

Double Infections with Avian A/H5N1 and Swine A/H1N1 Influenza Viruses in Chickens

Kazuhide Adachi¹, Tomoya Kato¹, Naoki Kirimura¹, Yuka Kubota¹, Hatsuki Shiba¹, Retno Damajanti Soejoedono², Ekowati Handharyani² & Yasuhiro Tsukamoto³

Abstract

The rapid outbreak of the highly pathogenic A/H5N1 avian influenza virus among domestic birds and its transmission to humans have induced world-wide fears of a new influenza pandemic. If a human-trophic strain of A/H5N1 is replicated in domestic animals, it might have high transmissivity and pathogenicity to humans. If the misassembling of both avian and swine influenza viruses occur in the same cells in domestic fowl, novel pandemic infections among humans might emerge due to human-fowl contacts. In the present study, examinations of mixed infections with A/H5N1 and A/H1N1 viruses were carried out using living chickens to elucidate the possibility of chimeric avian-swine influenza virus replication in domestic fowl. The sporadic strains of avian A/H5N1 and swine A/H1N1 viruses were co-infected into embryonated eggs and post-hatched chickens. A double staining method using the anti-A/H5N1 and anti-A/H1N1 antibodies indicated that A/H5N1 and A/H1N1 viruses were co-localized in the same cells in the chorioallantoic membrane of embryos, and in the lungs of chickens challenged by the double infections. This indicated that the avian influenza and swine influenza viruses might be assembling in the same cells of chickens, and chimeric viruses containing the characteristics of both viral strains might appear.

Keywords: Avian flu, swine flu, A/H5N1, A/H1N1, influenza virus, chicken

¹ Department of Animal Hygiene, Graduate School of Environmental & Biological Sciences, Kyoto Prefecture University, 1-5 Nakaragicho, Shimogamo, Kyoto 606-8522, Japan.

² Faculty of Veterinary Medicine, Bogor Agriculture University, JL. Agatis, Kampus IPB Darmaga, Bogor 16680, Indonesia

³ D.V.M., Ph.D. Professor, Department of Animal Hygiene, Graduate School of Biology and Environmental Sciences, Kyoto Prefecture University, 1-5 Nakaragicho, Shimogamo, Kyoto 606-8522, Japan. Tel/Fax: +81-75-703-5146, e-mail: ytsuka@kpu.ac.jp

1. Introduction

Influenza is recognized as a zoonotic disease, with the most commonly affected animals being humans, pigs, horses and species of aquatic birds (Alexander & Brown 2000, Brigel *et al.*, 2005, Rimmelzwaan & Osterhaus 2001, Normile 2005, CDC 2004). The influenza viruses belong to the family *Orthomyxoviridae*, and are divided into three types; A, B, and C. A-type viruses are responsible for major diseases in humans, as well as in avian species, and are further classified into subtypes on the basis of their antigenic properties, including the types of hemagglutinin (HA) and neuraminidase (NA) on the viral particle. Two A types (A/H1N1 and A/H3N2) and various B types are presently circulating in the human population. Approximately from 10% to 15% of people worldwide contract influenza annually, with rates as high as 50% during major epidemics.

In 1997, the A/H5N1 virus was transmitted to humans in Hong Kong, and thereafter spread to Africa, Indonesia, Vietnam and Egypt by means of domestic and wild birds. In Indonesia, over 200 people have been infected with A/H5N1, which resulted in a mortality rate of over 50% (Leroux-Roels *et al.*, 2007). This virus an imminent threat to humans, and to the poultry industry and potentially wild birds, and there is a high risk of a worldwide pandemic, which could thereby cause considerable mortality and economic disruption (Peiris *et al.*, 2004, Poland, 2006, Ungchusak *et al.*, 2005).

In April 2009, it became apparent to public health officials in Mexico City that an outbreak of influenza was in progress late in the influenza season. The virus from patients was determined to be a novel strain of influenza A of the A/H1N1 serotype. Detailed genetic examinations indicated that the virus was a novel reassortant containing genetic elements of influenza viruses found in swine, birds and humans (Bridges *et al.*, 2003, Gallaher 2009). Less than one month later, thousands of probable cases of infection by this novel virus, designated Influenza A/H1N1 2009, had been identified, and many deaths had occurred in Mexico. Sporadic cases, mostly associated with travel to Mexico, were subsequently noted in several other countries, including the USA, Canada and various countries in Europe, Asia and Africa. The World Health Organization (WHO) began to declare ever higher stages on its *Pandemic scale*, designating the novel influenza A/H1N1 2009 a potential threat to world-wide health. The infection of this pandemic influenza A/H1N1 virus expanded all over the world in 2009 and 2010.

In general, the influenza virus attaches onto the receptors on the cell surface and enters the cytoplasm by endocytosis, where it subsequently replicates using host enzymes and nucleotides, and a huge number of virions are produced by the host cells. Newly produced viruses distribute systematically via the blood stream and infect various organs expressing the viral receptors (Black *et al.*, 2007, Shinya *et al.*, 2006). Thus, various symptoms involving the respiratory, digestive and nervous systems appear, and resultant multiorgan failure leads to death in animals.

Fundamentally, new chimeric viruses possessing various influenza virus phenotypes can appear: cells with more than one strain of influenza viruses can produce new chimeric viruses through the misassembly during the viral replication processes. These misassemblies in mix-infected cells have led to world-wide pandemics with high pathogenicities in humans in the past 100 years. In addition, chimeric viruses have been easily produced artificially using cultured cells (Yamada *et al.*, 2006). Interestingly, chimeric influenza viruses were found in sporadic swine samples in Indonesia: in one case, the H5 gene was inserted with a part of the swine H1 gene (Lu *et al.*, 2014). If the chimeric influenza viruses with both phenotypes (A/H5 and A/H1) have a strong tropism for the receptor on human cells, then the effective transmission of the viruses among humans might occur. Accordingly, co-infections of avian and swine influenza viruses in the same cells are considered to be a first step in the assembly of such chimeric viruses. In all human cases of avian influenza, the patients were directly infected with avian A/H5N1 viruses from domestic birds, not from the pigs. If the re-assembling of both avian and swine influenza viruses occur in the same cell of domestic fowl, it has been hypothesized that novel pandemic infections among humans would emerge following direct contact between humans and fowl.

To elucidate the possibility of chimeric avian-swine influenza virus replication in domestic fowl, a mixed-infection with A/H5N1 and A/H1N1 viruses in living chickens was carried out, and the viral assembly sites were then traced immunohistochemically.

2. Materials and Methods

2.1 Experimental Challenge with Avian Influenza Virus A/H5N1 and Swine Influenza virus A/H1N1 in Embryonated Eggs

Embryonic chicken eggs at 10 days old were inoculated with A/H5N1 (A/Bogor2/FKH-IPB) (10^4 TCID₅₀) or A/H1N1 (A/Kyoto 27/2007) (10^4 TCID₅₀) into their chorioallantoic fluid (CAF) in a P3 room in Bogor Agricultural University, Indonesia. One group of eggs was inoculated with A/H5N1 only, one with A/H1N1 only and another one was double-inoculated with A/H5N1 and A/H1N1. After two to three days of incubation at 37°C, the embryos were inspected for death. The chorioallantoic membrane (CAM) was removed from survivors, and then was fixed with 10% neutral buffered formalin for further immunohistochemistry studies.

2.2 Experimental Challenge of Chickens with Avian Influenza A/H5N1 and Swine Influenza A/H1N1 Viruses

Newly-hatched broiler chicks were housed under controlled conditions in a BSL3 laboratory in Indonesia, and received food and water *ad libitum*. When they were 10 days old, the birds were inoculated intranasally with A/H5N1 (A/Bogor2/FKH-IPB) (10^4 TCID₅₀/ml) and/or A/H1N1 (A/Kyoto 27/2007) (10^4 TCID₅₀/ml); one group of birds was inoculated with A/H5N1 only, one with A/H1N1 only and the other was double-inoculated with A/H5N1 and A/H1N1 at the same time. At least three birds were prepared for each viral inoculation. At two days post-inoculation, the dead birds were counted and the lethality was scored in each group. The survivors were then sacrificed with pentobarbital solution, and the lungs were removed. The dead birds were also necropsied, and the lungs were removed and immersed in 10% neutral buffered formalin for further histopathology and immunohistochemistry studies.

2.3 Double Immunohistochemistry for Viral Antigens

After sufficient fixation in buffered formalin and washes in PBS, the CAM and lungs were soaked with 30% sucrose in PBS overnight. Thereafter, the organs were mounted in embedding liquid compound, frozen and cut into 5 µm sections with a cryostat. The frozen sections were attached onto mass-coated glass slides and were air-dried at room temperature.

After being washed in PBS, the slides were incubated with a rhodamine-conjugated anti-A/H5N1 polyclonal antibody (1:1000) and FITC-conjugated anti-A/H1N1 polyclonal antibody (1:1000) at 4°C overnight (Adachi *et al.*, 2008). After two washes with PBS, the slides were mounted with glycerol, and specific signals for viral antigens were examined under a florescent microscope with G and B filters (Nikon).

2.4 Histopathology

The formalin-fixed samples were embedded in paraffin and cut into 3- μ m sections with a microtome. Next, the sections were stained with hematoxylin and eosin (H&E) as routine procedures, and were observed under a light microscope.

3. Results

3.1 Infections of Chick Embryos with A/H5N1 and A/H1N1 Viruses

The viruses were injected into the CAF of embryonated eggs using a P3 system. At two days post-inoculation, the egg shells were opened, and the lethality of embryos was checked. The embryos infected with A/H5N1 all died in the culture. In contrast, all embryos infected with A/H1N1 survived. In the cases with double infections with the A/H5N1 and A/H1N1 viruses, all embryos were dead in the culture. Accordingly, A/H5N1 infection was highly pathogenic, but A/H1N1 was less pathogenic, in chick embryos. The high lethality in double-infected embryos was likely due to the A/H5N1 virus.

3.2 Infections of Post-Hatched Chickens with the A/H5N1 and A/H1N1 Viruses

The viruses were intranasally inoculated into the broiler chickens in a BSL3 room. At two days post-inoculation, the lethality of the infections was checked. The birds infected with A/H5N1 were all dead by two days post-inoculation. In contrast, all chickens infected with A/H1N1 survived. In cases of double infection with A/H5N1 and A/H1N1 viruses, three of the five birds had died. Accordingly, the A/H1N1 virus was concluded to have low pathogenicity in chickens. The high lethality in the double-infected birds seemed to have resulted from the high pathogenicity of the A/H5N1 virus.

Histopathologically, severe acute inflammation, hemorrhage and congestion, accompanied with edema and mucosal exudation, were predominately seen in the lungs of the A/H5N1-infected chickens (Fig. 1). In contrast, there were no obvious lesions in the pulmonary sections from A/H1N1-infected birds. Concerning the histopathology in double-infected birds, severe acute inflammation was prominent, which seemed to be consistent with the A/H5N1 infection.

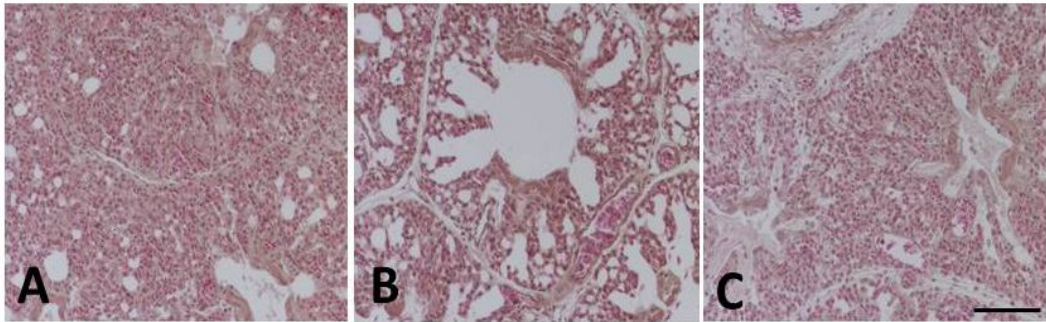


Fig. 1. The histopathology of the lungs from A/H5N1- and A/H1N1-infected chicken.

The pulmonary sections were cut and stained with H&E. In the lungs from A/H5N1-infected chickens obtained two days post-infection, heterophilic infiltrations, hemorrhage, edema and mucosal exudation were seen in the interstitium and parabronchial cavities (A). In contrast, A/H1N1-infected chicken lung sections showed no pathological lesions (B). Severe acute inflammation was also seen in the lungs of the chickens with A/H5N1 and A/H1N1 double infections (C). Bars, 200 μ m.

3.3 Localization of Viral Antigens in the CAM and Lungs of Infected Chickens

The CAM and chick lungs with co-infection by A/H5N1 and A/H1N1 viruses were sectioned, and the replication sites of each virus were examined by double staining with specific antibodies against each virus (Fig. 2). In the CAM of embryos, both A/H1N1 and A/H5N1 antigens were seen in the epithelial cells. In a merged view, co-localizations of both viral antigens were detectable in the epithelial cells. In addition, in the lungs of infected chickens, A/H1N1 and A/H5N1 antigens were seen in the pulmonary epithelial cells or cell debris, and then co-localization was noted in some cells in merge views.

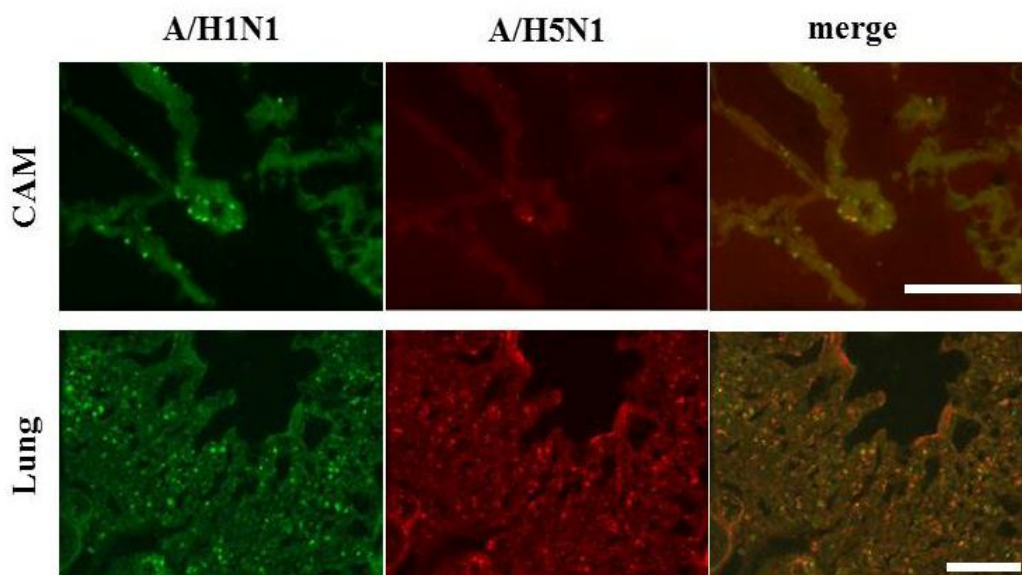


Fig. 2. Localization of viral antigens in the CAM and lungs of chickens with double infections by the A/H5N1 and A/H1N1 viruses

The infected samples of CAM and lungs were cryosectioned and double-stained with a FITC-conjugated anti-A/H1N1 antibody and rhodamine-conjugated anti-A/H5N1 antibody. In the infected CAMs, both A/H1N1 (green) and A/H5N1 (red) were seen in the epithelial cells. In a merged view, co-localization of both virus antigens was apparent in the epithelial cells (yellow). In the lungs of the infected chicken, A/H1N1 (green) and A/H5N1 (red) were seen in the pulmonary epithelial cells, and were co-localized in some cells in the merged view (yellow). Bars, 200 μ m

4. Discussion

The mechanism underlying sudden death due to A/H5N1 virus infections remains to be clarified in either poultry or humans (Chan *et al.*, 2005, Carr *et al.*, 1998, Cheung *et al.*, 2002, De Jong, 2006, Sakurai *et al.*, 1990, Salomon *et al.*, 2007, Shimoda *et al.*, 2002, Szretter *et al.*, 2007). It also remains unclear how the novel influenza viruses were generated. The pig has been thought to be a potential replication site for novel influenza viruses, because most of the influenza virus A subtype are infectious and replicate in this animal (Alexander *et al.*, 2000, Rimmelzwaan & Osterhaus 2001, Normile 2005). Clinical symptoms of influenza-infected pigs are limited, but the viral growth in pigs is very efficient. Accordingly, swine species are a suitable reservoir for influenza virus with respect to their amplification (Normile 2005). Migratory birds are also excellent contributors, especially to the wide range of world-wide epidemics of the viruses.

However, direct interactions between humans and domestic fowl were the apparent cause of the recent A/H5N1 infections in humans (Leroux-Roels *et al.*, 2007, Poland, 2006, Spickler *et al.*, 2008). Concerning the potential development of novel influenza pandemics in the near future, our research group has hypothesized the following steps: 1) first, an influenza virus adapted to the human receptor emerges and replicates in swine; 2) second, these swine viruses and avian viruses mix-infect an individual fowl via swine-avian and avian-avian interactions; 3) next, misassembly in the same cells during the process of viral replication occurs in the fowl; 4) then, the chimeric viruses emerge with human receptor-trophic HA antigens on their surface, 5) this chimeric virus enters and infect humans when humans come into contact with fowl at farms or poultry markets, 6) the virus replicates and obtain high pathogenicity in humans and 7) finally, there is viral transmission among humans, and a wide-range pandemic is initiated.

To confirm this paradigm, we carried out the mixed-infections of chickens with avian and swine influenza viruses. The clinical strain of A/H5N1 was infectious to MDCK and embryonated eggs, and was accompanied by high pathogenicity in the chickens. We could also adapt a vaccine strain of swine A/H1N1 virus to the chickens through considerable passages on MDCK cells and in infant chicks: this virus obtained the ability to infect and replicate in chickens. In addition, this virus had low pathogenicity in chickens. These characters of A/H5N1 and A/H1N1 were suitable for the present study with respect to establishing an experimental model for a putative natural outbreak of an A/H5N1 pandemic.

One particularly interesting and potentially important finding in the present study was that the A/H5N1 and A/H1N1 viruses were co-localized in the same cells of chickens with double infections by both viruses; double staining using the anti-A/H5N1 and anti-A/H1N1 antibodies clearly showed that the A/H5N1 and A/H1N1 viruses were co-localized in the same cells in both the CAM epithelia and in the pulmonary cells in chickens. This indicated that the A/H5N1 and A/H1N1 viruses might be assembling in the same cells of chickens. We are now attempting to pick up viral clones from the birds co-infected with both A/H5N1 and A/H1N1 influenza viruses. Once a chimeric virus is cloned, more adequate infectious examinations on viral transmission between birds and mammals can be carried out. Furthermore, we can produce a vaccine against the putative novel A/H5N1 using the chimeric virus. In addition, the tropism of the chimeric virus for the receptor on human cells will be clarified based on the mutation site(s) in the viral genome.

5. References

- Adachi, K., Handharyani, E., Sari, D.K., Takama, K., Fukuda, K., Endo, I., Yamamoto, R., Sawa, M., Tanaka, M., Konishi, I., & Tsukamoto, Y. (2008). Development of neutralization antibodies against highly pathogenic H5N1 avian influenza virus using ostrich (*Struthio camelus*) yolk. *Mol. Med. Rep.*, 1, 203-209.
- Alexander, D.J., & Brown, I.H. (2000). Recent zoonoses caused by influenza A viruses. *Rev. Sci. Tech.*, 19, 197-225.
- Beigel, J.H., Farrar, J., Han, A.M., Hayden, F.G., Hyer, R., de Jong, M.D., Lochindarat, S., Nguyen, T.K., Nguyen, T.H., Tran, T.H., Nicoll, A., Touch, S., & Yuen, K.Y. (2005). Writing Committee of the World Health Organization (WHO) Consultation on Human Influenza A/H5: Avian influenza A (H5N1) infection in humans. *N. Engl. J. Med.*, 353, 1374-1385.
- Black, S.M., Kumar, S., Wiseman, D., Ravi, K., Wedgwood, S., Ryzhov, V., & Fineman, J.R. (2007). Pediatric pulmonary hypertension: Roles of endothelin-1 and nitric oxide. *Clin. Hemorheol. Microcirc.*, 37, 111-120.
- Bridges, C.B., Kuehnert, M.J., & Hall, C.B. (2003). Transmission of influenza: implications for control in health care settings. *Clin. Infect. Dis.*, 37, 1094-1101.
- CDC. (2004). Experiences with influenza-like illness and attitude regarding influenza prevention- United States, 2003-04 influenza season. *M.M.W.R.*, 53, 1156-1158.
- Chan, M.C., Cheung, C.Y., Chui, W.H., Tsao, S.W., Nicholls, J.M., Chan, Y.O., Chan, R.W., Long, H.T., Poonm, L.L., Guan, Y., & Peiris, J.S. (2005). Proinflammatory cytokine responses induced by influenza A (H5N1) viruses in primary human alveolar and bronchial epithelial cells. *Respir. Res.*, 6, 135.
- Carr, M.J., Spalding, L.J., Goldie, R.G., & Henry, P.J. (1998). Distribution of immunoreactive endothelin in the lungs of mice during respiratory viral infection. *Eur. Respir. J.*, 11, 79-85.
- Cheung, C.Y., Poon, L.L., Lau, A.S., Luk, W., Lau, Y.L., Shortridge, K.F., Gordon, S., Guan, Y., & Peiris, J.S. (2002). Induction of proinflammatory cytokines in human macrophage by influenza A (H5N1) viruses: a mechanism for the unusual severity of human disease? *Lancet*, 360, 1831-1837.
- De Jong, M.D. (2006). Fetal outcome of human influenza A (H5N1) is associated with high viral load and hypercytokinemia. *Nat. Med.*, 12, 1203-1207.
- Gallaher, W.R. (2009). Towards a sane and rational approach to management influenza H1N1 2009. *Viol. J.*, 6, 51-59.
- Leroux-Roels, I., Borkowski, A., Vanwollegem, T., Dreame, M., Clement, F., Hons, E., Devaster, J.M., & Leroux-Roels, G. (2007). Antigen sparing and cross-reactive immunity with an adjuvanted rH5N1 prototype pandemic influenza vaccine: a randomized controlled trial. *Lancet*, 370, 580-589.
- Lu, L., Lycett, S.J., & Leigh Brown, A.J. (2014). Reassortment patterns of avian influenza virus internal segments among different subtypes. *BMC Evol. Biol.*, 24, 14.
- Normile, D. (2005). Infectious diseases. North Korea collaborates to fight bird flu. *Science*, 308, 175.

- Peiris, J.S.M., Yu, W.C., Leung, C.W., Cheung, C.Y., Ng, W.F., Nicholls, J.M., Ng, T.K., Chan, K.H., Lai, S.T., Lim, W.L., Yuen, K.Y. & Guan, Y. (2004). Re-emergence of fatal human influenza A subtype H5N1 disease. *Lancet*, 363, 617-619.
- Poland, G.A. (2006). Vaccines against avian influenza - a race against time. *N. Engl. J. Med.*, 354, 1411-1413.
- Rimmelzwaan, G.F., & Osterhaus, A.D. (2001). Influenza vaccine: new developments. *Curr. Opin. Pharmacol.*, 1, 491-496.
- Sakurai, T., Yanagisawa, M., Takuwa, Y., Miyazaki, H., Kimura, S., Goto, K., & Masaki, T. (1990). Cloning of a cDNA encoding a non-isopeptides-selective subtype of the endothelin receptor. *Nature*, 348, 732-735.
- Salomon, R., Hoffmann, E., and Webster, R.G. (2007). Inhibition of the cytokine response does not protect against lethal H5N1 influenza infection. *Proc. Natl. Acad. Sci. USA*, 104, 12479-12481.
- Shimoda, L.A., Sham, J.S., Liu, Q., & Sylvester, J.T. (2002). Acute and chronic hypoxic pulmonary vasoconstriction: a central role for endothelin-1. *Respir. Physiol. Neurobiol.*, 132, 93-106.
- Shinya, K., Ebina, M., Yamada, S., Ono, M., Kasai, N., & Kawaoka, Y. (2006). Avian flu: influenza virus receptors in the human airway. *Nature*, 440, 435-436.
- Spickler, A.R., Trampel, D.W., & Roth, J.A. (2008). The onset of virus shedding and clinical signs in chickens infected with high-pathogenicity and low-pathogenicity avian influenza viruses. *Avian Pathol.*, 37, 555-557.
- Szretter, K.J., Gangappa, S., Lu, X., Smith, C.W., Shieh, J., Zaki, S.R., Sambhara, S., Tumpey, T.M., & Katz, J.M. (2007). Role of hostcytokine responses in the pathogenesis of avian H5N1 viruses in mice. *J. Virol.*, 81, 2736-2744.
- Ungchusak, K., Auewarakul, P., Dowell, S.F., Kitphati, R., Auwanit, W., Puthavathana, P., Uiprasertkul, M., Boonnak, K., Pittayawonganon, C., Cox, N.J., Zaki, S.R., Thawatsupha, P., Chittaganpitch, M., Khontong, R., Simmerman, J.M., & Chunsutthiwat, S. (2005). Probable person-to-person transmission of avian influenza A (H5N1). *N. Engl. J. Med.*, 352, 333-340.
- Yamada, S., Suzuki, Y., Suzuki, T., Le, M.Q., Nidom, C.A., Sakai-Tagawa, Y., Muramoto, Y., Ito, M., Kiso, M., Horimoto, T., Shinya, K., Sawada, T., Kiso, M., Usui, T., Murata, T., Lin, Y., Hay, A., Haire, L.F., Stevens, D.J., Russell, R.J., Gamblin, S.J., Skehel, J.J., & Kawaoka, Y. (2006). Haemagglutinin mutations responsible for the binding of H5N1 influenza A viruses to human-type receptors. *Nature*, 444, 378-382.