

Short
Communication

A mutation in H5 haemagglutinin that conferred human receptor recognition is not maintained stably during duck passage

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A/Hong Kong/213/97 (HK213; H5N1), isolated from a human, binds to both avian- and human-type receptors, due to a haemagglutinin (HA) mutation probably acquired during adaptation to humans. Duck passage of this virus conferred lethality in ducks. Sequence analyses of the duck-passaged virus revealed that its HA gene reverted back to one recognizing only avian-type receptors, and consequently it bound human tissue to a lesser extent. This finding suggests that viruses with human-type receptor specificity are unlikely to be maintained in waterfowl, unlike those with the human-type PB2 mutation, such as H5N1 viruses of the Qinghai Lake lineage.

Received 26 November 2009

Accepted 2 February 2010

H5N1 influenza A viruses pose a major pandemic threat to humans worldwide. Since 2003, they have caused more than 262 deaths among 442 human cases (http://www.who.int/csr/disease/avian_influenza/en/). H5N1 viruses were previously non-pathogenic in wild aquatic birds, such as ducks, which are their natural hosts (Shortridge *et al.*, 1998). Since 2002, however, avian H5N1 viruses have caused lethal disease in wild aquatic birds (Chen *et al.*, 2005, 2006; Sturm-Ramirez *et al.*, 2004). Importantly, the viruses isolated at Qinghai Lake in western China in 2005 possessed Lys at position 627 in the PB2 protein, a substitution associated with mammalian adaptation of avian viruses (Chen *et al.*, 2005, 2006; Fornek *et al.*, 2009; Hatta *et al.*, 2001), indicating that viruses with this mutation could propagate efficiently and be maintained in waterfowl populations. If mammalian-adapted H5N1 viruses can be maintained in migrating waterfowl populations, it is possible for these viruses to spread, increasing the risk of outbreaks among mammals, including humans.

To assess the possibility that a human-adapted haemagglutinin (HA) mutation, like the Glu-to-Lys mutation at

position 627 in PB2 (PB2-E627K), can be maintained in waterfowl, we evaluated A/Hong Kong/213/03 (HK213), which was isolated from a boy in China in 2003 (Peiris *et al.*, 2004). Although most H5N1 viruses bind only to avian-type receptors containing sialic acid linked to galactose by α 2,3 linkages (SA α 2,3Gal), the HK213 virus binds to both human-type SA α 2,6Gal and avian-type receptors (Shinya *et al.*, 2005), a property believed to be important for efficient replication in humans. The amino acid mutation responsible for this dual binding ability involves the Asn residue at position 227 in the HA molecule (Gambaryan *et al.*, 2006; Shinya *et al.*, 2005). The HK213 virus shares a common lineage with several isolates from waterfowl during the 2002 outbreak in China, but is not virulent in ducks (Sturm-Ramirez *et al.*, 2004). We previously passaged HK213 virus five times in ducks and found that the resulting virus gained the ability to cause disease and highly lethal infection in these birds (Shinya *et al.*, 2005). Here, we evaluated the virological properties of this virulent duck-passaged HK213 virus (DP-HK213).

The entire genome of DP-HK213 was sequenced as follows: viral RNA was extracted from brain homogenate of one of ten dead ducks inoculated orally and intranasally with virus that had been passaged four times, by using an RNeasy Mini kit

The GenBank/EMBL/DDBJ accession numbers for the sequences of DP-HK213 determined in this study are AB557744–AB557750.

(Qiagen). RT-PCRs were performed with specific primers (Hoffmann *et al.*, 2001) and the products were cloned into the pT7Blue vector (Novagen). At least three independent clones were sequenced for each viral gene by using the automated sequencing facility at the University of Wisconsin-Madison Biotechnology Center. Confirmed sequences were processed for multiple alignments by accessing the Influenza Sequence Database (Macken *et al.*, 2001).

As shown in Table 1, there were 10 nucleotide/five amino acid differences between the original HK213 [GenBank accession numbers are AB212050 (PB2), AB212052 (PB1), AY576405 (PA), AB212054 (HA), AB212055 (NP), AB212056 (NA), AB212057 (M) and AB212058 (NS1)] and DP-HK213 viruses: a silent G to A mutation at position 576 (G576A) in the PB2 gene; A277G (the presumed amino acid change is Thr to Ala at position 85), G367A (Asp to Asn at 115) and a silent T789A mutation in the PA gene; A744G [Asn to Ser at 227 (H3 HA numbering)] in the HA gene; a silent G1032A mutation in the nucleoprotein (NP) gene; C412T (Thr to Ile at 131) and a silent T1292C mutation in the neuraminidase (NA) gene; a silent mutation A667G in the matrix (M) gene; and G636A (Asp to Asn at 209) in the non-structural (NS) gene. Asn at position 227 in HA, which is involved in dual receptor recognition (Gambaryan *et al.*, 2006; Shinya *et al.*, 2005), reverted back to the wild-type amino acid Ser in DP-HK213. Among the other amino acid mutations, none are known to be associated with avian adaptation (Table 1). Although the functional balance between the NA and HA proteins is known to be important, the mutation in NA was not in the vicinity of the active site. Further studies are needed to identify amino acid substitutions responsible for the high lethality of the DP-HK213 virus. These results show that the amino acid mutation for human receptor recognition in HK213 was not maintained during the duck passages.

To investigate whether the ability of DP-HK213 virus to bind to the human-type receptor was diminished with the loss of the Asn substitution at position 227 in HA, we

generated HK213 virus and a reassortant virus possessing the HA gene of DP-HK213 virus (DP-HK213 HA virus) in the background of the HK213 virus by reverse genetics (Neumann *et al.*, 1999). Briefly, the eight cDNAs derived from the genes of HK213 virus were cloned into plasmids for viral RNA production under the control of the human polymerase I promoter (pPoll). The HA gene possessing the mutation of the DP-HK213 virus was also inserted into pPoll. pCAGGS expression plasmids containing the chicken β -actin promoter were constructed to supply WSN (A/WSN/33) PA, PB1, PB2 and NP proteins. These eight pPoll (pPoll-HA was derived from HK213 or DP-HK213) and four pCAGGS plasmids were co-transfected into 293T cells by using Trans-IT LT-1 (Panvera) and, 48 h post-transfection, the viruses in the supernatants were harvested. The viruses were propagated in 11-day-old embryonated chicken eggs.

To assess the ability of virus with the parent HK213 or DP-HK213 HA to attach to human and duck tissues, sectioned duck colon and human bronchus paraffin tissues were prepared on aminopropylsilane-coated glass slides. They were deparaffinized, rehydrated and soaked in Tris-buffered saline until use. In parallel, we prepared UV-inactivated HK213 and DP-HK213 HA viruses in Tris-buffered saline. Each tissue section was covered with virus fluid (four HA units) and incubated with an anti-H5N1 rabbit polyclonal antibody as the primary antibody and Alexa 488-conjugated anti-rabbit IgG antibody as the secondary antibody at 4 °C. The attachment of DP-HK213 HA virus to human bronchus was appreciably lower than that of HK213 virus (Fig. 1c, d). In contrast, both viruses bound to duck intestinal mucosa (Fig. 1a, b). These data suggest that the amino acid reversion in the DP-HK213 HA, Asn to Ser at position 227, is responsible for the reduced binding of DP-HK213 HA virus to human bronchial mucosa.

In this study, we found that the amino acid mutation in HA involved in human adaptation is not maintained

Table 1. Nucleotide/amino acid substitutions identified in HK213 virus during duck passages

Viral protein	Nucleotide		Amino acid	
	Position of change	Position of change	Original HK213	DP-HK213
PB2	G576A	–		
PA	A277G	85	T	A
	G367A	115	D	N
	T789A	–		
HA	A744G	227*	N	S
NP	G1032A	–		
NA	C412T	131	T	I
	T1292C	–		
M1	A667G	–		
NS1	G636A	209	D	N

*H3 HA numbering.

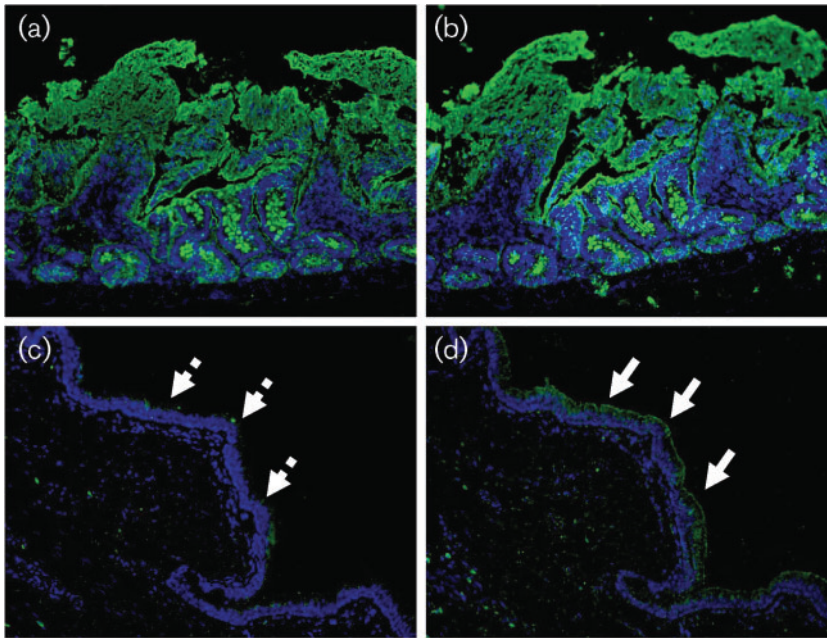


Fig. 1. Binding properties of HK213 and DP-HK213 virus to duck intestinal mucosa and human respiratory mucosa. (a, b) Binding of (a) DP-HK213 virus and (b) HK213 virus to duck intestinal mucosa. (c, d) Tissue binding of (c) DP-HK213 virus and (d) HK213 virus to human respiratory (bronchus) mucosa. Bound virus is shown as green fluorescence. Note that there is less binding of DP-HK213 virus (dashed arrows) to human respiratory tissue compared with that of HK213 virus (solid arrows).

during passage in ducks, which are the natural hosts of influenza A virus. Therefore, it is unlikely that the Ser-to-Asn substitution at position 227 in HA is maintained during virus replication in aquatic birds, as occurs with the Glu-to-Lys mutation in the PB2 of H5N1 viruses of the Qinghai Lake lineage (Chen *et al.*, 2005, 2006). Our study suggests that not all human-adaptive mutations are maintained in waterfowl, which may limit the number of amino acid residues that need to be monitored as molecular markers of the pandemic potential of avian viruses in humans.

Acknowledgements

We thank Susan Watson and Krisna Wells for editing the manuscript. This work was supported by a grant-in-aid for Specially Promoted Research (K.S., Y.K.), by the G-COE programme (K.S., A.M.) and by a contract research fund for the Program of Funding Research Centers for Emerging and Reemerging Infectious Diseases from the Ministries of Education, Culture, Sports, Science, and Technology (Y.K.) and by grants-in-aid of Health, Labor, and Welfare of Japan (Y.K.), by ERATO (Y.K.) and PREST (K.S.) (Japan Science and Technology Agency), and by National Institute of Allergy and Infectious Diseases Public Health Service research grants (Y.K.).

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