

# Human influenza A H5N1 virus related to a highly pathogenic avian influenza virus

Eric C J Claas, Albert D M E Osterhaus, Ruud van Beek, Jan C De Jong, Guus F Rimmelzwaan, Dennis A Senne, Scott Krauss, Kennedy F Shortridge, Robert G Webster

## Summary

**Background** In May, 1997, a 3-year-old boy in Hong Kong was admitted to the hospital and subsequently died from influenza pneumonia, acute respiratory distress syndrome, Reye's syndrome, multiorgan failure, and disseminated intravascular coagulation. An influenza A H5N1 virus was isolated from a tracheal aspirate of the boy. Preceding this incident, avian influenza outbreaks of high mortality were reported from three chicken farms in Hong Kong, and the virus involved was also found to be of the H5 subtype.

**Methods** We carried out an antigenic and molecular comparison of the influenza A H5N1 virus isolated from the boy with one of the viruses isolated from outbreaks of avian influenza by haemagglutination-inhibition and neuraminidase-inhibition assays and nucleotide sequence analysis.

**Findings** Differences were observed in the antigenic reactivities of the viruses by the haemagglutination-inhibition assay. However, nucleotide sequence analysis of all gene segments revealed that the human virus A/Hong Kong/156/97 was genetically closely related to the avian A/chicken/Hong Kong/258/97.

**Interpretation** Although direct contact between the sick child and affected chickens has not been established, our results suggest transmission of the virus from infected chickens to the child without another intermediate mammalian host acting as a "mixing vessel". This event illustrates the importance of intensive global influenza surveillance.

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See *Commentary page* ???

**Department of Virology and WHO National Influenza Centre, Erasmus University, PO Box 1738, 3000 DR Rotterdam, Netherlands** (ECJ Claas PhD, Prof ADME Osterhaus DVM, R Van Beek MS, G F Rimmelzwaan PhD); **Laboratory of Virology, National Institute of Health and the Environment, Bilthoven, Netherlands** (JC de Jong PhD); **National Veterinary Services Laboratories, United States Department of Agriculture, Animal and Plant Health Service, Veterinary Services, Ames, Iowa, USA** (DA Senne MS); **Department of Microbiology, University of Hong Kong, Queen Mary Hospital, Hong Kong Special Administrative Region, China** (Prof KF Shortridge PhD); and **Department of Virology and Molecular Biology, St Jude Children's Research Hospital, Memphis, Tennessee, USA** (S Krauss MS, Prof RG Webster PhD)

**Correspondence to:** Dr Eric C J Claas  
(e-mail: Claas@viro.fgg.eur.nl)

## Introduction

It is nearly 30 years since the last human influenza pandemic occurred, the Hong Kong pandemic of 1968. The influenza A H3N2 virus that was then introduced into the human population was shown to carry a new haemagglutinin, the major surface glycoprotein of the influenza virus. The influenza A H2N2 virus responsible for the previous 1957 pandemic carried new surface glycoproteins haemagglutinin and neuraminidase. Phylogenetic studies revealed that these newly emerging glycoproteins originated from avian viruses and had entered the human population after reassortment with human influenza virus strains.<sup>1–3</sup> However, the virus involved in the most devastating pandemic known to have occurred in human beings—the influenza A H1N1 virus of the 1918 pandemic, which killed over 20 million people worldwide—may have entered the human population without a reassortment event.<sup>4,5</sup> To date, as many as 15 different haemagglutinins and nine neuraminidases have been identified in avian species,<sup>6,7</sup> providing an extensive reservoir of influenza viruses that could be transmitted to other species.

In May, 1997, an influenza virus was isolated from a tracheal aspirate of a 3-year-old boy in Hong Kong, who died a few days after admission to hospital.<sup>8</sup> The child died from influenza pneumonia, acute respiratory distress syndrome (ARDS), Reye's syndrome, multiorgan failure, and disseminated intravascular coagulation, and had no known underlying diseases before admission. The virus could not be characterised in the haemagglutination-inhibition (HI) test with post-infection ferret antisera raised against recent human and swine influenza viruses. Further analysis revealed the virus to be an influenza A H5N1, a subtype previously not detected in human beings.<sup>8</sup> Here we present the genetic characterisation of this first human influenza A H5N1 isolate and the comparison with an influenza A H5N1 virus isolated from outbreaks of avian influenza in chickens in Hong Kong that preceded the human infection.

## Methods

The human virus A/Hong Kong/156/97 (H5N1) was propagated on Madin-Darby canine kidney (MDCK) cells.<sup>8</sup> Avian influenza outbreaks occurred in Hong Kong over the period late March to early May, 1997. Three chicken farms were separately affected and the overall mortality rate for the 6800 chickens exceeded 70%. On two of the farms the rate was close to 100%. From these three farms, avian influenza viruses of the H5 subtype were isolated by inoculation of pooled organ material of dead chickens into embryonated

	1				50
A/Hong Kong/156/97	-----	-----	-----	-----	-----
A/ck/Hong Kong/97	-----	-----	-----	-----	-----
A/ty/England/91	-----	-----	-----	---k---	--s-----
Consensus	DQICIGYHAN	NSTEQVDTIM	EKNVTVTHAQ	DILERTHNGK	LCDLNGVKPL
	51				100
A/Hong Kong/156/97	-----	-----	-----	-----	-----
A/ck/Hong Kong/97	-----	-----	-----	-----	-----
A/ty/England/91	-----	-----	1-----	--dn-v-g--	---d-----
Consensus	ILRDCSVAGW	LLGNPMCDEF	INVPEWSYIV	EKASPANDLC	<u>Y</u> PGNFNDYEE
	101				150
A/Hong Kong/156/97	-----	-----	-----	-----	-----
A/ck/Hong Kong/97	-----	-----	-----	-----	-----
A/ty/England/91	-----st-	-----r-	-----	-----n--	-----
Consensus	LKHLISRINH	FEKIQIIPKS	SWSNHDASSG	<u>VSS</u> ACPYLGR	SSFRRNVVWL
	151				200
A/Hong Kong/156/97	-----	-----	-----v--	-----	-----
A/ck/Hong Kong/97	---t-----	-----	-----	-----	-----
A/ty/England/91	---n-----	---s-----	-----	-----e-	-----v--
Consensus	<u>I</u> KKNSAYPTI	KRSYNNNTQE	DLVLWGIHH	PNDAA <u>E</u> QTKL	<u>Y</u> QNPTTYISV
	201				250
A/Hong Kong/156/97	-----	-----	-----	-----	-----
A/ck/Hong Kong/97	-----	-----	-----	-----	-----
A/ty/England/91	-----s-	-a-----	-----	-----	-----
Consensus	GTSTLNQRLV	PEIATRPKVN	<u>GOSGR</u> MEFFW	TILKPNDAIN	FESNGNFIAP
	251				300
A/Hong Kong/156/97	-----	-----	-----	-----	-----
A/ck/Hong Kong/97	-----	-----	-----	-----	-----
A/ty/England/91	-----	--a---g--	-----	-----	l-----
Consensus	EYAYKIVKKG	DSTIMKSELE	YGNCNTKCOT	PMGAINSSMP	FHNIHPLTIG
	301		330		
A/Hong Kong/156/97	-----	-----t	-----	- HA2	
A/ck/Hong Kong/97	-----	-----a	-----	- HA2	
A/ty/England/91	-----d-	-----p-v	---k--t	- HA2	
Consensus	ECPKYVKSNR	LVLATGLRN-	<u>PQ</u> <b><u>RE</u></b> RRRK R		

Figure 1: Alignment of the aminoacid sequences of the HA1 portion of the H5 haemagglutinin protein of HK97, CkHK97, and A/ty/England/50-92/91

Identical aminoacids are indicated as -. Dots (.) indicate absence of alignable aminoacids. The aminoacids thought to be involved in binding the receptor are underlined, with those forming the receptor-binding site at the tip of the haemagglutinin in bold. The potential N-linked glycosylation site difference is doubly underlined. The multiple basic residues at the cleavage site are in bold and italicised.

hens' eggs. A representative strain, A/chicken/Hong Kong/258/97 (CkHK97), was selected for comparison with the human virus isolate (HK97).

Propagation and identification of influenza viruses were conducted according to standard procedures.<sup>9</sup> The HI assay was done with 1% turkey erythrocytes. The neuraminidase-inhibition assay was done according to the method of Aymard-Henry et al.<sup>10</sup>—ie, inhibition of the fetuin cleavage activity of the viral neuraminidase by specific antisera.

Viral RNA extraction and amplification of the viral RNA by reverse transcription PCR (RT-PCR) was done as described previously.<sup>11</sup> In brief, the RNA was extracted with a guanidinium isothiocyanate solution and collected by precipitation with isopropanol. The viral RNA was then amplified with oligonucleotide primers that were selected from a consensus sequence of previously published sequences (GenBank). The amplified products were subjected to

nucleotide sequence analysis by cycle sequencing with an ABI dye terminator sequencing system (Warrington, UK). Sequences have been submitted to GenBank with the accession numbers AF028708, AF028709, and AF028710. Nucleotide and aminoacid sequences were aligned with sequences from GenBank with the GCG package (Madison, WI, USA). Phylogenetic trees were constructed as indicated in the legends to the figures.

## Results

An HI assay was done with the HK97 virus against a panel of 23 hyperimmune sera directed to variants of influenza viruses of 14 haemagglutinin subtypes (H1-H14). The anti-H5 serum, raised by immunisation with A/tern/South Africa/63 (H5N3), inhibited haemagglutination of the HK97 virus with turkey

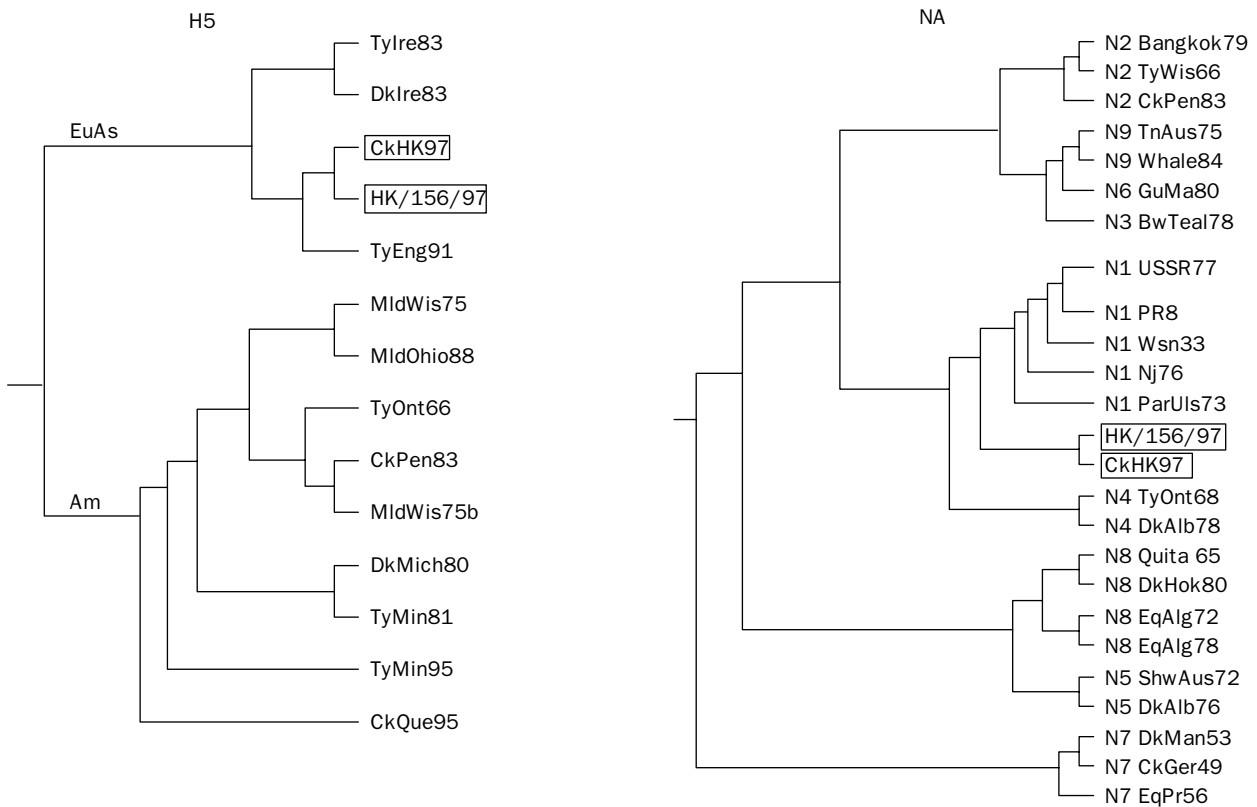


Figure 2: **Phylogenetic tree of the nucleotide sequences of the H5 haemagglutinins (H5) and neuraminidases (NA) of HK97 and CkHK97 compared with gene segments of other influenza viruses**

The GCG Wisconsin package was used to FETCH sequences from GenBank. Multiple sequence files were generated and analysed by DNAPARS in the PHYLIP package.<sup>14</sup> Subsequently, phenograms were generated with the DRAWGRAM program. Related sequences cluster together. The abbreviations have been published.<sup>4</sup> EuAs=Eurasian, Am=American.

erythrocytes up to a dilution of 1/2560. None of the other sera inhibited haemagglutination, indicating that the isolate was of the H5 subtype. Oligonucleotide primers were selected on the basis of published H5 gene sequences<sup>12,13</sup> and fragments of the expected length were amplified from the viral RNA in a RT-PCR assay. Nucleotide sequence analysis of the haemagglutinin gene confirmed the H5 genotype of the HK97 virus.

A comparison of the aminoacid sequences of the H5 genes from the human HK97 virus and the representative chicken virus, CkHK97, showed a high degree of homology in their respective H5 HA1 sequences (figure 1). Only three aminoacid differences were observed in the HA1 sequences, confirming a close genetic relation between these viruses, which belong to the Eurasian H5 subtype phylogeny (figure 2).

When the aminoacids expected to be involved in the assembly of the receptor binding site on the haemagglutinin<sup>15,16</sup> were examined, no differences were observed between the human and avian H5

haemagglutinins (figure 1).

The presence of multiple basic aminoacids in the sequence motif at the cleavage site of the haemagglutinin molecule of both the human and avian isolates (figure 1) is known to be associated with high virulence of avian influenza viruses.<sup>17-19</sup> Experimental infection of chickens with HK97 showed that even after passaging in mammalian cells—once in the child and twice in MDCK cells—the virus remained highly pathogenic for chickens: all eight chickens inoculated intratracheally with MDCK-cell-grown HK97 died within 3 days of infection. Also, CkHK97 was shown to be highly pathogenic in experiments in which seven of eight birds inoculated intravenously with 0.2 mL of a one in ten dilution of infectious allantoic fluids died within 24 h of inoculation and the remaining bird within 48 h.

When HK97 and CkHK97 were tested in HI assays against a panel of 17 monoclonal antibodies directed against A/chicken/Pennsylvania/83 (H5N2), the two viruses showed similar antigenic reactivities with all but

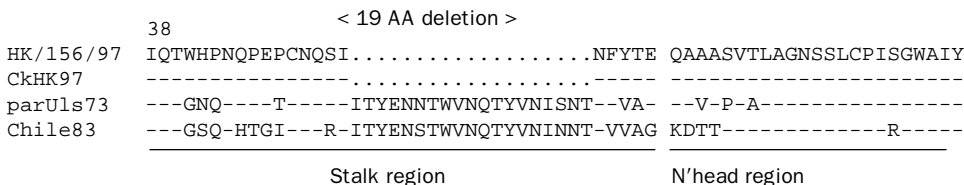


Figure 3: **Alignment of the aminoacid sequence of the N1 stalk region and N' part of the head region**

Identical amino acids are indicated as -. A deletion is seen in HK97 and CkHK97 compared with A/parrot/Ulster/73 (H7N1) and the human strain A/Chile/1/83.

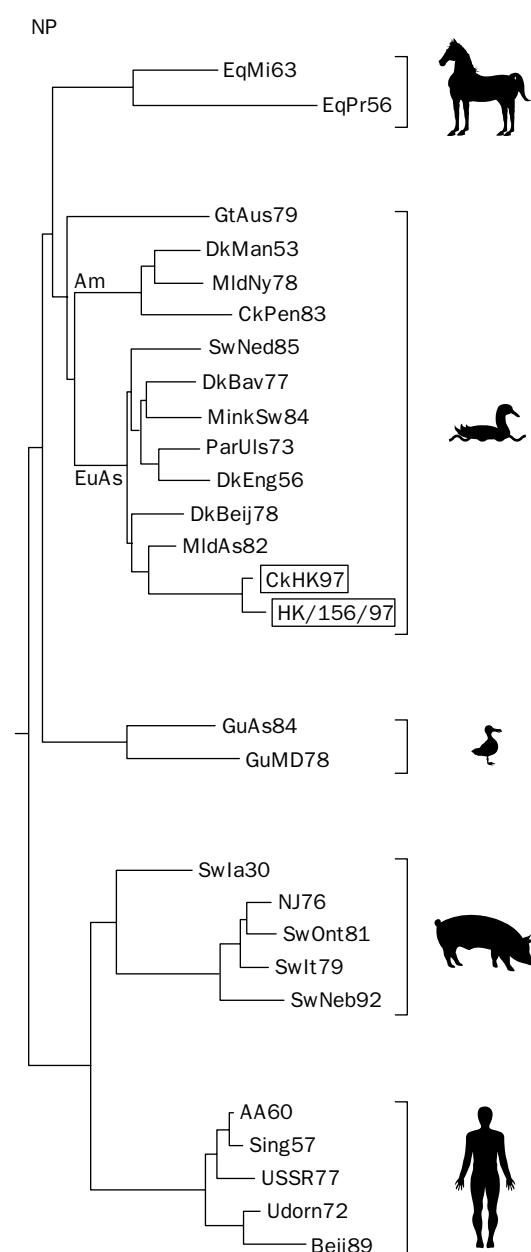


Figure 4: **Phylogenetic tree based on the nucleoprotein nucleotide sequences**

HK97 and CkHK97 sequences were aligned with nucleoprotein sequences from representative influenza viruses from different hosts. The phenogram was constructed with the DNADIST, FITCH, and DRAWGRAM programs from the PHYLIP package.<sup>14</sup> Only the horizontal distances are proportional to the number of differences, whereas vertical lines are for spacing the branches and labels. The abbreviations used have been published.<sup>4</sup> EuAs=Eurasian, Am=American.

one monoclonal antibody; a fourfold difference in HI titre was observed with the exceptional monoclonal antibody. However, a polyclonal antiserum directed against A/tern/South Africa/61 gave an HI titre of 1/40 with CkHK97 (both egg grown and MDCK-grown viruses) and of 1/640 with HK97, indicating clear differences in reactivity with HI antibodies.

The neuraminidase activity of HK97 and CkHK97 was inhibited by anti-N1 antiserum. RT-PCR with primer sets that amplified the 5' end of the neuraminidase gene segments further showed that these viruses were of the N1 genotype. Nucleotide sequence analysis and comparison with published neuraminidase sequences<sup>20</sup> confirmed this finding genetically (figure 2). The neuraminidase sequences showed unequivocally a molecular relation between HK97 and CkHK97 because a unique 57-nucleotide (19 aminoacids) deletion was seen in the stalk region of the N1 gene of both viruses (figure 3).

In addition to the glycoproteins haemagglutinin and neuraminidase, comparison of the nucleotide sequences of portions of the remaining six gene segments of HK97 and CkHK97 revealed a high homology between the viruses (table). The homology of the most closely related influenza-virus gene sequences in GenBank to the gene segments of HK97 and CkHK97 is about 90-95% for most genes. Phylogenetic trees, which were constructed by comparing the HK97 and CkHK97 nucleotide sequences with those in GenBank, showed that both HK97 and CkHK97 belong to the Eurasian lineage of avian influenza viruses. They also showed that HK97 is not the result of a reassortment event between a human and an avian influenza virus because all genes of the virus are apparently of avian origin. As an example, the phylogenetic tree for the nucleoprotein gene is shown in figure 4.

## Discussion

We have shown clearly the genetic similarity of HK97, the first human influenza virus isolate of the H5N1 subtype, and a virus that was isolated in outbreaks of avian influenza in Hong Kong that preceded human infection. Only three aminoacid differences were observed in the HA1 part of the H5 haemagglutinin, none of which affected directly the receptor-binding site. Therefore, the H5 haemagglutinin of HK97 probably had not acquired aminoacid mutations that have been associated with binding to sialic acids with an  $\alpha$ -2,6 linkage to the galactoside, the receptor preferred by human influenza viruses.<sup>21,22</sup> However, the loss of a potential N-linked glycosylation site in the haemagglutinin gene of HK97 at aminoacid 156Asn, close to the receptor-binding site, could affect binding to the cellular receptor. The altered potential glycosylation site at 156Asn may explain the altered HI reactivity with a polyclonal anti-H5 antiserum. In

Gene	Nucleotide			Aminoacid		
	Analysed	Mismatches	% homology	Analysed	Mismatches	% homology
PB2	394	1	99.8	130	3	97.7
PB1	434	13	97.0	143	10	93.0
PA	526	10	98.1	173	3	98.3
NP	1104	16	98.6	367	12	96.7
MA	401	4	99.0	108 (M1)	0	100
NS	266	2	99.2	127	2	98.4

### Nucleotide and aminoacid sequence homologies of the gene segments of HK97 and CkHK97

addition, the 19-aminoacid deletion in the neuraminidase stalk region of HK97 and CkHK97 demonstrates their genetic relatedness. Although direct contact between the child from whom HK97 was isolated and affected chickens has not been established, the results of this study as well as the closeness of the dates of the outbreaks in chickens and of the illness in the child suggest a link between the two events.

Previously, antibodies to avian influenza viruses, including the H5 subtype, have been detected in human sera from rural southern China,<sup>23</sup> and avian influenza viruses of the H7 subtype have been isolated from eye swabs of human beings in two cases of clinical conjunctivitis.<sup>24,25</sup> However, the HK97 virus is the first avian influenza virus isolated from a person with respiratory infection. The H5N1 subtype had not been found in any cases of avian influenza in the Hong Kong area until the recent outbreaks.

An important feature of HK97 is that it probably crossed the avian-human species barrier without prior adaptation in another mammalian species. Replication of avian influenza viruses has been observed in experimental human infection, but only when extremely high doses were used.<sup>26</sup> The most favoured hypothesis for the emergence of the pandemic viruses of 1957 and 1968 is that pigs acted as a "mixing vessel" for reassortment between avian and human influenza viruses.<sup>27,28</sup> Studies that identified influenza H3N2 human-avian reassortant viruses in pigs<sup>29</sup> and people<sup>30</sup> in Europe gave support to this hypothesis. In the case of influenza A viruses, only those carrying H1, H2, or H3 haemagglutinins and N1 or N2 neuraminidases have so far been shown to cause disease in man. Sero-archeological studies have suggested recycling of a limited number of viruses.<sup>31</sup>

The influenza A (H5N1) virus meets two of the three important criteria for a new pandemic influenza virus—ie, the ability to replicate in human beings and the absence of antibodies to the virus in the human population at large. The third criterium is the potential to rapidly spread from man to man, which has so far not been observed.

Approximately 6 months after the first human infection of influenza A H5N1 virus, 17 additional confirmed cases with five fatalities have been reported from Hong Kong.<sup>32</sup> Preliminary sequencing results of some of the more recent human virus isolates indicate that they are similar to, but distinguishable from, the first isolate. All genes are of avian origin (WHO press release, Dec 17, 1997), suggesting multiple independent transmissions from infected birds to people. The H5N1 viruses show no or only limited ability to spread among human beings. Since all the chickens in Hong Kong were killed, no additional cases have been reported so far.

As shown for the pandemic H2N2 viruses of 1957 and H3N2 viruses of 1968, reassortment of the avian virus with a human virus may be necessary to confer efficient man-to-man transmission. The present finding of avian H5N1 influenza viruses infecting a human host provides further insight into the epidemiology of influenza viruses: the close resemblance of HK97 to the avian virus CkHK97 indicates that it is unlikely that another mammal had acted as an intermediate host or "mixing vessel" in this incident.

If interspecies transmission occur in periods of

human influenza activity, man himself may act as a mixing vessel. Co-infection of the avian influenza H5N1 virus and a human H3N2 or H1N1 virus could result in a reassortant virus carrying the H5 haemagglutinin with additional gene segments of the human virus involved. Although there still has been no evidence of efficient spread of HK97, its detection illustrates the importance of the intensive global influenza surveillance system, in which China is included.<sup>33</sup>

#### Contributors

Eric C J Claas, Albert D M E Osterhaus, Ruud van Beek, Jan de Jong, and Guus Rimmelzwaan were responsible for the antigenic and molecular characterisation of the human H5N1 influenza virus from the child in Hong Kong, including the experimental design and analysis of data; Kennedy F Shortridge was involved in the isolation and antigenic analysis of the avian H5N1 viruses from chickens in Hong Kong; Dennis Senne carried out pathogenesis studies on the avian H5N1 influenza virus from Hong Kong in chickens and sequencing of the haemagglutinin connecting peptide; Scott Krauss and Rob Webster contributed to the experimental design of the study and to the antigenic and molecular analysis of the H5N1 influenza viruses from chickens in Hong Kong. Eric C J Claas wrote the manuscript. All other investigators were involved in editing the manuscript.

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