Antigenic and genetic conservation of the haemagglutinin in H1N1 swine influenza viruses

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We examined the level of antigenic conservation amongst the haemagglutinins (HAs) of H1 swine influenza viruses, recently isolated from a wide geographical area, in haemagglutination inhibition assays against a panel of four monoclonal antibodies (MAbs). We found a high degree of conservation with a dominant variant (52 of 54 isolates) that reacted with all MAbs. Only two minor variants, each failing to react with one MAb, were found. Using a one-step PCR technique followed by direct sequencing of the products, we examined the HA1 region of the HA RNA of two representative dominant variants. We found no amino acid substitutions relative to a reference strain. The sequences of the HA1 RNA of the two minor variants isolated here and of two other minor variants defined previously were also determined. Each contained inferred amino acid substitutions, all located at different positions on the HA. Finally, we sequenced HA1 RNA obtained from the original pig lung suspensions from which the two dominant and two minor variants had been isolated. Three of the parent viruses were identical to their progeny in eggs whereas the fourth parent virus contained four amino acid differences from its progeny.

Previous studies have investigated the haemagglutinin (HA) of swine influenza viruses isolated over a 24 year period using haemagglutination inhibition (HI) assays with a panel of monoclonal antibodies (MAbs) that identified three antigenic sites. These antigenic sites had remained unchanged over 24 years and only one antigenic variant was detected (Sheerar et al., 1989; Luoh et al., 1992). Since these studies had focused on viruses within an enzootic area of Wisconsin, U.S.A., we wanted to know whether or not more recent swine viruses isolated over a wider geographical area, i.e. epizootic viruses, exhibited the same degree of antigenic conservation. Using PCR directly on viral RNA (vRNA) and direct sequencing of the cDNA products we also studied the level of genetic conservation of RNA encoding the HA of these viruses.

A total of 110 pig lung tissue samples previously diagnosed as influenza virus-positive by examination with fluorescent antibody were kindly provided for this study by Dr Mary Lynn Vickers of the Animal Health Laboratory, University of South Dakota. These were macerated by a stomacher (Tekmar) in Hanks' buffered salt solution, clarified by low-speed centrifugation and supernatants were then inoculated into the allantoic cavity of 10-day-old embryonated chicken eggs and incubated at 35 °C for 72 h. Virus was isolated from 54 samples, from pigs from Kentucky (three samples), Iowa (13), Nebraska (seven), South Dakota (18), North Dakota (one), Minnesota (six), Illinois (one) and Wisconsin (five). Isolates were screened with an HI assay against the panel of MAbs described previously (Sheerar et al., 1989). ELISAs and virus neutralization assays were performed as described (Sheerar et al., 1989). All isolates were H1N1.

RNA was extracted either directly from pig lung suspensions or from pelletted virus using 4 M-guanidinium isothiocyanate followed by centrifugation on 5-7 M-CsCl. vRNA was then reverse-transcribed and amplified in a one-step PCR method adapted from Xu et al. (1990). To 1 μg of total RNA in a 18 μl sample we added 1 μl each of 10 μM specific forward and reverse 21-nucleotide primers. These primers, H1-121 and H1-1065R (Luoh et al., 1992), gave a product 966 bp in length spanning nucleotides 121 to 1085 of the HA1 region. The mixture was incubated at 65 °C for 4 min, chilled on ice to allow annealing and made up to a 100 μl volume with final concentrations in the reaction mixture of 60 mM-KCl, 35 mM-Tris–HCl pH 8.3, 1.5 mM-MgCl₂, 360 μM-DTT, 210 μM-dNTPs, 33 μg/ml gelatin, 2 units avian myeloblastosis virus reverse transcriptase (Pharmacia LKB), 1-7 units Taq DNA polymerase (AmpliTaq, Perkin-Elmer Cetus), and was then overlaid with 100 μl of mineral oil. Reaction tubes were incubated in a thermal cycler for one cycle at 42 °C for 45 min followed...
Table 1. Comparison of recently isolated H1N1 swine influenza viruses with MAbs to the H1 HA in HI, ELISA and neutralization assays

<table>
<thead>
<tr>
<th>Virus</th>
<th>HI titre*</th>
<th>ELISA titre†</th>
<th>Neutralizing titre‡</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2-15fl</td>
<td>7Blb</td>
<td>3F2c 1-6B2</td>
</tr>
<tr>
<td>Sw/IN/88</td>
<td>1024</td>
<td>&gt; 2048</td>
<td>512 &gt; 2048</td>
</tr>
<tr>
<td>52 Natural isolates</td>
<td>&gt; 8</td>
<td>&gt; 64</td>
<td>&gt; 8 &gt; 64</td>
</tr>
<tr>
<td>Sw/IA/88</td>
<td>&lt; 1</td>
<td>256</td>
<td>16 &gt; 512</td>
</tr>
<tr>
<td>Sw/SD/90</td>
<td>16</td>
<td>&lt; 1</td>
<td>32 &gt; 512</td>
</tr>
<tr>
<td>Sw/WI/88</td>
<td>&lt; 1</td>
<td>&gt; 2048</td>
<td>256 1024</td>
</tr>
<tr>
<td>v2-15fl</td>
<td>&lt; 1</td>
<td>&gt; 2048</td>
<td>32 1024</td>
</tr>
</tbody>
</table>

* HI titres are expressed as the reciprocal of the highest dilution (×10^2) of ascitic fluid inhibiting 4 HAU virus.
† ELISA titres are expressed as the reciprocal of the highest dilution (×10^-2) of the ascitic fluid providing an absorbance of 0.100 at 410 nm.
‡ Neutralizing titres are expressed as the reciprocal of the highest dilution of ascitic fluid neutralizing 1000 EID50 of virus.
§ ND, Not determined.

immediately by 30 cycles of 93 °C for 1·5 min, 53 °C for 1·5 min, 72 °C for 3 min and finally for 1 cycle at 72 °C for 10 min. cDNA was concentrated and purified using GeneClean (Bio 101).

PCR products were sequenced directly by the dideoxy-nucleotide chain-termination method using the primers H1-121, H1-345, H1-474, H1-685 and H1-809 (10 ng/lal) (Luoh et al., 1992), and the Sequenase kit (United States Biochemical) with [35S]dATP and adaptations to the protocol suggested by Bachmann et al. (1990). Nonidet P-40 (0·5%) (Sigma) was present in all reaction steps. Annealing reactions were boiled for 3 min followed by snap-cooling on solid CO2. Labelling reactions were carried out with the Mn²⁺ buffer provided in the Sequenase kit.

Of the 54 viruses isolated, 52 (> 96%) reacted with all of the MAbs in HI assay (Table 1) indicating a very high degree of antigenic conservation, in agreement with our previous work (Sheerar et al., 1989). These isolates were the dominant variant. Influenza A/Sw/IN/1726/88 (Sw/IN/88) is an isolate that has been well characterized in our laboratory and was used to generate the MAb panel, and was included here as a reference strain.

Only two minor variants were detected by HI assay: A/Sw/IA/6207/88 (Sw/IA/88) did not react (titre < 1:100) with MAb 2-15fl and A/Sw/SD/21026/90 (Sw/SD/90) did not react with MAb 7Blb. In addition, both A/Sw/WI/1915/88 (Sw/WI/88) and the MAb-selected variant v2-15fl from previous studies (Sheerar et al., 1989) did not react with MAb 2-15fl in the HI assay and were included in further investigations.

To investigate genetic variation of the dominant variant two representative viruses, A/Sw/SD/18582/88 (Sw/SD/88) and A/Sw/KY/1570/89 (Sw/KY/89), were selected. In an HI assay, these isolates had titres ≥ 1:6400 to all four MAbs. After amplification of cDNA derived from viral HA RNA, we directly sequenced the PCR products, from bases 160 to 1020, three times for each isolate. This covered the length of the HA1 region which had contained amino acid changes in previous escape mutants selected with the MAb panel (Luoh et al., 1992). We compared the HA sequences of Sw/SD/88 and Sw/KY/89 to that of Sw/IN/88 and found five silent nucleotide changes in each (data not shown) indicating a high degree of genetic conservation accompanying the antigenic conservation.

We further characterized the minor variant viruses in ELISAs and neutralization assays. Sw/IA/88, Sw/WI/88 and v2-15fl did not react with MAb 2-15fl in either the ELISA or the neutralization assay, but all three reacted with MAb 7Blb in both (Table 1). This indicated that, in each virus, only one epitope detected by our MAbs had changed. Sw/SD/90 did not react with MAb 7Blb in either ELISA or neutralization assay (as in the HI assay), but it also escaped neutralization by MAb 2-15fl even though it reacted with this MAb in the HI assay. This indicated changes in two epitopes. It is interesting to note that MAbs that react in HI assay but do not neutralize are not common but have been described before (Gitelman et al., 1986; Philpott et al., 1989).

Previously the HA1 region of both Sw/WI/88 and v2-15fl had been determined by conventional cloning and sequencing techniques (Luoh et al., 1992). Here we sequenced Sw/IA/88 and Sw/SD/90 from bases 160 to 1020 directly from the PCR products by methods developed in this study. When compared to Sw/IN/88 there were more nucleotide changes (Table 2) in both these minor variants than in the dominant variants and these resulted in a number of amino acid differences from the predicted sequence. Interestingly, none of these substitutions were the same in any two of the four minor variants. However, when mapped onto the H3 HA model there were substitutions from each variant around
one region of the molecule (data not shown) and it may be that escape from neutralization by MAb 2-15fl is due to these changes indirectly affecting the conformation of the epitope rather than the substitution of an amino acid directly involved in binding the antibody paratope. Such indirect effects of amino acid substitutions outside an epitope have been described for H3 influenza viruses (Brown et al., 1990), feline leukaemia virus (Nicolaissen-Strouss et al., 1987), foot-and-mouth disease virus (Parry et al., 1990) and human immunodeficiency virus (Reitz et al., 1988; Nara et al., 1990).

We returned to the original pig lung suspensions that had yielded the minor variants Sw/IA/88 and Sw/SD/90 and the dominant variants Sw/KY/89 and Sw/SD/88, in order to compare the HA1 sequences of viruses before and after passage in embryonated chicken eggs. After extracting RNA from these pig lung suspensions, we amplified and sequenced HA-derived cDNA three times from bases 160 to 1020 for the minor variants and from bases 160 to 660 for the dominant variants. Sw/SD/88 and Sw/KY/89 and one of the minor variants, Sw/SD/90, were all identical to the virus sequenced after passage in embryonated eggs (data not shown). This indicated that the degree of antigenic and genetic conservation we had seen was not due to selection of variants with a growth advantage in embryonated eggs and that the sequences we had defined were directly relevant to the viruses found in pigs. Sequence identity before and after passage in eggs has been described previously (Rajakumar et al., 1990). The other minor variant, Sw/IA/88, contained four point mutations which resulted in four amino acid substitutions compared with the virus passaged in embryonated eggs, suggesting the selection of a variant with a growth advantage. Other studies have shown that passage of human H1 (Robertson et al., 1987, 1991; Oxford et al., 1991) and H3 influenza viruses (Katz & Webster, 1992) in embryonated eggs can select for virus subpopulations. Also, the Sw/IA/88 virus in the pig lungs had only one amino acid substitution (146, Asn to Ser) compared with Sw/IN/88. This may mean that the virus present in pigs is not an antigenic variant and may further illustrate conservation amongst these viruses.

Finally, pigs were infected with either a dominant (Sw/IN/88) or minor (Sw/WI/88) variant virus in order to determine the relative levels of virus shedding. Two 6-week-old pigs that were antibody-negative (HI titre < 1:10) were inoculated intranasally with 2 ml of allantoic fluid containing 10^8 EID_{50}/ml of either Sw/IN/88 or Sw/WI/88. Nasal swabs were collected at 2, 3, 4, 5 and 7 days post-infection and were titrated in 10-day-old embryonated chicken eggs, as described above, to determine levels of virus shedding. Both pigs shed virus at high titres (> 10^5 EID_{50}/ml) on days 2 and 3. The pig infected with Sw/IN/88 shed virus at high titres (> 10^6 EID_{50}/ml) up to and including day 5, however, whereas the levels of virus shed for Sw/WI/88 dropped to 10^3 EID_{50}/ml by day 4 and was not detected after that. Additional studies on a large number of pigs are required to determine the significance of this observation.

These results show that the HA of H1N1 swine influenza viruses isolated between 1986 and 1991 are highly conserved, both antigenically and genetically. The viruses we studied were isolated from eight states of the U.S.A. indicating that this conservation is geographically widespread and agrees with our previous work with enzootic viruses (Sheerar et al., 1989). Similar findings have been described for H3 avian viruses (Kida et al.,...)
reported in human H1 influenza viruses (Nakajima et al., 1983; Raymond et al., 1986; Cox et al., 1989; Yamada et al., 1991). The reasons for the conservation amongst swine viruses are not known, but it may be that passage in large populations of young susceptible short-lived pigs results in little immune pressure on swine influenza viruses (Hinshaw et al., 1978).

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