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Replication of a Malaysian Strain Avian Influenza A Virus H5N1 in Madin-Darby Canine Kidney and African Green Monkey Kidney Cells

(Replikasi Virus Influenza Avian A Jenis H5N1 dalam Sel Ginjal Kanin Madin-Darby dan Monyet Hijau Afrika)

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ABSTRACT

The use of cell lines such as Madin-Darby Canine Kidney (MDCK) and African Green Monkey Kidney (Vero) cells in influenza vaccine production is much advocated presently as a safer alternative to chicken embryonated eggs. It is thus essential to understand the influenza virus replication patterns in these cell lines prior to utilizing them in vaccine production. The infectivity of avian influenza A virus (A/Chicken/Malaysia/5858/2004) H5N1 in MDCK and Vero cell lines was first assessed by comparing the cytopathic effect (CPE) caused by the virus infection. The viral loads in both of the infected media and cells were also compared. The results showed that both of the MDCK and Vero cells began to exhibit significant CPE ($p<0.05$) after 48 h post-infection (h p.i). The MDCK cell line was more susceptible to the virus infection compared to Vero cell line throughout the incubation period. A higher viral load was also detected in the host cells compared to their respective culturing media. Interestingly, after reaching its maximum titer at 48 h p.i, the viral load in MDCK cells declined meanwhile the viral load in Vero cells increased gradually and peaked at 120 h p.i. Overall, both cell lines support efficient H5N1 virus replication. While the peak viral loads measured in the two cell lines did not differ much, a more rapid replication was observed in the infected MDCK samples. The finding showed that MDCK cell line might serve as a more time-saving and cost-effective cell culture-based system compared to Vero cell line for influenza vaccine production.

Keywords: Cytopathic effect; H5N1; MDCK; Vero; viral load

ABSTRAK

Penggunaan kultur sel seperti sel ginjal kanin Madin-Darby (MDCK) dan monyet hijau Afrika (Vero) dalam penghasilan vaksin influenza adalah lebih selamat berbanding telur ayam berembrio yang disarankan pada masa ini. Maka, adalah penting untuk kita memahami corak replikasi virus influenza dalam sel-sel tersebut sebelum digunakan dalam penghasilan vaksin. Keboleh-jangkitan virus influenza jenis H5N1 (A/Chicken/Malaysia/5858/2004) dalam sel MDCK dan Vero dinilai dengan membandingkan kesan sitopatik (CPE) yang diakibatkan oleh jangkitan virus influenza. Titer virus dalam media dan sel terjangkit turut dibandingkan. Hasil kajian menunjukkan kedua-dua sel MDCK dan Vero mula mempamerkan CPE yang signifikan ($p < 0.05$) selepas 48 jam pasca-infeksi (h p.i). Sel MDCK adalah lebih rentan kepada jangkitan virus sepanjang tempoh eraman virus berbanding dengan sel Vero. Titer virus yang lebih tinggi diperoleh dalam sel perumah terjangkit berbanding dengan media kultur. Selepas mencapai titer maksimum pada 48 h p.i, titer virus dalam sel MDCK menurun manakala titer virus dalam sel Vero kekal meningkat secara perlahan dan memuncak pada 120 h p.i. Secara keseluruhan, kedua-dua sel perumah didapati mampu menyokong replikasi virus H5N1 dengan cekap. Meskipun titer virus maksimum yang dicapai antara kedua-dua sel perumah tidak berbeza, replikasi yang lebih pantas diperhatikan dalam sel MDCK. Hal ini mencadangkan penggunaan sel MDCK dalam penghasilan vaksin influenza adalah lebih jimat daripada segi masa dan kos berbanding dengan sel Vero.

Kata kunci: H5N1; kesan sitopatik; MDCK; titer virus; Vero

INTRODUCTION

Avian influenza A virus (AIV) is associated with yearly epidemics as well as sporadic pandemics (Sonnberg et al. 2013). Since the first recorded direct bird-to-human transmission of highly pathogenic avian influenza (HPAI) virus H5N1 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).

Information on the growth of influenza virus in cell lines provides insights in understanding the patterns of virus replication. This is particularly crucial for the development of influenza vaccine (Abdoli et al. 2013; Wanasawaeng et al. 2009). Knowledge on the AIV replication and subsequently its host response are of utmost importance in developing an appropriate intervention against the virus (Matsuoka et al. 2013).

Today, chicken-embryonated-egg (CEG) is a system of choice for large scale production of influenza vaccine.

However, this system has several downfalls. Besides being labor-intensive, it is also prone to microbiological contaminants (Pan et al. 2013; Youil et al. 2004). Moreover, CEG is likely to be in short supply in the event of an influenza pandemic considering most chickens will be culled to curb the spread of the virus (Murakami et al. 2008). Hence, the use of cell culture in influenza vaccine production is gradually gaining momentum. The egg-free system is known to support a rapid and larger scale of vaccine production (Murakami et al. 2012).

The development of cell culture system for virus propagation has brought to major advances in virus vaccine production. Till date, the frequently used cell lines for influenza virus propagation include MDCK and Vero cells (Donis 2014). MDCK-derived influenza vaccine has shown promising protection in mouse model and its efficacy is antigenically equivalent to egg-derived influenza vaccines (Nerome et al. 1999) whilst the immunogenicity of Vero cell-derived influenza virus vaccine was comparable to that of the egg-derived vaccine in the context of humoral and cellular responses (Brühl et al. 2000). These findings substantiate that MDCK and Vero are ideal cell lines for cell culture-based influenza vaccine development.

A thorough comparison of virus growth in cell lines might help to develop an optimized vaccine production strategy, as well as to assess quality differences concerning the virus strains and antigen produced (Genzel et al. 2010). In this study, a Malaysian isolate of HPAI H5N1 (A/chicken/Malaysia/5858/2004) virus was used (Balasubramaniam et al. 2011). The AIV H5N1 replication in MDCK and Vero cells was compared by measuring the virus CPE in terms of percentage of cell death and viral load in both of the infected media and cells at designated time points.

Materials and Methods

CELLS AND VIRUS

MDCK and Vero cells were obtained from American Type Culture Collection (ATCC) and were maintained in Roswell Park Memorial Institute medium (RPMI-1640, Gibco, Karlsruhe, Germany) supplemented with 10% (v/v) fetal bovine serum (FBS, Gibco, Karlsruhe, Germany) and 100 µg/mL ampicillin. The cells were grown at 37°C in a 5% CO_2 incubator. AIV H5N1 isolate (A/chicken/5858/2004/Malaysia) was propagated as described in Balasubramaniam et al. (2011). A serial dilution of the virus (10^{-1} to 10^{-8}) was done in RPMI-1640 medium.

MEASUREMENT OF VIRUS INFECTIVITY

50% tissue culture infectious dose ($TCID_{50}$) of the AIV H5N1 was determined in MDCK and Vero cells, as described previously (Hamilton et al. 2011) with minor modifications. In addition, plaque assay was conducted to calculate the multiplicity of infection (MOI) of the virus. Briefly, 200 µL of each virus dilution (10^{-1} to 10^{-8}) was inoculated into confluent cells and incubated for 1 h. The plates were shaken gently every 15 min. The media was then discarded, washed and replaced with an agar overlay (0.4% (w/v) agarose in RPMI-1640 media). On the fifth day, the cells were fixed with 4% (v/v) formaldehyde for 1 h. The formaldehyde was then aspirated out and the agar overlay was removed. The cells were stained with 0.5% (w/v) crystal violet for 5 min before plaque visualization and counting. Uninfected cells were prepared as negative control.

ASSESSMENT OF CPE PERCENTAGE

1×10^6 MDCK and Vero cells were grown in T-25 flasks until confluency. The cells were then transfected with 500 µL of AIV H5N1 at MOI 0.1 and incubated for 1 h. The media was then discarded, washed and replaced with new media. The cells were further incubated for 0, 4, 8, 12, 24, 48, 72, 96 and 120 h p.i. Uninfected cells were prepared along as the negative control. At each time point, CPE were assessed by calculating cell death using hemocytometer and 0.4% (w/v) trypan blue staining method.

VIRUS RNA ISOLATION

At each time point (0-120 h p.i), the infected culturing media and cell were isolated separately. Total RNA extraction from the samples was carried out by using Tri-RNA reagent (Favorgen, Pingtung, Taiwan) following the manufacturer's instructions. About 90 ng of total RNA was used to generate cDNA using a cDNA synthesis kit (PCR Biosystems, London, UK).

REAL TIME PCR

Real time PCR was carried out using the 2× qPCRBIO SyGreen Mix Hi-ROX (PCR Biosystems, London, UK) according to the manufacturer's instructions. For viral detection, forward primer (5'- AGA ATT CAG TCT TCT AAC CGA GGT CGA AAC GCC TAC CAG AAA CGA A -3') and reverse primer (5'- AGT CGA CCT CCA ATT CTA TGT TGA CAA AAT G -3') were used for the amplification of AIV H5N1 Matrix 2 (M2) gene. A 10-fold serial dilution of known concentration (2.53×10^8 copies) of a pure plasmid DNA, pRSET-NS1 (unpublished data), was performed to construct a 5-point standard curve. The forward primer (5'- AAG GAT CAT AGC TCG AGA GAT TCC AAC ACT GTG -3') and reverse primers (5'- GCA GTT TTC GAA TTC AAC TTC TGA CTC AAT -3') were used for the amplification of the standard. Thermal cycling was performed in the Step One Plus systems (Applied Biosystems, California, USA) with PCR cycling conditions: 90°C for 2 min, 40 cycles of 95°C for 30 s, 40 cycles of 60°C for 30 s and 72°C for 1 min. The primer-dimer melting step was included to monitor the amplification of the products. Gene copies of the test samples were calculated as gene copies/µL based on the standard curve and reported as log10 values.

STATISTICAL ANALYSIS

Data analysis was conducted with Statistics Package for Social Sciences (SPSS) version 20.0. Independent sample *t* test was used to examine the significance of CPE percentage between infected and uninfected cell. Spearman's (rho) correlation was used to assess the relationship between CPE percentage and the viral load in samples.

RESULTS

MEASUREMENT OF VIRUS INFECTIVITY

The $TCID_{50}$ was calculated based on the Reed-Muench method (Reed & Muench 1938). The $TCID_{50}$ of AIV H5N1 for MDCK and Vero cells were $10^{5.67}$/mL and $10^{3.5}$/mL, respectively. Meanwhile, the MOI of AIV H5N1 in MDCK cell was 0.944 while in Vero cell was 0.157. The nearly 100-fold higher $TCID_{50}$ value and nearly six-fold higher MOI value in MDCK cell indicates a higher viral titer production in the cell line compared to that by the Vero cell.

ASSESSMENT OF CPE PERCENTAGE

The replication of the AIV H5N1 was observed by assessing the CPE percentage in MDCK and Vero cells. As shown in Figure 1, both MDCK and Vero cells started to exhibit significant CPE after 48 h p.i ($p < 0.05$). MDCK cells appeared to be more susceptible to the virus infection than Vero cells throughout the viral incubation period. The CPE percentage, for MDCK and Vero cells, peaked at 90.55±1.00% and 80.48±0.34%, respectively, after 120 h p.i.

REAL TIME PCR

The primers specificity was verified in a melting curve analysis (data not shown). There was no noticeable amplification in negative control and non-template control samples. The viral loads of AIV H5N1 in MDCK and Vero cells were measured by absolute quantification of the target gene, M2 in the virus-infected culturing media and cells. It was found that the CPE percentage correlated positively and significantly with the viral load in the infected MDCK cells ($rho = 0.674$, $p < 0.001$) (Figure 2(a)) and Vero cells ($rho = 0.925$, $p < 0.001$) (Figure 2(b)). As shown in Figure 3, the viral load in the MDCK culturing media was higher than that of Vero throughout the incubation period, with 6.37±0.009 and 6.31±0.074 log10 gene copies/μL, respectively at 120 h p.i. However, the similar trend was not observed in the cells. The viral load in MDCK cells increased drastically after 12 h p.i and peaked at 48 h p.i with 7.08±0.025 log10 gene copies/μL. In the later time points, the viral load in MDCK cells showed a gradual decreasing trend and finally declined to 5.90±0.009 log10 gene copies/μL at 120 h p.i. Whereas in Vero cells, the increase in viral load was rather constant although slower. It continued to increase throughout 120 h p.i where the viral load peaked at 7.36±0.031 log10 gene copies/μL. By comparing the viral load in culturing media and infected cells, it was found that the viral loads in MDCK and Vero cells were significantly higher than in the culturing media. In MDCK samples, the viral load measured at 120 h p.i appeared to be slightly higher in the culturing media (6.37±0.009 log10 gene copies/μL) than that present in the cells (5.09±0.009 log10 gene copies/μL). Overall, the findings imply that a

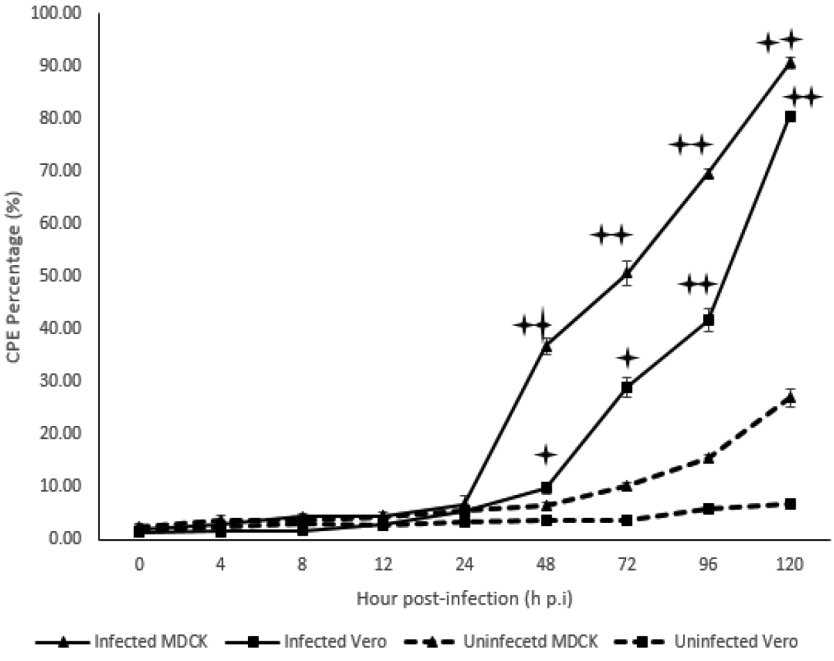

FIGURE 1. CPE percentage of infected and uninfected (negative control) MDCK and Vero cells. The data are reported in mean value ± SEM (n=3). The symbol indicates statistically significant ($p < 0.05$) whereas indicates statistically very significant ($p < 0.001$) compared to the negative control

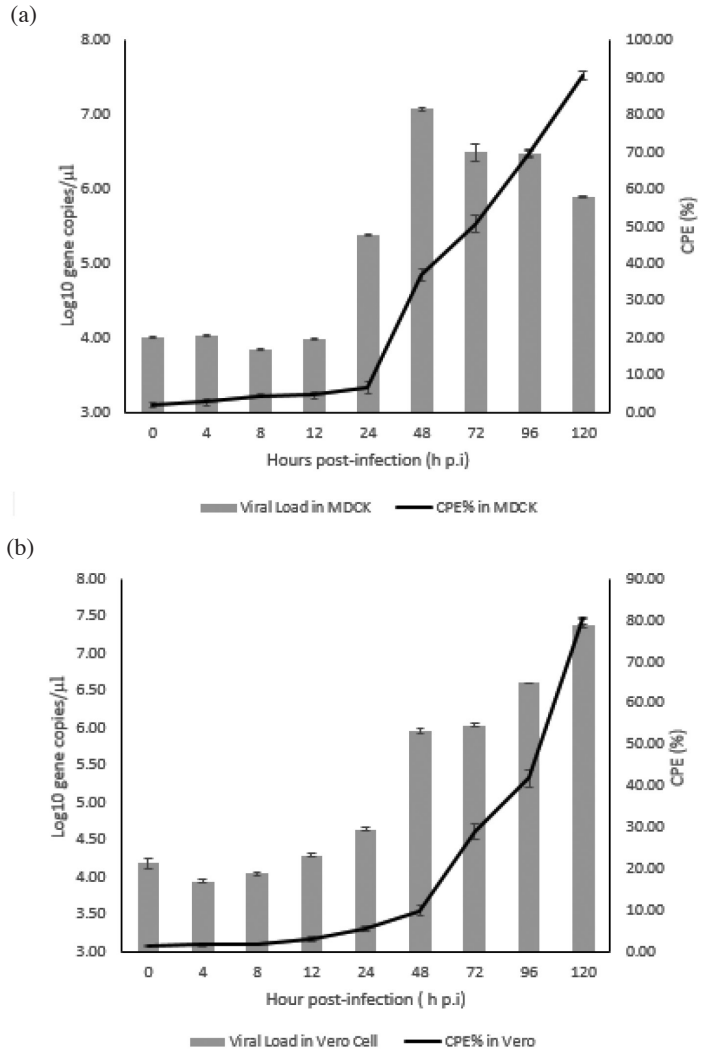

FIGURE 2. Correlation of viral load and CPE percentage in infected cells. The data are reported in mean value ± SEM (n=3). (A) The viral load in infected MDCK cells correlated positively and significantly with CPE (rho = 0. 674, $p < 0.001$). (B) The viral load in infected Vero cells correlated positively and significantly with CPE (rho = 0.925, $p < 0.001$)

faster virus replication is taking place in MDCK cells than that in Vero cells.

DISCUSSION

Substitution of the egg-based system by the cell culture approach for inactivated or attenuated influenza vaccine production has raised the question of which cell line is more suitable for virus cultivation. Theoretically, a higher virus titer produced in a cell line represents a larger scale of vaccine production is permitted by the particular cell line. Hence, this study aimed to compare the replication of a Malaysian strain AIV H5N1 in MDCK and Vero cell lines. The CPE exerted by the AIV H5N1 infection on the host cells and also the viral loads formed in the host cells and culturing media were evaluated at designated time points. MDCK and Vero cell lines were selected for virus replication in this study because they are recognized widely and are recommended by the World Health Organization (WHO) for the propagation of various influenza viruses (WHO 2005).

First and foremost, $TCID_{50}$ and MOI of the AIV H5N1 virus seed used in the study were determined. It was found that higher $TCID_{50}$ and MOI values of AIV H5N1 were achieved by propagating the virus in MDCK cells than in Vero cells. The higher $TCID_{50}$ and MOI values obtained from the MDCK samples suggest that the MDCK cell line could be a better cell-based system to produce AIV H5N1. In this light, a more reliable and sensitive quantification approach, real-time PCR was employed to measure the viral load in MDCK and Vero cells.

In order to ensure that the AIV H5N1 was provided with sufficient host cells for infection, the MDCK and Vero cells were infected with a relatively low virus titer at MOI 0.1. Both of the cell lines began to exhibit significant CPE, particularly cell death after 48 h p.i. The infected MDCK

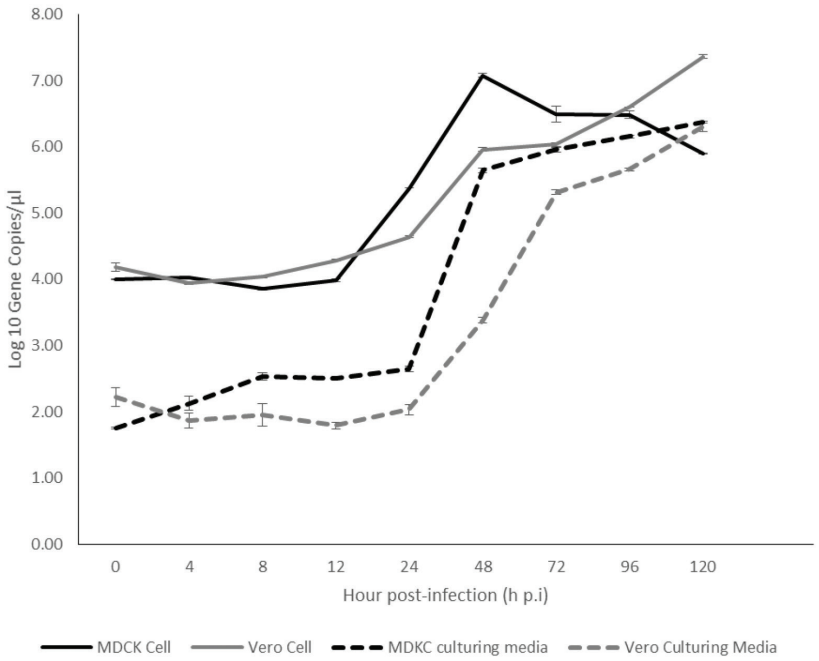

FIGURE 3. Comparison of AIV H5N1 viral load in the infected cells and their respective culturing media. The data are reported in mean value ± SEM (n=3)

cells appeared to be more vulnerable to the virus infection compared to Vero cells throughout the incubation period. This observation parallels several previous studies in which MDCK cells were shown to be highly susceptible to influenza virus compared to the other host cell types (Lugovtsev et al. 2013; Seitz et al. 2010). The higher percentage of cell death could also be explained by a higher viral load produced in MDCK cells at early time points of infection.

The level of viral load in patients has been demonstrated as one of the important indicators in influenza H5N1 pathogenesis (De Jong et al. 2006; Lau et al. 2010). The amount of virus that is present early in the course of infection reflects its direct CPE on host cells (El Saleeby et al. 2011). Generally, in this study, it was shown that the CPE percentage correlated positively and significantly with the viral load in both MDCK and Vero cells. In later time points, a gradual decline in the viral load was observed after 48 h p.i in MDCK cell. However, the CPE percentage continued to soar. Permissive host cells are important biological machinery for the production of virus particles (Whittaker 2001). Hence, the scarcity of viable cells due to high CPE in the later time points could account for reduction in the viral load produced in MDCK cells whereas in Vero cells, the CPE percentage and the viral load increased consistently but at a slower rate.

By comparing the culturing media of MDCK to that of Vero, a higher viral load was observed in MDCK culturing media throughout the incubation period. However, the maximum viral load measured in culturing media for both cell lines after 120 h p.i was almost identical (~6.30 log10 gene copies/µL). In infected host cells, a more rapid viral replication was observed in MDCK cells compared to Vero cells. The maximum viral load was achieved within 48 h p.i in MDCK cells whereas the maximum viral load was achieved later in Vero cell at 120 h p.i. The similar phenomenon was also observed in a number of previous studies (Govorkova et al. 1995; Youil et al. 2004). Overall, both of the cell lines have been proven to be capable of permitting efficient AIV H5N1 replication.

When analyzing the distribution of AIV H5N1 in the infected media and host cells, a higher viral load was detected in MDCK and Vero cells compared to their respective culturing media. This finding further ascertains a similar observation in a previous study which demonstrated the inefficiency of influenza viral shedding (Nayak et al. 2009). The virus particles were found not to be released readily from the host cells into the surrounding during the virus replication. However, the viral load in culturing media, albeit lower than that in host cell, is still remarkably important in producing influenza virions. Interestingly, a smaller viral load difference was detected between the host cells and culturing media at the later time points of infection. This scenario could be attributed to the release of more virus particles from the cells into the culturing media at the later time point, which is mostly due to apoptosis (Hinshaw et al. 1994). The accumulated infectious particles could not carry out further infection because more than 90% of the infected cells had died. In view of this, the viral load in the MDCK culturing media was therefore slightly higher than that in the infected MDCK cells after 120 h p.i.

CONCLUSION

In conclusion, both MDCK and Vero cell lines support efficient AIV H5N1 replication. Even though the maximum

viral load achieved in both cell lines did not seem to differ significantly, a more rapid replication was observed in infected MDCK host cell. This study suggests a more time-saving and hence cost-effective MDCK cell based influenza vaccine production.

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