Epidemiological Baseline of Influenza Virus in Wild Aquatic Birds in Hong Kong during the Pre-H5N1 Endemic Era

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SUMMARY

Influenza virus surveillance was conducted on wild ducks and shorebirds in Hong Kong at the Mai Po Nature Reserve to determine whether East Asian wild aquatic birds passing through or overwintering in Hong Kong are reservoirs of H5N1 influenza viruses and to establish an epidemiological baseline of influenza virus in wild aquatic birds during the pre-H5N1 endemic era. Three influenza viruses were isolated from 3178 faecal samples collected over three sampling periods from 1988 to 2001 during the southern and northern migration periods. The isolation rates and viruses were, respectively 0.08% (H10N5) in 1988 – 1990, 0.12% (H11N1) in 1998 and 0.09% (H3N8) in 1999 – 2001. Whereas tracheal and intestinal colon explants from representative shorebirds were susceptible to *in vitro* infection by the H10N5 virus, orally infected shorebirds were apparently not. Genetic analyses indicated that the nucleoprotein, matrix and nonstructural genes of the three viruses were related to those of aquatic bird viruses in Asia, but not to those of the human H5N1 virus. The present study provided epidemiological baseline information for future influenza virus surveillance in wild aquatic birds in southeast China.

INTRODUCTION

The ecology of influenza A viruses in wild aquatic birds in Asia has changed significantly in the past ten years. Surveillances of influenza viruses conducted in Asia in the 1990's and early 2000's have shown that wild aquatic birds harboured influenza viruses of a variety of haemagglutinin (HA) and neuraminidase (NA) subtypes but not the highly pathogenic H5N1 virus (*1-6*). On the contrary, studies from late 2003 and onward have demonstrated that highly pathogenic H5N1 virus was prevalent in Asian wild aquatic birds (*7-17*). These highly pathogenic H5N1 virus identified in wild aquatic birds are closely related to the H5N1 virus that affected human in 1997 (*18-20*) and the endemic H5N1 virus that affects human (*21, 22*) and domestic poultry (*23, 24*) in Southeast Asia.

In Asia, most wild aquatic birds use the Asian-Australasian migration system. This migration system extends from the Arctic tundra of Siberia and Alaska, southwards through Asia to the coasts and islands of the eastern Indian and western Pacific oceans, to the southern parts of Australia and to New Zealand (*25*). There is an estimated two to three million wild aquatic birds using flyways along the East Asian coastline every year to migrate from northern China, Mongolia and Siberia to Asia and Australasia during northern autumn (*26*). Therefore, characterizing the influenza virus in wild aquatic birds that use the wintering grounds and staging posts of this migration system will provide important information for studying the recent changes in the ecology of avian influenza viruses.

Despite of extensive surveillance on influenza virus in wild aquatic birds along the Asian-Australasian migration system over the past 20 years (*1-17*) , the ecology of influenza virus in wild aquatic birds in Hong Kong before the H5N1 endemic era (*21, 22*) is not available. Here, we present the findings on the occurrence of influenza viruses in wild aquatic birds in Hong Kong from late

1980's to early 2000's. Viruses isolated in the surveillance studies at the Mai Po Nature Reserve were characterised. We sought to determine whether East Asian wild aquatic birds passing through or overwintering in Hong Kong are reservoirs of H5N1 influenza viruses and to establish an epidemiological baseline of influenza virus in wild aquatic birds during the pre-H5N1 endemic era.

MATERIALS AND METHODS

Sampling site

The Mai Po Nature Reserve of Deep Bay on the northwestern coast of Hong Kong is part of the Pearl River Delta of southeastern China. It acts as a major site for birds to overwinter or refuel before they continue their southern or northern journey. Each winter, tens of thousands of aquatic birds, over 340 species, winter at the wetlands around the Mai Po Nature Reserve (*27*). The Mai Po Nature Reserve therefore, becomes an ideal site to study influenza viruses in wild aquatic birds that use Hong Kong as a wintering ground or staging post.

Virus sampling

There were three sampling occasions at the Mai Po Nature Reserve. Shorebirds and other birds at the marshes were studied from July 1988 to May 1990 during the northward migration in April and May and the southward migration from July to November as part of a banding exercise conducted by the World Wide Fund for Nature Hong Kong. Shorebirds were caught in mist nets and comprised adult, juvenile and first-stage age groups. Cloacal specimens were taken with fine cotton wool buds on toothpicks soaked in transporting medium (tissue culture medium M199 with antibiotic and antifungal agents) and transported on ice to the laboratory.

The second sampling was conducted in January 1998. Faecal samples on the banks of ponds at the Mai Po Nature Reserve were collected in transport medium and transported on ice to the laboratory.

The third viral surveillance was conducted from March 1999 to February 2001. Samplings were carried out weekly during the southward migration and overwintering period from October to February, and monthly from March to September. Faecal samples on the banks of ponds at the Mai Po Nature Reserve were collected in transport medium and transported on ice to the laboratory. In addition, cloacal specimens from captured birds were collected from October 1999 to February 2000. Samples were inoculated into the allantoic cavities of embryonated hen eggs. The infected allantoic fluids were used for subsequent studies.

Virus subtyping

The HA subtype of the haemagglutinating agent was subtyped in the haemagglutination inhibition test and the NA was subtyped by the neuraminidase inhibition test with a panel of reference antisera (*28*).

***In vivo* infection experiment**

Six trapped curlew sandpipers (*Calidris ferruginea*) and 12 redshanks (*Tringa totanus*) free of detectable virus on three successive daily cloacal swabings were inoculated orally with 100μl $10^{-4.0}$ EID_{50} of influenza virus as follow. Three curlews and three redshanks each were inoculated with the A/curlew sandpiper/HK/208/89 (H10N5) virus and a duck H4N6 virus (A/duck/HK/27/76 [Dk/HK/27/76]), a common subtype isolated from domestic ducks from southern China (*29*). Three redshanks each were inoculated with a duck H9N6 virus (A/duck/HK/147/77 [Dk/HK/147/77]), an

uncommon subtype, and a reference human H3N2 virus (A/HK/8/68 [HK/8/68]). Cloacal swabs were collected daily to detect virus replication.

***In vitro* infection experiment**

Wild caught curlew sandpipers and redshanks free of detectable influenza virus as described earlier and recently hatched domestic chicken and ducks raised in isolation were used to provide tracheal and intestinal colon ring explants. The same viruses used for *in vivo* infection, A/curlew sandpiper/HK/208/89 (H10N5), Dk/HK/27/76 (H4N6), Dk/HK/147/77 (H9N2) and HK/8/68 (H3N2), were used in this experiment. Six 1 mm deep explants of tracheal and colon rings each in 2 ml of complete MEM in vials on roller tubes (12rpm) at 37^{o}C were inoculated with 100 μl of viruses with range from 10^{-8} to 10^{-12} EID_{50}. Virus was allowed to absorb for 1 hour at 37^{o}C and washed 5 times with fresh medium. 500 μl of medium were withdrawn daily for four days and replaced each time with the same volume of fresh medium. The tracheal lining and colon villi shape remained the same as the uninoculated controls over this period.

Nucleotide sequences analysis

Viral RNA was extracted from infected allantoic fluids by using a QIAamp viral RNA mini kit (QIAGEN). Reverse transcription-PCR was performed with gene specific primers (sequences are available upon request). Amplified products of the expected sizes were purified by QIAquick PCR purification kit (QIAGEN). Purified amplicons were sequenced by using the BigDye Terminator Cycle Sequencing Ready Reaction (Applied Biosystems) and analysed on an ABI 377 automated sequencer (Applied Biosystems). Published sequences used for comparison in this study were obtained from

GenBank. Editing, analysis and alignment of sequence data were performed with Clustal X (*30*) and Evolutionary Genetics Analysis Program (MEGA 4) (*31*).

Phylogenetic analysis

Viral gene sequences obtained in this study were aligned with published sequences. Aligned gene sequences were used to infer the neighbour joining (NJ) phylogenetic trees using MEGA 4 and employed the proportion of differences between the sequences as the distance measure (*p*-distance) with 1000 bootstrap replicates.

RESULTS

Influenza virus surveillance at the Mai Po Nature Reserve

July 1988 – May 1990

Viral surveillance conducted in this period focused mainly on shorebirds. A total of 1246 cloacal swabs was collected and 1079 of them were from 30 species of shorebirds (Table 1). Out of 128 curlew sandpiper cloacal samples, one influenza virus, A/curlew sandpiper/HK/208/89 (H10N5) (Cs/HK/208/89), was isolated at the beginning of the southward migration in early July 1989 (Table 2). All the birds sampled were apparently healthy. The isolation rate of influenza virus of this surveillance period was 0.08%.

January 1998

After the H5N1 outbreak in 1997, viral surveillance at the Mai Po Nature Reserve was resumed with the possibility that the precursor virus of the H5N1 virus may have came from wild aquatic bird at the marshes adjacent to the Yuen Long farm where the H5N1 virus infected chicken.

In this surveillance study, 808 faecal samples were collected from the banks of the ponds in the Mai Po Nature Reserve where free flying ducks were overwintering (Table 2). One virus, A/aquatic bird/HK/603/98 (H11N1) (Ab/HK/603/98), was isolated in mid-January 1998 most probably from faeces of a duck. The isolation rate was 0.12%.

March 1999 – February 2001

The latest influenza virus surveillance at the Mai Po Nature Reserve was carried out mainly during the autumn and winter months from 1999 to 2001. Forty-seven cloacal swabs were collected from ducks (18 pintails and 11 wigeon), passerine birds (5 great-reed warblers) and other birds (7 Chinese bulbuls, 3 common kingfishers, 1 coot, 1 moorhen and 1 Japanese sparrow hawk). 1077 faecal samples were collected from the banks of ponds where free flying ducks (e.g. pintail, wigeon and teal) were overwintering (Table 2). Of the samples collected, two haemagglutinating agents were isolated from faeces most probably from ducks. One was identified as a Newcastle disease virus and the other an influenza virus. The Newcastle disease virus was isolated in April 1999. The influenza virus, A/aquatic bird/HK/399/99 (H3N8) (Ab/HK/399/99), was isolated in mid-November 1999 and the influenza virus isolation rate was 0.09%.

Phylogenetic analysis of the nucleoprotein gene of isolates from the Mai Po Nature Reserve

The structural protein of the replicating complex, nucleoprotein (NP), is an important determinant of host range restriction (*32-35*). Hence, in the present study 1389 bp of the NP genes (92.8% of the coding region) of the three viruses from the Nature Reserve were examined phylogenetically to determine their relationship with other Eurasian isolates. NJ analysis with *p*-distance of the NP genes revealed that the three viruses fall into avian-specific lineage but are not

closely related to each other (Figure 1). That of the shorebird virus, Cs/HK/208/89 (H10N5), grouped with several older viruses isolated from 1960 to 1980 while that of the Ab/HK/603/98 (H11N1) virus is closely related to contemporary viruses from ducks. The Ab/HK/399/99 (H3N8) virus showed an ancestral relationship to the 1980's avian viruses and contemporary ones. Based on the NP genes, the three viruses are related to avian influenza viruses isolated in Asia but did not seem to closely relating to the human H5N1 viruses of 1997 and 2003.

Sequence analyses of the H3 HA, N1 NA, NP, matrix and nonstructural genes

Phylogenetic analysis of the NP genes indicated that the three isolates from the Mai Po Nature Reserve do not closely related to the human H5N1 viruses. The H3 HA, N1 NA, matrix (M) and nonstructural (NS) genes were sequenced to confirm the above observations. Nucleotide sequences of the H3 HA, NP, N1 NA, M and NS genes showed that the three isolates were related to Eurasian avian viruses, particularly duck viruses from southeastern China (Table 3). The Cs/HK/208/89 (H10N5) virus has a closer relationship with older duck isolates whereas the Ab/HK/603/98 (H11N1) virus is related to the virus isolated in the late 1990's. This is consistent with previous report showing that the M and NS genes of the Ab/HK/603/98 (H11N1) virus are phylogenetically related to contemporary duck viruses in Hong Kong (*36*). The Ab/HK/399/99 (H3N8) virus, which has the NP gene more closely related to that of virus from northeastern China, possesses HA, M and NS genes resembling those of duck viruses in Japan.

***In vivo* infection experiment**

The Cs/HK/208/89 (H10N5) virus and the DkHK/27/76 (H4N6) virus were isolated from the cloacal swabs from one curlew sandpiper each on the first day post-infection but not on subsequent

days indicative of residual virus rather than multiplication by these two viruses (data not shown). Viruses were not isolated from the remaining four curlew sandpipers and any of the redshanks. Unless the curlew sandpiper and redshank used in this experiment were immune to the viruses under study arising from previous exposure to influenza viruses, it seems most likely that they are refractory to virus infection.

In vitro infection experiment

The four viruses, Cs/HK/208/89 (H10N5), Dk/HK/27/76 (H4N6), Dk/HK/147/77 (H9N2) and HK/8/68 (H3N2), grew in one or other or both of the tracheal and colon explants from the four types of bird (Table 4), although there was some variability with the curlew sandpiper and redshank explants. Thus, it might be inferred that the avian trachea and intestine are inherently capable of supporting the growth of a range of viruses.

DISCUSSION

The present study provided the first epidemiological baseline for influenza viruses in wild ducks and shorebirds of Hong Kong. Three influenza viruses were isolated from 3178 samples collected during the three surveillance periods between 1988 and 2001. The isolation rate of influenza viruses ranged from 0.08% to 0.12%. The aquatic birds characterised in this study came from their breeding sites in Northern China and Siberia (*25*). The higher incidence of infection of ducks in Siberia (1.2%) (*5*) is probably due to large numbers of juvenile birds in these areas at the end of the breeding season. When these ducks began migrating from the breeding grounds in summer and were examined in Hong Kong in winter, the isolation rates were much lower, possibly due to acquired immunity and dispersal of duck population over distance and time so that they

were below the critical levels necessary to maintain infections. Influenza surveillance of wild ducks in North America showed a similar observation along the Mississippi flyway. There was a high incidence of infection of ducks in Alaska (3.5%) (*37*) and Alberta (30.8% juvenile and 14.3% adult) (*38*) at the end of breeding season but a lower isolation rate in Arkansas (0.7%) (*39*) and on the Louisiana coast (3.1% in Sept. to 0.4% in Dec.) (*40*) in the migration and wintering periods.

The low incidence of infection in wild aquatic birds of Hong Kong suggested that these wild aquatic birds are unlikely to be responsible for the occurrence of influenza viruses that are prevailing in domestic poultry in the hypothetical influenza epicentre of southern China (Shortridge, 1992; Shortridge and Stuart-Harris, 1982). Phylogenetic and sequence analyses of the NP, N1 NA, M and NS genes of the three isolates from the Mai Po Nature Reserve indicated that these viruses are not closely related to the human H5N1 virus. Therefore, wild aquatic birds did not contribute significantly to the influenza virus gene pool in human and domestic poultry during the pre-H5N1 endemic era. In addition, it seems unlikely that the shorebirds migrating along the East Asian coastline, which are refractory to influenza virus infection possibly due to their acquired immunity, would be effective hosts for the transmission of influenza viruses over long distances in spite of the experimental demonstration that their tracheas and intestines (colon) were able to support virus growth. Insight into the apparent dearth of influenza viruses isolated from these birds was not resolved in this exercise and would require a more extensive longitudinal sampling and an examination of wider ecological aspects of bird migration.

In the light of these provisos, it appears that influenza viruses in southeasten China are established in domestic poultry largely by virtue of the agricultural practices of the region whereby domestic ducks are raised as an adjunct to rice farming (*29, 41*). Indeed, even if a large number of influenza viruses had been isolated from shorebirds, studies on the North American shorebirds

indicated that not all have the ability to replicate in ducks (*42*). Earlier longitudinal influenza virus surveillance on a Hong Kong duck farm adjacent to the Mai Po Nature Reserve where the present study was carried out in which viruses apparently introduced by wild birds had failed to become established in the duck population are in accord with that view (*43*).

In conclusion, we established an epidemiological baseline for influenza virus in wild aquatic birds in Hong Kong during the 1990's and early 2000's. The highly pathogenic H5N1 virus was not identified in these wild birds. Owing to the dearth of influenza viruses in wild aquatic birds and the high prevalence of influenza viruses in domestic poultry in southeastern China in the 1990's, it is possible that the highly pathogenic H5N1 influenza viruses could have transmitted from domestic poultry to wild aquatic birds acquired and being detected on numerous occasions recently in Asia (*7-17*).

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Table 1. Species of birds sampled during July 1988 – May 1990

Bird type	Common names	Scientific names	Number of cloacal samples
Shorebirds	Redshank	Tringa totanus	391
	Whimbrel	*Numenius phaeopus*	143
	Curlew sandpiper	*Calidris ferruginea*	128
	Dunlin	*C. alpina*	94
	Terek sandpiper	*Xenus cinereus*	74
	Greater sandpiper	*Charadrius leschenaultii*	59
	Pacific golden plover	*Pluvialis fulva*	27
	Wood sandpiper	*Tringa glareola*	17
	Bar-tailed godwit	*Limosa lapponica*	14
	Broad-billed sandpiper	*Limicola falcinellus*	14
	Common sandpiper	*Actitis hypoleucos*	13
	Grey-rumped sandpiper	*Heteroscelus brevipes*	13
	Marsh sandpiper	*Tringa stagnatilis*	13
	Great knot	*Calidris tenuirostris*	12
	Greenshank	*Tringa nebularia*	11
	Red knot	*Calidris canutus*	9
	Curlew	*Numenius arquata*	8
	Grey plover	*Pluvialis squatarola*	8
	Ruddy turnstone	*Arenaria interpres*	6
	Lesser sandplover	*Charadrius mongolus*	5
	Sharp-tailed sandpiper	*Calidris acuminata*	5
	Black-tailed godwit	*Limosa limosa*	4
	Asiatic dowitcher	*Limnodromus semipalmatus*	3
	Common snipe	*Gallinago gallinago*	2
	Black-winged stilt	*Himantopus himantopus*	1
	Green sandpiper	*Tringa ochropus*	1
	Kentish plover	*Charadrius alexandrinus*	1
	Pied avocet	*Avosetta recurvirostra*	1
	Red-necked stint	*Calidris ruficollis*	1
	Swinhoe's snipe	*Gallinago megala*	1
	Subtotal of shorebirds		1079
Ducks	Teal	*Anas crecca*	12
	Garganey	*A. querquedula*	4
	Yellow-nib duck	*A. poecilorhyncha*	1
	Subtotal of ducks		17

Table 1. Species of birds sampled during July 1988 – May 1990 (continued)[a]

Bird type	Common names	Scientific names	Number of cloacal samples
Other birds	Common kingfisher	*Alcedo atthis*	74
	Great-reed warbler	*Acrocephalus arundinaceus*	31
	Chinese bulbul	*Pycnonotus sinensis*	22
	Tree sparrow	*Passer montanus*	5
	White-breasted kingfisher	*Halcyon smyrnensis*	4
	Yellow bittern	*Ixobrychus sinensis*	4
	Crested bulbul	*Pycnonotus jocosus*	2
	Spotted dove	*Streptopelia chinensis*	2
	Chestnut bittern	*Ixobrychus cinnamomeus*	1
	Greater coucal	*Centropus sinensis*	1
	Little egret	*Egretta garzetta*	1
	Little green heron	*Butorides striatus*	1
	Moorhen	*Gallinula chloropus*	1
	Night heron	*Nycticorax nycticorax*	1
	Subtotal of other birds		150
		Total number of cloacal samples	1246

Table 2. Influenza virus surveillance at the Mai Po Nature Reserve

Periods	Samples collected and tested			Influenza viruses isolated	% influenza virus positive
	Faecal	Cloacal	Total		
Jul 1988 – May 1990	0	1246	1246	A/curlew sandpiper/HK/208/89 (H10N5)	0.08%
Jan 1998	808	0	808	A/aquatic bird/HK/603/98 (H11N1)	0.12%
Mar 1999 – Feb 2001	1077	47	1124	A/aquatic bird/HK/399/99 (H3N8)	0.09%

Table 3. Nucleotide similarity of isolates from the Mai Po Nature Reserve[a]

Isolates	Virus with greatest similarity (% similarity)[b] to:				
	H3 HA (1701 bp)	N1 NA (1389 bp)	NP (1497 bp)	M (982 bp)	NS (838 bp)
Cs/HK/208/89 (H10N5)	—[c]	—	Dk/Hong Kong/365/78 (H4N6) (97.3%)[d]	Dk/Nanchang/1749/92 (H11N2) (99.3%)	Dk/Nanchang/1944/93 (H7N4) (97.7%)
Ab/HK/603/98 (H11N1)	—	Sw/Hokkaido/55/96 (H1N1) (98.3%)	Dk/Hong Kong/P185/97 (H3N8) (98.7%)	WDk/ST/988/00 (H4N9) (97.9%)	Dk/Nanchang/8-174/00 (H11N9) (97.8%)
Ab/HK/399/99 (H3N8)	Dk/Tsukuba/28/06 (H3N8) (98.4%)	—	Ck/Jilin/hk/04 (H5N1) (98.8%)	Dk/Hokkaido/69/00 (H5N3) (99.4%)	Dk/Hokkaido/49/98 (H9N2) (99.6%)

[a] The accession number of the sequences reported in this paper are AJ427297 through AJ427303.

[b] Determined by BLAST in GenBank.

[c] Different subtype.

[d] Ck, chicken; Dk, duck; Sw, swan; WDk, wild duck.

Table 4. Growth of avian and human influenza A viruses in avian tracheal and colon explants

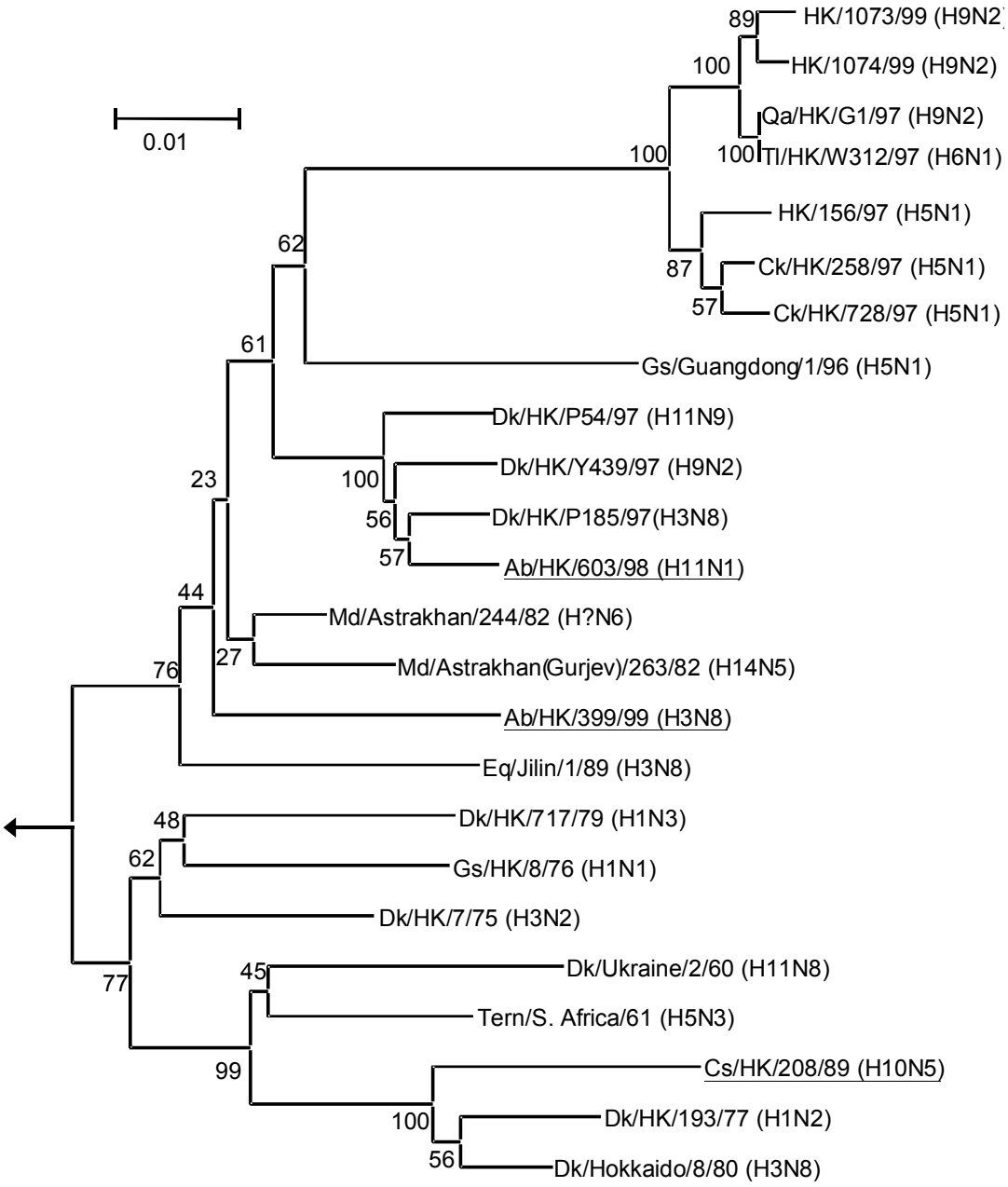
Type of bird providing explant	Age of bird	Log dilution of virus inoculum showing HA activity (EID_{50})								HA titre of the HA activity
		CS/HK/208/89 (H10N5)		Dk/HK/27/76(H4N6)		Dk/HK/147/77(H9N6)		HK/8/68 (H3N2)		
		T[a]	C[a]	T	C	T	C	T	C	
Curlew sandpiper	Adult	9[b]	-[c]	9	9	11	-	8	9	16-32
	Adult	-	9	10	10	-	10	NT[d]	NT	128-256
Redshank	Juvenile	10	-	10	10	9	-	8	8	64-128
Chicken	2 day	10	10	10	10	10	9	NT	NT	512-1024
	7 day	10	10	12	12	12	11	10	9	512-1024
Duck	7 day	10	9	9	9	9	9	10	10	16-32

[a] T, tracheal explant; C, colon explant

[b] Number denotes highest log dilution of virus inoculum showing HA activity over the four-day period

[c] HA activity not detected

[d] NT, not tested



LEGENDS

Figure 1. Neighbour joining tree of NP genes with the proportion of sequence difference as the distance measure. Bootstrap values are shown for each node. Nucleotides 1 to 1389 (1389 bp) of the NP gene were used to construct the tree. The tree is rooted to A/Equine/Prague/1/56 (H7N7) virus and the viruses characterised in this study are underlined. The lengths of the horizontal lines are proportional to the minimum number of nucleotide differences required to join the nodes. Vertical lines are for spacing branches and labels. Abbreviations: Ck, chicken; Dk, duck; Eq, equine; Gs, goose; Md, mallard; Qa, quail; Tl, teal.