

# Evolution of avian influenza viruses

David L. Suarez<sup>\*</sup>

*Southeast Poultry Research Laboratory, 934 College Station Road, Athens, GA 30605, USA*

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## Abstract

Although influenza viruses can infect a wide variety of birds and mammals, the natural host of the virus is wild waterfowl, shorebirds, and gulls. When other species of animals, including chickens, turkeys, swine, horses, and humans, are infected with influenza viruses, they are considered aberrant hosts. The distinction between the normal and aberrant host is important when describing virus evolution in the different host groups. The evolutionary rate of influenza virus in the natural host reservoirs is believed to be slow, while in mammals the rate is much higher. The higher rate of evolution in mammals is thought to be a result of selective pressure on the virus to adapt to an aberrant host species. Chickens and turkey influenza virus isolates have previously and incorrectly been lumped together with wild waterfowl, gull, and shorebird influenza viruses when determining rates of evolutionary change. To determine mutational and evolutionary rates of a virus in any host species, two primary assumptions must be met: first, all isolates included in the analysis must have descended from a single introduction of the virus, and second, the outbreak must continue long enough to determine a trend. For poultry, three recent outbreaks of avian influenza meet these criteria, and the sequences of the hemagglutinin and nonstructural genes were compared. Sequences from all three outbreaks were compared to an avian influenza virus consensus sequence, which at the amino acid level is highly conserved for all the internal viral proteins. The consensus sequence also provides a common point of origin to compare all influenza viruses. The evolutionary rates determined for all three outbreaks were similar to what is observed in mammals, providing strong evidence of adaptation of influenza to the new host species, chickens and turkeys. © 2000 Elsevier Science B.V. All rights reserved.

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## 1. Introduction

Influenza viruses have a high error rate during the transcription of their genomes because of the low RNA polymerase fidelity (Parvin et al., 1986; Stech et al., 1999). The

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<sup>\*</sup> Tel.: +1-706-546-3479; fax: +1-706-546-3161.

*E-mail address:* dsuarez@seprl.usda.gov (D.L. Suarez)

high error rate produces a quasispecies phenomenon where many different viral genotypes will cocirculate in the host, with each virus potentially having different levels of fitness for the host environment (Domingo et al., 1985). The advantage of a high error rate is the viruses' ability to rapidly adapt to a new environment. This may occur, for example, during infection of a new host species that requires adaptive genetic changes for optimal replication and transmission. The primary disadvantage of the high error rate is the production of many viral genotypes that are defective or have a reduced fitness for the current host. Most of the nucleotide changes are eventually eliminated from the gene pool because they provide either a neutral or a negative selection factor for the continued replication of the viral genotype. Sometimes these neutral or synonymous mutations can increase in frequency when they are linked to a nonsynonymous change that provides the viral genotype with a positive selection factor or because of random chance. These nonsynonymous and synonymous changes provide useful markers in the epidemiological tracking of viruses during an outbreak (Lindstrom et al., 1998a, b). For many viruses the rate of mutational change occurs at a steady predictable pace, frequently called a molecular clock (Buonagurio et al., 1986). The rate of mutational change, which includes both synonymous and nonsynonymous changes, can be determined for viruses from the same lineage as sampled over time. The evolutionary rate can also be estimated by examining the nonsynonymous or amino acid changes that occur in viruses from the same lineage as sampled over time.

Influenza viruses infect a wide variety of species often causing serious disease. However, in the natural hosts for avian influenza viruses, wild waterfowl, gulls, and shorebirds, the virus is considered avirulent (Slemons et al., 1974; Kawaoka et al., 1988; Stallknecht, 1998). When an influenza virus infects a new host species, it will often replicate and occasionally cause disease, but the virus rarely transmits well enough in the new species to cause an epidemic. For example, the Hong Kong H5N1 virus was believed to have been transmitted from chickens into humans at least 18 times during 1997, but little evidence is available for any human to human transmission (Mounts et al., 1999). Transmission between mammals also occurs regularly; for example, numerous reports of swine influenza viruses infecting humans have appeared (Dowdle and Hattwick, 1977; Rota et al., 1989; Wells et al., 1991; Kimura et al., 1998). Fortunately, epidemic disease outbreaks resulting from transmission of viruses between species are rare. A species crossover may result in the introduction of a new hemagglutinin and/or neuraminidase subtype into humans, an antigenic shift, which can result in a severe epidemic or pandemic, because the new host has no protective immunity to the recombinant influenza strain. This type of antigenic shift among influenza viruses has occurred in the human population three times this century (Webster et al., 1992).

Although introductions of influenza in poultry occur commonly, these outbreaks once discovered, usually do not continue for long because of control efforts or failure of the virus to adapt to the new host. However, at least three poultry outbreaks have occurred, which have extended for several years and for which there are multiple virus isolates available for study. These include the 1983–1989 Pennsylvania H5N2 outbreak (PA/83) (Suarez and Senne, 2000), the 1993-present Mexican H5N2 outbreak (Mex/93) (Garcia et al., 1997), and the 1994-present Northeast United States H7 Live Bird Market outbreak (NE LBM/94) (Suarez et al., 1999). These outbreaks provide us with a unique opportunity to study evolutionary change in poultry, primarily chickens and turkeys.

## 2. Materials and methods

Wild birds, including waterfowl, gulls, and shorebirds, are the natural hosts for influenza viruses; these are thought to be evolving slowly in the natural host reservoir (Webster et al., 1992), which should allow the determination of a consensus sequence that would approximate the viral sequence that most commonly circulates in the natural host. However, only a few genes have been sequenced from wild bird isolates, which does not provide a large sample to compare. To overcome this restriction, selected avian influenza viral gene sequences from poultry were also included in determining the consensus sequence. When influenza viruses are first transmitted from the natural reservoir species to poultry, the virus sequence in the new host is assumed to be the same or very similar as to what circulated in the donor host. If influenza viruses of the same lineage have been circulating in a poultry population for a long time, the virus will become less representative of the initial inoculum because of the rapid rate of influenza virus evolution. For this reason some poultry influenza isolates are excluded from contributing to the consensus sequence, and these viruses fall into two categories. The first category are isolates with a history of numerous passages in embryonated chickens eggs, as for example FPV/Rostock/34. The second category are multiple isolates from the same lineage. If viruses from the same lineage have been isolated over multiple years, we assume that the later isolates are less representative of the virus that came from the natural host than the earlier ones. For this reason, only the first isolate from a particular lineage is included in the analysis, for example A/CK/Pennsylvania/21525/83 to represent the PA/83 lineage (Suarez and Senne, 2000). Since it is not possible to know for certain how long influenza viruses of a particular lineage have been circulating in a poultry population, some sequences will be used to generate a consensus that are less representative of the natural reservoir viruses. These differences will not affect the final averaged results, since most of the nucleotide changes are random events that are unlikely to be seen in multiple lineages.

A consensus sequence was determined for each avian influenza virus gene of which we had sequences from at least 15 unique isolates. One exception was the H7 North American lineage. A consensus sequence was determined by choosing the most common nucleotide at each position of the coding sequence of the gene. The putative amino acid sequence was then determined for each gene. The consensus sequence was determined separately for isolates from the Eurasian (EA) and the North American (NA) lineage of viruses. The consensus sequence was then used as a common point of origin to calculate the mutational and evolutionary rates for different influenza outbreaks. Three avian influenza outbreaks were examined in this study. Each outbreak met two criteria for inclusion in the study: all isolates examined were related in one or more genes as determined by phylogenetic analysis and direct sequence comparison of discriminative nucleotide and amino acid changes (Garcia et al., 1997; Suarez et al., 1999; Suarez and Senne, 2000). Second, each outbreak lasted for a long period, with multiple virus isolates being available for study.

Initial multiple alignments of each gene for which a complete coding sequence was available were made with the Megalign (DNASTAR) program using the Clustal V algorithm. Phylogenetic trees were determined for the hemagglutinin and nonstructural

gene using the PAUP 3.1 program (Swofford, 1997). The distance matrix was calculated for each sequence compared to the appropriate coding sequence of the consensus to determine the number of nucleotide or amino acid differences between them. The sequence differences were then plotted with the Excel (Microsoft) program in a scattergram against the number of months after isolation from the index case for each outbreak (for example April 1983 for the Pennsylvania 83 H5N2 outbreak). Linear regression analysis was used to determine a trend line, and the slope was used to determine the rate of nucleotide or amino acid changes over time. The data were corrected for length of the sequence and converted to substitutions/site/year.

### 3. Results

The nucleotide and amino acid sequences of the nucleoprotein (NP), matrix (MA), nonstructural subtype (group) A (NS-A), and HA1 subunit of hemagglutinin subtypes H5 and H7 genes of available avian and avian-like influenza viruses were compared with selected influenza virus sequences from mammals to form phylogenetic trees using parsimony analysis. The nucleotide sequence trees for the NS-A, MA, and NP genes demonstrated the previously described groups of isolates including the human, classic swine, equine type 1, equine type 2, gull, avian NA, and avian EA lineages of viruses (Fig. 1). Both H5 and H7 hemagglutinin subtypes had only avian NA and EA groupings. Using phylogenetic analysis of amino acid sequence, the division between the avian NA and EA groups was lost (Fig. 2 and Table 1).

The consensus nucleotide and amino acid sequences were determined for both the EA and the NA lineages for the MA, NP, NS-A, and the hemagglutinin subtype H5 genes and compared by pairwise alignment to determine similarity. At the nucleotide level, the sequence similarity between the avian NA and EA lineages for the MA, NP, and NS-A genes was from 92.9 to 95.7%, and 83.1% for the H5 gene. However, when the amino acid sequences were compared, much higher similarities were found; 99 to 100% for the internal proteins and 93.1% for the H5 protein (Table 1).

Table 1  
Comparison of consensus sequences from avian influenza isolates isolated from either North America or Europe and Asia

|                         | Nucleotide length | % Similarity | Amino acid length | % Similarity |
|-------------------------|-------------------|--------------|-------------------|--------------|
| Nonstructural subtype A | 890               | 95.7         |                   |              |
| NS1                     |                   |              | 230               | 100          |
| NS2                     |                   |              | 121               | 100          |
| Matrix                  | 1027              | 94.8         |                   |              |
| M1                      |                   |              | 252               | 100          |
| M2                      |                   |              | 97                | 99           |
| Nucleoprotein           | 1565              | 92.9         | 498               | 99.7         |
| H5 Hemagglutinin HA1    | 996               | 83.1         | 332               | 93.1         |

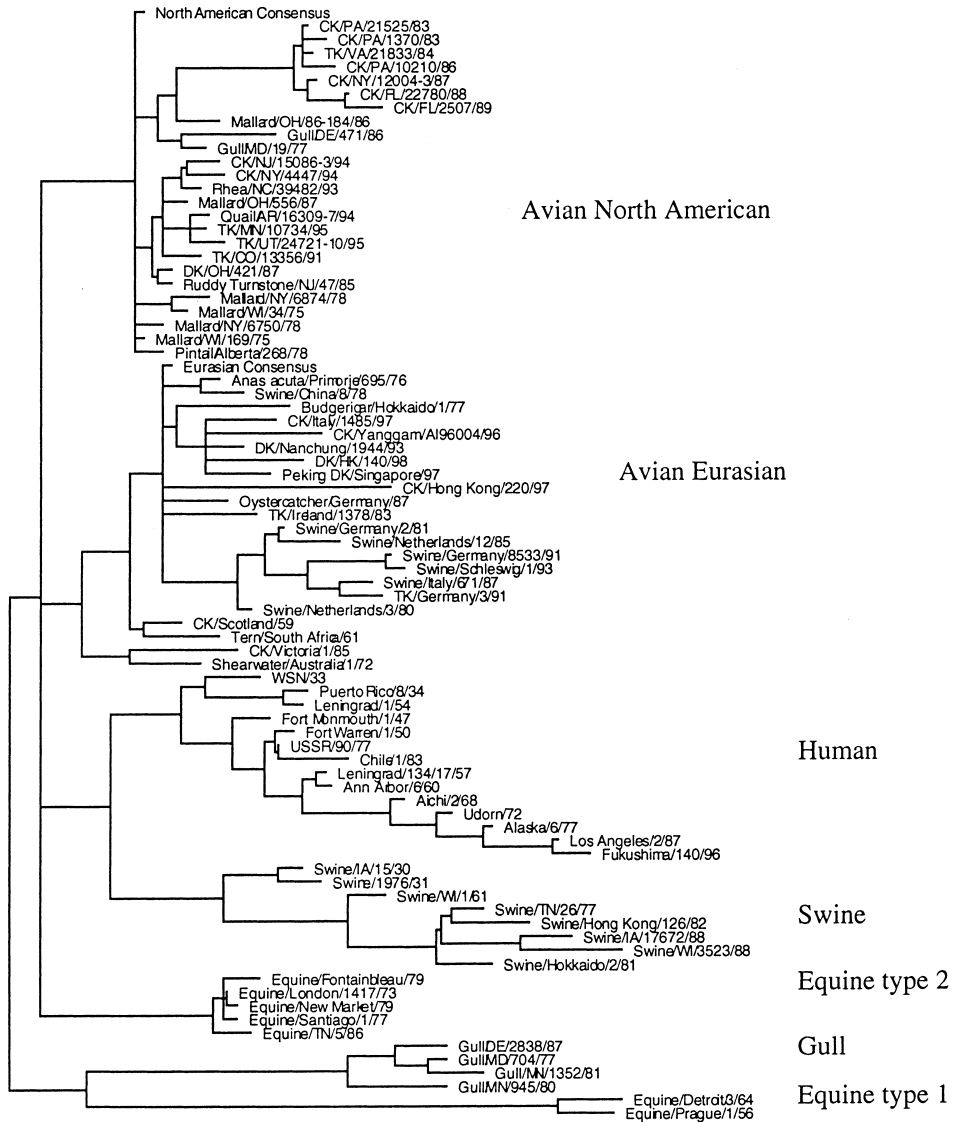


Fig. 1. Phylogenetic tree from the nonstructural subtype (group) A gene. The tree includes representative sequences from all seven major groups. The tree was made with PAUP 3.1 computer program using 100 bootstrap replicates in a heuristic search, and the tree is midpoint rooted. Abbreviations CK, chicken; TK, turkey; DK, duck. Standard two-letter abbreviations are used for states in the USA.

The consensus sequence was used as the point of origin in regression analysis to compare sequence changes among unrelated influenza virus isolates. The NP, M, and NS-A genes were examined for all avian and avian-like isolates from the EA lineage where the full coding sequence was available. Only the first isolate of related lineages was

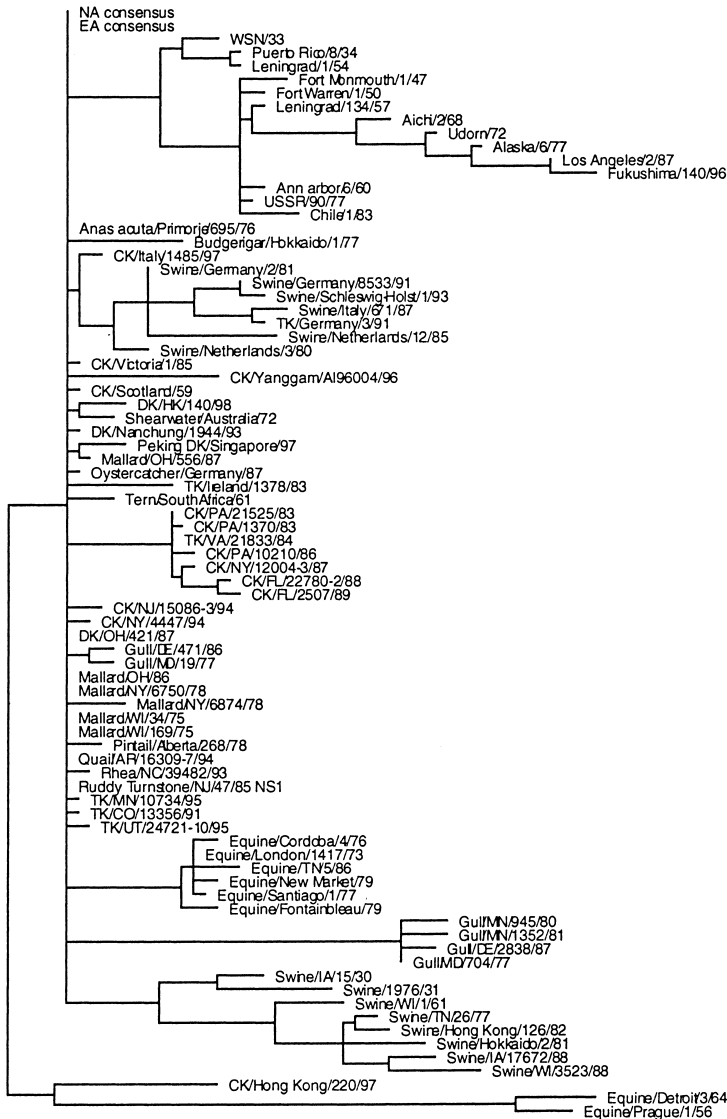


Fig. 2. Phylogenetic tree from the nonstructural subtype (group) A NS1 protein. The tree includes the same isolates as Fig. 1. The tree was made with PAUP 3.1 computer program using 100 bootstrap replicates in a heuristic search, and the tree is midpoint rooted. For abbreviations, see Fig. 1.

included; for example A/Swine/Netherlands/25/80, the first isolate for avian-like swine lineage was included, but all the other swine and turkey isolates from this lineage were not included. The number of nucleotide differences was determined using pairwise distance comparisons between the EA consensus sequence and each isolate, and the number of substitutions was then graphed against the year of isolation (Fig. 3). The trend

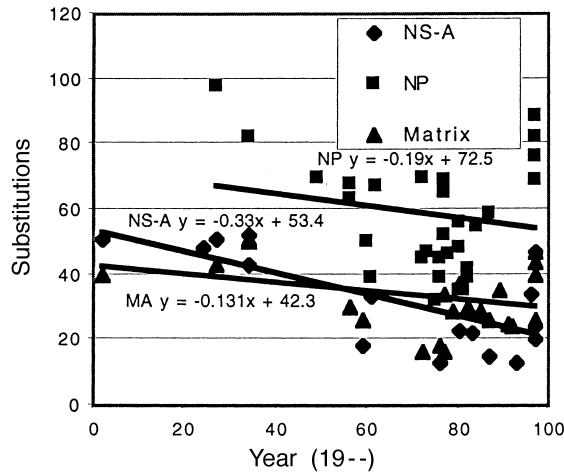


Fig. 3. Comparison of the mutation rates for the matrix (MA), nucleoprotein (NP) and the nonstructural subtype A (NS-A) genes of isolates with full coding sequence that grouped in the Eurasian lineage of viruses based on phylogenetic analysis. The number of nucleotide differences as compared to the Eurasian consensus sequence for the appropriate gene were plotted against the year of virus isolation. Linear regression analysis determined a trend line for each set of data points and the equation for the line is included in the graph.

line from the regression analysis and the equation for the line were added to the graph. For all three genes the trend line showed a negative slope, suggesting that the isolates from the EA group did not evolve over time. However, the trend can vary greatly, depending on which isolates are included in the analysis. In this example, the isolates before 1950 had many nucleotide differences from the consensus sequence probably for two reasons. First, these early viruses have probably been circulating in chickens long before they were first isolated in culture; past evidence demonstrates that avian influenza will rapidly evolve in poultry (Garcia et al., 1997; Suarez et al., 1999; Suarez and Senne, 2000). The second likely reason for the high number of sequence differences is that these early isolates have been extensively passed in embryonated eggs. This measure will introduce sequence changes as the virus becomes adapted for egg propagation (Robertson et al., 1987). Removal of these isolates from the comparison will make the slope positive, but for consistency all the data were calculated with a minimum of isolate selection.

The HA1 subunit from the three different poultry outbreaks was compared to the appropriate NA H5 or H7 consensus sequence. These included the PA/83, the Mex/93, and the NE LBM/94 outbreaks. The mutational and evolutionary rate for each isolate was determined by regression analysis (Fig. 4). A positive mutation rate was observed for all three lineages ranging from  $4.71 \times 10^{-3}$  to  $7.95 \times 10^{-3}$  nucleotide changes/site/year (Table 2). The evolutionary rate was  $1.08 \times 10^{-3}$  to  $2.41 \times 10^{-3}$  amino acid substitutions/site/year.

Data from the nonstructural gene is also presented for the PA/83 poultry outbreak (Table 2). However, the NE LBM/94 had multiple lineages for the nonstructural gene and could not be compared, and the Mex/93 lineage was NS subtype B. The data from four

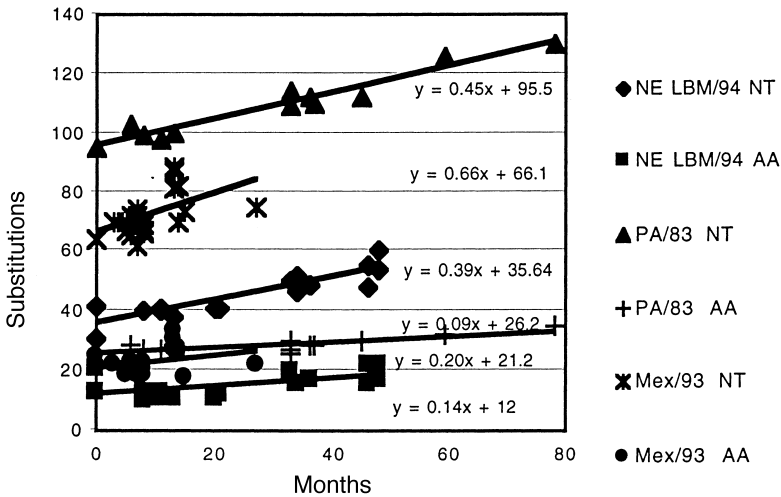


Fig. 4. Comparison of the mutation and evolutionary rates of the HA1 subunit from three influenza outbreaks in poultry. The number of nucleotide substitutions of each isolate was compared to the H5 or H7 consensus sequence and graphed using the month of isolation after the index case for each outbreak. Linear regression analysis determined a trend line for each set of data points and the equation for the line is included in the graph.

mammalian lineages, including human, classic swine, avian-like swine, and equine type 2, were also compared to the NA and EA consensus sequences. All lineages except for the avian-like swine had closer sequence similarities with the NA consensus sequence, and the regression analysis was performed using the consensus sequence with the highest similarity (Table 2).

Table 2

Mutational and evolutionary rates for the hemagglutinin and nonstructural genes as compared to North American or Eurasian avian influenza consensus sequence<sup>a</sup>

| HA1 Subunit      | HA1 NT | HA1 AA |
|------------------|--------|--------|
| PA/93            | 5.42   | 1.08   |
| Mex/93           | 7.95   | 2.41   |
| H7 LBM/94        | 4.71   | 1.7    |
| NS               | NS-A   | NS1    |
| PA/83            | 1.44   | 1.02   |
| Human            | 0.91   | 0.45   |
| Classic swine    | 0.79   | 0.17   |
| Avian-like swine | 2.67   | 0.84   |
| Equine type 2    | 0.43   | 0.22   |

<sup>a</sup>The number of nucleotide or amino acid substitutions were plotted against time and, using regression analysis, the slope of the best fitting line was determined which allowed the calculation of mutational and evolutionary rates for each lineage.

#### 4. Discussion

Influenza viruses have a complex relationship with many host species. Consequently, the mutation and evolutionary rates have to be determined separately for each major category, for example swine, human and equine groups. These major groups, however, are not completely defined in the avian world. The natural host reservoir is believed to include a wide variety of wild bird species, primarily ducks and gulls (Slemons et al., 1974; Kawaoka et al., 1988; Stallknecht, 1998). However, nucleotide sequence analysis of viruses from the natural host reservoir shows three distinct categories of avian viruses including the NA, EA, and gull lineages. Other classifications are possible, since little or no sequence data have been determined for avian influenza isolates from Africa, South America, Australia, and Antarctica, and the natural host range for influenza viruses is not truly known. The consensus sequences of the NA and EA lineages of the NP, MA and NS-A genes have 92.9% or greater sequence similarity, with an even higher amino acid identity (Table 1). The high degree of amino acid sequence conservation found in sequences from a wide variety of avian sources suggests that influenza viruses are well adapted to their natural hosts. One theory of host/parasite relationships predicts that well-adapted viruses will cause only subclinical or mild disease, but they will replicate and shed in sufficient concentrations to allow efficient transmission to other susceptible hosts (Knolle, 1989). This theory appears to describe avian influenza virus in migratory ducks where these conditions are fulfilled (Webster et al., 1978; Kida et al., 1980; Cooley et al., 1989). However, when influenza crosses into a new host species that it is not adapted to, the virus can often cause severe disease (Suarez et al., 1998).

The high sequence conservation of the internal genes, particularly at the amino acid level, provides a reasonable basis for using the consensus sequence as a common reference point to determine mutational and evolutionary rates for influenza in any host species. The use of a consensus sequence for all influenza genes is not possible because of a lack of sequence information for many of the hemagglutinin and neuraminidase subtypes and the polymerase genes. The consensus sequences for the other genes may also change as more sequence data becomes available, especially sequence data from wild birds. Ideally, a consensus sequence should be derived only from virus isolates from wild birds representing a variety of different years and different migratory flyways (ex. Eastern seaboard, Mississippi, and Western seaboard for North America).

Several assumptions need to be made to use the consensus sequence. Firstly, the source of all type A influenza outbreaks must ultimately be from the natural wild host reservoir. Based on available sequence data we have to limit ourselves to using the consensus sequences from the NA or EA reservoirs. Secondly, one can only compare isolates for mutational and evolutionary changes if all isolates are derived from a single introduction into the host population. For example, the classical swine and the avian-like European swine lineages cannot be compared in the same regression analysis. This is because the original source of infection was different for each lineage (Ludwig et al., 1995). This principle also applies to poultry outbreaks. For example, the Pennsylvania H5 outbreak in 1983–1989 cannot be compared to the Mexican H5N2 outbreak of 1993–present, because these outbreaks started with different introductions of influenza viruses. When one attempts to compare unrelated isolates, even within the same major groupings of viruses

(Fig. 3), the results will not be meaningful. For example, when the MA, NP, and NS-A genes were compared from avian and avian-like viruses from the EA group, the apparent trend is a nonsensical decrease in distance from the EA consensus sequence. Thirdly, representative viruses isolated over several years must be available to measure the mutational and evolutionary rates in an outbreak. For poultry this parameter severely limits the number of outbreaks available to study. During most outbreaks only one virus was isolated, and the outbreak was controlled. Currently, virus isolates to determine the mutational and evolutionary rates for poultry viruses are available only from the H5N2 PA/83, H5N2 Mex/93 and the H7 NE LBM/94 outbreaks.

These three poultry epizootics were reexamined using the NA consensus sequence as the point of comparison to determine the mutational and evolutionary rates for the HA and NS genes (Fig. 4, Table 2). For all three poultry influenza outbreaks, the mutational and evolutionary rates for the HA1 subunit were similar to each other and to the human, swine and equine type 2 influenza virus lineages (Sugita et al., 1991; Ludwig et al., 1995; Daly et al., 1996; Fitch, 1996; Fitch et al., 1997). The rates for the HA1 subunit were higher than that observed for the nonstructural gene, which has previously been observed (Buonagurio et al., 1986; Ludwig et al., 1995; Fitch, 1996; Lindstrom et al., 1998a, b). The higher rate of evolution of the hemagglutinin protein in the human influenza lineage has usually been linked to the viruses escaping the immune pressure of the host (Fitch et al., 1991). However, most chickens and turkey populations are naïve to influenza exposure (not vaccinated, no passively acquired antibody), and in view of the short production lifespans of the birds, another mechanism may be responsible for the mutational and evolutionary changes observed.

The importance of using the consensus sequences is to provide a common baseline to use the ever increasing influenza virus sequence information for comparisons. This common point of origin can allow an estimate of how long a virus has been circulating undetected in a species, by calculating the *y* intercept in the regression analysis. For the three poultry examples in this report, the PA/83 outbreak had the highest *y* intercept value for the HA1 subunit of 95.5 as compared to 66.1 and 35.6 for the Mexican H5 and H7 NE LBM outbreaks, respectively. This can be interpreted to mean that virus of the PA/83 lineage had been circulating in poultry for a longer period of time, which is in line with the high number of unique mutations found in this lineage (Suarez and Senne, 2000).

One commonly used method for determining mutational and evolutionary rates is to compare the number of substitutions in the genes of isolates as compared to the index case in an outbreak. For example, the sequence from the PA/83 lineage were compared to A/Chicken/Pennsylvania/21525/83 which was the first virus isolated from the PA/83 lineage. One problem with this method is that the index case may have extraneous mutations that are not representative of the lineage, which will affect the results. Using the consensus sequence should reduce the source of error, by providing a more stable baseline. The use of the consensus sequence tends to lower the mutation and evolutionary rates. For example in the NE LBM/94 H7 outbreak, using the index case to compare mutation rates the rate was  $7.0 \times 10^{-3}$  nucleotide changes/site/year while using the consensus sequence the rate was  $4.71 \times 10^{-3}$  nucleotide changes/site/year (Suarez et al., 1999).

The consensus sequence also allows a better understanding of what amino acids may be important for influenza viruses to cross species barriers. As more sequence data become available, the ability to discern important mutations should improve. For example, in the influenza viruses that caused the human H5N1 outbreak in Hong Kong, the ability to cross species was likely influenced by different viral genes. When the NS genes of the Hong Kong isolates were compared to the five mammalian influenza lineages, six amino acids were identified that differed from the EA consensus sequence but were the same as the predominant sequence of one or more of the swine or equine lineages (data not shown). This type of comparative analysis may help pinpoint specific amino acids that are responsible for the ability of a virus to cross species.

The evolution of avian influenza viruses remains complicated because of the large number of host species that can be infected by the virus. For veterinary researchers working on poultry diseases, an understanding of the evolution of influenza virus in chickens and turkeys is important for understanding disease pathogenesis and epidemiology. Understanding the role of wildlife is also important because influenza viruses regularly cross species from waterfowl to poultry. In migratory ducks, which are likely the primary source of influenza in poultry, these viruses appear to be evolving slowly, based on the highly conserved internal proteins. The hemagglutinin protein does not exhibit the same degree of sequence conservation that is observed for the internal proteins, and the evolutionary rate would appear to be higher for this gene (Table 1). The determination of an evolutionary rate in migratory waterfowl will remain difficult without having multiple low passage isolates available from different years. Poultry species, including chickens and turkeys, have often been lumped with other avian species and presumed to be part of a single population. However, turkeys in particular are not considered to be a natural host for avian influenza virus because of the lack of evidence for infection in wild turkey studies (Davidson et al., 1988; Hopkins et al., 1990). Also the chicken is probably an aberrant host, but no studies have been done with the red jungle fowl, the ancestor of the domestic chicken, to confirm this hypothesis. The mutational and evolutionary rates observed for the three poultry outbreaks also suggest that chickens and turkeys are aberrant hosts, because influenza viruses in poultry evolve at rates reported for mammals (Buonagurio et al., 1986; Sugita et al., 1991; Ludwig et al., 1995; Daly et al., 1996; Fitch et al., 1997; Lindstrom et al., 1998a, b). The continued surveillance and sequencing of isolates from migratory waterfowl and gulls remains an important goal for improving our understanding of the ecology of influenza viruses.

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