Arch Virol (1994) 135: 101–114

Archives of Virology

Influenza virus subtypes in aquatic birds of eastern Germany*

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Accepted October 18, 1993

Summary. We report the findings of a 12-year surveillance study (1977–89) of avian influenza A viruses in eastern Germany. Viruses were isolated directly from feral ducks (n = 236) and other wild birds (n = 89); from domestic ducks (n = 735) living on a single farm; and from white Pekin ducks (n = 193) used as sentinels for populations of wild aquatic birds; mainly sea birds. The efficiency of virus isolation was 9.9% overall, with considerable variability noted among species: 8.7% in wild ducks, 0.9% in other feral birds and 38% in Pekin ducks. Use of sentinel ducks in wild pelagic bird colonies improved virus detection rates fivefold, suggesting that this approach is advantageous in ecological studies. Among the 40 different combinations of hemagglutinin (HA) and neuraminidase (NA) subtypes we identified, H6N1 predominated (23.6% for all avian species), followed by H4N6 (11%). Among individual species, the frequency profiles favored H2N3 (20.8%) and H4N6 (20.3%) in feral ducks; H7N7 (22.3%), H4N6 (24.4%) and H2N3 (10.4%) in Pekin ducks used as sentinels; and H6N1 (34.8%) and H6N6 (15.1%) in domestic ducks maintained on a single farm. By relying on sentinel birds for serological assays, it was possible to trace an "influenza season" in feral swan populations, beginning in August and continuing through the winter months. Comparison of subtype distribution of influenza viruses for Europe and North America showed significant differences. This supports the fact of two geographically distinct gene pools of influenza viruses in birds

*This article is dedicated to the memory of Dr. Herbert Sinnecker who died in 1991 at the age of 61. He was the Director of the Institute of Viral Zoonosis in the former German Democratic Republic (GDR). It was Herbert Sinnecker's forsight and understanding of the need to resolve the origin of human and animal influenza pandemics that initiated the studies described in this article. He developed novel epidemiological and ecological methods that permitted definition of the influenza virus gene pool in central Europe. The unification of Germany made it possible to publish this article; otherwise, the studies encouraged and organized by H. Sinnecker would have been lost to the scientific community.

connected with their distinct flyways of each hemisphere. The high frequency of isolation of H2 influenza viruses is of considerable interest to those interested in the recycling of this subtype in humans. Similarly the frequent isolation of H7N7 influenza viruses raises concern about reservoirs of potentially pathogenic influenza virus for domestic poultry. Our results confirm the existence of a vast reservoir of influenza A viruses in European aquatic birds, which possesses sufficient diversity to account for strains that infect lower animals and humans.

Introduction

Influenza A viruses infect not only humans but also domestic animals, such as pigs and horses, as well as poultry and feral aquatic birds. Study of these infections can lead to improvements in veterinary medicine and perhaps will disclose the origin(s) of pandemic influenza A viruses in man [14, 24].

After two catastrophic outbreaks of influenza in 1957 and 1968, the World Health Organization recognized the need to identify the source(s) of human pandemic strains through the study of influenza viruses in lower animals and birds. Consequently, 14 different HA and 9 different NA subtypes of influenza viruses, as well as subtypes defined by combinations of these surface glycoproteins, have been isolated from apparently healthy aquatic birds, mainly ducks, in diverse geographic regions [7, 9, 10, 11, 19, 29, 32]. Phylogenetic studies of influenza virus genes indicate that human pandemic strains, and all current mammalian influenza A viruses, are derived either by direct transfer of avian influenza viruses or by genetic reassortment between human and avian viruses [25, 26, 38].

There have been many reports of influenza viruses isolated from aquatic birds [17], but there are only two reports about systematic, longitudinal studies; one in wild ducks in North America [11, 13], the other in domestic animals in Southeast Asia [28]. This paper presents the results of a 12 year surveillance study for systematic isolations of influenza viruses in birds. The results support the hypothesis that aquatic birds are the primordial source of all influenza viruses in other species [12, 23, 24]. Indeed, the gene pool of influenza A viruses in aquatic birds provides all the genetic diversity needed to generate pandemic influenza viruses capable of infecting humans, lower animals and birds [16, 23, 25]. Hinshaw et al. [13] showed that viruses isolated from ducks included those antigenically related to viruses that produce disease in birds and mammals.

Here we report the findings of our 12-year surveillance of avian influenza A viruses in eastern Germany, in which domestic ducks were used as sentinels to detect viruses in wild aquatic bird populations. The major aims of the study were to establish the extent of the influenza virus gene pool in Europe, to test ecological conclusions drawn from large populations of North American birds and to address a still-unanswered question about the avian influenza reservoir: are the virus populations in aquatic birds different from those in feral ducks and which virus population is the possible progenitor of viruses in domestic ducks and mammals including humans. The conclusion of our experiments is that the

predominant influenza virus gene pools of influenza A virus differ between Europe and North America and between aquatic birds and ducks.

Materials and methods

Feral birds

Figure 1 shows the 14 sites in eastern Germany where wild birds were captured for this study. Feral ducks of various species (mostly mallards) were fed with bread to which α-choralose had been added (1 g per kg). After cloacal and tracheal swabs were taken, the anesthesized birds were released into their natural habitats. Swans were seized directly; breeding gulls were caught by quickly placing wicker baskets over them or their nests and geese were trapped with rocket-assisted nets while they rested.

Domestic ducks

To investigate the distribution of influenza viruses among domestic ducks, we regularly checked the flocks on a duck farm in Wagun near Demmin (D1 on the map), which included 4 000–6 000 white Pekin ducks that were maintained continuously inside the farm buildings. Tracheal and cloacal swabs were taken every 14 days, beginning with 14 to 18 day-old birds. The Pekin ducks had contact with wild aquatic birds, such as gulls and feral ducks. After taking the last swabs, the ducks were killed on the 58th day.

Sentinels

Domestic ducks were also used as sentinels to detect influenza A viruses in wild aquatic bird populations in field studies conducted over seven years (1982–89). 4- to 5-week old white Pekin ducks, were maintained at three separate locations: (i) a gull-breeding site on Heuwiese Island near Rügen Island (S1, Fig. 1), where we had previously found influenza A viruses in gulls, feral ducks, swans and terns, (ii) a pond near Berlin (Heinersdorf) (S2), and (iii) a river site in Potsdam (S3). The young Pekin ducks were initially found to be free of influenza A viruses as shown by negative tracheal and cloacal swabs and no influenza virus antibody titers in their sera. The sentinels were kept from May-October in fenced-in habitats, each comprising an 80 m^2 grassy area with a small water pool. The ducks were inspected weekly, and food was provided whenever existing supplies were low. Tracheal and cloacal swabs were collected twice monthly.

Processing of samples

Tracheal and cloacal swabs were placed in 1 ml of phosphate-buffered saline (pH 7.2) containing 50% glycerol, penicillin, streptomycin and moronal (fungicide, Squibb factory). Samples were kept at 4 °C for 3–5 h and then at − 80 °C until tested. Blood samples were collected by peripheral venipuncture from sentinel ducks at approximately monthly intervals, and in May of 1980 and 1981 from swan populations living on a seabird-breeding island in the Baltic Sea and on rivers and lakes near Berlin. The sera were stored at − 20 °C until used.

Viruses were isolated in a building that had been specifically assigned for the processing of field specimens. A 0.1 ml volume of each sample was inoculated into the allantoic cavities of three 11-day-old embryonated chicken eggs. Each egg was incubated for 48 h at 36 °C before the allantoic fluid was tested for hemagglutinating activity; each sample was passaged up to three times.

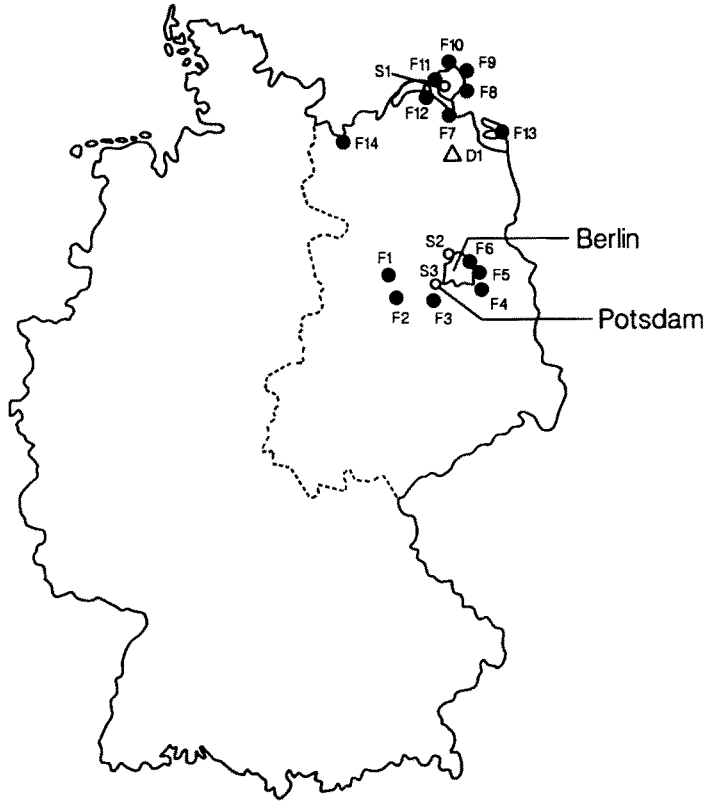

Fig. 1. Sites in eastern Germany where surveillance was done. *D* Domestic ducks, *S* sentinel ducks located at the seabird breeding island "Heuwiese" near Rügen Island (*S*1), Berlin-Heinersdorf (*S*2), and Potsdam (*S*3), *F* feral ducks captured at Rathenow (Gülper Lake) (*F*1), Brandenburg (*F*2), Caputh (*F*3), Schmöckwitz (*F*4), Berlin (Köpenik) (*F*5), Berlin (*F*6), Niederhof (*F*7), Rügen Island off the Baltic Coast (*F*8–10), Hiddensee Island (*F*11), Stralsund (*F*12), Usedom Island (*F*13), and Langenwerder near Wismar (*F*14)

Serologic tests and virus identification

Hemagglutination-inhibition (HI) and neuraminidase inhibition (NI) tests were performed as described by Palmer et al. [21], with the modification of Aminoff [1] used for the NI test. All sera were treated with a receptor-destroying enzyme before testing. Antisera to the HA and NA antigens of animal and avian reference strains were prepared in rabbits and ferrets; the results were verified with monospecific antisera to the isolated HA or NA.

Results

Virus isolation rate

In the present studies we utilized sentinel domestic ducks to detect influenza viruses in aquatic bird colonies as well as direct isolation of viruses from feral ducks and aquatic birds (Table 1). The highest rate of influenza virus isolation was from domestic ducks (38%) followed closely by a 37% isolation rate from sentinel ducks. The efficiency of isolation of influenza was lower in feral ducks

Table 1. Subtypes of influenza A viruses isolated during the eastern Germany surveillance study, 1977–89

Category of birds	Number tested	No. of viruses isolated	Rate of virus isolation	Prevalent subtypes
Domestic ducks[a] (*Anas platyrhynchos*)	1934	735	38%	H6N1 (35.0) H6N6 (15.0)
Sentinel ducks[b] (*Anas platyrhynchos*)	521[c]	193	37%	H4N6 (24.3) H7N7 (22.3)
Feral ducks (*Anas platyrhynchos, A. penelope, A. crecca, A. acuta, Clangula hyemalis, Melanitta fusca*)	2713	236	8.7%	H2N3 (20.8) H4N6 (20.3) H1N1 (13.6)
Swan (*Cygnus olor*)	812	13	1.6%	H3N3 (30.8) H7N3 (23.0) H10N8
Goose (*Anser anser*)	611	4	0.5%	H10N4 H1N1
Cormorant (*Phalacrocorax carbo*)	4500	18	0.4%	H6N1 (100)
Gull (*Larus ridibundus, L.canus*)	2182	13	1.1%	H7N3 (46.2) H11N6 (")
European knot (*Fulica atra*)	1312	13	1.0%	H4N7 (53.9)
Other waterbirds (*Arenaria interpres, Sterna paradiseaea, Tringa totanus, Calidris canutus*)	2059	13	1.7%	H3N6 (38.5)
Other birds (*Numeinus arquata, Numida meleagris, Tringa decaocto, Passer domesticus*)	5002	15	0.3%	H4N6 (66.6)

[a] Raised on a single farm in Wagun near Demmin in Mecklenburg-western Pomerania
[b] White Pekin ducks were used for detection of virus in water bird colonies
[c] Groups of 50 animals were tested repeatedly for 5–6 months

(8.7%) and lowest in direct sampling from aquatic birds (Table 1). In contrast to findings in humans and lower animals, different virus subtypes were often identified simultaneously within a single bird. For instance, about 8.2% of the Pekin ducks that were sampled showed dual infection, either with two different subtypes of influenza virus or with an influenza virus and a Newcastle Disease virus.

Distribution of influenza A subtypes

The frequency of isolation of the antigenically defined subtypes of influenza A virus over the entire study period is shown in Fig. 2. H6N1 isolates predominated

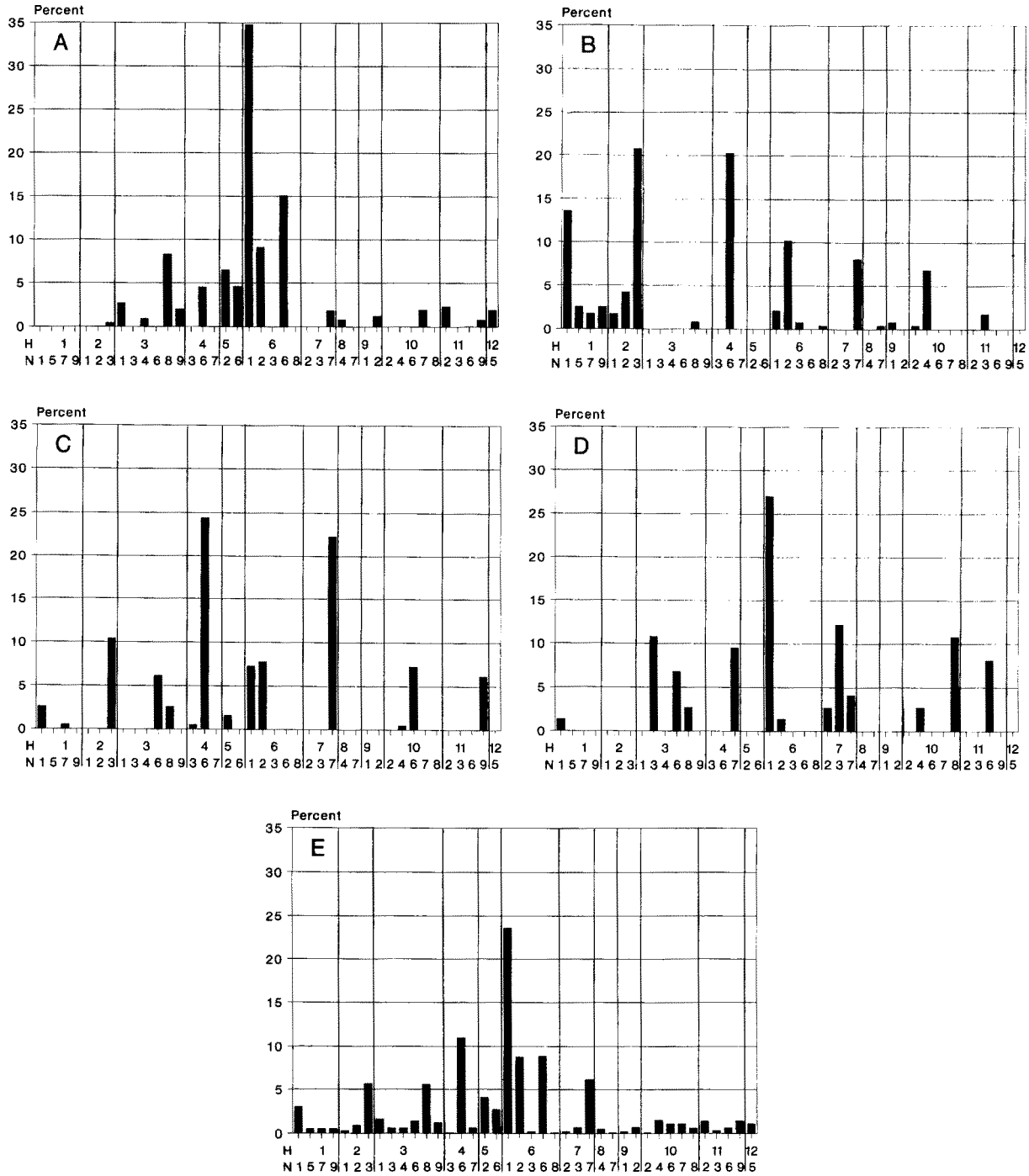

Fig. 2. Distribution of avian influenza A viruses collected from 1977–89 in eastern Germany. **A** Domestic ducks; **B** feral ducks; **C** sentinels; **D** swan, goose, cormorant, gull, European knot and other waterbirds (see Table 1); **E** all birds (in Table 1). *H1, 2, 3,* (etc.) refers to hemagglutinin subtypes in combination with *N1, 2, 3,* (etc.) neuraminidase subtypes

Table 2. Number and predominant subtypes of influenza A viruses isolated at different locations during the sentinel program in eastern Germany

Location map 1	Number of viruses isolated	Prevalent subtypes (%)
S1 "Heuwiese" Seabird breeding site near Rügen Island	16	H10N6 (87.5)
S2 Berlin-Heinersdorf	108	H7N7 (34.3) H4N6 (22.2)
S3 Potsdam	69	H4N6 (33.3) H6N1 (20.3)

(23.6%), followed by H4N6 viruses (11%). The subtype distribution among sentinel ducks clearly differed from that among feral ducks. In the latter population, where viruses were isolated directly from swabs, H2N3 and H4N6 isolates predominated (20.8% and 20.3%), followed by H1N1 and H6N2 (13.6% and 10.2%). Among sentinels, the H4N6 subtype was most prevalent (24.4%), with H7N7 and H2N3 viruses identified less often (22.3% and 10.4%). But there are differences in the predominant subtypes in three sentinel location sites (Table 2). Findings differed even further among domestic ducks living on a single farm, where the H6N1 and H6N6 subtypes predominated (34.8% and 15.1%) and there was no correlation between the dominant subtype in feral ducks and in domestic ducks. The subtyped isolates showed the following frequency distribution, in descending order: H6 (41.5%), H4 (11.7%), H3 (10.9%), H7 (7.1%), H2 (6.9%) and H5 (6.8%). Subtypes of interest are the H5 and H7 for these can be associated with highly pathogenic avian influenza in domestic poultry particularly in chickens. The H5 subtype was only detected in sentinel ducks and domestic ducks, not in wild ducks; H7 was detected most frequently in sentinels and was less frequent in feral ducks. Other subtypes predominating in sentinels are H10N6 and H11N9 indicating their presence in aquatic birds.

The subtype of greatest interest are H1, H2 and H3, for these are the subtypes that cause periodic pandemics in humans. H1 was detected mainly in feral ducks and other species, less often in aquatic birds and not in domestic ducks; H2 was a dominant subtype in feral and sentinel ducks and in other species but was rarely found in domestic ducks. H3 was less frequently isolated and was mainly from sentinel and domestic ducks.

Yearly variation of virus subtypes in domestic ducks

The role of domestic ducks in the perpetuation of influenza A viruses is controversial. They could represent an important source of pathogenic viruses for chickens, represent large sentinel flocks, or they may simply harbor a static group of subtypes which bear little resemblance to those in feral birds or other hosts. Access to a domestic duck farm in the northeastern part of Germany allowed us to address these issues. As shown in Fig. 3, the predominant subtype

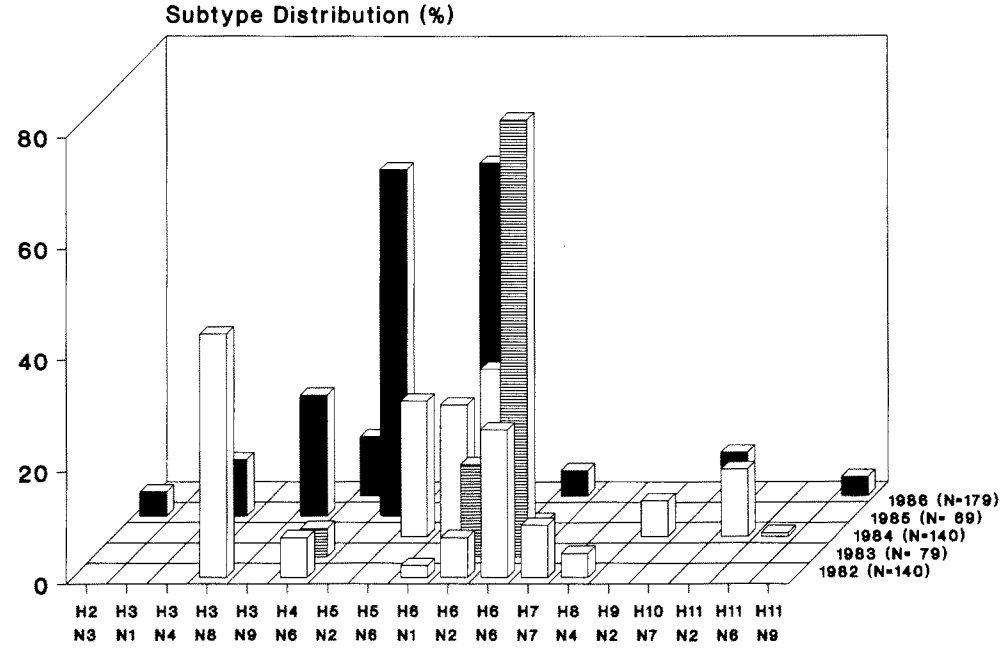

Fig. 3. Distribution of influenza A virus subtypes isolated from domestic ducks on a single farm in East Germany from 1982–1986 (see map location D1 in Fig. 1)

isolated from domestic ducks varied from year to year. In 1982, for instance, H3N8 isolates were most common (44%), shifting to H6N6 (81%) in 1983, H6N2 (31.5%) in 1984, H5N2 (64%) in 1985 and H6N1 (60%) in 1986. Some of these subtypes disappeared entirely from the duck population in the years following their initial detection. The H3N8 subtype, for example, was not detected during any of the four years after its high prevalence in 1982. Similarly, in 1985, the H5N2 subtype accounted for 57% of all viral isolates, yet this virus had not been identified in the preceding 3 years and was not detected in the following year. Other subtypes, by contrast, reappeared after the year of their initial isolation but at lower frequencies. Experience with the H6N6 subtype illustrates this generality well. Despite high isolation rates from many of the subtypes identified in domestic ducks, none caused influenza-like symptoms. These results demonstrate rapid yearly turnover of dominant influenza virus strains in a confined population of domestic ducks; moreover, some of the infecting strains did not have counterparts in the feral duck population (e.g., H3N1, H6N2 and H6N6), suggesting their derivation from other sources.

Seasonal detection of influenza epidemics in wild aquatic birds

We used white Pekin ducks as sentinels in water bird colonies or along lakes and rivers inside East Germany to improve virus detection rates in wild bird populations [30]. During the 12-year study period, 208 influenza A viruses, consisting of 13 different subtypes, were isolated from sentinel birds.

From May 1981 to April 1982, a total of 32 influenza A viruses comprising two subtypes (H8N5 and H4N6) was isolated from 13 sentinel ducks at the pond near Berlin. From July to December 1981, 19 influenza virus strains (H4N6, H6N2, H6N3 and H10N3) were isolated from 100 mallards (*Anas platyrhynchos*) captured at this pond. The rate of virus isolation from feral ducks was only 19%, but by using domestic ducks as sentinels, it was possible to improve this rate to about 25%.

Antibody studies

Antibodies to the same influenza viruses detected in mallards were recovered from sentinel ducks 1 month after the viruses had been isolated from swabs. Antibodies to the H1, H3, H7 and H11 subtypes were found in sentinels [30–32]. In general, NA antibodies appeared earlier than did HA antibodies. A combination of serologic findings and virus isolations from sentinel ducks and mallards indicated at least five different influenza A outbreaks in water birds during one year of observation. The outbreaks occurred in June, July, September, November and December. The repeated infection of sentinel ducks with different influenza A viruses indicates that natural infection of ducks does not provide cross protection between influenza A subtypes. Rises in antibodies were detected four weeks after virus isolation but the titers were low and declined to undetectable levels in two months [31].

Antibodies to the same influenza viruses were detected one month later confirming these outbreaks. We also tested, over prolonged periods, the viral antibody responses of a single species living in different geographic regions. Swans in the Baltic Sea had significantly higher titers to H7N7 viruses, whereas swans from lakes and rivers near Berlin had higher titers to H1N1 swine-like influenza A viruses. In serological studies, 90% to 100% of the swans living on the seabird-breeding island in the Baltic Sea had antibodies to all recognized animal subtypes of influenza A virus, compared with 31% (1980) and 46% (1981) of swans from rivers and lakes near Berlin. There was a rise in antibodies to the H7 and N8 antigens in Baltic swans from 1980 to 1981, but a decrease in antibodies to the H6, H7 and N3 antigens. Concomitantly, among swans living on lakes and rivers near Berlin, there were increases in titers to five antigens: H1 (swine), H11, N4, N8 and N2. These results indicate that influenza viruses can cause "epidemics" in avian populations without producing physical signs of disease [31].

Discussion

About 3 800 influenza A viruses were isolated among the 72 000 samples taken from avian species during our 12-year surveillance study. Analysis of the distribution of antigenic subtypes indicated the existence of a large reservoir of different influenza A viruses in aquatic birds living in East Germany. This gene pool was sufficiently diverse to account for new strains of viruses capable of infecting other birds, lower animals and humans. The 1253 influenza A virus

strains that could be fully characterized by serology represented 40 different HA/NA combinations, most frequently H6N1, H4N6, H6N2, H7N7, H3N8 and H2N3.

One of the notable findings in this study was the rapid yearly turnover of influenza virus subtypes in domestic ducks living on the same farm. During the 4-year observation period, some viruses were never seen again after their initial detection, even though they dominated other strains when first isolated. Thus, in a restricted area with some exposure to wild aquatic birds, there were a number of viruses isolated that were not detected in feral ducks, suggesting alternative sources of virus that could include non-aquatic birds. This finding agrees with the analysis of influenza in feral ducks in North America [27] that proposes that not all subtypes of influenza A are maintained in feral ducks. Similar findings have also been reported by Halvorson et al. [5].

Influenza viruses with a variety of HA and NA subtypes have been isolated from wild waterfowl in Russia [17], Southern China [28], Japan [35, 39], Israel [15], Europe [6, 19, 20, 34], North America [8, 10, 13, 36, 38] and Australia [18]. Recent phylogenetic studies indicate that the viruses identified in Eurasia and Australia were genetically distinct from those in North America [2, 3, 22, 38].

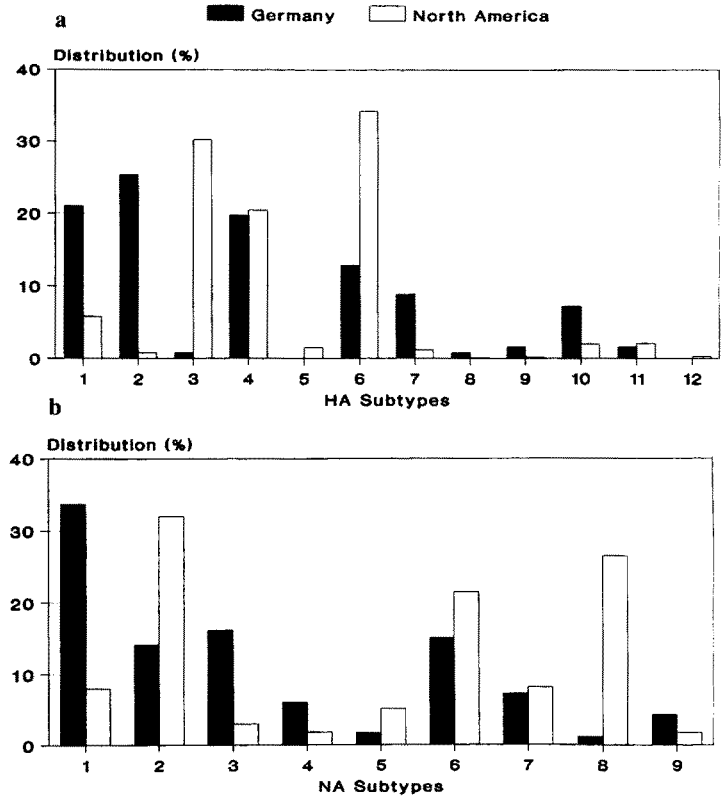

Fig. 4. Comparison of the HA (**a**) and NA (**b**) subtypes of influenza A viruses isolated from feral ducks in eastern Germany (this study) and North America [33]

These findings most likely reflect the distinct flyways of each hemisphere. Indeed, even in the same hemisphere, the predominant circulating viruses may differ strikingly if their avian hosts use separate migratory routes, as shown by Hinshaw et al. [13] in their study of North American waterfowl. Thus, we were not surprised to find that the prevalence rates for the HA and NA subtypes in East German isolation program contrasted sharply with rates reported by Webster et al. [38] for feral ducks in North America. Whereas H6, H3 and H4 were the predominant subtypes represented among feral ducks in North America, we most frequently identified H2, H1 and H4 in European feral duck populations (Fig. 4). A similar discrepancy was seen in the comparison of NA subtypes: N2 (28%), N8 (27%), N6 (22%) and N7 (8%) prevailed in North America, with N1 (33%), N3 (17%), N6 (16%) and N2 (13%) dominating in eastern Germany.

Ducks occupy a special niche in the hierarchy of ecological events leading to influenza virus infections in other birds and mammals. Because influenza A viruses replicate in the intestines of ducks without producing detectable disease, and are secreted in high concentrations in feces, these waterfowl serve as ideal reservoirs for the maintenance of a vast influenza gene pool. Experiments have shown that influenza viruses remain infectious in feces for more than 30 days at 4 °C and for 7 days at 20 °C [9, 37, 38]. Thus, fecal-oral transfer of viruses through water could explain how influenza viruses spread from ducks to other birds, including domestic species. Indeed, studies in turkey flocks in Minnesota have demonstrated the susceptibility of domestic birds to influenza viruses from wild aquatic ducks [4]. Our results with sentinel Pekin ducks reinforce this conclusion. Studies of Ludwig and Scholtissek [16] showed the emergence of H1 avian influenza viruses not only for domestic ducks but also for other domestic animals like swine. H1N1 avian viruses led to an outbreak in pigs in Europe, but later a reintroduction of the same virus to turkeys was found.

Virus isolation rates for the sentinels were consistently higher than the rates for wild ducks, illustrating the high susceptibility of domestic ducks to influenza A viruses. One of the difficulties in studying the ecology of influenza A viruses is the mobility of wild bird populations. The constant movement of birds to and away from single sites may seriously compromise sampling procedures, leading to biased profiles of antigenic subtypes. Moreover, serologic studies to detect antibody responses to influenza infection cannot be readily performed with wildfowl. For these reasons, we relied on sentinel ducks to improve the efficiency of surveillance. We realize that the susceptibility of ducks is likely to vary between virus subtypes and may be different from that of wild birds and could bias the sampling; regardless, it does provide a practical solution to sampling feral birds. We believe this approach was successful and recommend its use in future studies. The serologic data obtained with sentinel ducks indicate little, if any, long-term protection against influenza viruses from natural infection. Although the sentinels responded to natural influenza infection with a rise in antibodies four weeks after virus isolation, the titers were low and declined to insignificant levels within one to two months [31].

In attempts to discern a seasonal pattern of influenza infection, we simultaneously isolated virus from sentinel ducks on a seabird-breeding island in the Baltic Sea and from mallards on a pond near Berlin, in both August and October of 1981. Although four different subtypes were isolated from mallards, and only one from sentinels, the latter had antibody responses against all of the isolated subtypes, suggesting that each of these strains must have infected domestic ducks. We conclude that an "influenza season" begins in August in eastern Germany. The probability that the influenza A viruses detected in the fall continued to circulate during the winter (November to March/April) was high, as judged from the serologic reactions of sentinels at the pond near Berlin.

The frequent isolation of H1 and H2 subtypes are particularly relevant to human influenza and serve as an abundant source of viruses for future pandemics in humans. It is of interest that H2 influenza viruses have been relatively infrequently isolated in other regions of the world. The subtypes of particular interest to the poultry industry are H5 and H7, for they have the potential of being highly pathogenic in chickens and turkeys. The available evidence suggests that these viruses were frequently isolated especially from sentinel ducks in contact with sea birds. In earlier studies [33], our avian H7 isolates were tested for pathogenicity in one-day-old and 3-week-old chickens, young adult mice and Syrian hamsters and 10- to 12-week-old minipigs. Only one strain (A/chicken/Potsdam/79 H7N7) showed pronounced pathogenicity for chickens. Studies on the pathogenicity of the H5 isolates remain to be done.

These studies demonstrate the existence of a vast reservoir of influenza A viruses among feral aquatic birds and domestic ducks in eastern Germany. Although generally supporting the theory that all influenza viruses in lower animals and humans derive from an avian reservoir, the distribution of antigenic subtypes in our study showed marked differences from findings in large North American surveys. Thus, the contribution of avian influenza viruses to human epidemics and pandemics may vary among geographic regions of the world, especially if the regions do not share the same migratory flyways.

Acknowledgements

We gratefully acknowledge the expert field and laboratory work of the animal influenza virus group of the former Institute of Viral Zoonosis in Potsdam. We especially thank C. Scheunert, Dr. R. Sinnecker, Dr. D. Köhler, I. Zarse, B. Kriebel, H.-U. Dost, C. Scheefeld, R. Roske, and E. Zilske. J. Gilbert provided extensive assistance with manuscript preparation and D. Anderson typed the manuscript. The work done in Germany was supported by the Ministry of Health of the former GDR. The work done in the United States was supported in part by Public Health Service grants AI 29680, from the National Institute of Allergy and Infectious Diseases, by Cancer Center Support Grant (CORE) CA 21765, and by the American Lebanese Syrian Associated Charities.

Note added in proof

In the meantime 10 H13N2, 4 H13N6, and 2 H13N3 virus strains, collected in 1980, 1981 and 1982 from gull (6 H13N2 strains from sentinel ducks) at the seabird breeding island "Heuwiese" near Rügen Island and 5 H13N2 strains from mallard, captured at Berlin-Heinersdorf, were identified.

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Received August 23, 1993