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An Influenza A (H1N1) Virus, Closely Related to Swine Influenza Virus, Responsible for a Fatal Case of Human Influenza

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In July 1991, an influenza A virus, designated A/Maryland/12/91 (A/MD), was isolated from the bronchial secretions of a 27-year-old animal caretaker. He had been admitted to the hospital with bilateral pneumonia and died of acute respiratory distress syndrome 13 days later. Antigenic analyses with postinfection ferret antisera and monoclonal antibodies to recent H1 swine hemagglutinins indicated that the hemagglutinin of this virus was antigenically related to, but distinguishable from, those of other influenza A (H1N1) viruses currently circulating in swine. Oligonucleotide mapping of total viral RNAs revealed differences between A/MD and other contemporary swine viruses. However, partial sequencing of each RNA segment of A/MD demonstrated that all segments were related to those of currently circulating swine viruses. Sequence analysis of the entire hemagglutinin, nucleoprotein, and matrix genes of A/MD revealed a high level of identity with other contemporary swine viruses. Our studies on A/MD emphasize that H1N1 viruses in pigs obviously continue to cross species barriers and infect humans.

Influenza A viruses are ubiquitous in nature, and the H1N1 subtype infects many species, including, birds, pigs, and humans (14, 26). This subtype is believed to be responsible for the 1918 pandemic, in which more than 20 million people were killed. In the United States, a large reservoir of H1N1 influenza A viruses exists in pigs (7, 20). These viruses have been maintained in pigs in the United States for at least 60 years (40) and during this time have been transmitted to humans and birds in nature.

Since 1975, sporadic human infections with swine viruses have been documented. The first case in which a swine virus was isolated from a human occurred in a 13-year-old boy with Hodgkin's disease who had died of pneumonia (43). One of the best-known instances occurred among military recruits at Fort Dix, New Jersey, in 1976. During this outbreak, swine-like H1N1 influenza viruses were isolated from five of the recruits, one of these from the lung tissue of an individual who died with pneumonia (47). Since then, the transmission of swine H1N1 viruses to humans has been documented in Wisconsin in 1976 (20), Texas in 1979 and 1980 (11), Nevada in 1982 (32), and more recently Wisconsin in 1988 (36). In most of these cases, contact with pigs prior to illness was documented and there was little evidence of person-to-person spread (32); however, during the 1976 swine influenza outbreak at Fort Dix, serological evidence demonstrated that at least 230 military recruits were infected with the virus.

An additional concern is that pigs serve as a "mixing vessel" (1, 37), since they are susceptible to infection with viruses from birds and other mammals, thereby providing an opportunity for genetic reassortment between influenza viruses during a mixed infection (48). The isolation of H1N2 viruses from pigs in Japan (27), as well as reassortant H3N2 viruses from pigs in Italy (5), demonstrates that this type of reassortment does occur in nature, and such reassortment could yield a new human pandemic strain (37, 48). For this reason, any human infections with swine viruses should be examined for the possible emergence of a reassortant.

In July 1991, an H1N1 influenza virus was isolated from a 27-year-old man in Maryland; he was hospitalized with bilateral pneumonia and died of acute respiratory distress syndrome 13 days after admission. The patient was a laboratory animal caretaker in good health, initially reported to work only with birds, mice, and rabbits. The H1N1 virus isolated from this bronchial secretions was designated A/Maryland/12/91. In his report, we discuss the antigenic, genetic, and pathogenic characteristics of this isolate, as well as the results of the epidemiologic investigation.

MATERIALS AND METHODS

Case report and epidemiological investigation. The patient, a 27-year-old male from Maryland, was hospitalized on July 22, 1991, for bilateral pneumonia. On July 26, 1991, the bronchial secretions collected from the patient were inoculated into primary rhesus monkey kidney cells for virus isolation. Two days later, an influenza A (H1N1) virus designated as A/Maryland/12/91 (A/MD) was isolated. No other pathogen was recovered. Serum samples were taken from the patient on July 22, 1991, and on August 4, 1991. The patient died of acute respiratory distress on August 4, 1991.

A case investigation, including a review of the medical chart, interviews with household contacts and work supervisors, and a walk-through of the animal research facility where the patient worked, was conducted. Approximately 2 months after the onset of the patient's illness, all coworkers in the research facility, all hospital workers on the patients unit, and personal contacts of the patient were asked to answer questionnaires and submit blood specimens. Previously drawn sera were

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available for 20 of the coworkers through their employee health program.

Exposure to the patient was defined as any close contact (within 3 ft [ca. 91 cm]) with the patient within 5 days from onset of the patient's symptoms. Influenza-like illness was defined as the presence of two or more of the following symptoms within 1 month of the patient's onset of illness: fever, myalgia, cough, sore throat, and headache. Sera from the patient and potential contacts were tested by hemagglutination inhibition (HI) assay for antibody to H1 influenza viruses and were considered positive if titers were ≥1:20 or, if previously drawn sera were available, a fourfold titer rise was present. We defined a case of swine influenza illness as the presence of influenza-like illness and a positive titer. Frequencies, relative risks, and Yates corrected chi-square or Fisher exact *P* values were determined by using Epi Info software (12).

Virus growth and RNA isolation. All of the viruses used in this study were from the repositories at the University of Wisconsin—Madison and the Centers for Disease Control and Prevention. The viruses were grown in 9- to 11-day-old embryonated chicken eggs for 48 to 72 h at 35°C; the virus was purified, and the RNA was extracted (9, 24, 28).

Antigenic characterization. The virus isolated was characterized in HI and neuraminidase inhibition assays, using monospecific antisera to the different subtypes (31). HI assays were also done with postinfection ferret antisera and H1-specific monoclonal antibodies (MAbs). The H1-specific MAbs were previously generated in our laboratory against the HAs of A/Sw/IN/1726/88 (Sw/IN) and A/Sw/WI/27/86 (39), and postinfection ferret antisera (31) were generated against A/Sw/WI/1/67, A/NJ/8/76, A/WI/3523/88, and A/MD.

Oligonucleotide mapping. T_1 oligonucleotide mapping for the entire RNA genome was conducted (9, 52).

Reverse transcription and PCR. The primers used in this study correspond to the published sequence of Sw/IN (24) and were synthesized at the University of Wisconsin Biotechnology Center. The cDNA synthesis of the HA and NP genes was done in a one-step PCR method (28). Amplifications of the HA gene was done in two segments, using four primers. The first segment was from bases 1 to 1065, the second was from bases 1009 to 1770; these correspond to coding regions of the HA1 and HA2 portions, respectively. The primer pairs used were as follows: H1.1 5′d (AGCAAAAGCAGGGGAAAA TAA) and H1-1065R 5′d (AGCAATGGCTTCAAACAGA CC); and H1-1009 5′d (GCACAAAATTGAGAATGGCTA) and H1-1770R 5′d (CAAGGGTGTTTTTTCTCATGTCTC). Amplification of the NP gene was done by using two primers which span the entire coding sequence. This primer pair was NP-8 5′d (GCAGGGTAGATAATCACTCAC) and NP-1557R 5′d (CAAGGGTATTTTTCTTTAATTGTC).

Cloning and sequencing of HA and NP genes. The vector used was pBluescript II SK+ (Stratagene, La Jolla, Calif.) digested with *Eco*RV (New England Biolabs, Beverly, Mass.) and modified by the addition of a 3′ dTTP, using *Taq* polymerase (2). To compensate for errors introduced by reverse transcriptase, the PCR products from three separate reactions were mixed together, and an aliquot of this mixture was used for ligation into pBluescript II SK+. This was used to transform *Escherichia coli* DH10B (Gibco BRL, Grand Island, N.Y.) (18). Clones with the appropriate insert were selected and sequenced (24). To eliminate errors introduced by *Taq* or reverse transcriptase and obtain the consensus sequence data, we sequenced five clones generated from the pooled products of three PCRs, and only the conserved mutations were used in analysis.

RNA sequencing. Between 150 and 250 nucleotides were sequenced for RNA segments 1, 2, 3, 6, and 8 of A/MD. Sequencing primers used for these segments were primer 56 for RNA segment 1 (PB2), 5′d (TAATGTCACAGTCT CGCA); primer 13 (PB1), 5′d (CAAACCATTTGAATGG); primer 30 for RNA segment 3 (PA), 5′d (AGACTTTGTGC GACAATG); primer 692 for RNA segment 6 (NA), 5′d (GAACACAAGAGTCTG); and primer 596 for RNA segment 8 (NS), 5′d (ACAGTTCGAGTC). Primer numbers indicate the position of the first nucleotide of that primer in the sequence of each segment. Primers 5′d (GAAAGATGAG TCTTCTAA), 5′d (CGAGGACTGCAG), 5′d (CAGATT GCTGATTC), 5′d (ATGAGAACAATTGGGACT), and 5′d (GTGGATTCTTGATCGTCT), which correspond to sequences beginning at nucleotides 8, 239, 457, 646, and 808, respectively, of the M gene, were used for sequencing.

Experimental infection of swine. Four six-week-old, common-source weanling pigs seronegative for prior exposure to H1N1 swine viruses (by HI assays) were inoculated intranasally with 2×10^7 50% egg infectious doses of either A/MD or Sw/IN. The Sw/IN group was used as a control to determine the relative differences between A/MD and contemporary swine H1N1 viruses. Pigs were housed under P3 conditions at the University of Wisconsin—Madison according to the guidelines prescribed by the University of Wisconsin Research Animal Resources Center. The pigs were monitored daily, beginning 2 days prior to inoculation. Nasal swabs were collected daily from each pig for virus isolation and PCR starting 2 days after inoculation. The pigs were euthanized, in accordance with guidelines set by the American Veterinary Medical Association (42), on day 4 or 7 after inoculation, and complete postmortem examinations were conducted. Virus titration and histologic examination of lung tissues were conducted (8).

Sequence analysis. The sequence data were assembled, analyzed, compared, and translated by using programs from the Genetics Computer Group (GCG) (13).

Mapping of the mutations on the H1 HA. To locate the positions of amino acid differences between A/MD and Sw/IN, we aligned the HAs of A/MD and A/England/321/77 by using Bestfit and then located the equivalent amino acids on the established three-dimensional structure of the H3 HA. The diagram in Fig. 2 was generated with the MacInplot program, generously provided by Thomas J. Smith, Purdue University (44).

Nucleotide sequence accession numbers. The nucleotide sequences of the HA, NP, and M genes of A/MD are L24362, L24394, and M63532, respectively.

RESULTS

Epidemiologic investigation. The case investigation revealed that the patient had been in close contact with sick pigs at work. At least four swine had died unexpectedly from respiratory illness within days of the patient's onset of illness. Necropsies of the two pigs suggested viral pneumonia as the cause of death. Interviews with friends and family revealed no other recent exposure to pigs, turkeys, or influenza-like illness. The results in Table 1 show that the patient had a positive antibody reaction to swine influenza virus, with a 256-fold rise in titer in his second serum sample.

Coworkers exposed to sick pigs were more likely to report influenza-like illness than unexposed coworkers (9 of 24 exposed versus 0 of 10 unexposed; $P = 0.02$) but were no more likely to have positive titers (3 of 24 exposed versus 1 of 10 unexposed; relative risk [RR], 1.3; 95% confidence interval [CI], 0.2, 10.6). Also, they were no more likely to have swine

TABLE 1. Serological results from the index patient, Maryland, 1991[a]

Serum sample	HI titer		
	A/NJ/76	A/WI/88	A/MD
1	40	<5	<5
2[b]	1,280	1,280	1,280

[a] Positive reaction is a ≥4-fold rise in HI titer.
[b] Taken 13 days after serum sample 1.

influenza illness (1 of 24 exposed versus 0 of 10 unexposed; $P = 0.71$).

Coworkers exposed to the patient during the week prior to his illness were also somewhat more likely to report influenza-like illness than unexposed coworkers (8 of 21 exposed versus 1 of 13 unexposed; RR, 5.0; CI, 0.7, 35.2) but were not significantly more likely to have positive titers (3 of 21 exposed versus 1 of 13 unexposed; RR, 1.9; CI, 0.2, 16.0). The one worker who met the case definition for swine influenza illness had been exposed to the patient as well as to sick pigs.

Hospital workers potentially exposed to the patient's saliva or respiratory droplets during the first week of illness were not more likely to report influenza-like illness than hospital workers who were not exposed (6 of 40 exposed versus 8 of 36 unexposed; RR, 0.7; CI, 0.3, 1.8), nor were they more likely to have positive swine influenza antibody titers than unexposed workers (3 of 39 exposed versus 5 of 31 unexposed; RR, 0.5; CI, 0.1, 1.8) or swine influenza illness (0 of 39 exposed versus 3 of 31 unexposed; $P = 0.08$).

Of the nine exposed household contacts, four reported influenza-like illness, and one of the four had a positive titer. Thus, the one person with a positive titer met the case definition for swine influenza illness although his primary symptoms had been diarrhea and nausea.

Among the five subjects, other than the index patient, who met the case definition for swine influenza illness, antibody titer levels ranged from 1:20 to 1:160 and none had previously drawn sera available for comparison. All five had fully recovered from minor symptoms of influenza-like illness without complications.

Biological properties and antigenic characteristics of A/MD. The growth property of A/MD was examined by using hemagglutination (HA) assays. This isolate grows to high HA titers (1:512) in eggs and MDCK cells, which is atypical of contemporary swine H1N1 viruses, such as Sw/IN, which grows to relatively low HA titers (1:16) in eggs and tissue culture. Initial characterization of this virus by using HI and neuraminidase inhibition assays with monospecific antisera to each HA and NA subtype indicated that A/MD is an H1N1 virus. HI assays with postinfection ferret sera against a panel of characterized viruses and A/MD (Table 2) indicated that this virus is antigenically closely related to current swine H1N1 viruses. To examine the antigenic characteristics of A/MD more closely, we compared this isolate with other H1N1 viruses in HI assays using MAbs that define three antigenic sites on the H1 HA of Sw/IN (Table 3) (24).

The HI results in Table 3 show that this human isolate reacts with all of the MAbs except 1-6B2. Thus, it is antigenically related to, but distinguishable from, the swine viruses currently circulating, including the most recent human isolate of swine origin, A/WI/3523/88. This virus is related antigenically most closely to A/NJ/8/76, the human virus of swine origin which occurred among military recruits at Fort Dix, New Jersey, in 1976. MAb 1-6B2 has previously been shown to bind in site Sa, as defined by Caton et al. (6), which includes the receptor binding site. These MAbs show very little affinity for human H1N1 viruses, such as A/USSR/90/77 and A/Chile/1/83, and the avian HA from A/Dk/A1b/35/76 is readily distinguishable from swine viruses, based on its lack of reactivity with MAb 2-15f1 (site Ca) or MAb 3F2c (site Sb) (6, 24).

TABLE 2. Antigenic reactivities of influenza A (H1N1) viruses isolated from swine and humans with postinfection ferret serum against swine influenza viruses

Virus	HI titer with postinfection ferret[a] antiserum to[b]:				
	A/WI/1/67	A/NJ/8/76	A/NV/101/82[c]	A/WI/3523/88	A/MD
A/Sw/WI/1/67[d]	160	80	40	80	40
A/NJ/8/76[c]	160	640	160	320	160
A/WI/3523/88[e]	160	640	1,280	2,560	320
A/MD[e]	320	2,560	640	1,280	640

[a] Ferrets were inoculated intranasally with a 1:10 dilution of influenza virus in phosphate-buffered saline, and serum samples were obtained from the animals 14 days postinfection.
[b] Reciprocal crosses are underlined.
[c] See reference 11.
[d] Isolated from swine.
[e] Swine-like virus isolated from humans.

We also conducted HI assays with nonimmune horse serum, because horse serum contains a potent inhibitor (α_2-macroglobulin) of many influenza viruses which recognize sialic acid in the α2,6 linkage to galactose on oligosaccharide chains, rather than the α2,3 linkage. We compared the HI reactivity of A/MD with the reactivities of A/WI/3523/88 (H1N1), inhibitor-sensitive A/TX/1/77 (H3N2), and an inhibitor-resistant variant of A/TX/1/77. The HI titers were 1:4,800 for A/MD, 1:200 for A/WI/3523/88, 1:9,600 for A/TX/1/77, and <1:100 for the resistant variant of A/TX/1/77; this result suggests that A/MD is inhibitor sensitive like the H3 virus tested and thereby may recognize sialic acid in the α2,6 linkage.

Genetic characteristics of A/MD. The total genome of A/MD was examined by using oligonucleotide mapping with RNase T_1. We first compared T_1 maps of some reference swine influenza A (H1N1) viruses that were isolated either from

TABLE 3. Antigenic analysis of influenza type A (H1N1) viruses isolated from swine, humans, and birds with MAbs derived against a recently isolated swine influenza virus

Virus	HI titer with MAb[a]:			
	2-15f1	7B1b	3F2c	1-6B2
Swine H1N1				
Isolated from pigs				
Sw/IN	1,600	25,600	12,800	25,600
A/Sw/WI/1915/88	<100	25,600	12,800	51,200
Isolated from humans				
A/NJ/8/76	6,400	1,600	1,600	<100
A/WI/3523/88	1,600	12,800	51,200	25,600
A/MD	6,400	12,800	12,800	<100
Human H1N1				
A/USSR/90/77	<100	<100	<100	<100
A/Chile/1/83	<100	<100	<100	<100
Avian H1N1				
A/Dk/A1b/35/76	<100	6,400	<100	51,200

[a] HI titers are expressed as the reciprocal of the dilution of ascites fluid inhibiting four hemagglutinating doses of virus. MAbs 2-15F1 and 1-6B2 were prepared to the HA of A/Sw/WI/27/86; MAbs 3F2c and 7B1b were prepared to the HA of Sw/IN.

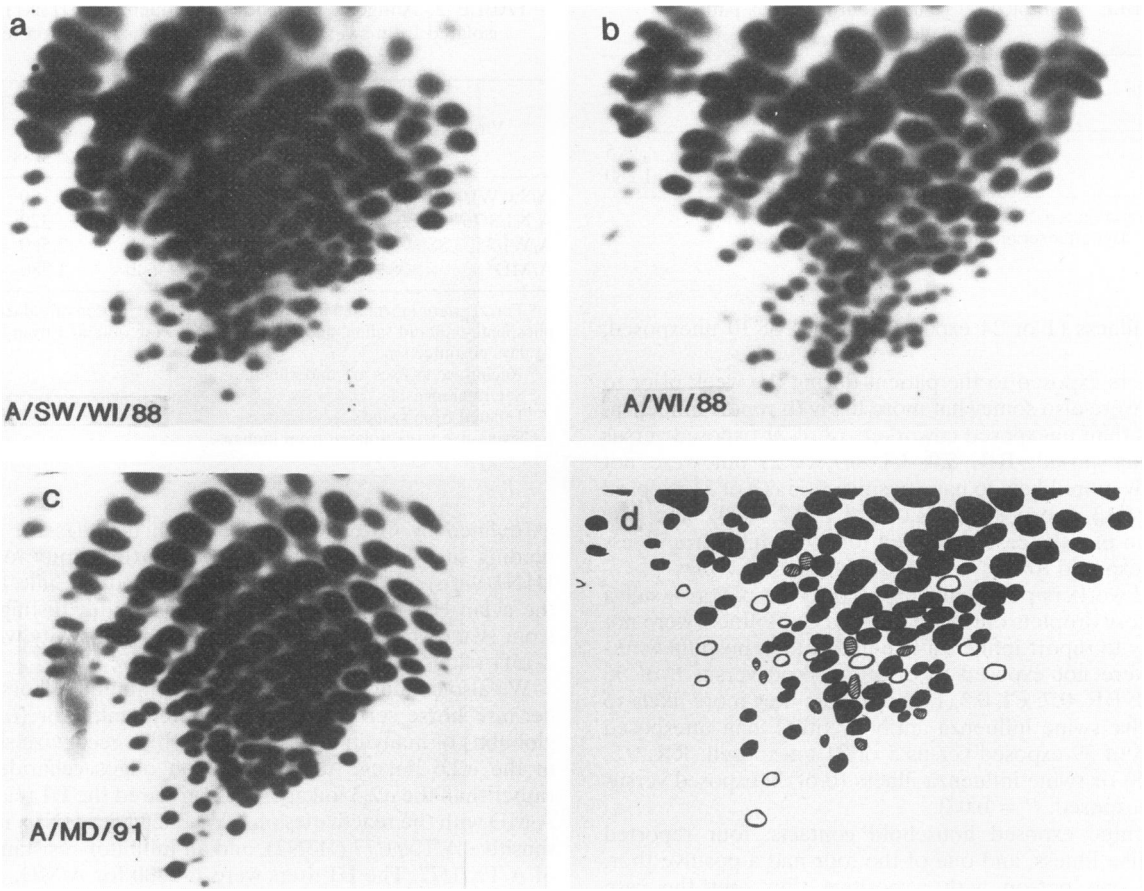

FIG. 1. T_1 oligonucleotide maps of total RNA of A/Sw/WI/88 (a), A/WI/88 (b), and A/MD (c) and a diagram of oligonucleotide comparison between panels b and c (d). The solid spots denote the oligonucleotide common to panels b and c, hatched spots denote the spots unique to panel b, and empty spots denote spots unique to panel c.

humans or from pigs between 1976 and 1988. T_1 maps of A/NV/82 (not shown), A/Sw/WI/88 (Fig. 1a), or A/WI/88 (Fig. 1b) differed from that of A/NJ/76 (not shown) by 14, 18, and 20 oligonucleotide spots, respectively, when 100 specific oligonucleotide spots were compared. Oligonucleotide differences between the T_1 maps of A/NV/82, A/Sw/WI/88, and A/WI/88 ranged from 6 (A/Sw/WI/88 versus A/WI/88) to 23 (A/NV/82 versus A/WI/88). These pairwise comparisons revealed that the evolution of swine influenza A (H1N1) virus generally results in an average of two to four (range, one to six) oligonucleotide changes per year, a rate that appears to be slightly lower than that observed for human influenza A (H1N1) viruses (10, 52). We then compared the T_1 map of A/MD (Fig. 1c) with those of representative swine influenza viruses. A total of 27 oligonucleotide differences (1.8/year) out of 100 were found between the T_1 maps of A/MD and A/NJ/76, which were isolated 15 years apart. This result indicates that the total genome of A/MD is closely related to that of A/NJ/76. Comparison of T1 maps of A/MD with other viruses currently circulating in pigs (such as A/WI/88 [Fig. 1d] and A/Sw/WI/88 [not shown]), however, revealed 24 oligonucleotide changes (8.3/year). This number of changes is certainly higher than expected for viruses of the same genotype isolated only 34 months apart (10, 52).

However, T_1 mapping alone might not have detected viruses having reassorted M and/or NS genes (36, 52). Therefore, to determine the origin of the RNA segments of A/MD, we sequenced the entire hemagglutinin, matrix, and nucleoprotein genes and also conducted partial sequence analysis of RNA segments 1 (PB2), 2 (PB1), 3 (PA), 5 (NA), and 8 (NS) (Table 4).

RNA sequence analysis demonstrated that partial PB2, PB1, PA, NA, and NS genes of A/MD had a 92 to 99% identity with swine influenza A (H1N1) viruses at the nucleotide level (Table 4), suggesting that these genes were closely related to those of typical swine H1N1 viruses.

We compared the HA of A/MD with H1 HAs of a current swine virus (Sw/IN), a swine-like virus isolated from soldiers at Fort Dix (A/NJ/11/76), a human H1N1 virus (A/USSR/90/77), and an avian H1N1 virus (A/Dk/Alb/35/76), using the GCG program Bestfit (Table 4). This Maryland isolate has a very high sequence identity with Sw/IN at both the nucleotide and amino acid levels. In fact, there was only one amino acid difference in the HA1 portion of the molecule (Table 5). The change was at amino acid 142 from an asparagine in Sw/IN to an aspartic acid in A/MD. When this change is aligned with the H1 HA of A/PR/8/34, it corresponds to amino acid 125, which is in site Sa (6). Three changes were found in the HA2 portion of the molecule (Table 5): one at amino acid 382 from an arginine (Sw/IN) to a glutamine (A/MD), another at 505 from an asparagine to a lysine, and the last at amino acid 544 from a serine to a leucine. The asparagine residue at 505 is in a favorable context (N-X-T/S) (46) for N-linked glycosylation in Sw/IN.

To locate these changes on the three-dimensional structure

TABLE 4. Sequence analysis of A/MD

Gene	% of A/MD sequenced	Strain used for comparison[a]	% Nucleotide identity[b]	% Amino acid identity[b]
PB2	10	A/Sw/TN/77	96	ND
	10	A/Sw/Germany/81	81.8	ND
	10	A/Gull/Astrakhan/227/84	83.5	ND
	10	A/Memphis/8/88	83.5	ND
	10	A/Eq/London/1416/73	87	ND
PB1	7	A/Sw/TN/77	93	ND
	7	A/Sw/Ontario/2/81	97.5	ND
	7	A/Sw/Hongkong/126/82	83	ND
	7	A/Memphis/8/88	81.9	ND
	7	A/Gull/MD/704/77	38	ND
	7	A/Dk/Hokkaido/8/80	37.5	ND
	7	A/Eq/TN/5/86	85	ND
PA	16	A/Sw/TN/77	94	ND
	16	A/Sw/Hongkong/126/82	79.5	ND
	16	A/Ty/MN/833/80	79.5	ND
	16	A/Dk/Hokkaido/5/80	79.7	ND
	16	A/Eq/TN/5/86	82.5	ND
HA	97.7	A/Sw/IN/1726/88	98.6	99.3
	97.7	A/NJ/11/76	93.6	95.4
	97.7	A/USSR/90/77	79.4	82.2
	97.7	A/DK/Alb/35/76	77.5	85.5
NP	98.9	A/Sw/IA/17672/88	98.9	99.4
	98.9	A/WI/3523/88	98.8	98.8
	98.9	A/TX/1/77	85.1	90.7
	98.9	A/USSR/90/77	85.7	90.4
	98.9	A/Sw/Germany/2/81	83.7	94.8
	98.9	A/Ty/MN/833/80	83.5	94.8
NA	18.3	A/Sw/IA/17672/88	99	ND
	18.3	A/Chile/1/83	84.8	ND
M1/M2	100	A/Sw/TN/77	97.1	95.9
	100	A/Sw/HongKong/126/82	98.3	95.9
	100	A/Sw/HongKong/127/82	88.1	85.8
	100	A/Sw/Netherlands/85	90.2	86.8
NS1/NS2	19.2	A/Sw/Ontario/2/81	97.7	ND
	19.2	A/Sw/US/37	92	ND
	19.2	A/Ann Arbor/6/60	89.9	ND
	19.2	A/Mallard/Alberta/827/78	77.5	ND
	19.2	A/Dk/Ukraine/63	89.6	ND
	19.2	A/Eq/Jilin/89	76.6	ND

[a] All sequences used for comparison were obtained from GenBank, except the sequence of the NS of A/Sw/Ontario/2/81, which was provided by Yoshihiro Kawaoka.

[b] Calculated by using the GCG program Bestfit. ND, not determined.

TABLE 5. Amino acid differences between the HAs of Sw/IN and A/MD

Position		Amino acid of:		Region
H1	H3	Sw/IN	A/MD	
142	129	Asn	Asp	HA1
382	367	Gln	Arg	HA2
505	490	Asn	Lys	HA2
544	528	Ser	Leu	HA2

of the HA (Fig. 2), the deduced amino acid sequence was aligned with the sequence of the H3 HA of A/England/321/77 (19), using Bestfit. This places amino acid 142 (N to D) at position 129 of the H3, which is five amino acids from the right edge of the receptor-binding site (29) and topographically close to amino acid 172 of the H1 that was previously shown to alter the binding of MAb 1-6B2 (24). Amino acid 382 (R to Q) is located near the fusion domain at position 367 of the H3; 505 is at the base of the HA at position 490 of the H3; and 544 is in the transmembrane region at 528 of the H3, which is not included in the crystal structure.

We compared the NP sequences of A/MD, a recent swine H1N1 virus (A/Sw/IA/17672/88), the most recent human H1N1 virus of swine origin (A/WI/3523/88), a human H3N2 virus (A/TX/1/77), a human H1N1 virus (A/USSR/90/77), an avian H1N1 virus (A/Sw/Germany/2/81), and an avian H4N2 virus (A/Ty/MN/833/80) (Table 4). For a review of the origin of all of these NPs, see reference 1. The sequence comparison was also done by using the GCG program Bestfit. The NP of A/MD shared very high nucleotide identity with A/Sw/IA/17672/88 and A/WI/3523/88, i.e., 98.9 and 98.8%, respectively. The NP of A/MD has a much lower sequence identity with the two human NP genes of A/TX/1/77 and A/USSR/90/77, 85.1 and 85.7%, respectively. The isolate has an even lower identity with the two avian NP genes of A/Sw/Germany/2/81 and A/Ty/MN/833/80, 83.7 and 83.5%, respectively. When we translated these sequences and compared the same NP segments at the amino acid level, they shared an even greater percent identity with A/MD. These results indicate that the NP of A/MD is of swine origin and was not the result of reassortment with either an avian or a human virus.

The matrix proteins are encoded by segment seven of influenza viruses. We compared this sequence with the sequences of a swine H1N1 virus (A/Sw/TN/77), a swine H3N2 virus (A/Sw/Hongkong/126/82), an avian H3N2 virus (A/Sw/Hongkong/127/82), and an avian H1N1 virus (A/Sw/Netherlands/85) (Table 4). Although segment 7 of A/MD shares a high sequence identity with the swine H1N1 virus (97.1%), it shares the greatest nucleotide identity with the swine H3N2 virus (98.3%). The isolate has a much lower sequence identity with both the H3N2 avian virus (88.1%) and the H1N1 avian virus (90.2%).

Infection of pigs. To determine whether this virus had any unusual characteristics in vivo, we compared the replication and lung pathology of the human isolate in pigs with that of Sw/IN. We inoculated, four 6-week-old seronegative piglets intranasally with A/MD or Sw/IN and examined virus shedding, fever, and lung pathology. Both groups had similar clinical signs; they were lethargic and had fevers (data not shown), but there was little coughing or sneezing. Pigs infected with either the current swine strain or the human isolate shed the virus for 6 days (data not shown). We also measured the amount of virus shed from each group and found that values for the two groups were very similar, i.e., approximately 10^5 50% egg infectious doses per ml of nasal sample. The viruses isolated from nasal swabs and lung tissues were further characterized by using HI assays with the MAbs against Sw/IN to determine whether any antigenic changes occurred as a result of passage in the pigs. The results (data not shown) indicated that the virus shed was antigenically identical to the inoculum, based on reactivity with our MAbs.

Necropsy examinations were performed on day 4 or 7 after infection to identify any differences between these two groups. On day 4, the pig infected with A/MD had gross pulmonary lesions characterized by areas of consolidation that were dark purplish red and palpably firm. On day 7, we observed similar lesions in a pig inoculated with Sw/IN. Histological examination of lung tissue from both groups indicated that the swine virus produced more severe damage than the human isolate on both days 4 and 7. We observed inflammatory cell infiltrates surrounding terminal and respiratory bronchioles in both groups. This infiltrate consisted primarily of mononuclear cells; however, plasma cells and a few polymorphonuclear leukocytes were also present. There was evidence of respira-

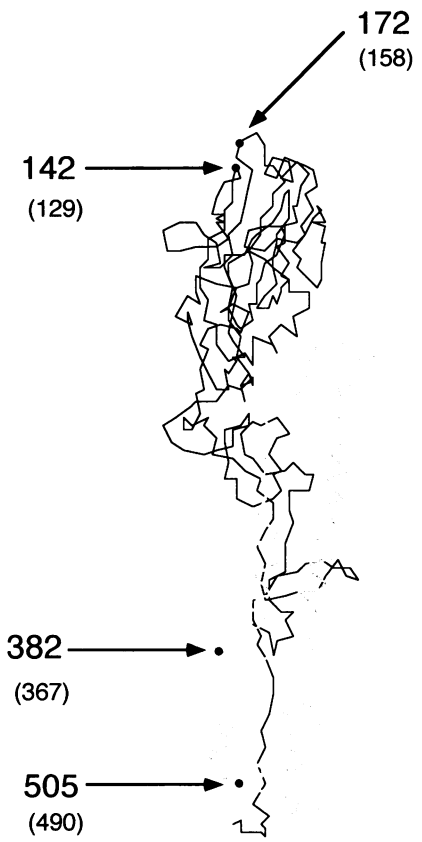

FIG. 2. Three-dimensional diagram of an HA molecule. The black line represents the HA1 portion of the molecule, and the grey line represents the HA2 portion. The positions of specific amino acids are indicated by the spheres. Amino acids 142, 382, and 505 are sites of amino acid differences between A/MD and Sw/IN. Amino acid 172 represents the site of the mutation in a MAb-selected variant virus (v1-6B2). This mutation was shown to be responsible for the lack of HI reactivity to MAb 1-6B2 by sequencing of the v1-6B2 variant (24). Numbers in parentheses indicate amino acid positions on the H3 HA.

tory epithelial cell damage, as well as mitotic figures, indicating cell proliferation.

DISCUSSION

In this study, we defined the antigenic, genetic, and pathogenic characteristics of A/MD, a swine-like H1N1 virus responsible for a fatal case of human influenza. The unique nature of the oligonucleotide maps and HA titers of this isolate suggested that this virus was a reassortant. However, our studies indicate that all genes of A/MD originated from swine viruses.

Our studies indicate that this human isolate is antigenically very similar to the viruses currently circulating in pigs in the United States. However, A/MD is an HA variant similar to a MAb-selected escape mutant, which is not typically isolated from pigs (28). Antigenically, the HA of A/MD is most closely related to the HA of A/NJ, another human virus of swine origin, as both have mutations (five amino acids apart) in site Sa as defined by Caton et al. (6).

After sequencing the entire HA to confirm its origin, we found only one mutation in the HA1 portion and three mutations in the HA2 portion of the molecule. The location of the mutation in the HA1 portion indicated that it was responsible for the HI reactivity pattern observed. Mutations in the HA often affect virus growth in eggs; however, mutations at this position (amino acid 142) have not been associated with egg adaptation (34, 35). The proximity of this charge change to the receptor-binding site suggests that it may have an effect on tissue tropism and thus species specificity of the virus. For example, our finding that A/MD is inhibitor insensitive suggests that this isolate binds a receptor in the α2,6 linkage, while the other H1, swine and swine-like, viruses tested bind an α2,3-linked receptor. The amino acid change at position 142 may affect this interaction, but additional studies would be required to establish this.

The other three amino acid differences between the HAs of these two viruses are in the HA2 portion of the molecule; this is rather unusual because the HA2 polypeptide is conserved among swine viruses (28, 49–51). One substitution is 34 amino acids from the amino-terminal end of the fusion protein. This change may influence protein structure; however, glutamine and arginine are both very polar amino acids, and many other H1 viruses also have this difference when compared with Sw/IN. These viruses include a swine virus (A/Sw/NE/92), a human virus of swine origin (A/NJ/11/76), a human virus (A/USSR/90/77), and an avian virus (A/Dk/A1b/35/76). These factors suggest that this substitution does not have an important phenotypic effect on this isolate. The other two changes are at the base of the HA2 molecule. One of these mutations, amino acid 505, results in the loss of a possible glycosylation site. This type of change may have a dramatic affect on the three-dimensional structure of the HA molecule. This lysine substitution at the base of the HA would induce a charge shift very close to the viral envelope, which may also affect the orientation of the trimer. Experiments with temperature-sensitive mutants (15) suggest that this domain of the HA is important for trimerization and for transport in or through the Golgi apparatus and may have signals which direct transport between the Golgi apparatus and the plasma membrane. The change in the transmembrane region to another aliphatic, more hydrophobic amino acid would result in little, if any, perturbation of the HA structure. Alterations in the cytoplasmic tail of the HA can alter virus infectivity (41) and intracellular transport (3, 15); however, it has not been shown that changes in the transmembrane region have this same effect.

We sequenced the entire NP to ascertain its origin, because the NP has been implicated as a major genetic determinant in the host range of influenza A viruses (1, 37, 45). Previous phylogenetic analysis of the NP has demonstrated that there are five host-specific lineages which probably evolved from an avian virus (17). Of these five, the classic swine and human NPs are considered to have the same lineage, while the avian, gull, and two equine lineages are considered separate. The results of our comparison between the NP genes of A/MD and those in the avian or human lineages indicate that the NP of A/MD shares the strongest sequence identity, i.e., 99.4% at the amino acid level, with a contemporary swine H1N1 NP. This indicates that the NP of A/MD is of swine origin and was not the result of reassortment with either an avian or a human virus.

The seventh segment of the influenza virus encodes two matrix proteins, M1 and M2 (23). The M1 protein has been shown to be involved in the regulation of the import and export of viral ribonucleoproteins across the nuclear membrane (25). The M2 protein is an integral membrane protein that accumulates in the membranes of infected cells and acts as an ion channel, important in viral replication (21, 33, 53). Although this segment is highly conserved among influenza viruses, it has been shown to be important in host restriction (4) and is responsible for the high-yielding property of influenza virus in

eggs (16, 30, 38). Klimov et al. (22) grouped M1 and M2 proteins of human influenza viruses into high-yielding and low-yielding types, on the basis of the amino acids present in five specific positions. Comparison of the deduced M1 and M2 amino acid sequences of A/MD with those of the high-yielding (A/PR/8/34) and low-yielding (A/Bangkok/1/79) reference strains revealed that A/MD has the same sequence as the high-yielding strain at only one of these five positions (data not shown). The high-yielding property of A/MD, therefore, could not be mapped to its M gene products.

The epidemiological investigation and laboratory examination demonstrated that the fatal case reported in this study was the result of a swine influenza virus infection of a healthy individual, apparently infected by direct contact with pigs. Among the coworkers, hospital workers, and personal contacts, neither exposure to sick pigs nor exposure to the patient was significantly associated with swine influenza illness overall. In fact, other than the index patient, only five people met the case definition for swine influenza illness. Three of the five were not exposed to the patient or to swine, and one had symptoms more consistent with gastroenteritis. The fifth person was a coworker who had a single serum sample with a titer of 1:160. Thus, at least four of the five people who met the case definition for swine influenza illness were probably not infected with the same virus as the index patient. This is not surprising given that the case definition was intended to be highly sensitive but not particularly specific. The fact that exposure to the patient or to sick pigs was associated with influenza-like illness, but not with a positive titer, may be due to recall bias.

This study further demonstrates that although swine influenza virus can infect humans, person-to-person transmission of swine viruses, with the exception of the New Jersey incident (47), has been infrequent. In previous reports (36, 43), fatal cases occurred in people who had underlying conditions, such as Hodgkin's disease or pregnancy; however, this patient had no other known coexisting illness. The possibility that the patient was coinfected by another pathogen does exist; however, no other pathogens were identified.

Since A/MD caused the death of a healthy, young person, there was a possibility that it was particularly virulent. That aspect cannot be examined in humans but can readily be investigated in the natural host, the pig. We found little difference between the clinical disease, gross pathology, or histopathology of pigs infected with A/MD or Sw/IN. Our initial gross impression was that lungs of pigs infected with A/MD were more severely damaged than those of pigs infected with the swine virus; however, histological examination revealed that the swine virus actually caused more tissue damage. Overall, there was no indication that A/MD was of increased virulence for pigs.

Our studies of this isolate reinforce the fact that people can be infected with swine influenza viruses. This has occurred in the past and is likely to continue in the future. We are identifying more of these cases now because of better surveillance. It is critical that all cases of this type be investigated for the possible emergence of reassortant strains of influenza virus, because of the epidemic potential of such a virus. In view of the zoonotic nature of influenza viruses, individuals working with animals, especially pigs, should be aware of the potential danger.

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