

Kertas Asli/Original Article

Significant Replication Time-points of Avian Influenza A Virus Strain H5N1 in Madin-Darby Canine Kidney Cells

(Corak Replikasi Virus Influenza A Avian Strain H5N1 dalam Sel *Madin-Darby Canine Kidney*)

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ABSTRACT

The occasional influenza pandemics and the seasonal influenza epidemics have destroyed millions of lives since the last century. It is therefore necessary to understand the virus replication patterns as this provides essential information on the virus infectivity, pathogenicity and spread patterns. This study aimed to investigate the replication of avian influenza A virus H5N1 (A/Chicken/Malaysia/5858/2004) in MDCK cells. In this study, the TCID₅₀ (50% tissue culture infectious dose) of AIV H5N1 was first determined. The MDCK cells were then infected with AIV H5N1 at TCID₅₀ for 0-48 h. The CPE (cytopathic effect) was observed and cell death was determined hourly. The virus-infected cells and media were subsequently collected for gene analysis. The results showed that the TCID₅₀ of AIV H5N1 was 10⁻⁹ dilution. The CPE percentage showed a strong and positive correlation with the infection period ($r = 1.0$, $n = 9$, $p < 0.01$). The amount of a highly conserved influenza viral gene, M2 gene amplified from infected media ($r = 0.471$, $n = 9$, $p > 0.05$) and infected cell ($r = 0.73$, $n = 9$, $p < 0.05$) were also positively correlated with the infection period. In conclusion, although CPE started to be observed in the early time points of infection, however, the M2 gene was only amplified from the infected media and cells after 48 h and 24 h, respectively. This signifies that AIV H5N1 used in this study is pathogenic and it is able to cause severe cytopathology to host cells even at low virus load.

Keywords: Replication; influenza; H5N1; MDCK cells

ABSTRAK

Pandemik dan epidemik influenza yang berkala telah meragut berjuta-juta nyawa sejak abad yang lalu. Hal ini memerihalkan kepentingan untuk memahami corak replikasi virus tersebut agar lebih memahami kebolehan untuk berjangkit, kepatogenan dan corak penyebaran virus tersebut. Kajian ini bermatlamat untuk mengkaji replikasi virus influenza avian (AIV) strain H5N1 (A/Chicken/Malaysia/5858/2004) dalam sel MDCK. Dalam kajian ini, TCID₅₀ (50% tissue culture infectious dose) AIV H5N1 telah ditentukan pada awalnya. Kemudian, sel MDCK dijangkiti dengan AIV H5N1 pada TCID₅₀ dari 0-48 jam. Kesan sitopatik (CPE) telah diperhatikan dan peratusan CPE ditentukan pada setiap jam pasca-infeksi. Sel dan media terjangkit seterusnya dikumpulkan untuk analisis gen. Hasil kajian menunjukkan bahawa TCID₅₀ AIV H5N1 ialah faktor pencairan 10⁻⁹. Peratusan CPE dalam sel MDCK berkorelasi secara positif dan signifikan ($r = 1.0$, $n = 9$, $p < 0.01$) dengan tempoh infeksi virus. Kuantiti gen terpelihara M2 yang diamplifikasikan daripada media ($r = 0.471$, $n = 9$, $p > 0.05$) dan sel terjangkit ($r = 0.73$, $n = 9$, $p < 0.05$) juga berkorelasi secara positif dengan tempoh infeksi virus. Konklusinya, walaupun CPE mula berlaku pada peringkat awal infeksi, namun gen M2 hanya dapat dikesan dalam media dan sel terjangkit selepas 48 jam dan 24 jam pasca-infeksi masing-masing. Hal ini menandakan AIV H5N1 yang digunakan dalam kajian ini amat patogenik dan mampu mengakibatkan kesan sitopatologi yang teruk pada sel perumah walaupun dalam titer virus yang rendah.

Kata Kunci: Replikasi; influenza; H5N1; sel MDCK

INTRODUCTION

Avian Influenza A virus (AIV) belongs to the *Orthomyxoviridae* family which carries negative-sense, single-stranded and segmented RNA genome. AIV is associated with yearly epidemics as well as sporadic pandemics. The natural reservoir of AIV has been identified as wild aquatic birds (Sonnberg et al. 2013).

Since the first recorded direct bird-to-human transmission of H5N1 virus in Hong Kong in 1997, the

virus has spread to countries in Asia, Middle East, Africa and Europe. The virus causes death and illness in domestic and migratory birds, as well as vulnerable human beings (Subbarao & Matsuoka 2013; Hatta et al. 2011).

Knowledge on the growth of influenza virus in cell culture provides insights in understanding the virus replication. This is particularly crucial for the development of influenza vaccine (Matsuoka et al. 2013; Wanasawaeng et al. 2009; Abdoli et al. 2013). To date, a few susceptible cell lines including *Madin-Darby Canine*

Kidney (MDCK) cell and human lung adenocarcinoma (A549) cell lines have been used to study the pathogenesis influenza virus. These cell lines are useful to study viral entry, replication and virus progeny production (Daidoji et al. 2008).

Today, chicken embryonated eggs is still the system of choice for mass production of influenza vaccine. However, this system comes along with several flaws, not only it is labour-intensive, but it is also prone to microbiological contamination (Youil et al. 2004; Pan et al. 2013). Hence, an egg-free system such as animal cell culture is gaining momentum in influenza vaccine production (Murakami et al. 2008).

In view of this, information on AIV H5N1 replication in MDCK cells will generate a better understanding on the viral pathogenicity. In this study, it was found that the replication of AIV H5N1 virus is time-dependent post-infection. The host cell was a better source to harvest the virus compared to its culturing media. The appropriate time to harvest the virus was after 48 hours when maximal cell death occurred. Overall, this study emphasizes on cell culture based influenza vaccine production using animal cell lines as susceptible host cells.

MATERIAL AND METHODS

TCID₅₀ ASSAY

Modified from Flint et al. (2008) and Lau et al. (2010), 1 x 10⁶ MDCK cells were grown on 12-well plates in RPMI-1640 medium at 37°C under 5% CO₂ until confluent. About 20 µl of each AIV H5N1 virus dilution (10⁻² to 10⁻¹²) was inoculated into four wells and incubated for 20 min at 37°C under 5% CO₂. The media was then discharged and replaced with fresh media. The cells were further incubated for 24 h before CPE determination. Uninfected cells were prepared as negative control. TCID₅₀ is a virus dilution where two of the four wells showing CPE.

ASSESSMENT OF CPE DEGREE

1 x 10⁶ MDCK cells in T-25 flasks were grown until confluent before infecting with 100 µl of AIV H5N1 at TCID₅₀. The infected cells were incubated in RPMI-1640 medium at 37°C under 5% CO₂ for 20 min. The media was discharged and replaced with fresh media. The cells were further incubated for 0, 2, 4, 6, 8, 10, 12, 24 and 48 hours post-infection (h p.i). Uninfected cells were prepared along as negative control. At each time point (0-48 h p.i), the CPE were assessed by quantitating the number of dead cells using a haemocytometer and 0.4% (w/v) trypan blue.

EXTRACTION AND AMPLIFICATION OF M2 GENE FROM INFECTED SAMPLES

At each time point (0-48 h p.i), infected media and cell were separated by spinning down the cell pellet at 3000xg for 5 min. Total RNA extraction from the samples were carried out by using Trizol LS (Invitrogen, New York, USA) in 1:3 ratio (0.25 ml sample:0.75 ml Trizol LS). The extracted total RNA was normalised to 38 ng/ul before proceeding to first strand cDNA synthesis with oligo-dT primers (Promega, Madison, USA) and AMV reverse transcriptase (Promega, Madison, USA). The first strand cDNA served as the template for amplification of AIV H5N1 M2 gene. The M2 gene amplification was carried out using EconoTaq Plus Green DNA polymerase master mix (Lucigen, Middleton, USA). The M2-specific primers are as follows:

M2 Forward: 5' AGAATT CAG TCT TCT AAC CGA GGT CGA AAC GCC TAC CAG AAA CGA A 3'

M2 Reverse: 5' AGT CGA CCT CCA ATT CTA TGT TGA CAA AAT G 3'

The PCR thermocycle was performed as follows: 95°C for 2 min, 95°C for 30 s (x 25 cycle), 55°C for 30 s (x 25 cycle), 72°C for 60 s (x 25 cycle) and 72°C for 10 min before cooling down to 4°C. The PCR products were analysed on a 1% (w/v) agarose gel electrophoresis at 100V for 30 min. The agarose gel was then stained with ethidium bromide for 5 min before observed under the Gel-Doc Imaging System (Biorad, California, USA) under which the intensity of the amplified M2 gene was determined.

STATISTICAL ANALYSIS

Results were expressed as mean ± SEM and analysed using *Pearson correlation* (for normally distributed data) or *Spearman correlation* (for not normally distributed data) to determine the relationship between virus replication period and CPE percentage in MDCK cell. These tests were also used to determine the relationship between the virus replication period and quantity of M2 gene amplified in the study. The statistical analysis was conducted using Statistics Package for Social Sciences (SPSS) version 20.0.

RESULTS

TCID₅₀ ASSAY

Table 1 shows the CPE percentage in MDCK cells caused by AIV H5N1 virus in a series of dilutions. The CPE decreased when the dilution factor increased. At dilution 10⁻⁹, two of the four wells inoculated with AIV H5N1 exhibited CPE hence the CPE percentage was 50%. The TCID₅₀ for AIV H5N1 virus used in the experiment was 10⁻⁹. It is therefore

concluded that the titre of the initial virus stock is 10^9 TCID₅₀/0.02 ml.

TABLE 1. CPE percentage for each AIV H5N1 virus dilution

Dilution (10-x)	Number of well showed CPE	CPE percentage (%)
2	4	100
3	4	100
4	4	100
5	4	100
6	4	100
7	4	100
8	3	75
9	2	50
10	1	25
11	0	0
12	0	0

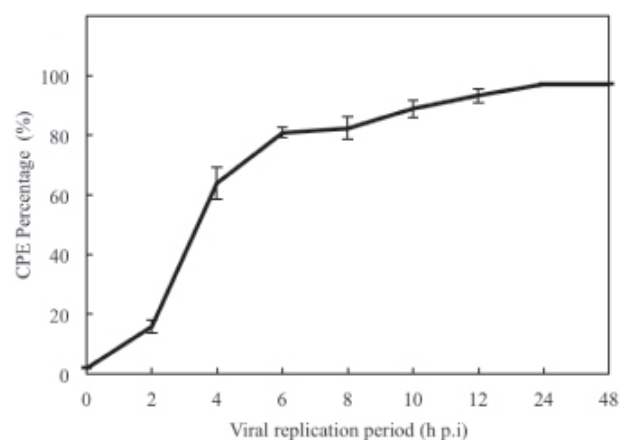


FIGURE 1. Line graph showing the CPE percentage versus viral replication period. The curve shows an ascending trend and peaks at 48 h.p.i. Data are reported in mean \pm SEM ($n = 3$)

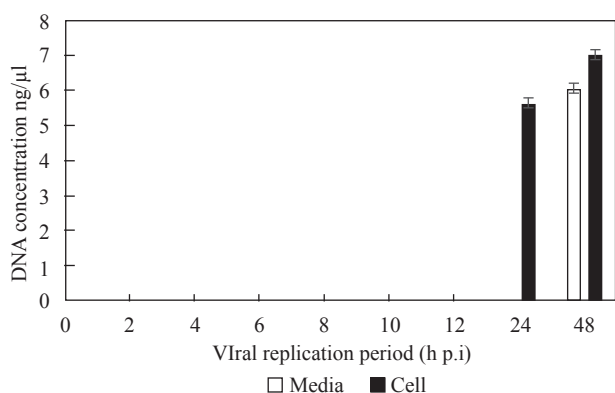


FIGURE 2. Bar chart showing the DNA concentration in infected media and MDCK cell versus viral replication period. Data are reported in mean \pm SEM ($n = 3$)

PERCENTAGE OF CELL DEATH

Figure 1 shows the CPE percentage versus viral replication period. The CPE percentage began as early as 2 h p.i ($15.93 \pm 2.31\%$) and increased tremendously and reached the peak at 48 h p.i. ($97.30 \pm 0.40\%$). The most remarkable increase in the CPE percentage occurred between 2 h p.i ($15.93 \pm 2.31\%$) to 4 h p.i ($64.06 \pm 5.44\%$). Overall, the curve shows an ascending trend and is consistent with statistical analysis in which CPE in MDCK cells due to AIV H5N1 infection correlates significantly ($p < 0.01$) with viral replication period. The correlation is positive and strong ($r = 1.0$).

QUANTIFICATION OF M2 GENE AMPLIFIED

Figure 2 shows that the M2 gene remained undetectable in infected media at 0-24 h p.i. The concentration of M2 gene amplified from infected media at 48 h p.i was 6.03 ± 0.059 ng/ μ l. The correlation between viral replication period and concentration of M2 gene in the infected media is positive but not significant ($r = 0.471$, $p > 0.05$). For infected MDCK cells, no M2 gene was detected from cells at 0-12 h p.i (Figure 2). The M2 gene was only able to be amplified from infected cells after 24 h p.i with a concentration of 5.56 ± 0.085 ng/ μ l and this increased consistently to 7.01 ± 0.213 ng/ μ l at 48 h p.i. The correlation between viral replication period and concentration of M2 gene in infected MDCK cells is significant ($p < 0.05$) and positive ($r = 0.73$).

DISCUSSION

The avian influenza virus (AIV) strain H5N1 used in this study was isolated in Malaysia in year 2004 [A/chicken/Malaysia/5858/2004(H5N1)]. MDCK cell line was chosen as a susceptible host cells in this study because it is globally recognised and recommended by WHO for cultivation of various influenza virus strains (WHO 2005).

TCID₅₀ (50% tissue culture infectious dose) is a virus infective dose that infects 50% of the monolayer cells after incubation for a certain period of time (Ward et al. 1984). Determination of infectivity of a virus is usually done by analysing cytopathic effects caused by the virus infection (Flint et al. 2008). In this study, it was found that the TCID₅₀ of AIV H5N1 used in the study was at dilution 10^{-9} . It is of paramount importance to determine TCID₅₀ of a virus infection as it suggests an appropriate virus dose to be used in following experiments.

The MDCK cells was infected with 0.1 ml of AIV H5N1 at TCID₅₀ (dilution 10^{-9}). CPE was observed and cell death represented as CPE percentage was calculated at each designated time point (0, 2, 4, 6, 8, 10, 12, 24 and 48 h p.i). CPE in infected MDCK cells started to manifest at 2 h p.i where it increased remarkably from a mere $1.97 \pm 0.32\%$ (0 h p.i) to $15.93 \pm 2.31\%$ (2 h p.i). The fact that CPE started

to be observed at such an early stage of infection indicates that this virus strain is considerably virulent.

The most severe CPE was observed at 48 h p.i when almost all infected MDCK cells detached from the flask. It is therefore recommended that the virus should be harvested after 48 h p.i for optimal virus derivation. This is supported by previous studies in which virus collection from MDCK cells was usually conducted after 48 h p.i (Aggarwal et al. 2011; Abdoli et al. 2013) or 72 h p.i (Wanasawaeng et al. 2009).

To analyse the distribution of infectious viral particles in infected media and MDCK cells, M2 gene was used as an indicator for the presence of AIV H5N1 in both the cell and media samples. AIV H5N1 M2 gene is highly conserved (Subbarao & Matsuoka 2013) and hence suitable to be used in virus detection using PCR. To ensure the accuracy and validity of M2 gene amplification, the total RNA extracted from both the media and MDCK cells were normalised to 38 ng/ μ l, which was also the lowest concentration among the collected samples.

The presence of M2 gene in infected media was only observed at 48 h p.i. The correlation between viral replication period and concentration of M2 gene is positive but not significant. This could be attributed to the lack of data for comparison since the M2 gene was only detected in media after 48 h p.i.

For infected MDCK cells, the presence of M2 gene was detected as early as 24 h p.i. The concentration of the M2 gene then increased from 5.56 \pm 0.085 ng/ μ l (24h p.i) to 7.01 \pm 0.213 ng/ μ l (48 h p.i). The correlation between viral replication period and concentration of M2 gene is strong and significant. This indicates that the virus titre in infected cells increased along with viral replication period.

During AIV H5N1 replication period, a few questions arose regarding the discrepancy in which the CPE in infected MDCK cells was observed much earlier than the time point where M2 gene was able to be amplified from the samples. This scenario has also been observed by Aggarwal et al. (2011). The authors reported that the degree of CPE could not be a definite indicator for the actual virus titre because it varies depending on the strain and virulence of the virus. The early CPE observed in this study proves that the virus strain used in this experiment is considerably virulent and therefore able to cause cytopathological harm in susceptible host cells over a short period of infection at low virus titre.

The titre of AIV H5N1 was also found to be lower in infected media compared to that found in infected cells. This is mainly because host cells provide important biological machinery for the production of virus particles and its genomes (Whittaker 2001). Besides, Nayak et al. (2009) also reported that budding of influenza virus from the host cell to its surrounding is rather inefficient. Only a small fraction of virus buds are released while the majority of virus progeny still attach to the host cell membrane even though they appear mature. As a result,

infectious viral particles of AIV H5N1 were less in the media than the cells. This study suggests that host cells are a more suitable source to harvest the virus compared to the media in cell-culture-based influenza vaccine production. The optimal harvesting time for influenza virus particularly in MDCK cells is after 48 h p.i.

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