

## Are Seals Frequently Infected with Avian Influenza Viruses?

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Received 19 March 1984/Accepted 30 May 1984

**Influenza A virus isolates of the H4N5 subtype (which has previously been detected only in birds) were recovered from harbor seals dying of viral pneumonia on the New England coast from June 1982 through March 1983. When these isolates were compared with other mammalian and avian viruses in serological assays and RNA-RNA competitive hybridization, it was found that the seal viruses were most closely related antigenically and genetically to recent avian virus strains and were readily distinguishable from mammalian viruses, including H7N7 isolates recovered from seals in 1980. Unlike any previous isolates from mammals, these recent seal viruses replicate in the intestinal tracts of ducks, a characteristic of avian viruses. The association of avian viruses with influenza outbreaks in seals suggests that transmission of avian viruses to seals is occurring in nature. Potentially, this may be an example of the adaptation of avian viruses to mammals, which would represent an intermediate step in the evolution of new mammalian strains.**

Avian species represent a major reservoir of influenza A viruses in nature (4). It has been postulated that these avian viruses play a role in the appearance of new strains in mammals, e.g., new human pandemic strains, by contributing genes via genetic reassortment between avian and human viruses (10). In support of this hypothesis are antigenic and genetic data that suggest that the hemagglutinin of the pandemic strain A/HK/1/68 (H3N2) originated from an avian virus like A/Dk/Ukr/1/63 (H3N8). In examining the role of avian viruses in disease outbreaks in mammals, we now describe the isolation and characterization of an H4N5 influenza A virus strain (previously detected only in birds) isolated from dead harbor seals in Massachusetts in 1982 to 1983.

During the winter of 1979 to 1980, an H7N7 influenza virus had been associated with a severe outbreak of pneumonia in the New England seal population (3). In view of this, we had been monitoring animals in this as well as other areas for increased mortality or evidence of influenza or both. From 1981 to 1982, there was no increase in seal deaths or strandings observed in Massachusetts; however, 16 lung samples from seals with evidence of pneumonia at autopsy were collected for virus isolation. One sample from an emaciated adult seal stranded on Plum Island, Mass., in June 1982 yielded an influenza A virus strain. Hemagglutination inhibition assays and neuraminidase inhibition tests (6) enabled the identification of the virus as H4N5 (Table 1). These surface antigens have previously been detected only on avian viruses (4). Comparisons with other avian strains (data not shown) by use of chicken and hyperimmune rabbit antisera indicated that the hemagglutinin of the seal virus was closely related to isolates from ducks, e.g., A/Dk/A1b/686/82 (H4N6), and turkeys, e.g., A/Ty/Mn/28/78 (H4N8), and that the neuraminidase was indistinguishable from the prototype for N5, i.e., A/Shearwater/Aust/1/75 (H6N5). These findings indicated that this new virus strain, A/Seal/Mass/133/82, found in seals, was most closely related antigenically to recent avian isolates and was clearly different

from the previous H7N7 seal isolates recovered from 1979 to 1980.

The H7N7 seal virus was shown to have derived all of its genome RNA segments from avian influenza strains (9). Although the surface proteins of the H4N5 seal isolate were clearly distinct from those of the earlier seal virus strain, it was not certain as to whether its internal genes were derived from the previous seal virus or from some other source. To clarify this question, competitive RNA-RNA hybridizations were performed with the RNA segments that code for the internal proteins of the H4N5 virus in comparison with a panel of avian and mammalian virus strains. With this assay, relative homologies are determined by the efficiency with which the various viral RNAs compete with the annealing of the labeled RNA segment and its homologous complementary RNA (1). The results of one of these assays, in which the nucleoprotein gene of Seal/82 was used, are shown in Fig. 1. Turkey/Minn/833/79 competed with the labeled Seal/82 RNA more efficiently than did the earlier seal virus. Two other avian strains, Mallard/Minn/39/79 (H4N2) and Duck/Alberta/60/76 (H12N5), competed at a level similar to that of Turkey/Minn/833/79. Equine and human influenza virus strains competed poorly with the nucleoprotein gene probe. These results (data not shown), as well as the relative homologies of Gull/Md/5/77 and Ty/Ore/1/71 with the other strains, are consistent with a recent analysis of a large number of influenza virus nucleoprotein genes (1).

With each of the other RNA segments, two to four of the seven avian virus strains competed with the labeled H4N5 viral RNA more efficiently than did the RNA from the H7N7 seal virus. These results indicate that the RNA segments coding for the internal genes of the H4N5 seal virus were derived from avian virus strains and not from the preceding H7N7 seal virus.

Since Seal/82 was antigenically and genetically most closely related to avian viruses, we examined its ability to replicate in an avian host. In ducks inoculated orally, Seal/82 replicated to high titers ( $10^{4.5}$  to  $10^{5.0}$  50% egg infective doses [EID<sub>50</sub>] per g of feces) in the intestinal tracts of ducks, a tropism common to avian but not mammalian viruses (11). This result contrasted markedly with those obtained with the

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TABLE 1. Comparison of disease outbreaks associated with influenza A virus in seals on the New England coast from 1979 to 1983

Characteristic	Time (yr) of disease outbreaks	
	1979-1980	1982-1983
Antigenic subtypes of influenza virus isolates	H7N7	H4N5
Estimated mortality	500	60
Histopathological findings	Necrotizing Bronchopneumonia	Necrotizing Bronchopneumonia
Virus recovery from the following tissues of dead seals	Lung, brain, hilar lymph nodes	Lung, brain, hilar lymph nodes
Documented human infection	Yes	No
Concurrent weather conditions	Mild winter	Mild winter
Virus recovery from experimentally inoculated animals		
Seals (nasal passage and eyes)	+	+
Ferrets (nasal passages)	+ ( $10^{8.0}$ EID <sub>50</sub> /ml)	+ ( $10^{6.5}$ EID <sub>50</sub> )
Ducks (intestinal tract)	-	+ ( $10^{5.5}$ EID <sub>50</sub> )

earlier H7N7 seal virus, which replicated poorly, if at all, in avian species and was not enterotropic in birds (9). The new H4N5 seal virus was, therefore, biologically more similar to avian viruses.

Since Seal/82 was essentially an avian virus and had, at that time, been recovered from only one animal, it was important to establish its ability to infect and replicate in seals. To test this, we inoculated six adult seals (1 to 9 years old) intranasally with approximately  $5.0 \times 10^7$  EID<sub>50</sub> of virus in a ground-lung suspension from which Seal/82 had been isolated. To determine whether different species of seals were susceptible to infection with the virus, we used one harbor seal (*Phoca vitulina*), two ringed seals (*Phoca hispida*), and three harp seals (*Phoca groenlandica*) for this study. Nasal, corneal, and anal swabs were collected daily for the first 3 days postinfection (p.i.) and then on alternate days until 9 days p.i. Two seals were sacrificed at 3 days p.i. and one at day 5 p.i., and tissues, including brain, spleen, lung, liver, kidney, colon, thymus, and different lymph nodes (gastric, bronchial, mesenteric, hilar, and mandibular) were collected for virus isolation. Although there were no overt disease signs or histological evidence of pneumonia in the experimental animals, virus was recovered from nasal and corneal swabs from five of the six animals; no virus was recovered from one harp seal. At 9 days p.i., virus was still being recovered from nasal and corneal swabs taken from two of the three remaining seals. Virus was detected in anal swabs from two of the six animals; however, the recovery was sporadic. From the sacrificed animals, low levels ( $10^{1.0}$  EID<sub>50</sub> per g of tissue) of virus were recovered from lung and lymph nodes (bronchial, mesenteric, and mandibular) from two of the animals, and no virus was recovered from either swabs or tissues from the other animal. Before inoculation, the seals had no antibodies to H4N5; by 2 weeks, however, the three remaining animals had detectable antibodies in both hemagglutination inhibition and neuraminidase inhibition assays; these antibodies were still present but declining by 30 days p.i. These findings indicate that Seal/82 infects and replicates in seals, the species from which it was recovered, and that at least three different species of seals are susceptible to infection with this virus.

To determine whether there was any prior evidence of H4N5 viruses in seals, sera from 200 seals collected from 1975 to 1982 were tested in hemagglutination inhibition tests for antibodies to the recent H4N5 virus; however, no antibodies (hemagglutination inhibition titer, <1:10) were detected. Individuals autopsying the seals and working with the viruses had no disease problems and had no detectable antibodies to the H4N5 virus in hemagglutination inhibition tests, suggesting that, in contrast to the H7N7 seal virus strain (8), there was no transmission of this virus to humans.

From January to March 1983, there was a three- to fourfold increase in the number of dead or dying seals observed on the New England coast, particularly in Massachusetts. Of the 60 dead seals reported to the New England Aquarium, 48 were autopsied, and 39 of these animals had evidence of pneumonia. Histopathological examination of the lungs indicated necrotizing bronchopneumonia characterized by extensive degeneration, necrosis, and desquamation of the bronchial and bronchial epithelium. Tissues (lung, lymph nodes, and brain) were collected at autopsy for virus isolation; 16 of the 29 animals tested yielded influenza A viruses, with the highest virus titers obtained from the lungs ( $10^{5.5}$  EID<sub>50</sub> per g of tissue) and lower titers ( $10^{3.5}$  EID<sub>50</sub> per g of tissue) obtained from the brain and lymph nodes. Antigenic characterization of these isolates showed that they were H4N5, indistinguishable from Seal/82, which had been isolated 6 months earlier. These findings suggest that an H4N5 virus was now involved in a disease outbreak in seals in that area.

To determine whether Seal/82 was the progenitor of the 1983 virus or represented a secondary introduction of an antigenically related virus, we compared viruses from 1982 and 1983 by oligonucleotide mapping (7) and found that they were virtually indistinguishable. Biologically, the Seal/83 strain was also enterotropic in birds, similar to Seal/82. Since it seems very unlikely that the same virus would be introduced on two separate occasions, it is more plausible that this virus remained in the seal population during this time. It is suggested that the virus is spreading and possibly adapting to seals and thereby increasingly causing disease in these animals.

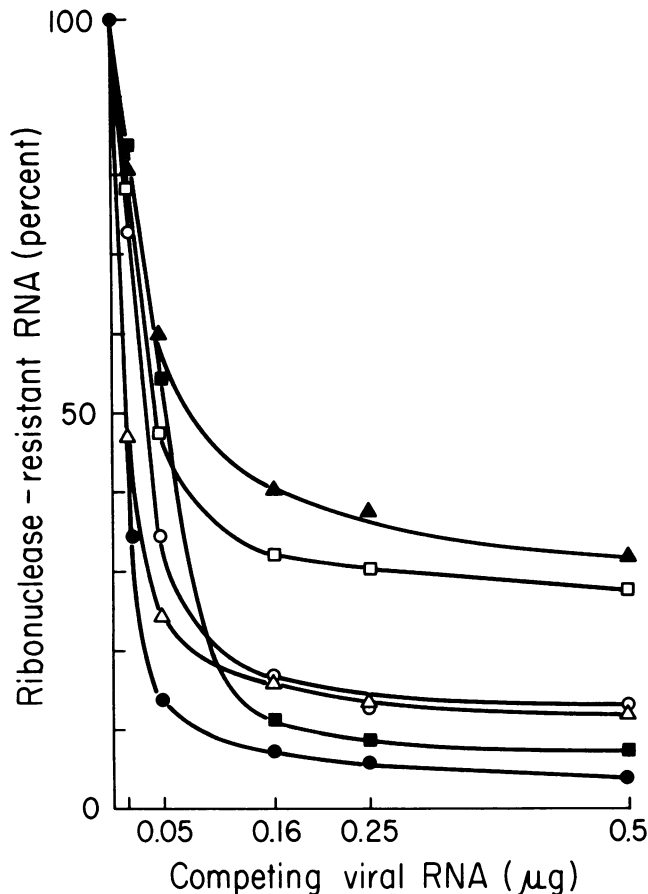


FIG. 1. Comparison of RNAs from avian virus strains and Seal/Mass/1/80 (H7N7) with RNA segment 5 from Seal/Mass/133/82 (H4N5) by competitive hybridization.  $^{125}\text{I}$ -Labeled RNA segment 5 was annealed with homologous complementary RNA in the presence of increasing amounts of competing RNA from the homologous virus strain and other strains. The degree of relatedness between the labeled probe and the corresponding RNA segment of the other virus strains is indicated by the efficiency with which each RNA competes with the annealing of the labeled RNA and its homologous complementary RNA. The isolation and iodination of the RNA segments have been described previously (2). The RNA mixtures were annealed for 24 h at  $10^\circ\text{C}$  below the melting temperature of the double strand formed by annealing the labeled probe with homologous complementary RNA. Other details were as described previously (1). Also included in the assay were: Mallard/Minn/39/79 (H4N2) and Duck/Alberta/60/76 (H12N5), which reacted similarly to Ty/Minn/833/79; Teal/Iceland/1/80 (H7N7), which reacted similarly to Gull/Md/5/77; and two equine strains and one human virus strain which competed poorly with this probe. With each of the other RNA segments, two to four of the seven avian virus strains tested competed with the labeled RNA more efficiently than did RNA from the H7N7 seal virus strain. Symbols:  $\blacktriangle$ , Ty/Ore/1/71 (H7N3);  $\square$ , Gull/Md/5/77 (H11N9);  $\circ$ , Mallard/NY/6874/78 (H3N2);  $\triangle$ , Seal/Mass/1/80 (H7N7);  $\blacksquare$ , Ty/Minn/833/79 (H4N2);  $\bullet$ , Seal/Mass/133/82 (H4N5).

Antigenic, genetic, and biological characterization of these H4N5 viruses indicates that these seal viruses are avian in all properties, including the ability to replicate in the intestinal tracts of birds. This is the second documented

outbreak of influenza in seals and the first evidence that an influenza virus with all of the properties of an avian influenza virus can be involved in naturally occurring influenza in mammals. The earlier epizootic of viral pneumonia in seals in 1979 to 1980 (3) involved H7N7 viruses which were antigenically and genetically related to avian strains but replicated poorly, if at all, in avian species and were not enterotropic in birds. However, this virus grew quite well in a variety of mammals, including squirrel monkeys (5), and caused conjunctivitis in humans (8), indicating that the host range of the H7N7 seal virus was not limited to marine mammals. The detection of the H4N5 strain in seals raises questions as to whether this avian virus has been newly introduced and, as a step in the evolution of influenza viruses in nature, is currently adapting to a mammalian host or whether avian viruses are frequently transmitted to seals and associated with disease outbreaks in these mammals.

We thank Jim and Mary Ann Bigelow and Janet Ulm for expert technical assistance.

This study was supported by Public Health Service grants AI 02649 and AI 16841 from the National Institute of Allergy and Infectious Diseases, by Childhood Cancer Center Support (CORE) grant CA 21765 from the National Cancer Institute, and by ALSAC.

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