

Imported Parakeets Harbor H9N2 Influenza A Viruses That Are Genetically Closely Related to Those Transmitted to Humans in Hong Kong

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In 1997 and 1998, H9N2 influenza A viruses were isolated from the respiratory organs of Indian ring-necked parakeets (*Psittacula krameri manillensis*) that had been imported from Pakistan to Japan. The two isolates were closely related to each other (>99% as determined by nucleotide analysis of eight RNA segments), indicating that H9N2 viruses of the same lineage were maintained in these birds for at least 1 year. The hemagglutinins and neuraminidases of both isolates showed >97% nucleotide identity with those of H9N2 viruses isolated from humans in Hong Kong in 1999, while the six genes encoding internal proteins were >99% identical to the corresponding genes of H5N1 viruses recovered during the 1997 outbreak in Hong Kong. These results suggest that the H9N2 parakeet viruses originating in Pakistan share an immediate ancestor with the H9N2 human viruses. Thus, influenza A viruses with the potential to be transmitted directly to humans may be circulating in captive birds worldwide.

Phylogenetic analysis of influenza A viruses, together with the presence of all 15 hemagglutinin (HA) and 9 neuraminidase (NA) subtypes of viruses in aquatic birds, suggests that these hosts are natural reservoirs of such viruses (31). Influenza A viruses have also been isolated from psittacines, among other avian species (3, 17), although little is known about their roles in the ecology, epizootiology, and epidemiology of these viruses. Because they are captured in the wild and distributed worldwide for sale as pets, psittacines might serve as biological vectors in the spread of influenza A viruses to animals in other countries. In fact, a Newcastle disease virus originating from imported psittacines caused outbreaks of the disease among domestic poultry in southern California in the early 1970s (30).

In recent years, H9N2 viruses have caused influenza outbreaks in poultry worldwide, including Europe, Pakistan, and Asia (3, 4, 10, 16). H9N2 viruses are genetically distinct in Asia, where at least three lineages defined by the nucleotide sequence of the NP gene are circulating (10). Some of these Asian H9N2 viruses have been transmitted to pigs and humans (10) in mainland China, as well as Hong Kong (13, 21, 22). For example, an H9N2 virus, A/quail/Hong Kong/G1/97(G1), that was genetically related to the H9N2 viruses from humans was isolated during surveillance for influenza A viruses in Hong Kong birds (9). Moreover, the genes encoding the internal proteins (PA, PB1, PB2, NP, M, and NS) of the H9N2 viruses isolated from two children in Hong Kong were genetically

closely related to those of the H5N1 viruses that had been directly transmitted from birds to humans, killing 6 of the 18 people infected (6, 11, 13, 26, 27, 28, 29).

During routine virologic diagnosis of birds imported to Japan, influenza A viruses were isolated in embryonated eggs from Indian ring-necked parakeets (*Psittacula krameri manillensis*) imported from Pakistan. The first virus, A/parakeet/Chiba/1/97, was isolated from the respiratory organ (trachea) of a bird that died at a pet shop within 10 days of importation in March 1997. The second virus, A/parakeet/Narita/92A/98, also isolated from respiratory organs (mixture of trachea and lung), came from a bird that died at the animal quarantine station at the Narita airport in Japan in June 1998. Both isolates were identified as influenza A viruses of the H9N2 subtype by conventional hemagglutination inhibition and neuraminidase inhibition assays (2, 14). The viruses were

TABLE 1. Nucleotide and amino acid differences between the two parakeet isolates

Segment	No. of different:	
	Nucleotides (% identity)	Amino acids (% identity)
PB2	7 (99.7)	3 (99.6)
PB1	17 (99.3)	4 (99.5)
PA	9 (99.6)	1 (99.9)
HA	14 (99.2)	9 (98.4)
NP	10 (99.3)	3 (99.4)
NA	9 (99.4)	5 (98.9)
M	5 (99.5)	1 (99.6) (M1), 0 (100) (M2)
NS	3 (99.6)	1 (99.6) (NS1), 0 (100) (NS2)

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TABLE 3. Potential glycosylation sites on HA proteins of parakeet and closely related H9N2 viruses

Virus	Glycosylation site ^a at position:							
	11	87	123	188	200	280	287	474
A/parakeet/Chiba/1/97	+	+	+	-	+	+	+	+
A/parakeet/Narita/92A/98	+	+	+	-	-	+	+	+
A/quail/Hong Kong/G1/97	+	+	+	+	+	+	+	+
A/Hong Kong/1073/99	+	+	+	+	+	+	+	+
A/Hong Kong/1074/99	+	+	+	+	+	+	+	+
A/chicken/Beijing/1/94	+	-	+	-	+	+	+	+
A/chicken/Hong Kong/739/94	+	-	+	-	+	+	+	+
A/chicken/Hong Kong/G9/97	+	-	+	-	+	+	+	+
A/duck/Hong Kong/Y439/97	+	-	+	-	+	+	+	+
A/quail/Hong Kong/AF157/93	+	-	+	-	+	+	+	+
A/turkey/California/189/66	+	-	+	-	+	+	+	+
A/turkey/Minnesota/1/95	+	-	+	-	+	+	+	+

^a +, glycosylation site present; -, glycosylation site absent.

quail viruses as well as human H5N1 Hong Kong viruses. A phylogenetic tree of the NP gene is shown as an example (Fig. 3).

A mutation at the initiation codon of the NP gene of A/parakeet/Narita/92A/98 virus (CUG instead of AUG) was de-

tected. Sequencing analysis of this region was repeated five times with identical results. The CUG initiation codon has not been observed in the genes of any influenza virus; however, it is the most efficient non-AUG initiation codon (15) and is used in the synthesis of both eukaryotic and viral proteins (7, 15, 24). Thus, we suggest that this CUG codon is used as an initiation codon for NP translation, although we cannot speculate on its biologic significance in the NP gene of A/parakeet/Narita/92A/98.

G1 quail virus replicated in chickens and ducks without producing any disease signs, but it spread to the brain in mice (10). We therefore examined the pathogenicities of our H9N2 parakeet viruses in chickens and mice by intranasally inoculating 6-week-old specific-pathogen-free chickens (approximately 10⁷ 50% egg infective doses/chicken; eight chickens/virus). Viruses were recovered from tracheal and cloacal swabs, but none of the infected chickens showed any disease signs. When 5-week-old female BALB/c mice (Japan Clea) were intranasally inoculated with virus (approximately 10⁷ 50% egg infective doses; 12 mice/virus), all survived without signs of disease. Of all organs tested, the lung was the only site from which virus

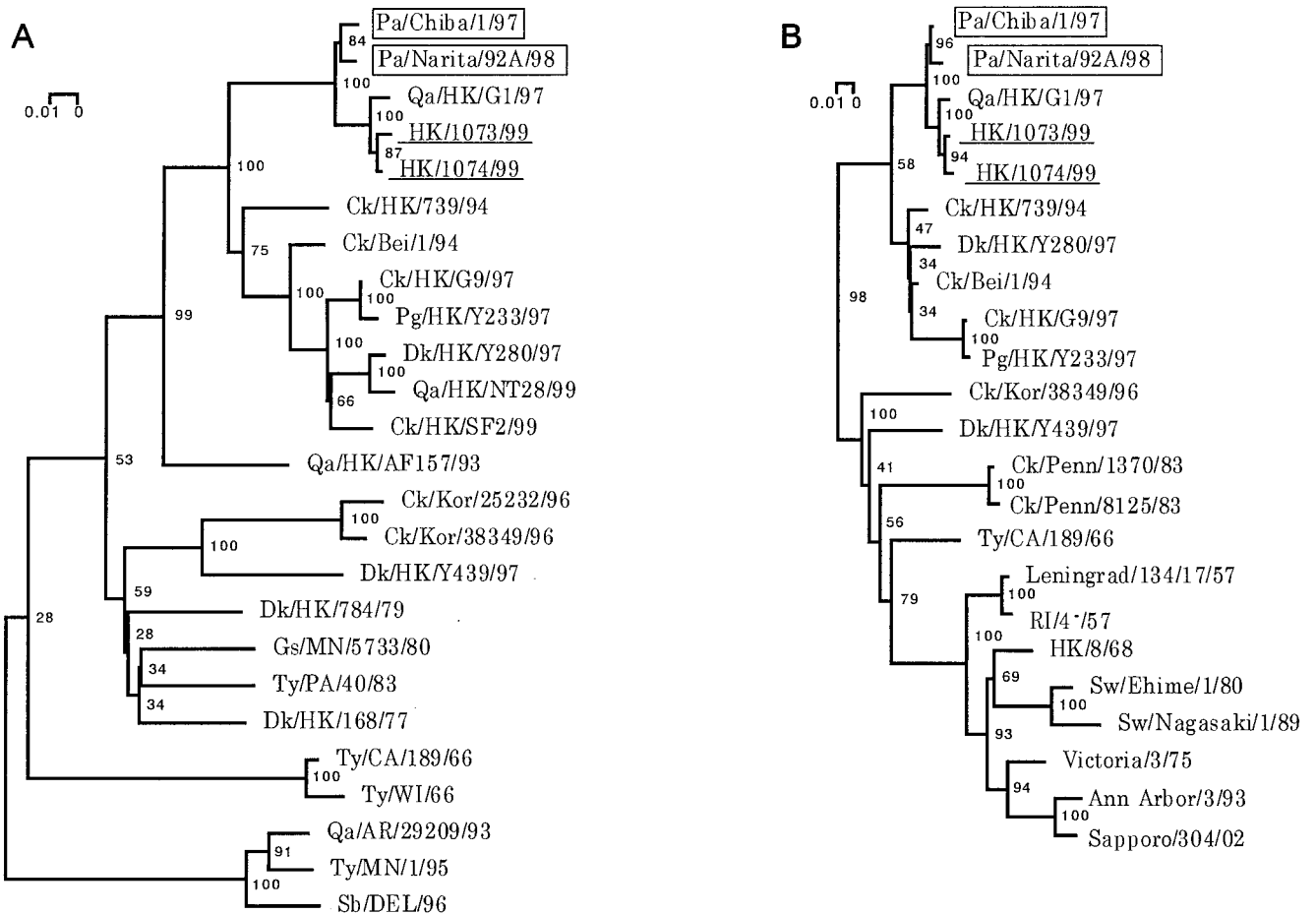


FIG. 2. Phylogenetic trees of the H9 HA (A) and N2 NA (B) genes of influenza A viruses. Nucleotides 46 to 1091 (1,046 bp) of the H9 HAs and nucleotides 1 to 1386 (1,386 bp) of the N2 NAs were used for phylogenetic analysis. Horizontal distances are proportional to the minimum number of nucleotide differences required to join nodes and sequences. Viruses isolated from parakeets or humans are boxed or underlined, respectively. Numbers at the nodes indicate confidence levels of bootstrap analysis with 1,000 replications as a percentage value. Abbreviations: Pa, parakeet; Qa, quail; HK, Hong Kong; Ck, chicken; Bei, Beijing; Pg, pigeon; Dk, duck; Kor, Korea; Gs, goose; MN, Minnesota; Ty, turkey; PA, Pavia; CA, California; WI, Wisconsin; AR, Arizona; Sb, shorebird; DEL, Delaware; Penn, Pennsylvania; Sw, swine.

TABLE 4. HA connecting peptide sequences of H9N2 viruses

Virus	Connecting peptide amino acid sequence	Reference
A/turkey/Wisconsin/66	P A V S S R	19
A/quail/Arizona/29209/93	P A A S N R	9
A/duck/Hong Kong/168/77	P A A S G R	9
A/duck/Hong Kong/784/79	P A A S D R	9
A/chicken/Beijing/1/94	P A R S S R	9
A/quail/Hong Kong/G1/97	P A R S S R	9
A/chicken/Hong Kong/G9/97	P A R S S R	9
A/Hong Kong/1073/99	P A R S S R	13
A/parakeet/Chiba/1/97	P A R S S R	This study
A/parakeet/Narita/92A/98	P A R S S R	This study

was recovered at 3 and 6 days postinfection (data not shown). These results indicate that the parakeet viruses differ from G1 quail virus in both tissue tropism and virulence in mice and hence contain amino acid alterations that would account for the discrepant phenotypes.

Taken together, our findings indicate that H9N2 viruses, genetically closely related to those transmitted to humans (13, 21, 22), were circulating in psittacines in Pakistan in 1997 to 1998 and were introduced into Japan during this period. Thus, viruses with the potential for bird-to-human transmission may still be circulating in countries other than China and may be spreading across geographical boundaries through the importation and sale of wild psittacine birds as pets.

An H9N2 influenza virus caused an influenza outbreak in poultry in Pakistan in 1999 (16). Although the causative virus was never fully characterized, the HA cleavage site sequence was identical to that of our viruses. Efforts to clarify the relationship between the Pakistan virus and ours will depend on more complete molecular characterization of the former.

Avian influenza A viruses are genetically divided into two geographically based lineages: Eurasian and American. The G1 quail strain and the 1997 H5N1 Hong Kong virus belong to the Eurasian lineage. Recent surveillance studies indicate that Eurasian avian viruses can be subdivided further and that the NP genes of the Hong Kong H5N1 and H9N2 genes belong to one of these sublineages (10, 20). Although earlier experimental attempts to infect human volunteers with avian influenza viruses had failed (5), the Hong Kong H5N1 and H9N2 viruses were clearly able to infect humans. Thus, the H5N1 and H9N2 Hong Kong viruses seem unique in their ability to replicate efficiently in humans. Although amino acid differences were found between these Hong Kong and other avian viruses, it is still not clear which differences account for the direct transmissibility of the former viruses to humans or, alternatively, which simply represent unique traits of the different avian lineages in nature. Thus, further genetic studies of Eurasian avian influenza viruses are needed to evaluate the potential of avian viruses to cross host range barriers.

The PB2, PA, NP, and M proteins of the 1997 H5N1 viruses contained human virus-like amino acid residues that might have been responsible for direct transmission of the avian virus to humans (32). Some of these residues are also found in the H9N2 parakeet viruses: position 661 (Ala [avian] and Thr [human]) in PB2, the residue located in the region responsible

for interaction with other polymerase components (23), and position 136 (Leu [avian] and Met [human]) in NP, the residue in the RNA binding domain of the protein (1). To unequivocally determine the contributions of these amino acid residues to the viruses' replicative capacity in mammals, one needs to generate viruses with specific mutations, using reverse genetics (12, 18).

In conclusion, the international trade of exotic pet birds carrying influenza A viruses may pose a serious health threat to humans. Previously, psittacine birds have not been thought to play a major role in the epizootiology and epidemiology of influenza A viruses. Since the trading of these birds across regional and international boundaries is extensive (over 400,000 nonpoultry birds [50,000 from Pakistan] were imported to Japan each year for the last 5 years), the risk of worldwide dissemination of potentially virulent influenza A virus is considerable. Thus, adequate quarantine and surveillance systems should be established in countries engaging in such trade.

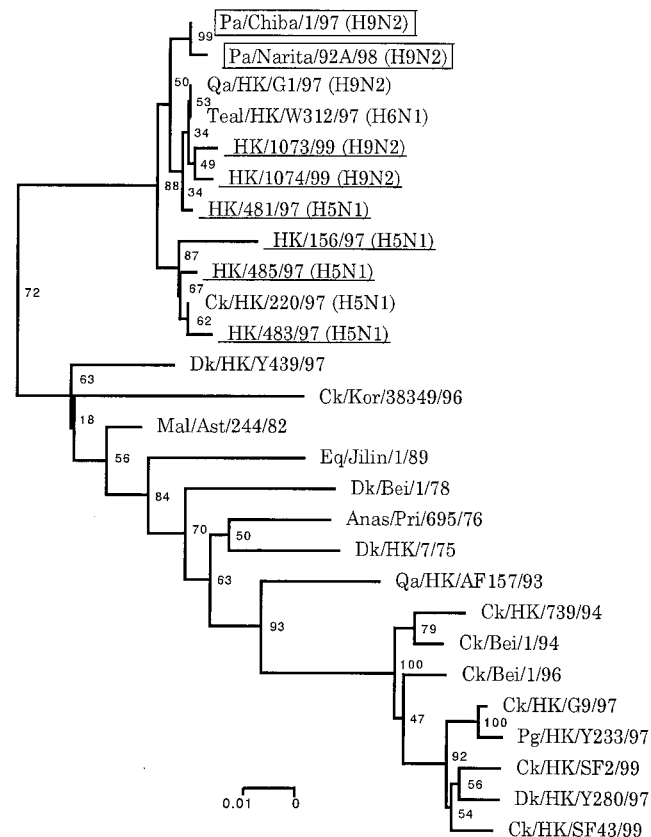


FIG. 3. Evolutionary relationship of influenza A virus NP genes. Nucleotides 741 to 1398 (658 bp) of the NP genes were used for phylogenetic analysis. All viruses underlined were isolated from humans. Horizontal distances are proportional to the minimum number of nucleotide differences required to join nodes and sequences. Viruses isolated from parakeets or humans are boxed or underlined, respectively. Numbers at the nodes indicate confidence levels of bootstrap analysis with 1,000 replications as a percentage value. Abbreviations: Pa, parakeet; Qa, quail; HK, Hong Kong; Ck, chicken; Dk, duck; Kor, Korea; Mal, mallard; Ast, Astrakhan; Eq, equine; Bei, Beijing; Anas, Anas acuta; Pri, Primorje; Pg, pigeon.

Nucleotide sequence accession numbers. All sequences used in this study were sent to DDBJ, and the accession numbers are AB049153 to AB049168.

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