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Continued circulation of reassortant H1N2 influenza viruses in pigs in Japan

Brief Report

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Summary. In 1991 and 1992, H1N2 influenza A viruses were isolated from the lungs of pigs with overt signs of respiratory disease at farms in the Chiba and Kanagawa prefectures of Japan. To determine the genetic origin of these isolates, we phylogenetically analyzed partial nucleotide sequences of their genes. The results indicate that influenza viruses possessing the N2 of human influenza virus and seven other gene segments of classical H1N1 swine influenza virus, which were first isolated in 1980, have become established in Japanese pigs.

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The genome of influenza A viruses consists of eight single-stranded RNA segments of negative sense [22]. Mixed infection with different influenza viruses in a single cell can lead to reassortment of the genomic segments, as demonstrated by experimental co-infection of ducks [14] and pigs [47]. In nature, the H2N2 Asian and H3N2 Hong Kong pandemic influenza strains provide striking examples of genetic reassortants involving human and avian viruses [41, 46]. Pigs are

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considered possible hosts for such reassortment events because they are sensitive to both types of viruses [15, 20, 33, 40]. Moreover, a naturally generated reassortant containing both human and avian viral genes continues to circulate among European pigs ([5], M. R. Castrucci, pers. comm.), and viruses have been transmitted from pigs to humans [6, 8, 36, 42].

Three types of influenza viruses are circulating in pigs: the classic H1N1 viruses (which have been maintained in this species for more than 70 years), the human-like H3N2 viruses (which have been introduced into pigs repeatedly since 1968 [5, 13]), and the avian-like H1N1 viruses (which were introduced from birds into European Pigs in 1979 [40]). Only the classic H1N1 viruses are currently circulating in the U.S. pig population [15, 48], whereas three types of swine influenza viruses are cocirculating in European pigs, provided an opportunity for genetic reassortment [5]. Still another mix of swine viruses can be found in Japan, where both the classic H1N1 and human-like H3N2 strains have cocirculated since 1980 [43].

Reassortant H1N2 swine viruses containing the hemagglutinin (HA) of classic H1N1 swine strains and the neuraminidase (NA) of human-like H3N2 virus were isolated in Japan in 1978 and again in 1980 [43, 50], in France in 1987 and 1988 [11]. In this report, we characterize influenza viruses isolated from pigs during the Japanese influenza outbreaks of 1991 and 1992, demonstrating that the H1N2 viruses have been maintained in this country for more than 12 years and continue to cause influenza outbreaks in Japanese pigs.

Viruses were initially isolated by inoculation of 10% suspensions of the diseased swine lung tissues into the amniotic cavity of 10-day-old chick embryonated eggs at the Kanagawa Laboratory of Animal Health, Kanagawa, and independently at the Zen-Noh Institute of Animal Health in Chiba. The possibility of genetic reassortment in embryonated eggs was eliminated by independently isolating viruses from lung homogenate at the Hokkaido University by direct plaque formation in Mardin-Darby canine kidney (MDCK) cells, as described previously [45]. Plaques were picked, suspended in 0.5 ml Eagle's modified minimum essential medium (Nissui Co. Ltd., Tokyo, Japan) containing antibiotics (100 units of penicillin and 100 g of streptomycin per ml) and 0.1% bovine serum albumin, and inoculated into the allantoic and amniotic cavity of 10-day-old chick embryos to obtain working stocks of virus. Hemagglutinating agents were subtyped in haemagglutination-inhibition (HI) [38] and neuraminidase-inhibition (NI) [3] tests with use of specific antisera to reference strains of influenza viruses [21]. Pig sera were collected from pigs of the same herd that the virus had been isolated in Chiba prefecture.

There was only limited epidemiologic information on the 1991 outbreak of swine respiratory disease in Chiba prefecture because of the reluctance of the pig farmer to cooperate with investigators. Nevertheless, seven diseased pigs were killed and their lungs were collected for laboratory analysis. A second outbreak, in January 1992, occurred at a pig farm in the Kanagawa prefecture, 73 km from the Chiba site. Clinical symptoms of respiratory disease, including cough and diaphragm cramps, were restricted to the fattening pig herd; neither the breeding

stock nor the suckling were affected. Lung tissue obtained from one of the three pigs that died contained chronic tubercula and *Actinobacillus pleuropneumoniae* (type 2).

The viruses were isolated by inoculating tissue suspensions (7 lung tissues in Chiba and 1 tissue in Kanagawa) into embryonated chicken eggs. The tissue collected in Kanagawa prefecture contained virus with a titre of 2×10^3 PFU/g, whereas that from Chiba prefecture failed to produce plaques; however, two out of ten eggs inoculated with the latter sample were virus-positive, indicating that it contained only a limited amount of virus. Five plaques picked from the Kanagawa sample were inoculated into eggs to obtain a working stock of virus. Subsequently, two viruses producing the highest HA titre were selected from the Chiba and Kanagawa samples: A/swine/Chiba/1/91 and A/swine/Kanagawa/1/92. There were no contacts between the Chiba and Kanagawa farms, or between the reporting laboratories, during the conduct of this study.

The isolates were characterized as H1, based on inhibition of their hemagglutinating activity by antisera (Table 1). They reacted to high titres with an antiserum to a classical swine H1N1 virus (A/swine/Wisconsin/11/80) and gave low titres with that to A/Aichi/2/68 (H3N2) and not at all with antisera to other reference strains. Neuraminidase activity was inhibited by antisera to viruses

Table 1. Antigenic analysis of swine H1N2 influenza isolates by HI tests with antisera against reference strains and sera from pigs on the Chiba farm

Antigen (subtype)	Antisera to				Pig sera[a]	
	Sw/Wis (H1N1)	PR/8 (H1N1)	Aichi (H3N2)	Bangkok (H3N2)	No. 4	No. 8
Reference strains						
Sw/Wis/80 (H1N1)	6400[b]	–[c]	–	–	640	640
Sw/Iowa/91 (H1N1)	6400	–	–	–	320	320
PR/8 (H1N1)	–	12800	–	–	–	–
Sw/Hok/85 (H3N2)	–	–	200	12800	–	–
Aichi (H3N2)	–	–	25600	200	–	–
Bangkok (H3N2)	–	–	400	25600	–	–
Isolates						
Sw/Chiba/1/91	6400	–	200	–	640	320
Sw/Kanagawa/1/92	3200	–	100	–	320	320

Abbreviations: Sw/Wis/80 = A/swine/Wisconsin/11/80; Sw/Iowa/91 = A/swine/Iowa/24297/91; PR/8 = A/PR/8/34; Sw/Hok/85 = A/swine/Hokkaido/10/85; Aichi = A/Aichi/2/68; Bangkok = A/Bangkok/79

[a] Pig sera were connected from pigs of the same herd from which the Sw/Chiba/91 virus had been isolated

[b] HI titre represents the reciprocal of the serum dilution inhibiting the activity of 4 hemagglutinating units of virus antigen

[c] Less than 100

Table 2. Antigenic analysis of swine H1N2 influenza isolates by NI tests with antisera against reference strains and sera from pigs on the Chiba farm

Antigen (subtype)	Antisera to						Pig sera[a]	
	Sw/Wis (H1N1)	PR/8 (H1N1)	Singapore (H2N2)	Aichi (H3N2)	Bangkok (H3N2)	Ty/Mass (H6N2)	No. 4	No. 8
Reference strains								
Sw/Wis/80 (H1N1)	640[b]	–[c]	–	–	–	–	–	–
Sw/Iowa/91 (H1N1)	320	–	–	–	–	–	–	–
PR/8 (H1N1)	160	1280	–	–	–	–	–	–
Sw/Hok/85 (H3N2)	–	–	–	–	80	–	–	–
Singapore (H2N2)	–	–	640	–	–	160	–	–
Aichi (H3N2)	–	–	–	1280	320	–	10	20
Bangkok (H3N2)	–	–	–	160	640	–	–	–
Ty/Mass (H6N2)	–	–	160	–	160	640	–	–
Isolates								
Sw/Chiba/1/91	–	–	160	320	–	320	40	40
Sw/Kanagawa/1/92	–	–	160	160	–	160	20	40

Abbreviations: Sw/Wis/80 = A/swine/Wisconsin/11/80; Sw/Iowa/91 = A/swine/Iowa/24297/91; PR/8 = A/PR/8/34; Sw/Hok/85 = A/swine/Hokkaido/10/85; Singapore = A/Singapore/57; Aichi = A/Aichi/2/68; Bangkok = A/Bangkok/79; Ty/Mass = A/turkey/Massachusetts/65

[a]Pig sera were collected from pigs of the same herd from which the Sw/Chiba/91 virus had been isolated

[b]NI titre represents the reciprocal of the serum dilution inhibiting 50% of the neuraminidase activity of virus antigen

[c]Less than 40

possessing N2 NA including A/Singapore/1/57 (H2N2), A/Aichi/2/68 (H3N2) and A/turkey/Massachusetts/65 (H6N2), but not by those to other reference strains (Table 2). Thus, the subtypes of both isolates were identified as H1N2. The 7 uncloned Chiba and 1 uncloned Kanagawa isolates were also identified as H1N2 (data not shown). Sera collected from pigs of the same herd in the Chiba prefecture also reacted with classical swine H1 virues (A/swine/Wisconsin/11/80 and A/swine/Iowa/24297/91) in HI test and to N2 viruses (e.g., A/Aichi/2/68) in NI test (Tables 1 and 2).

We then determined the genetic origin of the H1N2 isolates by phylogenetic analysis of nucleotide sequences (Fig. 1). Partial nucleotide sequences of the swine isolates were determined by reverse transcription and direct sequencing with the polymerase chain reaction (PCR) using viral RNA templates [17] and oligonucleotide primers (Table 3). Sequence data for each gene, together with information from GenBank, were phylogenetically analyzed by the Neighbor-Joining method [37] based on ODEN version 1.1.1. software (Yasuo Ina, National Institute of Genetics, Mishima, Japan). Evolutionary trees constructed by the Neighbor-Joining method indicated that genes other than the neuraminidase (NA) were closely related to the corresponding genes of classic H1N1 swine viruses

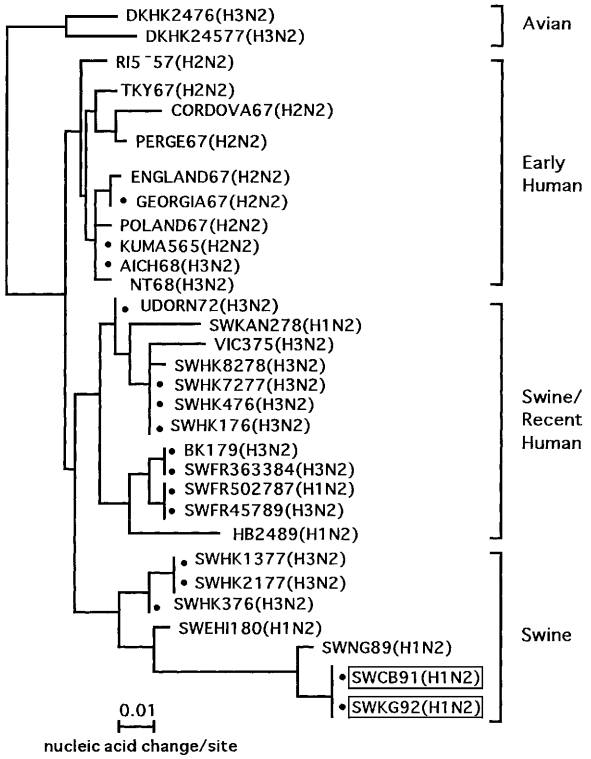

Fig. 1. Phylogenetic tree of influenza A virus N2 NA genes. Nucleotide residues 271–482 of each N2 NA were analyzed by the Neighbor-Joining method [37]. Horizontal distances are proportional to the minimum number of nucleotide differences required to join nodes and N2 NA sequences. Vertical lines are for spacing branches and labels. Swine H1N2 virus isolates are shown in boxes. The NA nucleotide sequences represent SWCB91 (A/swine/Chiba/1/91) and SWKG92 (A/swine/Kanagawa/1/92) from this study; SWNG89 (A/swine/Nagasaki/1/89), reported by Ouchi et al. [34]; SWFR363384 (A/swine/France/3633/84), SWFR502787 (A/swine/France/5027/87) and SWFR45789 (A/swine/France/457/89), reported by Gourrean et al. [11]; and HB2489 (A/Heibei/24/89) reported by Guo et al. [12]. Abbreviations of other viruses may be found in Nakao et al. [27] and Nerome et al. [30]. Previously published accounts of these N2 NA sequences may be found in Air et al. [1], Bently and Brownlee [4], Elleman et al. [7], Lentz et al. [23], Li et al. [24], Markoff and Lai [25], Martinez et al. [26], Nakao et al. [27], Nerome et al. [30, 31], and Rompuy et al. [35]

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(data not shown). Thus, the viruses most closely related to the H1N2 reassortants were all classic H1N1 swine viruses (Table 3), as had been found for H1N2 viruses isloated in 1978 and 1980 [28, 29]. A similar analysis was performed with the N2 NA gene to determine the genetic relation of the 1991 and 1992 isolates to H1N2 reassortants previously isolated from pigs in Japan and France. Two major lineages, including avian and human/swine viruses, were apparent (Fig. 1). The human/swine lineage could be further divided into three sublineages: early human (viruses isolated between 1957 and 1968), swine/recent human (including

Table 3. Genetic characterization of Sw/Chiba/1/91 (H1N2)[a]

Gene segment	Region[b]	Total bp[c]	Virus with the highest identity	Identity (%)
PA	76–201	126 (5.6)	A/swine/TN/1/77 (H1N1)	93.4
PB1	66–234	169 (7.2)	A/swine/TN/1/77 (H1N1)	94.1
PB2	45–233	189 (8.1)	A/swine/TN/1/77 (H1N1)	90.6
HA	204–352	149 (8.4)	A/swine/Nebraska/1/92 (H1N1)	89.9
NP	274–485	212 (14.2)	A/swine/TN/1/77 (H1N1)	95.5
NA	255–468	214 (15.1)	A/swine/Nagasaki/1/89 (H1N2)	98.6
M	145–289	145 (14.1)	A/swine/Ontario/81 (H1N1)	93.9
NS	610–812	203 (22.8)	A/swine/TN/1/77 (H1N1)	97.1

[a]The region indicated for each gene of Sw/Chiba/1/91 compared with the most closely related sequence in GenBank. A/swine/TN/1/77, A/swine/Nebraska/1/92, and A/swine/Ontario/81 belong to the classic swine lineage [Okazaki et al. [32] (PA); Kawaoka et al. [18] (PB1); unpublished data (H1HA and NS); Gorman et al. [9] (NP); [10] (PB2); Ito et al. [16] (M)]

[b]Region of gene sequenced

[c]Total basepairs sequenced (% of gene)

human viruses isolated after 1972), and swine. The swine sublineage included four H1N2 reassortants, A/swine/Ehime/1/80 (SWEHI180), A/Swine/Nagasaki/1/89 (SWNG89), A/swine/Chiba/1/91 (SWCB91), and A/swine/Kanagawa/1/92 (SWKG92), isolated in Japan. Thus, such H1N2 swine viruses have probably been maintained in Japan for more than 12 years since their generation and have evolved in the same lineage. The results also show that the 1991 and 1992 reassortants were genetically distinct from another Japenese H1N2 virus (A/swine/Kanagawa/2/78; SWKAN278) and French H1N2 strains (SWFR502787).

A reassortant whose HA and NA genes are derived from a human-like swine virus, with the remainder derived from an avian-like virus, was generated between 1983 and 1985 and continues to circulate among European pigs ([5], M. R. Castrucci, pers. comm.); it has even been transmitted to humans [6]. Thus, at least five stable lineages of viruses are circulating in pigs worldwide: classic H1N1, avian-like H1N1, human-like H3N2, European human-avain reassortants, and Japanese H1N2 reassortant viruses. This estimate does not include the multiple human-like H3N2 viruses, that were introduced into pigs at different times and continue to co-circulate in this species. Considering that influenza viruses are transmitted from pigs to humans relatively often [6, 8, 36, 42], the

presence of multiple, circulating influenza A strains in swine populations should raise concern over the potential for another major outbreak in humans, especially from avian-like H1N1 and early human-like H3N2 viruses, to which most or all of the human population lacks immunity.

The NA gene of the H1N2 swine virus isolated in France [11] was distinct from that of our 1991 and 1992 isolates (Fig. 1) and was most closely related to that of recent human viruses (e.g., A/Bangkok/1/79). The NA gene of another H1N2 isolate in Japan, A/swine/Kanagawa/2/78 [43], is also genetically distinct from other H1N2 swine reassortants. These results suggest that gentic reassortment between classic swine H1N1 and human-like H3N2 viruses has occurred on at least three separate occasions. Why, then, are all the swine-human reassortants H1N2 not H3N1? Possibly, the latter viruses, even though they can be generated in vitro [20], are not as competent as their parents or H1N2 reassortants to replicate and be transmitted among pigs in nature. Nonetheless, H3N1 reassortants have been isolated from humans [19, 49], suggesting that such viruses may appear in pigs in the future.

Ouchi et al. [34] reported the outbreak of swine influenza in Nagasaki, southern Japan, caused by H1N2 virus. The NA gene of A/swine/Nagasaki/1/89 (H1N2) belongs to the Japanese H1N2 reassortant lineage (Fig. 1). The Nagasaki prefecture is located 960 km south of the Chiba prefecture, where the current reassortants caused influenza outbreaks, indicating that H1N2 viruses are circulating widely among Japenese pigs.

Whether the H1N2 reassortants are capable of producing severe respiratory disease is unknown. At the Kanagawa farm, only three pigs died out of the 860 that were affected by respiratory illness, and *A. pleuropneumoniae* (type 2) was isolated from each. Thus, the severity of the disease produced by this reassortant virus may depend on coinfection with a bacterial pathogen. Some bacteria produce the HA cleavage enzyme, and therefore could be expected to exacerbate influenza infection [2, 39, 44]. Whether the *A. pleuropneumoniae* isloates secrete the protease remains unknown.

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