Role of domestic ducks in the propagation and biological evolution of highly pathogenic H5N1 influenza viruses in Asia

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Wild waterfowl, including ducks, are natural hosts of influenza A viruses. These viruses rarely caused disease in ducks until 2002, when some H5N1 strains became highly pathogenic. Here we show that these H5N1 viruses are reverting to nonpathogenicity in ducks. Ducks experimentally infected with viruses isolated between 2003 and 2004 shed virus for an extended time (up to 17 days), during which variant viruses with low pathogenicity were selected. These results suggest that the duck has become the ''Trojan horse'' of Asian H5N1 influenza viruses. The ducks that are unaffected by infection with these viruses continue to circulate these viruses, presenting a pandemic threat.

avian influenza | pathogenicity

From late 2003 through January 2004, H5N1 influenza viruses spread in an unprecedented manner across Asia, affecting poultry in Vietnam, Thailand, Indonesia, China, Japan, South Korea, Cambodia, and Laos. Hundreds of millions of chickens and ducks were culled in an effort to stop the spread. The outbreak appeared largely under control in March 2004. However, in July 2004 the virus reemerged in Thailand, Vietnam, and China and was isolated for the first time in Malaysia. The ongoing H5N1 outbreak in southeast Asia has caused the deaths of 12 humans in Thailand, 38 in Vietnam, and 4 in Cambodia (1). No human cases have been reported in Thailand in 2005. The available evidence shows that H5N1 infection is widespread among domestic ducks in southern China (2) and may therefore be endemic in domestic ducks throughout southeast Asia.

The biology of H5N1 influenza viruses in waterfowl appears to be changing. The first indication was the death of many domestic and exotic waterfowl in Hong Kong nature parks in late 2002; the birds had systemic viremia and showed signs of neurologic disease (3, 4). The only influenza viruses previously reported to cause the deaths of aquatic birds were A/tern/South Africa/61 (H5N3) (5) and a highly pathogenic H7N1 virus that caused an outbreak in Muscovy ducks in Italy in 1999–2000 (6). In all other reports, ducks infected with highly pathogenic H5 or H7 avian influenza viruses, including those isolated in Hong Kong in 1997–2002, consistently showed no disease signs or had very mild disease (7–12). In 2002, however, H5N1 influenza viruses of the Z and Z+ genotype emerged. These viruses became dominant in Vietnam and Thailand (13) and killed aquatic and terrestrial poultry (3, 4). Viruses isolated during outbreaks in Japan in 2003–2004 were also pathogenic to domestic ducks (14). Previous studies have shown that influenza viruses remain in evolutionary stasis in their natural hosts, to which they are normally nonpathogenic (15). Either this conclusion was wrong, the biology of influenza in domestic ducks differs from that in wild waterfowl, or the biology of influenza in domestic ducks has changed dramatically.

We hypothesized that the biological characteristics of H5N1 viruses circulating in ducks are evolving rapidly by mechanisms that have not previously been detected. This rapid evolution may provide a key to understanding the continuing epidemics. Here we show that in domestic ducks inoculated with the H5N1 viruses isolated in 2004, diminishing pathogenicity allows the shedding of detectable virus for long periods (facilitating virus transmission), and variant viruses are selected within a single passage. Because these H5N1 viruses become less pathogenic to domestic ducks but remain pathogenic to other domestic poultry and potentially to humans, the role of ducks in propagating these viruses raises great concern for veterinary and human health.

Materials and Methods

Viruses. The influenza virus isolates used in this study were received by the St. Jude Children's Research Hospital Influenza Repository from multiple collaborators, including the World Health Organization Influenza Laboratory Network. Stock viruses were grown in 10-day-old embryonated chicken eggs for 36–48 h at 35°C. All experimental work with the H5N1 viruses, including animal studies, was performed in an animal biosafety level 3+ laboratory approved for use by the U.S. Department of Agriculture and the U.S. Centers for Disease Control and Prevention.

Duck Infection Studies. Two 4-week-old mallard ducks (*Anas platyrhynchos*) were inoculated with 10^6 50% egg infectious doses (EID_{50}) of each stock virus in a 1-ml volume, as described in ref. 3 (0.5 ml was applied to the cloaca and 0.2 ml was applied to the trachea; 0.1 ml each was dripped into the throat, nares, and eyes). Four hours postinoculation (p.i.), two uninfected ducks were placed in the cage with the inoculated birds, sharing food and drinking water. All birds were observed daily for 21 days for cloudy eyes, lethargy, severe central nervous system dysfunction

Abbreviations: EID_{50}, 50% egg infectious dose; HA, hemagglutinin; HI, hemagglutination inhibition; p.i., postinoculation; IVPI, i.v. virus pathogenicity index; MDCK, Madin–Darby canine kidney.

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Table 1. H5N1 viruses characterized in mallard ducks

Isolation site	Virus	Inoculated dead/total	Contact dead/total	Pathogenicity	Days virus shed	No. amino acid differences in HA
Hong Kong	A/Hong Kong/156/97	0/2	0/2	Low	7	0
	A/chicken/Hong Kong/YU562/01	0/2	0/2	Low	7	0
	A/chicken/Hong Kong/SSP94/03	0/2	1/2	High	11	0
	A/chicken/Hong Kong/AP111/03	0/2	0/2	Low	11	0
Vietnam	A/mallard/Vietnam/16D/03	1/2	2/2	High	17	11
	A/duck/Vietnam/40D/04	1/2	0/2	High	13	4
	A/Vietnam/1203/04	2/2	1/2	High	13	2
	A/Vietnam/3046/04	0/2	0/2	Low	13	10
	A/chicken/Vietnam/48C/04	1/2	0/2	High	17	6
	A/chicken/Vietnam/133/04	1/2	1/2	High	11	2
Thailand	A/Thailand/1 (Kan-1)/04	0/2	0/2	Low	11	0
China	A/chicken/Anhui-Chaohu/85/04	0/2	0/2	Low	17	2
Indonesia	A/chicken/Pangkal Pinang/BPPV3/04	0/2	0/2	Low	13	0
Singapore	A/duck/Singapore/3/97 (H5N3)	0/2	0/2	Low	7	0

Two 4- to 6-week-old mallard ducks were inoculated with 10^6 EID_{50} of virus and placed after 4 hours with two contact ducks. The small number of ducks tested was because of the space constraints of the biosafety level 3+ facilities. High pathogenicity, at least one duck died; low pathogenicity, no ducks died. Days virus shed indicates the greatest number of days virus was detectable in at least one duck (inoculated or contact). All viruses that were shed at late time points were isolated from the cloaca. The number of amino acid differences in HA is a comparison of the HA gene sequence of virus shed by one duck at the latest time point and the HA gene sequence of the original virus sample. Changes occurred throughout the HA structure, with some occurring within antigenic sites. No changes were observed to occur within the multibasic cleavage site.

(violent tremors, uncontrollable shaking, marked loss of balance, and paralysis), and mortality. Tracheal and cloacal swabs were collected every other day starting on day 3 postinfection until virus was no longer isolated in embryonated chicken eggs (3, 16). The infectivity of positive samples was titrated by determining the EID_{50}. Viruses isolated on day 17 were used to inoculate additional ducks as described above with 10^6 EID_{50} of stock virus in a 1-ml volume. The ducks were examined and samples were collected as described above.

Hemagglutination Inhibition (HI) and Virus Neutralization Assays. The viruses were antigenically analyzed by HI with a panel of anti-H5 hemagglutinin (HA) polyclonal antisera (postinfection chicken antisera and hyperimmune goat and sheep antisera) and monoclonal antibodies (from mouse ascitic fluid). Antisera were treated with receptor-destroying enzyme, and the HI assays were performed as described in ref. 17. Sera collected from inoculated ducks on days 17 and 24 p.i. were tested by HI assay for antibodies to the HA of the virus with which the duck was inoculated. For consistency with previously reported studies, HI titers $\geq$40 were recorded as positive. For virus neutralization tests, diluted allantoic fluid containing 100 EID_{50} of the original virus and the day-17 isolates of A/mallard/Vietnam/16D/03, A/Vietnam/3046/04, and A/chicken/Vietnam/48C/04 were incubated for 60 min at 37°C with 2-fold serial dilutions (starting at 1:8) of hyperimmune duck serum, incubated, and injected into the allantoic cavity of embryonated chicken eggs. After incubation for 48 h at 37°C, virus in allantoic fluid was detected by HA assay and the virus neutralization titer was established by the Reed and Muench method (18).

Sequencing of Virus Samples, Plaque Purification of Viruses, and i.v. Virus Pathogenicity Index (IVPI). To sequence the virus samples, we used the procedure described by Guan *et al.* (16). We used influenza-specific universal primers to reverse-transcribe the HA gene and amplify it by PCR (19). PCR products were sequenced by using synthetic oligonucleotides produced by the Hartwell Center for Bioinformatics and Biotechnology at St. Jude Children's Research Hospital. Plaque purification of viruses was performed as described in ref. 20. The IVPI was determined in duplicate by the method of Capua and Mutinelli (21).

Results

Are the Currently Circulating H5N1 Viruses Uniformly Pathogenic in Ducks? In late 2002, H5N1 avian influenza viruses acquired the characteristic of high pathogenicity for waterfowl, killing many exotic water birds in Kowloon Park, Hong Kong, including ducks, geese, swans, and Greater flamingos (3, 4). In laboratory tests, three of these viruses were highly pathogenic to ducks, but a human isolate from that time [A/Hong Kong/213/03 (H5N1)] was not (3). We compared the pathogenicity and transmissibility in ducks of the H5N1 influenza viruses isolated from poultry and humans in 2003 and 2004 in various regions of Asia (two isolates from Hong Kong, one from mainland China, six from Vietnam, one from Thailand, and one from Indonesia) (Table 1) in mallards. Three additional viruses isolated in previous years were tested for comparison to the new 2003/2004 viruses: A/Hong Kong/156/97 (H5N1), A/chicken/Hong Kong/YU562/01 (H5N1), and A/duck/Singapore/3/97 (H5N3). Virus samples were not biologically cloned, because our intention was to examine the original virus isolates to approximate conditions found in nature.

All of the isolates tested, including the four human isolates, replicated in the inoculated ducks and were transmitted efficiently to the contact ducks, which shed virus at high titers. The viruses replicated to high titers by day 3 p.i. A wide spectrum of pathogenicity was observed in the ducks. For this study, we categorized pathogenicity on the basis of mortality: low pathogenicity was indicated by the absence of deaths, and high pathogenicity was indicated by the death of at least one duck. Within the high-pathogenicity category, there was a wide range of disease signs and mortality. Viruses that caused the death of at least one duck could cause very mild symptoms, such as cloudy eyes with no neurological signs (A/duck/Vietnam/40D/04) or could cause severe clinical signs, such as weight loss, cloudy eyes, and severe neurological dysfunction, in all ducks inoculated (A/mallard/Vietnam/16D/03).

A/Vietnam/1203/04 was the only human isolate tested that caused the deaths of ducks (Table 1). Several ducks infected with the human Vietnamese virus isolate A/Vietnam/3046/04 had cloudy eyes upon close examination but showed no other disease signs. The human virus isolate from Thailand and the chicken H5N1 isolates from mainland China and Indonesia were nonpathogenic in the ducks.

Table 2. Selection of H5N1 viruses in individual ducks as demonstrated by HI assay

		HI titer							
		Postinfection polyclonal antisera				mAb to H5 HA (A/ck/PA/83)			
Virus inoculated	Day isolated	A/HK/156/97	A/gs/HK/437-4/99	A/ck/VN/c58/04	Homologous virus†	CP24	CP58	176/26	406/7
A/mallard/Vietnam/16D/03	0*	640	160	<40	160	400	300	200	300
	7	640	320	<40	320	400	400	<100	300
	11	<40	80	<40	160	400	100	<100	100
	17	40	80	160	<40	400	<100	300	100
A/Vietnam/3046/04 (human isolate)	0*	1,280	1,280	2,560	320	500	400	<100	400
	11	1,280	<40	160	160	<100	<100	<100	<100
	13	1,280	40	320	80	<100	100	<100	<100
A/chicken/Vietnam/48C/04	0*	320	160	160	80	400	800	<100	300
	7	320	160	160	40	400	800	<100	300
	11	<40	<40	80	40	<100	<100	<100	100
	17	<40	<40	160	<40	<100	<100	<100	<100
A/chicken/Pangkal Pinang/BPPV3/04	0*	<40	160	40	40	<100	<100	<100	<100
	11	<40	80	80	80	<100	<100	<100	<100

*Refers to the original virus.
†Sera obtained from mallard ducks on day 17 after inoculation.

Wild waterfowl are generally thought to transmit influenza viruses primarily through fecal contamination of water (22). However, ducks inoculated with the 2002 H5N1 influenza viruses shed more virus from the trachea than from the cloaca (3). This property was retained in all of the viruses we tested.

Duration of Virus Shedding. In previous studies, nonpathogenic influenza viruses were shed by ducks for as long as 20 days (23, 24). In contrast, previous highly pathogenic H5N1 viruses have reportedly been shed by ducks for only 2 and 5 days (7, 12), with the exception of one virus isolated in 2002 (10 days) (3). To provide a better understanding of H5N1-shedding in ducks after infection, the duration of virus reisolation from ducks was investigated. In our study, all of the tested 2003 and 2004 isolates were shed in the feces by inoculated or contact ducks for a minimum of 11 days p.i. (Table 1). A range of virus-shedding was detected between days 11 and 17 p.i. for all of the viruses tested. Viruses in both pathogenicity groups were found to shed for as long as 17 days p.i. Fig. 1, which is published as supporting information on the PNAS web site, shows an example of the viral titers observed over the course of infection for one of the viruses tested, A/mallard/Vietnam/16D/03. All of the viruses tested showed similar results: viral titers were highest on day 3 and were consistently higher in the tracheal than in the cloacal swabs at that time. The titers then decreased progressively until day 11, after which the titers were consistently close to 10 EID_{50} per ml or were undetectable.

To compare the duration of shedding of these viruses with that of earlier H5N1 isolates, we inoculated ducks with an earlier human [A/Hong Kong/156/97 (H5N1)] and chicken [A/chicken/Hong Kong/YU562/01 (H5N1)] isolate. Because the newly acquired pathogenicity of H5N1 viruses in ducks may reflect reintroduction of the viruses into the duck population after circulation in another avian species, we included in this study a virus isolated from ducks that presumably has not undergone reintroduction [A/duck/Singapore/3/97 (H5N3)]. The human A/Hong Kong/156/97 virus isolate was shed for 7 days by inoculated ducks. Both contact birds were infected and both shed virus by day 3; virus titers were higher in the cloaca than in the trachea, as reported in ref. 3. Both inoculated birds and one of the contact birds had cleared the virus by day 7 p.i. The results were similar in ducks infected with A/chicken/Hong Kong/YU562/01 (H5N1) and A/duck/Singapore/3/97 (H5N3): Contact ducks and inoculated ducks shed virus by day 3, cloacal titers were higher than tracheal titers, and all of the birds cleared the virus by day 7 p.i. Interestingly, ducks infected with A/Hong Kong/156/97 (H5N1) and with A/chicken/Hong Kong/YU562/01 (H5N1) showed no clinical signs of disease, whereas ducks infected with A/duck/Singapore/3/97 (H5N3) became visibly sick, showing depression and weight loss; however, the birds recovered completely by day 7 p.i.

These results show that virus can be reisolated for a longer period from ducks infected with the newer (2003–2004) H5N1 isolates than from those infected with the tested viruses from 1997 and 2001. The longer period of virus-shedding appears to be another characteristic of the H5N1 viruses currently circulating in ducks.

Selection of Variant H5N1 Viruses in Ducks. To detect antigenic changes associated with the extended shedding of virus, we compared the ducks' immunity to viruses reisolated after prolonged shedding with their immunity to those in the original inocula. Serum obtained from individual ducks on day 17 p.i. was tested by virus neutralization assay against original stock virus and against virus isolated from a cloacal swab obtained from the same duck on the last day of detectable shedding (day 13 or day 17). Viruses from both pathogenicity groups (low, A/Vietnam/3046/04; high, A/chicken/Vietnam/48C/04 and A/mallard/Vietnam/16D/03) were tested. For all three viruses, the neutralization titer of the inoculated virus was higher than that of the virus isolated on the last day of shedding (A/Vietnam/3046/04, 640 vs. 320; A/chicken/Vietnam/48C/04, 320 vs. 80; A/mallard/Vietnam/16D/03, 320 vs. 20). We also analyzed day-17 serum by HI against virus samples obtained from the individual ducks at other times p.i. (Table 2). Each of the day-17 sera was reactive with the original isolate in both assays, indicating the immune competence of the ducks. HI assay results show that the original isolates react differently than the viruses isolated after extended shedding: Titers were higher for the original isolates when using homologous duck sera, except for A/chicken/Pangkal Pinang/BPPV3/04 isolated on day 11 after infection, which had a slightly higher HI titer than the original virus.

Because the viruses isolated after infection reacted differently from the inoculated viruses, we investigated whether this change reflected mutations leading to antigenic drift or selection of a virus present in the original inoculum. We sequenced the HAs

of viruses originally inoculated and those reisolated at different times p.i. in one duck from each of the groups that shed virus longest (11–17 days p.i.). We found that the viruses being shed had HA sequence differences over time. Table 1 shows the number of HA amino acid differences between the stock virus and virus isolated on day 11, 13, 15, or 17 p.i on the last day of detectable virus shedding. The A/mallard/Vietnam/16D/03 isolate showed the greatest number of HA differences. The four viruses from Hong Kong and the virus from Indonesia showed no amino acid differences. None of the amino acid differences observed occurred within the multibasic cleavage site of HA. The sequence data chromatograms of the stock samples showed a pattern of base peaks under the major base peaks that suggested a mixed population of virus (for example, see Fig. 2, which is published as supporting information on the PNAS web site). Sequence data of the viruses isolated from ducks after infection indicate that the minor constituent of the stock virus mixture becomes the dominant sequence. Therefore, the original sample contained a dominant virus and a smaller quantity of at least one other virus, and, during the course of infection, the smaller virus population became dominant. Such changes were not observed with the virus isolated in Indonesia (A/chicken/Pangkal Pinang/BPPV3/04); virus isolated on day 11 reacted strongly with antisera to the original isolate and had undergone only noncoding changes in its HA gene (nine base changes). It is noteworthy that sequence data for the original inoculum did not show the presence of another virus; therefore, the original sample was most probably not a mixture.

Selection of Less Pathogenic Variants in Ducks. To test whether the antigenic variants that emerged during infection remained pathogenic to ducks, we inoculated naive ducks with two viruses isolated on day 17 from ducks inoculated with A/mallard/Vietnam/16D/03 and A/chicken/Vietnam/48C/04 and with two viruses isolated on day 13 from ducks inoculated with A/duck/Vietnam/40D/04 and A/Vietnam/1203/04. Although all four viruses were originally highly pathogenic, the day-17 and day-13 isolates caused no mortality or signs of illness in inoculated naive ducks. The viruses replicated to high titers by day 3 p.i. in the trachea and cloaca and replicated until day 7 p.i. in inoculated and contact ducks. As observed previously, the titers were higher in the trachea than in the cloaca on day 3. The HI titers of the viruses isolated on day 7 (the last day of detectable shedding) were similar to those of the viruses with which the ducks were inoculated. The data indicate that, with the second passage in ducks, the virus that is isolated p.i. remains similar to that with which the ducks were inoculated. When we sequenced the HA genes of these viruses, we found base changes but no coding changes.

To determine whether the viruses isolated on day 17 remained pathogenic to chickens, we determined their IVPI. The A/mallard/Vietnam/16D/03 day-17 isolate had an IVPI score of 3.0 in chickens (the highest score possible, indicating that all inoculated chickens died within 24 h). The A/chicken/Vietnam/48C/04 day-17 isolate had an IVPI score of 2.88 in chickens. Although not all of the inoculated chickens died within 24 h, all died, and the virus would still be classified as highly pathogenic to chickens.

After observing that a nonpathogenic virus had been selected from an originally highly pathogenic virus mixture after one passage in ducks, we tested whether a highly pathogenic virus would be selected p.i. in ducks with a low-pathogenicity mixed virus population. Mallards were inoculated with the A/Vietnam/3046/04 day-13 isolate. The virus caused no signs of disease or mortality in the naive ducks. The inoculated and contact ducks shed virus by day 3 p.i. at high titers and continued to shed virus until day 9 p.i.

To ensure that the low-pathogenicity variant viruses had not been selected by chance, we repeated the above experiments with three of the viruses that were originally highly pathogenic to ducks but were nonpathogenic after one passage (A/mallard/Vietnam/16D/03, A/duck/Vietnam/40D/04, and A/chicken/Vietnam/48C/04). Infected ducks and contact ducks infected with the original sample of A/mallard/Vietnam/16D/03 died within 5 days; therefore, no long-term virus shedding occurred. The A/duck/Vietnam/40D/04 and the A/chicken/Vietnam/48C/04 viruses caused the deaths of all inoculated birds and one of the contact birds; the remaining contact bird in each group shed virus for 13 days. The day-13 viruses from A/duck/Vietnam/40D/04 and A/chicken/Vietnam/48C/04 were tested in an HI assay and reacted the same as the day-17 viruses from the first experiment. Sequence data indicated that the HA gene sequence of the viruses isolated on day 13 p.i. in this experiment was the same as the HA gene sequence obtained from the day-17 viruses in the first experiment.

***In Vitro* Selection of Variants.** To confirm that the original stock sample was a mixed population, we plaque-purified the A/Vietnam/1203/04 (H5N1) and A/mallard/Vietnam/16D/03 (H5N1) viruses on Madin–Darby canine kidney (MDCK) cells. Two morphologically distinct types of plaques (large and small) were generated by the A/Vietnam/1203/04 virus. Ten large and 10 small plaques were picked and passaged a second time on MDCK cells. The heterogeneous plaque morphology was observed after each passage. The original sample of A/Vietnam/1203/04 generated ≈71% large plaques (5 mm) and 29% small plaques (1 mm). When a large plaque was picked and passaged again on MDCK cells, 61% of the resulting plaques were large and 39% were small. Similarly, when a small plaque was picked and repassaged on MDCK cells, 59% of the resulting plaques were large and 41% were small. When the HA genes were sequenced, the original sample's HA showed a pattern of base peaks that indicated a mixed population of virus. The large-plaque virus isolated after two passages in MDCK cells had the same HA gene sequence as the original sample, but the pattern of base peaks indicated there was no longer a mixed population of virus. The small-plaque virus isolated after two passages in MDCK cells differed from the original virus in two amino acids in HA and was not a mixed population.

When ducks were inoculated with the original and two of the plaque-purified A/Vietnam/1203/04 viruses, the original sample caused mortality and neurological signs in the inoculated and contact ducks (Table 3, which is published as supporting information on the PNAS web site). The large-plaque virus caused the death of one duck and severe neurological signs in the remaining three ducks. The small-plaque virus caused no mortality, and only one duck showed signs of disease (Table 3). When the A/Vietnam/1203/04 virus was purified by limited dilution in chicken eggs before inoculation of ducks, no mortality or morbidity was observed. These findings suggest that the original stock samples contained a mixture of viruses that were variously pathogenic in ducks. Interestingly, ducks inoculated with the small-plaque isolate shed virus until day 7, whereas the large-plaque isolate was shed until day 13 by one duck.

A/mallard/Vietnam/16D/03 (H5N1) original virus isolate showed a similarly mixed plaque morphology. Again, large and small plaques were picked and passaged a second time on MDCK cells. The resulting plaques were again heterogeneous. No differences were found in the HA sequences of virus from the large and small A/mallard/Vietnam/16D/03 plaques; however, the original virus showed evidence of a mixed population, whereas the plaque-purified viruses did not. All of the plaque-purified viruses generated from the original A/mallard/Vietnam/16D/03 sample caused mortality in the inoculated and contact ducks similar to that caused by the original sample.

Discussion

The biology of H5N1 influenza viruses circulating in domestic poultry in Asia changed dramatically in late 2002; the virus acquired the ability to kill a large number of aquatic bird species (3, 4). The consequence of these findings was that regulatory authorities used disease signs as an indicator of the presence of the highly pathogenic H5N1 in both gallinaceous poultry and waterfowl. Our findings demonstrate the limitations of using only clinical signs to determine infection with highly pathogenic H5N1. The H5N1 viruses isolated from humans and poultry in Asia in late 2003 and early 2004 showed a trend toward decreased pathogenicity in ducks but remained highly pathogenic to chickens and presumably humans. In our studies, ducks that survived experimental infection with highly pathogenic H5N1 influenza virus shed virus for a prolonged period. Virus that was shed during the latter part of this period was nonpathogenic to ducks and was antigenically distinguishable from the input viruses; however, these viruses remained highly pathogenic to chickens. Sequence analysis of the HA confirmed that variants with amino acid changes had been selected. Analysis of the highly pathogenic avian H5N1 virus samples used to infect the ducks suggested the presence of a mixed virus population. Sequence analysis of the original material showed multiple double base peaks, further supporting the presence of mixed populations.

About half of the field isolates of H5N1 from poultry and humans used in this study were highly pathogenic to ducks. Genetic analysis of these viruses indicated that they all belong to the Z genotype (13). Our findings suggest a trend toward decreased pathogenicity of H5N1 viruses in ducks, although the small number of viruses and ducks tested (because of space constraints of the biosafety level 3+ facilities) precludes a firm conclusion. As reported by Chen *et al*. (2), H5N1 viruses isolated from healthy ducks (i.e., not pathogenic to ducks) remain pathogenic to chickens and to mammals (mice). Therefore, the duck may be resuming its role as a reservoir of H5N1 viruses, transmitting them to other bird species and potentially to mammals. Because all of the viruses tested were isolated during identified H5N1 outbreaks, they may not be representative of the endemic H5N1 virus population in the region. There may be many more ducks infected with low-pathogenicity viruses than are currently detected. Passive surveillance and outbreak reports will underestimate the prevalence of H5N1 viruses in duck populations and the role played by ducks in highly pathogenic avian influenza outbreaks. Region-wide influenza virus surveillance among healthy poultry throughout Asia is merited to determine whether highly pathogenic H5N1 is endemic in domestic birds. Such an initiative would elucidate the true impact and role of domestic ducks in the continual spread of H5N1 viruses.

The H5N1 viruses isolated since 2002 show other altered characteristics besides pathogenicity to ducks. Influenza A viruses have generally been found to replicate preferentially in the intestinal epithelial cells of ducks (25, 26). However, the newer H5N1 viruses are shed primarily from the upper respiratory tract (3, 4). Our findings of H5N1 virus isolates from 2003–2004 were similar. Previous studies of nonpathogenic H3N2 (23) and H7N2 (24) influenza viruses in ducks showed virus shedding for >2 weeks but no change in biological characteristics. In comparison studies with H5N1 in ducks, virus shedding was detected from 2 days (12) up to 5 days (7) after infection; however, one virus isolated in 2002 was reported to show virus shedding for 10 days after infection (3). In this study, we see virus shedding for up to 17 days after infection with the 2003–2004 H5N1 virus isolates. Therefore, it appears that the viruses that have emerged since 2002 have viral characteristics that have changed to a great extent compared with viruses isolated before 2002.

The cloacal shedding of virus observed in both pathogenicity groups indicates that long-term shedding is not infrequent or confined to one pathogenicity group. This characteristic is of great consequence especially in free-ranging ducks in that it increases the likelihood of transmission of virus to the environment, to other ducks, and, potentially, to other species. Water in which ducks swim, drink, and eat presents a high exposure risk to humans and domestic chickens. The risk is greatest in the rural areas of affected countries, where domestic ducks and chickens often mingle, frequently sharing the same water supply. The viruses are potentially transmitted to chickens under these conditions.

One possible explanation for the prolonged virus shedding is antigenic drift: The shed viruses may be variants that have escaped the immune response. Antigenic testing and sequence analysis proved that this explanation is incorrect; instead, results established that the original samples used to inoculate the ducks were composed of a mixture of viruses (genetic variants in a single isolate) that were probably in equilibrium. Two different plaque-purified viruses from the same original sample had different pathogenicity profiles in mallard ducks. As the virus replicates in the host, the predominant virus is targeted by host antibodies and is largely eliminated by the immune response. The minor virus population is then able to replicate and is shed at detectable titers over an extended period. The sera of the ducks, which were shown to be immunocompetent, neutralized the original input virus but had much less ability to neutralize the viruses isolated later in the course of infection. It is important to note that one of the plaque-purified viruses was shed through day 13 p.i. Therefore, the prolonged shedding of these 2003–2004 viruses may not have resulted from the fact that the inoculum was mixed. Instead, it may reflect a new characteristic acquired by these viruses. In either case, the prolonged shedding may be directly linked to the selection that occurs after a mixed virus population is inoculated.

The antigenic variants that arose from the original mixed populations were not pathogenic when inoculated into naive ducks. Viruses that had originally been pathogenic no longer caused morbidity or mortality in ducks, and virus that had been nonpathogenic remained nonpathogenic. These viruses appear to be returning to a host-adapted state in ducks. At the time of this report, influenza H5N1 viruses circulating in Thailand, Vietnam, and Indonesia are of the Z genotype (13). The genotype Z viruses may not be fully adapted to ducks and may continue to evolve through mutation and/or reassortment until they become well adapted (13). In the 2003 and 2004 isolates that were tested, we found a trend toward nonpathogenicity to ducks, indicating biological evolution toward equilibrium in their natural host.

A practical question that remains unanswered is whether it is safe to keep domestic poultry that survive after a flock has been reduced by clinically severe H5N1 infection. Our results indicate that it would be wise to cull all such flocks, for they could serve to select antigenic variants that are nonpathogenic to ducks, and ducks could then continue to spread these viruses and could potentially be reinfected with variants selected after amplification in contact ducks.

The phenomenon of heterogeneous plaque morphology that persists after plaque passaging has been described in European swine viruses that arose from an avian influenza A virus that crossed the species barrier to pigs in 1979 (27). Genetic instability (mutator mutations) might explain this heterogeneity, but the mutational rate of these swine viruses did not differ significantly from that of other influenza A viruses (28). The presence of partial heterozygotes, as suggested by Stech *et al.* (29), can also be excluded because the plaque-purified viruses showed no evidence of mixed virus populations (double base peaks in the sequence chromatogram, which should be seen in partial het-

erozygotes). Therefore, although we do not yet have an explanation for the plaque heterogeneity, it is interesting to note that this stable heterogeneity has been seen each time an avian influenza A virus has passed the species barrier to a mammal: to swine in 1979 (27), to mink in 1984 [A/mink/Sweden/E12665/84 (H10N4)], to seals in 1980 [A/seal/Massachusetts/1/80 (H7N7)], and to swine in 1998 [A/swine/Hong Kong/9A/98 (H9N2)] (C.S., unpublished observation). Correspondingly, the plaque heterogeneity of the recent highly pathogenic A/mallard/Vietnam/16D/03 (H5N1) virus may indicate a species-crossing event (from a terrestrial bird to ducks) that is establishing a new stable lineage in ducks.

The idea that H5N1 influenza viruses are not lethal to wild ducks (15) clearly has not applied in Asia since 2003. However, the fact that highly pathogenic H5N1 viruses in our study became nonpathogenic to ducks after extended shedding shows that these viruses are moving toward stability in this host. Because this mechanism increases the potential for spread of these viruses, it is biologically significant, helping to explain the origin of new H5N1 viruses and the manner in which they become the dominant viruses in a quasi-species population. This finding also has public health implications. There is a real possibility that if these H5N1 viruses continue to circulate, further human infection will occur, increasing the potential for human-to-human transmission. There is a high rate of H5N1 influenza virus infection in domestic ducks in the Vietnam region, and duck density has been strongly correlated with H5N1 outbreaks in Thailand (29). Therefore, the role of the duck in these outbreaks should be investigated further.

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