Arch Virol (1991) 118: 289–297

Archives of Virology

The first isolation of swine H1N1 influenza viruses from pigs in Thailand

Brief Report

S. Kupradinun[1], **P. Peanpijit**[1], **C. Bhodhikosoom**[1], **Y. Yoshioka**[2], **A. Endo**[2], and **K. Nerome**[2]

[1] Virus Research Institute, Department of Medical Sciences, 88/7 Soi Bamrasnaradura Hospital, Nonthaburi, Thailand

[2] Department of Virology and Rickettsiology, National Institute of Health, Shinagawa-ku, Tokyo, Japan

Accepted July 16, 1990

Summary. Two influenza A viruses were isolated from pigs in Thailand in January 1988 during the early febrile stage of an influenza-like illness. The isolates contained hemagglutinin and neuraminidase antigens related to those of swine H1N1 influenza virus. This result based on the virus isolation is compatible with the epizootiological evidence that, unlike the human influenza with peak activity in summer (May–July), swine influenza virus is prevalent in the winter season (November–January) in Thailand. The proportion of sera with hemagglutination-inhibiting antibody was higher to A/NJ/8/76 than to A/sw/Iowa/15/30. Likewise, hemagglutination-inhibition tests with monoclonal antibodies indicated that hemagglutinin antigen of the isolates was very similar to that of A/NJ/8/76 virus. In agreement with the serological survey and antigenic characteristic, genetic relatedness between the isolates from Thailand and A/NJ/8/76 virus was also demonstrated by the oligonucleotide mapping of RNA, suggesting that they may be of the same origin.

*

In January 1976, approximately 500 men were infected with influenza virus containing hemagglutinin (HA) and neuraminidase (NA) antigens similar to those of swine H1N1 virus [16–18]. Since then, ample evidence has been documented for the prevalence of H1N1 influenza virus among swine populations in many parts of the world [4, 7, 13]. In large scale serological surveillance of swine and genome characterization of the isolates, it was shown that swine H1N1 virus was introduced to Japan around May 1977 through pigs imported from the U.S.A. [7, 9]. Especially, a recent event in the U.S.A. that a pregnant woman died of infection with the swine influenza viruses [18] suggests the possibility of future appearance of this virus in man again.

A/Hong Kong (H3N2) influenza virus which suddenly appeared in man in 1968 in southern China is suggested to arise by genetic reassortment between human and avian influenza viruses [6, 11]. Subsequent virological surveillance of swine showed that numerous H3N2 variants, in addition of H1N1 viruses, have been conserved in the swine populations in Hong Kong [12–14]. Following the first isolation of a H1N2 reassortant from a pig in 1978 in Japan [8], the finding of another swine H1N2 reassortant in 1980 in Japan underlines the importance of pigs as potential reservoirs for future pandemics [10].

In the course of epizootiological surveillance of swine in Thailand, two A influenza viruses were isolated in 1988. In this communication, we will describe antigenic and genome characteristics of the isolates through the comparison with those of the reference swine H1N1 influenza viruses.

For virus isolation, nasal swabs were obtained from swine herds in the vicinity (Rachaburi, Chonburi) of Bangkok and placed in bacterial broth containing penicillin (500 U/ml), streptomycin (1 mg/ml), fungizon (0.03 mg/ml) and gentamycin (0.06 mg/ml). Virus isolation was peformed by intra-allantoic inoculation of 10-day-old embryonated hen's eggs.

For serological surveillance, a total of 217 sera were collected in swine herds in the vicinity of Bangkok in 1976. Before examination, all sera were treated as described previously [5].

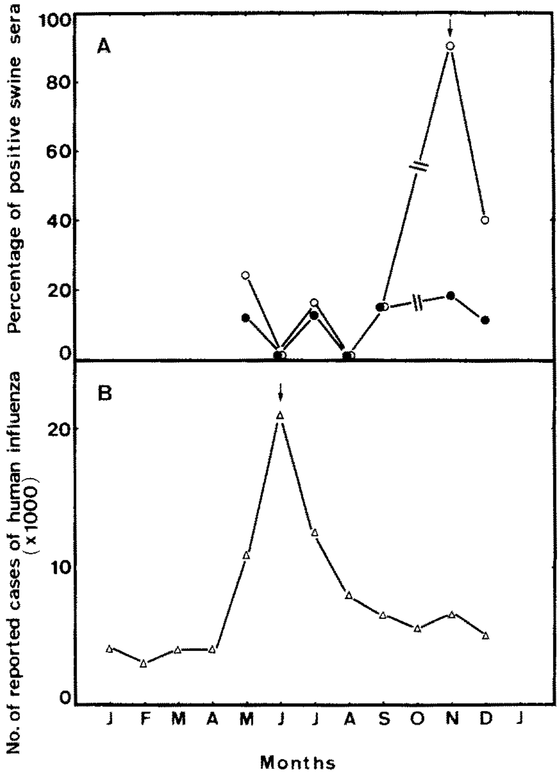

Fig. 1. Proportion of swine sera with HI antibodies of 64 or higher to two swine viruses from 1986 (**A**) and surveillance of human influenza based on the reported cases of influenza in Thailand in 1985 (**B**). In the serological surveillance an HI titer of 64 or greater was selected as positive sera and the percentages of sera with HI antibody were calculated. ○——○ Antibody titer to A/NJ/8/76; ●——● antibody titers to A/sw/Iowa/15/30. Arrow indicates a peak incidence of influenza

The following influenza A viruses were used: A/sw/Iowa/15/30 (H1N1), A/sw/North Ireland/38 (H1N1), A/sw/Cambridge/39 (H1N1), A/sw/Illinois/1/63 (H1N1), A/sw/Hong Kong/1/74 (H1N1), A/sw/Wisconsin/1/76 (H1N1), A/NJ/8/76 (H1N1), A/sw/Shizuoka/1/78 (H1N1), and A/Aichi/2/68 (H3N2). All viruses were grown in 11-day-old fertile hen's eggs and purified as described previously [8]. Hemagglutination-inhibition (HI) tests were done in disposable microtiter-U-plates using 0.5% chicken red blood cells. Neuraminidase-inhibition (NI) tests were performed according to the methods recommended by the WHO Expert Committee [1], but diluent contained 0.5% Triton X-100 to avoid steric hindrance by antibody to HA. Hyperimmune sera were prepared in rabbit by frequent intravenous injection of the purified viruses. Monoclonal antibodies to HA of A/NJ/8/76 were kindly provided by R. G. Webster.

The incidence of antibody with recent H1N1 virus (A/NJ/8/76) and the classical strain of swine H1N1 virus (A/sw/Iowa/15/30) in serum samples were examined in HI tests and results are shown in Fig. 1. There was obvious variation in the proportion of positive sera depending upon the month of collection. All sera collected between May and September gave a lower proportion of HI reaction with both the strains, A/NJ/8/76 and A/sw/Iowa/15/30, suggesting that this period (mainly summer) is not the major occurrence of swine virus infection. However, the peak incidence of HI distribution was detected in sera collected in November 1987. Similarly, the mean HI activity also tended to increase after October 1987 and reached the peak distribution in winter season (data not shown). In addition, the geometric mean HI titer and the proportion of sera with HI antibody were relatively higher to A/NJ/8/76 than to A/sw/Iowa/15/30, suggesting that A/NJ/8/76-like variants are prevalent in the swine populations in Thailand.

Even though the Division of Epidemiology, Ministry of Public Health issues the annual epidemiological surveillance report, the number of cases of influenza would be a good index of the prevalence of influenza infection in the community because there is a good correlation between the number of reported cases of influenza by month and number of virus isolation [14]. The number of reported cases of influenza in Thailand each month was also presented in Fig. 1, and it was shown that the peak incidence of seasonal outbreaks of influenza infection occurred in summer (May–June). This exhibited a sharp contrast to the outbreak of swine influenza, which was similar to that of human influenza in winter season in the temperate zone.

In order to confirm this from view of virological surveillance, nasal swabs were collected and subjected to the virus isolation. Two hemagglutinating agents were isolated from nasal swabs taken from pigs in Chonburi, located 90 km east of Bangkok in January, 1988. Antigenic characterization of the two isolates was done by HI tests with hyperimmune sera, post-infection ferret serum and monoclonal antibodies to reference strains of swine H1N1 viruses. The results were summarized in Table 1. Hemagglutinin antigens of the two isolates reacted to high titers with all the antisera used in the tests, suggesting that these viruses

Table 1. Antigenic characterization of the hemagglutinin and neuraminidase antigens of influenza A viruses isolated from swine in Thailand by HI and NI tests

	HI titers									NI titers	
	Antisera to				Monoclonal antibodies to A/NJ/8/76					Antisera to	
	sw/Iowa/30[a]	sw/Cam/39[a]	sw/Ill/63[a]	NJ/76[b]	6/1a	117/2	72/3	40/3	36/3	sw/Iowa/30[a]	Aichi/2/68[c]
Reference strains											
A/sw/Iowa/15/30 (H1N1)	*2,048*	128	1,024	32	1,000	1,600	200	–	400	512	<100
A/sw/Cambridge/39 (H1N1)	512	*32,768*	2,048	256	–	–	–	–	–	ND	ND
A/sw/Illinois/1/63 (H1N1)	1,024	256	*4,096*	64	1,024	2,048	1,024	128	1,024	ND	ND
A/NJ/8/76 (H1N1)	2,048	256	ND	*2,048*	4,096	4,096	2,048	512	512	ND	ND
A/Aichi/2/68 (H3N2)	ND	ND	ND	ND	ND	ND	ND	ND	ND	<4	*32,768*
Isolates											
A/sw/Bangkok/1/88	1,024	4,096	8,192	2,048	256	4,096	128	64	2,048	64	<100
A/sw/Bangkok/2/88	1,024	2,048	4,096	2,048	64	4,096	256	64	2,048	64	<100

[a] Hyperimmune rabbit serum

[b] Post-infection ferret serum

[c] Antiserum monospecific for the isolated neuraminidase

Values shown in italics represent the homologous antibody titers

– HI titer less than 12

ND Not done

contain hemagglutinin subunits similar to that of swine H1N1 viruses. However, it is difficult to interpret in detail the antigenic relationships between the isolates and the four reference strains used, particularly when HI tests were performed with hyperimmune sera.

In order to resolve this problem, five monoclonal antibodies to HA of A/NJ/8/76 were also employed in the tests. As shown in Table 1, HI patterns and HI titers of A/sw/Iowa/15/30 and A/sw/Cambridge/39 with monoclones were different from those of the isolates, especially the latter virus failed to react with all monoclones used. In contrast to this, two isolates from Thailand appeared to be similar in their reaction patterns to those of A/sw/Illinois/1/63 and A/NJ/8/76 viruses.

In NI test with antiserum monospecific for the isolated neuraminidase of A/Aichi/2/68 and hyperimmune serum to A/sw/Iowa/15/30, it was shown that the swine isolates contain neuraminidase antigen belonging to N1 subtype. Coupled with the evidence obtained from HI tests, these results were compatible with the above speculation based on the serological surveillance that A/NJ/8/76-like virus may circulate in the swine populations in Thailand.

Swine H1N1 influenza viruses were first isolated in Thailand, and the similarity of surface glycoprotein antigens of the isolates A/sw/Bangkok/1/88 and A/sw/Bangkok/2/88, and recent swine H1N1 viruses led us to compare the genome structure of the six viruses including isolates from Thailand by oligonucleotide mapping. The RNAs of A/sw/North Ireland/38, A/sw/Cambridge/39, A/sw/Hong Kong/1/74, A/sw/Tennessee/1/75, A/NJ/8/76, A/sw/Shizuoka/1/78, A/sw/Bangkok/1/88, and A/sw/Bangkok/2/88 viruses were digested with ribonuclease T1 (Sankyo Co. Ltd.). Resulting oligonucleotides were 5′-end labeled with γ-[^{32}P]ATP (NEN) using T4 polynucleotide kinase (Boehringer) according to the method described previously [2], and two dimensional electrophoresis were done as described previously [3].

In the oligonucleotide maps produced by two viruses isolated in Thailand, the distribution patterns of the oligonucleotide spots were identical to each other except for 1 spot (Fig. 2 E and F), indicating common origin of the two viruses. In addition, close examination showed that, with the exception of A/sw/Tennessee/1/75 (Fig. 2 B), a large proportion of the oligonucleotide spots of the remaining viruses were also identical to each other. To assess the extent of homology of each RNA in more detail, about 60 large oligonucleotides were selected and analyzed because these nucleotides were precisely distinguishable and seem likely to represent unique sequences.

As shown in Table 2, comparative analysis of the above five viral RNAs showed that the two isolates from Thailand were genetically closer to A/sw/Hong Kong/1/74 (Fig. 2 A), A/NJ/8/76 (Fig. 2 C) and A/sw/Shizuoka/1/78 (Fig. 2 D), according to the considerable number of common spots (84.9–85.5%).

Although the above three virus RNAs were similar to the same degree to that of the isolates on the basis of common spots, the calculated minimum

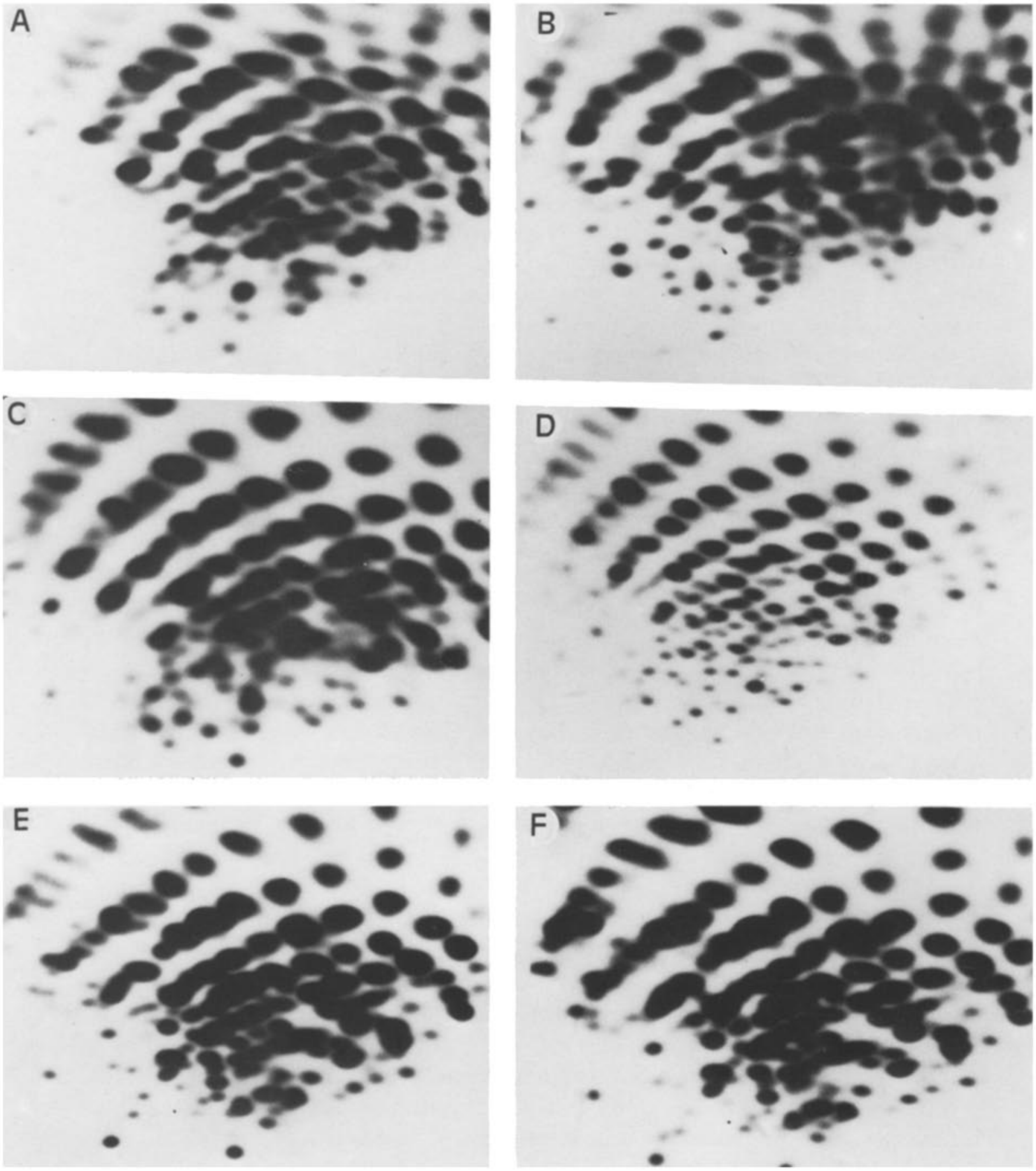

Fig. 2. Comparison of the oligonucleotide maps of the entire RNAs between four reference strains and two isolates from Thailand. **A** RNAs from A/sw/ Hong Kong/1/74, **B** A/sw/ Tennessee/1/75, **C** A/NJ/8/76, **D** A/sw/Shizuoka/1/78, **E** A/sw/Bangkok/1/88, and **F** A/sw/ Bangkok/2/88

Table 2. Comparison of the base changes amongst large oligonucleotides of the RNAs of swine (H1N1) influenza viruses isolated in Thailand and other countries

RNAs from the following strains	No. of oligonucleotide spots			
	common (%)	additional	missing	minimum no. of base change
A/sw/Bangkok/1/88 vs A/sw/Bangkok/2/88	98.4	1	0	1.0
A/sw/Bangkok/1/88 vs A/sw/Shizuoka/1/78	84.9	9	8	13.0
A/sw/Bangkok/1/88 vs A/NJ/8/76	85.5	8	8	12.0
A/sw/Bangkok/1/88 vs A/sw/Tennessee/1/75	62.9	15	17	24.5
A/sw/Bangkok/1/88 vs A/sw/Hong Kong/1/74	85.5	13	13	19.5

The minimum number of base changes was calculated according to the following formula: (no. of spots missing) × 1.5 + (difference between no. of spots missing and no. of additional spots); for example, comparison of oligonucleotide spots of A/sw/Bangkok/1/88 with those of A/sw/Shizuoka/1/78 revealed 8 missing and 9 additional spots presented and the minimum no. of base changes was calculated to be $8 \times 1.5 + 1 = 13.0$

number of base change (12.0) demonstrated that A/NJ/8/76 virus (Fig. 2 C) was closest to the viruses isolated in Thailand (Fig. 2 E and F) in oligonucleotide patterns. Migration profiles of oligonucleotides produced by A/sw/North Ireland/38 (H1N1) and A/sw/Cambridge/39 were nearly identical to each other (data not shown), but they were greatly different from A/sw/Bangkok/1/88 and A/sw/Bangkok/2/88 viruses.

Serological surveillance strongly suggested that swine H1N1 influenza viruses, but not the classical strain, i.e., A/sw/Iowa/15/30, are prevalent in swine populations in Thailand in the winter season. In agreement with this evidence, two swine H1N1 viruses isolated in Thailand appeared to be clearly related to recent swine H1N1 viruses which have been prevalent in the U.S.A., Japan and Hong Kong. In recent years, comparative studies on antigenic characteristics have been made by Hinshaw et al. [4], and it was shown that swine H1N1 viruses from Hong Kong, Italy, Japan, and the U.S.A. contain a hemagglutinin indistinguishable from that of A/NJ/8/76. In this study, swine H1N1 viruses were divided into two groups, i.e., the U.S.A.-Asian and the European types.

In a previous paper [9], we demonstrated that swine H1N1 viruses which have been prevalent in Japan since May 1977 were introduced from the U.S.A. through imported pigs. Coupled with this evidence, swine H1N1 virus, similar to A/NJ/8/76, appeared to be already causing outbreaks even in the swine populations in Thailand. Probably, the isolation of swine H1N1 viruses in Thailand reflects the introduction of A/NJ/8/76-like variants through pigs imported from the U.S.A. These results suggest that epizootiological study on influenza virus in the swine population may provide an important evidence with respect to the origin of future pandemic viruses in man.

Acknowledgement

This work was supported by Research Promotion Project in National Institute of Health, Thailand, from Japan International Cooperation Agency. The authors would particularly like to thank Dr. K. Kanai, and Mr. K. Nakajima from Japan International Cooperation Agency for providing numerous facilities.

References

1. Aymard-Henry M, Coleman MT, Doudle WR, Laver WG, Schild GC, Webster RG (1973) Influenza virus neuraminidase and neuraminidase inhibition test procedures. Bull WHO 48: 199–202
2. Billeter MA, Parson JT, Coffin JM (1974) Nucleotide sequence complexity of avian tumor virus RNA. Proc Natl Acad Sci USA 71: 3560–3564
3. Desselberger U, Nakajima K, Alfino P, Pederson FS, Haselstein WA, Palese P (1978) Biochemical evidence that "new" influenza virus strains in nature may arise by recombination (reassortant). Proc Natl Acad Sci USA 75: 3341–3345
4. Hinshaw VS, Alexander DJ, Aymard M, Bachmann PA, Easterday BC, Hannoun C, Kida H, Lipkind M, MacKenzie JS, Nerome K, Schild GC, Scholtissek C, Senne DA, Shortridge KF, Skehel JJ, Webster RG (1984) Antigenic comparisons of swine-influenza-like H1N1 isolates from pigs, birds and humans: an international collaborative study. Bull WHO 62: 871–878
5. Jensen KF (1961) Diagnosis of influenza by serologic methods. Am Rev Respir Dis 83: 120–124
6. Laver WG, Webster RG (1973) Studies on the origin of pandemic influenza. III. Evidence implicating ducks and equine influenza viruses of human influenza. Virology 51: 383–391
7. Nerome K, Ishida M, Oya A, Oda K (1982) The possible origin of H1N1 (Hsw1N1) virus in the swine population of Japan and antigenic analysis of the isolates. J Gen Virol 62: 171–175
8. Nerome K, Sakamoto S, Yano N, Yamamoto T, Kobayashi S, Webster RG, Oya A (1983) Antigenic characteristics and genome composition of a naturally occurring recombinant influenza virus isolated from a pig in Japan. J Gen Virol 64: 2611–2620
9. Nerome K, Ishida M, Sakamoto S, Sako M, Nonaka S, Webster RG, Oya A (1983) The possible origin of swine (H1N1) influenza virus in the swine population of Japan and genome composition of a recombinant (H1N2) virus. In: Laver WG (ed) The origin of pandemic influenza viruses. Elsevier, Amsterdam, pp 201–210
10. Nerome K, Yoshioka Y, Sakamoto S, Yasuhara H, Oya A (1985) Characterization of a 1980-swine recombinant influenza virus possessing H1 hemagglutinin and N2 neuraminidase similar to that of the earliest Hong Kong (H3N2) virus. Arch Virol 86: 197–211
11. Scholtissek C, Rohde W, von Hoynigen V, Rott R (1978) On the origin of the human influenza virus subtypes H2N2 and H3N2. Virology 87: 13–20
12. Shortridge KF, Webster RG, Butterfield WK, Campbell CH (1977) Persistence of Hong Kong influenza virus variants in pigs. Science 196: 1454–1455
13. Shortridge KF, Webster RG (1979) Geographical distribution of swine (H1N1) and Hong Kong (H3N2) influenza virus variants in pigs in South East Asia. Intervirology 11: 9–15
14. Shortridge KF, Cherry A, Kendal AP (1979) Further studies of the antigenic properties of H3N2 strains of influenza A isolated from swine in South East Asia. J Gen Virol 44: 251–254
15. Stuart-Harris CH, Schild GC (1976) The epidemiology of influenza. In: Stuart-Harris

CH, Schild GC (eds) Influenza, the viruses and the diseases. Arnold, London, pp 112–120
16. U.S. Department of Health, Education, and Welfare (1976) Influenza – United States. MMWR 25: 47–48
17. U.S. Department of Health, Education, and Welfare (1976) Influenza. MMWR 25: 63–64
18. U.S. Department of Health, Education, and Welfare (1988) Human infection with swine influenza virus-Wisconsin. MMWR 37: 661–663

Authors' address: K. Nerome, National Institute of Health, 10-35, Kamiosaki 2-chome, Shinagawa-ku, Tokyo 141, Japan.

Received December 18, 1989