Investigation of in vitro replication features of wild ducks and turkey H7N3 influenza viruses: possibile role in interspecies transmission.

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Short Communication

Comparison of *in vitro* replication features of H7N3 influenza viruses from wild ducks and turkeys: potential implications for interspecies transmission

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In previous work, it was shown that turkey H7N3 influenza viruses, presumably derived '*in toto*' from interspecies transmission of duck viruses in Northern Italy, had only 2 aa differences in haemagglutinin and a few amino acid differences as well as a 23 aa deletion in neuraminidase compared with duck viruses. Here, the replication of these duck and turkey viruses in Madin–Darby canine kidney cells was investigated with respect to virus–cell fusion and viral elution from red blood cells. Duck viruses showed similar receptor-binding properties to turkey viruses but possessed a higher pH of fusion activation than the turkey viruses. Conversely, turkey viruses were not able to elute from red blood cells. These data confirm that neuraminidase-stalk deletion impairs the release of virions from cells and also confirm existence of naturally occurring viruses with different pH fusion activities, raising the possibility that these features may play a role in the evolution of influenza viruses in different hosts.

In recent years, avian influenza viruses, a main concern for the poultry industry, have also been recognized as a human health concern because of their ability to transmit, and cause (sometimes deadly), disease directly to man (Alexander, 2000; Lipatov *et al.*, 2004). This ability is likely to depend on the functional integrity and optimal combination of each genome constellation (Horimoto & Kawaoka, 2001). In this context, a balance in haemagglutinin (HA) and neuraminidase (NA) viral glycoprotein activities plays a role and may also be linked to the pathogenicity of avian influenza viruses (Mitnaul *et al.*, 2000; Hulse *et al.*, 2004). In spite of considerable recent advances on the structural and functional features involved in virus–cell interaction during cell entry (Skehel & Wiley, 2000; Huang *et al.*, 2003; Russell *et al.*, 2004), the determinants of avian influenza virus interspecies transmission and emergence of potentially pandemic influenza viruses remains poorly understood.

In the presence of limited indications of influenza virus transmission to humans directly from the wild bird reservoir (Kurtz *et al.*, 1996), it is hypothesized that terrestrial poultry (chickens, quail, etc.) could act as an intermediate host where virus from wild waterfowl may acquire mutations that render it more able to transmit to humans (Perez *et al.*, 2003). Recently, a very close relationship has been described between H7N3 viruses isolated from wild ducks in 2001 and H7N3 viruses circulating since the autumn 2002 in turkeys and chickens in a large number of poultry farms in Northern Italy (Capua *et al.*, 2002a, b). Circumstantial evidence suggested direct '*in toto*' derivation of the low pathogenicity H7N3 turkey viruses from the avian influenza strains circulating in wild waterfowl 1 year earlier. Moreover, serological evidence in humans indicated that the same poultry strains were able to cause infection in poultry workers during the 2002–2003 avian epidemics (Puzelli *et al.*, 2005). Sequence comparison of HA and NA genes of viruses isolated in embryonated fowl's eggs had shown only 2 aa differences at positions 261 (R→S) in the HA1, corresponding to position 271 on the H3 molecule, and 161 (K→R) in the HA2, and few amino acid differences as well as a 23 aa deletion in the NA gene, between the duck and poultry viruses, respectively (Campitelli *et al.*, 2004). Therefore, it seemed important to compare the two groups of duck and turkey viruses, each group with identical HA and NA sequences, with regards to their receptor binding and NA activities, as well as virus replication, with specific attention to virus–cell fusion.

Here, the receptor-binding properties of these H7N3 duck and turkey viruses were first investigated, even though neither of the two HA amino acid changes were located in

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the receptor-binding region (Campitelli *et al.*, 2004). Before testing, confirmation of amino acid differences previously described and absence of additional mutations were assessed for all viral samples used. The two H7N3 duck viruses (A/Mallard/IT/33/01 and A/Mallard/IT/43/01) and the two H7N3 turkey viruses (A/Turkey/IT/214845/02 and A/Turkey/IT/220158/02), grown in 10-day-old embryonated fowl's eggs, were titrated on Madin–Darby canine kidney (MDCK) cells by observing cytopathic effect at 3 days after infection, and 100 $TCID_{50}$ of allantoic fluid was analysed in haemagglutination tests using human and chicken red blood cells (RBC). All four viruses had similar HA activity with both types of substrates, and titres ranged from 128 to 256. Furthermore, when viruses were normalized by HA unit and an ELISA assay was carried out using fetuin, which possesses features resembling influenza virus receptor-analogues (Gambaryan & Matrosovich, 1992), no differences were observed (data not shown). Together, these results showed that the two groups of viruses had similar receptor-binding properties under the experimental conditions used.

Of the two HA amino acid differences observed between the duck and turkey viruses, the one at position 271 located within the 'hinge region' on the HA1 stalk, is in close contact with aa 90 and 91 in the globular head and also to residue 284 in the HA stalk (H3 numbering is used throughout). Because the substitution R→S changes both the charge and size of aa 271 it could affect atomic interactions between the HA head and stalk in this region during fusion activation within the cellular endosome. To investigate the effect of endosomal acidification during virus replication in MDCK cells, bafilomycin A (Alexis Biochemicals), a selective inhibitor of the vacuolar-type proton-ATPase, was used. Here, the replication ability of the two groups of viruses was tested in MDCK cells in the presence of different concentrations of bafilomycin A (Fig. 1). Fifty $TCID_{50}$ of each virus was inoculated in quadruplicate onto the wells of 96-well flat-bottom plates containing MDCK cells with or without bafilomycin A in modified Eagle's medium. After 2 h at 37 °C, the inocula were removed and replaced with fresh medium supplemented with TPCK (L-1-tosylamide-2-phenylethyl chloromethyl ketone) treated trypsin (2 μg ml^{-1}; Sigma) in the presence or absence of the inhibitor. Infected cells were analysed on day 4, when HA production in control wells inoculated with virus alone was evident by the ELISA fetuin-binding assay (Gambaryan & Matrosovich, 1992). As shown in Fig. 1(a), at increasing concentrations of bafilomycin A the growth of all four viruses was profoundly impaired compared with that of the control, confirming previous observations (Guinea & Carrasco, 1995). However, duck viruses required a concentration of bafilomycin A 10-fold higher than that necessary for the turkey viruses to achieve the same level of inhibition. Bafilomycin A had no detectable effects on MDCK cell viability up to 500 nM concentration under the experimental conditions used (data not shown). Subsequently, experiments performed in the same manner as described above, but maintaining the antibiotic only during the 2 h of the virus cell adsorption, were carried out

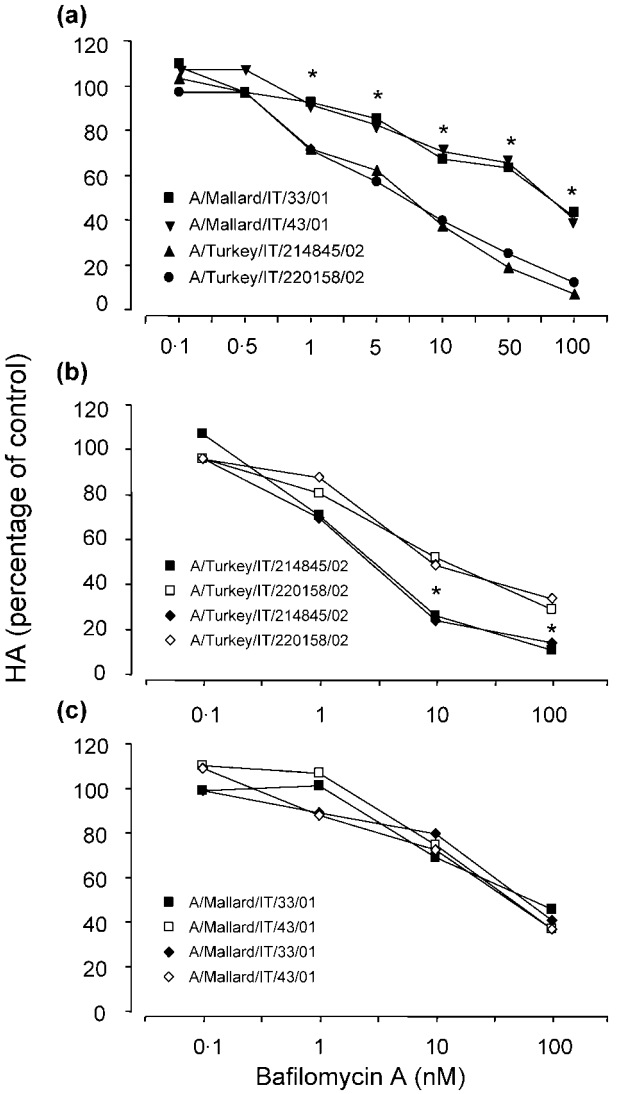

Fig. 1. Effect of treatment with bafilomycin A on virus replication on MDCK cells. (a) Virus growth curves of duck and turkey H7N3 viruses in the presence of bafilomycin A throughout the 4 day replication period. (b and c) Virus growth curves of turkey and duck viruses in which bafilomycin A treatment was restricted to 2 h of virus adsorption. Treatments were conducted at 4 °C to estimate the effect on virus attachment (open symbols) and at 37 °C to measure effect on viral internalization (filled symbols), respectively. Virus growth is expressed as the relative levels of HA found in the supernatant fluids of virus grown in the presence of bafilomycin A compared with that obtained in its absence. Values shown are the mean of three independent experiments. Asterisks indicate that inhibition of duck viruses was significantly different from that of the viruses isolated from turkeys and that inhibition of turkey viruses, under treatment restricted to the virus adsorption, was significantly affected by the temperature at which was conducted (Student's *t*-test) at $P<0{\cdot}05$.

to restrict the effects of bafilomycin A to the early steps of virus replication. Experiments with the initial incubation time of 2 h at 4 °C, a temperature that allows virus cell adsorption but not entry, were also performed to investigate the inhibitory effect on binding to or internalization into cells. When treatment of cells with bafilomycin A was carried out at 4 °C during the initial infection of the cells, virus growth of both turkey and duck viruses was only partially affected by bafilomycin A as measured following 4 days of incubation at 37 °C. However, turkey viruses showed a significant, temperature related, increased inhibition when incubation with bafilomycin A was carried out at 37 °C, whereas the duck viruses displayed no difference in their virus replication whether the bafilomycin A treatment of cells was at 37 or 4 °C (Fig. 1b and c). Overall, these results indicate that the two groups of H7N3 viruses differ in the replication properties restricted to the early steps of virus entry into a cell immediately after cell adsorption.

Acidification of virus-containing endosomes promotes the HA-mediated fusion of the virus with the endosome membrane, and activates the M2 virus ion-channel, inducing the dissociation of the M1 protein from the nucleocapsid (Martin & Helenius, 1991; Skehel & Wiley, 2000). Since no amino acid differences were observed in the M2 transmembrane region (the ion-channel domain) of the duck and turkey H7N3 viruses (L. Campitelli, unpublished data), we investigated whether the differences in the susceptibility of H7N3 duck and turkey viruses to the bafilomycin A inhibitor were related to their HA fusion properties. A fusion assay, based on fluorescence dequenching of octadecyl rhodamine (R18)-labelled (Molecular Probes) purified virus was performed as described using MDCK cells (Takeda *et al.*, 2003; Chu & Whittaker, 2004). One hundred microlitres of labelled gradient-purified virus (100 μg ml^{-1}) was added to 2×10^6 MDCK cells at 4 °C for 1 h in binding buffer constituted of PBS with 0·2 % BSA. Unbound virus was removed by washing with PBS, and cells were resuspended in binding buffer with 5 mM HEPES pH 7·0. Fusion of virus with the cell membrane was triggered by adding a predetermined amount of 250 mM HCl to obtain a final pH of 6·0 and 5·0, and then incubated for 10 min at 37 °C. Then, after washing with PBS, cells were resuspended in PBS and fluorescence dequenching was measured by using a FACScan flow cytometer (Becton Dickinson). Fig. 2 shows that the two groups of viruses exerted a similar fusion activity at pH 5·0, i.e. the experimental conditions that correspond to the physiological environment in which virus fusion is presumed to occur inside the endosome. However, when the experiments were performed at a higher pH, pH 6·0, the two H7N3 duck viruses maintained a significant fusion activity, whereas the turkey viruses did not. In particular, cell fusion activity of duck viruses at pH 6·0 was fivefold higher than that exerted by turkey viruses. As expected, none of the four viruses had significant fusion activity at pH 7·0.

Since the NA active domain of both duck and turkey viruses was conserved and no additional glycosylation sites were

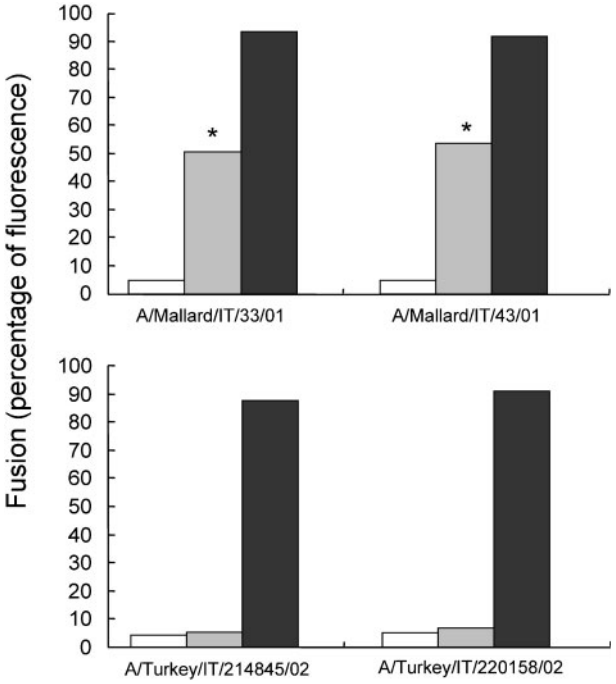

Fig. 2. Effect of pH on virus–cell fusion. Virus fusion of duck and turkey H7N3 viruses at pH 7·0 (white bars), 6·0 (grey bars) and 5 (black bars) is expressed as level of fluorescence observed. Fusion efficiency was expressed as a percentage of fluorescence following the addition of Triton X-100 to a final concentration of 1 %. The experiment was repeated at least twice, with comparable results. Asterisks indicate that fusion of duck viruses was significantly different from that of turkey derived viruses (Student's *t*-test) at $P<0·05$.

present on the turkey virus HA, we decided to investigate whether the amino acid deletion in the NA stalk of turkey viruses affected the release of progeny virions from cells, by observing their ability to elute from RBCs compared to the duck strains. Eight HA units of virus were allowed to agglutinate chicken or human RBCs at 4 °C for 1 h, and the NA activity was monitored at 37 °C by checking the time for virus elution from the RBCs. Similar experiments were performed in parallel but in the presence of 10-fold serial dilutions of the NA inhibitor Zanamivir at an initial concentration of 10 nM, as a control of NA activity. The results in Table 1 indicate that turkey viruses were not able to elute from either type of RBC during an overnight incubation at 37 °C, whereas duck viruses eluted completely within 1 h. When the elution experiments were performed entirely at 4 °C, a temperature at which NA activity is blocked, none of the viruses were able to elute, even after an overnight incubation. Conversely, when the experiments were performed at 37 °C in the presence of receptor-destroying enzyme (Sigma), all viruses eluted, beginning from 30 min incubation (data not shown). Furthermore, elution of the duck viruses was inhibited during incubation at 37 °C in presence of Zanamivir (Table 1), thus demonstrating that the observed effect was specifically determined

Table 1. Elution times of duck and turkey H7N3 viruses from specific RBCs at 37 °C

The experiment was repeated three times with comparable results. ND, Not determined.

Virus	Elution time (h)			
	Chicken RBCs		Human RBCs	
	Zanamivir−	Zanamivir + (nM)*	Zanamivir−	Zanamivir + (nM)*
Grown in eggs				
A/Mallard/IT/33/01	2	>24 (0·1)	1	>24 (0·1)
A/Mallard/IT/43/01	2	>24 (0·1)	1	>24 (0·1)
A/Turkey/IT/214845/02	>24	ND	>24	ND
A/Turkey/IT/220158/02	>24	ND	>24	ND
Passed in MDCK cells				
A/Mallard/IT/33/01	1	>24 (>10)	1	>24 (1)
A/Mallard/IT/43/01	1	>24 (>10)	1	>24 (1)
A/Turkey/IT/214845/02	>24	ND	>24	ND
A/Turkey/IT/220158/02	>24	ND	>24	ND

*Elution performed in presence (+) or absence (−) of Zanamivir. Numbers in parentheses are the concentration of Zanamivir at which virus elution was completely inhibited during an overnight incubation.

by the NA activity. Finally, because the type of cell substrates on which influenza virus is grown may affect some molecular characteristics of the virus, a similar experiment was conducted using virus passed on MDCK cells. As shown in Table 1, impairment of NA activity on turkey virus did not depend on the cells in which the virus was propagated.

Our results indicate that a higher pH of fusion activation of H7N3 duck influenza viruses on MDCK cell substrate, than that required by turkey viruses, are in accord with studies showing that amino acid changes in HA found in naturally occurring and *in vitro* selected variants can raise the pH in which virus fusion occurs (Daniels *et al.*, 1985; Doms *et al.*, 1986; Grambas & Hay, 1992). The reason for selection of HA mutants that mediate membrane fusion at elevated pH has not been established yet, but it is hypothesized that may be a consequence of differences in endosomal pH within different cell types (Lin *et al.*, 1997). On the other hand, very little is known about the appropriate pH requirements of influenza viruses within different tissues of birds such as ducks and turkeys. Tissue tropism of influenza viruses in such hosts can be quite different, since duck viruses grow typically on the intestine epithelium, whereas in turkeys the main target organ is the respiratory apparatus, although virus is also excreted in faeces (Swayne *et al.*, 1992). Whether the *in vitro* characteristics we observed could be related to different tropism requirements implying host adaptation is not known. It should be noted that, although the amino acid R and S found at position 271 is unique within the entire H7 HA database, this position in duck viruses is often occupied by polar charge residues compared with aliphatic uncharged residues usually found in turkey viruses.

The H7N3 turkey viruses showed a reduced ability to release virions from the cell substrate, in keeping with the presence of a short-stalked NA. An NA stalk deletion appears to be an early phenomenon of adaptation of duck viruses to domestic poultry, but the biological significance of this is unclear (Castrucci & Kawaoka, 1993; Wagner *et al.*, 2000; Banks *et al.*, 2001). In fact, this feature is often associated to changes in HA protein that in turn reduce HA receptor affinity to counterbalance the reduced NA activity (Baigent & McCauley, 2001). In our study, the absence of additional glycosylation sites as well as other amino acid changes on the HA globular head of the turkey viruses, isolated not only at the time of the presumed initial virus introduction into poultry but also 2, 5, 6 and 7 months later (L. Campitelli, unpublished data), suggest the existence of an alternative mechanism of compensation. Proving this latter aspect, as well as the involvement of amino acid positions 271 of the HA1 and 161 of HA2 in the fusion process, will require development of HA and NA reassortant and mutagenized viruses.

Also in the light of recent serological evidence of human infection with the low pathogenicity H7N3 turkey viruses in Italy (Puzelli *et al.*, 2005), expanding our understanding of the molecular factors that influence replication properties of duck and turkey viruses in different avian species could provide a better insight into some aspects involved in the interspecies transmission.

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References

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

Baigent, S. J. & McCauley, J. W. (2001). Glycosylation of haemagglutinin and stalk-length of neuraminidase combine to regulate the growth of avian influenza viruses in tissue culture. *Virus Res* **79**, 177–185.

Banks, J., Speidel, E. S., Moore, E., Plowright, L., Piccirillo, A., Capua, I., Cordioli, P., Fioretti, A. & Alexander, D. J. (2001). Changes in the haemagglutinin and the neuraminidase genes prior to the emergence of highly pathogenic H7N1 avian influenza viruses in Italy. *Arch Virol* **146**, 963–973.

Campitelli, L., Mogavero, E., De Marco, M. A. & 11 other authors (2004). Interspecies transmission of an H7N3 influenza virus from wild birds to intensively reared domestic poultry in Italy. *Virology* **323**, 24–36.

Capua, I., Marangon, S., Cordioli, P., Bonfanti, L. & Santucci, U. (2002a). H7N3 avian influenza in Italy. *Vet Rec* **151**, 743–744.

Capua, I., Mutinelli, F., Pozza, M. D., Donatelli, I., Puzelli, S. & Cancellotti, F. M. (2002b). The 1999–2000 avian influenza (H7N1) epidemic in Italy: veterinary and human health implications. *Acta Trop* **83**, 7–11.

Castrucci, M. R. & Kawaoka, Y. (1993). Biologic importance of neuraminidase stalk length in influenza A virus. *J Virol* **67**, 759–764.

Chu, V. C. & Whittaker, G. R. (2004). Influenza virus entry and infection require host cell N-linked glycoprotein. *Proc Natl Acad Sci U S A* **101**, 18153–18158.

Daniels, R. S., Downie, J. C., Hay, A. J., Knossow, M., Skehel, J. J., Wang, M. L. & Wiley, D. C. (1985). Fusion mutants of the influenza virus hemagglutinin glycoprotein. *Cell* **40**, 431–439.

Doms, R. W., Gething, M.-J., Henneberry, J., White, J. & Helenius, A. (1986). Variant influenza virus hemagglutinin that induces fusion at elevated pH. *J Virol* **57**, 603–613.

Gambaryan, A. S. & Matrosovich, M. N. (1992). A solid-phase enzyme-linked assay for influenza virus receptor-binding activity. *J Virol Methods* **39**, 111–123.

Grambas, S. & Hay, A. J. (1992). Maturation of influenza A virus hemagglutinin – estimates of the pH encountered during transport and its regulation by the M2 protein. *Virology* **190**, 11–18.

Guinea, R. & Carrasco, L. (1995). Requirement for vacuolar proton-ATPase activity during entry of influenza virus into cells. *J Virol* **69**, 2306–2312.

Horimoto, T. & Kawaoka, Y. (2001). Pandemic threat posed by avian influenza A viruses. *Clin Microbiol Rev* **14**, 129–149.

Huang, Q., Sivaramakrishna, R. P., Ludwig, K., Korte, T., Böttcher, C. & Herrmann, A. (2003). Early steps of the conformational change of influenza virus hemagglutinin to a fusion active state: stability and energetics of the hemagglutinin. *Biochim Biophys Acta* **1614**, 3–13.

Hulse, D. J., Webster, R. G., Russell, R. J. & Perez, D. R. (2004). Molecular determinants within the surface proteins involved in the pathogenicity of H5N1 influenza viruses in chickens. *J Virol* **78**, 9954–9964.

Kurtz, J., Manvell, R. J. & Banks, J. (1996). Avian influenza virus isolated from a woman with conjunctivitis. *Lancet* **348**, 901–902.

Lin, Y. P., Wharton, S. A., Martin, J., Skehel, J. J., Wiley, D. C. & Steinhauer, D. A. (1997). Adaptation of egg-grown and transfectant influenza viruses for growth in mammalian cells: selection of hemagglutinin mutants with elevated pH of membrane fusion. *Virology* **233**, 402–410.

Lipatov, A. S., Govorkova, E. A., Webby, R. J., Ozaki, H., Peiris, M., Guan, Y., Poon, L. & Webster, R. G. (2004). Influenza: emergence and control. *J Virol* **78**, 8951–8959.

Martin, K. & Helenius, A. (1991). Nuclear transport of influenza virus ribonucleoproteins: the viral matrix protein (M1) promotes export and inhibits import. *Cell* **67**, 117–130.

Mitnaul, L. J., Matrosovich, M. N., Castrucci, M. R., Tuzikov, A. B., Bovin, N. V., Kobasa, D. & Kawaoka, Y. (2000). Balanced hemagglutinin and neuraminidase activities are critical for efficient replication of influenza A virus. *J Virol* **74**, 6015–6020.

Perez, D. R., Lim, W., Seiler, J. P., Yi, G., Peiris, M., Shortridge, K. F. & Webster, R. G. (2003). Role of quail in the interspecies transmission of H9 influenza A viruses: molecular changes on HA that correspond to adaptation from ducks to chickens. *J Virol* **77**, 3148–3156.

Puzelli, S., Di Trani, L., Fabiani, C., Campitelli, L., De Marco, M. A., Capua, I., Aguilera, J. F., Zambon, M. & Donatelli, I. (2005). Serological analysis of humans exposed to avian H7 influenza viruses in Italy between 1999 and 2003. *J Infect Dis* **192**, 1318–1322.

Russell, R. J., Gamblin, S. J., Haire, L. F., Stevens, D. J., Xiao, B., Ha, Y. & Skehel, J. J. (2004). H1 and H7 influenza haemagglutinin structures extend a structural classification of haemagglutinin subtypes. *Virology* **325**, 287–296.

Skehel, J. J. & Wiley, D. C. (2000). Receptor binding and membrane fusion in virus entry: the influenza hemagglutinin. *Annu Rev Biochem* **69**, 531–569.

Swayne, D. E., Picken, M. D. & Guy, J. S. (1992). Immunohistochemical demonstration of influenza A nucleoprotein in lungs of turkeys with natural and experimental influenza respiratory disease. *Avian Pathol* **21**, 547–557.

Takeda, M., Leser, G. P., Russell, C. J. & Lamb, R. A. (2003). Influenza virus hemagglutinin concentrates in lipid raft microdomains for efficient viral fusion. *Proc Natl Acad Sci U S A* **100**, 14610–14617.

Wagner, R., Wolff, T., Herwig, A., Pleschka, S. & Klenk, H.-D. (2000). Interdependence of hemagglutinin glycosylation and neuraminidase as regulators of influenza virus growth: a study by reverse genetics. *J Virol* **74**, 6316–6323.