Isolation and characterization of influenza A viruses from avian species in Hong Kong

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Surveillance of apparently healthy ducks, chickens, and geese at a poultry dressing plant in Hong Kong yielded 51 haemagglutinating viruses 25 of which were influenza A viruses. Of these, 24 were subtyped into 13 combinations based on haemagglutinin and neuraminidase surface antigens. Of the 13 different influenza A viruses isolated, 7 possessed combinations of haemagglutinin and neuraminidase subunits that have not been reported previously—i.e., Hav2N1, Hav2Nav5, Hav4N2, Hav7N2, Hav7Nav1, Hav7Nav3, and Hav7Nav6. Four of the isolates were non-avid: they were not neutralized by antisera to any of the reference subtypes of influenza A viruses, yet antisera to each isolate inhibited both that virus and a known reference strain. The large number of combinations of haemagglutinin and neuraminidase and the isolation of two different influenza A viruses from one duck suggests that recombination may be occurring in nature.

The surveillance of lower animals and birds for influenza viruses is aimed at elucidating the ecology and natural history of influenza viruses (*9*, *14*). In addition, these studies may help to elucidate the source of new human pandemic strains as well as serving as an early warning system for influenza in domestic animals.

The isolation of influenza viruses from lower animals and birds (for a review see *5*) and of a swinelike influenza virus—A/New Jersey/8/76 (Hsw1N1)—from man at Fort Dix, NJ, USA, in February 1976 emphasizes the importance of animal surveillance for influenza viruses.

South-east Asia has been the point of initial isolation of at least two of the recent pandemic strains of human influenza. The Asian/57 (H2N2) strain was first detected in western Kweichow and eastern Yunnan, China, and the Hong Kong/68 (H3N2) strain was first isolated in south-east China. The present report describes the isolation and initial characterization of influenza A viruses isolated in Hong Kong from ducks, chickens, and geese; many of these were raised in the People's Republic of China and the others in the New Territories of Hong Kong.

MATERIALS AND METHODS

Samples

Tracheal and cloacal swabs were collected at random from domestic ducks, geese, and chickens at a poultry dressing plant in Hong Kong. Sampling was carried out, from November 1975 to June 1976, from apparently normal birds the majority of which originated from unknown localities in the People's Republic of China, while some were from the New Territories, Hong Kong. Each swab was placed in 1.5 ml of transport medium consisting of Earle's Minimal Essential Medium with added bicarbonate, penicillin, streptomycin, and gentamycin. Swabs collected in this manner were held at 4°C for 2–4 h before assay in embryonated chicken eggs.

Virus isolation

A 0.2-ml volume of sample was inoculated into the allantoic cavity of a 10-day-old chick embryo; when available, two eggs were used for each sample. The eggs were incubated at 37°C for 48 h and tested individually for haemagglutination activity. For subsequent studies, the haemagglutinating agents were grown in the allantoic cavity of 11-day-old chick embryos and were purified by centrifugation on a preformed sucrose gradient (*10*). Formalin-inac-

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tivated influenza virus vaccines were prepared as previously described (*15*).

Antisera

Antisera specific for the isolated haemagglutinin and neuraminidase subunits of the reference strains of influenza A viruses (*19*) were prepared in goats (*17*). Antisera to each of the avian influenza A subtypes and to Newcastle disease virus (NDV) were prepared in guinea-pigs (*1*). Antisera were also prepared against some of the influenza A viruses isolated from avian species in Hong Kong. Each guinea-pig was given a subcutaneous injection with 1.0 ml of formalin-inactivated gradient-purified virus together with Freund's incomplete adjuvant. Three weeks later the guinea-pigs were hyperimmunized by the intramuscular inoculation of 0.5 ml of inactivated virus without adjuvant. The guinea-pigs were exsanguinated two weeks after the second inoculation.

Serological tests

Haemagglutinin (HA) titrations and haemagglutination inhibition (HI) tests were performed in plastic trays containing receptor-destroying enzyme-treated sera (*6*). Neuraminidase (NA) titrations and neuraminidase inhibition (NI) tests were carried out as described previously (*20*).

Group-specific internal antigens of the haemagglutinating agents were identified by complement fixation and by incorporating specific goat antisera to the ribonucleoprotein (RNP) of influenza A and B viruses into agarose in single-radial-diffusion assays. The antigens were precipitated from infected allantoic fluid at low pH and taken up in sodium laurylsarcosinate as described in detail elsewhere (*12*).

RESULTS

Isolates

A total of 51 haemagglutinating agents were isolated from 588 samples taken from ducks, geese, and chickens (Table 1). Ducks provided the largest number of isolates numerically and proportionately, twice as many being derived from the cloaca as from the trachea. No marked differences were noted in the frequency of isolation of haemagglutinating agents from the two sampling sites for geese and chickens. Of the 51 isolates, 25 reacted in complement fixation or single-radial-diffusion tests with specific antisera to influenza A RNP and were designated type A influenza viruses. Most of the influenza viruses were isolated from the cloacae of ducks. Twelve of the isolates were serologically related to known NDV strains. Fourteen haemagglutinating agents were neither influenza A or B nor NDV and were not identified. The frequency of isolation was relatively uniform over the 8-month period of the study.

Antigenic analysis of influenza virus isolates with reference antisera

Of the 25 influenza A viruses, 20 could be subtyped with antisera prepared against the isolated haemagglutinin and neuraminidase subunits of the prototype human, avian, equine, or porcine strains (Table 2). In the haemagglutination inhibition test, 13 of these isolates reacted specifically with a particular antiserum clearly identifying the haemagglutinin subtype. Six isolates—duck/Hong Kong/7/76,

Table 1. Haemagglutinating (HA) agents isolated from domestic poultry at a Hong Kong dressing plant

Type of bird	Source of samples	No. of samples	No. of HA isolates	No. positive for influenza A RNP	No. serologically related to known NDV strains	Unidentified agents
Duck	trachea	164	13	6	2	5
	cloaca	137	26	16	3	7
Goose	trachea	86	2	1	1	0
	cloaca	80	3	1	0	2
Chicken	trachea	54	4	1	3	0
	cloaca	67	3	0	3	0

Table 2. Identification of the surface antigens on avian influenza A viruses from Hong Kong

Virus isolate	Haemagglutinin [a] (HI titre with prototype antisera)	Neuraminidase [b]
A/duck/HK/7/76	Hav7 (320) H3 (160)	N2
A/duck/HK/8/76	Hav6 (640)	N1
A/duck/HK/9A/76	Hav4 (160)	Nav1
A/duck/HK/11/76	Hav4 (320)	Nav1
A/duck/HK/13/76	Hav6 (640)	N1
A/duck/HK/14/76	Hav4 (160)	Nav1
A/duck/HK/15/76	Hav2 (160)	Nav5
A/duck/HK/16/76	Hav4 (160)	Nav1
A/duck/HK/22A/76	Hav7 (320) H3 (80)	Nav3
A/duck/HK/22B/76	Hav7 (1280) H3 (320) Heq2 (40)	Nav1
A/duck/HK/23/76	Hav5 (160) HO (20)	Nav2
A/duck/HK/27/76	Hav4 (320)	Nav1
A/duck/HK/29/76	H3 (80) Hav7 (10)	Neq2
A/duck/HK/30/76	Hav4 (160)	Nav1
A/duck/HK/31/76	Hav6 (640)	N2
A/duck/HK/32/76	Hav6 (640)	N2
A/duck/HK/34/76	Hav7 (160) H3 (40)	N2
A/duck/HK/37/76	Hav4 (40)	N2
A/chicken/HK/1/76	H3 (640) Hav7 (320)	Nav6
A/goose/HK/1/76	Hav4 (160)	Nav1

[a] HI tests with specific antisera to the haemagglutinin subunits of each recognized subtype of influenza A. The figure in parenthesis is the HI titre.

[b] NI tests with specific antisera to the neuraminidase subunits of each recognized subtype of influenza A.

duck/22A, duck/22B, duck/29, duck/34, and chicken/Hong Kong/1/76—reacted with Hav7 and H3 antisera and, in the case of duck/22B, weak reactivity was observed also with Heq2 antiserum. Cross-reactivity between Hav7, H3, and Heq2 is well documented (*3*, *11*, *13*); the extent of cross-reactivity suggests that these haemagglutinins are of the Hav7 subtype. Each isolate reacted specifically with a single antiserum in the neuraminidase inhibition test (Table 2).

The duck/22A and duck/22B isolates, which provided the Hav7Nav3 and Hav7Nav1 subtypes, respectively, were from a single swab inoculated into two separate eggs. A similar observation was made as regards the isolates from duck/9A and duck/9B, when Hav4Nav1 and NDV isolates were isolated from the same duck. In both cases the isolates from the individual eggs were antigenically homogeneous.

Antigenic analysis of influenza A strains not inhibited by prototype antisera

Five influenza A virus isolates did not react with specific antisera to either the haemagglutinin or the neuraminidase of the prototype viruses (Table 3). Thus, in initial screening, A/duck/HK/24/76 was inhibited with specific antisera to N2 in neuraminidase inhibition tests, but the haemagglutinin was not inhibited by any of the prototype sera in HI tests. Similarly, A/duck/HK/28/76 was not inhibited in either the HI or the NI test with any of the prototype antisera.

Antisera were prepared in guinea-pigs to the intact purified viruses and were tested in HI and NI tests with each of the prototype influenza viruses and with the strains used in the preparation of antisera (Table 3). The antiserum prepared in guinea-pigs inhibited the homologous virus and prototype viruses to the same titres (results not shown). Thus, antisera to A/duck/HK/24/76 inhibited A/duck/Ukraine/1/63 (Hav7Neq2) and A/duck/HK/24/76 to similar titres in HI tests. These results show that some of the influenza viruses isolated recently from avian species are non-avian and do not react with specific prototype antisera. Furthermore, as shown in Table 2, A/duck/HK/29/76 reacted to higher titres with antisera to H3 than with antisera to Hav7, but when A/duck/HK/29/76 and A/duck/Ukraine/63

Table 3. Identification of influenza A viruses not inhibited by prototype antisera

Virus	Antigens identified with reference antisera		Antigenic analysis with antisera [a] to homologous strain	
	HI	NI	HI	NI
A/duck/HK/24/76	H?	N2	Hav7	N2
A/duck/HK/28/76	H?	N?	Hav4	Neq2
A/duck/HK/33/76	Hav2	N?	Hav2	N1
A/duck/HK/35/76	Hav2	N?	Hav2	N1
A/duck/HK/36/76	H?	N?	ND [b]	N1

[a] Antisera were prepared in guinea-pigs with purified viruses and tested in HI and NI tests with each of the prototype influenza viruses and with the strain used for vaccination.

[b] Sera not prepared but neuraminidase reacted with antisera to A/duck/HK/35/76.

viruses were tested against antiserum to A/duck/HK/24/76 they were inhibited to the same titres.

Antigenic subtypes of influenza A viruses isolated from birds in Hong Kong

Thirteen different influenza viruses were detected among the twenty-four isolates from birds in Hong Kong. The viruses possessed 5 different avian haemagglutinin subtypes in combination with 8 different neuraminidase subtypes (Table 4). The virus most frequently isolated possessed Hav4Nav1 and was isolated from 6 ducks and 1 goose; 7 influenza A viruses possessing Hav7 in combination with 5 different neuraminidase subunits (N2, Neq2, Nav1, Nav3, and Nav6) were isolated from ducks and chickens.

Several of the combinations of haemagglutinin and neuraminidase are novel in that they have not been reported previously. Seven isolates with new combinations of haemagglutinin and neuraminidase were recognized: Hav2N1, Hav2Nav5, Hav4N2, Hav7N2, Hav7Nav1, Hav7Nav3, and Hav7Nav6. All were isolated from ducks, with the exception of Hav7Nav6, which was isolated from a chicken.

Table 4. Antigenic subtypes of influenza A viruses isolated from avian species in Hong Kong

Virus subtype	Influenza A isolates	Know influenza A virus prototype
Hav2N1 [a]	A/duck/HK/33/76 A/duck/HK/35/76	A/chicken/Germany "N"/49 (Hav2Neq1)
Hav2Nav5 [a]	A/duck/HK/15/76	
Hav4Nav1	A/duck/HK/9A/76 A/duck/HK/11/76 A/duck/HK/14/76 A/duck/HK/16/76 A/duck/HK/27/76 A/duck/HK/30/76 A/goose/HK/1/76	A/duck/Czech/56 (Hav4Nav1)
Hav4/Neq2	A/duck/HK/28/76	
Hav4N2 [a]	A/duck/HK/37/76	
Hav5Nav2	A/duck/HK/23/76	A/tern/S. Africa/61 (Hav5Nav2)
Hav6N2	A/duck/HK/31/76 A/duck/HK/32/76	A/turkey/Mass/65 (Hav6N2)
Hav6N1	A/duck/HK/8/76 A/duck/HK/13/76	
Hav7Neq2	A/duck/HK/29/76	A/duck/Ukraine/63 (Hav7Neq2)
Hav7N2 [a]	A/duck/HK/7/76 A/duck/HK/34/76 A/duck/HK/24/76	
Hav7Nav1 [a]	A/duck/HK/22B/76	
Hav7Nav3 [a]	A/duck/HK/22A/76	
Hav7Nav6 [a]	A/chicken/HK/1/76	

[a] Novel combinations of haemagglutinin and neuraminidase not previously reported.

DISCUSSION

A total of 51 haemagglutinating agents were isolated from 588 tracheal or cloacal samples collected from apparently healthy ducks, chickens, or geese at a poultry dressing plant in Hong Kong. Of the 51 isolates, 25 were type A influenza viruses and 12 were Newcastle disease virus; 14 of the haemagglutinating agents were not identified. Previous studies (*18*) showed that about half of the haemagglutinating agents isolated from birds were paramyxoviruses that could not be classified. Some of the unidentified agents may fall into this category.

Of the 25 influenza A viruses, 20 were identified with specific antisera to the isolated haemagglutinin and neuraminidase subunits of the reference strain of each subtype; 5 of the influenza A viruses failed to react with antisera to either the haemagglutinin or the neuraminidase (or both) subunits of the subtype influenza reference strains. Antisera made to these isolates reacted with both the homologous isolate and one of the reference strains, which suggests that these five isolates were non-avid. The isolation of influenza viruses that are non-avid and fail to react to high-titred sera makes surveillance studies time-consuming, for it means that antisera must be prepared against these strains in order to place them into subtypes.

Among the 25 influenza A isolates 13 different influenza viruses were detected, but no new haemagglutinin or neuraminidase subtype was detected. The virus most frequently isolated possessed Hav4Nav1 and was similar to the A/duck/Czechoslovakia/56 reference strain.

The A/duck/HK/23/76 (Hav5Nav2) strain possessed the same surface antigens as the A/duck/HK/826/69 virus isolated in Hong Kong in 1969 (*7*). The Nav5 neuraminidase on A/duck/HK/15/76 (Hav2Nav5) had been detected earlier (*4*) on a virus from shearwaters in Australia: A/shearwater/Australia/73 (Hav6Nav5). More recently, viruses possessing Hav6Nav5 have been isolated from turkeys and feral ducks in the USA (Y. Hinshaw, unpublished observations, 1977); the present report is the first time that the Nav5 neuraminidase has been found in combination with Hav2.

An influenza virus characterized as Hav4Neq2 (*8*) was isolated during an outbreak of respiratory disease in chickens in Alabama, USA, in 1975. An influenza virus isolated from *Gracula religiosa* (A/myna bird/Massachusetts/71) by Butterfield et al. (*2*) has also been shown to possess Hav4Neq2 (W. K. Butterfield, personal communication, 1977). These viruses possess the same surface antigens as A/duck/Hong Kong/28/76 (Hav4Neq2). The isolation of influenza viruses with identical surface antigens from different species from widely separated areas of the world emphasizes the wide distribution of these viruses and the possible role of feral birds in the spread of these viruses to domestic species.

Of the 7 isolates with new combinations of haemagglutinin and neuraminidase recognized, 6 were from ducks and the other from a chicken. Two different influenza viruses with Hav7Nav3 and Hav7Nav1 subunits were isolated from the sample from one duck—which shows that mixed infection can occur in nature. The recombination and selection of new strains of influenza viruses have been demonstrated in animals under laboratory conditions (*16*). The occurrence of mixed infection and the large number of new haemagglutinin and neuraminidase combinations among the isolates from Hong Kong suggest that recombination may occur in nature.

The 13 different influenza viruses isolated from birds possessed 5 different haemagglutinin subunits and 8 different neuraminidase subunits. The human N1 and N2 neuraminidases were found in combination with 4 different haemagglutinin subunits. Further studies are needed to determine if any of the above-mentioned influenza viruses causes disease in birds.

Over the past few years a large number of influenza A viruses have been isolated from birds, especially from the cloacas of ducks ([*18*] and review by Easterday [*5*]). In the present study, most of the virus isolates came from ducks, which suggests that the duck may play an important role in the ecology of influenza viruses. The significance of these viruses and of the new combinations of haemagglutinin and neuraminidase described above remains to be elucidated in order to clarify the natural history of influenza viruses in birds and the origin of new strains pandemic for man.

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RÉSUMÉ

ISOLEMENT ET CARACTÉRISATION DE VIRUS GRIPPAUX DE TYPE A À PARTIR D'ESPÈCES AVIAIRES À HONG KONG

La surveillance de canards, poulets et oies apparemment sains dans une usine d'habillage de volailles à Hong Kong a donné 51 virus hémagglutinants, dont 25 étaient des virus grippaux de type A. Parmi ces derniers, 24 étaient sous-typés en 13 combinaisons basées sur les antigènes de surface hémagglutinine et neuraminidase.

Parmi les 13 différents virus grippaux de type A isolés, 7 possédaient des combinaisons de sous-unités hémagglutinine et neuraminidase n'ayant jamais été signalées—Hav2N1, Hav2Nav5, Hav4N2, Hav7N2, Hav7Nav1, Hav7Nav3, et Hav7Nav6.

Quatre des isolements obtenus étaient non avides: ils n'étaient neutralisés par les immunsérums d'aucun des sous-types de référence de virus grippaux de type A; cependant, les immunsérums dirigés contre chacun de ces isolements inhibaient à la fois le virus correspondant et une souche de référence connue. Etant donné le grand nombre de combinaisons d'hémagglutinine et de neuraminidase et l'isolement de deux virus grippaux A différents chez un même canard, il se pourrait qu'une recombinaison se produise dans la nature.

REFERENCES

1. BUTTERFIELD, W. K. & GRAVES, J. H. *Avian diseases*, **19**: 267 (1975).
2. BUTTERFIELD, W. K. ET AL. *Avian diseases*, **17**: 155 (1973).
3. COLEMAN, M. T. ET AL. *Lancet*, **2**: 1384 (1968).
4. DOWNIE, J. C. & LAVER, W. G. *Virology*, **51**: 259 (1973).
5. EASTERDAY, B. C. *In:* Kilbourne, E. D., ed. The influenza viruses and influenza. New York, Academic Press, 1975, p. 449.
6. FAZEKAS DE ST GROTH, S. & WEBSTER, R. G. *Journal of experimental medicine*, **124**: 331 (1966).
7. HIGGINS, D. A. & SCHILD, G. C. *Bulletin of the World Health Organization*, **47**: 531 (1972).
8. JOHNSON, D. C. ET AL. *Journal of the American Veterinary Medical Association*, **169**: 1138 (1976).
9. KAPLAN, M. & BEVERIDGE, W. I. B. *Bulletin of the World Health Organization*, **47**: 439 (1972).
10. LAVER, W. G. *In:* Habel, K. & Salzman, N. P., ed. Fundamental techniques in virology. New York, Academic Press, 1969, p. 371.
11. LAVER, W. G. & WEBSTER, R. G. *Virology*, **51**: 383 (1973).
12. PALMER, D. F. ET AL. Advanced laboratory techniques for influenza diagnosis. Washington, U.S. Department of Health, Education and Welfare, 1975 (Immunology Series No. 6).
13. TUMOVA, B. & EASTERDAY, B. C. *Bulletin of the World Health Organization*, **41**: 429 (1969).
14. WEBSTER, R. G. *In:* Selby, P., ed. Influenza: virus, vaccines, and strategy. New York, Academic Press, 1976, p. 33.
15. WEBSTER, R. G. & CAMPBELL, C. H. *Virology*, **48**: 528 (1972).
16. WEBSTER, R. G. & CAMPBELL, C. H. *Virology*, **62**: 404 (1974).
17. WEBSTER, R. G. ET AL. *Bulletin of the World Health Organization*, **51**: 325 (1974).
18. WEBSTER, R. G. ET AL. *Journal of general virology*, **32**: 217 (1976).
19. *Bulletin of the World Health Organization*, **45**: 119 (1971).
20. *Bulletin of the World Health Organization*, **48**: 199 (1973).