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Isolation and Serological Characterization of Influenza A Viruses From Birds That Were Dead on Arrival at Tokyo Airport

By

K. Nerome[1], M. Nakayama[1], M. Ishida[1], H. Fukumi[1], W. K. Butterfield[2], R. G. Webster[3], and C. H. Campbell[2]

[1] Department of Virology and Rickettsiology, National Institute of Health, Shinagawa-ku, Tokyo, Japan
[2] The Plum Island Animal Disease Laboratory, Agriculture Research Service, U.S. Department of Agriculture, Greenport, New York, U.S.A.
[3] Division of Virology, St. Jude Children's Research Hospital, N. Lauderdale, Memphis, Tennessee, U.S.A.

With 2 Figures

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Summary

Twenty-two strains of influenza A virus were isolated from caged birds which had been imported into Japan from India and Thailand and had died during transportation to Tokyo.

Serological tests divided these strains into two groups. Viruses in the first group contained Hav7 hemagglutinin and were related antigenically to A/duck/Ukraine/1/63 [Hav7 Neq2]; viruses in the second group contained Hav4 hemagglutinin and were related to A/duck/Czech/56 (Hav4 Nav1]. All strains contained Neq2 neuraminidase that was closely related to that of A/equine/Miami/1/63 [Heq2 Neq2] and A/duck/Ukraine/1/63 [Hav7 Neq2]. It was conlcuded that the strains in the first group were Hav7 Neq2 and those in the second group were Hav4 Neq2; both groups of viruses showed antigenic drift from the prototype strains.

Introduction

The mechanism by which new strains of human influenza virus arise is not fully understood. The results of recent studies showed that various serological types of influenza A virus are distributed in domestic and wild birds (6—8, 10 to 13, 18, 20, 21, 24, 25, 29, 30, 33). Both direct and indirect evidence suggests that birds may play an important role in the epidemiology of human influenza, especially in the appearance of new subtypes. There are two suggested alternatives; the first is that new pandemic strains might originate from animals and birds which

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act as reservoirs of influenza viruses and these viruses may subsequently become adapted to man (2, 11); the second is that recombination between human and animal influenza viruses might give rise to new strains, the surface antigens of which are derived from animal influenza virus strains, while other biological characteristics such as infectivity, pathogenicity and virulence, come from human viruses (14, 18). There is an increasing body of evidence that supports the second suggestion, especially the fact that several experiments have revealed that frequent recombinations occur *in vivo* between different types of influenza A viruses of avian, swine and human origin in mixedly infected hosts (26, 27, 28).

The hemagglutinin antigen of the Hong Kong influenza virus which suddenly appeared in man in 1968 was characterized in immunological tests and peptide analysis; it was shown that Hong Kong influenza virus possesses a hemagglutinin subunit which has a partially common structure with those of some strains isolated from birds and horses in 1963 (5, 12, 14, 24). From the above findings, it was suggested that the avian influenza viruses containing surface antigens related to those of human strains may be possible progenitors of human pandemic strains.

In the present paper we describe the antigenic properties of hemagglutinin (HA) or neuraminidase (NA) antigens of 22 influenza A strains isolated from mynah birds *(Gracula religiosa)* and banded parakeets *(Psittacula alexandria faciata)*.

Materials and Methods

Virus Strains

The following virus strains were used: A/swine/Iowa/15/30 [Hsw 1 N 1], A/equine/Prague/1/56 [Heq 1 Neq 1], A/equine/Miami/1/63 [Heq 2 Neq 2], A/PR/8/34 [H 0 N 1], A/FM/1/47 [H 1 N 1], A/RI/5+/57 [H 2 N 2], A/Aichi/2/68 [H 3 N 2], A/chicken/Germany "N"/49 [Hav 2 N 1], A/duck/England/56 [Hav 3 Nav 1], A/duck/Czech/56 [Hav 4 Nav 1], A/tern/S. Africa/61 [Hav 5 Nav 2], A/turkey/Mass/65 [Hav 6 N 2], A/duck/Ukraine/1/63 [Hav 7 Neq 2] and A/turkey/Ontario/6118/68 [Hav 8 Nav 4]. Influenza type B (B/Gifu/2/73) and C (Taylor/1233) viruses were also employed in serological tests. All strains except influenza C virus were propagated in the allantoic cavity of 11-day-old fertile hen's eggs; influenza C virus was propagated in the amniotic cavity of 8-day-old fertile hen's eggs.

Antisera

Antisera for characterization of the hemagglutinin (HA) were prepared in rabbits and chickens by repeated intravenous and intradermal injections of either purified virions or HA fractions derived from them. HA of the isolates No. 7 and 23 were released from purified virions with sodium dodecyl sulfate (SDS). After the removal of SDS, HA was adsorbed to chicken erythrocytes. The erythrocytes coated with HA were injected intravenously into chickens (3, 16). Antisera to Hav 7 and Neq 2 had been prepared by R.G.W. by the method described elsewhere (29, 30). Antiserum to the ribonucleoprotein (RNP) of influenza A viruses, was kindly provided by Dr. A. Sugiura from the Institute of Public Health, Tokyo, and had been prepared by immunizing rabbits with RNP derived from detergent-disrupted NWS virions (22). For complement fixation (CF) tests for influenza B and C viruses, ferret and human convalescent sera were used, respectively.

Virus Isolation

Respiratory organs, including trachea and lungs, as well as cloacal tissue, were removed from dead birds and were ground into 50 per cent suspension in broth containing antibiotics and inoculated into the amniotic cavity of 9-day-old fertile hen's

eggs. After incubation at 34° C for 3 days, amniotic and allantoic fluids were harvested and tested for hemagglutinating activity.

Serological Tests

Hemagglutination inhibition (HI) tests were performed as described (9) but in reduced volumes in microtiter plates. Sera were treated with RDE before the test (4).

For neuraminidase inhibition (NI) tests, the method recommended by the WHO Expert Committee was followed (32).

CF tests were done in checkerboard titrations by the method described (15).

Double immunodiffusion tests were carried out in 1 per cent agarose in 0.01 M phosphate-buffered saline containing 0.1 per cent sodium azide but the salt concentration was increased to 8 per cent. Virions disrupted with 0.5 per cent SDS were used as antigen.

Cell Culture and Plaque Formation

Infectivity assay was carried out by plaque assay in an established line of canine kidney (MDCK) cells as previously described (23).

Electron Microscopy

The structure of viruses was examined by electron microscopy in a Hitachi H-500 type by staining of virus with phosphotungstic acid as described previously (17).

Results

Isolation of Influenza Viruses From Birds

During the period from May to August, 1976, over 200 birds that were dead on arrival at the Tokyo Branch of the Animal Quarantine Service were tested for the presence of virus. These birds had been captured in India and Thailand, held for an unknown period, and then shipped by aeroplane.

Many hemagglutinating agents were isolated from the respiratory organs. Among them, 22 strains grew in the allantoic cavity of fertile hen's eggs as well as in MDCK cells. They possessed neuraminidase (NA) activity when tested with fetuin as substrate. Electron microscopic examination of several isolates showed particles morphologically similar to influenza virus.

Serological Characterization

RNP Antigen

RNP serotype was determined by CF tests. None of the isolates reacted with sera convalescent from influenza B and C, but they all reacted with the antiserum to influenza A virus RNP. In double immunodiffusion tests against antiserum to influenza A-RNP, the strains tested formed a precipitin line which was continuous with the line formed between A/Aichi/2/68 and the reference serum (Fig. 1 A). All 22 isolates, therefore, were influenza A viruses.

Hemagglutinin Antigen

Table 1 shows the results of cross HI tests with antisera to either whole virions or the purified HA of prototype strains and newly isolated viruses. Antisera to two isolates (No. 7 and 23) delineated all 22 isolates into two groups. The HA of one group, comprising 14 strains (Nos. 7, 9, 11, 67, 73, 81, 125, 127, 129, 131, 133, 137, 169, 179), was related, to various extent, to Hav7 contained in A/duck/Ukraine/1/63. The HA of these isolates also cross-reacted with antisera to Aichi/

2/68 and equine/Miami/1/63. The cross-reaction between the hemagglutinin from isolate No. 7 and H3 was asymmetrical, as shown by strong inhibition of the latter by the antiserum to the former. This was also the case between Hav7 and H3.

Several of the isolates in this group showed antigenic relationships with the three prototype strains (duck/Ukraine/1/63, Aichi/2/68, equine/Miami/1/63) both in HI tests and in double immunodiffusion tests. The hemagglutinin of Nos. 7, 9, 11 and 179 viruses formed a continuous precipitin line with duck/Ukraine/1/63 when tested against Hav7 serum. Spurs in Figures 1 b and 1 c indicated partial identity of these strains with Aichi/2/68 and equine/Miami/1/63. It was concluded, therefore, that the viruses in this group contained antigenic determinants of the Hav7 subtype.

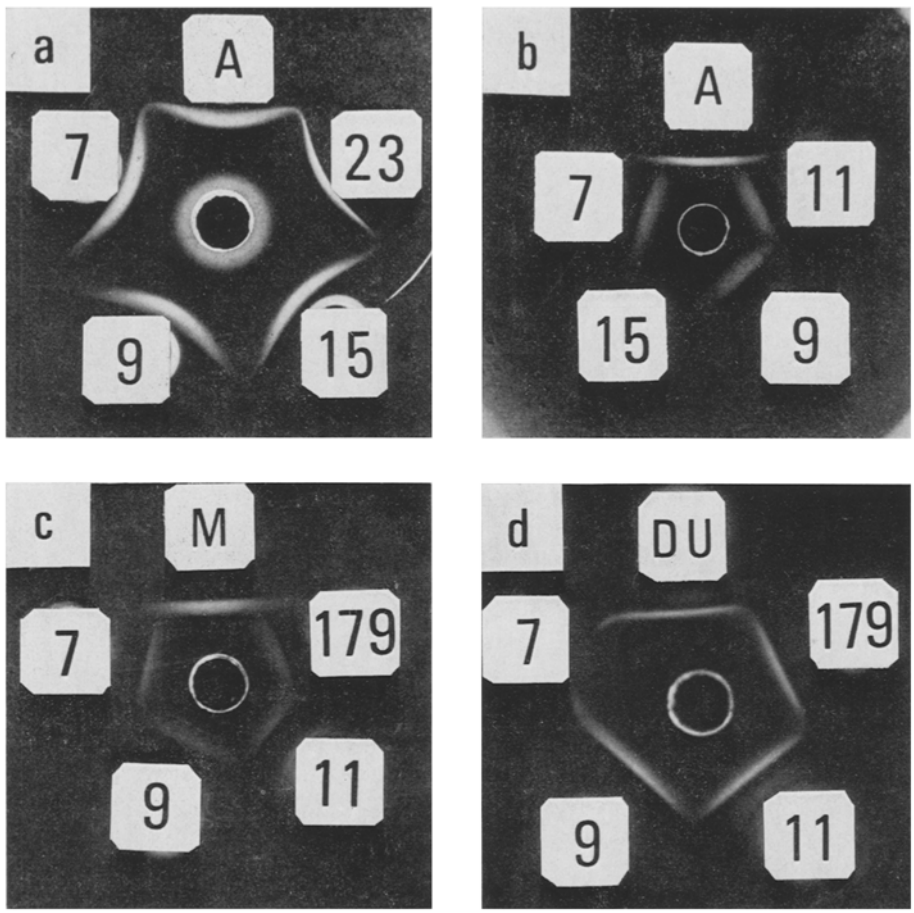

Fig. 1. Double immunodiffusion reactions of influenza virus isolates and prototype strains

Center wells contain antisera to: (a) ribonucleoprotein of A/NWS; (b) hemagglutinin of A/Aichi/2/68 strain [H3]; (c) hemagglutinin of A/equine/Miami/1/63 [Heq2]; (d) hemagglutinin of A/duck/Ukraine/1/63 [Hav7]. The outer wells contain viruses: (A) A/Aichi/2/68 [H3N2]; (M) A/equine/Miami/1/63 [Heq2 Neq2]; (DU) A/duck/Ukraine/1/63 [Hav7 Neq2]; and virus isolates numbers 7, 9, 11, 15, 23 and 179

The second group consisted of eight isolates (Nos. 15, 23, 87, 89, 91, 93, 95 and 97). All eight strains reacted in a similar way with antiserum prepared to the HA of No. 23 virus. However, the extent of cross-reaction with the antiserum to duck/Czech/56 varied from strain to strain (Table 1).

Table 1. *Hemagglutination inhibition (HI) and neuraminidase inhibition (NI) tests with the isolates and prototype strains of influenza viruses*

	HI tests						
	Antisera to						NI tests
Test virus	Aichi/2/68[a]	eq/Miami/1/63[a]	Hav 7[b]	duck/Czech/56[a]	7-HA[c]	23-HA[c]	Neq 2[d]
A/Aichi/2/68 [H 3 N 2]	4,096	512	16,384	<*	16,384	<	<8
A/equine/Miami/1/63 [Heq 2 Neq 2]	<	4,096	512	<	256	<	4,096
A/duck/Ukraine/1/63 [Hav 7 Neq 2]	128	64	4,096	<	128	<	8,192
A/duck/Czech/56 [Hav 4 Nav 1]	<	<	<	2,048	<	512	<8
Isolate Number							
7	64	128	1,024	<	1,024	<	2,048
9	64	64	1,024	<	1,024	<	8,192
11	64	64	1,024	<	1,024	<	8,192
67	64	32	32	<	1,024	<	8,192
73	32	<	64	<	512	<	8,192
81	32	<	128	<	1,024	<	2,048
125	64	32	256	<	1,024	<	8,192
127	64	<	128	<	1,024	<	2,048
129	128	<	256	<	1,024	<	8,192
131	128	<	128	<	1,024	<	2,048
133	64	<	256	<	1,024	<	8,192
137	32	<	256	<	1,024	<	8,192
169	32	32	128	<	512	<	2,048
179	64	64	512	<	1,024	<	8,192
15	<	<	<	256	<	4,096	8,192
23	<	<	<	512	<	4,096	8,192
87	<	<	<	32	<	4,096	8,192
89	<	<	<	128	<	4,096	2,048
91	<	<	<	128	<	4,096	8,192
93	<	<	<	256	<	4,096	8,192
95	<	<	<	32	<	4,096	8,192
97	<	<	<	32	<	4,096	8,192

* < = less than 32
[a] Rabbit antiserum to whole virion
[b] Rabbit antiserum to purified hemagglutinin
[c] Chicken antiserum to purified hemagglutinin
[d] Rabbit antiserum to purified neuraminidase

Double immunodiffusion tests with antiserum to the No. 23 hemagglutinin suggested that viruses of this group contained at least two antigenic determinants, one of which was common to the hemagglutinin of duck/Czech/56 (Fig. 2). Reciprocal tests using antiserum to duck/Czech/56 failed to reveal the antigenic rela-

tionship between duck/Czech/56 and the second group of isolates, probably because the antiserum employed was not potent enough. The antigenic relatedness between duck/Czech/56 and isolate No. 23 led us to the interpretation that the viruses of this group contained Hav4, but that their hemagglutinins had drifted antigenically from the original prototype strain of Hav4.

Neuraminidase Antigen

The neuraminidase activity of all 22 isolates was inhibited by antiserum to Neq2, but not by antisera to N1, N2, Nav1, Nav2, Nav4 and Neq1. The identity of their neuraminidases with Neq2 was also demonstrated by double immunodiffusion tests (Fig. 2d).

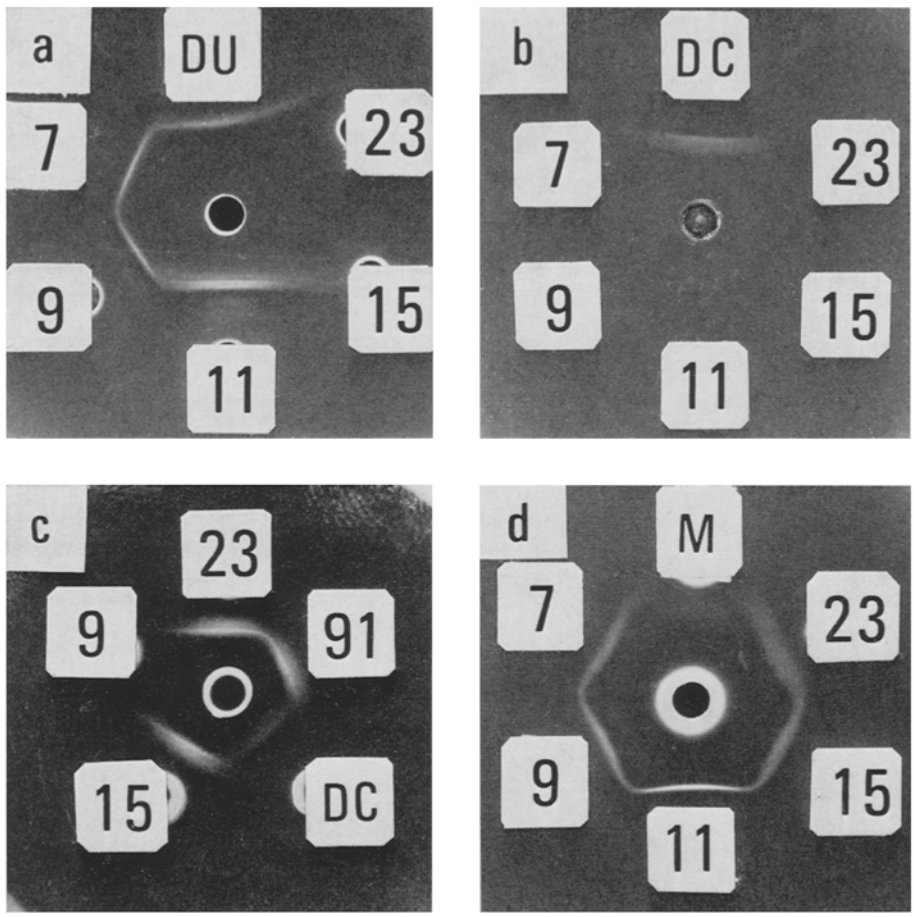

Fig. 2. Double immunodiffusion reactions of influenza virus isolates and prototype strains

Center wells contain antisera to: (a) hemagglutinin of A/duck/Ukraine/1/63 [Hav7]; (b) hemagglutinin of A/duck/Czech/56 [Hav4]; (c) hemagglutinin of 23; (d) neuraminidase of A/equine/Miami/1/63 [Neq2]. The outer wells contain viruses: (DU) A/duck/Ukraine/1/63 [Hav7 Neq2]; (DC) A/duck/Czech/56 [Hav4 Nav1]; (M) A/equine/Miami/1/63 [Heq2 Neq2]; and virus isolates numbers 7, 9, 11, 15, 23 and 91

Discussion

Twenty-two virus strains isolated from birds imported into Japan from Thailand and India during a 3-month period in 1976, were classified as Hav7 Neq2 and Hav4 Neq2. The antigenic composition of the former group was the same as duck/Ukraine/1/63. The virus of the latter combination has previously been reported from Japan, but some of us (W.K.B., R.G.W., and C.H.C.) have recently isolated virus strains of this antigenic type from birds in Hong Kong (19). This strain might have been generated recently as a result of genetic recombination (26, 29) or may have escaped detection until this time. The HA of both kinds of isolates, although related to Hav4 and Hav7, show antigenic drift from the prototype strains. Antigenic drift in avian influenza viruses has recently been described for the Hav6 subgroup by WEBSTER *et al.* (31). This report shows that antigenic drift occurs in the Hav4 and Hav7 subtypes and may well occur among all avian influenza strains.

In contrast, the NA of both types of isolates was Neq2 and was antigenically closely related to the two prototype strains, equine/Miami/1/63 and duck/Ukraine/1/63. Antigenic drift in the NA might be more gradual than in the HA, for all of the neuraminidase antigens on the human strains prevalent from 1957 to 1977 are of a single type [N2] and antigenic drift has only been apparent since 1972.

Table 2. *Source of influenza A viruses used in this study*

Isolate number	Country of origin	Species of bird	Tissues[a]	Antigenic type		
				Type	HA	NA
7	India	Mynah	R	A	av7	eq2
9	India	Mynah	R	A	av7	eq2
11	India	Mynah	R	A	av7	eq2
15	Thailand	Mynah	R	A	av4	eq2
23	Thailand	Mynah	R	A	av4	eq2
67	India	Banded Parakeet	R	A	av7	eq2
73	India	Banded Parakeet	R	A	av7	eq2
81	India	Banded Parakeet	R	A	av7	eq2
87	Thailand	Mynah	R	A	av4	eq2
89	Thailand	Mynah	R	A	av4	eq2
91	Thailand	Mynah	R	A	av4	eq2
93	Thailand	Mynah	R	A	av4	eq2
95	Thailand	Mynah	R	A	av4	eq2
97	Thailand	Mynah	R	A	av4	eq2
125	India	Mynah	R	A	av7	eq2
127	India	Mynah	R	A	av7	eq2
129	India	Mynah	R	A	av7	eq2
131	India	Mynah	R	A	av7	eq2
133	India	Mynah	R	A	av7	eq2
137	India	Mynah	R	A	av7	eq2
169	Thailand	Mynah	R	A	av7	eq2
179	Thailand	Mynah	R	A	av7	eq2

[a] R = respiratory organ including trachea and lung

Table 2 summarizes the antigenic type of the isolates, species of hosts, the tissues from which the viruses were isolated, and the countries from which the birds originated. Apparently species boundaries do not exist in the distribution of these viruses, for both types of viruses were found in parakeets as well as in mynahs. On the other hand, the geographical prevalence may be different for the two types of viruses. Birds from India yielded only one type (Hav7 Neq2], while those from Thailand yielded both types [Hav7 Neq2 and Hav4 Neq2]. Recently 42 strains of influenza A virus were isolated from dead birds originating from India and all of the virus strains were characterized as A/duck/Ukraine/1/63 [Hav7 Neq2] (1). This evidence obviously coincides with our results. Whether these results reflect differt epizootiological backgrounds in the two countries must await further study.

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Authors' address: Dr. K. NEROME, Department of Virology and Rickettsiology, National Institute of Health, 10-35, 2-Chome, Shinagawa-ku, Tokyo, Japan.

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