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Reassortment and Interspecies Transmission of North American H6N2 Influenza Viruses

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H6N2 influenza viruses were isolated from California chickens in 2000 and 2001. Here we report the characterization of these H6N2 viruses, one of the few descriptions of non-H5, non-H7 subtype influenza viruses in this host. The H6N2 viruses were nonpathogenic in experimentally infected chickens and could be divided into three genotypes. All three genotypes of virus had similar surface glycoproteins and all contained an 18 amino acid deletion in the neuraminidase, a characteristic of other chicken influenza viruses. Differences were apparent, however, in the complement of replicative protein genes between the genotypes. The presence of multiple H6N2 genotypes suggests that independent transmission and/or reassortment events may have taken place between aquatic bird and chicken influenza viruses.

Key Words: influenza; H6N2; interspecies transmission; avian influenza.

INTRODUCTION

Aquatic birds are the primary reservoir of influenza A viruses throughout the world. Together, the species of ducks and shorebirds harbor all 15 hemagglutinin (HA) and all 9 neuraminidase (NA) influenza A subtypes (for review see Ito and Kawaoka, 1998). Although stable lineages of some influenza A subtypes are present in mammals, phylogenetic evidence suggests that all influenza A viruses have ancestral links to viruses within the aquatic bird reservoir (Webster *et al.,* 1992). The transfer of gene segments from avian viruses to human viruses has caused at least two human pandemics of the 20th century (Beare and Webster, 1991).

A crucial factor that limits the transfer of viruses from aquatic birds to humans is the low potential that aquatic avian viruses have for growth in primates (Beare and Webster, 1991; Murphy *et al.,* 1982). One mechanism to overcome this species barrier is the replication of viruses in intermediate hosts. Pigs have been implicated as a likely intermediate host (Scholtissek, 1990) due to their susceptibility to both avian and human viruses (Kida *et al.,* 1994), a trait that is, in part, attributable to the presence of both human and avian virus receptors in the porcine respiratory tract (Ito *et al.,* 1998).

Events in Hong Kong during 1997 and 1999, however, demonstrated that avian influenza viruses do not require adaptation in a mammalian host before they can infect humans. In 1997, 18 persons were confirmed infected with avian H5N1 viruses after contact with infected birds (Claas *et al.,* 1998; Subbarao *et al.,* 1998). In 1999, two persons were infected with an avian H9N2 virus that had been identified as previously circulating in quail (Lin *et al.,* 2000; Peiris *et al.,* 1999). Although these avian viruses infected humans, they did not have the capacity for human-to-human spread. The H5N1 and H9N2 events have highlighted the need that exists for the characterization of viruses that circulate in alternative influenza avian hosts such as chicken and quail (Matrosovich *et al.,* 1999, 2001).

Isolations of influenza viruses from chickens have been infrequent in comparison to those from other domestic poultry species such as duck and turkey (Alexander, 2000; Lin *et al.,* 1994; Shortridge *et al.,* 1977, 2000). Many isolates collected from chickens are of the H5 or H7 subtypes, which are seemingly unique in their ability to cause substantial mortality. The mortality of H5 and H7 subtypes corresponds to the acquisition of polybasic residues at the HA cleavage site (Bosch *et al.,* 1981; Horimoto and Kawaoka, 1994; Klenk *et al.,* 1977; Steinhauer, 1999). Despite the isolation of influenza viruses from chicken, they have been considered only transient hosts of viruses from the reservoir in aquatic birds. Emerging evidence, particularly from studies of H9N2 (Cameron *et al.,* 2000; Guan *et al.,* 2000) and H7 (Suarez *et al.,* 1999) viruses, suggests that this hypothesis may no longer be true and that nonpathogenic strains of virus can be maintained and spread in poultry species.

During 2000 and 2001, nonpathogenic H6N2 viruses were isolated from six chicken flocks located in Califor-

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TABLE 1

Summary of Viral Isolations

Virus isolate	Date of isolation	County in which virus was isolated	Source of isolate	Disease or disease signs	Concurrent pathogens
A/Ck/CA/431/00	February 2000	Los Angeles	Tracheal swab of dead rooster	• Upper respiratory tract infection	• Herpesvirus (presumptive) • Secondary bacteria
A/Ck/CA/465/00	February 2000	San Bernardino	Pooled tissue of killed chickens	• Decreased egg production • Respiratory distress	• Multiple bacteria species
A/Ck/CA/1002a/00	March 2000	San Bernardino	Pooled tissue of killed chickens	• Decreased egg production • Respiratory distress • Increased flock mortality	• *Pasteurella* sp. • *Mycoplasma* sp. • Multiple bacteria species
A/Ck/CA/139/01	March 2001	Stanislaus	Pooled trachea and lung tissue of killed chickens	• Increased bird huddling • Weight loss • Increased flock mortality • Respiratory distress	• Herpesvirus (Marek's disease) • Multiple bacteria species
A/Ck/CA/905/01	March 2001	San Bernardino	Pooled tissue of killed chickens	• Decreased egg production • Respiratory distress	• None identified
A/Ck/CA/6643/01	June 2001	San Diego	Pooled trachea and cecal tonsil tissue of killed chickens	• Respiratory distress	• *Haemophilus paragallinarum* and *Mycoplasma gallisepticum*

nia. Limited data are available on the composition of nonpathogenic chicken viruses and on the H6 influenza virus subtype in general. This report describes the genetic and biological characteristics of these H6N2 viruses isolated from chickens in California.

RESULTS

Virus isolations and clinical symptoms

Respiratory distress and/or decreased egg production, both of which are signs of influenza in chickens, were observed in six flocks of chickens in California between February of 2000 and June of 2001. Orthomyxoviruses were isolated from all six flocks (Table 1), and the viruses were designated A/Chicken/California/431/00 (A/Ck/CA/431/00), A/Chicken/California/465/00 (A/Ck/CA/465/00), A/Chicken/California/1002a/00 (A/Ck/CA/1002a/00), A/Chicken/California/139/01 (A/Ck/CA/139/01), A/Chicken/California/905/01 (A/Ck/CA/905/01), and A/Chicken/California/6643/01 (A/Ck/CA/6643/01). The primary cause of the observed symptoms was difficult to determine because of the presence of multiple avian pathogens in most infected flocks. Results of HA inhibition (HI) and NA inhibition assays (data not shown) confirmed that all six isolates were H6N2 viruses.

Growth of A/Ck/CA/139/01 in chickens

The A/Ck/CA/139/01 virus was isolated from a flock of Cornish cross chickens. This flock displayed increased mortality but whether the influenza infection played a role in the higher mortality was unclear. To assess the pathogenicity of this isolate and its ability to replicate, we intravenously inoculated eight chickens across two age groups and then monitored them for 12 days. None of the chickens displayed any obvious signs of clinical disease. Although of low pathogenicity, the virus was able to replicate and all birds were seropositive at 14 days after infection (Table 2). Three days after infection, virus was recovered from the cloacae and tracheae of 8-week-old birds infected intravenously, whereas virus was recovered from the trachea of only one 4.5-week-old bird. A second group of 4.5-week-old chickens was infected by a combination of tracheal, ocular, oral, and nasal routes. Virus was isolated only from the trachea of these birds.

Chickens whose nares, pharynges, eyes, and tracheae were inoculated with A/Ck/CA/431/00 or A/Ck/CA/465/00 did not show any signs of disease, although all birds seroconverted (data not shown).

Antigenic analysis

Because of the spatial and temporal distribution of the six Californian virus isolates, it was unclear whether these viruses represent a single lineage that was introduced into a chicken flock in California and continued to spread, or whether the viruses represented multiple lineages that infected multiple chicken populations. Multiple antigenic groups would suggest multiple transmissions of viruses from their reservoir in aquatic birds,

TABLE 2

Growth of A/Ck/CA/139/01 in Experimentally Infected Chickens

Bird No.	Age at time of inoculation (week)	Route	Viral growth in cloaca[a] ($TCID_{50}$)	Viral growth in trachea ($TCID_{50}$)	Serum HI[b] titer 14 days after infection
871	8	IV	Yes ($<1 \times 10^2$)	Yes ($1 \times 10^{3.5}$)	640
866	8	IV	Yes ($1 \times 10^{4.7}$)	Yes ($<1 \times 10^2$)	1280
867	8	IV	Yes ($1 \times 10^{4.5}$)	Yes ($1 \times 10^{3.7}$)	1280
863	8	IV	Yes ($1 \times 10^{4.5}$)	Yes ($1 \times 10^{2.7}$)	1280
861	4.5	IV	Yes (1×10^4)	No	640
872	4.5	IV	Yes ($<1 \times 10^2$)	No	80
862	4.5	IV	Yes ($1 \times 10^{4.5}$)	No	640
898	4.5	IV	Yes (1×10^4)	Yes ($<1 \times 10^2$)	1280
870	4.5	NR[c]	No	Yes (ND)	640
869	4.5	NR	No	Yes (ND)	320
864	4.5	NR	No	Yes (ND)	640
865	4.5	NR	No	Yes (ND)	320

[a] Virus growth was determined 3 days after infection.
[b] Abbreviations: HI, hemagglutination inhibition; IV, intravenous inoculation; NR, natural routes; ND, not done.
[c] Natural routes of infection included oral, nasal, tracheal, and orbital.

whereas a more conserved pattern would indicate the spread of a single viral lineage. To clarify this issue, we tested the isolates with antisera to A/Turkey/Massachusetts/65 (H6N2), to A/Teal/HongKong/w312/97 (H6N1), and to A/Shorebird/Delaware/127/97 (H6N2), and with postinfection antisera to A/Ck/CA/431/00 and to A/Ck/CA/139/01 (Table 3). All of the viruses isolated from San Bernardino County (A/Ck/CA/465/00, A/Ck/CA/1002a/00, and A/Ck/CA/905/01) had the same antigenic profile. A/Ck/CA/431/00, which was isolated from Los Angeles County, could be distinguished from the Bernardino County isolates by a lower HI titer to A/Turkey/Massachusetts/65 antisera. A/Ck/CA/139/01 and A/Ck/CA/6643/01, from Stanislaus and San Diego Counties, respectively, reacted to equivalent titers to the panel of test antisera, but could be distinguished from the other chicken viruses by a lower titer to A/Teal/HongKong/w312/97 antisera.

Genetic analysis of the surface glycoproteins

Before this study, the Influenza Sequence Database contained the sequences of HA genes of only six H6 viruses, and only one of those was collected from North American aquatic birds. To further assess the genetic relatedness of the chicken viruses, and to compare them with other North American viruses, we sequenced the HA gene from the Californian chicken viruses and that of eight H6 viruses isolated from North American duck and shorebird species. Analysis of these data and of the available database sequences revealed that the HA genes of A/Ck/CA/431/00, A/Ck/CA/465/00, A/Ck/CA/

TABLE 3

Antigenic Comparison of the Californian Chicken Viruses with Other H6 Viruses by Hemagglutination Inhibition Assay

Virus	Titer of antisera to virus				
	A/Ty/MA/65 (H6N2)	A/Tl/HK/w312/97 (H6N1)	A/Sb/DE/127/97 (H6N2)	A/Ck/CA/431/00 (H6N2)	A/Ck/CA/139/01 (H6N2)
A/Ck/CA/431/00	20	40	<20	**160**[a]	160
A/Ck/CA/465/00	80	80	20	160	160
A/Ck/CA/1002a/00	80	40	20	160	160
A/Ck/CA/905/01	80	80	20	160	320
A/Ck/CA/139/01	80	<20	80	160	**640**
A/Ck/CA/6643/01	160	<20	80	160	640
A/Ty/MA/65	**640**	40	80	80	ND
A/Tl/HK/w312/97	160	**640**	20	20	ND
A/Sb/DE/127/97	<20	<20	**160**	<20	ND

Abbreviations: Ty, turkey; MA, Massachusetts; Tl, teal; HK, Hong Kong; Sb, shorebird; DE, Delaware; Ck, chicken; CA, California; ND, not done.
[a] Boldface type indicates results of assays in which we used antiserum and the virus against which the antiserum was originally raised.

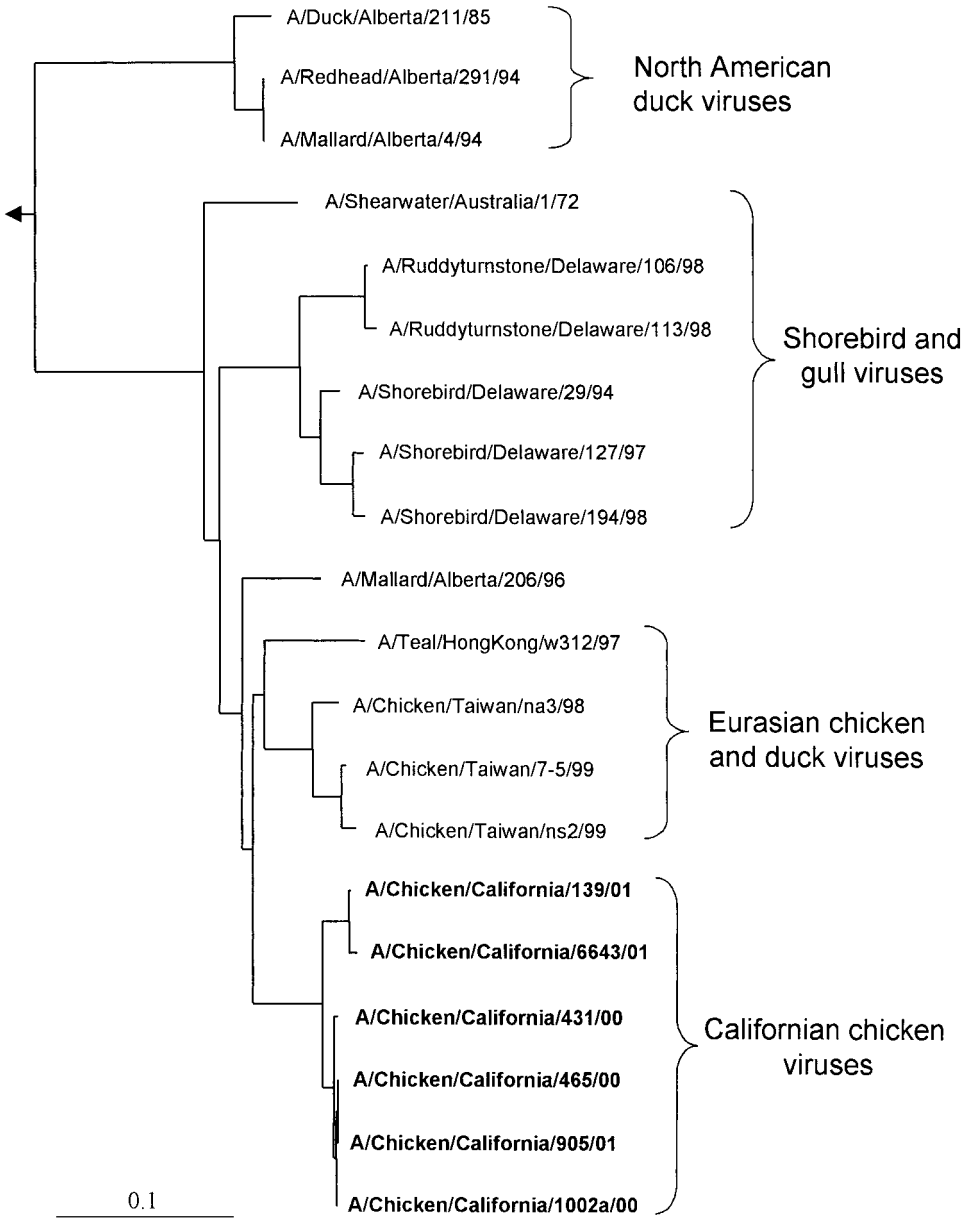

FIG. 1. Phylogenic distance tree of genes that encode the HA of H6 influenza viruses. The sequences used in the study were the 1059-bp region of HA that corresponds to positions 49 through 1107 of the HA open reading frame of A/Teal/HongKong/w312/97. Horizontal distances are proportional to genetic distance, and the tree is rooted to the HA gene of A/Mallard/Pensylvania/10218/84 (H5N2). The scale is in number of nucleotide substitutions per site.

1002a/00, and A/Ck/CA/905/01 were more than 99.5% identical to each other. The HA genes of A/Ck/CA/139/01 and A/Ck/CA/6643/01 were 99.6% identical to each other, but 97.1–97.5% identical to the other Californian viruses. Phylogenetic analysis of the H6 gene sequences available in the Influenza Sequence Database and those obtained in this study show that the Californian chicken viruses cluster together (Fig. 1). Of the other viruses analyzed, A/Mallard/Alberta/206/96 was the most similar (90–91% identity) to the Californian chicken viruses.

All of the viruses had a sequence very similar to the avirulent consensus sequence at the HA cleavage site (Table 4). The seven potential glycosylation sites (positions 26, 27, 39, 182, 306, 498, and 557) within the HA were conserved among all of the Californian H6N2 isolates, and the five shorebird viruses whose genes were sequenced in this study, the three duck viruses lacked the signal sequence at position 182.

The NA genes of the H6N2 chicken viruses were more than 98.5% identical to each other over the 1326 bp analyzed. Database searches showed that the Californian viruses were most similar [91–92% identical (data not shown)] to H5N2 viruses isolated from chickens in Mexico. The predicted sequence of the NA protein of all

TABLE 4

Sequence of the Hemagglutinin Cleavage Site in H6 Viruses

Virus	Cleavage site sequence
Avirulent avian consensus	PQRETR/G
A/Chicken/California/1002A/00	PQIETR/G
A/Chicken/California/431/00	PQIETR/G
A/Chicken/California/465/00	PQIETR/G
A/Chicken/California/905/01	PQIETR/G
A/Chicken/California/139/01	PQIATR/G
A/Chicken/California/6643/01	PQIATR/G
A/Redhead/Alberta/291/94	PQAETR/G
A/Mallard/Alberta/4/94	PQAETR/G
A/Mallard/Alberta/206/96	PQIETR/G
A/Ruddyturnstone/Delaware/106/98	PKVETR/G
A/Ruddyturnstone/Delaware/113/98	PKVETR/G
A/Shorebird/Delaware/127/97	PKVETR/G
A/Shorebird/Delaware/194/98	PKVETR/G
A/Shorebird/Delaware/29/94	PKVETR/G

six Californian chicken viruses contained an 18 amino acid deletion in the stalk region. No large deletions have been found in the stalk region of aquatic bird viruses; thus, our present finding indicates that this deletion may represent an adaptation for growth in chickens.

Genetic analysis of the genes that encode internal proteins

Genetic and serological data of the surface glycoproteins showed that the Californian chicken H6N2 viruses share a recent common ancestor. To determine whether viruses of the same genotype were present in all six chicken flocks, we sequenced the genes that encode the internal proteins of the H6N2 chicken viruses. All of the genes sequenced were most homologous to those of American avian viruses with identities between 89 and 98% (data not shown). The sequence data showed that genes encoding the internal proteins of A/Ck/CA/431/00, A/Ck/CA/465/00, A/Ck/CA/1002a/00, and A/Ck/CA/905/01 were more than 99% identical. These viruses were isolated from separate flocks over a 14-month period; thus, this finding indicates that this virus genotype has been transmitted between flocks or introduced multiple times from other virus reservoirs. The M and PA genes of all six Californian viruses were similar (>98.8% identity) over the 990 and 2185 bp analyzed, respectively. Differences were seen, however, in the origin of the PB2, PB1, NP, and NS genes. The NS gene of A/Ck/CA/139/01 was only 64.9% identical to that of the other chicken viruses and belonged to a different NS allele (Fig. 2). A/Ck/CA/139/01 and A/Ck/CA/6643/01 had similar PB2 and PB1 genes (>99% identical), but these group to phylogenetically distinct lineages when compared to the corresponding genes of the other Californian viruses (<93% identity) (Fig. 3). Additional heterogeneity was seen among the Californian viruses in the origin of the NP gene of A/Ck/CA/6643/01, which was only 93% identical to those of the other viruses (Fig. 4). These findings show that three different genotypes of H6N2 have been isolated from Californian chickens (Fig. 5).

GenBank accession numbers

The nucleotide sequences presented in this article have been submitted to GenBank under the Accession Numbers AF457663–AF457716.

DISCUSSION

Chickens are generally not considered to be a primary host of influenza viruses (Suarez, 2000). This hypothesis is supported by the increased mutational rates of many chicken viruses compared to the rates of viruses isolated from aquatic birds, a trait consistent with the adaptation of a virus to a new host (Suarez, 2000). The confined outbreaks of influenza seen in domestic poultry are thought to have originated from the transfer of viruses from feral animals (Alexander, 2000, 1998; Halvorson *et al.*, 1983). The sporadic and infrequent isolation of nonpathogenic influenza viruses from chickens supports the idea that influenza is not enzootic in these birds. The isolation of six H6N2 viruses from Californian chickens during 2000 and 2001 seemed inconsistent with the hypothesis that chicken populations are only transient hosts. Of the six viruses characterized in this study, the eight gene segments of four were nearly identical and all four viruses were of the same lineage. Although the infected flocks were from a small geographic area, the flocks were distinct, and the isolates were obtained during a period of 14 months. This relatively long period of virus isolation suggests that low-pathogenic viruses can circulate in chickens. Many chicken producers in California are multiage operations that obtain new birds frequently from pullet ranges. Thus, in addition to biosecurity factors brought about by human movement between flocks, the transfer of birds could disperse viruses throughout an area.

Two likely explanations are consistent with the presence of three genotypes of H6N2 viruses in Californian chickens. The first possibility is that a H6N2 virus was introduced into Californian chickens from aquatic bird reservoirs. This virus spread, and subsequently reassorted with other viruses present in chicken populations. The similarities of the PA, HA, NP, NA, and M genes from all three genotypes of H6N2 viruses suggest that these viruses share a recent common progenitor virus. An alternative, although not necessarily mutually exclusive, possibility is that all three genotypes of virus exist in the aquatic avian reservoir and that three independent transmissions from this reservoir to chickens have occurred. There have been infrequent reports of influenza isolations in chickens in California in the last 15 years (Pearson *et al.,* 1993, 1998), although a H10N7 subtype virus

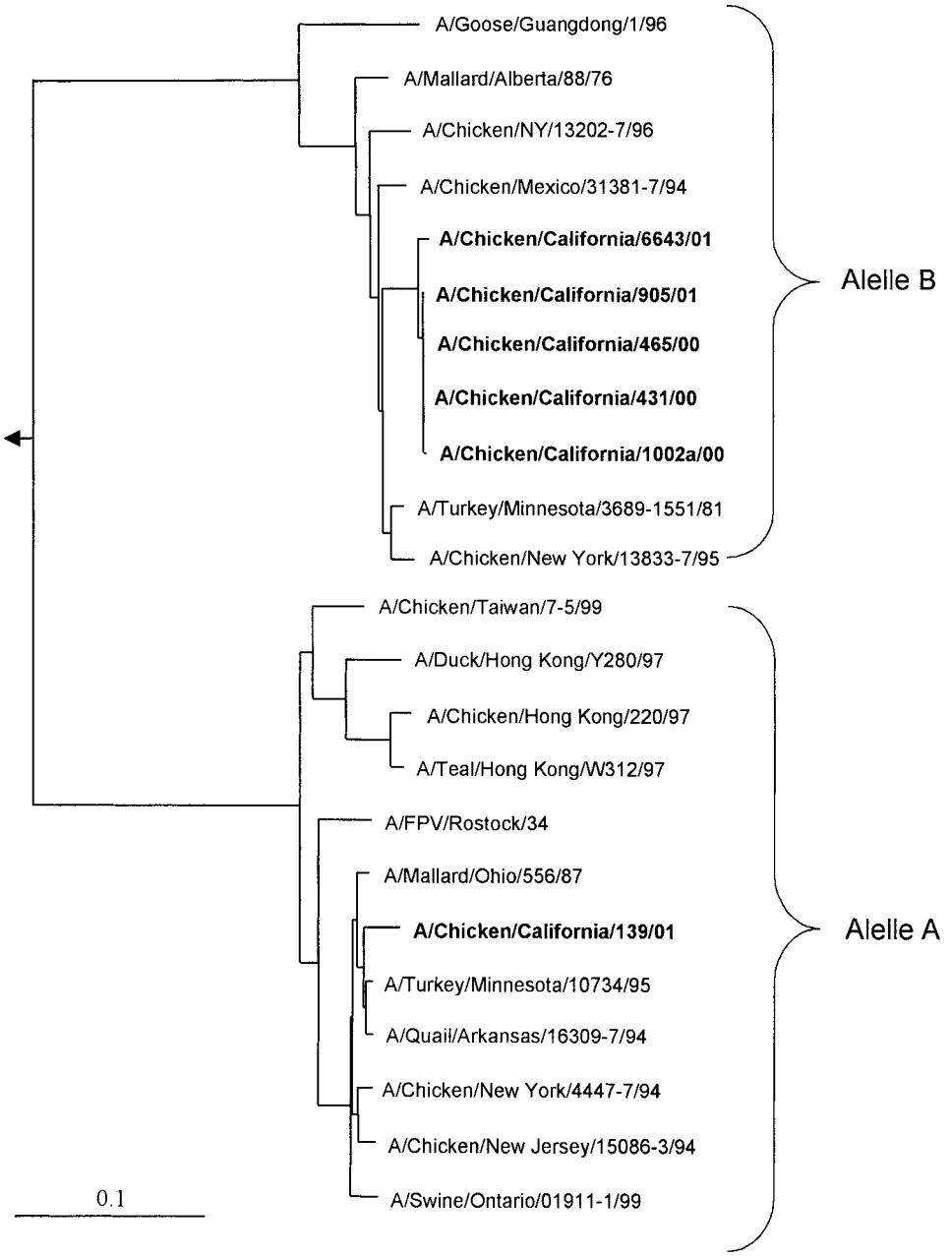

FIG. 2. Phylogenetic distance tree resulting from the comparison of 831-bp of avian influenza nonstructural (NS) genes. Horizontal distances are proportional to genetic distance, and the tree is rooted to the NS gene of A/Equine/Prague/1/56. The scale is in number of nucleotide substitutions per site.

was isolated in 1999. If these infrequent reports of influenza isolations are a true reflection of the prevalence of circulating viruses, as opposed to a lack of surveillance, then the opportunity for reassortment in this population would be rare. As such, the more plausible explanation for the presence of three H6N2 genotypes would be that multiple introductions have occurred from the aquatic bird reservoir. If this hypothesis is true, then one could assume that H6N2 viruses with the capacity to infect chickens have been relatively common in feral birds in California during the last few years and that reassortment is occurring in these feral bird populations.

The phylogenetic analysis of H6N2 viruses isolated from North American birds was inconclusive in determining which bird population contained the donor reservoir. The HA of the H6N2 viruses were most closely related to a virus isolated from a mallard duck in Alberta during 1996. No recent H6 viruses were available from other United States West Coast aquatic bird species for comparison. Interestingly, the HA genes of the Californian

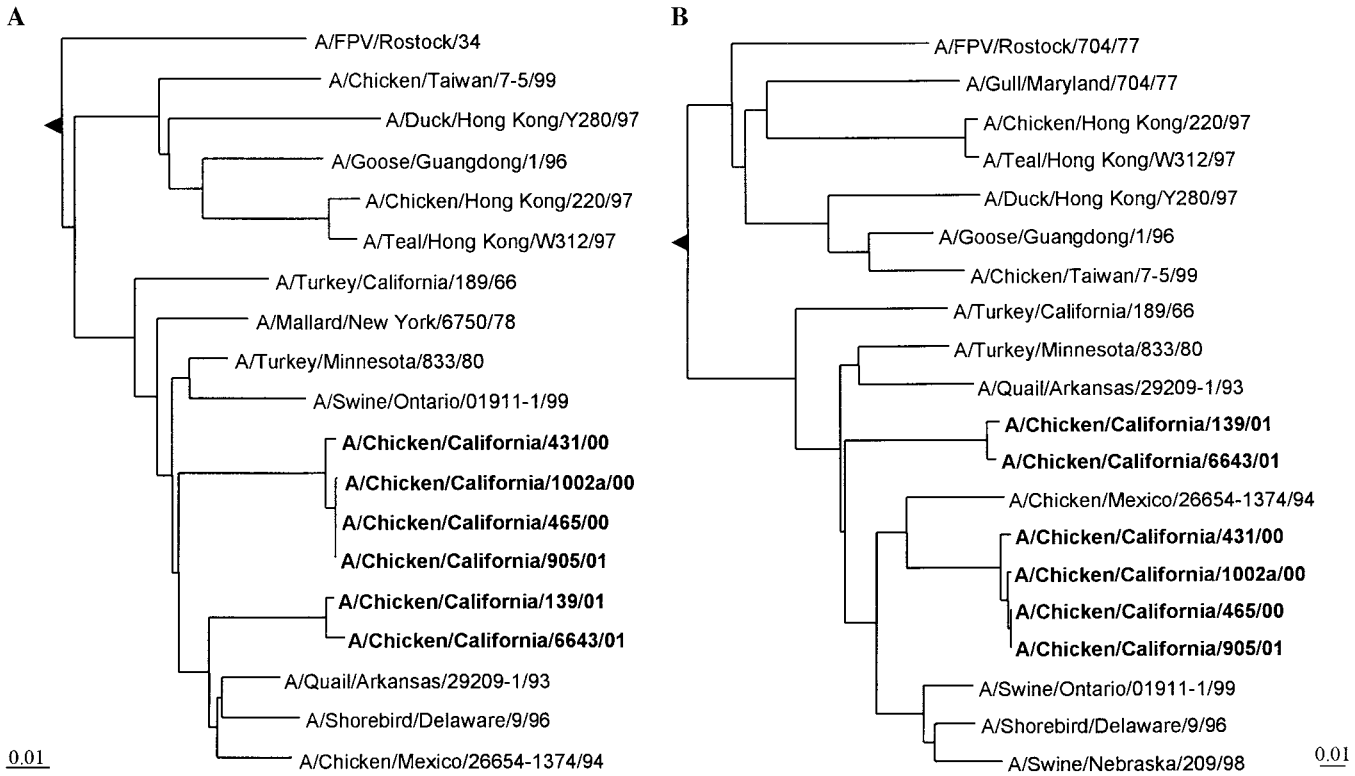

FIG. 3. Phylogenetic distance trees resulting from the comparison of the partial nucleotide sequences of PB1 (2187 bp) (A) and PB2 (1975 bp) (B) genes of selected influenza viruses. Within each tree, horizontal distances are proportional to genetic distance. The PB1 tree is rooted to the PB1 gene of A/Equine/London/1416/73, and the PB2 tree is rooted to the PB2 gene of A/Equine/Prague/1/56. The scale is in number of nucleotide substitutions per site.

viruses were more similar to those viruses isolated from chickens in Eurasia than to those H6 viruses isolated from shorebirds on the U.S. East Coast (Fig. 1). These greater similarities to Eurasian chicken viruses may indicate convergent evolution in which common mutations arise as viruses adapt to the chicken host.

Unlike some pathogenic H5 and H7 chicken viruses (Matrosovich *et al.,* 1999), the H6N2 viruses in this study did not contain any additional glycosylation signal sequences in the globular head of the HA. However, distinct patterns of sites of consensus glycosylation sequences were seen among duck, chicken, and shorebird viruses. A glycosylation consensus signal at position 182 was present in all H6 molecules of chicken and shorebird viruses but was absent from the H6 molecules of duck viruses. The association between host and potential sites of glycosylation suggests that some biological activity that is conserved in chicken and shorebird viruses is absent in duck viruses.

The Californian H6N2 viruses have an 18 amino acid deletion in their NA proteins, a trait that is typical of chicken viruses (Matrosovich *et al.,* 1999). NA deletions have been detected in H5N2 viruses isolated from chickens in Mexico (Zhou *et al.,* 1999) and in Pennsylvania (Deshpande *et al.,* 1985), but they are absent in H9N2 viruses from chickens in Korea and Hong Kong (Guan *et al.,* 1999). The deletion in the Californian virus N2 molecules is two amino acids shorter than that seen in the H5N2 viruses. The functional relevance of the NA deletions remains unknown.

With the exceptions of the M, NS, and PB1 genes, the genes of the chicken H6N2 viruses do not have any particularly close homologues in the Influenza Sequence Database (Macken *et al.,* 2001). This finding more likely reflects a lack of data rather than a true lack of similar genes in other circulating avian viruses; however, this finding does not exclude the possibility that viral gene segments undergo defined genetic changes in their adaptation for growth in chickens. All of the California H6N2 virus genes sequenced belong to the American lineage, and none of these genes are similar to those of H6N1 and H6N2 viruses that circulate in avian species in Hong Kong (Chin *et al.,* 2002; Hoffmann *et al.,* 2000). Two distinct alleles of the NS gene are present in avian influenza viruses (Garcia *et al.,* 1997; Scholtissek and von Hoyningen-Huene, 1980; Treanor *et al.,* 1989), although no association between alleles and geographic location, avian species, or year of isolation has been observed. A/Ck/CA/431/00, A/Ck/CA/465/00, A/Ck/CA/1002a/00, A/Ck/CA/905/01, and A/Ck/CA/6643/01 have NS genes belonging to the B allele, whereas A/Ck/CA/139/01 contained the A allele. Influenza viruses containing B allele

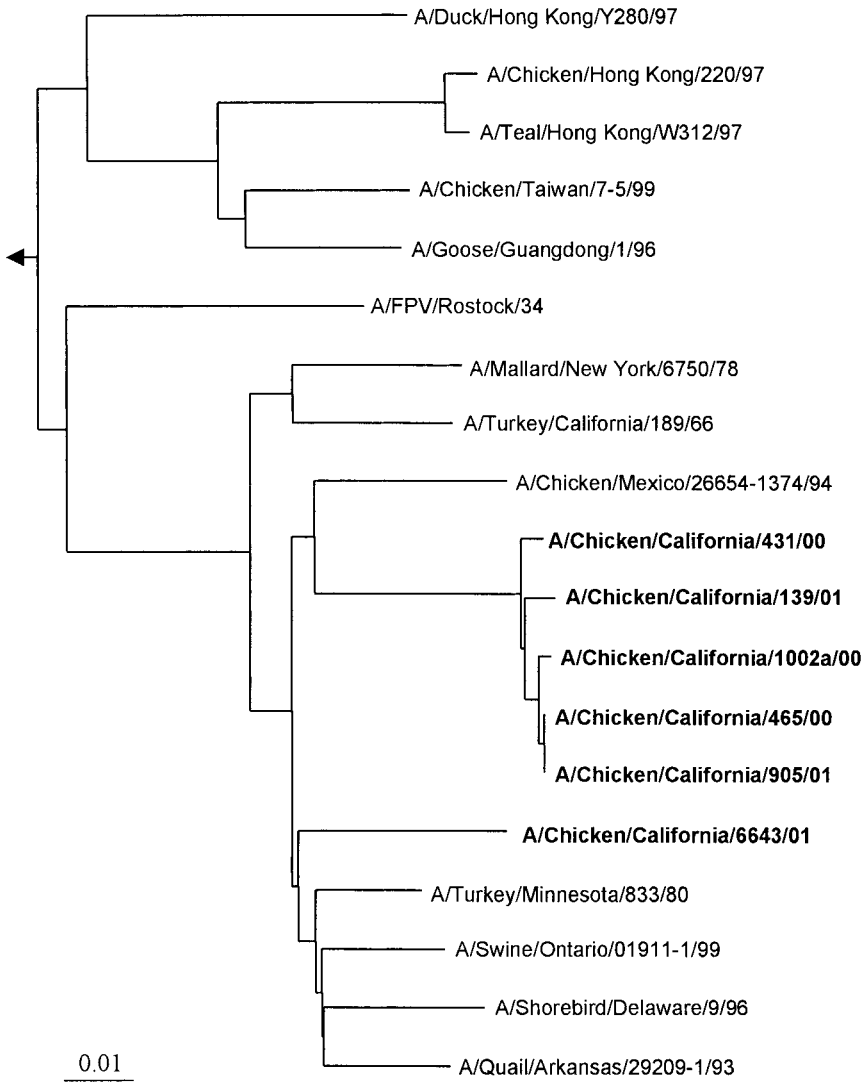

FIG. 4. Phylogenetic distance tree resulting from the comparison of 1454 bp of avian influenza nucleoprotein (NP) genes. Horizontal distances are proportional to genetic distance, and the tree is rooted to the NP gene of A/Equine/Prague/1/56. The scale is in number of nucleotide substitutions per site.

NS genes are attenuated for growth in squirrel monkeys (Treanor *et al.,* 1989), and the lack of this allele in viruses from mammals (Kawaoka *et al.,* 1998) suggests they may not replicate well in mammalian hosts in general. The presence of both alleles in the California H6N2 viruses demonstrates that no such NS host restriction occurs in chickens.

Although the Californian chicken viruses are poorly pathogenic on their own, they may predispose birds to infection with other potentially harmful microbes. Even without the confirmed presence of other pathogens, A/Ck/CA/905/01 induced respiratory distress and a decreased egg production. If stable lineages of nonpathogenic viruses establish in chicken, these viruses could have a substantial economic effect on the industry. Also, because the symptoms of these viruses are less noticeable, the viruses may be much harder to eradicate than

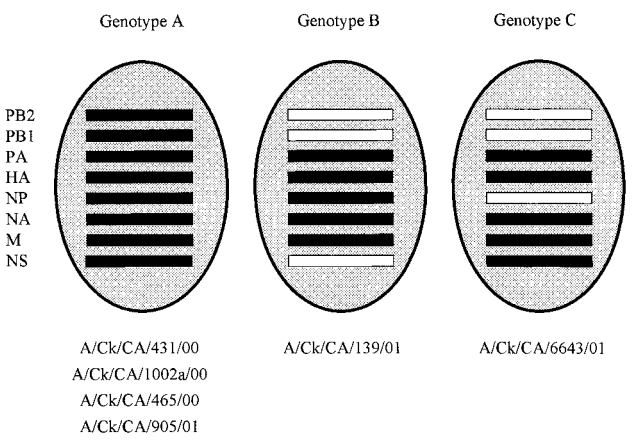

FIG. 5. Schematic diagram representing the three different genotypes of H6N2 viruses in Californian chickens. Individual genes of the same color are more than 97% similar.

the highly pathogenic isolates. H7 and H5 viruses are the only influenza subtypes that acquire the polybasic HA cleavage site that is associated with high virulence in chickens. This does not, however, relegate the other subtypes to insignificant pathogens.

To fully understand the ecology and biology of influenza, it is necessary to examine viruses from all hosts of the influenza A virus. The present description of H6N2 viruses from chickens in California is one of the few characterizations of nonpathogenic chicken viruses and of H6 viruses in general. Additional studies of chicken viruses will help to identify the molecular features that are associated with viral adaptation and maintenance in alternative hosts.

MATERIALS AND METHODS

Growth of viruses in chickens

White leghorn chickens (four 8-week-old birds and four 4.5-week-old birds) were inoculated intravenously with 200 μl of diluted virus (1:10 dilution of infected allantoic fluid or 2×10^4 $TCID_{50}$). Birds were examined daily for disease signs and cloacal and tracheal swabs were taken 3 days after infection. Swabs were placed in 1 ml of sample medium [50% glycerol in phosphate-buffered saline (PBS) that contained 1000 U/ml penicillin, 200 μg/ml streptomycin, 50 U/ml mycostatin, 100 U/ml polymyxin B, and 250 μg gentamycin). Three embryonated chicken eggs were inoculated with 0.1 ml of the sample medium and incubated for 2 days at 37°C. HA assays of chicken red blood cells were performed to verify virus growth in the harvested allantoic fluid. Viral titers in the samples were calculated as $TCID_{50}$s for MDCK cells using standard procedures. Four additional 4.5-week-old birds were each given a total of 1 ml of virus dilution via the tracheal, ocular, nasal, and oral routes. Blood samples were taken from all birds before and at 14 days after infection, and HI assays (Palmer *et al.,* 1975) were used to analyze seroreactivity to the inoculant virus.

Serology

The H6N2 chicken viruses were compared antigenically by using the HI assay as previously described (Palmer *et al.,* 1975). All sera were pretreated with the receptor-destroying enzyme from *Vibrio cholerae* (Denka Seiken, Tokyo) to abolish interference by nonspecific serum inhibitors. The viruses, which came directly from infected allantoic fluid, were diluted to four hemagglutination doses for use in the assay.

RNA extraction, RT-PCR, and DNA sequencing

Viral RNA was extracted from allantoic fluid by using the RNeasy kit (Qiagen, Santa Clara, CA) according to the manufacturer's instructions. Reverse transcription and PCR were performed under standard conditions by using primers specific for the various genes of influenza virus. PCR products were purified by using a QIAquick PCR purification kit (Qiagen). Sequencing reactions were performed by the staff of the Hartwell Center for Bioinformatics and Biotechnology at St. Jude Children's Research Hospital. Template DNA was sequenced by using rhodamine or dRhodamine dye-terminator cycle sequencing-ready reaction kits with AmpliTaqDNA polymerase FS [Perkin–Elmer, Applied Biosystems, Inc. (PE/ABI), Foster City, CA] and synthetic oligonucleotides. Samples were subjected to electrophoresis, detection, and analysis on PE/ABI model 373 or model 377 DNA sequencers.

DNA sequence analysis

DNA sequences were compiled and edited by using the Lasergene sequence analysis software package (DNASTAR, Madison, WI). Multiple sequence alignments were made by using CLUSTAL W (Thompson *et al.,* 1994), and phylogenetic trees were generated by using the neighbor-joining algorithm in the PHYLIP version 3.57C software package (Felsenstein, 1993).

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REFERENCES

Alexander, D. J. (1998). Avian influenza in the Eastern Hemisphere (excluding the Pacific Basin) during 1992–1997. *In* "Proceedings of the Fourth International Symposium on Avian Influenza," pp. 9–13. Athens, GA.

Alexander, D. J. (2000). A review of avian influenza in different bird species. *Vet. Microbiol.* **74,** 3–13.

Beare, A. S., and Webster, R. G. (1991). Replication of avian influenza viruses in humans. *Arch. Virol.* **119,** 37–42.

Bosch, F. X., Garten, W., Klenk, H. D., and Rott, R. (1981). Proteolytic cleavage of influenza virus hemagglutinins: Primary structure of the connecting peptide between HA1 and HA2 determines proteolytic cleavability and pathogenicity of avian influenza viruses. *Virology* **113,** 725–735.

Cameron, K. R., Gregory, V., Banks, J., Brown, I. H., Alexander, D. J., Hay, A. J., and Lin, Y. P. (2000). H9N2 subtype influenza A viruses in poultry in Pakistan are closely related to the H9N2 viruses responsible for human infection in Hong Kong. *Virology* **278,** 36–41.

Chin, P. S., Hoffmann, E. H., Webby, R. J., Webster, R. G., Guan, Y., Peiris, J. S. M., and Shortridge, K. F. (2002). Molecular evolution of H6 influenza viruses from poultry in Southeastern China: Prevalence of H6N1 influenza viruses possessing seven A/HK/156/97 (H5N1)-like genes in poultry. *J. Virol.* **76,** 507–516.

Claas, E. C., Osterhaus, A. D., van Beek, R., de Jong, J. C., Rimmelzwaan, G. F., Senne, D. A., Krauss, S., Shortridge, K. F., and Webster, R. G. (1998). Human influenza A H5N1 virus related to a highly pathogenic avian influenza virus. *Lancet* **351,** 472–477.

Deshpande, K. L., Naeve, C. W., and Webster, R. G. (1985). The neur-

aminidases of the virulent and avirulent A/Chicken/Pennsylvania/83 (H5N2) influenza A viruses: Sequence and antigenic analyses. *Virology* **147,** 49–60.

Felsenstein, J. (1993). PHYLIP (phylogenetic I reference package) version 3.5. Department of Genetics, University of Washington, Seattle, WA. [Distributed by the author.]

Garcia, M., Suarez, D. L., Crawford, J. M., Latimer, J. W., Slemons, R. D., Swayne, D. E., and Perdue, M. L. (1997). Evolution of H5 subtype avian influenza A viruses in North America. *Virus Res.* **51,** 115–124.

Guan, Y., Shortridge, K. F., Krauss, S., Chin, P. S., Dyrting, K. C., Ellis, T. M., Webster, R. G., and Peiris, M. (2000). H9N2 influenza viruses possessing H5N1-like internal genomes continue to circulate in poultry in southeastern China. *J. Virol.* **74,** 9372–9380.

Guan, Y., Shortridge, K. F., Krauss, S., and Webster, R. G. (1999). Molecular characterization of H9N2 influenza viruses: Were they the donors of the "internal" genes of H5N1 viruses in Hong Kong? *Proc. Natl. Acad. Sci. USA* **96**, 9363–9367.

Halvorson, D., Karunakaran, D., Senne, D., Kelleher, C., Bailey, C., Abraham, A., Hinshaw, V., and Newman, J. (1983). Epizootiology of avian influenza-simultaneous monitoring of sentinel ducks and turkeys in Minnesota. *Avian Dis.* **27,** 77–85.

Hoffmann, E., Stech, J., Leneva, I., Krauss, S., Scholtissek, C., Chin, P. S., Peiris, M., Shortridge, K. F., and Webster, R. G. (2000). Characterization of the influenza A virus gene pool in avian species in southern China: Was H6N1 a derivative or a precursor of H5N1? *J. Virol.* **74,** 6309–6315.

Horimoto, T., and Kawaoka, Y. (1994). Reverse genetics provides direct evidence for a correlation of hemagglutinin cleavability and virulence of an avian influenza A virus. *J. Virol.* **68,** 3120–3128.

Ito, T., Couceiro, J. N., Kelm, S., Baum, L. G., Krauss, S., Castrucci, M. R., Donatelli, I., Kida, H., Paulson, J. C., Webster, R. G., and Kawaoka, Y. (1998). Molecular basis for the generation in pigs of influenza A viruses with pandemic potential. *J. Virol.* **72,** 7367–7373.

Ito, T., and Kawaoka, Y. (1998). Avian influenza. *In* "Textbook of Influenza" (K. G. Nicholson, R. G. Webster, and A. J. Hay, Eds.), pp. 126–136. Blackwell Sci., Oxford.

Kawaoka, Y., Gorman, O. T., Ito, T., Wells, K., Donis, R. O., Castrucci, M. R., Donatelli, I., and Webster, R. G. (1998). Influence of host species on the evolution of the nonstructural (NS) gene of influenza A viruses. *Virus Res.* **55,** 143–156.

Kida, H., Ito, T., Yasuda, J., Shimizu, Y., Itakura, C., Shortridge, K. F., Kawaoka, Y., and Webster, R. G. (1994). Potential for transmission of avian influenza viruses to pigs. *J. Gen. Virol.* **75,** 2183–2188.

Klenk, H. D., Rott, R., and Orlich, M. (1977). Further studies on the activation of influenza virus by proteolytic cleavage of the haemagglutinin. *J. Gen. Virol.* **36,** 151–161.

Lin, Y. P., Shaw, M., Gregory, V., Cameron, K., Lim, W., Klimov, A., Subbarao, K., Guan, Y., Krauss, S., Shortridge, K., Webster, R., Cox, N., and Hay, A. (2000). Avian-to-human transmission of H9N2 subtype influenza A viruses: Relationship between H9N2 and H5N1 human isolates. *Proc. Natl. Acad. Sci. USA* **97,** 9654–9658.

Lin, Y. P., Shu, L. L., Wright, S., Bean, W. J., Sharp, G. B., Shortridge, K. F., and Webster, R. G. (1994). Analysis of the influenza virus gene pool of avian species from southern China. *Virology* **198,** 557–566.

Macken, C., Lu, H., Goodman, J., and Boykin, L. (2001). The value of a database in surveillance and vaccine selection. *In* "Options for the Control of Influenza IV. Proceedings of the World Congress on Options for the Control of Influenza IV, Crete, Sept. 23–28, 2000." (A. Osterhaus, N. Cox, and A. Hampson, Eds.) pp. 103–106. Excerpta Medica, Amsterdam (International Congress Series 1219).

Matrosovich, M., Zhou, N., Kawaoka, Y., and Webster, R. (1999). The surface glycoproteins of H5 influenza viruses isolated from humans, chickens, and wild aquatic birds have distinguishable properties. *J. Virol.* **73,** 1146–1155.

Matrosovich, M. N., Krauss, S., and Webster, R. G. (2001). H9N2 influenza A viruses from poultry in Asia have human virus-like receptor specificity. *Virology* **281,** 156–162.

Murphy, B. R., Hinshaw, V. S., Sly, D. L., London, W. T., Hosier, N. T., Wood, F. T., Webster, R. G., and Chanock, R. M. (1982). Virulence of avian influenza A viruses for squirrel monkeys. *Infect. Immunol.* **37,** 1119–1126.

Palmer, D. F., Coleman, M. T., Dowdle, W. R., and Schild, G. C. (1975). Advanced laboratory techniques for influenza diagnosis. Immunology Series No. 6, pp. 51–52. U.S. Department of Health, Education, and Welfare, Washington, DC.

Pearson, J. E., Senne, D. A., and Panigrahy, B. (1992). Avian influenza in the Western Hemisphere including the Pacific Basin. *In* "Proceedings of the Third International Symposium on Avian Influenza," pp. 22–31. University of Madison, Wisconsin, WI.

Pearson, J. E., Senne, D. A., and Panigrahy, B. (1998). Avian influenza in the Western Hemisphere including the Pacific Basin 1992–1996. *In* "Proceedings of the Fourth International Symposium on Avian Influenza," pp. 14–17. Athens, GA.

Peiris, M., Yuen, K. Y., Leung, C. W., Chan, K. H., Ip, P. L., Lai, R. W., Orr, W. K., and Shortridge, K. F. (1999). Human infection with influenza H9N2. *Lancet* **354,** 916–917.

Scholtissek, C. (1990). Pigs as the "mixing vessel" for the creation of new pandemic influenza A viruses. *Med. Princ. Pract.* **2,** 65–71.

Scholtissek, C., and von Hoyningen-Huene, V. (1980). Genetic relatedness of the gene which codes for the nonstructural (NS) protein of different influenza A strains. *Virology* **102,** 13–20.

Shortridge, K. F., Butterfield, W. K., Webster, R. G., and Campbell, C. H. (1977). Isolation and characterization of influenza A viruses from avian species in Hong Kong. *Bull. WHO* **55,** 15–20.

Shortridge, K. F., Gao, P., Guan, Y., Ito, T., Kawaoka, Y., Markwell, D., Takada, A., and Webster, R. G. (2000). Interspecies transmission of influenza viruses: H5N1 virus and a Hong Kong SAR perspective. *Vet. Microbiol.* **74,** 141–147.

Steinhauer, D. A. (1999). Role of hemagglutinin cleavage for the pathogenicity of influenza virus. *Virology* **258,** 1–20.

Suarez, D. L. (2000). Evolution of avian influenza viruses. *Vet. Microbiol.* **74,** 15–27.

Suarez, D. L., Garcia, M., Latimer, J., Senne, D., and Perdue, M. L. (1999). Phylogenetic analysis of H7 avian influenza viruses isolated from the live bird markets of the Northeast United States. *J. Virol.* **73,** 3567–3573.

Subbarao, K., Klimov, A., Katz, J., Regnery, H., Lim, W., Hall, H., Perdue, M., Swayne, D., Bender, C., Huang, J., Hemphill, M., Rowe, T., Shaw, M., Xu, X., Fukuda, K., and Cox, N. (1998). Characterization of an avian influenza A (H5N1) virus isolated from a child with a fatal respiratory illness. *Science* **279,** 393–396.

Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1994). CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* **22,** 4673–4680.

Treanor, J. J., Snyder, M. H., London, W. T., and Murphy, B. R. (1989). The B allele of the NS gene of avian influenza viruses, but not the A allele, attenuates a human influenza A virus for squirrel monkeys. *Virology* **171,** 1–9.

Webster, R. G., Bean, W. J., Gorman, O. T., Chambers, T. M., and Kawaoka, Y. (1992). Evolution and ecology of influenza A viruses. *Microbiol. Rev.* **56,**152–179.

Zhou, N. N., Shortridge, K. F., Claas, E. C., Krauss, S. L., and Webster, R. G. (1999). Rapid evolution of H5N1 influenza viruses in chickens in Hong Kong. *J. Virol.* **73,** 3366–3374.