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Characterization of Avian H5N1 Influenza Viruses from Poultry in Hong Kong

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The transmission of avian H5N1 influenza viruses to 18 humans in Hong Kong in 1997 with six deaths established that avian influenza viruses can transmit to and cause lethal infection in humans. This report characterizes the antigenic and biological properties of the H5N1 influenza viruses isolated from chickens, ducks, and geese from farms and poultry markets in Hong Kong during 1997 and compares them with those of virus isolated from the index human case. Each of the H5N1 viruses from Hong Kong poultry markets that were tested were lethal in chickens, possessed polybasic amino acids at the carboxy-terminus of HA1, and by definition were highly pathogenic in poultry. The available nonpathogenic H5 influenza viruses and the pathogenic H5N1 virus from Hong Kong were analyzed with monoclonal antibodies prepared to A/chicken/Pennsylvania/1370/83 (H5N2). The analysis revealed limited antigenic drift in 15 years and established that monoclonal antibodies are useful reagents for identification and antigenic analysis of avian strains that may transmit to humans in the future. One of the monoclonal antibodies permitted separation of the H5N1 influenza viruses from poultry into two groups that correlated with the presence or absence of a carbohydrate at residue 158 adjacent to the receptor binding site on HA. The H5N1 viruses examined replicated in geese, pigs, rats, and mice, but to only a very limited extent in ducks. It is noteworthy that all infected geese shed virus and that the H5N1 viruses caused disease signs and death in a portion (3 of 16) of the geese, with evidence of systemic spread to the brain. The tropism for geese is unusual and may provide insight into the origin of these viruses. In mice, the H5N1 virus caused lethal pneumonia and spread systemically to the brain. Mice would thus provide an ideal model system for studying immune responses and pathogenesis. Transmission experiments in chickens revealed that the H5N1 viruses are spread by fecal–oral transmission rather than by aerosol, and that the viruses are inactivated by drying of feces at ambient temperature. However, infectivity is maintained for at least 4 days in wet feces at 25°C. There were differences in the morphology of the H5N1 viruses isolated from birds and humans. The perpetuation of H5N1 influenza viruses in the poultry markets in Hong Kong and the transmission of these viruses to humans emphasize the importance of these markets in the epidemiology of influenza. The poultry markets are of critical importance in the perpetuation and transmission of influenza viruses to other avian species and to mammals, including humans.

INTRODUCTION

In late March and early May 1997, an H5N1 influenza virus caused high mortality on three chicken farms in the New Territories, Hong Kong SAR, China. Approximately 75% mortality occurred on the three farms, with a loss of over 6500 chickens. Also in early May, a descendant of the H5N1 virus contracted by a young child caused fatal viral pneumonia with severe complications. This was the first reported avian influenza to cause clinical respiratory illness in humans (de Jong *et al.*, 1997; Claas *et al.*, 1998; Subarrao *et al.*, 1998). Following a lag phase of about 6 months, a second "wave" of infection in humans in November and December 1997 caused 17 additional cases and 5 deaths. The ages of the 18 patients ranged from 1 to 60 years. The clinical features of the first 12 cases, described by Yuen *et al.* (1998), included an onset typical of classical influenza, with fever and upper respiratory tract infection. However, a high percentage of patients (7 of 12 cases) had severe complications with pneumonia; gastrointestinal manifestations, elevated liver enzymes, and renal failure were also usually prominent. The authors noted that except for the index case, all of the children infected fared better than the adults; the children under 13 years had uneventful recoveries, whereas all 7 of the older patients had severe disease and four died.

By early December 1997, there was great concern that an incipient pandemic of influenza was emerging in Hong Kong. Human influenza cases were occurring with

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apparently random distribution, and the viruses isolated were avian H5N1 strains. Because it was urgent to determine the source of the viruses, an international task force of virologists was assembled for surveillance of the poultry markets. Influenza H5N1 was isolated from approximately 20% of fecal samples from chickens and from approximately 2% of fecal samples from ducks and geese. It became apparent that poultry markets were the source of H5N1 influenza viruses, setting the stage for the decision to depopulate Hong Kong's poultry.

Prior to this outbreak, H5 influenza viruses had been isolated only from avian species. The H5 influenza viruses are perpetuated in a nonpathogenic form in wild aquatic birds in different regions of the world and in domestic ducks in Southern China (Hinshaw *et al.,* 1980; Sharp *et al.,* 1993; Shortridge, 1992; Süss *et al.,* 1994). Only the A/tern/South Africa/61 (H5N3) influenza virus has been associated with mortality in its natural host (Becker, 1966). Highly pathogenic, H5 influenza viruses previously isolated from lethal outbreaks in domestic poultry (i.e., chickens and turkeys) include A/chicken/Scotland/59 (H5N1) (Pereira *et al.,* 1965), A/turkey/Ontario/7732/66 (H5N9) (Narayan *et al.,* 1969), A/chicken/Pennsylvania/1370/83 (H5N2) (Bean *et al.,* 1985), and A/chicken/Queretaro/14588/95 (H5N2) (Horimoto *et al.,* 1995). Available evidence indicates that the highly pathogenic avian H5, as well as the H7 strains, evolve from nonpathogenic precursors (Röhm *et al.,* 1995). Although previous studies found no evidence that A/chicken/Pennsylvania/1370/83 (H5N2) virus had been transmitted to poultry workers (Bean *et al.,* 1985), more recent studies in southern China indicated low levels of antibodies to all avian influenza virus subtypes tested in the rural human population (Shortridge, 1992).

The present study characterizes H5N1 influenza viruses isolated from domestic poultry and from the market environment in Hong Kong during the second wave of H5N1 human influenza in November and December 1997. The H5N1 influenza viruses initially isolated from chickens in March and May 1997 were biologically characterized by antigenic analysis with monoclonal antibodies and polyclonal sera, measurement of their stability at environmental temperatures, and assessment of their abilities to replicate in ducks, geese, pigs, rats, and mice under experimental conditions. The studies established that there were two antigenically distinguishable subgroups of H5N1 influenza virus circulating in poultry in Hong Kong. Both groups were lethal for chickens and replicated in experimental animals, but failed to transmit between mammals.

RESULTS

Pathogenicity of H5N1 influenza viruses isolated from poultry in Hong Kong

One of the initial requirements after an influenza virus transmits to humans is to find a suitable vaccine strain. There was an urgent need to isolate nonpathogenic H5N1 viruses that could serve as a surrogate vaccine strain. The pathogenicity of isolates from poultry in Hong Kong was assessed by inoculation into chicken embryos and sequencing of the connecting peptide of the HA. All of the viruses examined killed 10- to 12-day-old chicken embryos and the chicken embryo infectious dose corresponded to the chicken embryo lethal dose (EID_{50}–ELD_{50}). Viruses isolated from chickens ($N = 39$), ducks ($N = 7$), and geese ($N = 2$) were inoculated either intravenously or intranasally into chickens. After intravenous injection, some viruses killed the inoculated birds within 16 h (range, 16–4 days; results not shown). After inoculation into the nares, the time to death was longer (2 to 3 days; Table 1). The lethal chicken dose (CLD_{50}) of human isolate (HK156-97) and the initial chicken isolate (CHK258-97) was 0.5 egg infectious doses (EID_{50}). Chickens inoculated with either isolate developed disease signs typical of highly pathogenic avian influenza, including swelling of the head and leg joints, generalized hemorrhage, loss of ability to stand, and general paralysis. Each of the chicken, duck, and geese isolates studied were lethal for chickens and showed similar pathological features to the index human (HK156-97) and chicken viruses (CHK220-97; CHK258-97) that have been described (Suarez *et al.,* 1998). Sequences through the connecting peptide region of the HA of each of the chicken, duck, and goose isolates showed that there was no variation in the sequence at the connecting peptide and that all viruses possess a series of basic amino acids (RERRRKKR), but CHK258-97 has Ala at −11 while the others have Thr at this position (Table 1). Thus, each of the H5N1 viruses examined was lethal in chickens, possessed multiple basic amino acids at the connecting peptide of HA, and met the definition of highly pathogenic avian influenza virus (Senne *et al.,* 1996). Therefore, none of the H5N1 influenza viruses examined were suitable as a surrogate vaccine strain.

Antigenic analysis

To determine the antigenic diversity of the H5N1 influenza viruses isolated from poultry in Hong Kong, the viruses, including the index human case (HK156-97), were analyzed with monoclonal antibodies (MABs) and monospecific polyclonal goat and ferret antisera to the H5 hemagglutinin in HI tests. A panel of 6 MABs was selected from our pool of 17 MABs based on discriminative reactivities between isolates (results not shown). Each of the viruses included in the study reacted with the monospecific reference goat antisera, establishing that they belonged to the H5 subtype (Table 2). Fourteen of the viruses had the same reactivity pattern shown by A/Hong Kong/156/97. Four viruses had reactivity patterns resembling A/chicken/Hong Kong/258/97. The difference between these two groups was loss of reactivity with one

TABLE 1

H5 Influenza A Viruses Used in the Study of Hong Kong Isolates

Host	Virus strain	Subtype	Abbreviation	Lethality for chickens: Route of inoculation	Lethality for chickens: Time to death (days)	Nucleotides of HA sequenced	Connecting peptide sequences	Carbohydrate at residue 158
			Pathogenic					
Human	A/Hong Kong/156/97	H5N1	HK156-97	IN	3	8-1767	TPQRERRRKKR	−
Chicken	A/chicken/Hong Kong/220/97	H5N1	CHK220-97	IN	2	38-1746	TPQRERRRKKR	+
	A/chicken/Hong Kong/258/97	H5N1	CHK258-97	IN	2–3	21-1746	APQRERRRKKR	+
	A/chicken/Hong Kong/728/97	H5N1	CHK728-97	IN	2	21-1746	TPQRERRRKKR	−
	A/chicken/Hong Kong/786/97	H5N1	CHK786-97	IN	2	21-1746	TPQRERRRKKR	+
	A/chicken/Hong Kong/915/97	H5N1	CHK915-97	IN	2–3	21-1746	TPQRERRRKKR	−
	A/silky chicken/Hong Kong/p17/97	H5N1	CHK17-97	IN	2–3	21-1746	TPQRERRRKKR	−
	A/chicken/Hong Kong/y385/97	H5N1	CHK385-97	IN	2	21-1746	TPQRERRRKKR	+
	A/chicken/Hong Kong/y388/97	H5N1	CHK388-97	IN	2	21-1746	TPQRERRRKKR	+
	A/chicken/Hong Kong/1203/97	H5N1	CHK1203-97	IN	2	21-1111	TPQRERRRKKR	−
	A/chicken/Hong Kong/976/97	H5N1	CHK976-97	ND[a]	—	21-1111	TPQRERRRKKR	−
	A/chicken/Hong Kong/990/97	H5N1	CHK990-97	ND[a]	—	21-1111	TPQRERRRKKR	−
	A/chicken/Hong Kong/w31/97	H5N1	CHK31-97	IN	2	295-1111	TPQRERRRKKR	−
	A/chicken/Hong Kong/w307/97	H5N1	CHK307-97	IN	2	295-1111	TPQRERRRKKR	−
	A/chicken/Hong Kong/w308/97	H5N1	CHK308-97	IN	2	295-1111	TPQRERRRKKR	−
	A/chicken/Hong Kong/p21/97	H5N1	CHK21-97	IN	2–3	295-1111	TPQRERRRKKR	−
	A/chicken/Hong Kong/w608/97	H5N1	CHK608-97	IN	2	295-1111	TPQRERRRKKR	−
	A/chicken/Hong Kong/w609/97	H5N1	CHK609-97	IN	2	295-1111	TPQRERRRKKR	−
	A/chicken/Hong Kong/w162/97	H5N1	CHK162-97	IN	2	449-1111	TPQRERRRKKR	−
Duck	A/duck/Hong Kong/p46/97	H5N1	DHK46-97	IN	2–3	21-1746	TPQRERRRKKR	−
	A/duck/Hong Kong/y283/97	H5N1	DHK283-97	IN	2–3	21-1111	TPQRERRRKKR	−
Goose	A/goose/Hong Kong/w355/97	H5N1	GHK355-97	IN	2	21-1746	TPQRERRRKKR	−
	A/goose/Hong Kong/w374/97	H5N1	GHK374-97	IN	2	295-1111	TPQRERRRKKR	−
			Nonpathogenic					
Duck	A/duck/Potsdam/2216-4/84	H5N6	DP84	ND	—	21-1718	VPQRETR	−
	A/duck/Potsdam/1402-6/86	H5N2	DP86	ND	—	21-1718	VPQRETR	−
	A/duck/Minnesota/1525/81	H5N1	DMN81	ND	—	21-1721	VPQRETR	−
	A/duck/Hong Kong/205/77	H5N3	DHK205-77	ND	—	21-1700	VPQRETR	−
	A/duck/Hong Kong/698/79	H5N3	DHK689-79	ND	—	21-1700	VPQRETR	−
Turkey	A/turkey/Ramon/73	H5N2	TRAM73	ND	—	64-1091	VPQRETR	−
Gull	A/gull/Pennsylvania/4175/83	H5N1	GPA83	ND	—	21-1721	VPQRETR	−

[a] RNA analysis only. ND, not determined.

MAB. One virus demonstrated a unique reactivity pattern: A/duck/Hong Kong/Y283/97 reacted with 4 of the 6 MABs. One of the H5N1 virus isolates gave variable reactivity patterns with the MABs on initial isolation and second egg passages, indicating a mixture of viruses. Cloning of this isolate A/chicken/Hong Kong/728/97 at limiting dilution in chicken embryos resulted in a reactivity profile similar to A/Hong Kong/156/97. The postinfection ferret antiserum to the index human case (HK156-97) discriminated between itself and all of the other H5N1 isolates (Table 2), but did not differentiate them into distinguishable groups. Similarly, the monospecific goat anti-H5 serum did not discriminate between the two groups of viruses that are separable by MAB CP46.

Antigenic analysis of nonpathogenic H5 influenza viruses isolated from different regions of the world showed reactivity with the panel of MABs and with the reference polyclonal goat antiserum. This panel of antibodies did not discriminate between the earlier H5 isolated from Hong Kong [A/duck/Hong Kong/698/79 (H5N3)] and the index human case, and there was considerable cross reactivity between A/duck/Potsdam/1402-6/86 (H5N2), A/duck/Minnesota/1525/81 (H5N1) and the index human case. The other strains show less reactivity. Each of the nonpathogenic viruses were considered potential surrogates for vaccine preparation and were provided to the Centers for Disease Control and The World Influenza Center for evaluation.

Characterization of the HA of the index human and chicken isolates (Subbarao *et al.*, 1998; Claas *et al.*, 1998) revealed the presence of a mutation at amino acid residue 158 of HA1. We sequenced each of the HAs through

TABLE 2

Antigenic Analysis of H5N1 Influenza Viruses from Poultry in Hong Kong

VIRUSES	Monoclonal antibodies HI titers to A/chicken/PA/1370/83 CP24	CP25	CP46	CP58	Monoclonal antibodies HI titers to A/chicken/PA/8125/83 176/26	406/7	Polyclonal sera Anti-A/tern/Sa/61 (goat)	Anti-A/HK/156/97 (ferret)	Glycosylation at amino acid 158 (+/−) CHO
Pathogenic strains									
A/Hong Kong/156/97	400	400	12800	6400	3200	1600	240	640	−
A/silky chicken/Hong Kong/P17/97	400	400	800	3200	1600	400	40	40	−
A/chicken/Hong Kong/W31/97	400	400	3200	3200	3200	1600	320	80	−
A/goose/Hong Kong/W374/97	400	400	3200	6400	3200	800	160	80	−
A/duck/Hong Kong/Y283/97	<	<	6400	6400	6400	3200	640	40	−
High path Hong Kong[a]	200–800	200–800	1600–12800	3200–12800	1600–3200	400–3200	80–640	40–640	−
A/chicken/Hong Kong/258/97	800	400	<	3200	1600	200	40	20	+
A/chicken/Hong Kong/220/97	800	400	<	≥12800	3200	200	40	40	+
A/chicken/Hong Kong/786/97	800	400	<	3200	3200	400	80	40	+
A/chicken/Hong Kong/Y385/97	800	400	<	3200	3200	400	40	80	+
A/chicken/Hong Kong/Y388/97	800	800	<	3200	NT	400	80	NT	+
Nonpathogenic strains									
A/duck/Singapore/3/97	100	100	3200	6400	6400	3200	160	80	NT
A/duck/Hong Kong/698/79	400	800	1600	6400	6400	3200	160	NT	NT
A/duck/Potsdam/1402-6/86	100	400	6400	12800	6400	6400	2560	NT	NT
A/duck/Minnesota/1525/81	800	1600	6400	12800	12800	6400	640	NT	NT
A/duck/Potsdam/2216-4/84	<	100	12800	12800	6400	3200	2560	NT	NT
A/turkey/Ramon/73	<	<	6400	100	200	<	5120	NT	NT
A/gull/Pennsylvania/4175/83	<	<	<	12800	6400	1600	640	NT	NT
Reference strains									
A/tern/SA/61	<	<	1600	400	400	<	80	10	−
A/chicken/Pennsylvania/1370/83	3200	6400	6400	6400	3200	3200	160	40	−

Note. NT, not tested. <, less than 100.

[a] Range of antibody titers for 11 isolates.

this region and found that the viruses could be separated into two groups on the basis of the presence or absence of a potential carbohydrate at this site (Table 1) and that the reactivity of one of our monoclonal antibodies (CP46) correlated with the presence or absence of this mutational change.

The neuraminidase of each of the H5N1 viruses were identified with a panel of monospecific antisera to the nine neuraminidase subtypes. Each of the viruses reacted with the N1 antisera and partial sequencing of each gene encoding the NA confirmed that they belonged to the N1 subtype (results not shown).

Animal studies

Since the H5N1 influenza in Hong Kong transmitted to and caused severe infection in humans, studies were done to establish the properties of these viruses in experimental animals.

Replication in ducks. Since wild ducks are regarded as a possible reservoir for influenza viruses, and virus was isolated from fecal samples collected under duck cages in the poultry markets, studies were done in this species to determine the extent of replication. The virus from the index human case (HK156-97), an early chicken isolate (CHK258-97), and a duck isolate (DHKY283-97) were inoculated into groups of Pekin ducks by the oral and nasal routes. Both the human and the chicken viruses grew to very low titers (2.5 vs 1.5 for CHK258-97 and HK156-97) in the trachea and were shed in the feces of less than half the ducks, but the duck isolate failed to replicate (Table 3). None of the viruses caused disease signs and food intake was not diminished. The CHK258-97 (H5N1) virus was shed for a longer period of time from the trachea than was the human H5N1 virus, but the shedding occurred in only two of the six ducks inoculated. Thus, both the chicken and human H5N1 viruses tested have the ability to replicate in ducks, but the duck isolate's failure to replicate raises the possibility that these are not duck isolates.

Replication in geese. Since H5N1 influenza viruses were isolated from apparently healthy geese in the poultry markets in Hong Kong, isolates from humans, chick-

TABLE 3

Replication of H5N1 Influenza Viruses in Domestic Ducks

Virus	Day	Infectivity titers in chicken embryos: Trachea Shedding (N = 6)	Trachea Titer $\log_{10}$/ml	Cloaca Shedding (N = 6)	Cloaca Titer $\log_{10}$/ml
A/Hong Kong/156/97	1	0	0	1	~1.5
	2	2	<1.5	3	0–1.5
	3	0	0	2	~1.5
	4	0	0	0	0
	5	0	0	0	0
A/chicken/Hong Kong/258/97	1	1	<1.5	1	~1.5
	2	2	<1.5	1	~1.5
	3	1	2.6	0	0
	4	2	1.5–2.5	0	0
	5	1	2.5	0	0
A/duck/Hong Kong/Y283/97	1	0	0	0	0
	2	0	0	0	0
	3	0	0	0	0
	4	0	0	0	0
	2	0	0	0	0

ens, ducks, and geese were inoculated into groups of geese. Each of the viruses replicated to modest titers in geese and was shed in the feces (Table 4). The CHK258-97 and GHKW355-97 viruses were shed for an extended period (13 days). Each of the H5N1 viruses tested replicated in geese and was shed in modest infectivity titers from both the trachea and the feces (1.0–4.0 $\log_{10}$/ml). It is noteworthy that the chicken isolate (CHK258-97) caused disease signs in 3 of 6 and mortality in 1 of 6 geese, and that the goose isolate (GHKW355-97) caused mortality in 1 of 5 geese. The disease signs included inflammation and red hemorrhage streaks on the leg joints and reddening of the beak. The lungs of the dead birds were severely hemorrhaged with virus titers of 3.5 $\log_{10}$/ml; higher titers of virus were detected in the brain (5.8 $\log_{10}$/ml), and the birds showed general

TABLE 4

Replication of H5N1 Influenza Viruses in Geese

Virus	Day	Infectivity titers ($\log_{10}$/ml): Trachea No shedding	Trachea Titer (range)	Cloaca No shedding	Cloaca Titer (range)	Disease: Signs	Disease: Death
HK156-97	3	2/2	2.5–3.2	1/2	2.5	0/2	
	5	2/2	3.0–4.0	2/2	1.0–3.25	0/2	
CHK258-97 (E1)[a]	3	1/1	3.0	1/1	4.2	2/2	1/2
	5	1/1	2.3	1/1	3.8	1/1	
CHK258-97 (E2)[b]	3	1/4	3.8	2/4	2.2–2.5	0/4	
	5	4/4	2.2–3.5	4/4	2.5–4.0	1/4[c]	
	13	0/4	—	1/4	3.2	0/4	
DHKY283-97	3	3/3	2.5–2.8	3/3	2.5–3.5	0/3	
	5	1/3	2.5	2/3	2.75–4.0	0/3	
GHKW355-97	3	3/5	2.2–3.6	2/5	0–2.5	0/5	
	5	2/5	1.0–4.0	2/5	~2.2	1/5[d]	1/5
	13	0/5	—	2/4	~2.2	0/4	

[a] E1, experiment 1.
[b] E2, experiment 2.
[c] Red hemorrhage on legs.
[d] Red hemorrhage on legs and beak.

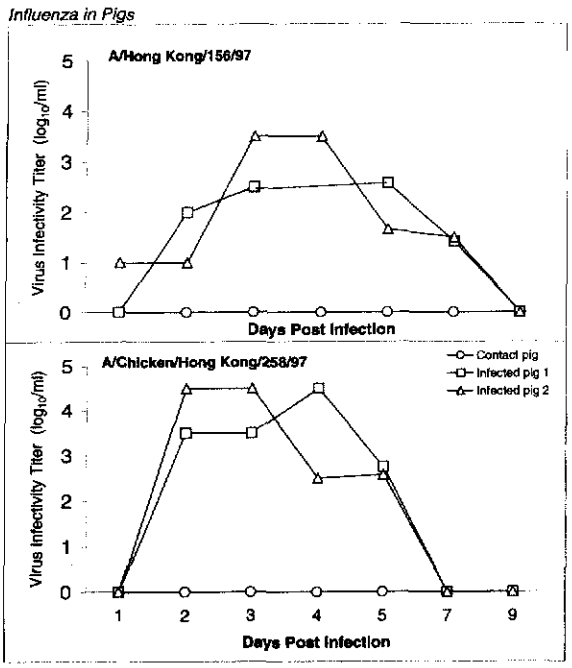

FIG. 1. Replication of H5N1 influenza viruses in pigs. (Top) Infectivity titers in the nasal tract of pigs infected with A/Hong Kong/156/97 (H5N1). (Bottom) Infectivity titers in the nasal tract of pigs infected with A/chicken/Hong Kong/258/97 (H5N1).

malaise before death. Thus, these H5N1 viruses showed a higher propensity to infect and cause disease in geese than in ducks.

Replication in pigs. Pigs are considered the intermediate host in the transmission of influenza viruses between avians and humans (Scholtissek *et al.*, 1993; Ito *et al.*, 1998) so they were tested for their ability to replicate the H5N1 viruses. Pigs were infected with the index human H5N1 strain (HK156-97) and an initial chicken isolate (CHK258-97) by the oral and nasal routes, and the results are given in Fig. 1. Both the human and chicken H5N1 viruses replicated in pigs, but the chicken isolate replicated to higher titers (4.5 log_{10}/ml) in pigs than did the index human virus (~3.6 log_{10}/ml). Neither the human nor the chicken isolates transmitted to contact pigs in the same pen. Thus, pigs support the replication of the H5N1 virus to only modest titers, and there was no detectable transmission to contact animals.

Replication in mice. Mice serve as a useful animal model system for studying influenza viruses, and the index human H5N1 virus was inoculated into mice. Balb/c mice were infected intranasally with the virus and the results are given in Fig. 2. The virus replicated in the lungs on initial infection and killed the animals. Infectivity titers ranged from 5.5–5.6 log_{10}/ml (results not shown), and virus was detected at very low titers in the brain (~1.0 log_{10}/ml). On the second passage from the lungs of mice, high titers of virus were detected in the lungs (7.8–8.5 log_{10}/ml), and virus was detected in blood (3.5 log_{10}/ml) and in the brain (2.5–3.3 log_{10}/ml). The mice lost weight (Fig. 2) beginning on the second day after infection. The twice-passaged virus killed mice by the fourth day postinfection, and all of the mice had died by the fifth day. The virus caused severe pneumonia and hemorrhaging in the lungs, but there was no evidence of gross pathology in other organs. The A/chicken/Hong Kong/258/97 (H5N1) virus isolate was also inoculated into mice and caused 100% mortality on the initial passage (results not shown). Neither the chicken nor the human H5N1 isolates required adaptation to mice. Thus, these H5N1 viruses have a suprisingly high pathogenicity for mice. Contact mice kept in cages with infected mice did not become infected, despite high titers of virus in the lungs and the death of all inoculated animals.

Replication in rats. The poultry markets in Hong Kong had a large rat population having direct contact with feces containing H5N1 influenza viruses. To determine their susceptibility to infection, Sprague–Dawley rats were inoculated experimentally with the viruses listed in Table 5. The H5N1 influenza viruses from humans, chickens, and ducks all replicated to low titers in the lungs, but only a portion of animals shed virus (Table 5). Virus was detected in the lungs on day 3 but not on day 5, postinfection. The goose strain tested failed to replicate in rats. The viruses caused no disease signs, and the rats gained weight during the experiment. It is noteworthy that the chicken isolate (CHK258-97) replicated to higher titers (5.0 log_{10}/ml) than did the other viruses.

Stability of H5N1 virus in the environment. The detection of H5N1 influenza virus in bird feces in the poultry

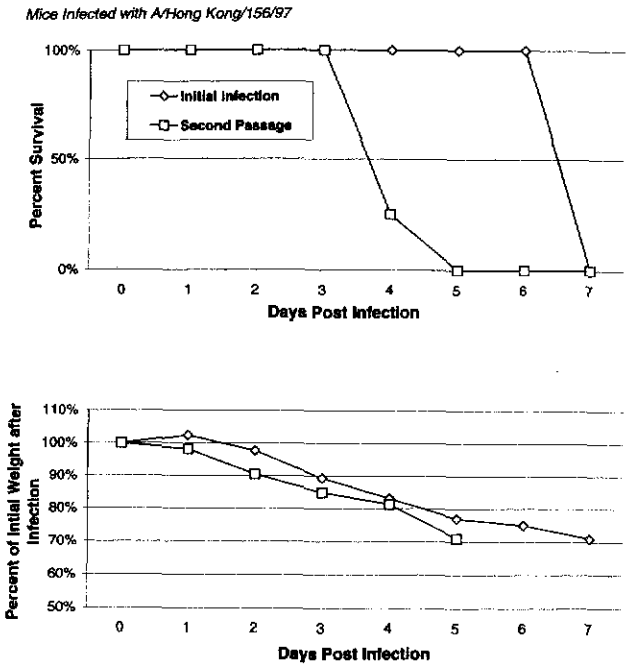

FIG. 2. Replication of A/Hong Kong/156/97 (H5N1) in mice. (Top) Percentage survival after first and second passage in Balb/c mice. (Bottom) Percentage of weight loss of the first and second passage in Balb/c mice.

TABLE 5

Replication of H5N1 Influenza Virus in Rats

Viruses	Detection of virus on day 3 after infection: No. shedding/ No. inoculated	Titer $\log_{10}$/ml (lung)	Disease signs
HK156-97	1/2	0–2.8	0/10
CHK258-97	2/2	~2.5–5.0	0/10
DHKY283-97	1/2	1.0–2.5	0/10
GHKW355-97	0/2	0	0/10

markets raised the question of the stability of the virus. Studies were done on the stability of H5N1 in feces collected from infected birds. The titer of virus in feces collected from infected birds was 3.5 to 4.5 $\log_{10}$ EID_{50} per g. When feces were dried at room temperature (~25°C), infectivity declined to nondetectable levels by day 1. When wet feces were held at 25°C the infectivity declined to 2.5 $\log_{10}$/ml by the first day and to 1.5 $\log_{10}$/ml after 4 days (Fig. 3). At higher temperatures (35°C), infectivity in moist feces dropped to undetectable levels by the second day. When moist chicken feces were stored at low temperatures (4°C), virus remained viable for an extended time with no detectable loss of infectivity over 40 days.

Transmission of H5N1 viruses between chickens. Although H5N1 viruses were isolated from poultry markets in Hong Kong and were found to be highly pathogenic to chickens after experimental infection in the laboratory, there was very limited evidence of high mortality in the poultry markets (approximately 1000 markets) in Hong Kong. To determine the transmissibility of these viruses between chickens, birds were infected and put into cages with uninfected birds. Virus was detected in the trachea and in the feces of inoculated chickens 2 days after infection with a low dose of virus (5 LD_{50}). The contact chickens began shedding virus on the 4th day after contact, and showed disease signs. They died on the 5th and 6th days with virus in feces (>4.5 $\log_{10}$/ml) and trachea (3.5 $\log_{10}$/ml).

In a second experiment, four chickens were housed in a cage below infected birds (5 LD_{50}). The pan used to collect feces under the top cage was removed to allow feces to fall into the lower cage. One of the contact birds in the lower cage began shedding virus on the fifth day after birds in the cage above were infected. Three contact birds died (days 5 and 7), and one was not infected and survived. When contact birds were housed in cages adjacent to infected birds, but without direct contact, there was no detectable transmission to the contact birds and the birds remained healthy. These studies indicate that the CHK258-97 H5N1 influenza virus is most effectively transmitted by the fecal–oral route and that aerosol transmission was ineffective under the conditions tested.

Morphology. The H5N1 viruses isolated from chickens and humans showed slight differences in morphology (Fig. 4). On electron microscopy, the chicken virus showed a mixture of long filamentous (35%; $N = 161$) and spherical virions. The virus isolated from humans was predominantly spherical, with a few filaments (16%; $N = 220$).

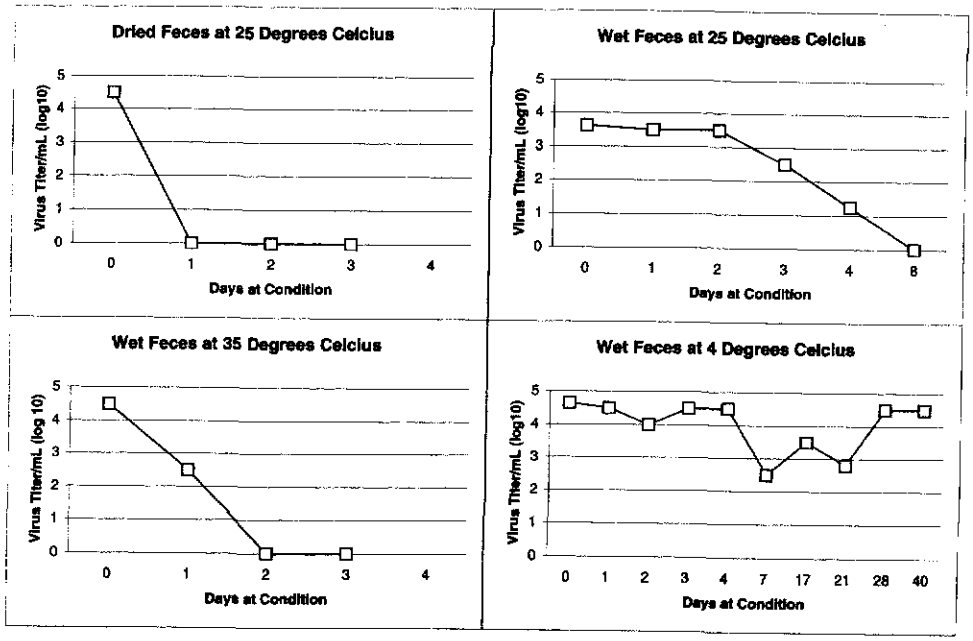

FIG. 3. Stability of A/Hong Kong/156/97 (H5N1) at environmental temperatures.

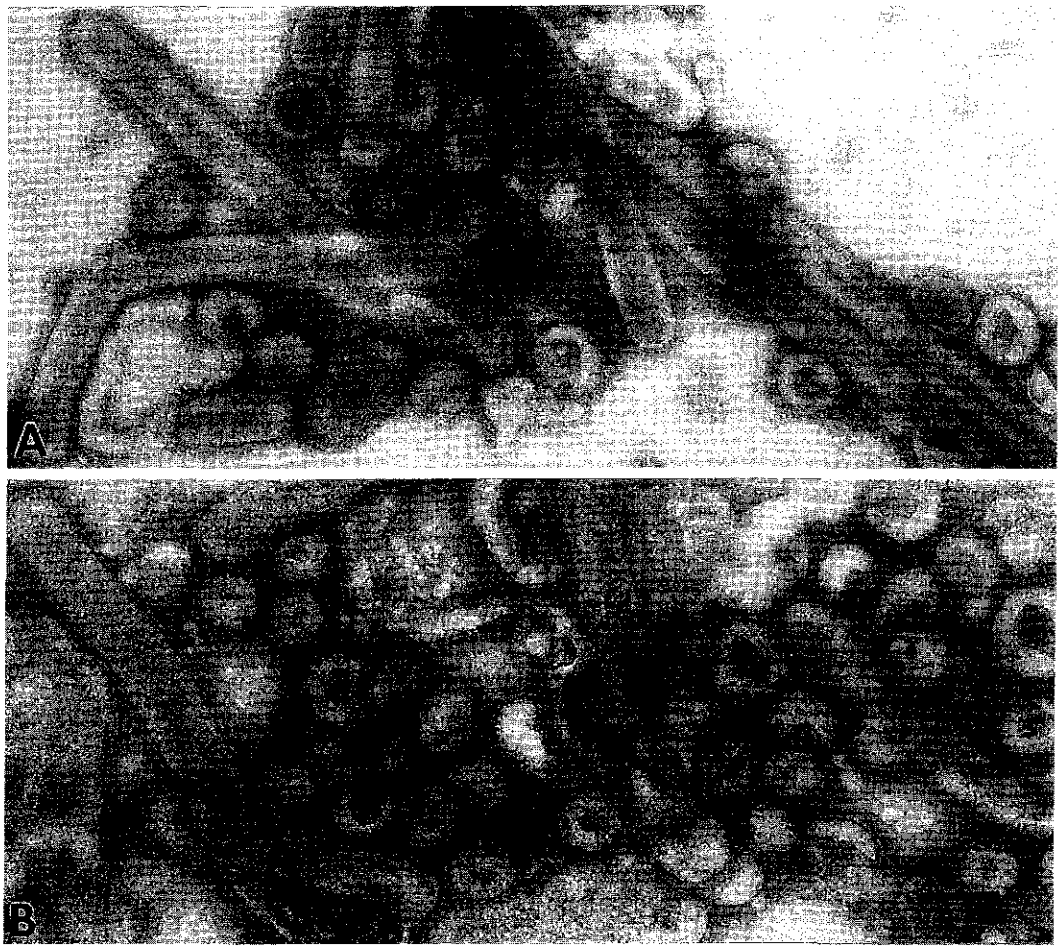

FIG. 4. Electron micrographs of H5N1 viruses from (A) chickens (CHK258-97) and (B) humans (HK156-97). See Materials and Methods for details. Bar equals 1 nm.

DISCUSSION

Analysis of pathogenicity by inoculation of chickens showed that all of the H5N1 isolates examined caused high (100%) mortality. Sequence analysis revealed that each isolate possessed a series of six basic amino acids at the cleavage site (TPQRERRRKKR) and all except one had Thr at the −11 position. Thus, all of the isolates meet the definition of highly pathogenic avian influenza viruses. It is noteworthy that these viruses caused rapid death in chickens: after intravenous infection, some of the isolates killed birds within 16 h, making this one of the most lethal avian influenza viruses, similar to A/turkey/England/91 (H5N1). The H5N1 influenza viruses isolated from ducks and geese in the poultry markets were also highly pathogenic and were not distinguishable from chicken isolates. Thus, none of the isolates was suitable for use as a surrogate nonpathogenic vaccine strain for use in humans and lower animals. The absence of nonpathogenic strains, which are considered the precursors of pathogenic strains (Röhm *et al.,* 1995), suggests that these H5N1 viruses had been present in Hong Kong markets for some time, but had not been detected for lack of surveillance.

Antigenic analysis of H5N1 viruses from domestic poultry in Hong Kong established the subtype of the isolates, showing that they reacted to high titers with the panel of monoclonal antibodies to A/chicken/Pennsylvania/1370/83 (H5N2) and to the monospecific H5 goat antiserum. One of the monoclonal antibodies (CP46) detected antigenic differences among the H5N1 viruses that correlated with the presence or absence of a carbohydrate at residue 158 of the HA. The majority of monoclonal antibodies did not separate the isolates into different groups; the majority of the panel of 17 monoclonal antibodies prepared to the HA of A/chicken/Pennsylvania/1370/83 (H5N2) and A/chicken/Pennsylvania/8125/83 (H5N2) reacted with H5N1 isolates from Hong Kong, demonstrating that little antigenic drift occurs in avian influenza viruses (data not shown). The experience in Hong Kong established the value of having such monoclonal antibodies available as reference reagents. Some of the original H5N1 isolates gave different reactivities with the antibodies after subsequent passages,

and biological cloning indicated that they were mixtures of viruses.

Antigenic analysis of the nonpathogenic H5 influenza viruses with monoclonal antibodies established that the viruses examined shared epitopes. The nonpathogenic Eurasian viruses most closely related to the index human case were A/duck/Singapore/3/97, A/duck/Hong Kong/698/79, and A/duck/Potsdam/1402-6/86, and these were considered possible surrogate vaccine strains for use in humans. Overall, there was limited antigenic drift between the H5 viruses isolated between 1979 and 1997; however, the presence of a carbohydrate residue adjacent to the receptor binding site may influence their antigenicity, and its importance in selection of vaccine strains for humans is still being evaluated.

Studies of the ability of the avian H5N1 viruses to replicate in mammals indicate that, like other H5 avian strains tested (Kida *et al.,* 1994), these strains from Hong Kong can replicate in pigs. The virus titers in pigs were modest (3.5–4.5 $\log_{10}$/ml), produced no disease signs, and neither the human nor the avian H5N1 isolate tested transmitted to contact animals. Whether transmission would occur under field conditions is as yet unresolved. Pigs were being raised in Hong Kong within meters of the infected chickens, but there was no disease in pigs.

It is noteworthy that experimental inoculation of Pekin ducks resulted in infections of only $\frac{1}{3}$ of the birds with human and chicken H5N1 isolates. The duck isolate did not replicate when inoculated into ducks. The possibility must be considered that these are not duck isolates, but that they originated from chickens in the same markets. In contrast, geese supported the replication of each H5N1 virus tested, and ~12% of animals developed disease signs and died. It is unusual for influenza viruses to cause disease in aquatic birds. Inoculation of geese with H7N7 isolates from geese that were highly pathogenic in chickens produced no disease signs (Röhm *et al.,* 1996). The report of a severe outbreak of disease in geese in Guandong Province with 40% mortality (Xu *et al.,* 1998) associated with an H5 influenza virus, A/goose/Guandong/1/96 (H5N1), may throw light on the origin of the H5N1 outbreak in Hong Kong. This virus was isolated from geese in 1996 and may be the precursor of the virus that appeared in chickens in Hong Kong. Guandong Province is adjacent to Hong Kong and provides much of the poultry for the markets.

The H5N1 viruses from Hong Kong were inactivated within 1 day when dried and held at 25°C but were more stable in wet feces, with infectivity being detectable for more than 4 days. At lower temperature (4°C) there was minimal drop in virus titers in wet feces in 40 days. This indicates that air drying is an efficient method of decontaminating an area after all fecal material has been removed. It also suggests that human infection requires contact with freshly deposited contaminated feces.

Despite the presence of the H5N1 virus in the majority of the poultry markets tested, only 18 humans were infected, and there was no convincing evidence for human-to-human spread. Thus, H5N1 viruses lacked the property of transmissibility from pig to pig, mouse to mouse, and human to human. Even in chickens, the virus transmitted only by the fecal–oral route, and aerosol transmission was not demonstrated. The failure to transmit by aerosol may be due to the relatively low titers in the respiratory tract of chickens (3.5 $\log_{10}$/ml), but not in pigs (3.6–4.5 $\log_{10}$/ml) or in mice (5.5–8.5 $\log_{10}$/ml). The molecular basis for transmissibility is still poorly understood. The HA has been associated with pathogenicity and ability to replicate in the intestinal tract of ducks (Naeve *et al.,* 1984), but its contribution to transmissibility is unresolved. The receptor specificity of avian influenza viruses (Paulson, 1985) (α2-3 binding to terminal sialic acid) is clearly not a strict host-range determinant but may be important for spread from human to human. Pigs have receptors for both kinds of viruses (α2-3 and α2-6) (Ito *et al.,* 1998), but only a subset of virus subtypes replicate in pigs, and few transmit from pig to pig. Studies in pigs suggest that the NP, NA, M, and NS genes may contribute to the restriction of replication of avian influenza viruses in this species (Kida *et al.,* 1994). It must be kept in mind that host-range transmission is a polygenic trait and that an optimal constellation of genes is required (Rott *et al.,* 1979; Webster and Rott, 1987).

The role of ducks in the natural history of influenza A viruses is well established (Webster *et al.,* 1992). However, the available evidence suggests that wild ducks do not maintain H5 influenza viruses in nature (Sharp *et al.,* 1993). Studies in seabirds, particularly in shorebirds, indicate that H5 influenza viruses are more consistently isolated from these species (Süss *et al.,* 1994; Webster, unpublished data). In 1991, H5N2 viruses were isolated from up to 20% of surfbirds tested in Delaware Bay, mainly from Ruddy Turnstone (*Arenaria interpres*) and Red Knots (*Calidris canatus*). Descendents of these viruses were detected in chickens in poultry markets in New York and in chickens in Mexico (Horimoto *et al.,* 1995), suggesting that the migrating shorebirds may be the reservoir of H5 influenza viruses. The presence of influenza viruses in migratory birds in Asia has not been studied, so we do not know if the shorebirds that migrate from Australia to Siberia through Hong Kong are the harbingers of H5 influenza virus. Extensive studies in domestic poultry in Hong Kong and SE Asia from 1975–1987 established that each of the known subtypes of influenza A is isolated year-round from domestic ducks (Shortridge *et al.,* 1977). However, it is noteworthy that H5 influenza viruses were previously isolated only from ducks and geese and were not isolated from chickens. Thus, H5N1 is a relatively new introduction into chickens in Hong Kong. Lethality and the spread of the virus to the brain after initial intranasal inoculation are unusual findings in mice. It usually requires multiple passages to

adapt influenza viruses to mice, and few strains [A/NWS/34 (H1N1)] are neurotropic after intranasal inoculation. The mouse will provide a useful model system for the resolution of high pathogenicity and the immune response of mammals to a highly pathogenic avian influenza virus.

It is still not known why these H5N1 influenza viruses transmitted to humans when earlier studies on H5N2 in Pennsylvania provided no evidence for transmission (Bean *et al.,* 1985). Since there was no evidence for human-to-human transmission (MMWR 1998), each of the 18 human cases must have originated from domestic poultry. Since most poultry markets examined in Hong Kong in December 1997 contained H5N1-infected birds, we can speculate that the markets were the probable source of virus.

There is serological evidence that influenza subtypes other than H1, H2, and H3 have transmitted to humans previously in Southern China (Shortridge 1992). However, we do not know if transmission has been limited to the Eurasian lineage of influenza viruses. The transmission of H5N1 influenza viruses to humans and the high percentage of complications in humans (Yuen *et al.,* 1998) should serve to alert pandemic planning authorities that all subtypes of influenza A viruses can potentially become pandemic strains. The other important lesson from Hong Kong in 1997 was that the separation of host species can influence interspecies transmission. Thus, chickens are now marketed separately from aquatic birds. Marketed aquatic birds are limited to ducks and geese, which are killed at a separate wholesale market. There have been no additional cases of H5N1 detected in humans or in live bird markets since the domestic poultry was destroyed on December 29–30, 1997.

MATERIALS AND METHODS

Viruses

The viruses were isolated in chicken embryos as described previously (Shortridge *et al.,* 1977), and some were cloned at limit dilution in eggs or plaqued in MDCK cells or chick embryo fibroblasts. All viruses were handled in a BL3+ facility approved for use by the United States Department of Agriculture, and the research staff wore fitted HEPA-filtered masks and took prophylactic rimantadine.

Virus and serological assays

The viruses used in this study are listed in Table 1. Hemagglutination titrations and hemagglutination inhibition (HI) assays were performed in microtiter plates (Palmer *et al.,* 1975). Monospecific goat and postinfection ferret antisera, and ascitic fluids from mice containing monoclonal antibodies were used in the HI tests; hyperimmune goat serum was prepared to the HA isolated from A/tern/South Africa/61 (H5N1). Monoclonal antibodies were prepared to the HA of A/chicken/Pennsylvania/1370/83 (H5N2) and A/chicken/Pennsylvania/8125/83 (H5N2), as described (Kawaoka *et al.,* 1987). Ferret antiserum to A/Hong Kong/156/97–A/Turkey/Wisconsin/68 [R] was kindly provided by the Centers for Disease Control and Prevention.

RNA extraction and PCR

Viral RNA was extracted from allantoic fluid with the RNeasy extraction kit (Qiagen, Santa Clara, CA). Amplification of the viral RNA was carried out by reverse transcription–PCR, as described previously (Shu *et al.,* 1993). After purification with the QIAquick PCR Purification Kit (Qiagen), the PCR products were sequenced by the Center for Biotechnology at St. Jude Children's Research Hospital using rhodamine dye-terminator cycle sequencing ready reaction kits with AmpliTaq DNA Polymerase FS (Perkin–Elmer, Applied Biosystems Inc. [PE/ABI], Foster City, CA) and synthetic oligonucleotides. Samples were electrophoresed on PE/ABI model 377 DNA sequencers.

Infection studies

All studies were carried out in BL3+ conditions using animals that were serologically and virologically free of detectable influenza virus. The dose of infecting virus was approximately 10^6 EID_{50}.

Poultry

Chickens. Three-week-old specific pathogen-free (SPF) chickens were inoculated with 1.5 ml virus by intravenous injection or with 0.1 ml into the nares.

Ducks. Pekin ducks (young adults, 2 months old) were inoculated with 1.0 ml of virus by the tracheal and oral routes and into the nares and eyes.

Geese. White geese (Embden or Chinese, 4 weeks old) were inoculated with 1.0 ml of virus by the tracheal and oral routes and into the nares and eyes.

All poultry were observed for disease signs, and food and water intake was monitored. Tracheal and cloacal samples were collected at the intervals shown and titrated for virus in embryonated eggs. Blood samples were collected from chickens at 14 and 21 days postinfection.

Mammals

Pigs. Yorkshire white pigs (weanlings; approximately 4 weeks old) were inoculated intranasally with 1.0 ml administered into each nostril with a plastic pipet. An uninoculated littermate was housed in the same pen with two inoculated pigs to test for pig-to-pig transmission of virus. Temperatures were taken daily beginning 2 days before infection, and food consumption was recorded.

Swabs from each nostril were collected daily and titrated for virus in embryonated eggs.

Mice. Balb/c mice (approximately 8 weeks old) were anesthetized and inoculated intranasally with 100 μl of 10-fold dilutions of virus. The mice were weighed daily. A portion of each group of mice was exsanguinated at intervals after infection, and organs were removed in the order blood, brain, and lung. The organs were ground, and 10% suspensions were prepared and titrated for infectious virus in embryonated eggs and in MDCK cells.

Rats. Sprague–Dawley rats (approximately 8 weeks old) were inoculated intranasally and orally with 100 μl of virus. The rats were weighed daily. A portion of each group was exsanguinated at intervals after infection, and organs were removed in the order blood, brain, and lung. The organs were assayed for virus infectivity in embryonated eggs.

Stability of the H5N1 virus in chicken feces

Chickens were infected with the virus into the nares with 0.1 ml containing approximately 100 CLD_{50}. Fecal samples were collected from pans under the cages daily and made into 10% suspensions with PBS or dried at room temperature (~25°C). Aliquots were stored at different temperatures and titrated in embryonated eggs for residual infectious virus. Dried samples were rehydrated and similarly titrated.

Transmission of H5N1 virus between chickens

Two White Leghorn SPF chickens (6–8 weeks old) were infected into the nares with 5 LD_{50} of H5N1 influenza virus. Infected birds were placed in a cage with two susceptible contact birds of the same age. A group of four chickens was placed in a cage directly below the infected birds and the fecal dropping tray was removed from the upper cage. Other groups of birds were housed in cages immediately adjacent to the infected birds but at a distance where the beaks of the birds could not touch.

Electron microscopy

The H5N1 influenza viruses were grown in chicken embryos and were examined after the second (chicken strain) and the third passage (human strain). The viruses were absorbed to freshly glow-discharged carbon-coated grids and negatively stained with 2% phosphotungstic acid (pH 7.2). The samples were examined in a Phillips EM301 electron microscope operated at 60 kV.

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