JOURNAL OF VIROLOGY, Apr. 2007, p. 4315–4322 Vol. 81, No. 8
0022-538X/07/$08.00+0 doi:10.1128/JVI.02458-06

Antigenic and Genetic Evolution of Swine Influenza A (H3N2) Viruses in Europe▽

J. C. de Jong,[1,2]* D. J. Smith,[1,3] A. S. Lapedes,[4] I. Donatelli,[5] L. Campitelli,[5] G. Barigazzi,[6] K. Van Reeth,[7] T. C. Jones,[3] G. F. Rimmelzwaan,[1] A. D. M. E. Osterhaus,[1,2] and R. A. M. Fouchier[1]

National Influenza Center, Department of Virology, Erasmus Medical Center, Rotterdam, The Netherlands[1]; ViroClinics BV, Rotterdam, The Netherlands[2]; Department of Zoology, University of Cambridge, Cambridge, United Kingdom[3]; Theoretical Division, Los Alamos National Laboratory, Los Alamos, New Mexico[4]; Division of Virology, Istituto Superiore di Sanità, Rome, Italy[5]; Istituto Zooprofilattico Sperimentale, Parma, Italy[6]; and Laboratory of Virology, Faculty of Veterinary Medicine, Ghent University, Ghent, Belgium[7]

Received 8 November 2006/Accepted 31 January 2007

In the early 1970s, a human influenza A/Port Chalmers/1/73 (H3N2)-like virus colonized the European swine population. Analyses of swine influenza A (H3N2) viruses isolated in The Netherlands and Belgium revealed that in the early 1990s, antigenic drift had occurred, away from A/Port Chalmers/1/73, the strain commonly used in influenza vaccines for pigs. Here we show that Italian swine influenza A (H3N2) viruses displayed antigenic and genetic changes similar to those observed in Northern European viruses in the same period. We used antigenic cartography methods for quantitative analyses of the antigenic evolution of European swine H3N2 viruses and observed a clustered virus evolution as seen for human viruses. Although the antigenic drift of swine and human H3N2 viruses has followed distinct evolutionary paths, potential cluster-differentiating amino acid substitutions in the influenza virus surface protein hemagglutinin (HA) were in part the same. The antigenic evolution of swine viruses occurred at a rate approximately six times slower than the rate in human viruses, even though the rates of genetic evolution of the HA at the nucleotide and amino acid level were similar for human and swine H3N2 viruses. Continuous monitoring of antigenic changes is recommended to give a first indication as to whether vaccine strains may need updating. Our data suggest that humoral immunity in the population plays a smaller role in the evolutionary selection processes of swine H3N2 viruses than in human H3N2 viruses.

Since their pandemic emergence in 1968, human influenza A (H3N2) viruses have infected pigs frequently. Sometimes they initiated epidemics among pigs, for instance in Hong Kong in 1968 and 1975, Czechoslovakia in 1975, Italy in the early 1980s, Belgium and France in 1984, Great Britain in 1987, Canada in 1988, and the United States in 1998 (1, 4, 13, 18, 19, 22, 24, 28, 29). Serological and molecular studies showed that most of the swine H3N2 virus strains isolated in Europe, Hong Kong, and Canada in the period from 1977 to 1995 were closely related to each other and to the human H3N2 strains A/England/42/72, A/Port Chalmers/1/73, A/Victoria/3/75, and A/Bangkok/1/79 (2, 5, 14, 21). Serological examinations indicate the introduction of an A/Port Chalmers/1/73-like human influenza virus into the pig population in 1974 at the latest (14).

Exploiting the antigenic similarity with swine H3N2 viruses since the late 1970s, the human H3N2 strain A/Port Chalmers/1/73 is currently included in swine influenza vaccines widely in use in Europe (12). The swine population of the United States remained free from H3N2 viruses until 1998 (29). Phylogenetic analyses demonstrated that the swine H3N2 viruses which emerged in 1998 in the United States were heterogeneous and related to the human H3N2 strains A/Wuhan/359/95, A/Finland/339/95, and A/Sydney/5/97 (26).

Sporadic virus isolation, serological evidence, and sequence analysis showed that after they colonized the European pig population in the early 1970s, the A/Port Chalmers/1/73-like swine H3N2 viruses were circulating for about 10 years at a low level (1, 6, 24). An Italian study showed that all eight RNA fragments of the early swine H3N2 viruses isolated between 1977 and 1983 were human-like, whereas swine viruses isolated thereafter had obtained the six fragments coding for internal and nonstructural proteins from the avian-like H1N1 virus introduced into European pigs in 1979 (6). Around 1984, coincident with this genetic mixing, the swine H3N2 viruses developed high epidemic potential and were frequently isolated from pigs in many European countries, as mentioned above. In hemagglutination inhibition (HI) assays, these epidemic viruses were proved to have retained their serological relationship with A/Port Chalmers/1/73 during their 10-year-long silent circulation. However, swine H3N2 viruses circulating in The Netherlands and Belgium lost most of this cross-reactivity during the early 1990s (8).

Because the geographical spread of swine influenza viruses can be heterogeneous (3), we investigated whether this antigenic change was restricted to Northern European countries by analyzing a number of swine H3N2 viruses isolated in Italy during the period from 1977 to 1999. In addition, the intro-

* Corresponding author. Mailing address: National Influenza Center, Department of Virology, Erasmus Medical Center, P.O. Box 1738, 3000 DR Rotterdam, The Netherlands. Phone: 31 (10) 408 8067. Fax: 31 (10) 408 9485. E-mail: jc.de.jong@wxs.nl.

▽ Published ahead of print on 7 February 2007.

duction of an A/Port Chalmers/1/73-like human influenza virus strain into the European swine population offered us a unique opportunity to compare the antigenic and molecular evolution of swine H3N2 influenza viruses with that of their human counterparts over a period of about 20 years, starting from a similar ancestral human virus. We analyzed the antigenic drift of the European swine H3N2 viruses of the A/Port Chalmers/1/73 lineage using recently developed antigenic cartography methods (23). These methods facilitate the quantitative analysis and visualization of the antigenic properties of influenza viruses as measured by, e.g., HI assays. The results were compared with the genetic changes occurring in swine and human H3N2 viruses since 1983. Increased understanding of the antigenic and genetic evolution of influenza A viruses upon transmission to new hosts is important in times of pandemics, pandemic threats, and outbreaks, including those caused by the highly pathogenic avian influenza H5N1 virus currently circulating in large parts of the world.

MATERIALS AND METHODS

Viruses. Several swine H3N2 viruses from Belgium and The Netherlands used in this study have been described previously (8). Additional virus isolates were obtained from respiratory samples of pigs in Italy during the period from 1977 to 1999. All virus isolates were propagated in MDCK cells as described previously (8).

HI assays. HI assays using postinfection ferret antisera were performed to compare the antigenic properties of influenza A virus isolates as described previously (7, 10, 23). All antiserum samples were treated overnight with receptor-destroying enzyme and subsequently incubated at 56°C for 1 h. Twofold serial dilutions of each antiserum, starting at a 1:20 dilution, were tested for their ability to inhibit the agglutination of turkey erythrocytes by 4 hemagglutinating units of influenza A virus. All HI assays were performed in duplicate.

Antigenic cartography. The quantitative analyses of the antigenic properties of swine H3N2 viruses were performed using antigenic cartography methods as described previously for human H3N2 viruses (23). Briefly, antigenic cartography is a way to visualize, and increase the resolution of, HI data (or any binding assay data). In an antigenic map, the distance between antiserum point S and antigen point A corresponds to the difference between the $\log_2$ of the maximum titer observed for antiserum S against any antigen and the $\log_2$ of the titer for antiserum S against antigen A. Thus, each titer in an HI table can be thought of as specifying a target distance for the points in an antigenic map. Modified multidimensional scaling methods are then used to arrange the antigen and antiserum points in an antigenic map to best satisfy the target distances specified by the HI data. The result is a map in which the distance between points represents antigenic distance as measured by the HI assay. Because antisera are tested against multiple antigens, and antigens tested against multiple antisera, many measurements can be used to determine the position of the antigen and antiserum points in an antigenic map. In the case of the human H3N2 viruses, the resolution is 0.8 of a twofold dilution in the HI assay. The three-dimensional graphics were generated using the PyMOL Molecular Graphics System (9).

Genetic analyses. A phylogenetic tree based on the hemagglutinin 1 (HA1) domain was constructed to analyze the genetic evolution of swine H3N2 viruses. Data for this tree were partly taken from GenBank, partly from a previous publication (8), and partly determined for the present study. Reverse transcriptase PCR and sequencing of HA1 were performed essentially as described previously (8). PCR products were purified from agarose gels using the QiaQuick gel extraction kit (QIAGEN, Leusden, The Netherlands) and sequenced using the Big Dye terminator sequencing kit, version 3.0 (Amersham Pharmacia Biotech, Roosendaal, The Netherlands) and an ABI PRISM 3100 genetic analyzer (Applied BioSystems, Nieuwerkerk aan den IJssel, The Netherlands), according to the instructions of the manufacturer. All primer sequences are available upon request. Nucleotide and amino acid sequences were aligned using the ClustalW program running within the BioEdit software package, version 5.0.9 (15). Nucleotide sequence alignments were bootstrapped 100 times using the Seqboot package of PHYLIP, version 3.6 (11), and trees were constructed with the Dnaml package, using three jumbles. The consensus tree was calculated using the Consense package of PHYLIP 3.6, and this tree was used as the usertree in Dnaml to recalculate the branch lengths from the nucleotide sequences. Trees were visualized with the Treeview 1.6.6 program distributed with BioEdit, version 5.0.9. Amino acid sequence alignments were used to calculate the number of amino acid substitutions between pairs of strains and to produce "genetic maps" as described previously (23).

Nucleotide sequence accession numbers. The new sequences determined for this study are available from GenBank under accession numbers DQ975252 to DQ975267.

RESULTS

Analysis of antigenic properties of swine H3N2 viruses by HI assay. For the analyses presented here, we used 20 HI tables that included 30 ferret antisera to human and swine H3N2 viruses and 48 influenza A(H3N2) viruses, including 41 isolates of swine origin and 7 prototype human strains. Table 1 is a composite HI table, which includes 7 ferret antisera which were used in most of the HI tables and which were titrated against 7 prototype human strains and a selection of 22 swine influenza viruses from Belgium, The Netherlands, and Italy. The Italian swine H3N2 viruses from the period of 1984 to 1999 showed antigenic changes similar to those observed in The Netherlands and Belgium in the early 1990s. The Italian strains isolated in the period 1984 to 1993 were antigenically similar to A/Port Chalmers/1/73 and A/Victoria/3/75 and, to a lesser degree, A/England/42/72. These observations confirm those of an earlier study in which A/Swine/Italy/520/85 and A/Swine/Italy/635/87 were characterized as antigenically and genetically closely related to the early human prototype H3N2 viruses (5). In contrast, but similar to the contemporary Northern European swine H3N2 viruses, the Italian strains isolated in the period 1993 to 1999 displayed reduced reactivity with antisera to A/Port Chalmers/1/73, A/England/42/72, and A/Victoria/3/75.

Three Italian strains isolated before 1984, A/Swine/Italy/1850/77, A/Swine/Italy/6/81, and A/Swine/Italy/7/81, reacted with ferret antiserum to A/Port Chalmers/1/73 only weakly in comparison to the homologous virus. Antisera to A/Hong Kong/1/68 and A/England/42/72 showed high HI titers against A/Swine/Italy/1850/77 and A/Swine/Italy/6/81, whereas A/Swine/Italy/7/81 proved to be closely related antigenically to A/Victoria/3/75 and A/Philippines/2/82.

Phylogenetic analysis of swine and human H3N2 viruses. We determined the HA1 nucleotide sequences of a number of representative swine viruses from those listed in Table 1 and constructed a DNA maximum-likelihood phylogenetic tree in which human and swine H3 HA1 sequences available from public databases were also included (Fig. 1). In accordance with the HI data, the H3N2 viruses isolated from pigs in Italy from 1983 to 2001 appear in the phylogenetic tree mixed with the contemporary swine H3N2 viruses from Northern Europe. These European swine virus sequences, and sequences obtained from swine viruses in Hong Kong from 1999 to 2002, form a virus lineage (the A/Port Chalmers/1/73-like Eurasian swine H3N2 lineage, called swine PCh lineage below) which has evolved separately from the human H3N2 virus lineage since around 1973. In agreement with the HI data, virus isolates A/Swine/Italy/1850/77, A/Swine/Italy/6/81, and A/Swine/Italy/7/81 did not belong to the swine PCh lineage. The first two strains are also genetically more closely related to A/Bilthoven/16190/68 (A/Hong Kong/1/68-like) and are lo-

TABLE 1. HI titrations with postinfection ferret antisera against influenza A(H3N2) virus isolates of swine and human origin

Virus	Titer of ferret antiserum to antigen[a]						
	HK68	EN72	PC73	VI75	PH82	SwBR84	SwOE97
A/Hong Kong/1/68	**2560**	1280	160	<20	80	160	640
A/England/42/72	640	**2560**	1280	160	40	320	80
A/Port Chalmers/1/73	80	640	**1280**	320	80	640	80
A/Victoria/3/75	40	160	320	**1280**	80	640	40
A/Texas/1/77	<20	40	<20	160	320	320	160
A/Bangkok/1/79	<20	<20	<20	320	2560	40	40
A/Philippines/2/82	<20	<20	<20	40	**2560**	20	20
A/Swine/Italy/1850/77	2560	1280	160	80	40	160	40
A/Swine/Italy/6/81	640	640	80	40	20	80	40
A/Swine/Italy/7/81	<20	20	<20	320	640	320	40
A/Swine/Brabant/84	80	320	1280	1280	40	**5120**	1280
A/Swine/Ghent/1/84	20	80	320	320	<20	1280	1280
A/Swine/Italy/520/85	80	640	640	640	80	2560	1280
A/Swine/Italy/635/87	<20	320	640	320	<20	2560	1280
A/Swine/Italy/729/88	<20	160	160	320	<20	1280	1280
A/Swine/Belgium/379/91	40	160	640	160	<20	640	640
A/Swine/Italy/1188/92	<20	160	640	320	<20	2560	640
A/Netherlands/35/93	40	640	1280	640	40	2560	2560
A/Swine/Netherlands/849/93	<20	<20	<20	80	20	320	640
A/Swine/Netherlands/L2/93	<20	80	160	80	<20	640	640
A/Swine/Belgium/231/94	<20	20	<20	80	<20	640	2560
A/Swine/Italy/1367-2/94	20	160	160	160	<20	1280	640
A/Swine/Italy/1380-2/95	<20	20	40	40	<20	640	1280
A/Swine/Italy/1453/96	<20	<20	<20	80	<20	320	1280
A/Swine/Oedenrode/7C/96	20	80	80	160	20	640	**5120**
A/Swine/Oedenrode/7K/96	<20	<20	<20	80	<20	320	2560
A/Swine/Ommel/97	<20	<20	<20	80	<20	320	5120
A/Swine/Flanders/1/98	<20	20	20	160	40	640	2560
A/Swine/Italy/1619/99	<20	20	<20	160	20	320	2560

[a] Homologous HI titers are indicated in bold.

cated at the top of the tree, whereas A/Swine/Italy/7/81 is more closely related to A/Netherlands/233/82 (A/Bangkok/1/79-like) and appears in the human branch at the bottom of the tree.

Antigenic cartography. Recently, antigenic cartography methods were developed to provide a quantitative framework for the analysis of binding assay data, such as those generated by HI assays, and were used to map the antigenic evolution of human H3N2 viruses from 1968 to 2003 based on data from multiple HI tables. It was found that the H3N2 virus isolates aggregated as points in 11 antigenic clusters (23). We used the same methods to analyze the HI tables for swine H3N2 viruses described above (Fig. 2A). The antigenic map revealed that the swine PCh-lineage viruses (shown in Fig. 2 as dots changing from blue to red over time, from 1984 to 1999; the color code is given in the lower right panel) evolved differently from the human influenza viruses (shown as green dots). The earliest viruses of the swine PCh lineage (blue) were antigenically closely related to A/Port Chalmers/1/73 and are located within the same human cluster, while later swine strains of this lineage (purple to red) were antigenically distinct and distant from the human lineage.

The antigenic evolution of swine H3N2 viruses was found to be slower than the antigenic evolution of human H3N2 viruses (see below for a quantitative analysis). In addition, swine PCh-lineage viruses and human H3N2 viruses have followed different pathways in antigenic space since 1973. As expected, the earliest Italian swine viruses (represented as yellow dots in the antigenic map) were not in close proximity to viruses of the swine PCh lineage in the antigenic map; A/Swine/Italy/1850/77 and A/Swine/Italy/6/81 appeared close to A/Hong Kong/1/68, while A/Swine/Italy/7/81 was located close to A/Bangkok/1/79 (Fig. 2A).

While the antigenic evolution of human H3N2 viruses from 1968 to 2003 was found to be mostly two-dimensional (23), a three-dimensional antigenic map was required for the combined swine/human H3N2 HI data set. Although the two- and three-dimensional maps proved to be quite similar, the swine PCh-lineage viruses appeared as two distinct clusters in the three-dimensional map (Fig. 2B), which were distorted in the two-dimensional map. The gap between the swine clusters did not appear to be related to a period of less-intensive sampling of strains (see Table 1). Swine H3N2 viruses therefore display a clustered antigenic evolution, as was previously reported for human H3N2 viruses (23).

The same algorithms used to generate antigenic maps were subsequently applied to make genetic maps, using a distance matrix representing the number of amino acid differences between all pairs of HA1 sequences. The strains in the genetic map were color-coded the same as in the antigenic map, to facilitate the side-by-side comparison of the antigenic and genetic maps (Fig. 2C). In contrast to their appearance in the antigenic map, the swine PCh-lineage viruses were not visible as two separate clusters in the genetic map. In agreement with the phylogenetic tree (Fig. 1), the genetic map revealed rather gradual amino acid changes over time, starting from the ancestral A/Port Chalmers/1/73-like viruses.

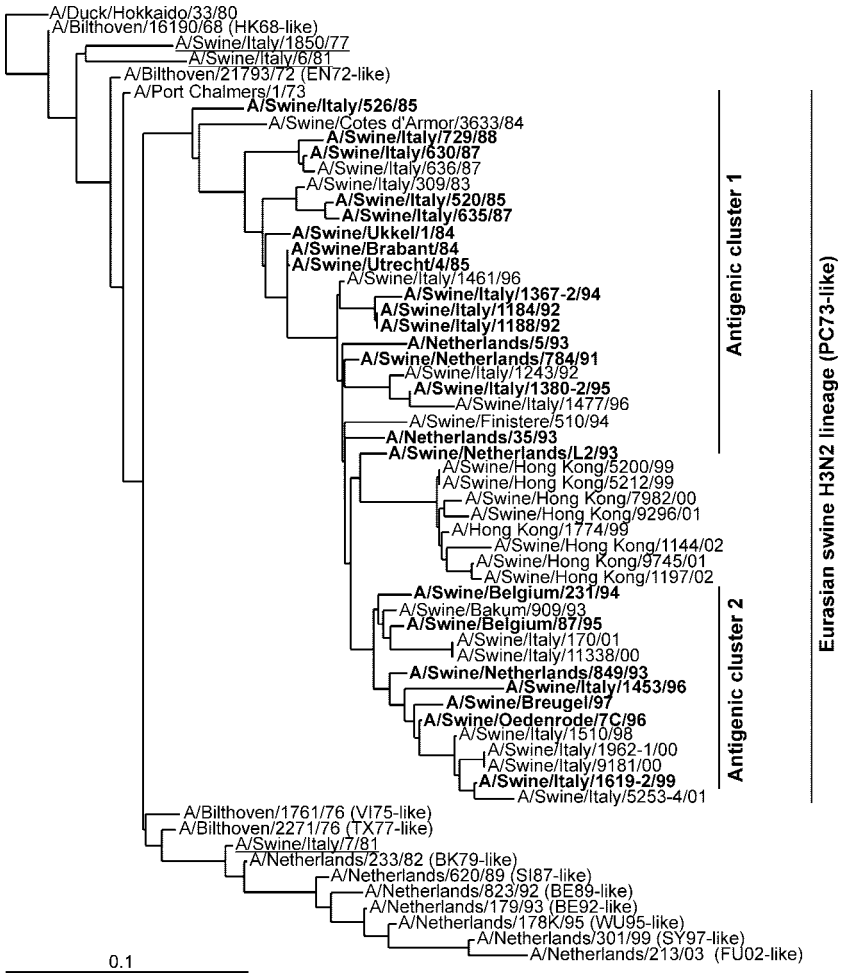

FIG. 1. Phylogenetic tree for HA1 of swine influenza A(H3N2) viruses and human reference strains. HA1 nucleotide sequences, 987 nucleotides in length, which were sequenced for the current study or collected from public databases were aligned and bootstrapped 100 times, after which trees were constructed with a maximum-likelihood algorithm, using A/Duck/Hokkaido/33/80 as the outgroup. The consensus tree was generated, for which the branch lengths were subsequently recalculated. Strains in bold were used for antigenic characterization, and the antigenic cluster to which they belong (see Fig. 2) is indicated. The underlined swine strains did not belong to the swine PCh lineage. The scale bar represents approximately 10% nucleotide change between close relatives.

Comparison of evolutionary rates of swine and human H3N2 viruses. We next compared the rates of genetic and antigenic change for human and swine H3N2 viruses in the period from 1982 to 2002 (Fig. 3). Influenza virus A/Port Chalmers/1/73 was used as the common ancestor for all post-1982 human and swine viruses. For each swine strain, the antigenic distance to the postulated ancestor was calculated from the antigenic map, and the genetic distance was calculated from the phylogenetic tree in Fig. 1. The same was done for the post-1982 subset of human H3N2 viruses included in previous work (23), to match the years of isolation of the swine virus collection.

The genetic evolution in terms of nucleotide substitutions of swine and human H3N2 viruses proved to have occurred at approximately the same rate (Fig. 3A and B), confirming earlier observations (20, 27). At the nucleotide level, swine and human H3N2 viruses have evolved on average at a rate of change in maximum-likelihood (ML) distance of 0.0047 and 0.0060 per year (~4.6 and 5.9 nucleotide substitutions per year), respectively, since 1982. Similar data were obtained when comparing distances between human and swine H3N2 HA sequences at the amino acid sequence level from genetic maps as represented in Fig. 2C and phylogenetic trees (data not shown).

In sharp contrast, the rates of antigenic change of swine and human H3N2 viruses were very distinct (Fig. 3C and D). While human H3N2 viruses have evolved at a rate of about 2.0 antigenic units per year since 1982, swine H3N2 viruses have evolved more than six times more slowly, about 0.3 antigenic units per year. Thus, compared to human H3N2 viruses, swine H3N2 influenza viruses have accumulated many more nucleotide and amino acid substitutions in HA1 that had little effect on the antigenic properties of the virus.

Correlation between amino acid changes and antigenic clusters of swine H3N2 viruses. To investigate the potential molecular basis for the differences in antigenic properties, we performed a statistical analysis using the Recursive Partitioning and Regression Trees (rpart) program of the statistical

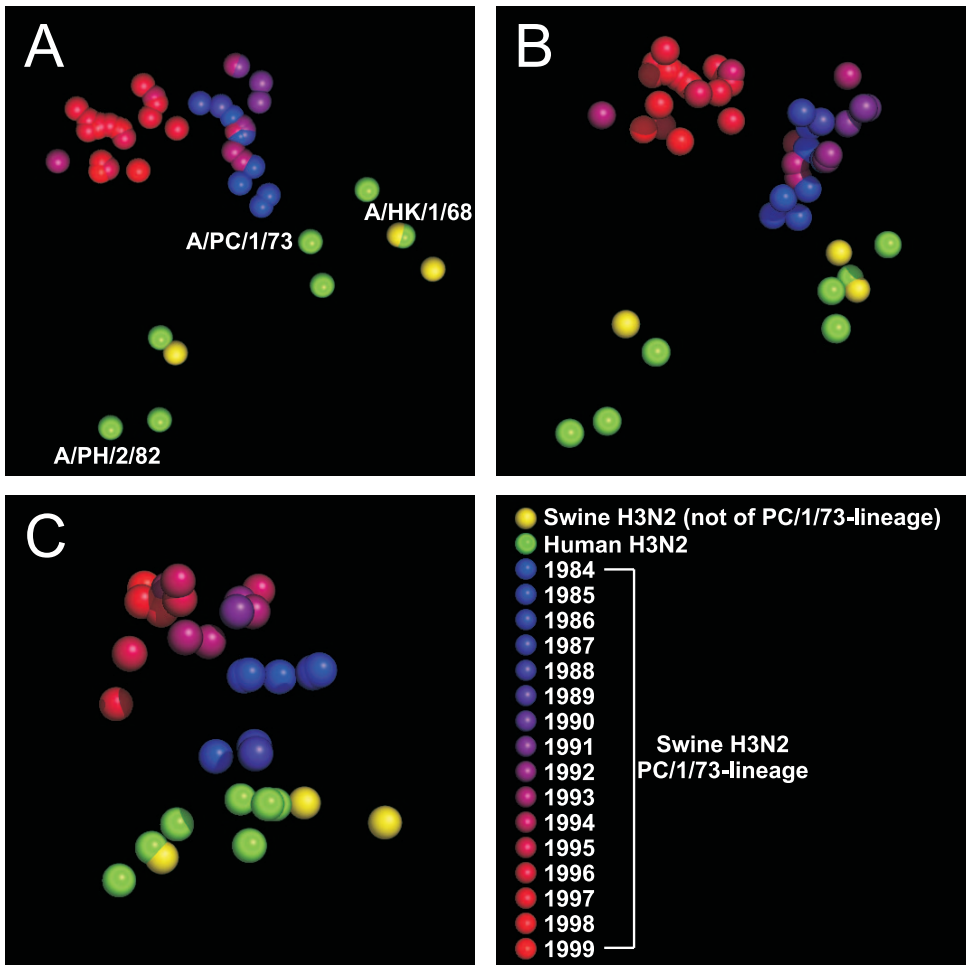

FIG. 2. Antigenic and genetic maps of swine and human H3N2 viruses. The relative positions of strains (colored spheres) and antisera (not shown) in the maps were computed such that the distances between strains and antisera in the map represent the corresponding HI measurements with the least error. Because only the relative positions of antigens can be determined, the orientation of the map within the axes is free. A two-dimensional map was generated on the basis of 20 HI tables, which included 30 ferret antisera and 48 influenza H3N2 viruses (A). The same analysis was repeated for the same strains to allow antigens and antisera to be positioned in a three-dimensional space (B). For the strains shown in panels A and B for which the HA1 amino acid sequence had been determined, a genetic map was generated based on an amino acid Hamming distance matrix (C). The color-coding of the viruses is the same in the three maps, and these colors are explained in the lower right panel.

package "R" (17) to identify amino acid substitutions that differed systematically between the HA1 sequences of strains belonging to the early and late antigenic clusters. Sequence data were available for 23 swine H3N2 strains, of which 15 strains belonged to the early cluster and 8 to the late cluster. We found one position in HA1 that distinguished the two clusters without exception; at position 145 we found serine (S) in all early-cluster strains and asparagine (N) or arginine (R) in all late-cluster strains (Table 2).

Allowing one exception, at position 278 we observed S in all early-cluster strains and N in 7 of 8 late-cluster strains. One of the late-cluster strains had 278S just like the early-cluster strains, but had alanine (A) at position 10, whereas all early-cluster strains had threonine (T) at this position. Allowing two exceptions, at position 137, we observed N in 13 of the 15 early-cluster strains and S in all late-cluster strains. The two exceptions from the early cluster had S at position 137, just like all late-cluster strains, but had T at position 34, whereas all late-cluster strains had isoleucine (I) at this position. Further analysis detected other combinations of mutations that could also explain the cluster transition. However, since such increasingly complicated combinations of mutations have a higher probability of being specific to the given data set and a smaller probability of being a generic feature, we just mention here the three simplest combinations of possibly explanatory mutations. Of note, whether the described cluster-differentiating mutations are causally related to the cluster transition is not yet known.

Antigenic drift measured with porcine sera. To investigate the relevance of the described antigenic drift for the efficacy of influenza A(H3N2) vaccination of pigs, we examined porcine sera collected 4 weeks after vaccination with a commercial swine influenza vaccine containing A/Port Chalmers/1/73 virus. The sera were titrated in HI assays against influenza viruses A/Port Chalmers/1/73 and A/Swine/Brabant/84 as representatives of the early antigenic cluster and A/Swine/Oedenrode/7C/96 and A/Swine/Flanders/1/98 as representatives of the late antigenic cluster (Table 3). Similar to the results with the ferret antisera, the porcine sera showed lower HI titers against the

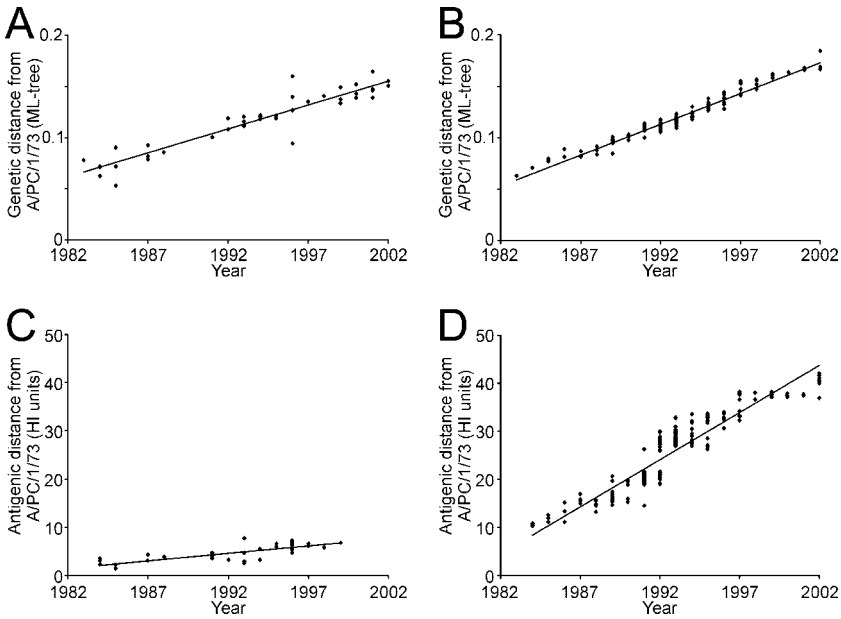

FIG. 3. Rates of genetic and antigenic evolution of swine and human H3N2 viruses. For all swine PCh-lineage strains shown in Fig. 1, the genetic distance to A/Port Chalmers/1/73 was calculated from the phylogenetic tree and was plotted as a function of time (A). The same was done for the post-1982 subset of human H3N2 strains from an earlier study (23) (B). The regression lines for panels A ($R^2 = 0.88$) and B ($R^2 = 0.95$) had similar slopes, of 0.0047 and 0.006, respectively. For the same strains, the antigenic distance to A/Port Chalmers/1/73 was computed from the two-dimensional antigenic maps as described previously (23). The antigenic distances from A/Port Chalmers/1/73 through antigenic cluster centroids (23), as a function of time, were plotted both for the swine strains (C) and the human strains (D). The regression lines for panels C ($R^2 = 0.64$) and D ($R^2 = 0.86$) had different slopes, of 0.3 and 2.0, respectively.

most recent swine virus, A/Swine/Flanders/1/98, than against the early A/Swine/Brabant/84 virus, although the ferret and porcine sera did not distinguish equally well between A/Port Chalmers/1/73 and early strain A/Swine/Brabant/84 on the one hand and the late strains A/Swine/Oedenrode/7C/96 and A/Swine/Flanders/1/98 on the other hand.

DISCUSSION

The present study confirmed the close antigenic relationship between the swine H3N2 viruses isolated in The Netherlands and Belgium during the period 1983 to 1993 and the human A/Port Chalmers/1/73 virus by using antigenic cartography methods based on HI assays. Among the Italian strains isolated before 1983, we detected strains that were not serologically related to A/Port Chalmers/1/73 but to A/Hong Kong/1/68 and A/England/42/72 or to A/Philippines/2/82 (Table 1, Fig. 2).

TABLE 2. Cluster-differentiating amino acid substitutions in HA1 of swine PCh-lineage viruses[a]

Possibility	Early cluster	Late cluster
1	145S (15/15)	145N/R (8/8)
2	278S and 10T (15/15)	278N and 10I/T (7/8) or 278S and 10A (1/8)
3	137N and 34I/V (13/15) or 137S and 34T (2/15)	137S and 34I (8/8)

[a] Only the three simplest possibilities as determined using the Recursive Partitioning and Regression Trees (rpart) program of the statistical package "R" are listed. Cluster-differentiating substitutions are listed with the HA1 amino acid position and residue indicated. The numbers of strains in each antigenic cluster with the specific cluster-differentiating mutation(s) are indicated in parentheses.V, valine.

These findings indicate that soon after the emergence of subtype H3N2 in the human population in 1968 and again around 1982, independent introductions of human H3N2 viruses in the swine population took place, leading to endemic circulation in swine that persisted after the first introduction at least until 1981. Because no similar viruses have been isolated from pigs since 1981, these viruses apparently have become extinct in pigs. It is possible that these two lines were out-competed by the swine PCh-lineage viruses.

The post-1983 swine H3N2 viruses from Italy were shown to be closely related to A/Port Chalmers/1/73 virus in the beginning and to exhibit a pattern of antigenic drift similar to that observed for the A/Port Chalmers/1/73-like swine viruses in The Netherlands and Belgium during the early 1990s, forming a new antigenic cluster (Table 1; Fig. 2). It may be suspected that, around this time, another transmission of avian H3N2 virus occurred, replacing the former swine PCh lineage. However, phylogenetic analyses proved that the newer swine H3N2 viruses gradually evolved from the early part of the swine PCh lineage (Fig. 1).

TABLE 3. HI test titers of pig antisera collected 4 weeks after vaccination with a commercial A/Port Chalmers/1/73 vaccine[a]

Virus strain	Titer range	Median titer
A/Port Chalmers/1/73	**640–1280**	**640**
A/Swine/Brabant/84	160–640	160
A/Swine/Oedenrode/7C/96	80–640	160
A/Swine/Flanders/1/98	20–160	40

[a] Range and median for 11 vaccinated animals are given. Homologous HI titers are indicated in bold.

Phylogenetic analyses confirmed that an A/Port Chalmers/1/73-like human influenza virus strain is the most probable ancestor of the swine PCh lineage and show A/Victoria/3/75 virus, or even an avian H3N2 virus, to be a much less likely candidate for this role. Phylogenetic analyses also confirmed the parallel evolution of the swine H3N2 viruses in Belgium, The Netherlands, and Italy after 1983 (Fig. 1). In general, the Northern European strains are located closer to the trunk of the tree than the Italian strains, suggesting that the Italian viruses were derived from the swine PCh-lineage strains circulating and evolving in The Netherlands and Belgium during the 1990s. Subsequently, these variants reached Italy and followed independent evolutionary pathways in that country. The spread of swine viruses from The Netherlands to Italy may have been facilitated by the extensive commercial transport of live pigs by this route, amounting to about a million animals per year. On the other hand, the magnitude of the transport of live swine in the reverse direction is negligible. This large, continuous stream of pigs may have created opportunities for multiple introductions of Northern European swine viruses into Italy.

Using antigenic cartography methods, we showed that the antigenic drift of swine PCh-lineage viruses followed a different direction than the antigenic drift of human H3N2 viruses and, like the human viruses, appeared to be punctuated rather than gradual, creating two clusters of strains. Within a cluster, there are also limited antigenic differences, but there is a clear gap between the two clusters.

The positions of human H3N2 viruses isolated since 1968 could be placed in a mostly two-dimensional plane, and it was speculated that this was because there is a selective advantage for viruses moving linearly away from previous clusters as they most effectively escape existing population-level immunity (23). The swine PCh-lineage viruses isolated since 1984 can also be accommodated in such a two-dimensional plane, which differs, however, from the plane of the human map, and when the two maps are combined, three dimensions are required to avoid distortion. We conjecture that the difference in evolutionary paths is the consequence of the introduction of the ancestral A/Port Chalmers/1/73-like virus into another species. Because of intrinsic differences between hosts (e.g., cellular receptors) and selection pressures (innate and acquired humoral and cellular defense systems), different changes in the antigenic structure of the virus are allowed or provoked in swine than in humans. As a consequence, the antigenic drift of the swine PCh-lineage virus follows different pathways in swine than in humans.

Using statistical methods, we identified several amino acid substitutions in HA1 that could be responsible for the antigenic differences between strains of the early and later swine clusters. Interestingly, the three major mutations of the three simplest combinations of such substitutions (S145N, S278N, and N137S; see Table 2) occurred at the same amino acid positions as some of the cluster-differentiating mutations for the transition from the cluster of A/England/42/72–A/Port Chalmers/1/73-like H3N2 viruses—which is closely related to the early swine cluster—to the cluster of A/Victoria/3/75-like viruses in the human H3N2 virus antigenic map; two of these mutations were even identical (23). The fact that the antigenic drift still takes different directions in the two hosts could be explained by differences in the numerous other mutations in the two lineages. However, before causally relating cluster-differentiating mutations to the corresponding antigenic drift, experimental introduction of these mutations separately or in combination into the HA1 gene by reverse genetics should confirm that they can achieve the cluster transitions concerned. Such experiments were beyond the scope of the present study but are currently in progress. As the antigenic drift in human H3N2 viruses is usually ascribed to the immune pressure mounted in the human population because of previous infections with earlier variants, the common potential cluster-differentiating mutations may suggest that the described antigenic change in the swine PCh-lineage viruses is also due to immune pressure (see below). Alternatively, these mutations may have been selected in both hosts on the basis of advantages other than immune evasion or may have been incidentally coupled to other mutations having this effect.

During the period from 1983 to 1999, the antigenic drift of the swine PCh-lineage viruses was approximately sixfold slower than that of the human counterparts (Fig. 3). This difference is especially striking because the evolution of the two lineages started in the same cluster of swine PCh-lineage viruses. The slower antigenic drift of the swine viruses is not the consequence of a lower rate of genetic evolution of HA1 at the nucleotide or amino acid level (Fig. 1 and Fig. 3); as has been described previously (20, 27) and confirmed in the present study, the genetic evolution of swine and human H3N2 viruses occurs at approximately the same rates. This means that, in the swine viruses, fewer of the observed amino acid substitutions affect the antigenic properties of the HA protein than in human H3N2 viruses. This could be the consequence of a lower selective pressure in pigs for amino acid changes that influence antigenic reactivity.

Because of the short average life span of pigs, swine influenza virus evolution may be determined to only a limited extent by immune pressure, the driving force of antigenic drift of influenza viruses in humans. Whereas the human H3N2 virus requires frequent antigenic changes of HA to ensure that a sufficiently large pool of immunologically susceptible hosts is available, the vast majority of pigs are killed at the age of 6 months, and the susceptible pig population is continuously renewed, thus limiting the buildup of immune pressure. Only adult sows used for pig breeding live long enough to experience more than one influenza season, and thus, we speculate that these animals are essential for the endemicity of swine influenza viruses and may create some degree of immune pressure, leading to the (slow) antigenic drift described in the present report. Such an immune pressure could be mounted by the frequent occurrence of local outbreaks of swine influenza A (H3N2) virus, as is indicated by the seroprevalences of anti-A/Port Chalmers/1/73 antibodies in sow sera, reported to be between 28 and 61% in the period from 1975 to 1984 (14) and 39% in the period from 1991 to 1992 (3). Also, vaccination of sows with A/Port Chalmers/1/73-containing vaccines, estimated at about 10% for Northwest Europe, could have contributed to a limited extent to the immunity concerned.

Because the human influenza virus strain A/Port Chalmers/1/73 (H3N2) is currently widely used in commercial influenza vaccines for pigs in Europe, the observed antigenic drift could be relevant to the influenza vaccination policy for swine. We

showed that the porcine immune system recognizes in a qualitatively similar way the antigenic changes that were identified in HI assays using postinfection ferret antisera (Table 3), confirming earlier observations (25). The ferret and porcine sera did not distinguish equally well between A/Port Chalmers/1/73 and early strain A/Swine/Brabant/84 on the one hand and the late strains from 1996 and 1998 on the other hand, but such quantitative discrepancies can be expected for antibody responses mounted by two different species after exposure to an antigen in different forms (live virus leading to infection versus inactivated virus) administered by different routes (intranasally versus intramuscularly).

Despite the demonstrated increase in the antigenic distance between the swine H3N2 viruses and A/Port Chalmers/1/73 virus in the early 1990s, vaccination challenge studies in pigs have not provided sufficient arguments for updating the A/Port Chalmers/1/73 vaccine strain to more recent strains. Pigs immunized with a commercial A/Port Chalmers/1/73-based adjuvanted swine influenza vaccine were protected from fever and did not transmit virus to sensitive contact pigs upon challenge with the recent swine H3N2 strain A/Swine/Oedenrode/7C/96 (16). However, vaccine-induced protective immunity was incomplete, because four of seven vaccinated pigs excreted influenza virus for 1 to 4 days after challenge, whereas naturally immune pigs were completely protected. In another experimental study, the same commercial vaccine provided complete protection against disease after challenge with the recent A/Swine/Flanders/1/98 strain, although, in results similar to those of the above-mentioned study, four of eight vaccinated pigs allowed some virus replication in the lungs (25). Antigenic analyses as such, therefore, are insufficient to decide whether vaccine updates for pigs are needed. The challenge studies showed that the antigen dose and the adjuvant in swine influenza vaccines may also be important determinants of vaccine potency. Nevertheless, the antigenic cross-reactivity to A/Port Chalmers/1/73 virus may eventually fall to a level such that the current vaccine is no longer effective. Therefore, regular clinical monitoring of the H3N2 vaccine's efficacy is to be advised. In general, continuous and more-intensive monitoring of potential antigenic changes in swine influenza viruses is strongly recommended and will give a first indication as to whether vaccine strains may need updating.

ACKNOWLEDGMENTS

We thank L. van der Kemp, C. Baas, T. M. Bestebroer, and S. Puzelli for performing serological and molecular determinations and H. Geerligs for supplying sera from vaccinated pigs and data for influenza vaccination of pigs in Europe.

D.J.S. was supported by the NIH Director's Pioneer Award program, part of the NIH roadmap to medical research, through grant number DP1-OD000490-01.

REFERENCES

1. **Aymard, M., J. M. Gourreau, C. Kaiser, M. Fontaine, F. Madec, and J. P. Tillon.** 1985. Les marqueurs immunovirologiques du risque d'influenza A H3N2 chez les porcs. Rev. Epidémiol. Santé Publique **33:**283–291.
2. **Bikour, M. H., E. H. Frost, S. Deslandes, B. Talbot, J. M. Weber, and Y. Elazhary.** 1995. Recent H3N2 swine influenza virus with haemagglutinin and nucleoprotein genes similar to 1975 human strains. J. Gen. Virol. **76:**697–703.
3. **Brown, I. H., P. A. Harris, and D. J. Alexander.** 1995. Serological studies of influenza viruses in pigs in Great Britain 1991–2. Epidemiol. Infect. **114:**511–520.
4. **Campitelli, L., I. Donatelli, E. Foni, M. R. Castrucci, C. Fabiani, Y. Kawaoka, S. Krauss, and R. G. Webster.** 1997. Continued evolution of H1N1 and H3N2 influenza viruses in pigs in Italy. Virology **232:**310–318.
5. **Castrucci, M. R., L. Campitelli, A. Ruggieri, G. Barigazzi, L. Sidoli, R. Daniels, J. S. Oxford, and I. Donatelli.** 1994. Antigenic and sequence analysis of H3 influenza virus haemagglutinins from pigs in Italy. J. Gen. Virol. **75:**371–379.
6. **Castrucci, M. R., I. Donatelli, L. Sidoli, G. Barigazzi, Y. Kawaoka, and R. G. Webster.** 1993. Genetic reassortment between avian and human influenza A viruses in Italian pigs. Virology **193:**503–506.
7. **De Jong, J. C., F. M. de Ronde-Verloop, T. M. Veenendaal-van Herk, T. F. Weijers, K. Bijlsma, and A. D. M. E. Osterhaus.** 1988. Antigenic heterogeneity within influenza A(H3N2) virus strains. Bull. W. H. O. **66:**47–55.
8. **De Jong, J. C., A. P. van Nieuwstadt, T. G. Kimman, W. L. A. Loeffen, T. M. Bestebroer, K. Bijlsma, C. Verweij, A. D. M. E. Osterhaus, and E. C. J. Claas.** 1999. Antigenic drift in swine influenza H3 haemagglutinins with implications for vaccination policy. Vaccine **17:**1321–1328.
9. **DeLano, W. L.** 2002. The PyMOL Molecular Graphics System. DeLano Scientific, San Carlos, CA. http://www.pymol.org.
10. **Dowdle, W. R., and A. P. Kendal.** 1979. Influenza viruses, p. 585–609. *In* E. H. Lennette and N. J. Schmidt (ed.), Diagnostic procedures for viral, rickettsial and chlamydial infections, 5th ed. American Public Health Association, Washington, DC.
11. **Felsenstein, J.** 1989. PHYLIP: phylogeny inference package (version 3.2). Cladistics **5:**164–166.
12. **FIDIN.** 1996/1997. Varkensvaccins (vaccines for pigs), p. 286–296. *In* FIDIN Repertorium diergeneesmiddelen, 6th ed. Amsterdam Medical Press, Alkmaar, The Netherlands. www.fidin.nl/repertorium.
13. **Haesebrouck, F., P. Bironi, M. B. Pensaert, and J. Leunen.** 1985. Epizootics of respiratory tract disease in swine in Belgium due to H3N2 influenza virus and experimental reproduction of disease. Am. J. Vet. Res. **46:**1926–1928.
14. **Haesebrouck, F., and M. Pensaert.** 1988. Influenza in swine in Belgium (1969–1986): epizootiologic aspects. Comp. Immunol. Microbiol. Infect. Dis. **11:**215–222.
15. **Hall, A.** 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp. Ser. **41:**95–98.
16. **Heinen P. P., A. P. van Nieuwstadt, E. A. de Boer-Luijtze, and A. T. J. Bianchi.** 2001. Analysis of the quality of protection induced by a porcine influenza A vaccine to challenge with an H3N2 virus. Vet. Immunol. Immunopathol. **82:**39–56.
17. **Ihaka, R., and R. Gentleman.** 1996. A language for data analysis and graphics. J. Compu. Graphi. Stati. **5:**299–314.
18. **Kundin, W. D.** 1970. Hong Kong A-2 influenza virus infection among swine during a human epidemic in Taiwan. Nature **228:**857.
19. **Morin, M., C. Girard, Y. ElAzhary, R. Fajardo, R. Drolet, and A. Lagacé.** 1990. Severe proliferative and necrotizing pneumonia in pigs: a newly recognized disease. Can. Vet. J. **31:**837–839.
20. **Nerome, K., Y. Kanegae, K. F. Shortridge, S. Sugita, and M. Ishida.** 1995. Genetic analysis of porcine H3N2 viruses originating in southern China. J. Gen. Virol. **76:**613–624.
21. **Ottis, K., L. Sidoli, P. A. Bachmann, R. G. Webster, and M. M. Kaplan.** 1982. Human influenza A viruses in pigs: isolation of a H3N2 strain antigenically related to A/England/42/72 and evidence for continuous circulation of human viruses in the pig population. Arch. Virol. **73:**103–108.
22. **Shortridge, K. F., R. G. Webster, W. K. Butterfield, and C. H. Campbell.** 1977. Persistence of Hong Kong influenza virus variants in pigs. Science **196:**1454–1455.
23. **Smith, D. J., A. S. Lapedes, J. C. de Jong, T. M. Bestebroer, G. F. Rimmelzwaan, A. D. Osterhaus, and R. A. Fouchier.** 2004. Mapping the antigenic and genetic evolution of influenza virus. Science **305:**371–376.
24. **Tůmová, B., D. Věžíková, J. Menšík, and A. Štumpa.** 1980. Surveillance of influenza in pig herds in Czechoslovakia in 1974–1979. 1. Introduction of influenza epidemic A(H3N2) viruses into pig herds. Zentbl. Vet. Med. B. **27:**517–523.
25. **Van Reeth, K., S. De Clercq, and M. Pensaert.** 2001. The significance of antigenic evolution for swine influenza vaccine efficacy: learning from vaccination-challenge studies in pigs, p. 99–106. *In* B. Dodet and M. Vicari (ed.), Emergence and control of zoonotic ortho- and paramyxovirus diseases. John Libbey Eurotext, Paris, France.
26. **Webby, R. J., S. L. Swenson, S. L. Krauss, S. M. Goyal, K. D. Rossow, and R. G. Webster.** 2001. Evolving H3N2 and emerging H1N2 swine influenza viruses in the United States, p. 241–249. *In* A. D. M. E. Osterhaus, N. Cox, and A. W. Hampson (ed.), Options for the control of influenza IV. Elsevier Science Publishers, Amsterdam, The Netherlands.
27. **Webster, R. G., W. J. Bean, O. T. Gorman, T. M. Chambers, and Y. Kawaoka.** 1992. Evolution and ecology of influenza A viruses. Microbiol. Rev. **56:**152–179.
28. **Wibberley, G., C. Swallow, and D. H. Roberts.** 1988. Characterization of an influenza A(H3N2) virus isolated from pigs in England in 1987. Br. Vet. J. **144:**196–201.
29. **Zhou, N. N., D. A. Senne, J. S. Landgraf, S. L. Swenson, G. Erickson, K. Rossow, L. Liu, K.-J. Yoon, S. Kraus, and R. G. Webster.** 1999. Genetic reassortment of avian, swine, and human influenza A viruses in American pigs. J. Virol. **73:**8851–8856.