Published in final edited form as:

J Virol Methods. 2014 June 15; 202: 28–33. doi:10.1016/j.jviromet.2014.02.023.

Proteolytic enzymes in embryonated chicken eggs sustain the replication of egg-grown low-pathogenicity avian influenza viruses in cells in the absence of exogenous proteases

Ahmed Kandeil^a, Ola Bagato^a, Hassan Zaraket^b, Jennifer Debeauchamp^b, Scott Krauss^b, Rabeh El-Shesheny^a, Richard J. Webby^b, Mohamed A. Ali^a, and Ghazi Kayali^{b,*}

^aEnvironmental Research Division, National Research Centre, Dokki 12311, Giza, Egypt

^bDepartment of Infectious Diseases, St. Jude Children's Research Hospital, Memphis, TN 38105

Abstract

Low pathogenic influenza viruses grow readily in embryonated chicken eggs but require the addition of exogenous proteases to grow in MDCK cell culture. In this study, we found that influenza viruses propagated previously in eggs, can grow for up to two passages in cell culture without the addition of exogenous proteolytic enzymes. These results indicate that the reason for virus propagation in cells during the first two passages may be due to proteases from egg allantoic fluid carried over from egg culture. The ability of influenza viruses to grow in cells in the absence of trypsin is currently considered as a hallmark of highly pathogenic influenza viruses. Our data indicate that differentiating between high and low pathogenicity using cell culture only is not appropriate and other indicators such as sequence analysis and in-vitro pathogenicity index should be performed.

Keywords

Influenza; protease; cell culture

1. Introduction

Influenza A virus is a single-stranded negative sense RNA virus of the *orthomyxoviridae* family. Its genome is made of 8 segments encoding at least 11 known proteins (Nelson and Holmes, 2007). Influenza A virus is subtyped based on its surface glycoproteins; the hemagglutinin (HA) and the neuraminidase (NA) proteins. Aquatic birds are considered the main natural reservoir of influenza A viruses, of which there are currently 16 HA subtypes and 9 NA subtypes (Salomon and Webster, 2009; Webster et al., 1992). Only two subtypes, the H3N2 and the H1N1, currently circulate in humans. More recently, influenza A viral

© 2014 Elsevier B.V. All rights reserved.

*Corresponding author. 262 Danny Thomas Place, Memphis, TN 38105. Phone: +1 901 595 3400. Fax: +1 901 595 8559. ghazi.kayali@stjude.org.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

genetic material was isolated from bats, leading to the designation of additional antigenically distinct subtypes, H17N10; however, the N10 neuraminidase has not been shown to possess actual neuraminidase activity (Garcia-Sastre, 2012; Tong et al., 2012).

The HA protein is a class I membrane fusion protein. It is cleaved post-translationally into mature metastable protein made of two subunits; HA1 and HA2 (Skehel and Wiley, 2000; Wilson and Cox, 1990). The HA1 subunit harbors the receptor binding pocket which regulates the $\alpha(2, 3)$ versus $\alpha(2, 6)$ -linked sialic acid receptor binding preference. The HA2 subunit harbors a fusion peptide at its N-terminal that mediates virus-cell membrane fusion. Cleavage of the HA protein occurs at a basic amino acid linker between the HA1 and HA2 subunits (Bertram et al., 2010; Klenk and Garten, 1994). In low pathogenic influenza viruses (LPIV), this linker consists of a single amino acid residue that is recognized by a limited number of serine-like proteases that are present in the respiratory (in mammalian and avian species) and intestinal (in avian species) tracts (Klenk and Garten, 1994; Webster and Rott, 1987). In case of highly pathogenic avian influenza viruses (HPAIV), the cleavage site is polybasic and is recognized readily by ubiquitous subtilisin-like proteases enabling systemic replication of the virus (Stieneke-Grober et al., 1992; Webster and Rott, 1987). In humans, host cells and bacteria in the airway epithelium play a role in HA cleavage (Bottcher-Friebertshauser, Klenk, and Garten, 2013). In tissue culture, replication of LPIVs but not HPAIVs requires the addition of exogenous proteases such as tosyl phenylalanyl chloromethyl ketone (TPCK)-treated trypsin to cleave the HA protein. Replication of LPIVs and HPAIVs in embryonated chicken eggs is supported by proteases present in allantoic fluid (Horimoto and Kawaoka, 1997). Since avian LPIVs are grown commonly in eggs, the aim of this study was to investigate the effect of residual proteases present in the allantoic fluid on replication of egg-grown influenza viruses representing a broad array of subtypes in cell culture in the absence of exogenous trypsin.

2. Methods

2.1. Viruses and Cell Culture

A panel of egg-grown LPIVs H4-H16 was used in this study (Table 1). The H5N1 and H7N7 viruses were highly pathogenic viruses that were rendered low pathogenic by reverse genetics (Webby et al., 2004). This was accomplished by deleting the polybasic cleavage site from the HA gene and creating reassortant viruses containing the altered HA and NA of the wildtype virus and the 6 internal genes from the A/Puerto Rico/38 virus. Viruses were grown initially in the allantoic fluids of 10-day old embryonated SPF chicken eggs following standard procedures (WHO, 2002). Each egg-propagated virus was inoculated into 6-well tissue culture plates (100 μ l/well) (Greiner, Kremsmunster, Austria) containing 80-90% confluent MDCK cells with and without the addition of TPCK-treated trypsin in the infection media and grown for 72hrs (WHO, 2002). Confluent cells were washed twice with PBS before the addition of the virus inoculum. After an 1hr incubation at 37 °C, the inoculum was removed and the cells were washed once with PBS and then incubated for 72hrs. Following that, the viruses were propagated twice more in MDCK cells by adding 100 μ l of the previous passage per well. We recorded cytopathic effect (CPE) subjectively,

and conducted a hemagglutination assay (HA) using chicken RBCs, HA titers were recorded as the reciprocal of the virus dilution that caused agglutination to RBCs (WHO, 2002).

2.2. Plaque Assay

In order to quantify virus replication, plaque assays were used for virus titration. Six-well tissue culture plates were seeded with MDCK cells (10^5 cells/well). At 90-100% confluence (one day post-seeding), the cells were washed twice with PBS. Viruses were diluted 10-fold in DMEM (Lonza, Basel, Switzerland) and 100 μ l of undiluted virus and each dilution were mixed with 400 μ l DMEM and inoculated into MDCK cells. The plates were incubated at 37 °C for 1 hr. The wells were then aspirated to remove residual inoculum. Each well was then immediately covered with 2 ml of DMEM overlay medium containing 1% agarose type 1 (Lonza), 1% antibiotic-antimycotic mixture (Lonza), and 1 μ g/ml TPCK-treated trypsin (Worthington, Lakewood, NJ). Plates were then incubated at 37 °C with 5% CO₂ for 2 days. The formation, number, and growth rate of the plaques were microscopically observed daily. Once clear plaques could be visualized, 1ml of 10% formaldehyde was added to each well for 1hr for cell fixation and virus inactivation. The formaldehyde was then discarded and the plates rinsed with water and dried. For visualization of the plaques, 1ml of the staining solution, consisting of 1% crystal violet and 20% methanol in distilled water, was added to each well and incubated at room temperature for 5min, the dye was then discarded and the wells were rinsed with water and dried. Viral plaques were then counted and virus titer was calculated using the Reed and Muench method (Reed and Muench, 1938).

2.3. Western blotting

In order to qualify cleavage of HA in the presence and absence of trypsin, 2 consecutive passages of avian influenza H9N2 virus were subjected to western blotting. Propagated viruses were analyzed by SDS-PAGE as described previously (Ruppel et al., 1985); the only modification was that 1% BSA in PBS-0.3% Tween20 was used to block the protein-free binding sites on the nitrocellulose membrane. Immunorecognition was performed on cut membrane strips carrying chicken anti-H9N2 sera (dilution 1:100). Immune detection was carried out with peroxidase-conjugated goat anti-chicken IgG (KPL, Gaithersburg, MD) diluted 1:2000 in PBS-0.3% Tween20.

2.4. Zymogram

The presence of serine proteases in egg uninfected allantoic fluid and cultured viruses was determined by zymography (Heussen and Dowdle, 1980). In this assay, serine proteases will degrade gelatin at their specific molecular weights and the gel at that site will not absorb the dye, thus the protease activity will be visualized as an unstained band on a stained background. Gelatin was used as a protease substrate and was added to the separating gel before polymerization of acrylamide. A volume of 10 μ l of infected or uninfected allantoic fluid was added to 10 μ l of sample buffer containing 0.1M Tris-HCl, 4% SDS, 10% glycerol, and 50mg bromophenol blue at pH 6.8. The sample was then loaded to wells of an acrylamide stacking gel. A volume of 5 μ l of 10-200 kDa protein marker (Lonza) was applied to one of the gel wells and allowed to run parallel to the samples for detection of the molecular weight(s) corresponding to the proteases. Electrophoresis was conducted at 60V for 2hrs. After electrophoresis, the marker was cut and Coomassie-stained then destained

while the rest of the gel was soaked in renaturing (2.5% triton-X-100) solution with gentle shaking for 30 min at room temperature to remove SDS from the gel. The gel was then incubated overnight at 37°C with gentle shaking in substrate buffer (30 mM tris-HCl, 60 mM NaCl, CaCl₂ (Serva, Heidelberg, Germany)) at pH 5, 7 and 10 then stained with 0.5% Coomassie blue dye (in 10% acetic acid, 5% methanol (Sigma, St. Louis, MO)) and de-stained using 60% methanol. The molecular weight of the proteases present was determined by comparison with the molecular weight marker.

3. Results

At passage 1, all viruses produced an HA titer at 48hrs with or without using trypsin. At 72hrs (Fig. 1.), titers increased to 32 or 64 for the viruses propagated without trypsin while the viruses that grew with trypsin had HA titers between 8 and 64. At passage 2, viruses propagated with trypsin continued to grow. However, only 6 viruses, mostly having higher titers in passage 1, were able to grow without the presence of trypsin and had low titers (HA 2-16). This trend continued into passage 3 where all viruses except H16 grew with trypsin while none propagated in its absence. To confirm these results, 2 consecutive passages of H4, H9, and H10 viruses were repeated in the presence or absence of trypsin. HA titers measured were within 1-fold difference of the measurements obtained earlier.

We also observed CPE at 48hrs and 72hrs post-infection (Fig. 2.). In the presence of trypsin, all viruses produced high CPE in passage 1 (>80%). In passage 2, high CPE was observed for all viruses except for H6, H13, and H16 that had lower CPE (30-50%). The same observation was made in passage 3 and H6, H13, and H16 had even lower CPE (0-20%). In the absence of trypsin, all viruses had high CPE in passage 1. In passage 2, viruses H4, H5, H7, H8, H9, and H11 had a CPE on cells (40-100%), while the other viruses did not show CPE. In passage 3, none of the viruses had CPE.

The results of the plaque assays are shown in figure 3. All viruses except H13 provided high titers in the plaque assay in all passages with trypsin. The H13 virus only provided a titer by the third passage. For the other viruses titers remained the same or increased by the 3rd passage. In the absence of trypsin, all viruses except for H13 and H16 yielded a titer at passage 1 (4-7 log₁₀ PFU/ml). Titers dropped by the second passage but H13 and H16 did not plaque (3-6 log₁₀ PFU/ml). Only three viruses (H4, H9, and H10) yielded a titer by the third passage and these titers were 4 log₁₀ PFU/ml.

Western blot data (Fig. 4.) showed that the H9N2 HA was being cleaved into HA1 and HA2 efficiently in the presence of trypsin. This was also evident in the first MDCK passage in the absence of trypsin.

Zymogram data of uninfected allantoic fluid showed several egg protease activities at neutral and alkaline pH. Clear proteolytic bands were visualized at 155, 133, 69, 62, 54, 39, and 36 kDa at pH 7 and 10. An additional proteolytic band at MW of about 107 kDa was detected at pH 7 (Fig. 4.). Zymogram of MDCK-grown H4, H5, H6, and H7 viruses was conducted. Bands at molecular weight around 130 kDa for all passages of these viruses were visualized, the intensity of the band decreased with increased passaging. Trypsin is visualized at around 24 kDa in passages where trypsin was used. Figure 5 illustrates this

finding for MDCK-grown H5 virus. In order to verify the potential of egg allantoic fluid as a protease-replacement, the first cell passage (without trypsin) of H4, H5, H10, H14, and H15 were passaged in MDCK cells without trypsin but with the addition of 10% to 50% allantoic fluid or trypsin in the infection media. All these viruses propagated successfully with trypsin as verified by HA titers between 128 and 256. However, only two viruses grew in the presence of allantoic fluid (H5 and H10) but had low titers of 4.

4. Discussion

These results demonstrated that most LPIVs propagated in embryonated chicken eggs can grow readily for up to 2 passages in MDCK cells without the need for the addition of TPCK-treated trypsin albeit to titers that were lower than those obtained in the presence of trypsin. We hypothesized that endogenous trypsin-like serine proteases found in the eggs' allantoic fluids sustain the propagation of LPIVs in MDCK cells and once depleted, TPCK-treated trypsin becomes essential for MDCK culture of these viruses. By zymography, several egg proteases that may be involved in viral HA activation were visualized. Furthermore, one of these proteases was visualized in the MDCK-grown viruses further supporting the egg-protease involvement in virus propagation on cells. However, propagating viruses by replacing trypsin with egg protease was not successful as only 2 viruses grew but to very low titers. It remains important to verify the involvement of such proteases in virus MDCK cultures and explain the means by which such proteases may aid HA cleavage, and further research is required if allantoic enzymes are to be used as a replacement for trypsin.

Other researchers were not able to detect protease-mediated cleavage for the H13 and H16 Has (Galloway et al., 2013). In the H16 HA, the cleavage site that would create the HA1/HA2 complex displays an α -helical structure and hides in the negatively charged cavity, in contrast to the flexible loop conformations found in other known structures, and more importantly, this unusual structure is correlated with its inefficient cleavage by trypsin (Lu et al., 2012). In our data, H16 only yielded a high titer in the plaque assay in the first passage in the presence of trypsin (Fig. 3.) and had the lowest HA titer. This might be explained by Lu et. al findings. The HA titer after the first cell passage in the absence of trypsin was one-fold higher than that in the presence of trypsin. Since the difference is minimal, we do not believe there is an underlying mechanism for this finding and, most likely, it is due experimental error. The H13 virus had higher HA and plaque assay titer in the third passage with trypsin. This surprising finding may be due to the H13 virus adapting to cell culture as suggested by others (Keawcharoen et al., 2010).

One weakness of this study was that different HA titers for different viruses prior to MDCK infection was not adjusted for. However, since the aim was not to compare viruses to each other but rather compare the different passages of the same virus, we do not believe that adjusting titers would have altered the findings. If this had an effect, it will be mostly affecting our subjective CPE reading as higher content of viral particles should provide more CPE. However, all assays that we conducted led to the same conclusion. On another hand, we did not wash the cells extensively after the initial 1 hr incubation to allow the inoculum to adsorb on the cells. Extensive washing of the cells post this incubation period may reduce

carried-over allantoic fluid and hence inhibit the propagations of LPIVs in the absence of exogenous proteases.

Current influenza protocols state that only few avian influenza viruses of the H5 or H7 subtypes are able to grow on MDCK cells in the absence of exogenous trypsin (Krauss, Walker, and Webster, 2012). The inability of some AI viruses, especially reassortants, to grow in MDCK cells in the absence of trypsin is commonly used as a differentiation method to determine their pathogenicity. In light of our results, this cannot be used as a single method to determine pathogenicity in case the viruses were passaged in eggs. Other assays, such as pathogenicity testing in chickens and HA sequence data (APHIS, 2011), are needed to determine whether viruses have low or high pathogenicity.

TPCK has been shown to induce mammalian cell death through various mechanisms (Fabian et al., 2009). The addition of TPCK-treated trypsin to MDCK cells has a cytotoxic effect that may decrease viral titers in cell culture. Thus, investigating alternatives to TPCK-treated trypsin such as serine proteases from chicken eggs is warranted.

Acknowledgments

This work was funded by the National Institute of Allergy and Infectious Diseases, National Institutes of Health, Department of Health and Human Services, under contract number HHSN266200700005C, and supported by the American Lebanese Syrian Associated Charities (ALSAC).

References

1. APHIS, U. Guidelines for Avian Influenza Viruses. 2011
2. Bertram S, Glowacka I, Steffen I, Kuhl A, Pohlmann S. Novel insights into proteolytic cleavage of influenza virus hemagglutinin. *Reviews in medical virology*. 2010; 20:298–310. [PubMed: 20629046]
3. Bottcher-Friebertshauser E, Klenk HD, Garten W. Activation of influenza viruses by proteases from host cells and bacteria in the human airway epithelium. *Pathogens and disease*. 2013; 69:87–100. [PubMed: 23821437]
4. Fabian Z, O'Brien P, Pajacka K, Fearnhead HO. TPCK-induced apoptosis and labelling of the largest subunit of RNA polymerase II in Jurkat cells. *Apoptosis: an international journal on programmed cell death*. 2009; 14:1154–64. [PubMed: 19653099]
5. Galloway SE, Reed ML, Russell CJ, Steinhauer DA. Influenza HA subtypes demonstrate divergent phenotypes for cleavage activation and pH of fusion: implications for host range and adaptation. *PLoS pathogens*. 2013; 9:e1003151. [PubMed: 23459660]
6. Garcia-Sastre A. The neuraminidase of bat influenza viruses is not a neuraminidase. *Proc Natl Acad Sci U S A*. 2012; 109:18635–6. [PubMed: 23100536]
7. Heussen C, Dowdle EB. Electrophoretic analysis of plasminogen activators in polyacrylamide gels containing sodium dodecyl sulfate and copolymerized substrates. *Analytical biochemistry*. 1980; 102:196–202. [PubMed: 7188842]
8. Horimoto T, Kawaoka Y. Biologic effects of introducing additional basic amino acid residues into the hemagglutinin cleavage site of a virulent avian influenza virus. *Virus Res*. 1997; 50:35–40. [PubMed: 9255933]
9. Keawcharoen J, Spronken MI, Vuong O, Bestebroer TM, Munster VJ, Osterhaus AD, Rimmelzwaan GF, Fouchier RA. Repository of Eurasian influenza A virus hemagglutinin and neuraminidase reverse genetics vectors and recombinant viruses. *Vaccine*. 2010; 28:5803–9. [PubMed: 20600474]
10. Klenk HD, Garten W. Host cell proteases controlling virus pathogenicity. *Trends Microbiol*. 1994; 2:39–43. [PubMed: 8162439]

11. Krauss, S.; Walker, D.; Webster, RG. Influenza Virus Isolation. In: Kawaoka, Y.; Neumann, G., editors. *Influenza Virus Methods and Protocols*. Humana Press; London, UK: 2012. p. 11-24.
12. Lu X, Shi Y, Gao F, Xiao H, Wang M, Qi J, Gao GF. Insights into avian influenza virus pathogenicity: the hemagglutinin precursor HA0 of subtype H16 has an alpha-helix structure in its cleavage site with inefficient HA1/HA2 cleavage. *Journal of virology*. 2012; 86:12861–70. [PubMed: 22993148]
13. Nelson MI, Holmes EC. The evolution of epidemic influenza. *Nat Rev Genet*. 2007; 8:196–205. [PubMed: 17262054]
14. Reed LJ, Muench H. A simple method of estimating fifty percent endpoints. *The American Journal of Hygiene*. 1938; 27:493–497.
15. Ruppel A, Rother U, Vongerichten H, Lucius R, Diesfeldt HJ. *Schistosoma mansoni*: immunoblot analysis of adult worm proteins. *Experimental parasitology*. 1985; 60:195–206. [PubMed: 3896836]
16. Salomon R, Webster RG. The influenza virus enigma. *Cell*. 2009; 136:402–10. [PubMed: 19203576]
17. Skehel JJ, Wiley DC. Receptor binding and membrane fusion in virus entry: the influenza hemagglutinin. *Annu Rev Biochem*. 2000; 69:531–69. [PubMed: 10966468]
18. Stieneke-Grober A, Vey M, Angliker H, Shaw E, Thomas G, Roberts C, Klenk HD, Garten W. Influenza virus hemagglutinin with multibasic cleavage site is activated by furin, a subtilisin-like endoprotease. *EMBO J*. 1992; 11:2407–14. [PubMed: 1628614]
19. Tong S, Li Y, Rivallier P, Conrardy C, Castillo DA, Chen LM, Recuenco S, Ellison JA, Davis CT, York IA, Turmelle AS, Moran D, Rogers S, Shi M, Tao Y, Weil MR, Tang K, Rowe LA, Sammons S, Xu X, Frace M, Lindblade KA, Cox NJ, Anderson LJ, Rupprecht CE, Donis RO. A distinct lineage of influenza A virus from bats. *Proc Natl Acad Sci U S A*. 2012; 109:4269–74. [PubMed: 22371588]
20. Webby RJ, Perez DR, Coleman JS, Guan Y, Knight JH, Govorkova EA, McClain-Moss LR, Peiris JS, Rehg JE, Tuomanen EI, Webster RG. Responsiveness to a pandemic alert: use of reverse genetics for rapid development of influenza vaccines. *Lancet*. 2004; 363:1099–103. [PubMed: 15064027]
21. Webster RG, Bean WJ, Gorman OT, Chambers TM, Kawaoka Y. Evolution and ecology of influenza A viruses. *Microbiological reviews*. 1992; 56:152–79. [PubMed: 1579108]
22. Webster RG, Rott R. Influenza virus A pathogenicity: the pivotal role of hemagglutinin. *Cell*. 1987; 50:665–6. [PubMed: 3304656]
23. WHO. WHO Manual on Animal Influenza Diagnosis and Surveillance. Geneva, Switzerland: 2002.
24. Wilson IA, Cox NJ. Structural basis of immune recognition of influenza virus hemagglutinin. *Annual review of immunology*. 1990; 8:737–71.

Egg-grown avian influenza viruses grew in cell culture without exogenous proteases.

Egg proteases may have enabled viruses to grow for up to 2 passages in cells.

Egg proteases were visualized by zymogram in uninfected allanotic fluid.

Similar proteases were visualized in cell cultured viruses.

Fig. 1. Chicken RBCs hemagglutination assay titers of influenza viruses grown in MDCK cells at 72hrs post-inoculation. 13 low pathogenic avian influenza viruses were propagated in cell culture for 3 consecutive passages in the presence or absence of trypsin. P# indicates passage number, -T indicates absence of trypsin, +T indicates presence of trypsin, 72 indicates 72 hours post inoculation.

Fig. 2. cytopathic effect (%) exerted by influenza viruses on MDCK cells at 72hrs post-inoculation. 13 low pathogenic avian influenza viruses were propagated in cell culture for 3 consecutive passages in the presence or absence of trypsin. Cytopathic effect was qualitatively recorded. P# indicates passage number, -T indicates absence of trypsin, +T indicates presence of trypsin, 72 indicates 72 hours post inoculation.

Fig. 3.

Plaque assay titers of influenza viruses grown on MDCK cells at 72hrs post inoculation. 13 low pathogenic avian influenza viruses were propagated in cell culture for 3 consecutive passages in the presence or absence of trypsin. Plaque assay was conducted in the presence of trypsin. P# indicates passage number, -T indicates absence of trypsin, +T indicates presence of trypsin, 72 indicates 72 hours post inoculation.

Fig. 4. Western blot showing HA cleavage of H9N2 at different passages in the presence or absence of trypsin. P# indicates passage number, -T indicates absence of trypsin, +T indicates presence of trypsin.

Fig. 5. Visualization of various proteolytic enzymes in allantoic fluid of un-infected embryonated chicken eggs by Zymography. Clear bands in the gel indicate the presence of proteolytic enzymes. M denotes protein marker, pH# indicates pH at which the assay was carried out.

Fig. 6.

Visualization of proteolytic enzymes in different passages of MDCK-cultured H5 virus by Zymography. Clear bands in the gel indicate the presence of proteolytic enzymes. Egg proteases appear in all passages at molecular weight around 130 kDa while trypsin appears in passages that included the addition of trypsin at molecular weight 24 kDa. P# indicates passage number, -T indicates absence of trypsin, +T indicates presence of trypsin.

Table 1
Egg-grown influenza viruses used in this study

Virus Name	Subtype	HA Titer
A/duck/Hong Kong/365/78	H4N6	256
RG-A/turkey/Egypt/7/2007	H5N1	1024
A/quail/Hong Kong/YU 421/02	H6N1	256
RG-A/Netherlands/219/2003	H7N7	512
A/turkey/Ontario/6118/68	H8N4	128
A/quail/Lebanon/272/09	H9N2	1024
A/chicken/Germany/N/49	H10N7	1024
A/duck/Hong Kong/P50/97	H11N9	512
A/duck/Alberta/60/76	H12N5	32
A/gull/Astrachan/458/85	H13N6	256
A/mallard duck/Astrachan/263/82	H14N5	256
A/wedge-tailed shearwater/Western Australia/2576/79	H15N9	512
A/black-headed gull/Sweden/5/99	H16N3	64