Bull. Org. mond. Santé
Bull. Wld Hlth Org. } 1973, **49**, 559-566

Characterization and ecology of a type A influenzavirus isolated from a shearwater

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An influenzavirus isolated from a shearwater bird nesting on Tryon Island on the Australian Great Barrier Reef in 1971 has been more extensively characterized. Haemagglutinin subunits were isolated from the shearwater virus and from the antigenically related avian influenzaviruses A/turkey/Mass./65(Hav6N2) and A/duck/Penn./69(Hav6N1). Maps of the tryptic peptides from the heavy polypeptides (HA1) of the haemagglutinin subunits of the three viruses showed a number of differences, but peptide maps of the light polypeptides (HA2) were almost identical, suggesting that these had almost the same amino acid sequence. Extensive tests confirmed that the neuraminidase of the shearwater virus was not related antigenically to any known neuraminidase. The sera collected from pelagic birds nesting on islands in the Capricorn–Bunker group in 1970 were devoid of any antibodies to the shearwater virus, while a high proportion of the sera collected from birds on the same islands in 1972 (one year after the isolation of the shearwater virus) had antibodies to the haemagglutinin and neuraminidase of the shearwater virus, some to a high titre. Thus, the shearwater virus appeared to have only recently been introduced into the area from where it was isolated.

Pandemics of type A influenza are caused by "new" viruses, which appear at intervals of 10–15 years in the human population, and the evidence suggests that these new viruses may be formed by genetic recombination between established human influenzaviruses and animal or avian strains of influenzavirus type A (for a review, see Webster (13)). Consequently, the antigens of future human pandemic influenzaviruses may already exist in nature in viruses infecting animals or birds, both wild and domesticated.

Previous studies (2, 9) showed that sera from pelagic birds nesting on islands on the Great Barrier Reef off the eastern Australian coast contained antibody directed specifically against the neuraminidase of the A/Asian/57/(H2N2) strain of the human influenzavirus, suggesting that the birds had been infected with a virus carrying this antigen. Attempts to isolate this virus from the birds have so far been unsuccessful, but a type A influenzavirus was isolated from an apparently healthy shearwater bird, which had haemagglutinin subunits of subtype Hav6 and neuraminidase subunits antigenically unrelated to those of any known influenzavirus (3).

This paper describes a more detailed characterization of the haemagglutinin and neuraminidase subunits of the shearwater virus and the distribution of antibodies to this virus in the sera from pelagic birds.

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MATERIALS AND METHODS

Viruses

The isolation of the shearwater virus, A/shearwater/E.Aust./1/72(Hav6Nav5), has already been de-

scribed (3). Other type A influenzaviruses of human, animal, or avian origin and the strains of influenza type B viruses were from the collection of the World Influenza Centre in London.

Sera

" Monospecific " sera to the isolated haemagglutinin and neuraminidase subunits of the shearwater virus were prepared as already described (3), while sera to other type A and type B influenzaviruses were prepared by standard methods. Some sera were also collected from wedge-tailed shearwaters (*Puffinus pacificus*) and noddy terns (*Anous minutus*), nesting on islands in the Capricorn and Bunker groups on the Great Barrier Reef. The sera were stored without refrigeration for periods of up to 3 weeks before they were tested, sodium azide being added to prevent bacterial growth.

Serologic tests

Haemagglutinin-inhibition (HI) tests were carried out by the method of Fazekas & Webster (4), and neuraminidase-inhibition (NI) tests were carried out as described by Dasen & Laver (2). Immuno-double-diffusion tests were performed as described by Schild & Pereira (12).

Isolation and peptide mapping of the haemagglutinin polypeptides

The haemagglutinin subunits of the shearwater virus were isolated from a recombinant virus containing these subunits and A/Bel/42 virus neuraminidase. The virus particles were disrupted with sodium dodecyl sulfate (SDS) and the haemagglutinin subunits were isolated by electrophoresis on cellulose acetate strips as described by Downie & Laver (3).

Haemagglutinin subunits were isolated in a similar way from particles of A/duck/Penn./69 virus and from a recombinant virus containing A/turkey/Mass/65 haemagglutinin and A/Bel/42 neuraminidase. A/duck/Penn./69 virus contained neuraminidase subunits, which were denatured by SDS at room temperature; consequently it was not necessary to segregate genetically the haemagglutinin and neuraminidase subunits before isolating the former electrophoretically from the SDS-disrupted virus particles.

The heavy and light polypeptides (HA1 and HA2) of the haemagglutinin subunits from the 3 viruses were separated by centrifugation on guanidine hydrochloride–dithiothreitol density gradients (8). These were then digested with trypsin, and the peptides soluble at pH 6.5 were mapped by two-dimensional electrophoresis and chromatography (7).

RESULTS

Antigenic relationship of the shearwater virus with other influenzaviruses

Antisera to the isolated haemagglutinin and neuraminidase subunits of the shearwater virus were tested in double-immuno-diffusion tests with a number of influenzaviruses and the results are summarized in Tables 1 and 2. The only viruses to react with antiserum to the haemagglutinin of the shearwater virus were those of subtype Hav6 (Table 1) and none reacted with antiserum to the shearwater neuramini-

Table 1. Reactions in double immuno-diffusion tests of rabbit antiserum to purified haemagglutinin of A/shearwater/EA/1/72

Isolate	Test virus	Double immuno-diffusion reaction
human	A/PR8/34(HON1)	0
	A/FM1/47(H1N1)	0
	A/Singapore/1/57(H2N2)	0
	A/Hong Kong/1/68(H3N2)	0
	B/Lee/40	0
	B/Hong Kong/8/73	0
porcine	A/swine/Iowa/15/30(Hsw1N1)	0
equine	A/equine/Prague/56(Heq1Neq1)	0
	A/equine/Miami/63(Heq2Neq2)	0
avian	A/shearwater/EA/1/72	+
	A/FPV/Dutch/27(Hav1Neq1)	0
	A/chick/Germany'N'/49(Hav2Neq1)	0
	A/chick/England/56(Hav3Nav1)	0
	A/duck/Czech/56(Hav4Nav1)	0
	A/tern/S. Africa/61(Hav5Nav2)	0
	A/chick/Scotland/59(Hav5N1)	0
	A/turkey/Mass/65(Hav6N2)	+[a]
	A/duck/Penn./69(Hav6N1)	+[a]
	A/turkey/Canada/63(Hav6Neq2)	+[a]
	A/duck/Ukraine/1/63(Hav7Neq2)	0
	A/turkey/Ont/6118/68(Hav8Nav4)	0

[a] The precipitin lines given by turkey/Mass/65, duck/Penn./69, and turkey/Canada/63 showed continuity with that of A/shearwater/1/72 virus.

dase (Table 2 and Fig. 1). The absence of any antigenic relationship between the neuraminidase of the shearwater virus and that of other known influenzaviruses was confirmed by neuraminidase-inhibition tests (Table 3). On the basis of these tests the neuraminidase of the shearwater virus was assigned to a new subtype, Nav5.

Peptide maps

Maps of the tryptic peptides of the heavy and light polypeptides (HA1 and HA2) of the haemagglutinin subunits of the shearwater virus and of the avian influenzaviruses of subtype Hav6 (A/turkey/Mass/65 and A/duck/Penn./69) are shown in Fig. 2 and 3. These were obtained by electrophoresis at pH 6.5, followed by ascending chromatography in a mixture of pyridine, isoamyl alcohol, and water (35 : 35 : 30). The peptides were stained with ninhydrin. The maps of the heavy chains from these 3 viruses show a number of differences, but the maps of the light chains are almost identical suggesting that the light polypeptides of the haemagglutinin subunits from the 3 viruses had almost the same amino acid sequences.

Table 2. Reactions in double immuno-diffusion tests of rabbit antiserum to purified neuraminidase of A/shearwater/EA/1/72

Isolate	Test virus	Double immuno-diffusion reaction
human	A/PR8/34(H0N1)	0
	A/FM1/47(H1N1)	0
	A/Singapore/1/57(H2N2)	0
	A/Hong Kong/1/68(H3N2)	0
	B/Lee/40	0
	B/Hong Kong/8/73	0
porcine	A/swine/Iowa/15/30(Hsw1N1)	0
equine	A/equine/Prague/56(Heq1Neq1)	0
	A/equine/Miami/63(Heq2Neq2)	0
avian	A/shearwater/EA/1/72	+
	A/FPV/Dutch/27(Hav1Neq1)	0
	A/chick/Germany'N'/49(Hav2Neq1)	0
	A/chick/England/56(Hav3Nav1)	0
	A/duck/Czech/56(Hav4Nav1)	0
	A/tern/S. Africa/61(Hav5Nav2)	0
	A/chick/Scotland/59(Hav5N1)	0
	A/turkey/Mass/65(Hav6N2)	0
	A/duck/Penn/69(Hav6N1)	0
	A/turkey/Canada/63(Hav6Neq2)	0
	A/duck/Ukraine/1/63(Hav7Neq2)	0
	A/turkey/Ont/6118/68(Hav8Nav4)	0

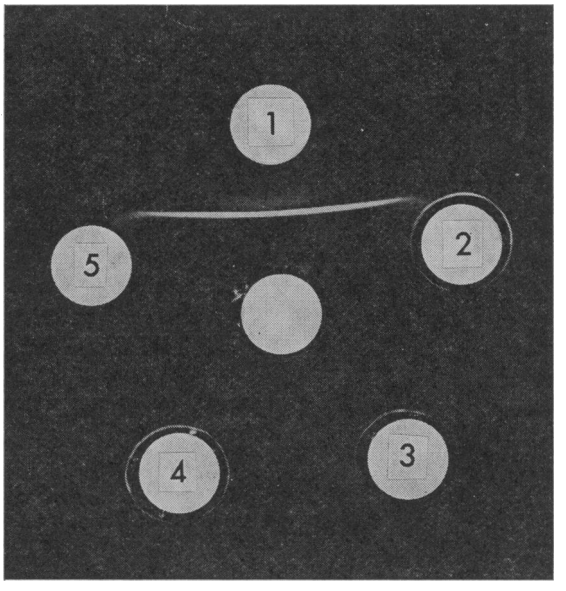

Fig. 1. Double-immunodiffusion tests showing the reaction between antibody to the shearwater virus neuraminidase and various representative avian influenzaviruses. The well in the centre contained the antiserum to shearwater virus neuraminidase and the surrounding wells contained virus particles disrupted with sarkosyl. (1) A/shearwater/EA/72; (2) A/tern/SA/61; (3) A/chicken/Scot/56; (4) A/turkey/Mass/65; (5) A/duck/ Penn./69.

Antibodies to the shearwater virus in sera from pelagic birds

Sera were collected from 164 shearwaters and 57 noddy terns nesting on islands in the Capricorn and Bunker groups in December 1970, and from 595 shearwaters and 96 noddy terns nesting on the same islands in December 1972. These sera were tested for antibodies to the haemagglutinin and neuraminidase of the shearwater virus, which was isolated from a tracheal swab taken from a bird on Tryon Island in December 1971.

Table 3. Antigenic character of the neuraminidase of A/shearwater/EA/1/72 virus by neuraminidase-inhibition tests

Influenzavirus (type A)	Hyperimmune serum to	NI_{50} titre with homologous virus	NI_{50} titre with A/shearwater/1/72
avian	A/FPV/Rostock/34(Hav1N1)	150	< 10
	A/chicken/Scotland/59(Hav5N1)	500	< 10
	A/duck/Germany/210/67(Hav4N1)	> 1 000	< 10
	A/duck/Germany/1868/68(Hav6N1)	300	< 10
	A/duck/Pennsylvania/486/69(Hav6N1)	500	< 10
	A/turkey/Mass/65(Hav6N2)	250	20
	A/FPV/Dutch/27(Hav1Neq1)	300	< 10
	A/chicken/Germany/N/49(Hav2Neq1)	> 1 000	< 10
	A/turkey/Canada/63(Hav6Neq2)	> 1 000	< 10
	A/quail/Italy/1117/65(Hav2Neq2)	500	< 10
	A/duck/Ukraine/1/63(Hav7Neq2)	200	< 10
	A/duck/England/56(Hav3Nav1)	> 1 000	< 10
	A/duck/Czech/56(Hav4Nav1)	> 1 000	< 10
	A/duck/England/62(Hav4Nav1)	300	< 10
	A/tern/South Africa/61(Hav5Nav2)	800	< 10
	A/turkey/England/63(Hav1Nav3)	300	< 10
	A/turkey/Ontario/6118(Hav8Nav4)	800	< 10
	A/shearwater/EA/1/72(Hav6Nav5)	—	400
	A/shearwater/EA/1/72(anti-pure neuraminidase) [a]	—	1 000
mammalian	A/PR8/34(H0N1)	200	< 10
	A/Bel/42(H0N1)	400	< 10
	A/FM1/47(H1N1)	100	< 10
	A/Singapore/1/57(H2N2)	800	< 10
	A/Singapore/1/57(anti-pure neuraminidase) [a]	400	< 10
	A/Hong Kong/1/68(H3N2)	1 000	< 10
	A/England/42/72(H3N2)	1 500	< 10
	A/equine/Prague/56(Heq1Neq1)	500	< 10
	A/equine/Miami/1/63(Heq2Neq2)	500	< 10
	A/swine/Iowa/15/30(Hsw1N1)	300	< 10
	A/swine/Cambridge/39(Hsw1N1)	1 000	< 10

[a] The rabbit antisera were made against the purified neuraminidase subunits of A/shearwater/1/72 and A/Singapore/57 viruses isolated by electrophoretic separation of detergent-disrupted influenzaviruses on cellulose acetate strips (6).

Antiserum to the purified neuraminidase of A/shearwater/1/72 (NI_{50} titre 1 : 1 000) was tested against the other strains mentioned in the above table. In no case was an NI_{50} titre greater than 1 : 20 detected.

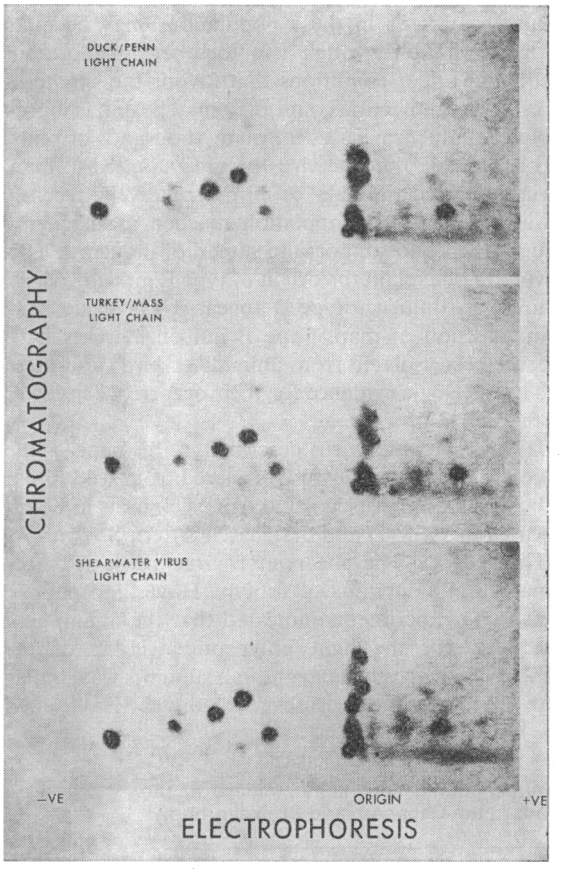

Fig. 2. Maps of the tryptic peptides (soluble at pH 6.5) from the light polypeptide chains of the haemagglutinin subunits of A/duck/Penn./69, A/turkey/Mass/65, and A/shearwater/E.Aust./72 viruses.

None of the sera collected in 1970 contained detectable antibodies to either the haemagglutinin or the neuraminidase of the shearwater virus, but a high proportion of sera collected in 1972, particularly those from noddy terns, contained antibodies to the shearwater virus antigens (Table 4). Many of the sera contained antibodies to both the haemagglutinin and neuraminidase of the shearwater virus, but some contained antibody to only one or other of these antigens (Table 4). Table 5 shows the haemagglutination-inhibition and neuraminidase inhibition titres for some of the sera that contained both kinds of antibody.

The distribution of antibodies in sera from different birds nesting on the various islands (Table 4) shows that a higher proportion of sera from noddy terns contained antibodies to the shearwater virus. The sera from shearwaters nesting on the same islands as the noddy terns also contained antibodies to the shearwater virus, but the sera from shearwaters nesting on islands without any noddy terns were practically devoid of antibodies to the shearwater virus.

These findings suggest that the shearwater virus was more likely a noddy tern virus, infecting and spreading readily in these latter birds. Shearwaters could be infected, but it appeared that they could not spread the virus readily.

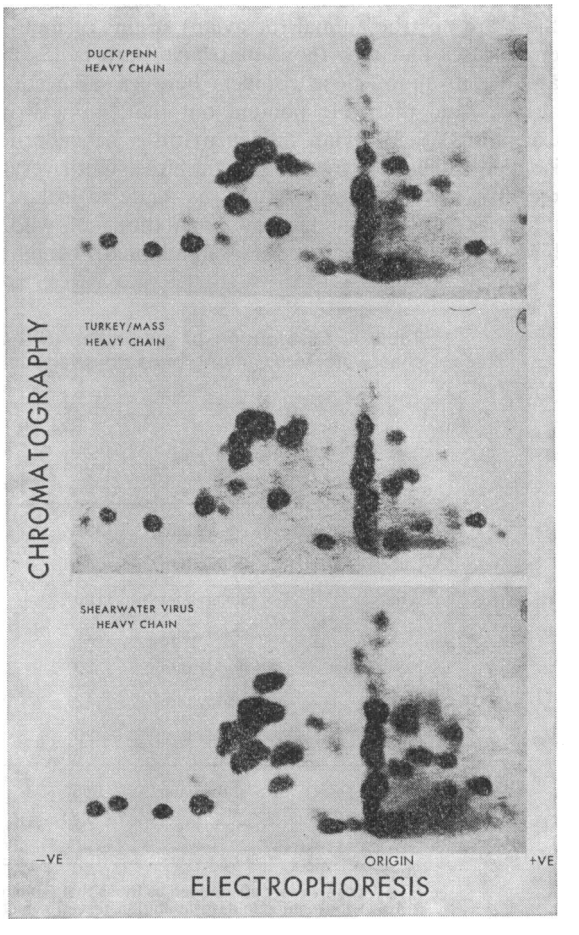

Fig. 3. Maps of the tryptic peptides (soluble at pH 6.5) from the heavy polypeptide chains of the haemagglutinin subunits of A/duck/Penn./69, A/turkey/Mass/65, and A/shearwater/E.Aust. /72 viruses.

DISCUSSION

The origin of the " new " type A influenzaviruses that cause the great human influenza pandemics has not been established. However there is evidence to indicate that at least one " new " human influenzavirus (A/Hong Kong/68) may have arisen as the result of genetic recombination between a human influenzavirus (the A/Asian strain) and an animal or avian influenzavirus related to A/duck/Ukraine/63(Hav7Neq2) and A/equine/Miami/63(Heq2Neq2) viruses (10, 11, 14).

Future pandemics could originate in a similar way and the surface antigens of future human pandemic viruses may already exist in influenzaviruses infecting animals or birds. Kilbourne (5) has suggested that there are no true animal (or avian) strains of influenzaviruses and that those that have been isolated are the result of close contacts between domestic animals and man. He pointed out that, with two exceptions (1, 3), avian influenzaviruses have been isolated exclusively from domestic birds. Until very recently, however, no attempt was made to isolate influenzaviruses from healthy populations of wild birds and the incidence of well-adapted, benign influenzaviruses in these populations may be quite high. Wild birds often live together in enormous colonies under conditions that favour the spread of respiratory infections, and they may have harboured type A influenzaviruses for many thousands of years. It is possible that the virus became adapted to man, via domestic animals or birds, relatively recently when the human population reached a sufficiently high density to support the spread of influenza. This would not explain the origin of type B influenzavirus, however. Influenza type B appears to be exclusively an infection of man. Type B influenzaviruses have never been isolated from animals or birds and there is no serologic evidence for their occurrence in lower creatures.

The shearwater virus described in this paper is the second influenzavirus to be isolated from wild birds; the first was a virus isolated from sick terns in South Africa in 1961 (1) and designated A/tern/S.Africa/61 (Hav5Nav2). The shearwater virus possessed haemagglutinin subunits of subtype Hav6, and peptide mapping experiments indicated that the amino acid sequence of the light polypeptide (HA2) of the shearwater virus haemagglutinin subunits was similar to that of other viruses of subtype Hav6, i.e.,

Table 4. Distribution of antibodies to the haemagglutinin and neuraminidase of the shearwater virus among birds from various islands in the Capricorn and Bunker groups [a]

Island [b]	Type of bird	No. of sera tested	No. of sera with antibody to:		
			haemagglutinin and neuraminidase	haemagglutinin only	neuraminidase only
Lady Musgrave	shearwater	297	3	0	13
Lady Musgrave	noddy tern	34	10	4	5
Wreck	shearwater	120	0	0	1
Tryon	shearwater	178	1	1	1
North-West	noddy tern	20	7	2	3
Fairfax	noddy tern	22	11	0	4
Hoskyn	noddy tern	20	3	2	5
total from all islands	shearwater	595	4	1	15
	noddy tern	96	31	8	17

[a] Positive sera are defined as those that inhibited the neuraminidase by 30 % or more at a dilution of 1/10 in the neuraminidase-inhibition test or which had a haemagglutination-inhibition titre of 1/20 or greater. Recombinant viruses which contained either the haemagglutinin or the neuraminidase of the shearwater virus were used in these tests to overcome the problem of "steric inhibition" by antibody encountered when the parental virus containing both antigens was used.

[b] Lady Musgrave island had large numbers of both shearwaters and noddy terns; Wreck and Tryon islands had many shearwaters but were devoid of noddy terns. Fairfax and Hoskyn islands had no shearwaters. North-West island had very large numbers of both noddy terns and shearwaters, but only the noddy terns were sampled on this island.

A/turkey/Mass/65(Hav6N2) and A/duck/Penn./69 (Hav6N1). The neuraminidase of the shearwater virus, on the other hand, was not related to that of either A/turkey/Mass/65 or A/duck/Penn./69 viruses, nor to that of any other known influenzavirus.

The serologic survey that was carried out suggested that the shearwater virus may have a greater capacity to infect and spread in noddy terns than in shearwaters. This would explain why very few of the shearwaters on Tryon Island (where the virus was isolated) had any antibodies to the shearwater virus, since very few noddy terns nest on that island. The isolation of this virus from a shearwater was therefore an extraordinarily fortuitous event. On the other hand, on Lady Musgrave Island which has both shearwaters and noddy terns in great abundance, a much higher proportion of the shearwaters had antibodies to the shearwater virus. About 50% of the noddy tern sera from all islands on the other hand had antibodies to the shearwater virus.

The shearwater virus may have been introduced by migratory birds into the pelagic birds nesting on the islands in the Capricorn and Bunker group sometime between 1970 and 1971. The virus was isolated from a bird on Tryon Island in December 1971, but sera taken from birds on adjacent islands in 1970 had no detectable antibodies to the shearwater virus. However, in 1972, a high proportion of sera collected from birds on the same islands had antibodies present. Many of these sera contained antibodies to both the haemagglutinin and the neuraminidase of the shearwater virus, but some possessed antibodies to only one of these antigens (Table 4). It seems likely that the occurrence of sera containing antibody to only one of the shearwater virus antigens was due to variation among individual birds in their response to the two antigens on the virus. It is also evident from the data in Table 5 that the sera from birds responding to both antigens contained widely different titres of antibodies to the haemagglutinin and neuraminidase of the shearwater virus. An alternative explanation is that, following the introduction of the shearwater virus by migratory birds into the Australian pelagic birds, genetic recombination may have occurred with avian influenzavirus already present in these birds. In this event the haemagglutinin and neuraminidase of the shearwater virus would be segregated into recombinant viruses and the sera containing only one kind of antibody could be the result of infection by these recombinants.

Table 5. Haemagglutination-inhibition and neuraminidase-inhibition titres of selected sera containing antibodies to both antigens of the shearwater virus [a]

Type of bird	Island	HI titre	NI titre
shearwater	Lady Musgrave	20	86
	Lady Musgrave	60	85
	Tryon	160	325
noddy tern	Lady Musgrave	120	50
	Lady Musgrave	640	10
	Fairfax	40	200
	Fairfax	60	25
	Fairfax	240	465
	Fairfax	120	140
	Hoskyn	120	250
	Hoskyn	400	1 000
	North-West	240	100
	North-West	240	45

[a] Titres are expressed as the reciprocals of the serum dilution giving 50 % inhibition of neuraminidase activity under the conditions of the test or showing 50 % inhibition of 4 haemagglutinating units of virus.

ACKNOWLEDGEMENTS

We thank the World Health Organization for financial support. Part of the work was also supported by US Public Health Service Research Grant AI-08831 from the National Institute of Allergy and Infectious Disease and by ALSAC. We thank also our many colleagues, who accompanied us to the Coral Islands of the Great Barrier Reef, for their help in catching the birds and in collecting the sera.

RÉSUMÉ

CARACTÉRISATION ET ÉCOLOGIE D'UN VIRUS GRIPPAL DE TYPE A ISOLÉ CHEZ UN PUFFIN

Les virus grippaux « nouveaux » responsables des pandémies de grippe chez l'homme peuvent avoir pour origine une recombinaison génétique entre des virus grippaux humains déjà en circulation et des souches grippales animales ou aviaires. Il est donc possible que les antigènes des virus grippaux qui provoqueront les futures pandémies

existent déjà dans la nature, ce qui justifie l'étude des virus grippaux infectant les oiseaux sauvages.

On a isolé en 1971 un virus grippal de type A chez un puffin nichant sur l'île Tryon dans la Grande Barrière australienne. L'hémagglutinine de ce virus était antigéniquement apparentée à celles des virus aviaires A/turkey/-Mass./65 (Hav6N2) et A/duck/Penn./69 (Hav6N1). Les cartes des peptides des chaînes lourdes de ces trois hémagglutinines étaient différentes à maints égards, mais les cartes des peptides des chaînes légères étaient quasi identiques suggérant une similitude presque complète des séquences d'amino-acides et confirmant les données immunologiques. Par contre, les épreuves d'inhibition de la neuraminidase ont montré que la neuraminidase du virus isolé chez le puffin étaient antigéniquement différente de toutes les neuraminidases connues.

Des sérums prélevés en 1970 chez des oiseaux marins nichant sur des îles de la région étaient dépourvus d'anticorps pour le nouveau virus récemment identifié, mais en 1972 (un an après l'isolement), une forte proportion des sérums recueillis dans les mêmes conditions donnaient des réactions positives, à des titres parfois élevés, avec l'hémagglutinine et la neuraminidase de ce virus. Il semble donc que ce dernier n'ait été introduit que récemment, probablement par des oiseaux migrateurs, dans la région où il a été isolé.

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