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Potential for transmission of avian influenza viruses to pigs

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Pandemic strains of influenza A virus arise by genetic reassortment between avian and human viruses. Pigs have been suggested to generate such reassortants as intermediate hosts. In order for pigs to serve as 'mixing vessels' in genetic reassortment events, they must be susceptible to both human and avian influenza viruses. The ability of avian influenza viruses to replicate in pigs, however, has not been examined comprehensively. In this study, we assessed the growth potential of 42 strains of influenza virus in pigs. Of these, 38 were avian strains, including 27 with non-human-type haemagglutinins (HA; H4 to H13). At least one strain of each HA subtype replicated in the respiratory tract of pigs for 5 to 7 days to a level equivalent to that of swine and human viruses. These results indicate that avian influenza viruses with or without non-human-type HAs can be transmitted to pigs, thus raising the possibility of introduction of their genes into humans. Sera from pigs infected with avian viruses showed high titres of antibodies in ELISA and neutralization tests, but did not inhibit haemagglutination of homologous viruses, cautioning against the use of haemagglutination-inhibition tests to identify pigs infected with avian influenza viruses. Co-infection of pigs with a swine virus and with an avian virus unable to replicate in this animal generated reassortant viruses, whose polymerase and HA genes were entirely of avian origin, that could be passaged in pigs. This finding indicates that even avian viruses that do not replicate in pigs can contribute genes in the generation of reassortants.

Introduction

The first isolation of a H1N1 human influenza virus occurred in 1933 and since then new subtypes of pandemic human type A influenza viruses have appeared in 1957 (H2N2 Asian virus), in 1968 (H3N2 Hong Kong virus) and in 1977 (reappearance of H1N1 strain). Serological and virological evidence suggests that since 1889 there have been six instances of the introduction of a virus bearing a haemagglutinin (HA) subtype that differs from the previously circulating strains. For HA, there has been a cyclical appearance of three human subtypes, with the sequential emergence of H2 viruses in 1890, H3 in 1900, H1 in 1918, H2 again in 1957, H3 in 1968 and H1 in 1977 (Murphy & Webster, 1990). Thus, pandemic human influenza A viruses have so far been limited to the H1, H2 and H3 subtypes.

The H2N2 Asian/57 and H3N2 Hong Kong/68 human pandemic influenza viruses are genetic reassortants between human and avian viruses (Webster & Laver, 1972; Scholtissek *et al.*, 1978; Kawaoka *et al.*, 1989), raising a pivotal question: how and where could such genetic reassortment occur? Avian influenza virus genes might be introduced into human viruses by direct transmission or possibly by transmission through an intermediate host susceptible to both human and avian viruses. There is ample evidence, both *in vivo* and *in vitro*, to support genetic reassortment between human and animal influenza A viruses. 'New' influenza viruses were produced in pigs after mixed infection with human and swine viruses (Webster *et al.*, 1971, 1973; Webster & Campbell, 1972). Genetic reassortants have also been found in nature in pigs (Sugimura *et al.*, 1980; Castrucci *et al.*, 1993) and in humans (Cox *et al.*, 1983; Guo *et al.*, 1992). Indeed, pigs afford many features that would favour the emergence of new pandemic influenza virus strains through genetic reassortment, leading Scholtissek *et al.* (1985) to propose a swine 'mixing vessel' hypothesis.

For pigs to serve as intermediate hosts in genetic reassortment events, they should ideally support replication of both human and avian influenza viruses.

There is compelling evidence for the transmission of human viruses to pigs (Kundin, 1970; Mancini, 1985; Tumova *et al.*, 1980; Wibberley *et al.*, 1988) but the information on avian influenza viruses is less comprehensive, although H1N1 viruses in European pigs have possibly originated from birds (Scholtissek *et al.*, 1983). The available evidence indicates that pigs support replication of some avian influenza viruses (Hinshaw *et al.*, 1981) but the tests to date have been limited to only a few subtypes. To assess the potential for avian-to-pig transmission, we tested the growth of a large number of avian influenza viruses, including those with non-human-type HAs, in swine hosts.

Methods

Viruses. The viruses used in this study are listed in Tables 1, 2 and 3. They were propagated in the allantoic cavities of 11-day-old embryonated chicken eggs at 35 °C for 2 days.

Experimental infection of pigs with influenza viruses. Thirty- to 40-day-old pigs, an F1 cross between the Landrace and Durock breeds, were purchased and quarantined for at least 10 days. Their sera were checked for the absence of antibodies to influenza A virus by ELISA using disrupted Aichi/2/68 (H3N2) or Sw/Hok/2/81 (H1N1) strains as antigens. The pigs were used at 50 to 80 days of age. Each virus was diluted in PBS and 0·5 ml [approximately 10^7 50 % egg infectious doses (EID_{50}) or 10^5 to 10^7 p.f.u.] was dropped into each of the pig's nostrils. Two to four pigs were tested with each virus. Each group of pigs was kept in a separate pen. Nasal swabs were taken once a day for 7 days. In experiments to examine the site of replication of viruses in pigs, tracheal swabs, blood and faecal samples as well as nasal swabs were taken twice a day for 14 days. Each of these specimens was put into 1 ml of broth containing 10000 units of penicillin and 10 mg of streptomycin. Each sample (0·1 ml) was inoculated into allantoic cavities of two to four 11-day-old embryonated eggs. To determine the infectivity titres, each of the 10-fold serial dilutions of the sample was inoculated into four embryonated chicken eggs or onto Madin-Darby canine kidney (MDCK) cell monolayers. For plaque formation on MDCK cells, 5 to 10 μg/ml of trypsin (1:250; Gibco) was added into the agar overlay (Tobita *et al.*, 1975).

Serological tests. Haemagglutination-inhibition (HI) tests were performed according to Webster & Laver (1967); ELISA was carried out according to Kida *et al.* (1982). Neutralization of infectivity of viruses was determined in embryonated eggs using 10^4 EID_{50} of the viruses and by plaque inhibition on MDCK cells using 10^2 p.f.u. of the viruses (Kida *et al.*, 1982).

Identification of the gene segments of reassortants. The dideoxynucleotide chain termination procedure of Sanger *et al.* (1977) was used to determine the origin of gene segments in reassortants. The primers used were PA-TM149 (TGGAAGTCTGTTTCATGT), PB1-1489 (ACATTTGAATTCACAAG), PB2-1 (AAAGCAGGTCAA), NP-213 (GATCCAGAACAG), NP-1429 (GGAGTCTTCGAG), M-WSN8 (GCAAGGTAGATATT) and NS-540 (GAGGATGTCAA-AAAT).

Results

Replication of avian influenza viruses in pigs

To determine the time points for maximum virus shedding in pigs, we first infected two pigs with strain Sw/Hok/2/81 (H1N1) or Aichi/2/68 (H3N2), intranasally. Samples were taken from the nose, trachea, blood and faeces, twice a day, at 9:00 a.m. and at 5:00 p.m., and were injected into the allantoic cavities of four eggs before titration of infectivity. Each virus was recovered from the nasal swabs of both pigs of the respective groups for 6 to 7 days and also detected to a lesser extent in the tracheal swabs. Virus was never isolated in the blood or faecal samples. Maximum virus titres in the nasal swabs were 10^3 to 10^5 EID_{50}/ml of swab suspension on days 2 to 4 post-inoculation (p.i.). A serum antibody response of these pigs to the inoculated viruses was clearly demonstrated by ELISA, neutralization and HI tests on day 14 p.i. The results are summarized in Table 1 with infectivity titres per ml of the morning samples on days 3 and 4 p.i. None of these pigs showed any clinical signs of infection during the 14 day observation period or had virus-induced lesions on necropsy. These results indicate that cofactor(s) may be involved in the manifestation of disease signs of pig influenza, in addition to virus infection.

We then examined the replication of influenza viruses of all HA subtypes except H1 and H14. On the basis of the above results, virus replication was monitored by inoculation into embryonated chicken eggs with nasal swab suspensions taken every day for 14 days after infection and determining the virus titres of day 3 and 4 p.i. samples. Of 40 strains tested, 33 strains replicated in pigs (Table 1). At least one strain of each HA subtype was represented in this total. Virus was recovered from pigs for 4 to 7 days p.i. Approximately one-third of the avian viruses replicated to a similar level as swine and human viruses (10^4 to 10^5 EID_{50}/ml). None of the infected pigs had clinical signs of disease or virus-induced lesions upon necropsy.

The receptor specificity of HA differs widely among influenza viruses. Most avian influenza viruses preferentially bind the *N*-acetylneuraminic acid-α-2,3-galactose (NeuAcα2,3Gal) linkage on cell surface sialyloligosaccharides, whereas human and swine influenza viruses preferentially bind the NeuAcα2,6Gal linkage (Rogers & Paulson, 1983). To determine whether the receptor specificity of HA plays a role in restriction of virus replication in pigs, we infected pigs with a variant of strain Aichi/2/68 (VIR/Aichi/2/68), which is resistant to horse serum inhibitor, contains a single amino acid change at residue Leu-226 (changed to glutamine) and binds the NeuAcα2,3Gal linkage (Rogers *et al.*, 1983). The inhibitor-resistant virus replicated to a level similar to that of the parent Aichi/2/68 virus (Table 1), suggesting that the difference in receptor specificity (NeuAcα2,3Gal compared with NeuAcα2,6Gal) does not constitute a major obstacle to virus replication in pigs.

Table 1. *Susceptibility of pigs to the H1 to H13 influenza viruses*

Virus strain	Virus titre p.i. per ml nasal swab*		Duration of virus shedding (days)	Antibody titre in serum†			Virus strain	Virus titre p.i. per ml nasal swab*		Duration of virus shedding (days)	Antibody titre in serum†		
	Day 3	Day 4		ELISA	NT	HI		Day 3	Day 4		ELISA	NT	HI
Sw/Hok/2/81 (H1N1)	3·0	3·5	7	40960	384	128	Dk/HK/342/78 (H5N2)	–	–	0	–	–	–
	4·0	3·0	6	20480	128	128		–	–	0	–	–	–
Aichi/2/68 (H3N2)	4·0	5·0	6	10240	384	256	Dk/HK/820/80 (H5N3)	3·0	4·0	5	5120	32	–
	2·0	3·3	6	20480	64	128		5·5	4·0	4	5120	32	–
Dk/HK/273/78 (H2N2)	–	–	0	–	–	–	Dk/HK/825/80 (H5N3)	–	–	0	–	–	–
	–	–	0	–	–	–		–	–	0	–	–	–
Dk/HK/278/78 (H2N9)	3·0	2·0	4	2560	16	–	Dk/HK/716/79 (H6N1)	–	–	0	–	–	–
	3·3	2·1	5	5120	32	–		–	–	0	–	–	–
Dk/TW/1010/86 (H2N8)	2·3	2·5	5	2560	24	–	Dk/HK/960/80 (H6N2)	2·0	5·3	5	10240	16	–
	2·8	2·0	4	2560	24	–	Dk/HK/959/80 (H6N2)	2·0	3·5	4	20480	48	–
VIR/Aichi/2/68 (H3N2)	2·0	3·3	6	40960	64	320	Dk/HK/301/78 (H7N1)	2·0	2·3	4	10240	32	–
	2·0	2·0	6	10240	32	160		2·3	2·0	4	20480	64	–
Sw/Hok/10/85 (H3N2)	3·0	5·0	6	12800	512	640	Dk/HK/293/78 (H7N2)	2·0	2·0	4	10240	64	–
Dk/HK/7/75 (H3N2)	2·3	4·0	7	40960	32	–		2·0	2·0	6	5120	16	–
	3·0	4·3	7	20480	32	–	Dk/TN/1/76 (H7N3)	5·5	4·0	5	40960	48	–
Gs/HK/10/76 (H3N2)	2·0	2·3	7	20480	24	–		2·0	2·5	4	10240	16	–
	2·0	2·0	4	20480	32	–	Ty/TX/1/79 (H7N3)	2·0	2·5	4	10240	96	–
Dk/HK/24/76 (H3N2)	–	–	0	–	–	–		5·5	3·5	5	40960	64	–
	–	–	0	–	–	–	Ty/OT/6118/68 (H8N4)	4·5	5·5	7	40960	64	–
Dk/HK/64/76 (H3N2)	2·0	2·0	4	10240	24	–		2·0	2·5	6	81920	128	–
	2·0	2·0	4	20480	24	–		3·3	2·0	5	40960	96	–
Dk/HK/231/77 (H3N2)	2·3	3·0	7	40960	64	–	Dk/HK/448/78 (H9N2)	3·0	4·0	5	10240	16	–
	2·0	2·0	5	20480	32	–		2·0	3·5	4	10240	32	–
Dk/HK/526/79 (H3N6)	–	–	0	–	–	–	Dk/HK/702/79 (H9N5)	3·0	4·0	7	20480	24	–
	–	–	0	–	–	–		2·0	4·0	6	10240	16	–
Dk/Hok/8/80 (H3N8)	–	–	0	–	–	–	Dk/HK/938/80 (H10N1)	5·0	2·0	4	20480	32	–
	–	–	0	–	–	–		2·0	3·0	5	10240	32	–
Dk/Hok/7/82 (H3N8)	–	–	0	–	–	–	Dk/HK/873/80 (H10N3)	4·0	3·0	4	10240	96	–
	–	–	0	–	–	–		4·0	5·0	6	20480	128	–
Dk/HK/668/79 (H4N5)	3·3	5·0	5	2560	32	–	Dk/HK/560/79 (H10N8)	2·0	2·0	4	10240	64	–
	2·5	4·3	5	2560	32	–		2·0	2·0	4	5120	16	–
Dk/HK/365/78 (H4N6)	3·8	4·0	4	2560	512	–	Dk/HK/562/79 (H10N9)	5·0	4·3	5	10240	64	–
	2·7	3·5	5	2560	96	–		6·0	4·8	5	10240	96	–
Dk/HK/980/81 (H4N6)	–	–	0	–	–	–	Dk/HK/272/78 (H11N9)	2·0	2·3	4	10240	16	–
	–	–	0	–	–	–		2·0	3·3	5	20480	32	–
Dk/HK/438/78 (H4N8)	2·4	2·3	4	1280	16	–	Dk/HK/563/79 (H11N9)	2·0	3·3	4	40960	64	–
	3·3	4·0	7	1280	32	–	Dk/HK/838/80 (H12N5)	2·0	2·3	4	51200	32	–
Dk/HK/835/80 (H4N9)	2·6	3·3	6	1280	16	–		2·3	2·0	5	51200	32	–
	3·3	4·0	7	1280	32	–	Gull/MD/704/77 (H13N6)	3·3	2·0	4	10240	32	–
Dk/PN/10128/83 (H5N2)	4·3	3·3	5	10240	32	–		2·0	3·5	5	10240	32	–
	5·5	3·0	5	10240	32	–							

* Virus titres are infectivity per ml of virus-positive samples and are expressed as $\log_{10}$ EID_{50}. Dashes (–) indicate negative findings in either sample. Samples collected from blood and faeces were uniformly free of virus. Each result represents the outcome of inoculation in a single pig.

† Serum antibodies were titrated 14 days p.i. by ELISA, neutralization test (NT) and HI test with the homologous viruses. Titres below 40, 2 and 8, respectively, were considered negative.

Sera from pigs infected with avian viruses had high antibody titres in ELISA (1:1280 to 1:40960; preinfection sera titres < 1:40) and neutralization tests (1:16 to 1:512) with the homologous strains. These sera, however, did not cause complete inhibition of haemagglutination of the homologous strains, although partial HI patterns were observed in some cases (Table 1). The lack of HI activity of sera from pigs infected with avian viruses contrasts with the results obtained from pigs infected with H1N1 swine or H3N2 human viruses. To examine whether this disparity stems from structural differences in the viral HA, we studied sera from pigs infected with H3 avian viruses for HI activity with a human virus, strain Aichi/2/68. The sera showed HI titres of 1:160 to 1:640, suggesting that the structure of avian HA molecules does differ from that of human and swine viruses.

These results demonstrate the potential of avian influenza viruses, including those with non-human-type HAs (H4 to H13), to be transmitted to pigs. They could therefore participate in genetic reassortment events with human viruses. The lack of HI activity of sera from pigs

Table 2. *Serological results from recovered virus clones of a pig inoculated concurrently with Dk/8/80 (H3N8) and Sw/2/81 (H1N1) influenza viruses*

Time (days p.i.)	Virus (p.f.u./ml) from nasal swab	Number of plaques examined	Number of recovered virus clones identified as			
			H3N8	H3N1	H1N8	H1N1
1	19500	12	6*	0	0	6
2	550	12	0	0	0	12
3	1785	11	8*	0	0	3
4	16500	11	0	2*	0	9

* Two clones of each subtype were studied for their genetic origins (see Table 3).

infected with avian viruses cautions against the use of HI tests for seroepidemiological surveys of avian virus infection in pigs.

Mixed infection of a pig with avian and swine influenza viruses

It was determined that nine out of the 38 avian influenza viruses did not replicate in pigs, since no serum antibody response of these pigs to the inoculated viruses was detected by any of the three different tests and no virus was recovered from the animals. It was also confirmed that Dk/Hok/8/80 (H3N8) did not replicate in an additional four pigs (data not shown). In order to test whether the genes of such non-replicating avian viruses could be rescued upon co-infection of pigs with a swine virus, non-replicating strain Dk/Hok/8/80 was inoculated simultaneously with the swine strain Sw/Hok/2/81 (H1N1) into a pig, intranasally. Virus was recovered from the nasal swabs of the pig for 6 days and directly assayed by plaque formation in MDCK cells. Some viruses were then subtyped (Table 2). When we determined the genotype of these isolates by partial sequencing, all eight gene segments of H3N8 isolates tested were from the avian virus (Table 3). These viruses, however, did not replicate upon re-inoculation into seronegative pigs, indicating that Dk/Hok/8/80 virus requires Sw/Hok/2/81 as a helper virus for replication in pigs. In addition to the neuraminidase (NA) gene, two of the recovered reassortant H3N1 viruses contained the nucleoprotein (NP) and matrix protein (M) or non-structural protein (NS) genes of the swine virus. These reassortants replicated upon re-inoculation into seronegative pigs and could be passaged in pigs. Thus, the avian viruses unable to replicate in pigs could contribute genes for reassortments. The results also suggest that the NP, NA and M or NS genes may contribute to the restriction of replication of Dk/Hok/8/80 virus in pigs.

Discussion

Pigs have been suggested to serve as intermediate hosts for the generation of human pandemic strains of influenza virus (Webster *et al.*, 1971, 1973; Webster & Campbell, 1972; Scholtissek *et al.*, 1985; Kida *et al.*, 1988; Yasuda *et al.*, 1991). This hypothesis is supported by interspecies transmission of virus from birds and humans to pigs and from pigs to humans (Pensaert *et al.*, 1981; Ottis *et al.*, 1982), and by genetic reassortment between human and avian viruses in pigs in nature (Castrucci *et al.*, 1993). Despite these observations, the extent of susceptibility of pigs to avian influenza viruses remains in question. Here we demonstrate the replicative capacity of avian influenza viruses in pigs, including those with HA subtypes found in humans. The duration and level of replication of at least one-third of the avian viruses were similar to results for swine viruses suggesting, despite the lack of direct evidence, that a subset of avian viruses can be directly introduced and perhaps even maintained in pigs. Moreover, even the avian viruses that did not replicate in pigs contributed genes to

Table 3. *Genome segment origin and replication of virus clones recovered from a co-infected pig*

Virus clone*	Genome segment origin†								Replication in pigs
	PB2‡	PB1‡	PA‡	HA	NP	NA	M	NS	
3 (H3N8)	D	D	D	D	D	D	D	D	–
4 (H3N8)	D	D	D	D	D	D	D	D	–
10 (H3N8)	D	D	D	D	D	D	D	D	–
17 (H3N8)	D	D	D	D	D	D	D	D	–
6 (H3N1)	D	D	D	D	S	S	S	D	+
7 (H3N1)	D	D	D	D	S	S	D	S	+
Dk/Hok/8/80 (H3N8)	D	D	D	D	D	D	D	D	–
Sw/Hok/2/81 (H1N1)	S	S	S	S	S	S	S	S	+

* Clones correspond to those noted in Table 2.

† D indicates the gene segment was derived from Dk/Hok/8/80 (H3N8) and S indicates it was derived from Sw/Hok/2/81 (H1N1). The origin of the segments was determined by partial sequencing of each gene segment.

‡ Polymerase genes.

reassortant viruses. Thus, the present findings are consistent with the proposed role of pigs as permanent or intermediate hosts in genetic reassortment events. The present results, on the other hand, also showed that a quarter of the avian viruses tested did not replicate in pigs at all.

The influenza virus genes responsible for restriction of host range have been studied only in birds and squirrel monkeys. The HA and NA genes restrict replication of a human virus in ducks (Hinshaw *et al.*, 1983), whereas the NP and M genes are responsible for the restriction of avian viruses in squirrel monkeys (Murphy *et al.*, 1982). In this study, we showed that NA, NP and M or NS genes restrict the replication of an avian (duck) virus Dk/Hok/8/80 in pigs. Because the NP gene of some swine viruses rescued infectivity of a temperature-sensitive avian virus, it was suggested to be 'a possible major factor in determining the host specificity of influenza H3N2 viruses' (Scholtissek *et al.*, 1985). However, the importance of the NP gene in restricting virus replication in pigs had not been defined consistently before this study. In addition, the lack of isolation of H1N8 reassortant viruses upon co-infection with the H3N8 avian and H1N1 swine viruses may indicate the importance of the NA gene in host range restriction, although this result might also be explained by incompatibility between the HA and NA.

Despite the difference in receptor specificity between human, swine and avian viruses, pigs supported both the growth of two-thirds of the avian viruses tested, and that of a horse serum inhibitor-resistant human virus, which preferentially bound the NeuAcα2,3Gal linkage (Rogers *et al.*, 1983). Baum & Paulson (1990) reported the presence of NeuAcα2,6Gal but not NeuAcα2,3Gal sialyloligosaccharides on the surface of epithelial cells of human trachea, which corresponds to the receptor specificity of human influenza viruses, thus supporting the idea that receptor specificity plays a role in determining the host range restriction of influenza viruses in humans. Our results, therefore, suggest that, unlike epithelial cells of human trachea, such cells in the upper respiratory tract of pigs may contain both NeuAcα2,6Gal and NeuAcα2,3Gal sialyloligosaccharides. It has, in fact, recently been shown that the cells lining the upper respiratory tract of pigs possess both receptor specificities (H. Kida *et al.*, unpublished results).

At least one avian strain from each HA subtype productively replicated in pigs. The virus titres in the nasal swabs were high (more than 10^4 EID_{50}/ml) for the majority of the virus strains with the HA subtypes H4 to H10, which have not been detected in humans. These results suggest that avian viruses with such non-human-type HAs have the potential to be introduced into pigs, and hence into humans. However, only H1 and H3 viruses have been found in pigs so far. This may indicate that additional factors are involved in establishing avian viruses in the pig population or that the appearance of such viruses may be only a function of time.

None of the sera from pigs infected with avian influenza viruses inhibit haemagglutination of the homologous strains, even though they showed high titres in ELISA and neutralization assays. Such sera, however, did inhibit haemagglutination of a human virus of the same subtype. Similar findings were previously reported (Kida *et al.*, 1982; Yoden *et al.*, 1982). Such discrepancy between the virus HAs suggests that they share common epitopes but that the tertiary structures may differ, since one or two carbohydrate attachment sites are missing on the H3 HA molecule of influenza virus strains of avian origin compared to those of human origin (Kida *et al.*, 1987; Yasuda *et al.*, 1991). The lack of HI activity of sera from pigs infected with avian influenza viruses suggests that seroepidemiological surveys with HI tests could fail to detect infection with avian influenza viruses in some mammalian hosts.

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