Newcastle disease virus (NDV) and an avian paramyxovirus (APMV-IM) were isolated during the 21 days following experimental infection.

Virus	Swab site	Days after inoculation																				
		0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
NDV[a]	Pharynx	0	2	1	2	NT[b]	2	2	1	2	0	3	2	2	2	0	2	NT	NT	NT	NT	NT	1
	Cloaca	0	0	0	2	NT	0	3	0	2	2	3	2	1	1	0	0	NT	NT	NT	NT	NT	0
APMV-IM[a]	Pharynx	0	3	2	3	2	3	2	2	0	0	1	0	0	NT	NT	NT	NT	NT	NT	NT	NT	NT
	Cloaca	0	0	0	1	1	3	3	2	2	1	1	1	1	0	0	0	0	0	0	0	0	0

[a] Three penguins were inoculated with each virus.
[b] NT = not tested.

virus from pharyngeal and cloacal swabs (Table 1). Newcastle disease virus was isolated for up to 21 days after inoculation of virus. The birds developed no clinical disease during the course of the infection and virus specific antibody was detected on day 21 after inoculation of virus. The serum antibody titers ranged from 2^4 to 2^5. One penguin had a serum antibody titer of 2^4, while two had specific antibody titers of 2^3 to APMV-IM prior to inoculation of NDV. All birds inoculated with NDV excreted virus despite the presence of this antibody. Avian PMV was isolated from experimentally infected penguins for up to 21 days after administration of virus. The infected birds developed no clinical disease.

Experiment #2: Penguins inoculated with SAZ virus became depressed and anorectic, developed dysentery and died 9 and 13 days after administration of the virus. Post-mortem examination showed a pale, friable liver, edema of the gizzard and petechial and ecchymotic hemorrhages on the serosal surface of the gastrointestinal tract. Histologically the affected penguins exhibited widespread periacinal necrosis of the liver, nephrosis, mild proventiculitis and severe enteritis characterized by necrosis of the epithelium, dilation of the crypts and attenuation of the crypt epithelium. SAZ virus was re-isolated from the liver and spleen of both penguins after death.

Experiment #3: The penguin inoculated with strain AUST-MI-411 died 3 days after inoculation of virus. It had been depressed and anorectic for 1 day. The bird showed no gross lesions at post-mortem, although the liver may have been congested. The only remarkable histological lesion was an acute hepatic necrosis. Two penguins inoculated with the liver suspension from the above penguin developed a similar clinical disease. They became depressed and stopped eating 2–3 days after inoculation of the tissue suspension and

died 4–5 days after the development of clinical disease. Both birds showed a widespread acute periacinal necrosis, nephrosis and mild proventiculitis. The penguin inoculated with the splenic suspension died of a ruptured liver 1 day after inoculation of virus. The penguins inoculated with virus passaged through mouse brain failed to develop clinical disease. One of these birds was killed 7 days after inoculation of virus and showed no remarkable gross or histological lesions. The other was depressed and losing body condition for 5 days prior to inoculation of virus and died 1 day later. Virus was re-isolated from the first penguin inoculated with AUST-MI-411 and from two penguins given liver suspension. No clinical disease was observed in the penguin inoculated with strain AUST-MI-418.

DISCUSSION

Serological studies showed that little blue penguins captured on the coast of Victoria, Australia had been infected with strains of avian paramyxovirus and flavivirus. Earlier studies by Morgan and Westbury (1981) and Morgan et al. (1981) demonstrated the widespread prevalence of serum antibody to APMV strains distinct from NDV on Macquarie Island and Antarctica. The widespread distribution of infection with APMV strains among penguins from three distinct geographical regions suggests that these viruses are part of the normal microbiological experience of penguins. Numerous APMV strains has been isolated around the world in recent years (Alexander, 1980). Some of these strains have been associated with highly lethal disease in psittacine birds and domestic turkeys (Smit and Rondhuis, 1976; Nerome et al., 1978; Liplund et al., 1979), while others may have a synergistic effect with other pathogenic microorganisms (Lang et al., 1975).

The results of experiments demonstrated that APMV-IM, originally isolated from royal penguins on Macquarie Island could multiply in the respiratory and gastrointestinal tract of little blue penguins. However, its growth was not associated with clinical disease. Penguins used in these transmissions were tested for the presence of APMV strains prior to experimental infection by virus isolation and serological tests. No virus or specific antibody was detected and it was therefore assumed that the penguins were susceptible. However, two penguins used in NDV transmission test had specific antibody to APMV prior to inoculation of NDV. The penguins used in the APMV transmission study were trapped in the same locality as the birds used in the NDV study. It is possible that the penguins used in the APMV transmission study may have been infected with APMV strains sometime in the past and serum antibody to APMV waned to undetectable levels. Little is known of the persistence of APMV antibody in penguins.

NDV similarly multiplied in the respiratory and gastrointestinal tract of experimentally infected penguins. No disease developed, but this was not an unexpected finding as strain V4 is non-pathogenic for chickens (Simmons, 1967). The trial nevertheless demonstrated the susceptibility of penguins to infection with NDV. The presence of low NDV antibody titers in one penguin emphasized the difficulties involved in using wild animals in transmission studies as there is no control on the diseases or infections they may have acquired and to which they may therefore have partial and effective immunity.

At least 32% of the penguins sampled at Phillip Island had group specific antibody to flavivirus. These viruses multiply in arthropods, vertebrates becoming infected through a bite from an infected vector. Ticks were considered by us to be the most likely vector of flavivirus infection in little blue penguins since flavivirus strains have been isolated from ixodid ticks

collected from sea birds in Australia (St. George et al., 1977) and from ticks on Macquarie Island (Morgan et al., 1981). The failure to isolate an arbovirus from about 800 specimens of *Ixodes kohlsi* collected during this study suggested that other *I. kohlsi* might not be the vector of the flavivirus infecting little blue penguins as no virus was present at the time of the collection of the birds. Ectoparasites such as the fleas *Parapsyllus autraliacus* and *P. taylor*, the louse *Austrogonoides watersoni* and the tick *I. percavatus* have been found on little blue penguins (Reilly and Balinford, 1975; Obendorf and McColl, 1980) and one of these may be a vector. Alternatively, hematophagous insects such as mosquitoes or midges could be the vector since they have been incriminated as the vectors of flavivirus infection in sea bird environments (Lvov et al., 1971).

The failure to isolate a flavivirus prevented an assessment of the significance of flavivirus infection in penguins. However, a flavivirus SAZV isolated from ticks infesting birds in Australia and Macquarie Island (St. George et al., 1977) was used to experimentally infect penguins. Two penguins infected with this virus died 9 and 13 days after inoculation with the virus and the virus was isolated from the birds after death. This provided evidence that SAZ virus had multiplied in the birds and the time course of the disease and death suggested that the virus was associated with the death of the penguins. Similarly, the arbovirus designated AIS-MI-411 was associated with disease and mortality in experimentally infected penguins. The failure of strain AIS-MI-418 to induce disease could indicate that it was non-pathogenic for penguins. The small number of penguins used in these tests and the lack of control birds prevented us from drawing definite conclusions from these trials. The difficulties associated with the husbandry and maintenance of these wild birds under laboratory conditions prevented experimentation with larger groups. Nevertheless, these results provided indications that some flavivirus infections could be lethal for penguins. Consequently virus isolations should be attempted in any investigation of mortality in penguins because there is evidence of flavivirus serum antibody in penguins and indications that some flavivirus strains are pathogenic for penguins.

ACKNOWLEDGMENTS

We would like to thank the Fisheries and Wildlife Division, Department of Conservation, Forests and Land for permission to capture and house the penguins. We must also thank Dr. K. E. Harrigan for his assistance in examining the histopathological slides and M. Pase, G. Hancock, A. Halford and J. Crooks for assistance with field and laboratory work.

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