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Influenza infection in humans and pigs in southeastern China

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Summary. The three last pandemic strains of influenza A virus – Asian/57, Hong Kong/68 and Russian/77 – are believed to have originated in China. The strains responsible for the 1957 and 1968 human pandemics were reassortants incorporating both human and avian influenza viruses, which may have arisen in pigs. We therefore undertook a population-based study in the Nanchang region of Central China to establish the prevalence, types and seasonal pattern of human influenza infection and to screen serum samples from animals and humans for evidence of interspecies transmission of influenza viruses.

Two definite influenza seasons were demonstrated, one extending from November to March and the other July to September. The profile of antibodies to commonly circulating human influenza viruses was no different in Nanchang and neighboring rural communities than in Memphis, Tennessee, USA. In particular, Chinese women who raised pigs in their homes were no more likely to have been exposed to influenza virus than were subjects who seldom or never had contact with pigs. However, we did obtain evidence using isolated H7 protein in an enzyme-linked immunoabsorbent assay for infection of pig farmers by an avian H7 influenza virus suggesting that influenza A viruses may have been transmitted directly from ducks to humans. The results of the serological survey also indicated that pigs in or near Nanchang were infected by human H1N1 and H3N2 influenza viruses, but not with typical swine viruses. We found no serological evidence for H2 influenza viruses in humans after 1968.

Introduction

Influenza is the paradigm of a viral disease in which continual mutation and reemergence of the causative virus result in recurrent epidemics and pandemics, many with high rates of morbidity and mortality [9, 26]. New subtypes of human

type A influenza viruses have appeared at irregular intervals since isolation of the first human strain by Smith et al. in 1993 [22]. In 1957, the H2N2 subtype (Asian influenza) replaced the H1N1 as the major subtype circulating in humans, producing an outbreak of influenza A H2N2 in Guizhou Province, near Guiyang, in Southern China [23]. Similarly, in mid-July of 1968, there was an influenza epidemic in Hong Kong that was later attributed to a new subtype of influenza A virus, characterized by a unique H3 hemagglutinin but sharing the N2 neuraminidase of the previous Asian virus [2]. Finally, in 1977, the H1N1 virus, which had disappeared in 1957, reemerged in Northern China. Repeated isolation of new subtypes of influenza A viruses in China raises the possibility that this country serves as an influenza epicenter.

The influenza A viruses responsible for the 1957 and 1968 human pandemics arose by genetic reassortment between previous human viruses and avian viruses [17]. The 1957 Asian virus acquired three of its genes – PB1, HA, and NA – from avian viruses, while the 1968 Hong Kong virus acquired two such genes – PB1 and HA1, its remaining genes coming from previously circulating human viruses [11, 25]. Reassortment of avian and human viral genes, preceding the generation of human pandemic strains, could occur by several mechanisms. One is direct introduction of an avian virus into humans; alternatively, other species of animals susceptible to both human and avian viruses might serve as intermediate hosts. Because of their ability to act as hosts for viruses from either birds or humans [6, 10], swine have been considered a logical vehicle for the reassortment of influenza viruses and for their transmission to humans [18]. This hypothesis lacked direct support until Castrucci et al. [1] demonstrated reassortment of avian-like H1N1 and human-like H3N2 viruses in a natural population of Italian pigs and until Claas et al. [3] showed that these viruses could also be transmitted to humans.

To better understand the link between China and the generation of human pandemic strains of influenza viruses, we investigated the frequency of interspecies transmission and reassortment of influenza A viruses among pigs and humans living in or near Nanchang city. A further goal was to establish seasonal variants in the prevalence of influenza in the Nanchang region, as compared to findings in other countries. Located in the southeastern part of China, Nanchang has a network of irrigation canals over its suburban area that create a favorable environment for aquatic birds, the reservoir for most avian influenza viruses [5]. Swine husbandry in Nanchang is usually a family business, in which pigs as well as poultry are raised in the yards and houses of farmers. Thus, the study site possessed many of the features known to be conducive to frequent exchanges of influenza virus between species [19].

Materials and methods

Virus isolation

From January to December of 1994, we collected 15 throat swabs every week from outpatients with upper respiratory tract distress attending a local children's hospital in

Nanchang. The patients ranged in age from 2 months to 10 years and resided in the city or nearby rural communities, where they had daily contact with domestic animals, including pigs, ducks and chickens. In accord with recommendations of the WHO [27], all clinic samples were treated with antibiotics and then inoculated into 10-day-old embryonated chicken eggs and Madin-Darby canine kidney (MDCK) cells for isolation of influenza virus. The inoculated eggs were incubated for 3 days at 35 °C, while MDCK cells were cultured for 7 days and observed daily for cytopathic effects (CPE). Culture media and allantoic and amniotic fluids were tested for hemagglutinin from eggs using red blood cells from chickens, guinea pigs, or both. All of the isolates were identified by HI and NI tests with a panel of reference antisera prepared from hyperimmunized goats or rabbits; postinfection ferret antisera was used to identify human isolates.

Study groups for serologic investigation

Four groups were selected for the serologic investigations: (i) people who lived or worked in close contact with pigs (268 slaughterhouse workers and 200 women who raised pigs). Their ages ranged from 18 to 50 years old, with a mean of 31.3 years. The slaughterhouse workers represented nine different businesses in Nanchang city. Most of the women who raised pigs were farmers from rural communities around Nanchang; besides pigs, they raised ducks and chickens and worked in rice fields. (ii) People who had little or no contact with pigs (200 university students at the provincial medical school). The age distribution of this cohort ranged from 19 to 22 years old, with a mean of 20.7. None of the humans in the above groups in China had ever been vaccinated against influenza. (iii) People living in the United states who had not received recent influenza vaccinations in the current year (32 employees of St. Jude Children's Research Hospital ranging from 29 to 55 years of age (mean, 37.3 years)). (iv) Slaughtered pigs from suburban areas or neighboring counties of Nanchang city, or from several slaughterhouses. They were usually 8 or 10 months old when slaughtered.

Serologic studies were performed from December 1993 to June 1994. Blood samples were collected twice, at 6-month intervals, from the slaughterhouse workers, and once from other subjects.

Viruses

The influenza A virus strains used in the serologic study included Nanchang/3332/93 (H3N2); Texas/36/91 (H1N1); Swine/Beijing/47/91 (H1N1), which is a classical swine virus; Swine/Italy/786/88 (H1N1), which is a strain of recent avian origin; Swine/Italy/809/89 (H3N2) which is a Port Chalmers/73 H3N2-like strain; Duck/Nanchang/1681/93 (H3N8); Duck/Nanchang/1904/93 (H7N4); Duck/Nanchang/1941/93 (H4N4); Duck/Nanchang/1749/93 (H11N2); Japan/305/57-A/Bel/42 (H2N1), R a reassortant strain, Duck/Nanchang/1904/93-A/Bel/42 (H7N1), [R] a reassortant strain. This reassortant was used to assay antibodies to H2 and to avoid any cross reactions with current N2 strains. The A/Bel/42 (N1) neuraminidase was used, for it is sufficiently different antigenically from current N1 strains.

Nanchang viruses were isolated from humans and ducks in the region where this study was done. A reassortant influenza virus possessing the neuraminidase subunits of the A/BEL/42 (H0N1) strain and the hemagglutinin subunits of Duck/Nanchang/1904/93 was prepared as described by Webster [24]; all other viruses were from the repository at St. Jude Children's Research Hospital.

Viruses were grown in 11-day-old embryonated chicken eggs for 2 days, after which allantoic fluids were harvested, measured for hemagglutination titers, and used as antigens in HI and NI tests.

Purification of hemagglutinin (HA)

The antigenic reassortant Duck/Nanchang/1904/93-A/Bel/42 was concentrated using an ultrafiltration cartridge and purified by centrifugation through a 25–70% sucrose gradient. To purify the HA protein, we followed the method of Laver and Webster [13], mixing the purified Duck/Nanchang/1904-BEL virus (hemagglutinin titer, 10 000 units/ml) with SDS so that the final concentration of detergent was 1%. Electrophoresis of the SDS-disrupted virus was carried out on cellulose acetate strips in Tris-boric acid-EDTA buffer, pH 9, containing 0.4% SDS. The HA band was excised and eluted in water at 4 °C. Cold ethanol (−20 °C) was then added, and the mixture was allowed to stand at −20 °C overnight. The HA protein was then precipitated by ultracentrifugation and redissolved in cold water. The purified HA contained no other viral proteins detectable by gel electrophoresis or ELISA assays.

Serologic assays

Hemagglutination inhibition (HI) and neuraminidase inhibition (NI) assays were used to test for the antibodies against human, swine and avian influenza viruses. The HI test was performed with serum samples treated with receptor-destroying enzyme (RDE) according to recommendations of the WHO [27]. Standard WHO procedures were also used with the NI test.

A modified ELISA method [14] was employed for detection of influenza virus antibodies of low titer, especially avian virus antibodies in humans and pigs. Briefly, purified HA stored frozen at −70 °C was diluted in carbonate buffer to a concentration of 2000 HAU/ml. One hundred μl of the solution was added to each well of flat-bottomed ELISA plates (Corning Glass Works, Corning, NY), which were incubated overnight at 4 °C. Reactions were stopped with superblock buffer (Pierce Chemical Co., Rockford, IL). Control wells received only carbonate buffer. A fresh frozen HA sample was used for each assay.

The same test was performed after addition of the following sequence of reagents: human serum samples or pig serum samples; goat antihuman IgG or rabbit antipig IgG conjugated with peroxidase (Sigma Chemical Company, St. Louis, MO), and finally ABTS substrate (Pierce Chemical Company, Rockford, IL). After each reagent was added, the plate was incubated at room temperature for 1 h; an exception was substrate, for which incubation was only 30 min. A microplate reader (Model 3550, Bio-Rad Laboratories, Richmond, CA) was used to measure the green chromogen produced by enzymatic cleavage of the substrate. Remaining optical diversity (OD) values were calculated by subtracting the OD value of the mean value for respective negative controls (samples in wells containing no antigen) from those of the test wells. ELISA titers were calculated from the remaining OD values.

Results

Seasonality of influenza virus infection

To determine the seasonality of virus infection, fifteen throat swab specimens were collected each week from pediatric patients showing respiratory symptoms. Two clusters of infection – one extending from November to March (11 isolates), and one from July to September (6 isolates) – were apparent (results not shown). The first outbreak was primarily associated with two strains of influenza B virus (B/Panama/45/90-like and B/Guangdong/05/94-like) with a single A/Beijing/32/92-like isolate. A-type strains were isolated most often in July and

August (A/Guangdong/25/93-like); a single A/Beijing/32/92-like isolate (H3N2) was recovered in February. Although the sampling size was small, the results indicated that influenza occurs in both winter and summer months in Central China.

Seasonal changes in antibody titers to human H3N2 influenza viruses supported the finding in children. Of 268 Nanchang slaughterhouse workers sampled in January, 1994 (35%) had detectable antibodies to H3N2, a prevalence that fell to 20% by June of 1994.

Antibodies to human influenza viruses

An important aspect of this study was to assess the exposure of populations living in or near Nanchang to commonly circulating or presumably extinct human viruses. The viral antigens selected for this comparative test included A/Texas/36/92 (H1N1), the most prevalent virus in human populations worldwide; A/Nanchang/3332/93, a previously isolated H3N2 virus; and A/Japan/305/57-Bel; an H2 virus which disappeared from humans in 1968. The results (Table 1) show remarkably similar rates of positivity among the study groups, regardless of the site where the virus was initially isolated. One exception was the lack of antibody to A/Japan/305/57-Bel (H2N1) in students. Since H2 is known to have disappeared from humans in 1968, it would not be expected to have infected persons younger than 27, including the vast majority of students. Occasional high titers of antibody to A/Japan/57-Bel were found only in persons older than 27 years. Overall, the profile of antibodies to human influenza viruses did not indicate different exposures to influenza A viruses among occupational groups (exposed or not exposed to pigs) in the Nanchang region, or between persons living in Nanchang and Memphis, TN.

Antibodies to swine and avian viruses

Three subtypes of influenza viruses – the classic swine H1N1, avian-like H1N1 and human-like H3N2 – are currently circulating in pigs in Europe [1]. We therefore tested the same four study groups for antibodies to swine viruses, using Sw/Beijing/47/91 (classic H1N1), Sw/Italy/786/92 (avian-like H1N1), and Sw/Italy/809/89 (human-like H3N2). As shown in Table 2, reactivity rates were uniformly high to Sw/Italy/809/89, which most closely resembles the human A/Port Chalmers/73 virus. Few subjects had detectable antibody to the classic swine and avian-like viruses; titers of 20 are at the lower limit of specificity and are of doubtful reliability. Since results for the human-like swine virus may reflect the triggering of antibody memory generated in response exposure to recent H3N2 strains, the serologic data do not indicate transmission of swine viruses to humans in Nanchang.

We also considered the possibility of direct transmission of avian viruses to humans. Thus, HI and NI assays, as well as an ELISA method, were used to screen serum samples from the Nanchang and Memphis populations. The highest reactivity rates to an H7 duck virus were found in women raising pigs

Table 1. Exposure of Nanchang populations to human influenza viruses

Group	No. of samples	Sampling time	No. (%) of positive samples and distribution of antibody titers				
			A/Texas/36/92 (H1N1)		A/Nanchang/3332/93 (H3N2)		A/Japan/305/57-Bel (H2N1)
			HI	NI	HI	NI	HI
Slaughter-house workers	268	Jan., 1994	85 (32%) 320 1 160 1 80 5 40 28 20 50	72 (27%) 80 12 40 18 20 42	93(35%) 160 4 80 8 40 26 20 55	117(44%) 80 32 40 39 20 46	ND[a]
	186	Jun., 1994	71(38%) 80 2 40 15 20 54	23 (12%) 80 1 40 5 20 17	37(20%) 160 1 80 1 40 9 20 26	17(9%) 80 2 40 4 20 11	55(30%) >160 1 80 7 40 14 20 33
Women raising pigs	216	Dec., 1993	49 (23%) 80 2 40 6 20 41	29 (13%) 80 4 40 7 20 18	81 (37%) >160 1 80 7 40 15 20 58	38 (18%) 80 5 40 11 20 22	50 (23%) 40 7 20 43
Student controls	205	Mar., 1994	37 (18%) 40 8 20 28	ND	93 (45%) 160 1 80 7 40 40 20 45	ND	0
Memphis controls	32	May, 1993	12 (38%) 80 2 40 2 20 8	5 (16%) 80 1 40 2 20 2	11 (34%) 80 1 40 5 20 5	8 (25%) 80 2 40 2 20 4	16 (53%) 80 1 40 4 20 11

[a] Not done

Table 2. Serum antibody responses of Nanchang populations to swine virus

Groups	No. of samples	Sampling date	No. (%) positive samples and distributions of antibody titers					
			Sw/BJ/47/91 (H1N1)		Sw/Ita/786/88 (H1N1)		Sw/Ita/809/89 (H3N2)	
			HI	NI	HI	NI	HI	NI
Slaughter-house workers	186	June, 1994	2 (1%) 20 2	0	3 (1.6%) 20 3	11 (6%) 80 1 40 4 20 6	151 (81%) >160 3 160 2 80 26 40 51 20 69	108 (58%) 80 19 40 35 20 54
Women raising pigs	191	May, June, August, 1994	6 (3%) 20 6	2 (1%) 20 2	0	6 (3%) 40 3 20 3	149 (76%) >160 4 160 3 80 22 40 43 20 77	138 (72%) 80 46 40 30 20 62
Student controls	205	March, 1994	3 (1.5%) 20 3	ND[a]	10 (5%) 40 1 20 9	ND	175 (85%) >160 39 80 60 40 42 20 34	ND
Memphis Controls	32	May, 1993	5 (16%) 80 1 40 2 20 2	2 (6%) 80 1 40 2	3 (9%) 80 1 40 1 20 1	2 (6%) 80 1 40 1	27 (84%) >160 4 80 6 40 10 20 7	27 (84%) 80 7 40 10 20 10

[a] Not done

(25%, with a maximum titer of 800); the remaining groups had low or negligible rates (Table 3). A 26% rate of positivity was demonstrated with an H11N2 virus and the NI assay in samples collected from Nanchang slaughterhouse workers and the Memphis controls. This result may reflect cross-reactivity with human N2 strains. Finally, reactivity with the N8 antigen of Nanchang/1681/93 was detected in two slaughterhouse workers. Since the virus used in this test was of the H3N8 subtype, we repeated the assay with a reassortant virus, possessing the HA of A/equine/Prague/1/56 and N8 NA (H7N8), as the antigen. The result confirmed reactivity in these human sera to N8 antibodies.

Antibodies to human, swine and avian viruses in slaughtered pigs

If pigs serve as hosts for genetic reassortment of human and avian influenza viruses [7, 15, 20], it should be possible to demonstrate mixed infection of these animals with human and avian viruses. Using ELISA, we failed to detect antibody as H7 duck virus in serum samples from 200 pigs slaughtered in Nanchang (Table 4). Reactivity to three swine viruses was either weak or absent altogether; however, three pigs out of 200 had both HI and NI antibodies to human H3N2 virus with titers greater than 40 suggesting limited interspecies transmission.

Discussion

The historical record and the appearance of the Asian, Hong Kong, and Russian pandemic strains of influenza virus in China this century suggest that the majority of pandemics of human influenza since 1850 have originated in China [21]. However, the reason has not been defined. Since pigs were postulated to be the "mixing vessels" for the transmission of viruses between humans and birds [19], we chose as subjects a population with close contact with pigs in Nanchang. We then conducted a serological investigation to find the relationship between influenza infection and exposure to pigs, and to describe the characteristics of influenza epidemiology in Nanchang.

According to a report from the WHO collaborating center for influenza [16], most virus isolation and outbreaks of disease in northern China occurred during winter months. In contrast, Shanghai, which is about midway between Beijing and Guangdong, often experiences two peaks of influenza activity each year. One occurs in summer-autumn and the other in winter-spring. The influenza isolation patterns in Guangdong of southern China indicate that viruses are isolated year around, with peak activity in summer. To determine the seasonality of human influenza epidemic in Nanchang, the isolation of influenza virus in a local children's hospital was monthly reviewed. We found a biannual pattern of influenza peak season in Nanchang, which is similar to the isolation pattern in Shanghai which is about five hundred miles away from Nanchang. In addition, we analyzed the incidences of the antibodies to human viruses in two bleedings at different time of the same slaughterhouse workers. We noticed that the number of seropositives and the titers of antibodies to human H3N2 virus were decreased

Table 3. Serum antibody responses of Nanchang populations to avian viruses

Groups	No. of samples	Sampling time	No. of positive (percentage) and distribution of antibody titers								
			D/Nanchang/1681/93 (H3N8)		D/Nanchang/1749/93 (H11N2)		D/Nanchang/1904/93 (H7N4)		D/Nanchang/1941/93 (H4N4)		D/Nanchang/1904/93-Bel (Purified H7)
			HI	NI	HI	NI	HI	NI	HI	NI	ELISA[a]
Slaughter-house workers	268	January, 1994	18(7%) 4 0 3 20 15	2(1%) > 8 0 2	0	70(26%) 80 9 40 19 20 42	0	2(1%) 80 1 40 1	0	0	12(5%) 400 9 800 3
Women raising pigs	100	December, 1993	ND[b]	1(0.5%) 20 1	ND	ND	ND	3(3%) 20 3	ND	0	25(25%) 400 21 800 4
Student controls	100	March, 1994	ND	0	ND	ND	ND	0		0	6(6%) 400 6
Memphis controls	32	May, 1994	7(19%) 40 2 20 5	0	0	18(56%) 80 3 40 7 20 8	0	0	0	ND	1(3%) 400 1

[a] ELISA titers were calculated by subtracting the OD value of respective negative controls from those of test wells. Remaining OD values ranging from 0.200–0.299 were regarded as a 400 ELISA titer; those from 0.300–0.399 were regarded as an ELISA titer of 800

[b] Not done

Table 4. Serum antibody responses of slaughtered pigs in Nanchang to human, swine and avian viruses

No. of samples	Sampling time	No. (%) positive samples and distribution of antibody titers										No. of positive samples
		human viruses				swine viruses						avian virus
		A/Nanchang/ 3332/93 (H3N2)		A/Texas/36/91 (H1N1)		Sw/Beijing/47/91 (H1N1)		Sw/Italy/786/88 (H1N1)		Sw/Italy/809/89 (H3N2)		D/Nanchang/ 1904/93-Bel (Purified H7)
		HI	NI	HI	NI	HI	NI	HI	NI	HI	NI	ELISA
200	April, 1994	10(5%) 80 1 40 2 20 7	4(2%) 80 1 40 1 20 2	20(10%) 40 1 20 19	0	0	0	0	1(0.5%) 40 1	1(0.5%) 20 1	1(0.5%) 20 1	0

in June compared with those in January. This may only reflect the first peak season of human influenza in winter in Nanchang.

If China is in fact an epicenter for the generation of highly virulent strains of influenza, one might predict a quite different profile of serum antibodies to human influenza viruses in Nanchang populations compared to those of Western countries. This difference should be especially pronounced in groups having daily contact with known animal carriers of human influenza viruses (pigs, for example). Our HI and NI assay results indicate remarkably similar patterns of reactivity to three human influenza viruses in Nanchang populations and the control group in Memphis, TN. The lack of antibodies to an H2 in a group of students indicates that the Asian H2N2 pandemic virus, which appeared suddenly in 1957 and persisted until 1968, has not re-emerged in this region of China.

Since the early 1970s, at least a dozen individual cases or outbreaks of human influenza caused by swine influenza virus have been recognized in North America and Europe [21]. Oddly, there are no reports of human infections by swine viruses in China. Since two of our study groups were in frequent contact with pigs, they were likely candidates for pig-to-human transmission of virus. However, rates of reactivity and antibody titers with characteristic swine viruses were essentially the same in slaughterhouse workers and pig farmers as in students who were not exposed to pigs.

Studies have shown that avian influenza viruses can cross species barriers to infect pigs [20], horses [4], and sea mammals [8]. Claas et al. (1994) also reported the infection of children with an avian-human reassortant from European pigs. These findings raise a central question: Are avian influenza viruses able to infect humans directly? In Southern China, domestic farmers often raise ducks as an adjunct to rice growing, creating numerous opportunities for human-duck contact. The likelihood of human exposure to virus-containing duck feces is perhaps greater in Southern China than anywhere else in the world.

Shortridge [21], using a single-radial hemolysis test to screen 950 serum samples from southeastern Chian, found presumptive serologic evidence for human exposure to avian influenza viruses. Since avian viruses may not provoke a strong immune response in humans, conventional methods of antibody detection such as the HI tests are unreliable. Thus, to test serum samples in the present study, we relied on the ELISA method to avoid false-positive results.With this assay, the proportions of subjects showing positive reactions to the H7 antigen of duck viruses isolated in Nanchang were consistent with the frequency of their contacts with ducks. The highest positivity rates were found in samples from women who raised pigs and ducks in their homes, and who spent considerable time in rice fields. Slaughterhouse workers who lived in suburban areas of Nanchang, where numerous duck farms are located, had the second highest rates, followed by university students and Memphis controls, neither of whom had contact with living ducks. The NI assay yielded evidence of antibodies to N4 and N8 avian subtypes. Taken together, these findings support the theory of direct avian-to-human transmission of influenza viruses.

The 1918 human pandemic virus is believed to be derived from avian sources that crossed host barriers, infected pigs and humans and has remained in swine populations until recent times [7]. The H3N2 swine virus, derived from the 1968 human pandemic strain, was passed to pigs presumably through contact with infected herdsmen [12, 20]. Testing for antibodies to both human influenza viruses and swine influenza viruses in slaughtered pigs in Nanchang, we found human virus antibodies in Nanchang pigs, but failed to detect antibody to swine virus. This suggests that commonly circulating swine viruses are not present in Nanchang pigs. Because swine husbandry in Nanchang is usually a family business, the size of each pig farm is relatively small and farms are usually isolated from each other; perhaps explaining the apparent low incidence of antibody to swine influenza virus in pigs and in Nanchang residents having contact with pigs.

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