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# 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

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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 the 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.



## 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 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 et al., 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

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**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 |

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 μl/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 72 h (WHO, 2002). Confluent cells were washed twice with PBS before the addition of the virus inoculum. After an 1 h incubation at 37 °C, the inoculum was removed and the cells were washed once with PBS and then incubated for 72 h. Following that, the viruses were propagated twice more in MDCK cells by adding 100 μl 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 μl of undiluted virus and each dilution were mixed with 400 μl DMEM and inoculated into MDCK cells. The plates were incubated at 37 °C for 1 h. 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 1ug/ml TPCK-treated trypsin (Worthington, Lakewood, NJ, USA). Plates were then incubated at 37 °C with 5% $CO_2$ for 2 days. The formation, number, and growth rate of the plaques were microscopically observed daily. Once clear plaques could be visualized, 1 ml of 10% formaldehyde was added to each well for 1 h for cell fixation and virus inactivation. The formaldehyde was then discarded and the plates rinsed with water and dried. For visualization of the plaques, 1 ml 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 5 min, 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 μl of infected or uninfected allantoic fluid was added to 10 μl of sample buffer containing 0.1 M Tris–HCl, 4% SDS, 10% glycerol, and 50 mg bromophenol blue at pH 6.8. The sample was then loaded to wells of an acrylamide stacking gel. A volume of 5 μl 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 60 V for 2 h. 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, $CaCl_2$ (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, USA)) 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 48 h with or without using trypsin. At 72 h (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.