EXPERIMENTAL EXPOSURE OF FRANKLINS' GULLS (Larvus pipixcan) AND MALLARDS (Anas platyrhynchos) TO A TURKEY INFLUENZA A VIRUS A/Turkey/Minn/BF/72 (Hav6Neq2) 1

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EXPERIMENTAL EXPOSURE OF FRANKLINS' GULLS (*Larvus pipixcan*) AND MALLARDS (*Anas platyrhynchos*) TO A TURKEY INFLUENZA A VIRUS A/Turkey/Minn/BF/72 (Hav6Neq2)[1]

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Abstract: Gulls (*Larvus pipixcan*) and mallards (*Anas platyrhynchos*) were experimentally exposed to a turkey influenza A isolant, A/turkey/Minn/BF/72 (Hav6Neq 2). No clinical signs of disease were observed in either species. Tracheal shedding of virus from the gulls persisted for 24 days post-inoculation but virus later than 6 days post exposure could not be demonstrated in either tracheal or cloacal samples from the mallards. Precipitating antibodies were not detected. Hemagglutination-inhibition antibodies were demonstrated in inoculated gulls but antibody levels were low and erratic in ducks.

INTRODUCTION

Migratory wild birds possibly may act as reservoirs of influenza viruses and therefore may play a role in dispersal of viruses over thousands of kilometers.[9] Serologic surveys have shown that some birds have circulating antibodies against type A influenza viruses.[9,10,18,20,21] Isolation of these viruses from many species of birds strongly supports this theory.[1,6,14,16] Free-ranging wild birds also may provide optimum conditions for genetic interaction of type A influenza viruses.[19]

Worldwide distribution of these viruses suggests that wild birds may play a role in the natural history of influenza infections for both animal and human populations. Thus, efforts have been made to obtain more information on the epizootiology of influenza in wild birds. Various workers have exposed avian species to influenza viruses.[8,11,17,20] Evaluations of these data suggest a marked variation among birds in response to a specific avian influenza virus. Narayan, *et al.*[11] found that A/turkey/Ont/7732/66 (Hav5N6) caused acute disease in chickens and turkeys, but was non-pathogenic for ducks, geese and pigeons. Homme and Easterday[8] noted that pheasants responded with higher levels of hemagglutination-inhibition (HI) antibody to A/turkey/Wis/66 (Hav9N2) than ducks and geese. Virus was reisolated from ducks, pheasants and domestic geese, but not from Canada geese. Winkler, *et al.*[19] exposed geese to A/turkey/Wis/66 (Hav9N2) and A/turkey/Wis/68 (Hav5N2) and detected low levels of HI antibody and anti-ribonucleo-protein (anti-RNP) antibody, but they were unable to recover the virus. They were able to recover A/turkey/Wis/66 from experimentally infected birds and detect low levels of HI antibody, but agar gel-precipitin (AGP) tests were negative for anti-RNP antibody. Slemons and Easterday[17] experimentally determined the response of pheasants, ducks, turkeys, pigeons and Japanese quail (*Coturnix coturnix japonica*) to A/turkey/Ont/7732/66. Clinical signs were not observed among pheasants and ducks but turkeys developed a severe disease and died. One of 19 pigeons and three of 20 quail died. Pheasants and quail developed a marked antibody response, but the response in ducks was poor. Virus recovery was erratic.

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Our study was undertaken to determine the response of Franklins' gulls and mallards to a pathogenic influenza virus isolated from turkeys (unpublished data). Of particular interest were the clinical signs of disease, the shedding of virus, and the nature of antibody response.

MATERIALS AND METHODS

Laboratory procedures

Virus

The influenza virus used was a 6th allantoic cavity passaged stock virus of A/turkey/Minn/BF/72 maintained at −70 C with a titer of $8.5x10^3$ EID/50. The virus was resuspended in veal-infusion (VI) broth, diluted 1:1000 and 0.2 ml amounts were inoculated into the allantoic sacs of 10-day-old embryonated chicken eggs. After 72 h. of incubation at 37 C the allantoic fluids were collected, pooled and frozen at —70 C. Infectivity and end-points expressed as 50% embryo infective dose (EID/50) were calculated by the method of Reed and Muench.[12] The virus had a hemagglutination (HA) titer of 1/560 and $3.1x10^3$ EID/50.

Blood collection

Blood samples were collected by peripheral venipuncture and the serum was stored at —20 C with one drop of 1% sodium azide as preservative in 1 ml of serum.

Microhemagglutination-inhibition (MHI) test

The microhemagglutination (MHA) test[5] was used to determine the MHA titer and to determine the level of microhemagglutination-inhibition (MHI) antibody in serum. Chicken erythrocytes (CRBC) were used at a concentration of 0.5% in 0.01M phosphate buffered saline, pH 7.2.

Agar gel-precipitin (AGP) test

The AGP test for ribonucleoprotein (RNP) antibody was performed as described by Beard.[3]

Experimental birds

Franklins' gulls *(Larvus pipixcan)* chicks were captured at Agassiz National Wildlife Refuge, Middle River, Minnesota. The chicks were only a few days old and still fledglings when captured. The chicks were housed and raised to 6-7 months age in wire mesh cages 2.5x1.5x 1.8 m in an isolation unit. At this time they were divided into two colonies and maintained in separate cages in isolation units.

One-day-old domesticated, commercially-raised mallards *(Anas platyrhynchos)* were obtained from Ridgeway Hatchery, LaRue, Ohio. The ducklings were raised in Horsfall-Bauer units until 5 weeks of age. At this time they were divided into two colonies and maintained in separate isolation units.

Experimental infection

All birds were exposed by intratracheal instillation of 0.2 ml. of infectious allantoic fluid with EID/50 $3.1x10^3$.

Tracheal and cloacal swabs

Tracheal and cloacal swabs were placed in tubes containing 2 ml VI broth, penicillin (1,000 units/ml), streptomycin (10 mg/ml) and mycostatin (10 mg/ml). The tubes were frozen on dry ice after collection. When ready for inoculation, the samples were thawed, let stand at room temperature for 60 min. and 0.2 ml was injected into the allantoic cavity of 9-11-day-old chicken embryos. Three eggs were used for each swab. Eggs were checked twice daily to detect embryo deaths. When death was detected, or at 4 days, the allantoic fluid was collected from each egg and checked for hemagglutinating activity.

Experimental procedures

Experiment One

Five Franklins' gulls were exposed to the virus by intratracheal inoculation. Twenty-four hours later five unexposed gulls were placed in this room; the latter

gulls thus became the contact exposed group. Fourteen gulls in another room served as negative controls. On 0, 1, 3, 6, 9, 13, 24, 37, and 49 days post-challenge, tracheal and cloacal swabs were collected from all birds in the exposed group. Similar samples were taken from all the gulls in the negative control group on 0, 6, 13, 37 and 49 days. Blood samples were collected for antibody assay on 0, 9, 24 and 49 days post-challenge from all gulls.

Experiment Two

Four mallards were exposed to the virus at 6 weeks of age, and 24 h later 5 other mallards were added to serve as contact controls. Three mallards in another isolation room served as negative controls. On days 0, 1, 3, 6, 9, 13, 24 and 43 days post-challenge, tracheal and cloacal swabs were collected from all exposed mallards. Similar samples were collected from mallards in the negative control group on days 0, 1, 9, 24, and 43. Blood samples were collected from all ducks for antibody assay on days 0, 9, 24 and 43.

RESULTS

Experiment One

All gulls exposed intratracheally had HI antibody by 9 days post exposure. Considerably higher titers were evident on day 24 and day 49 post-exposure. No anti-RNP antibody was demonstrated in any of the gulls. Virus was recovered from the tracheal swabs of the exposed gulls for 6 days post-exposure. HI and anti-RNP antibody was not found in the 5 contact gulls, but virus was recovered intermittently from their trachea for 23 days post-exposure. Virus was recovered from the cloaca of exposed birds for up to the 13th day. Results are summarized in Table 1. The results of the cloacal swab samplings of 37 and 49 days post-challenge are not included in this table.

The negative control gulls remained negative serologically and virus was not recovered.

Experiment Two

Virus was recovered intermittently from the trachea and cloaca of the exposed and contact ducks for 6 days following exposure. Virus also was recovered from dead ducks but no gross pathology was observed.

The ducks did not develop clinical signs of disease. Only 1 had antibody 9 days after exposure (titer 1:80). Anti-RNP antibody could not be demonstrated. Virus and antibody were not detected in the negative control ducks. Results are summarized in Table 2. The results of the tracheal and cloacal swab samplings of 9, 13, 24 and 43 days post-challenge are not included in this table.

DISCUSSION

Zakestel'skaja[21] reported reactors to HI test against certain avian and equine strains of influenza A viruses. Laver and Webster[10] found that some seabirds nesting on the islands off the coast of Australia had neuraminidase - inhibition (NI) antibodies against human A/Singapore/1/57 (H2N2). The possibility that some of these titers are non-specific has not been ruled out. Efforts of other workers[2,11,17,20] have indicated considerable variation among avian species in response to a single strain of avian influenza virus.

Becker[4] injected swift tern *(Sterna bergii)* intramuscularly with A/tern/South Africa (Hav5Nav2) and showed that the virus had no apparent effect on swift tern, although it did stimulate high levels of circulating antibody. Tern virus had been isolated[13] from a European common tern *(Sterna hirundo)* epizootic that caused a high mortality. Samadieh and Bankowski[15] infected starlings *(Sturnus vulgaris)* with A/turkey/Calif/ Meleagrium/64 and A/turkey/Calif/5162/66 but the birds did not sero-convert either to intramuscular or intravenous inoculations of virus. Moreover, they could not demonstrate replication of virus in starling tissues. However, Bahl and Pomeroy (unpublished data) experimentally infected starlings (*S. vulgaris*) and

TABLE 1. Experiment One: Susceptibility of Franklin's Gulls to A/turkey/Minn/BF/72 Virus. Results of Virus Recovery From Tracheal and Cloacal Swabs and Serum HI Test.

	SWABS FOR VIRUS RECOVERY												SEROLOGY HI TEST			
	Day 0		1		3		6		9		13		24					
Gull #	TS	CS	TS	CS	TS	CS	TS	CS	TS	CS	TS	CS	TS	CS	Day 0	9	24	49
56	0	0	+	0	+	NA	0	NA	0	0	0	+	0	0	0	32	32	128
57	0	0	+	0	+		+		0	0	0	0	0	0	0	8	8	64
58	0	0	+	0	+		0		0	0	0	0	0	0	0	16	16	64
59	0	0	+	0	+		0		0	0	0	0	0	0	0	32	32	128
60	0	0	+	+	+		0		0	0	0	+	0	0	0	32	16	Dead
	Day 0		1		2		5		8		12		23		Day 0	8	23	48
51	0	0	0	0	0	NA	0	NA	+	0	0	0	+	0	0	0	0	0
52	0	0	+	0	0		+		0	0	0	0	+	0	0	0	0	0
53	0	0	+	0	0		+		0	0	0	0	0	0	0	0	0	0
54	0	0	0	0	0		0		+	0	0	0	0	0	0	0	0	0
55	0	0	0	0	0		0		0	0	0	0	+	0	0	0	0	0

TS = Tracheal swab, CS = Cloacal swab

#51-55 Contact gulls placed in contact with exposed gulls, #56-60 one day after challenge

#56-60 Exposed gulls

0 = No virus isolation

+ = Virus isolation

NA = Not analyzed — contamination

TABLE 2. Experiment Two: Susceptibility of Mallard Ducks to A/Turkey/Minn/BF/72 Virus. Results of Virus Recovery from Tracheal and Cloacal Swabs and Serum HI Tests.

	SWABS FOR VIRUS RECOVERY								SEROLOGY HI TEST			
	Day 0		1		3		6					
Duck #	TS	CS	TS	CS	TS	CS	TS	CS	Day 0	9	24	43
D 80	0	0	+	0	+	+	0	0	0	0	0	0
D 81	0	0	0	0	+	+	+	+	0	0	0	0
D 82	0	0	+	0	+	+						
D 83	0	0	+	0	+	+	+*	+*				
	Day 0		2		4		7					
D 84	0	0	0	0	+	+	0	+	0	0	0	0
D 85	0	0	0	0	+	+	+	0	0	0	0	0
D 86	0	0	0	0	+	+	0	+	0	1:80	0	0
D 87	0	0	0	0	+	0	0	+	0	0	0	0
D 88	0	0	0	0	+*	+*						

TS = Tracheal swab CS = Cloacal swab

D 80-83 = Exposed ducks

D 84-88 = Contact ducks. Placed in contact with exposed D 80-83 one day after challenge

0 = No isolation

+ = Virus isolation

* Dead

redwinged blackbirds (*Agelaius phoeniceus)* with A/turkey/Minn/BF/72 (Hav-6Neq2) and recovered the virus for 7 days post-inoculation. They demonstrated HI antibody but no anti-RNP antibody.

In our experiments the absence of clinical disease on exposure of Franklins' gulls and mallards to A/turkey/Minn/BF/72 indicates that this virus is not likely to cause disease in nature. However, experimental conditions do eliminate stress factors like migration, severe weather conditions, scarcity of feeding habitat and concurrent infections. Captivity eliminates some of the above stresses, but imposes others like environmental stresses of ambient temperature, humidity, feed and feeding habits. These in turn would inadvertently affect the normal intestinal flora. This change in normal flora may partly explain the death of one gull in Experiment One and 2 ducks in Experiment Two. No significant gross lesions were observed in these birds, neither was any pathogenic microorganisms isolated upon necropsy. Cultures taken from trachea, liver and lungs yielded pure cultures of *Proteus* and *Pseudomonas.*

HI antibody levels in the experimentally infected birds were low and of short duration. Serologic conversion was not demonstrable in the contact controls in either experiment in spite of virus isolations from the trachea and the cloaca. In these birds probably no systemic infection occurred, hence the lack of any measurable anti-RNP antibody.

Natural infection of migratory ducks and pelagic birds with influenza viruses is potentially important. Carrier migratory waterfowl may spread the virus to other wild bird populations during their annual migrations and therefore provide excellent conditions for inter - species transfer of influenza viruses. A multiple circulation of varied influenza viruses among wild birds provides greater opportunity for genetic recombination. Evidence has accumulated that viruses causing or responsible for pandemics of influenza in man might be derived from influenza viruses infecting other mammals and birds.[19]

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