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An epidemiological study of influenza viruses among Chinese farm families with household ducks and pigs

L. L. SHU[1], N. N. ZHOU[1,2], G. B. SHARP[1,3], S. Q. HE[2], T. J. ZHANG[2], W. W. ZOU[2] AND R. G. WEBSTER[1]*

[1] *Department of Virology/Molecular Biology, St. Jude Children's Research Hospital, 332 North Lauderdale, P.O. Box 318, Memphis, TN 38101 USA*
[2] *Department of Microbiology, Jiangxi Medical College, 162 Ba Yi Road, Nanchang, Jiangxi, China*
[3] *Department of Hematology/Oncology, St. Jude Children's Research Hospital, 332 North Lauderdale, P.O. Box 318, Memphis, TN 38101 USA*

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SUMMARY

To examine the possibility of interspecies transmission and genetic reassortment of influenza viruses on farms in Southern China, we surveyed 20 farm families living outside the city of Nanchang who raised pigs and ducks in their homes. Weekly interviews of family members and virus isolation studies of throat swabs and faecal samples, collected from September 1992 to September 1993, established the seasonal pattern of respiratory tract infections in these families and identified 11 influenza viruses (6 in humans and 5 in ducks). Most of the human isolates were type A of H3N2 subtype. Serologic studies of farm pigs indicated infection by the same human viruses circulating in family members, but there was no evidence that either swine or avian viruses had been transmitted to pigs. Eight of 156 human serum samples inhibited the neuraminidase activity of two of the duck isolates, raising the possibility of interspecies transmission of these avian viruses. Genotype analysis of duck and human isolates provided no evidence for reassortment. Our findings support the concept that intermingling of humans, pigs and ducks on Chinese farms is favourable to the generation of new, potentially hazardous strains of influenza virus.

INTRODUCTION

Influenza viruses are negative-sense RNA viruses with a single-stranded genome comprising eight segments [1]. They can be divided into 14 hemagglutinin (HA) and 9 neuraminidase (NA) subtypes based on differences in the surface glycoproteins. All known subtypes of influenza viruses are found among wild avian species, which serve as reservoirs for the virus [2]. In general, influenza viruses are species specific; however, surveillance work has shown that some avian viruses can apparently cross the species barrier directly to horses [3], pigs [4] and sea mammals [5] sometimes with fatal consequences. These observations raise an intriguing question: Could the viruses be transmitted directly to humans from avians?

Previous studies have established clear links between interspecies transmission and genetic reassortment of influenza viruses and the appearance of pandemic influenza [6, 7]. In human influenza pandemic strains of 1957 (Asian/57, H2N2) and 1968 (Hong Kong/68, H3N2), three genes (HA, NA and polymerase (PB1]) of the Asian/57 strain were of avian influenza virus origin, while the remaining genes were of human virus origin [7, 6]. The Hong Kong/68

* Corresponding author.

pandemic strain obtained its HA and PB1 genes from an avian virus and the other genes were characteristic of human influenza viruses [8]. Phylogenetic analysis of the gene segments of these human pandemic viruses [9] affirmed earlier sequence analyses. Viruses whose genomes consisted of a mixture of influenza segments of both human and avian origin have been isolated from pigs in Italy [10]. Moreover, direct transfer and replication of a human influenza-like virus has been demonstrated in pigs [4], and interspecies transmission of influenza viruses from pigs to humans is well documented [11–13].

Despite abundant evidence supporting interspecies transmission and genetic reassortment of influenza viruses, little is known about the conditions that favour these events. Since Southern China has been proposed as an epicentre for the emergence of pandemic influenza virus [14], we elected to study 20 farm families in this region who raise pigs and ducks in their homes. The purpose was to establish the seasonal pattern of respiratory illness and the prevalence and types of influenza viruses among humans, ducks and pigs crowded together in one household and determine if any influenza A viruses were passed directly from ducks or pigs to humans. Serum samples collected from humans and pigs were tested for evidence of interspecies transmission of influenza viruses between these species.

MATERIALS AND METHODS

Isolation and identification of viruses. Twenty farm families that raised pigs and ducks at their residences in the suburbs of Nanchang, Jiangxi Province, China were studied from September 1992 to September 1993 (Table 1). Each week a researcher visited each of the 20 families to determine if anyone in the family had evidence of respiratory infection. We chose one family member each week who had respiratory disease symptoms which included sore throat, cough, headache and fever and recorded their recent exposures to animals. A throat swab was collected from the person with the most severe respiratory signs. When no one in the household was sick, we sampled the person who worked most closely with the pigs. Additional samples were obtained from the local hospital whenever influenza virus was isolated from one of these families. For example, in April 1993, we collected an additional 45 samples from patients in a local hospital who complained of respiratory distress.

Obtaining swab samples from pigs at each of these residences was not feasible because of their large size and because farmers were reluctant to allow weekly nasal swabbings of pigs. Instead, serological studies were conducted on a random sample of pigs from the 20 households, from which we collected blood sample shortly before slaughter. Ducks from the 20 households were permitted to wander free during the day in areas near each house, returning to the house each night. To determine which influenza A viruses, if any, these ducks were infected with, each week we also collected one fresh duck faeces sample from each of 10 duck sites, collecting a total of 540 faeces samples over the course of the study.

All samples from humans and ducks were inoculated into embryonated chicken eggs for isolation of influenza viruses. Isolates were then identified by hemagglutinin inhibition (HI) and neuraminidase inhibition (NI) testing with a panel of monospecific antisera [15].

Serologic study. Periodically we collected blood samples from the person at each of the 20 residences who were identified as being responsible for care of pigs and ducks. We sampled 20 people in September 1992, 18 people in November 1992 and 18 people in June 1993. Because animal care duties were not always performed by the same person in each family, blood samples were not collected from the same person each time. In November 1993, 2 months after the 1 year of surveillance, we collected blood samples from 5 people and 5 pigs at each of the 20 residences, a total of 100 human and 100 pig samples. An additional 51 blood samples were collected from patients seen at the local hospital because of respiratory symptoms; 55 serum samples were also collected from pigs in a local slaughterhouse in July 1993. All specimens were tested with the HI and NI assays for antibodies against human, pig, and duck influenza viruses (15). An HI or NI titre of 20 or more was considered positive.

Hybridization assay. In order to determine the genotypes of the influenza viruses isolated in this study, hybridization assays were done. Since the surface genes were identified by antigenicity analysis (HI and NI), we focused on the six viral genes coding for internal proteins. Details of the hybridization assay have been described previously [16]. Briefly, viral RNA was extracted from allantoic fluid containing influenza viruses. The cDNAs of all RNA

Table 1. *Details of the study group in Nanchang raising pigs and ducks*

Family (ID no.)	No. of family members	No. of children in the family	No. of persons handling animals	No. of pigs raised	No. of pigs bled	No. of ducks raised*
1	3	0	3	24	5	4
2	5	2	2	18	5	4
3	4	3	1	16	5	4
4	7	3	2	28	5	4
5	4	0	2	23	5	4
6	7	4	2	12	5	4
7	5	2	2	22	5	4
8	4	1	3	18	5	4
9	5	2	2	23	5	4
10	10	3	2	8	5	4
11	9	4	2	15	5	4
12	8	2	2	16	5	4
13	8	3	3	24	5	4
14	7	2	2	17	5	4
15	7	3	2	18	5	4
16	10	3	2	15	5	4
17	4	1	2	21	5	4
18	6	2	2	20	5	4
19	8	3	3	32	5	4
20	8	3	2	12	5	4

* Each family enrolled in the study raised four ducks; however, during the day ducks were free to roam and the number present at sampling time would vary.

segments were synthesized with use of reverse transcriptase (Life Sciences, St. Petersburg, FL) and a 12-base oligodeoxynucleotide primer (5_AGCAAAAGCAGG) that is common to all segments of influenza viruses. The cDNAs were amplified by the polymerase chain reaction (PCR) using pairs of primers specific for the gene under study. For dot blot hydridization, the PCR products were cross-linked to nylon membranes (Zeta Probe, BioRad, Richmond, CA) by ultraviolet light (Stratalinker, Stratagene, La Jolla, CA). The sequences and nucleotide locations for the primers and probes have been reported previously [16]. Oligonucleotide probes approximating 20 nucleotides in length were prepared for each of the 6 genes encoding the internal proteins of influenza viruses: nonstructural (NS), and matrix (M), nucleoprotein (NP), polymerase (PA), polymerase B1 (PB1), and polymerase B2 (PB2). Four probes were prepared for each gene: (1) a control probe that would bind to regions conserved by all influenza viruses, (2) a probe specific for viruses from humans, (3) a probe specific for viruses from pigs, and (4) a probe specific for viruses from avian hosts. Probes were tailed with digoxigenin (Boehringer Mannheim, Indianapolis, IN). Probe binding was detected by a calorimetric reaction with nitroblue tetrazolium and X-phosphate (Boehringer Mannheim, Indianapolis, IN).

Genetic analysis. To determine the genetic relationships of the viruses isolated in this study, phylogenetic analysis were done. To facilitate a phylogenetic analysis, we partially sequenced the NP genes of one human H3N2 virus and four duck viruses of different subtypes by fmol sequencing methods (Promega, Madison, WI) [17]. Briefly, the primers were end-labelled with [^{32}P]ATP by incubation at 37 °C for 10 min and then at 100 °C for 2 min. The extension and termination reactions were performed in the Programmable Thermal Controller by incubation at 95 °C for 2 min, followed by denaturation at 95 °C for 30 s, annealing at 42 °C for 30 s, and extension at 70 °C for 1 min, for a total of 30 cycles. After addition of the stop solution and heating at 100 °C for 2 min, 3 μl of each reaction mixture was loaded on a polyacrylamide sequencing gel.

All partial nucleotide sequences were analysed by computer with the FastDB program to find the best matches in GenBank. The phylogenetic analysis was performed with PAUP (Phylogenetic Analysis Using Parsimony) software package (version 2.4, David

Swofford, Illinois Natural History Survey, Champaign, IL), which relies on maximum parsimony to generate the phylogeny.

RESULTS

Seasonality of respiratory infections. The results of family interviews to determine the seasonal pattern of respiratory infections in a defined location in Southern China are shown in Fig. 1A. During the 1-year surveillance period (September 1992–September 1993), there were 74 reports of respiratory symptoms among the 20 families, including headache, fever, sore throat and cough. Headache was a common complaint (40/74, 54·1%), but reports of fever were rare (3/74, 4·1%). As expected, the peak incidence of respiratory symptoms occurred during the winter (November, December, January and February) with a minor peak in May (Fig. 1A).

Isolation of influenza viruses from humans and ducks. Four H3N2 type A and two type B influenza viruses were isolated from throat swabs of these 20 families between April and July 1993. The sites and dates of virus isolation are presented in Table 2. Two additional H3N2 type A viruses and one type B virus were isolated from the 45 samples collected at a local hospital in April 1993. Five duck influenza viruses of various subtypes were isolated from faecal samples collected between October 1992 and January 1993.

The farmer at site H2 bought ducks from other farmers in the village and slaughtered and dressed them for sale at the local market. The A/Nanchang/3542/93 (H3N2) virus was isolated from this family on 28 June 1993. A duck virus [A/Duck/Nanchang/1681/92 (H3N8)] was isolated from a faeces sample collected on 17 October 1992 from the site (D1) where ducks from this household usually gathered. A second duck virus [A/Duck/Nanchang/1941/93 (H4N4)] was isolated from a faeces sample collected at this same site on 14 January 1993. A type B influenza virus (B/Nanchang/3451/93) was isolated on 25 May 1993, at site H11 from a 5-year-old child with a cough and sore throat; 2 months later a type A influenza virus, A/Nanchang/3631/93 (H3N2), was isolated from the child's father. Two H7N4 duck viruses (A/Duck/Nanchang/1904/92 and A/Duck/Nanchang/1944/93) were isolated from site D4 at an interval of 2 weeks (31/12/92 and 14/1/93).

The nine human viruses were isolated from April to July in 1993 (Fig. 1B), while the five duck viruses were isolated from October 1992 to January 1993 (Fig. 1C). Thus, influenza viruses were circulating at different times in humans and ducks living in close proximity on family firms.

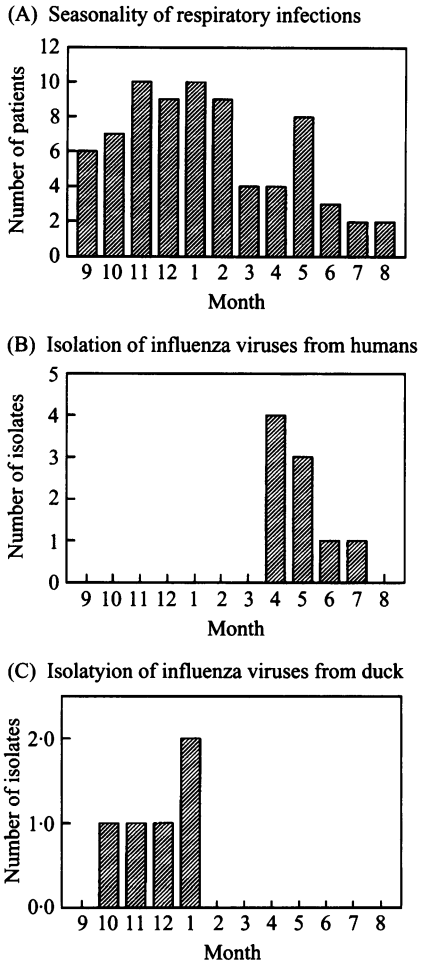

Fig. 1. Seasonal patterns of respiratory illness and influenza virus isolation among farm families living in Southern China. (A) Respiratory infections by month. The symptoms included any combination of sore throat, cough, headache, and fever. (B and C) Isolation of human and duck viruses by month.

Serologic analyses of humans and pigs. Five pigs at each residence (total of 100) were bled in November 1993 and blood was collected from 55 pigs at a local slaughterhouse in July 1993. Antibodies to H1 human influenza viruses were detected in 2% (titre, 40) of slaughterhouse pigs and 12% (titre, 20–80) of household pigs; H3 antibodies were found in 11% (titre, 20–80) and 12% (titre, 20–80) of these groups, respectively (Table 3). Serologic evidence of N1 pig viruses was limited to two slaughterhouse pigs. Antibodies to duck viruses were not detected in any

Table 2. *Isolation of influenza viruses from farm families in the suburbs of Nanchang, China*

Samples		Virus isolation			
		Virus	Type or subtype	Site†	Isolation date
Human	Family (n* = 1175)	A/Nanchang/3332/93	H3N2	H12	4/15/93
		A/Nanchang/3396/93	H3N2	H16	5/3/93
		A/Nanchang/3542/93	H3N2	H2	6/28/93
		A/Nanchang/3631/93	H3N2	H11	7/28/93
		A/Nanchang/34262/93	B	H6	5/17/93
		A/Nanchang/3451/93	B	H11	5/25/93
	Hospital (n = 45)	A/Nanchang/20/93	H3N2	Hospital	4/14/93
		B/Nanchang/26/93	B	Hospital	4/14/93
		A/Nanchang/69/93	H3N2	Hospital	4/25/93
Duck	Family homes and yards (n = 540)	Duck/Nanchang/1681/92	H3N8	D1	10/17/92
		Duck/Nanchang/1749/92	H11N2	D9	11/7/92
		Duck/Nanchang/1904/92	H7N4	D4	12/31/92
		Duck/Nanchang/1944/93	H7N4	D4	1/14/93
		Duck/Nanchang/1941/93	H4N4	D1	1/14/93

* n, total sample number; H, human site; D, duck site.

Table 3. *Serologic survey of swine for influenza viruses*

Source of sample	No. of samples	Sampling time	No. of positive samples*							
			Human viruses				Swine viruses		Duck viruses A/Duck/Nanchang/1749/92 (H11N2) A/Duck/Nanchang/1941/93 (H4N4) A/Duck/Nanchang/1904/92 (H7N4) A/Duck/Nanchang/1681/91 (H3N8)	
			H1N1 A/Texas/36/91		H3N2 A/Nanchang/3332/93		H1N1 A/Swine/Beijing/47/91			
			HI	NI	HI	NI	HI	NI	HI	NI
Family study	100	November 1993	12 (80) 12%	0	12 (80) 12%	0	0	0	0	0
Slaughterhouse	55	July 1993	1 (40) 2%	0	6 (80) 11%	4 (20) 7%	0	2 (40) 4%	0	0

* Maximum antibody titres given in parentheses.

pigs, nor was there evidence of HI antibodies to classical H1N1 swine influenza virus, although two pigs had antibodies to N1 that could be explained by cross reactivity with current human H1N1 viruses.

Serologic analyses with the HI and NI assays were also performed on the total of 156 blood samples collected from the 20 farm families during the study period (Table 4). Both assays detected antibodies to the currently circulating human H1N1, H3N2 and influenza B viruses. Antibodies to the HA of A/Texas/36/91 (H1N1) were found in 64% of the 100 persons sampled in November 1993; 32% of these samples were positive for H3N2 antibodies and 6% for B antibodies. Failure to isolate H1N1 influenza viruses from the 20 families suggests that the positive assay results represent previous exposure to the virus. The peak frequency of antibodies to H3N2 virus in sera taken in November 1993 corresponds well to

Table 4. *Detection of HI and NI antibodies in farm families raising pigs and ducks in Southern China*

		No. of persons positive for different types and subtypes of influenza virus														
		Human					Swine		Duck							
		B	H1N A/Texas/ 36/91		H3N2 A/Nanchang. 3332/93		A/swine Beijing/ 47/91		H11N2 A/duck Nanchang/ 1749/92		H4N4 A/duck Nanchang/ 1941/93		H7N4 A/duck Nanchang/ 1904/92		H3N8 A/duck Nanchang/ 1681/92	
Sampling date	No. of samples	HI	HI	NI	HI	NI	HI	NI	HI	NI	HI	NI	HI	NI	HI	NI
September 1992	20	2 (40)*	7 (40)	3 (20)	2 (20)	0	0	0	0	1 (40)	0	0	0	0	0	0
November 1992	18	3 (20)	8 (40)	8 (20)	3 (20)	3 (20)	0	0	0	9 (80)	0	0	0	1 (20)	0	0
June 1993	18	2 (40)	8 (40)	7 (40)	2 (80)	1 (20)	0	0	0	5 (40)	0	0	0	1 (40)	0	0
November 1993	100	6 (80) 6%	64 (160) 64%	18 (80) 18%	32 (160) 32%	26 (40) 26%	0	6 (40) 6%	0	23 (40) 23%	0	2 (20) 2%	0	6 (40) 6%	0	0
July 1993 (hospital)	51	3 (20) 6%	15 (200) 29%	NT†	26 (640) 51%	NT	NT	NT	NT	6 (80) 12%	NT	0	NT	0	NT	0

* Maximum titre.

† Not tested, insufficient serum.

isolation of the virus in preceding months (April–July). Antibodies specific for swine H1 were not detected in humans; the antibodies to swine N1 probably reflect cross reactivity with human H1N1 viruses.

Antibodies to the N2 and N4 neuraminidases of duck influenza viruses were detected in some of the families. The N2 antibodies were probably induced by the human H3N2 strain; however, the presence of N4 antibodies cannot be explained by a similar mechanism. It is noteworthy that the prevalence of N4 antibodies (6%) to H7N4 in the group of 100 subjects in November 1993 was higher than the prevalence of N4 antibodies (2%) to H4N4. The two persons who were seropositive for H4N4 antibodies were also positive for H7N4 antibodies, confirming the specificity of these antibodies. Although H4N4 and H7N4 viruses were isolated from two duck sites where one or more humans showed evidence of infection with one of these duck viruses, these were not isolated from five duck sites associated with residences where humans showed serologic evidence of infection with these viruses. The relative risk of one or more family members being seropositive for H4N4 or H7N4 viruses for exposure to ducks testing positive for one of these viruses was 1·1 (95% confidence interval: 0·3–3·9).

Genetic analysis of human duck viruses. To determine if any of the isolates were reassortants of human, avian or swine viruses, we identified the host of origin of each viral gene segment. Sixty-six genes encoding the internal proteins of six H3N2 human viruses and five duck viruses were identified by dot blot hybridization. The PB1, PB2, PA, NP, NS and M genes of six human isolates bound only the human virus probe. Similarly, the internal genes of the five duck viruses proved to have an origin in ducks. The NP gene of one of the human viruses (A/Nanchang/3332/93, H3N2) and of five duck viruses were phylogenetically analysed by joining the partial sequences to the published NP sequences [18, 19]. The resulting tree (Fig. 2) shows that the A/Nanchang/3332/93 virus belongs to the human lineage and is closely related to A/Hong Kong/83. The five duck viruses are members of the Asian branch of the avian lineage.

The human viruses isolated during the study were most like the recent Hong Kong isolate; the duck viruses were most closely related to duck influenza viruses isolated in Hong Kong in 1975. There was no evidence of genetic reassortment between human and duck viruses.

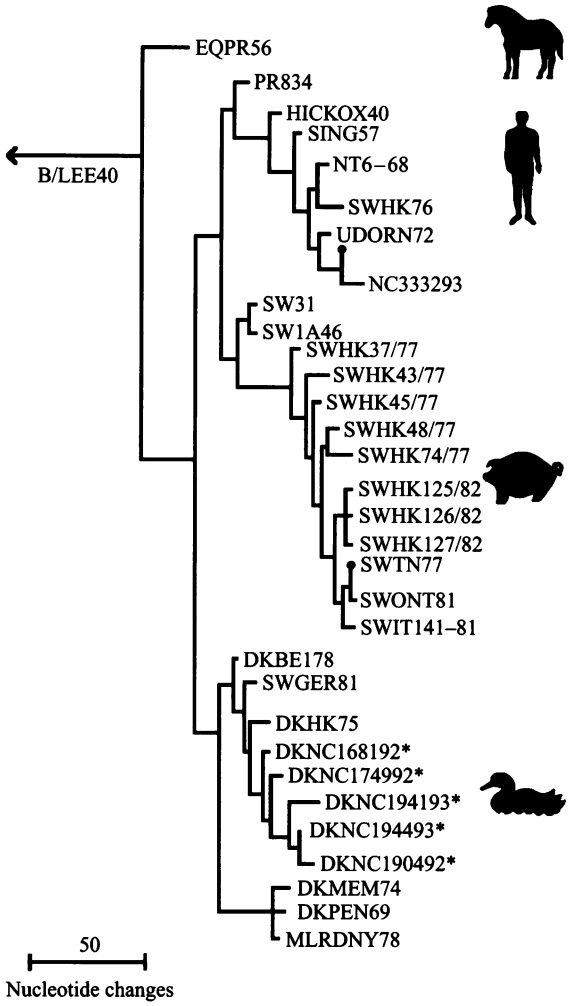

Fig. 2. Evolutionary tree based on the NP genes of influenza A viruses. The genes from isolates marked by asterisks (*) were sequenced in this study. Others were described by Gorman et al. (30) and Shu et al. (19). The tree is rooted in the NP gene of B/Lee/40. Other details are given in Materials and Methods.

DISCUSSION

Southern China has been implicated as an epicentre for the generation of pandemic influenza viruses [14]. The precise mechanism for the emergence of such viruses is unknown but is believed to involve interspecies transmission and genetic assortment. Our study is the first to document the presence of influenza viruses in household ducks in Southern China and to present evidence for their transmission to family members.

Symptoms of respiratory tract infections in 20 farm families living near Nanchang in Southern china reached a major peak in winter (November–February) and a minor peak in May. The latter corresponded well to the detection of influenza viruses in throat swab samples taken at regular intervals from family members. These results are consistent with surveillance results in Guangdong Province (1982–7) and Hong Kong (1978–88) indicating peak influenza virus activity in May–July [20]. Because we did not isolate influenza A viruses from humans until April, it is doubtful that influenza virus accounted for more than a small percentage of the symptoms noted in winter. Rhinovirus, parainfluenza virus, adenovirus, respiratory syncytial virus and certain bacteria are all potential causative agents for the types of complaints recorded in this survey [21, 22].

The rate of isolation of duck viruses, 0·9% (5/540), is much lower than the 6·4% reported for domestic poultry in an epidemiologic study conducted over 5 years in Hong Kong and Southern China to ascertain the prevalence of influenza viruses in birds. In that survey, the isolation rate was generally higher in the hot, humid summer months than in the cooler, drier months of winter [23]. All five of the duck viruses identified in the present study were isolated in fall or winter. The discrepancies between these surveys can also be attributed, in part, to differences in the sources of samples. Our avian samples were only collected from ducks being raised by the 20 farm families, whereas the Hong Kong investigators concentrated on many different species of birds crowded together in live bird markets, where conditions would likely amplify virus isolation rates compared to those in the field.

The serologic study showed that human influenza viruses were transmitted to pigs both raised on family farms and slaughtered in Nanchang, but there was no evidence for transmission of swine or avian influenza viruses to pigs in this area of Southern China.

Of 156 human serum samples collected from farm families, eight inhibited the NA activity of A/Duck/Nanchang/1904/92 (H7N4) with two of these samples also inhibiting the activity of A/Duck/Nanchang/1941/93 (H4N4) influenza viruses. Results of these N1 assays were specific for N4 neuraminidase and did not inhibit N8 neuraminidase. Since the NI assay is not inhibited by nonspecific factors in human serum, our findings raise the possibility that family members were infected with the virus isolated from ducks. The absence of HI antibodies is not surprising, for earlier studies also failed to detect HI antibodies in humans experimentally infected with avian influenza viruses [24]. Future analyses should include ELISA assays, although isolated and purified HA antigens are not currently available in China.

The family most likely to have had repeated exposures to duck influenza virus lived at site 2, where the farmer purchased live ducks from different families in the area, killed them on the open balcony of his house, and prepared them for market. He also raised pigs and ducks. Two duck viruses (A/duck/Nanchang/1681/92, H3N8; A/duck/Nanchang/1941/93, H4N4) were isolated from fecal specimens collected at the duck site near this residence on 10/17/92 and 1/14/93. Five family members had antibodies against H3 human viruses, and a 10-year-old child had an antibody titre of 40 to N4 duck virus. The delayed collection of serum, in November 1993, or 10 months after the isolation of the H4N4 duck virus, may explain why the child's antibody titre was low. Domestic ducks are the principal reservoir of influenza A viruses in Southern China [23], and our data from site 2 suggest that these avian viruses are occasionally transmitted to humans who have frequent contact with ducks. Other studies of human sera from Southern China have detected antibodies to avian H4-H13 in farm workers [23] by the single radial haemolysis test, but these results have not been confirmed.

Serologic evidence of direct transmission of a duck virus to humans has important health implications. It would afford the opportunity for human and avian influenza A viruses, as a mechanism thought to be responsible for the appearance of new human pandemic strains [6]. Moreover, certain avian viruses may produce disease without prior reassortment. For example, an H3N8 virus with avian genes transmitted directly from avians was the cause of severe respiratory disease in horses in Northeast China, with mortality rates approaching 20% in some herds [25]. Similarly, there is evidence of direct avian to mammal infection of seals [26, 27] and whales [5, 28].

Our failure to isolate reassortant viruses from humans or ducks may be related to differences in the seasonal distribution of duck and human viruses within the Nanchang region or in the year chosen to conduct the survey or the fact that we surveyed only a small number of families. Genetic reassortment requires cocirculation of different viruses within the same host population. Thus, as concluded by Lin and colleagues [29], reassortment of avian influenza viruses

with strains from other hosts in nature are rare events an are not likely to be detected without extended surveillance studies in favourable settings.

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