Short Communication

Growth Characteristics of the H5N1 Avian Influenza Virus in Chicken Embryonic Eggs and MDCK Cells

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Abstract

Chicken embryonic eggs and Madin-Darby canine kidney (MDCK) cell line were used for comparing the propagation of avian influenza A (H5N1) virus (C2105Dx1, a Thai isolate). The growth of the H5N1 virus was determined by using HA test and 50% infectivity dose to assess the suitability of the systems supporting the propagation of the virus. The results indicated that the Thai H5N1 virus was propagated better in the chicken embryonic eggs, which should be considered as a system of choice for the avian H5N1 virus isolation. The optimal time for harvesting the selected Thai H5N1 virus was at 24 hours after inoculation in the chicken embryonic eggs, yielding the virus titer of at least 9 log2 HAU/$_{50}$µl or 107.0 TCID$_{50}$/ml.

Keywords : avian influenza virus, chicken embryonic eggs, H5N1, MDCK cells

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บทคัดย่อ

ลักษณะการเจริญเติบโตของเชื้อไวรัสไข้หวัดนกสับไทป์ H5N1 ในตัวอ่อนลูกไก่ และเซลล์เพาะเลี้ยงชนิด MDCK Cells

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การเปรียบเทียบลักษณะการเจริญเติบโตของเชื้อไวรัสไข้หวัดนกสับไทป์ H5N1 (C2105Dx1) ที่แยกเชื้อไวรัสได้จากประเทศไทยในตัวอ่อนลูกไก่ในไข่ฟัก และเซลล์เพาะเลี้ยงชนิด Madin-Darby canine kidney (MDCK) cell line โดยการทดสอบด้วยเทคนิค Hemagglutination (HA) test และ 50% Infectivity dose เพื่อประเมินประสิทธิภาพในการส่งเสริมการเจริญเติบโตของเชื้อไวรัสในโฮสต์แต่ละชนิด ผลการศึกษาพบว่า เชื้อไวรัส H5N1 สายพันธุ์ดังกล่าวสามารถเพิ่มจำนวนได้ดีในตัวอ่อนลูกไก่ในไข่ฟักซึ่งเป็นโฮสต์ที่เหมาะสมสำหรับการแยกเชื้อไวรัสโดยระยะเวลาที่เหมาะสมสำหรับการเก็บเชื้อไวรัสที่แยกได้จากประเทศไทยครั้งนี้คือ ภายหลังการฉีดเชื้อไวรัสในตัวอ่อนลูกไก่ในไข่ฟักเป็นเวลา 24 ชั่วโมงซึ่งได้ปริมาณเชื้อไวรัสมากที่สุดอย่างน้อย 9 log2 $HAU/_{50}\mu l$ หรือ 107.0 $TCID_{50}$/ml

คำสำคัญ : เชื้อไวรัสไข้หวัดนก ตัวอ่อนลูกไก่ H5N1 MDCK cells

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Introduction

Influenza viruses are members of the family Orthomyxoviridae composing of 4 genera, A, B, C and Thogotovirus based on the basis of the nucleocapsid or matrix antigen (Brown, 2000). However, only type A influenza viruses are able to infect a variety of avian and mammalian hosts and can cause severe disease in many species (Lee et al., 2004).

Influenza virus is an enveloped RNA virus containing 8 segments of single stranded negative-sense RNA genomes. The envelope contains haemagglutinin (HA) and neuraminidase (NA) proteins. Sixteen serotypes of HA (H1-H16) and nine (N1-N9) of NA have been identified in both mammalian and avian influenza A viruses (Stevens et al., 2006). The viral particles are approximately 50-120 nm in diameter for spherical forms (Brown, 2000). Most laboratory-adapted influenza viruses existing in the spherical morphology of approximately 100 nm in diameter are grown in the cell culture system. However, influenza viruses isolated from the clinical specimens are believed to be predominantly filamentous particles (Sieczkarski and Whittaker, 2005). In addition, the internalization of the filamentous influenza virus particles is delayed according to their spherical particles.

The laboratory techniques based on isolation and propagation of influenza viruses are important in the surveillance, studies of host range, pathogenesis and vaccine production (Seo et al., 2001). Avian influenza virus isolation often uses chicken embryonic eggs. However, the cell culture system is an alternative method in some laboratories. Cultivation of influenza viruses in the embryonated chicken eggs (CE) is also the system of choice for generating of large quantities of virus used in the laboratory studies (Murphy and Webster, 1996). However, the virulent strains of type A influenza virus after inoculating into the allantoic cavities of chicken embryos rapidly kills the embryos and yields a low virus titer.

The disadvantages of using chicken embryonic eggs are that the eggs may contain various microbiological contamination and residual endotoxin (Oxford et al., 2003), and the eggs may be unavailable in some laboratories. The alternative techniques using tissue culture system may be considered in some laboratories since it is easy to obtain and maintain the culture system. Attempting to propagate the influenza virus in the tissue culture system has been done using primary chick embryo kidney cell (Austin et al., 1978), Vero cells (Youil et al., 2004), Hep2 and RD cells (WHO, 2005). Currently Madin-Darby canine

kidney cell (MDCK) is the cell culture of choice using for a wide variety of influenza A viruses propagation, comprising of human, equine, porcine and avian origins (Tobita et al. 1975). WHO (2005) also recommends MDCK cells as the preferred cell line for culturing the influenza viruses. The advantage of the MDCK cell line is the availability from the cell bank system and free of other microbiological contaminants (Oxford et al., 2003). In addition, the MDCK cells are also used for large quantities of the H5N1 virus production especially for vaccine production.

The objectives of this study are to compare the two systems for a Thai first isolated H5N1 virus (C2105Dx1) propagation and to learn more on the nature of this Thai isolate. The results might be very useful for the avian influenza research when using this virus and other related H5N1 virus.

Materials and Methods

Virus: Avian influenza A (H5N1) virus used in this study was isolated from 25-day-old broiler chickens in Thailand during the first outbreak in 2004 and named C2105Dx1 (Veterinary Diagnostic Laboratory, Faculty of Veterinary Science, Chulalongkorn University, Thailand). The stock virus from the second passage of 10-day-old embryonating chicken eggs, containing the titer of 7.78 log10EID50/ml, was prepared as a described by OIE (2004). All viral manipulations were performed under the appropriate biosafety level 3 laboratory conditions.

MDCK cells: MDCK cells used in this study (passage number 53) were kindly provided by Dr. Christopher Olsen from the Veterinary School, University of Wisconsin-Madison.

1. Viral propagation in MDCK cells

Flask preparation: Confluent monolayer of MDCK cell line was prepared in a 75 cm^2 flask. After washing MDCK cells with 10 ml of PBS, pH 7.0, 1 ml of trypsin was added to the flask to detach the cells and discarded. The flask again filled with 3 ml of trypsin and shaked until all cells detached from the plastic surface. After that, 3 ml of 5% FBS (Fetal Bovine Serum, GIBCO, Invitrogen Corp) of MEM (Modified Eagle Medium, GIBCO, Invitrogen Corp was added to inactivate the trypsin digestion.

Inoculation of the virus: 5% FBS of MEM from the flask was discarded and the flask was washed 2-3 times with 3 ml of MEM. The inoculum was prepared by diluting the virus with MEM to multiplicity of infection (MOI) of 1.8, then inoculated onto the monolayer of MDCK cells and incubated for 1 hr at 37°C and in 5% CO2 incubator to allow the viral adsorption. After that, the inoculum was descanted and 10 ml of 3% FBS of MEM were added to the monolayer.

Harvesting of the virus: The flask containing virus was freezed and thawed twice before harvesting the supernatant at 0, 8, 16, 24, 32, 40, 48, 56, 64 and 72 hrs post inoculation (hpi) and kept at -80°C before virus titration.

2. Viral propagation in chicken embryonic eggs: Stock virus containing the titer of 7.78 log $10EID_{50}$/ml was inoculated approximately 0.2 ml/egg in the 9-days old embryonic chicken eggs. Four eggs at each incubation period of 0, 4, 8, 12, 16, 20, 24, 28 and 32 hpi were collected and the allantoic fluid was harvested aseptically for virus titration.

3. Virus titration: To determine the haemagglutination titer, 1% (v/v) chicken red blood cells (RBCs) was used in 96 wells V-bottom micro titer plates (NUNC, Denmark) (OIE, 2004). Briefly, 0.05 ml of PBS was dispensed into each well of a micro titer plate. Then, 0.05 ml of the infected allantoic fluid was placed in the first well and two-fold dilutions of 0.05 ml volume of the suspension was performed across the plate. The 0.05% of RBCs was added to each well, mixed by tapping the plate and settled for 30 min at room temperature. HA was determined by tilting the plate and observed the presence or absence of the agglutination. The titration was read to the highest dilution yielding complete agglutination.

To determine of the infectivity titer, MDCK cell line was used in 96 well microplates (NUNC, Denmark). The harvested virus from each incubation period was diluted in a ten-fold dilution manner. The diluted virus was transferred to the monolayer of MDCK cells microplates and allowed to absorb at 37°C for 1 hr in 5% CO_2 incubator. Then, the inoculum was discarded and washed with 150 µl of PBS twice. A hundred and eighty microlitres of 3% MEM was added to all wells. Cells were incubated at 37°C, 5% CO2 incubator for 72 hr. The plates were observed daily for cytopathic effect (CPE) under the inverted microscope. The CPE characterized

as a rounding up of infected cells was microscopically recognized. Then, the viral titer was calculated in $TCID_{50}$/ml of log10 values as described in Reed and Munch (1938).

Results and Discussion

The isolation and propagation of influenza viruses are important in epidemiological surveillance, studies of host range, pathogenesis, diagnosis and vaccine production. Therefore, chicken embryonic eggs and Madin-Darby canine kidney (MDCK) cell line were used to compared the propagation ability of avian influenza A (H5N1) virus (C2105Dx1), a Thai isolate.

Using the MDCK monolayer at the MOI of 1.8, the H5N1 virus initially grew between 8 to 16 hours after inoculation and reached maximum titer between 40 to 48 hrs after inoculation. The infectivity titer of viral propagation in MDCK cells was between 2.4 to 4.2 $TCID_{50}$/ml, whereas, the HA titer was between 2.0 to 2.5 log2 (Figure 1 and 2). Morphological change of cytopathic effect (CPE) was firstly observed at 16 hpi in accordance with viral infectivity and HA determination (Figure 3). The infected chicken embryonic eggs died within 32 hpi according to the characterization of virulent strains as described by Park et al. (2001), thus the study was performed until 32 hpi. The virus initially grew between 12 to 16 hours after inoculation and reached maximum titer between 24 to 28 hrs after inoculation. The infectivity titer of viral propagation in chicken embryonic eggs was between 5.7 to 7.4 $TCID_{50}$/ml and between 7.3 to 9.0 log2 of HA titer (Figure 4 and 5). Thus, for the Thai H5N1 virus studied in this work, the infectivity and HA titers of the H5N1 virus in chicken embryonic egg were better than those of MDCK cells (>3 log10 and >5 log2, respectively). The poor replication efficiency of the Thai H5N1 virus in MDCK cells was similar to the previous study (Seo et al., 2001) that the replication efficiencies of the 1997 H5N1 viruses ranged from 1.5 to 5.0 log10$TCID_{50}$/ml. The virus initially appears initially on the surface epithelium of the allantoic membrane, then in the vascular endothelial cells of chorioallantoic membrane and the visceral organs of the embryos, before spreading to the parenchymal cells of many organs. In contrast to the virulent strains, non-virulent virus strain confines in the allantoic membrane and sometimes may not kill the embryos (Park et al., 2001).

The binding property of the virus to the host cell is determined by two factors, the receptor binding affinity of the virus and the receptor density on the host cell surface (Asaoka et al., 2006). These binding specificities correspond to the types of sialic acid linkages within those hosts. Avian influenza viruses preferentially bind to 5-N-acetylneuraminic acid α-2, 3-galactose (Neu5Ac α-2,3Gal) linkage, while human influenza viruses preferentially bind to Neu5Acα-2,6Gal (Roger et al., 1983). The allantoic cells of chicken embryonated eggs contain Neu5Acα2,3Gal but no Neu5Acα2,6Gal, while amniotic cells and MDCK cells contain both linkages (Ito et al., 1997). The H5N1 virus in Thailand contained a glutamine 222 (226 in H3) and a glycine at position 224 (228 in H3) in HA1, which are preferential related to avian cell-surface receptor or Neu5Acα-2,3Gal (Kaewcharoen et al., 2004). Therefore, the allantoic cavities should be the most preferential sites of viral replication.

Since, cell surface sialyloligosaccharides play an important role in the selection and maintenance of the receptor specificities of influenza viruses (Ito et al., 1997), cultivation of the H5N1 virus in the Neu5Acα2,3Gal-riched allantoic cavities will not select the virus variant with mutations as previously described in human influenza viruses (Widjaja et al., 2006; Gambaryan et al., 1999; Ito et al., 1997; Hardy et al., 1995). Undoubtedly, the chicken embryo still remains the best system of choice for the isolation and propagation of the Thai H5N1 virus. The optimal time for harvesting the selected Thai isolate from our study was 24 hours after inoculation, which yield the titer of at least 9.0 log2 HAU/$_{50}$µl or $10^{7.0}$ $TCID_{50}$/ml.

In this study, the replication efficiency of the H5N1 virus was greater in chicken embryonic eggs than MDCK cells due to the binding property between the virus from the avian origin and host cell. To culture large scale of the H5N1 virus such as vaccine production or antigen preparation for further researches, the chicken embryonic eggs are the most appropriate system with minimal viral selection. However, diagnostic laboratories receiving the specimens from various species may consider MDCK cell as an alternative system for a wide variety of influenza A virus isolation.

Figure 1 Growth curves of the virus in MDCK cells based on HA test determination (HAU/$_{50}$µl). The HA titers were between 4.0 to 5.7 HAU/50µl (2.0 to 2.5 log2 HAU/50µl).

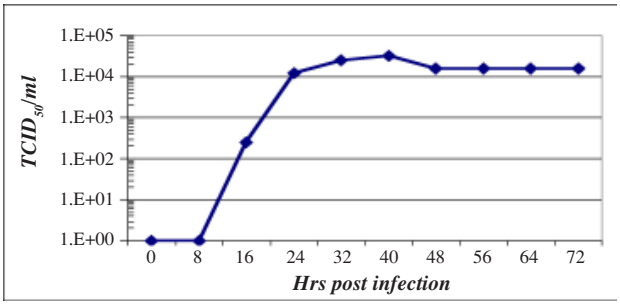

Figure 2 Growth curves of the virus in MDCK cells based on 50% infectivity dose determination (TCID$_{50}$/ml). The infectivity titers were between 2.4 to 4.2 log10 TCID$_{50}$/ml.

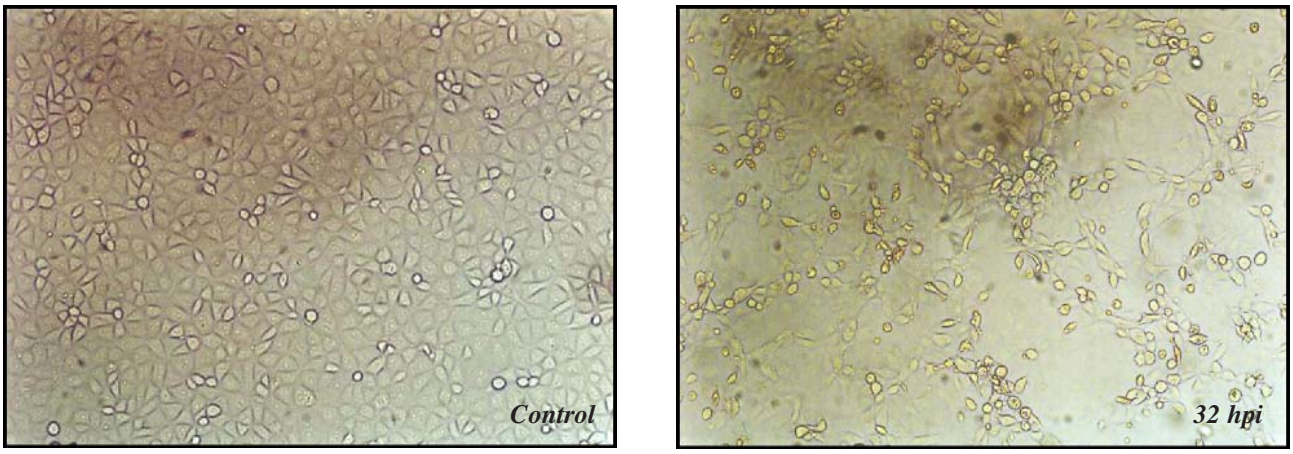

Figure 3 Photomicrographs of normal MDCK cells cultured (left) and sequential changes after viral infection 32 hpi (right). Morphological change of CPE was firstly observed at 16 hpi in accordance with viral infectivity and HA determination.

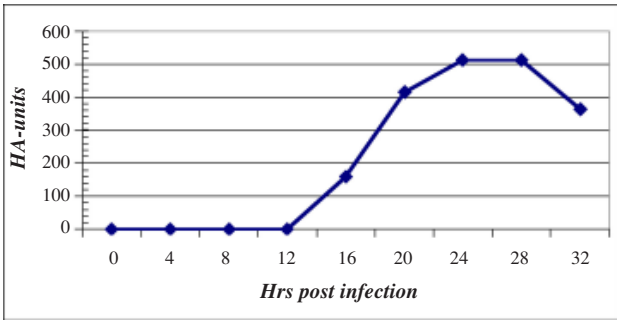

Figure 4 Growth curves of the virus in chicken embryonic eggs based on HA test determination (HAU/$_{50}$µl). The HA titers were between 158 to 512 HAU/$_{50}$µl (7.3 to 9.0 log2 HAU/$_{50}$µl).

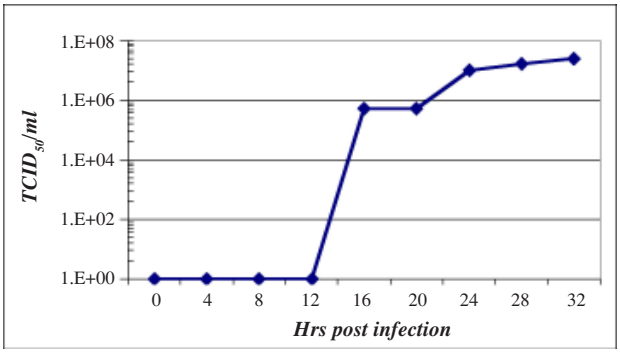

Figure 5 Growth curves of the virus in chicken embryonic eggs based on 50% infectivity dose determination (TCID$_{50}$/ml). The infectivity titers were between 5.7 to 7.4 log 10 TCID$_{50}$/ml.

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