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# Coinfection of Wild Ducks by Influenza A Viruses: Distribution Patterns and Biological Significance

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**Coinfection of wild birds by influenza A viruses is thought to be an important mechanism for the diversification of viral phenotypes by generation of reassortants. However, it is not known whether coinfection is a random event or follows discernible patterns with biological significance. In the present study, conducted with viruses collected throughout 15 years from a wild-duck population in Alberta, Canada, we identified three discrete distributions of coinfections. In about one-third of the events, which involved subtypes of viruses that appear to be maintained in this duck reservoir, coinfection occurred at rates either close to or significantly lower than one would predict from rates of single-virus infection. Apparently, the better adapted an influenza A virus is to an avian population, the greater is its ability to prevent coinfections. Conversely, poorly adapted, nonmaintained viruses were significantly overrepresented as coinfectants. Rarely encountered subtypes appear to represent viruses whose chances of successfully infiltrating avian reservoirs are increased by coinfection. Mallards (*Anas platyrhynchos*) and pintails (*A. acuta*) were significantly more likely to be infected by a single influenza A virus than were the other species sampled, but no species was significantly more likely to be coinfected. These observations provide the first evidence of nonrandom coinfection of wild birds by influenza A viruses, suggesting that reassortment of these viruses in a natural population does not occur randomly. These results suggest that even though infections may occur in a species, all subtypes are not maintained by all avian species. They also suggest that specific influenza A virus subtypes are differentially adapted to different avian hosts and that the fact that a particular subtype is isolated from a particular avian species does not mean that the virus is maintained by that species.**

Migratory ducks and other waterfowl appear to serve as the reservoir for most influenza A viruses, including the hemagglutinin (HA) and neuraminidase (NA) subtypes of previous pandemic strains (13, 32, 35, 36, 39). Although humans do not appear to be directly infected by avian influenza viruses, swine can support the replication of both human and avian viruses (16). Coinfection of swine by a human and an avian virus is thought to favor genetic reassortment, which may generate hybrid viruses capable of infecting humans (5, 6, 30, 42). This link between human influenza pandemics and avian reservoirs of influenza A virus has stimulated considerable interest in the virus strains that infect these birds and the mechanisms responsible for their maintenance.

Over the past three decades, influenza A viruses of various HA and NA subtypes have been detected in wild waterfowl throughout the world. Investigators have detected such viruses in ducks and whistling swans from Siberia that overwinter in Japan (22–25, 40), in North American ducks that overwinter in the southern United States (37), and in waterbirds sampled in South Africa (4), Israel (18), western Europe (2, 34, 38), Russia (19, 21), southern China (33), and Australia (7).

Influenza A viruses preferentially replicate in the cells lining the intestinal tracts of ducks and are excreted in high concentrations in their feces, resulting in the infection of both wild and domestic birds when water supplies are contaminated (12, 35, 36, 43). Genetic reassortment between two different avian influenza viruses can occur in the duck intestinal tract, partially explaining the wide spectrum of influenza virus subtypes that have been isolated from these waterfowl (10). Such infections are symptomless and produce only minimal antibody responses (17); however, despite their lack of detectable antibodies, ducks shed virus for a maximum of 14 days and are immune to subsequent influenza infections for at least 96 days after the first episode (17). Influenza A viruses that infect avian species accumulate, to a large extent, only synonymous mutations, i.e., those that do not result in amino acid changes. This evolutionary stasis at the amino acid level is in contrast to the rapid evolution seen in mammalian influenza A viruses (8, 9, 14, 28, 31, 41).

The major aim of this study was to determine if coinfection of North American wild ducks by influenza A viruses occurs randomly or in patterns that might reflect the intrinsic ability of the viruses to reassort and thereby to adapt to avian species. Such well-adapted viruses would be more likely to be maintained by an avian reservoir and thus would be available for possible emergence from such a reservoir for potential reassortment with human and other mammalian viruses during the annual migrations of the ducks from Canada to the south.

## MATERIALS AND METHODS

**Sample collection.** Annually from 1976 to 1990, Canadian Wildlife Service personnel sampled ducks in Alberta, Canada, after the end of the breeding season in June, when the birds were assembling at marshalling lakes to begin their migration south. Cloacal specimens were collected from late July through early September at lakes near Vermilion (1976 to 1978), Grande Prairie (1979 to 1984), and Edmonton (1985 to 1990), as reported previously (32). A subset of

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samples were collected from ducks in April 1984, shortly after the birds had returned from their overwintering locations. Although three different sampling sites were used over the course of the study, a high percentage of ducks visited each site during the spring and summer and traveled to the same wintering grounds. Thus, our results represent a single population of North American migratory ducks. Any bias introduced by the use of multiple sampling sites would probably be slight, as all comparisons of influenza A viruses present in coinfections and single infections involved ducks sampled at the same location.

Over the 15-year study period, cloacal specimens were randomly collected from 14,392 (20.6%) of 69,892 ducks caught and banded by wildlife personnel, who also recorded the gender, age, and species of the birds (1, 32).

**Virus identification.** We processed 12,321 (85.6%) of the 14,392 samples that were collected. All specimens were stored in liquid nitrogen upon their arrival at St. Jude Children's Research Hospital and were removed randomly for processing. Mallards (*Anas platyrhynchos*) accounted for 62.6% of processed samples, pintails (*Anas acuta*) accounted for 19.8%, and blue-winged teals (*Anas discors*) accounted for 11.7%. The remaining processed samples (5.7%) were collected from 11 other duck species. Since 94.3% of processed samples were from mallards, pintails, and blue-winged teals, our results apply primarily to these species.

Viruses were grown in the allantoic cavities of 11-day-old embryonated chicken eggs (11). HA titer determinations and HA inhibition tests were performed in microtiter plates with receptor-destroying enzyme-treated sera as previously described (26). NA titer determinations and NA inhibition tests were performed by the methods of Aymard-Henry et al. (3). Viruses were classified according to the antigenic characteristics of their HAs and NAs, as previously described (11). All hemagglutinating agents were identified in inhibition tests with monospecific antisera to the isolated surface antigens of reference influenza viruses (11). Antisera to selected avian isolates were prepared in chickens by standard procedures (26). Coinfection was defined as positivity for more than one HA or NA. In addition, as described in more detail elsewhere (10), all 863 influenza A virus isolates from 1978, 29.4% of the study's total 2,937 isolates, were mixed with specific antisera to the detected virus and then reinoculated into chicken embryos to allow the detection of any virus that might not grow well or might be masked in the presence of another virus. Viruses that grew in the presence of antisera were then characterized. For example, in one instance, a cloacal sample that had yielded an H6N2 virus was passaged in chicken embryos with antiserum to H6N2. A virus was isolated and identified as H3N8, indicating that the sample contained both H3N8 and H6N2 viruses. Since the first virus detected was blocked in the second cycle of testing, the use of this technique enabled us to determine specific HA-NA pairings. A third cycle of this process was required in 28 instances when dual coinfectants were detected; a fourth cycle was required twice when triple coinfections were detected. Although we did not use this technique with isolates collected in years other than 1978, in most of these instances (83%) a single HA was found with two NAs or a single NA was found with two HAs, making determination of the two coinfecting viruses straightforward.

**Epidemiologic analysis.** All recorded coinfections were analyzed to determine if certain pairs of influenza A virus subtypes were more or less likely to coinfect ducks and thus, presumably, to reassort. The influence of age on susceptibility to coinfection was determined by comparing the prevalence rates of coinfection among juvenile and adult ducks over the entire study period. Samples for which age was not recorded were excluded from this analysis. We also compared the prevalence rates of single and multiple infections among the three major duck species and a group of the other 11 species of ducks sampled.

To assess the distribution of coinfections (whether random or nonrandom), we calculated their total number as well as the total number of single infections during each week when at least one pair of coinfecting viruses was found. We then determined the number and proportion of weekly samples positive for each virus found as a coinfectant during such weeks. The expected proportion of weekly samples harboring coinfecting viruses was calculated by multiplying the proportion for one coinfecting virus by the proportion for the other, and the result was multiplied by the total number of samples obtained in that week to calculate the number of times each coinfection would be expected to occur during the week it was found, assuming that coinfection was random.

An observed-to-expected ratio (the actual frequency of each coinfection divided by its predicted frequency) was used to summarize the degree to which specific subtype combinations were more or less likely to coinfect wild ducks. For dual coinfections, differences between the probability of coinfection and the product of the probabilities of a duck being infected by each coinfecting virus singly were tested for significance by Fisher's exact test. Two-sided *P* values were calculated unless otherwise specified. To facilitate comparisons, we grouped the viruses according to whether they occurred as often as expected ($P > 0.05$) or significantly more often or less often than expected ($P \leq 0.05$). To determine if species had an effect on the ratios associated with each coinfection, the above analysis was repeated, taking into account the species of duck in which the coinfection was detected. The method outlined above was used to compare the numbers of dual and single infections occurring in a particular duck species and to calculate species-specific observed-to-expected ratios for each coinfection.

A week-long sampling period was used in most calculations, because all the ducks in a previous experiment shed virus from 4 to 14 days after infection (10). Thus, the viral infections that might be related to a coinfection would be most likely to occur during the week of the coinfection. To determine if the length of the sampling period affected our results, we repeated all calculations with the entire season as the base of analysis. All calculations were performed with the Statistical Analysis System, version 6 (29).

TABLE 1. Numbers of the 58 different HA-NA subtypes isolated during study[a]

| HA-NA subtype(s) | Total no. of isolations of each virus | % of total isolates accounted for by each virus |
|---|---|---|
| H3N8, H6N2 | 745 | 26.3 |
| H4N6 | 414 | 14.6 |
| H6N5, H6N6 | 168 | 5.9 |
| H6N8 | 92 | 3.2 |
| H1N1 | 73 | 2.6 |
| H3N6 | 72 | 2.5 |
| H4N2 | 47 | 1.7 |
| H4N8 | 32 | 1.1 |
| H3N1, H3N2 | 27 | 0.9 |
| H2N3 | 19 | 0.7 |
| H10N7, H11N9 | 18 | 0.6 |
| H7N3 | 17 | 0.6 |
| H1N2 | 14 | 0.5 |
| H6N4 | 13 | 0.5 |
| H1N4 | 12 | 0.4 |
| H8N4 | 10 | 0.4 |
| H12N5 | 9 | 0.3 |
| H9N1 | 7 | 0.2 |
| H4N4, H6N1 | 6 | 0.2 |
| H1N3, H3N5, H3N9, H4N1, H5N2 | 5 | 0.2 |
| H4N3, H4N5, H6N9 | 4 | 0.1 |
| H2N5, H3N4, H6N3, H9N2, H10N6 | 3 | 0.1 |
| H1N6, H1N9, H2N4, H3N3, H7N8, H11N4, H12N1 | 2 | 0.07 |
| H1N5, H1N8, H2N9, H4N9, H5N3, H7N2, H7N5, H10N1, H10N3, H11N3, H11N6, H11N8, H12N4, H13N6 | 1 | 0.04 |
| Total[b] | 2,839 | 100.0 |

[a] Excludes 98 virus isolates for which the HA, NA, or both have not been characterized.

[b] The total number and percentage of each virus subtype isolated, e.g., 745 (26.3%) H3N8 isolations plus 745 (26.3%) H6N2 isolations, plus 414 (14.6%) H4N6 isolations, etc.

## RESULTS

**Influenza A viruses infecting the wild-duck population.** H3N8 and H6N2 viruses were each isolated 745 times during the study, with each virus accounting for 26.3% of all isolations (Table 1). Together with H4N6, which was isolated 414 times, these viruses comprised about two-thirds of the identified isolates. By comparison, the 29 different viruses that were each isolated four or fewer times during the study accounted for only 1.9% of all isolates, with 14 of these viruses being isolated only once (Table 1). Of the 15 recognized HA subtypes, 13 were isolated at least once from the wild-duck population. The exceptions were H14 viruses, which have been detected in European ducks (15, 20), and H15 viruses, which have recently been found in Australia (27). Each of the nine known NA subtypes was isolated from 18 (N7) to 873 times (N8) during the study. The viral isolates represented 58 different combinations of HAs and NAs (Table 1). In addition to samples containing a single virus, 54 samples containing more than one virus (47 containing two viruses and 7 containing three) were identified. Four of these coinfections involved one or more unidentified viruses, leaving 50 coinfections that could be analyzed. All but two of these coinfections occurred in mallards,

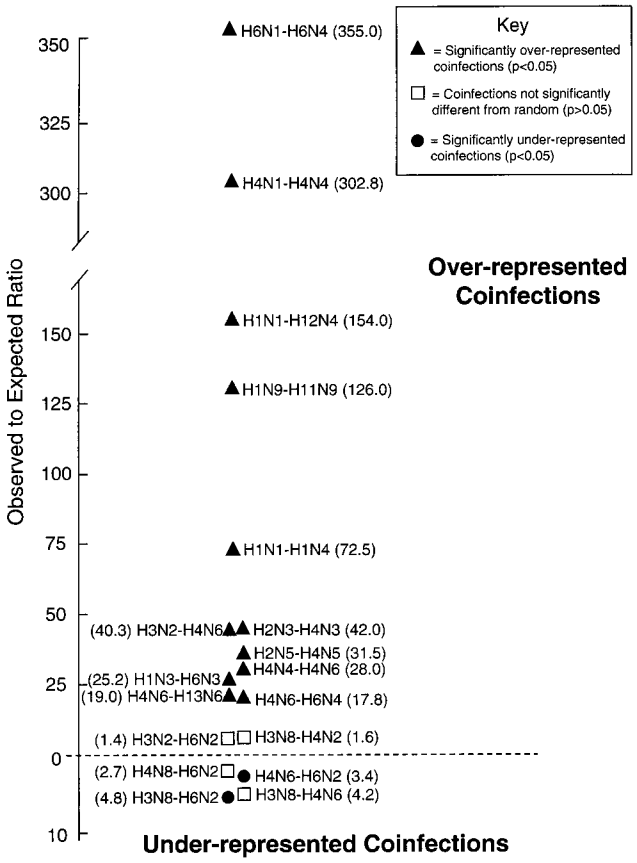


FIG. 1. Ratios of observed to expected frequencies of dual influenza A virus coinfections in wild ducks. Coinfections above the line occurred more often than expected during the study, compared to the number of times the coinfecting viruses were isolated during the week of each coinfection. These ratios indicate that the coinfection occurred $x$ number of times more often than was expected. The coinfections below the line occurred less often than expected. These ratios, which originally had values less than 1.0, were transformed into reciprocals by dividing them into 1.0. These ratios indicate that these coinfections occurred $x$ times less often than expected. When a coinfection with two viruses occurred during more than 1 week of the study, we calculated the mean observed-to-expected ratio. Solid triangles and circles indicate that the over- or underrepresentation was statistically significant.

pintails, or blue-winged teals, the species that accounted for 97.5% of isolates.

**Species- and age-specific prevalences.** Juvenile ducks were 2.5 (95% confidence interval, 2.3 to 2.8) times more likely to be singly infected with influenza A viruses than were adults ($P < 0.0001$, one sided) but only 1.6 (0.9 to 3.0) times more likely to be coinfected ($P = 0.96$, one sided). Mallards and pintails were 2.1 (1.9 to 2.4) times more likely to be singly infected than blue-winged teals and all 11 other species of ducks combined ($P < 0.0001$). A total of 26% of mallards and 29% of pintails were singly infected compared with 14% of blue-winged teals and 9% of the remaining species. There was no apparent association between species and prevalence of coinfection ($P = 0.77$). Coinfection prevalences were 0.49% for mallards, 0.41% for pintails, 0.42% for blue-winged teals, and 0.25% for the group of ducks of other species.

**Likelihood of coinfection.** To assess the variability of coinfection with different influenza A viruses, we determined whether a particular virus pair appeared significantly more often or less often than expected ($P \leq 0.05$), assuming that coinfection is a random event. As shown in Fig. 1, the 18 different dual coinfections which were detected over the course of the study had a wide range of observed-to-expected ratios. The highest ratio was 355.0 for the overrepresented coinfection of H6N1 with H6N4 in 1979, indicating that this coinfection occurred 355 times more often than was expected. The lowest ratio was 4.8 for the underrepresented coinfection of H3N8 with H6N2 in 1978; this ratio indicates that the coinfection occurred about five times less often than was expected (4.8 is the reciprocal of the mean of the three ratios listed in Table 2 for this coinfection).

Most of the coinfections we detected were isolated during a single week of the study, but four were detected during two or three different weeks. As shown in Table 2, for 4 of the 18 coinfections, all of which occurred in 1978, we were unable to reject the null hypothesis of random occurrence; that is, these coinfections occurred about as often as would be expected if coinfections occurred randomly ($P > 0.05$). All of these coinfections involved viruses with the three HAs and the three NAs that appear to consistently infect both adult and juvenile ducks (mallards, pintails, and blue-winged teals) in this population of North American ducks over extended periods (H3, H4, and H6; N2, N6, and N8) (32). The other 14 coinfections had observed-to-expected ratios indicative of nonrandom variability (i.e., $P \leq 0.05$). Two different coinfections, both of which occurred in three different weeks of 1978, were detected less often than one would predict from their single-isolate prevalence rates during the week or weeks of identification. H6N2 viruses participated in both of these coinfections, with H3N8 and H4N6 strains serving as partner coinfectants. Like their apparently random counterparts, these underrepresented coinfections involved the HAs and NAs that are maintained by mallards, pintails, and blue-winged teals in this avian population and could be detected only when the first detected virus was blocked with antisera. In contrast, 12 of the nonrandom coinfections were significantly overrepresented, with observed-to-expected ratios ranging from 17.8 to 370.0. Although some of the HAs and the NAs that appear to be maintained by this avian population (i.e., H3, H4, H6, N2, and N6) were also represented in this group, many of these coinfections involved nonmaintained HAs and NAs.

**Duck species-specific estimates.** To determine if our findings were influenced by the species of duck in which each coinfection was detected, we calculated species-specific observed-to-expected ratios. For example, all three coinfections of H1N1 with H1N4 (Table 2) were detected in mallards in one week of 1976, and we recalculated the observed-to-expected ratio excluding all samples not obtained from mallards that week, thereby reducing the ratio from 72 to 54. Because the number of ducks sampled during the week of each coinfection was reduced (because of the exclusion of all other species except for the one of interest), the effect of this reanalysis was to reduce the magnitude of each ratio; i.e., the species-specific ratio was closer to unity than was the overall ratio. However, in each instance, the species-specific ratio was in the same direction as the overall ratio; thus, this reanalysis was congruent with the original classification of coinfections as underrepresented, randomly occurring, or overrepresented.

As shown in Table 2, three different coinfections occurred in more than one species. In each instance, the species-specific observed-to-expected ratios were similar when the sample size was adequate. For example, the pintail-specific ratio for the H3N8-H6N2 coinfection in 1978 was 0.32, compared to mallard-specific ratios of 0.21, 0.16, and 0.19. The H3N2-H6N2 coinfection in 1978 had a mallard-specific ratio of 1.0 compared to pintail-specific ratios of 1.7 and 3.1. The H1N3-H6N3 coinfection in 1990 had a mallard-specific ratio of 26.7, a pin-

TABLE 2. Distribution pattern and frequency of dual coinfections[a]

| Dual coinfection | Yr and wk of virus isolation | No. of ducks sampled during wk | Species in which detected[b] | Observed frequency of dual coinfection | Frequency of: | | Ratio of observed to expected frequencies of coinfection[c] | $P^d$ |
|---|---|---|---|---|---|---|---|---|
| | | | | | First virus strain ($f1$) | Second virus strain ($f2$) | | |
| Underrepresented | | | | | | | | |
| H3N8-H6N2 | 1978, 32 | 820 | M, P | 11 | 84 | 335 | 0.32 (11/34.3) | <0.0001 |
| | 1978, 33 | 309 | M | 1 | 38 | 71 | 0.11 (1/8.7) | 0.0007 |
| | 1978, 34 | 545 | M | 1 | 33 | 85 | 0.20 (1/5.1) | 0.04 |
| H4N6-H6N2 | 1978, 32 | 820 | M | 2 | 17 | 335 | 0.29 (2/6.9) | 0.01 |
| | 1978, 33 | 309 | M | 1 | 15 | 71 | 0.29 (1/3.44) | 0.21[e] |
| | 1978, 34 | 545 | M | 3 | 68 | 85 | 0.28 (3/10.6) | 0.004 |
| Randomly occurring | | | | | | | | |
| H3N2-H6N2 | 1978, 32 | 820 | M, P | 2 | 3 | 335 | 1.63 (2/1.2) | 0.57 |
| | 1978, 33 | 309 | P | 1 | 4 | 71 | 1.09 (1/0.9) | 1 |
| H3N8-H4N2 | 1978, 32 | 820 | M | 1 | 84 | 6 | 1.6 (1/0.61) | 0.48 |
| H3N8-H4N6 | 1978, 34 | 545 | M | 1 | 33 | 68 | 0.24 (1/4.12) | 0.11 |
| H4N8-H6N2 | 1978, 34 | 545 | W | 1 | 17 | 85 | 0.37 (1/2.7) | 0.49 |
| Overrepresented | | | | | | | | |
| H1N1-H1N4 | 1976, 32 | 943 | M | 3 | 13 | 3 | 72.5 (3/0.04) | <0.0001 |
| H1N1-H12N4 | 1984, 34 | 154 | M | 1 | 1 | 1 | 154.0 (1/0.007) | 0.007 |
| H1N3-H6N3 | 1990, 35 | 126 | M, P, G | 3 | 5 | 3 | 25.2 (3/0.119) | <0.0001 |
| H1N9-H11N9 | 1990, 35 | 126 | M | 1 | 1 | 1 | 126.0 (1/0.008) | 0.008 |
| H2N3-H4N3 | 1990, 35 | 126 | M | 1 | 1 | 3 | 42.0 (1/0.02) | 0.024 |
| H2N5-H4N5 | 1990, 35 | 126 | B | 2 | 2 | 4 | 31.5 (2/0.06) | 0.0008 |
| H3N2-H4N6 | 1985, 33 | 282 | M | 1 | 1 | 7 | 40.3 (1/0.24) | <0.025 |
| H4N1-H4N4 | 1976, 32 | 943 | M | 1 | 2 | 2 | 235.7 (1/0.004) | 0.004 |
| | 1977, 35 | 370 | M | 1 | 1 | 1 | 370.0 (1/0.003) | 0.003 |
| H4N4-H4N6 | 1977, 32 | 727 | M | 1 | 1 | 26 | 28.0 (1/0.036) | 0.036 |
| H4N6-H6N4 | 1982, 33 | 389 | M | 1 | 22 | 1 | 17.8 (1/.056) | 0.057 |
| H4N6-H13N6 | 1989, 34 | 19 | B | 1 | 1 | 1 | 19.0 (1/0.05) | 0.053 |
| H6N1-H6N4 | 1979, 33 | 355 | P | 1 | 1 | 1 | 355.0 (1/0.003) | 0.003 |

[a] A total of 54 coinfections were detected during the study. Four involved one or more unidentified viruses, and seven involved three viruses. The table lists only dual coinfections for which both viruses were identified and excludes triple coinfections.

[b] Duck species abbreviations: B, blue-winged teal (*Anas discors*); G, green-winged teal (*Anas crecca*); P, pintail (*Anas acuta*); M, mallard (*Anas platyrhynchos*); W, widgeon (*Anas americana*).

[c] Expected frequency of coinfection = $[(f1/n)\ (f2/n)] \times n$, where $n$ is the number of ducks sampled during the week when coinfection was detected (excluding triple coinfections). The median observed-to-expected ratios for underrepresented, randomly occurring, and overrepresented coinfections were 0.29, 1.09, and 42.0, respectively. The numbers from which the ratios were calculated are given in parentheses.

[d] Fisher's exact test, two sided.

[e] This coinfection is included with the underrepresented coinfections, even though its associated $P$ value exceeds 0.05, because its associated ratio is consistent with those for two other weeks when the coinfection was detected.

tail-specific ratio of 15.0, and a green-winged teal (*Anas crecca*) ratio of 2.0, although just two ducks of the last species were sampled that week.

**Season versus week sampling estimates.** To determine if our findings were affected by the unit of time selected as the base of analysis, we repeated the calculations with prevalence rates over the entire summer season rather than during the week of sample collection. This approach resulted in slight changes in the magnitude of some of the observed-to-expected ratios and $P$ values but generally did not change the classification of coinfections as randomly occurring, significantly underrepresented, or significantly overrepresented. The only exceptions to this were the H3N8-H4N6 and H4N8-H6N2 coinfections classified as randomly occurring in Table 2. Based on their associated $P$ values, these would be classified as underrepresented coinfections if the season were used as the unit of analysis. Otherwise, the results based on seasonal prevalences were consistent with those reported in Table 2.

**Consistency of observed-to-expected ratios over time.** We would stress that pairs of significantly nonrandom viruses isolated in multiple weeks of the study were associated with similar observed-to-expected ratios of coinfection. For example, the ratios for the underrepresented H4N6-H6N2 combination, isolated in each of three different weeks during 1978, were 0.29, 0.29, and 0.28 ($P$ = 0.01, 0.21, and 0.004, respectively [Table 2]). Moreover, the ratios for an overrepresented combination, H4N1-H4N4, found during two different weeks of 1976 and 1977 were consistently high (235.7 and 370.0), indicating that these viruses were aggressive coinfectants whenever they appeared together in the wild-duck population.

**Triple coinfections.** Table 3 lists the six different triple coinfections that were detected during the study. The observed-to-expected ratios for these coinfections ranged from 1.5 to 7938.0, in each instance indicating that these combinations of viruses occurred more often than expected. The H1N2-H6N5-H6N2 coinfection was found twice during week 34 of 1979.

**Frequency of isolation of over- and underrepresented coinfectants.** Many of the overrepresented coinfecting viruses in this study were not found as single isolates during the week(s) of their collection. In August (week 35) 1977, for example, H4N1 and H4N4 strains were isolated together but not singly (Table 2). Similar findings were recorded for H1N1 and H12N4 strains in 1984, H1N9 and H11N9 in 1990, H4N6 and H13N6 in 1989, and H6N1 and H6N4 in 1979. An additional three of the overrepresented coinfections involved at least one virus that was not found as a single infectant during the week

TABLE 3. Distribution pattern and frequency of triple coinfections

| Triple coinfection | Yr and wk of virus isolation | No. of ducks sampled during week | Observed frequency of triple coinfection | Frequency of: | | | Ratio of observed to expected frequencies of coinfection[a] |
|---|---|---|---|---|---|---|---|
| | | | | First virus strain (*f*1) | Second virus strain (*f*2) | Third virus strain (*f*3) | |
| H1N2-H6N5-H6N2 | 1979, 34 | 395 | 2 | 2 | 152 | 37 | 28.6 (2/0.07) |
| H1N4-H6N5-H6N8 | 1979, 34 | 395 | 1 | 1 | 152 | 60 | 17.1 (1/0.5) |
| H3N1-H3N4-H4N6 | 1983, 31 | 289 | 1 | 8 | 10 | 1 | 1,044.0 (1/0.001) |
| H3N2-H3N8-H6N2 | 1978, 32 | 821 | 1 | 4 | 85 | 336 | 5.9 (1/0.17) |
| H3N8-H4N6-H6N2 | 1978, 34 | 546 | 1 | 69 | 34 | 86 | 1.5 (1/0.68) |
| H4N1-H4N4-H11N4 | 1983, 33 | 126 | 1 | 1 | 1 | 2 | 7,938.0 (1/0.0001) |

[a] Expected frequency of coinfection = $[(f1/n)\,(f2/n)\,(f3/n)] \times n$, where $n$ is the number of ducks sampled during the week when coinfection was detected. The numbers from which the ratios were calculated are given in parentheses.

of its coinfection. It should be emphasized that most of the viruses found only as coinfectants during the week of their isolation were never detected as single isolates over the entire summer when they occurred and were rarely found as single infectants during the 15 years of study.

The rarity of these viruses and their proclivity for coinfection are illustrated in Table 4, which provides information about the prevalence of the 17 significantly overrepresented dual coinfectants as single infectants and as dual or triple coinfectants during the entire study. H4N6, which was classified as an overrepresented coinfectant in Table 2, was excluded from Table 4 because it was also classified as a randomly occurring and underrepresented coinfectant. The prevalences of the 17 viruses included in Table 4 were low in the seasons of their isolations as dual infectants (median, 3.35 per 1,000 samples), and they were rarely isolated during the 15-year study (median number of isolations, 5). Of the 17 viruses, 8 (H1N3, H1N9, H2N5, H4N1, H4N4, H6N3, H12N4, and H13N6) were found as coinfectants at least as often as they were found as single isolates; 3 of these viruses (H6N3, H12N4, and H13N6) were detected only as coinfectants.

Table 5 lists the two different viruses (H3N8 and H6N2) that were found to be both significantly underrepresented and randomly occurring coinfectants and the three other viruses classified as randomly occurring coinfectants. H4N6 is also excluded from this table, because it was involved in all three classifications of coinfections. Over the study period, H3N8 and H6N2 were the two most frequently isolated viruses, together accounting for about half of the total isolations in the study. As shown in Table 2, when H3N8 and H6N2 interacted with each other or with H4N6, which together were the three most prevalent viruses in the study, they had significantly reduced chances of coinfecting. However, when H3N8 and H6N2 interacted with H3N2, H4N2, or H4N8, which also appear to be maintained by mallards, pintails, and blue-winged teals in this avian population but which less frequently infected them, H3N8 and H6N2 had higher rates of coinfection, approaching expected levels based on the numbers of single infections. As shown in Table 2, H3N8 and H6N2 were found as coinfectants only when the special blocking technique used for 1978 samples was employed.

Prevalence rates during the season of isolation, up to and including the week of the coinfection, were markedly lower for the overrepresented coinfectants than for the underrepresented or randomly occurring group (median, 3.35 versus 201.5 and 98.6 isolations per 1,000 samples [Tables 4 and 5]). The

TABLE 4. Influenza A viruses overrepresented as coinfectants[a]

| Coinfecting virus | Yr and wk of isolation as coinfectant | Prevalence(s) of virus in season of coinfection[b] | Total no. of isolations during study | No. of times detected as coinfectant[c] | % of isolations detected as coinfectant |
|---|---|---|---|---|---|
| H1N1 | 1976, 32; 1984, 34 | 13.8, 2.5 | 73 | 4 | 5.5 |
| H1N3 | 1990, 35 | 39.7 | 5 | 3 | 60 |
| H1N4 | 1976, 32 | 3.2 | 12 | 4 | 33.3 |
| H1N9 | 1990, 35 | 7.9 | 2 | 1 | 50 |
| H2N3 | 1990, 35 | 7.9 | 19 | 1 | 5.3 |
| H2N5 | 1990, 35 | 15.9 | 3 | 2 | 66.7 |
| H3N2 | 1985, 33 | 3.5 | 27 | 5 | 18.5 |
| H4N1 | 1976, 32; 1977, 35 | 2.1, 0.4 | 5 | 3 | 60 |
| H4N3 | 1990, 35 | 23.8 | 4 | 1 | 25 |
| H4N4 | 1976, 32; 1977, 32, 35 | 2.1, 0.7, 0.8 | 6 | 4 | 66.7 |
| H4N5 | 1990, 35 | 31.7 | 4 | 2 | 50 |
| H6N1 | 1979, 33 | 1.2 | 6 | 1 | 16.7 |
| H6N3 | 1990, 35 | 23.8 | 3 | 3 | 100 |
| H6N4 | 1979, 33; 1982, 33 | 1.2, 1.2 | 13 | 3 | 23.1 |
| H11N9 | 1990, 35 | 7.9 | 18 | 1 | 5.9 |
| H12N4 | 1984, 34 | 1.3 | 1 | 1 | 100 |
| H13N6 | 1989, 34 | 15.9 | 1 | 1 | 100 |
| Median values, range (minimum, maximum) | | 3.35 (0.41, 39.7) | 5 (1, 73) | 2 (1, 5) | 50.0 (5.3, 100) |

[a] The analysis was limited to coinfections with observed-to-expected ratios greater than 1.0 and two-sided Fisher exact $P$ values of $\leq 0.05$. The table excludes H4N6, which was found as a significantly underrepresented, significantly overrepresented, and randomly occurring coinfectant.

[b] Seasonal prevalences per 1,000 samples were calculated up to and including the week of virus isolation.

[c] Includes triple coinfections.

TABLE 5. Influenza A viruses underrepresented or randomly occurring as coinfectants[a]

| Coinfecting virus | Yr and wk of isolation as coinfectant | Prevalence of virus in season of isolation[b] | Total no. of isolations during study | No. of times detected as coinfectant | % of isolations detected as coinfectant |
|---|---|---|---|---|---|
| Significantly underrepresented | | | | | |
| H3N8 | 1978, 32, 33, 34 | 103.5, 108.8, 93.7 | 745 | 17 | 2.3 |
| H6N2 | 1978, 32, 33, 34 | 409.3, 360.2, 294.2 | 745 | 28 | 3.8 |
| Median | | 201.5 | 745 | 22.5 | 3.0 |
| Apparently randomly occurring | | | | | |
| H3N2 | 1978, 32, 33 | 3.7, 7.1 | 27 | 5 | 18.5 |
| H3N8 | 1978, 32, 34 | 103.4, 93.7 | 745 | 17 | 2.3 |
| H4N2 | 1978, 32 | 7.3 | 47 | 1 | 2.1 |
| H4N8 | 1978, 34 | 11.3 | 32 | 1 | 3.1 |
| H6N2 | 1978, 32, 33, 34 | 409.3, 360.2, 294.2 | 745 | 28 | 3.8 |
| Median | | 98.6 | 47 | 5 | 3.1 |

[a] Analysis limited to influenza A viruses classified as significantly underrepresented or randomly occurring coinfectants, excluding H4H6, which was found as a significantly underrepresented, significantly overrepresented, and randomly occurring coinfectant.
[b] Seasonal prevalences per 1,000 samples were calculated up to and including the week of virus isolation.

total numbers of isolations were also substantially lower for overrepresented coinfectants (median, 5) than for underrepresented or randomly occurring coinfectants (median isolations, 745 and 47, respectively [Tables 4 and 5]). Considered together, these observations strongly suggest an inverse relationship between the prevalence of a virus and its coinfection rate.

In this study, the underrepresented and apparently randomly occurring coinfections were characterized almost uniformly by combinations of viruses with different HAs and NAs; hence, they could be expected to give rise to reassortants with new combinations of HAs and NAs. In contrast, 75% of the viruses that were significantly overrepresented as dual coinfectants were isolated with viruses possessing either the same HA or the same NA gene segment (Table 2). For example, in 1976, H1N1 was found with a virus with the same HA but a different NA (H1N4).

It may also be important that overrepresented coinfectants found more than once during the study as coinfectants were usually found with the same virus on each occasion. For example, H1N4 was detected as a dual coinfectant three times during the study, each time with H1N1 (Table 2). H1N3 and H6N3 were detected three times as dual infectants during the study, in each instance being found together; H6N3, in fact, was isolated only as a coinfectant with H1N3, never alone or with another virus (Tables 2 and 4). Similarly, H2N5 and H4N5 were found together in two isolations but rarely alone and never with any other virus (Tables 2 and 4). These results indicate that not only were some influenza A viruses more likely to be isolated as dual coinfectants than singly but also many were likely to be paired with the same virus in subsequent dual coinfections.

## DISCUSSION

**Viral adaptation to an avian species.** Compared to the influenza A viruses that infect mammals, influenza A viruses that infect birds have much lower rates of amino acid changes. Avian influenza viruses are so well adapted to their hosts that mutations are rarely beneficial; hence, the viruses would appear to be locked in evolutionary stasis (8, 9, 14, 28, 41). Therefore, the only way for new viruses to emerge in the avian population is through coinfection followed by reassortment. Viruses persisting in the wild-duck population showed a coinfection rate that was either similar to or substantially lower than one would predict from rates of single-virus infections.

Although H3N8, H6N2, and H4N6 were the most prevalent viruses detected in this study, coinfections between them were observed only when the original isolate was mixed with an antiserum to the first detected virus and reinoculated into chicken embryos. While this technique permitted us to detect these coinfections, we found that they occurred from 11 to 32% as often as would be expected based on the number of single infections with these viruses during the same week. This suggests that the three viruses most frequently found in mallards, pintails, and blue-winged teals in this population and which, presumably, are the best adapted to these duck species all have a selective advantage that prevents their growth as coinfectants both in chicken eggs in the laboratory and in these wild ducks in nature. Experimentally, reassortants have been produced in the laboratory when one duck is infected at the same time with two influenza viruses at the same titer (10). It is reasonable to speculate that the viruses that are most well adapted to an avian population replicate more rapidly than other viruses, so that if infections with two well-adapted viruses occur at different times, the first infectant may prevent the growth of later infectants. This would explain why the most well-adapted and consequently most common viruses are very much underrepresented and cannot even be detected as coinfectants without the extraordinary measures we used for the 1978 isolates. This reasoning would also predict that coinfections between well-adapted and poorly adapted viruses would be negatively affected, although such coinfections might occur if the poorly adapted virus infected the duck first.

**Maladapted viruses infecting an avian population.** Interestingly, rare viruses were actually found as coinfectants substantially more often than their frequencies of single isolation would indicate. However, these coinfections tended to occur between viruses with the same HAs or NAs, so that any reassortants would have at least one surface protein to which the avian population had already been exposed. The evolutionary benefit of coinfection by nonmaintained, infrequently isolated viruses and viruses sharing one of their surface proteins is not immediately clear. We propose that these rare viruses are

poorly adapted to the species of avians sampled but gain a brief survival advantage by participating in superinfections with other viruses. The rarity of these viruses, which were overrepresented as coinfectants, implies that they must either mutate or reassort with other viruses if they are to become adapted to this avian population. This suggests that while an influenza virus may be in evolutionary stasis in a particular avian host to which it is well adapted, it may not be in stasis in all avians. Apparently, such a virus must change if it is to adapt to a new avian host. Thus, coinfection allows a rare virus to temporarily infect an avian population but offers no clear potential for genetic reassortment of its surface proteins because these coinfectants tend to share either their HA or NA. It should be noted that these coinfections were found in years when we did not use the special blocking technique used with the 1978 isolates. We do not know if the use of this method with all isolates would increase the numbers of overrepresented coinfectants. If so, the observed-to-expected ratios and *P* values reported in this study would be conservative, meaning that such coinfections would be even more overrepresented than indicated by our results.

We found that even though juvenile ducks were significantly more likely to be infected with influenza A viruses than were adults, they were not significantly more likely to be coinfected with such viruses. This suggests that the duck immune system plays a less important role in coinfection. We found that influenza A virus prevalences differed significantly in this duck population according to species, even though coinfection prevalences did not. This may suggest that viral subtypes are differentially adapted to different species of ducks and thus are better able to infect certain species or that some species of ducks have increased exposure to these viruses. Although the same pair of influenza A virus coinfectants may be overrepresented in one duck species and underrepresented in another, our results showed that coinfecting pairs were classified in the same categories regardless of whether species was considered in the calculation of observed-to-expected ratios. On the few occasions when the same coinfections were detected in both mallards and pintails, species-specific ratios were similar, but there were too few such occurrences for us to determine if coinfection patterns in general were similar in these species or in the other species of ducks sampled.

The consistency of our findings over time and the fact that many of the results were highly significant statistically is reassuring. When coinfections involving the same subtype combinations occurred more than once during the study, the patterns of coinfection were similar at each time point and the results were consistent regardless of whether prevalence rates were calculated on a weekly basis or over the entire summer sampling season.

**Coinfection versus reassortment.** Although we studied influenza A virus coinfection and were not able to directly study reassortment in a natural population of wild avians, these results apply to reassortment, since coinfection of one duck with two viruses must occur before the reassortment can take place. Thus, if there are mechanisms which prevent or limit coinfection, they also prevent and limit reassortment; similarly, if there are mechanisms that increase the likelihood of coinfection, they are also likely to increase the likelihood of reassortment.

**Mechanisms favoring virus survival in species-specific avian hosts.** The results of this analysis help to define the mechanisms responsible for the maintenance of influenza A viruses in an avian population in nature. Overrepresented coinfections generally involved a rare virus and a partner with a matching HA or NA surface protein. Although useful in allowing poorly adapted viruses to infect susceptible ducks, this mechanism probably does not contribute to productive reassortment. In contrast, well-maintained, frequently isolated viruses coinfected at expected or less than expected levels, with the most successful viruses appearing to have the greatest ability to prevent coinfections. Thus, while coinfections between influenza A viruses in an avian population can provide the opportunity for the generation of novel reassortants capable of surviving in such a reservoir and, ultimately, of being transmitted to mammalian hosts, coinfection and reassortment do not occur at random in a natural avian population, and there are limits on the types and quantities of reassortants that can be produced. Our results suggest that different influenza A virus subtypes are maintained by different avian species. While a particular subtype may infect more than one avian species, they appear to have differential, species-specific levels of adaptation. Thus, an influenza A virus subtype which may be overrepresented as a coinfectant in one species may be underrepresented in another. It appears that the pattern of influenza A virus maintenance by avians is complex, with particular avian species being the major hosts for certain subtypes and other species being the major hosts for others.

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