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Growth Characteristics of Chikungunya Virus Isolate from Indonesia in Various Human Cell Lines *in vitro*

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Chikungunya (CHIK) fever, a febrile illness caused by Chikungunya virus (CHIKV) infection, is one of mosquito-borne viral diseases affecting people living in the tropical and subtropical regions in the world. The pathogenesis of the disease is yet to be completely unraveled, and research on CHIK has been conducted by employing various methods, including using cell lines to investigate the biological characteristics of CHIKV *in vitro*. To assess the suitability of human cell line model for CHIK study, various human cell lines including A549, Huh7, and HepG2 were infected with CHIKV and assayed for their susceptibility to infection. The MTT and plaque assay methods were performed to measure cell viability and virus growth kinetics, respectively. Fluorescence-activated Cell Sorting (FACS) and immunofluorescence assay were performed to measure the proportion of infected cells in the system and their morphological visualization. Both A549 and Huh7 human cell lines showed stable high cell viability upon infection while CHIKV growth kinetics were significantly lower in these cells compared to Vero-CCL81, a monkey cell line that is routinely used in other arboviruses research. Interestingly, we observed significantly different results in HepG2 human cell line, in which cell viability and CHIKV growth kinetics were significantly higher. FACS and immunofluorescence assay confirm the higher infection rate of CHIKV in HepG2 than A549 human cell line. We concluded here that human hepatocytes HepG2 cell line was susceptible to Asian Genotype of CHIKV and proposed as an alternative cell for the *in vitro* CHIKV studies to the commonly used A549 and Vero cells.

Keywords: cell viability, Chikungunya, FACS, growth kinetic, HepG2

Penyakit demam Chikungunya yang disebabkan oleh infeksi virus Chikungunya (CHIKV) adalah penyakit tular-vektor yang menjangkiti penduduk di daerah tropis dan subtropis. Patogenesis penyakit ini masih belum sepenuhnya diketahui, dan penelitian terus berlangsung menggunakan berbagai metode, termasuk menggunakan galur sel untuk melihat karakteristik CHIKV secara *in vitro*. Untuk mengetahui kemampuan berbagai galur sel manusia yang sesuai untuk penelitian chikungunya secara *in vitro*, sel A549, HepG2 dan Huh7 diinfeksi dengan CHIKV dan dilihat kemampuannya dalam merespon infeksi virus. Uji MTT dan *plaque assay* dilakukan untuk memeriksa viabilitas dan kinetika pertumbuhan CHIKV pada sel yang telah diinfeksi. Analisis secara *Fluorescence-activated Cell Sorting (FACS)* dan *immunofluorescence assay* dilakukan untuk mengukur proporsi sel terinfeksi dan visualisasi secara morfologi. Sel manusia A549 dan Huh7 menunjukkan viabilitas sel lebih tinggi dan stabil namun kinetika pertumbuhan virus lebih rendah dibandingkan dengan Vero-CCL 81, galur sel monyet yang biasa digunakan pada penelitian arbovirus. Berbeda dengan sel lainnya, sel HepG2 menunjukkan viabilitas sel, kinetika pertumbuhan virus, dan titer virus yang tinggi. Hasil analisis *FACS* dan *immunofluorescence assay* mengkonfirmasi tingginya infeksi CHIKV pada sel HepG2 dibandingkan sel A549. Dari hasil tersebut terbukti bahwa galur sel manusia HepG2 dapat terinfeksi CHIKV Genotipe Asian dan dapat digunakan sebagai sel model alternative untuk penelitian CHIKV *in vitro* selain sel A549 dan Vero yang umum digunakan dalam penelitian arbovirus.

Kata kunci: Chikungunya, *FACS*, HepG2, kinetika pertumbuhan, viabilitas sel

Chikungunya fever (CHIK) is an acute febrile illness caused by infection of Chikungunya virus (CHIKV). The disease is notably characterized by a febrile illness typically associated with debilitating joint pain (Gasque *et al.* 2015; Horwood and Buchy 2015). The virus is a positive-stranded single strand RNA virus belongs to the genus of *Alphavirus* in *Togaviridae* family. The virus is transmitted to humans through mosquito vectors *Aedes aegypti* and *Ae.*

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albopictus. Symptoms of disease starts 4-7 days after mosquito bite and characterized by fever, headache, myalgia, rash, and joint pain (Laras *et al.* 2005). First-time isolated in Tanzania in 1952, CHIKV has now spread to tropical and subtropical regions in the world where the vector is prevalent. Chikungunya cases have been detected in various countries in West Africa and the virus's re-emergence has caused outbreak in Kenya in 2004 (Pialoux *et al.* 2007; Jain *et al.* 2008). The virus reached Indian Ocean and India in 2006-2007 which cause massive outbreak with significant economic and social impact. In Southeast Asia, chikungunya epidemic has been reported in Indonesia, Thailand, Cambodia, Vietnam, Myanmar and Philippines (Kumar *et al.* 2014). Cases have also been reported in Europe and America following the Indian outbreak in 2006-2007 (Amraoui and Failloux 2016; Yactayo *et al.* 2016).

The first reported case of CHIK in Indonesia was in Sumatera in 1982 which then spread to Java, Bali, Kalimantan, Flores and Sulawesi. The virus re-emerged in sporadic outbreaks in Java during the period of 2000-2002 (Laras *et al.* 2005) and sporadic cases have been reported from several provinces (Sari *et al.* 2017; Sasmono *et al.* 2017). Although the number of CHIK case was relatively lower than dengue (DEN), it should be interpreted with caution because similar symptoms of CHIK and DEN might cause the underreported cases in Indonesia especially with logistic constrain for viral diagnostics (Laras *et al.* 2005). In 2015, a dengue outbreak occurred in Jambi province in Sumatra, Indonesia (Haryanto *et al.* 2016) where from 103 non-dengue samples, 8 were reported to be CHIK (Perkasa *et al.* 2016; Sasmono *et al.* 2017).

With no suitable animal model that can fully reproduces the CHIK syndrome, the understanding of CHIK pathogenesis may relies much on *in vitro* studies (Gasque *et al.* 2015). Persistent liver tissues infection, liver tissue death occurs in non-human primate models, this indicates that's hepatocyte involvement in this infectious disease (Labadie *et al.* 2010). For this purpose, mosquito and vertebrate cell lines has been routinely used and among them certain cells have been proposed for their use in CHIKV studies, such as A549 and HepG2 (Wikan *et al.* 2012; Khongwichit *et al.* 2016). Moreover, studies have reported the role of liver as target organ for CHIKV infection and the associated severe clinical manifestations (Chua *et al.* 2010; Hoz *et al.* 2015; Chan and Leung 2017). In this study, we studied the growth characteristics of Indonesian CHIKV isolate on three different cell lines *in vitro* using local Indonesian strain of CHIKV. We aimed to assess the suitability of human cell lines for CHIKV studies *in vitro*.

MATERIALS AND METHODS

Cell Culture and Virus Propagation. Cells were originally obtained from American Type Culture Collection (ATCC) and have been maintained in Eijkman Institute's cells repository. The monkey kidney Vero (CCL-81) cell was maintained in MEM medium supplemented with 5% of Fetal Bovine Serum (FBS), 1% antibiotic/antimycotic (100 U mL^{-1} penicillin, 100 µg mL^{-1} streptomycin and 0.25 µg mL^{-1} Amphotericin B), and 2 mM of l-glutamine. The human cell lines A549 (lung epithelial alveolar), HepG2 (hepatocytes), and Huh7 (hepatocytes) cells were maintained in RPMI medium supplemented with 10% of FBS, 1% antibiotic/antimycotic, and 2 mM of l-glutamine (all from Gibco-Thermo Fisher Scientific). All cell lines were cultured at 37˚C and 5% Co_2.

CHIKV strain JMB-192 and JMB-230 (designated as CHIKV-JMB-192 and CHIKV-JMB-230), isolated from dengue-suspected patients from Jambi in 2015, have been characterized as Asian genotype (Sasmono *et al.* 2017). The DENV-1 strain JMB-034 (designated as DENV-1 JMB-034) isolated from dengue-suspected patients in Jambi in 2015, have been characterized as Genotype I (Haryanto *et al.* 2016). Viruses were propagated in Vero cells and harvested when 70-80% of CPE observed and stored in aliquots at -80°C freezer. Virus titer was measured by standard plaque assay as mentioned elsewhere (Wikan *et al.* 2012).

Cell Viability Assay. Cell viability assay were measured by classical 3-(4,5-Dimethyl-thiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay. Vero-CCL 81, A549, HepG2 and Huh 7 were seeded 1×10^5 cells/well in 96 well plate. After overnight incubation, the cells were infected with CHIKV-JMB-230 at multiplicity of infection (MOI) of 0.1 (theoretically one virus particle per 10 cells). The infection was allowed for 1 hour at 37°C, 5% CO_2. The inoculation medium was removed from wells and replenished by fresh medium. The plates were incubated at 37°C, 5% CO_2 and tested for cells' viability daily from day 1 to day 8.

The supernatant was collected from the designated wells and used in a growth kinetic analysis. The wells

with attached cells were replenished with 100 µL of fresh medium and analyzed for cell viability performed using classical MTT assay (Vybrant-Thermo Fisher Scientific), according to the manufacturer's instructions. Briefly, 10 µL of 12 mM MTT stock were added to wells containing cells and then incubated at 37°C for 2 h. Following the incubation period, 100 µL of the 10% SDS in 0.01 M HCl solution was added and continued for incubation at 37°C, overnight (approximately 16 h). After thorough mixing, absorbance from each well was measured at 570 nm using a microplate reader.

Growth Kinetic of CHIKV by Plaque Assay. The supernatant collected from the designated wells was used in a growth kinetic analysis using standard plaque assay method in Vero-CCL-81 cells, as mentioned elsewhere (Wikan *et al.* 2012). Virus titer was defined as the number of plaque forming unit per mL (PFU ml^{-1}).

Infection Rate of CHIKV by Fluorescence-activated Cell Sorting (FACS). To confirm and analyze the infection rate of CHIKV, fluorescence-activated cell sorting (FACS) was performed. A549 and HepG2 cell lines were seeded in 6-well plate. After overnight incubation, the cells were infected at MOI of 1 with CHIKV-JMB-192 or DENV-1 JMB-034 at 37˚C and 5% CO_2 for 2 hours. Uninfected cells were used as control where medium only added. Following the incubation period, inoculant was removed from each well and replenished with fresh medium. The plates were then incubated at 37°C, 5% CO_2 for 24, 48, and 72 hours post-infection. At the designated time point, cells were collected, washed with 1× PBS pH 7.2 (Amresco), and simultaneously fixed and permeabilized using BD Cytofix/Cytoperm (Becton Dickinson). The permeabilized cells were then incubated with alphavirus monoclonal antibody (Santa Cruz) or dengue serotype-specific purified IgG monoclonal antibody to the envelope protein for DENV-1 (Biotem), according to the infecting virus, for 30 minutes at room temperature. Cells were then washed and incubated with fluorescein isothiocyanate (FITC)-labelled affinity purified goat anti-mouse IgG (Thermo Fisher Scientific) for 30 minutes at room temperature. After incubation, the cells were washed and analyzed using FACS Accuri C6 Plus instrument (Becton Dickinson) where a total of 100,000 events were analyzed for each read.

Immunofluorescence Assay of CHIKV. Immunofluorescence assay was performed to visualize CHIKV- and DENV-1 infected cells. A549 and HepG2 cell lines were grown on sterile cover slips put in wells of 6-well plate containing complete RPMI medium and incubated overnight at 37°C incubator supplemented with 5% CO_2. The attached cells were infected with either CHIKV-JMB-192 at MOI of 1, or DENV-1 JMB-034 at MOI of 0.01. Following the 48 hrs incubation, the medium containing inoculant was removed from wells and the attached cells (in the surface of cover slip) were washed with PBS. The cells were then fixed using 3.7% formaldehyde and incubated at room temperature for 10 minutes. Cell permeabilization was done using 2% Triton-X in PBS and incubated at room temperature for 10 minutes, continued with blocking in PBS containing 1% FBS and 0.5% Tween-20. Subsequently, cells were incubated with a mouse anti-alphavirus monoclonal antibody (Santa Cruz) for CHIKV infection and dengue serotype-specific purified IgG monoclonal antibody to the envelope protein for DENV-1 (Biotem) for DENV-1 infection at room temperature for 1 hour. After washing steps, cells were incubated with fluorescein isothiocyanate (FITC)-labelled affinity purified goat anti-mouse IgG (Thermo Fisher Scientific) at room temperature for 30 minutes. The cells were then counter-stained with DAPI and washed with PBS. Following the counterstaining step, the cover slips were removed from wells and mounted into object glasses using Prolong antifade reagent (Thermo-Fisher Scientific) and then directly observed under a Nikon Eclipse 80i fluorescent microscope (Nikon).

RESULTS

Cell Viability Assay. Cell viability assay showed vary upon infection with CHIKV, high cell viability was observed in A549 and Huh7 cell lines up to 8 days of CHIKV infection (Fig 1). In contrast, the HepG2 and Vero cell lines showed the decreasing cell viability over time. The Vero cells exhibited the lowest cell viability with the reduced number of viable cells since day 2 post infection. We observed that HepG2 cells viability was sustained above 80% up to day 3 post infection and significantly decreased during the course of infection, showing the susceptibility of this hepatocyte to CHIKV infection (Fig 1).

Growth Kinetic of CHIKV by Plaque Assay. In regards to the number of replicated virus produced and released to supernatant by infected cells, HepG2 cells facilitated the production of the highest virus titer (measured as PFU mL^{-1}) during the course of CHIKV infection in which the titer reached maximum at day 5 post infection (Fig 2). Similar pattern was also

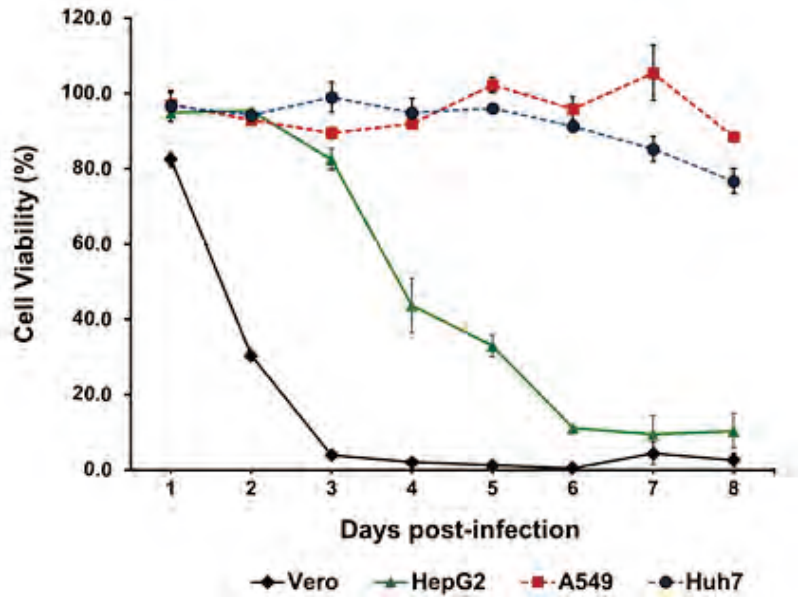

Fig 1 Cell viabilites of three different human cell lines upon infection with CHIKV. The monkey cell line Vero-CCL81 was used as a reference cell line.

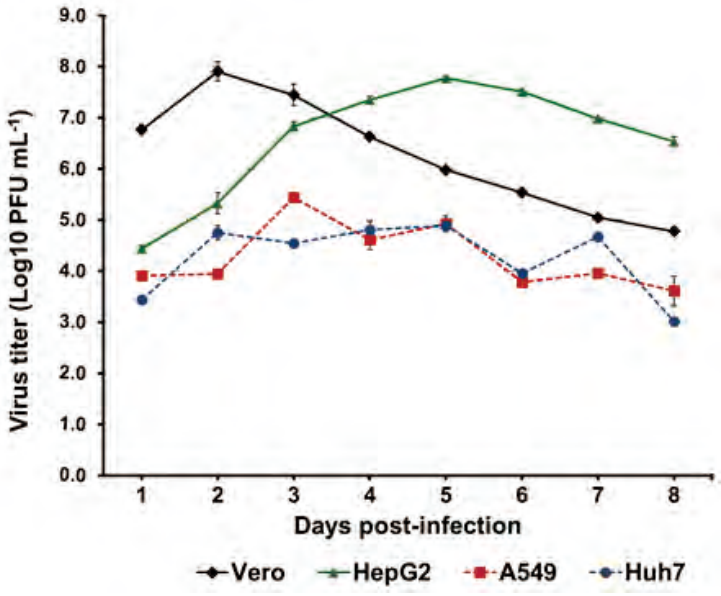

Fig 2 CHIKV growth kinetics in three different human cell lines. The monkey cell line Vero was used as a reference cell line.

observed in Vero cell line but the highest titer was observed earlier at day 2 post infection. Other cell lines showed lower virus titers with the trend of decreasing titers over the course of infection. Low virus production was observed in A549 and Huh7 cells. Therefore, growth kinetic of CHIKV in HepG2 cell line was the highest among human cell line tested.

Infection Rate of CHIKV Analysis by Fluorescence-Activated Cell Sorting (FACS) and Immunofluorescence Assay. To further examine the susceptibility of HepG2 and A549 cell lines to different of arboviruses infection, we performed FACS assay to compare CHIKV and DENV-1 infection rates in those cells. Our results showed that HepG2 cell line was more susceptible to CHIKV infection than A549 cells. The proportion of CHIKV-infected cells was significantly higher in HepG2 cell line which peaked at 48 hpi with 52.6% of infected cells. In contrast, the proportion of DENV-1-infected cells were significantly lower (Fig 3).

A contrasting result was observed in A549 cells, in which less CHIKV was detected in this cell line compared to HepG2. Higher infection rate of DENV-1

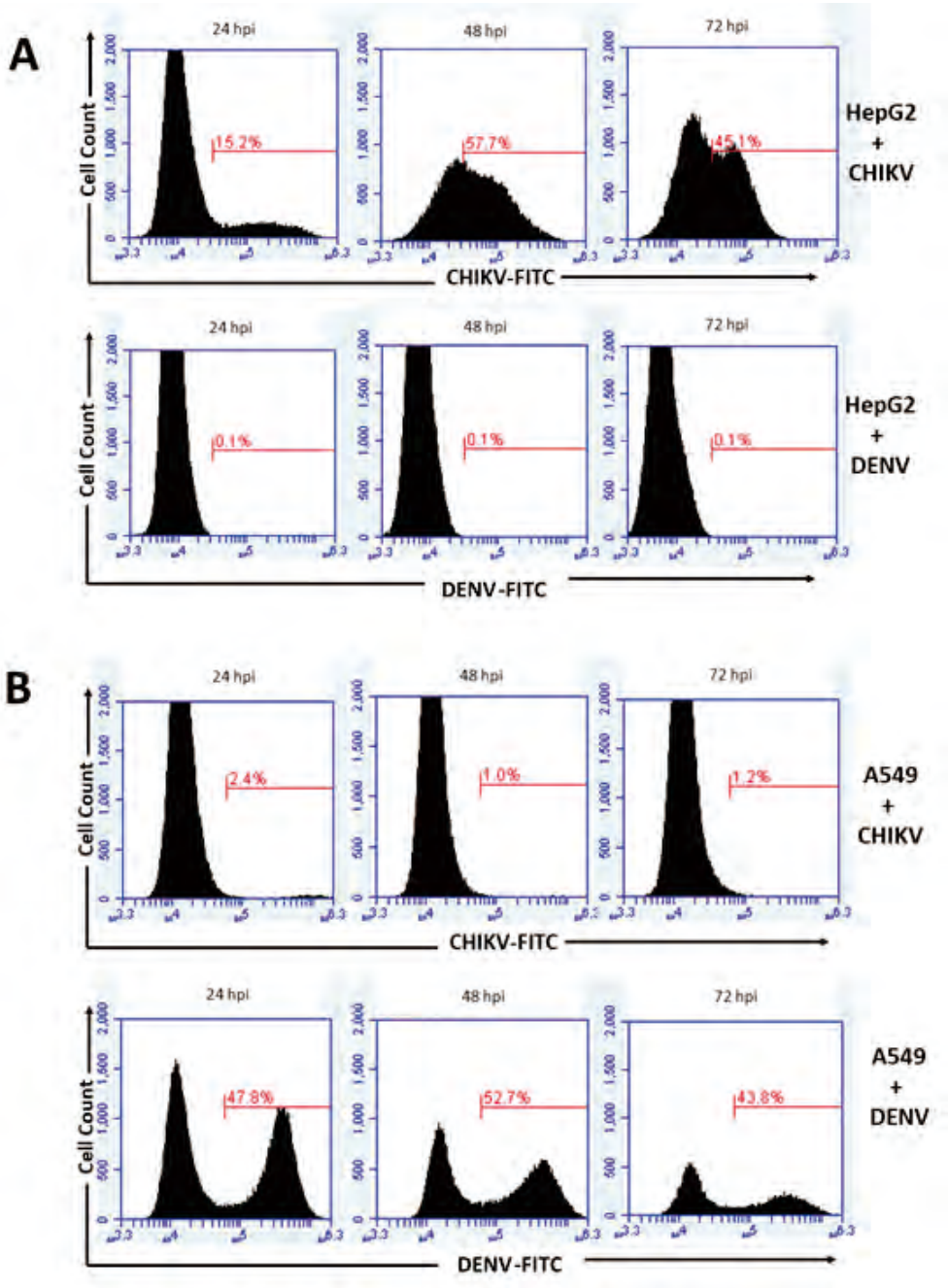

Fig 3 Proportion of CHIKV- and DENV-1-infected cells as measured by FACS analysis. Virus infection percentage of CHIKV and DENV in HepG2 (A) and A549 (B) cells at 72-hours post-infection.

in A549 cell line was prominent compared to HepG2 (Fig 3). The DENV-1 infection was detectable at 24 hours post infection (h.p.i) by 47.6% and peaked at 48 h.p.i by 52.4% infected cells.

The FACS results were also confirmed by immunofluorescence assay. CHIKV was abundantly detected in HepG2 cell line, while detection was very dim in A549. In constrast, DENV-1 was strongly detected in A549 cell line, while barely detected in HepG2 cells (Fig 4).

DISCUSSION

The human tissue-tropism of CHIKV in the pathogenesis of CHIK disease is still to be fully defined (Wikan *et al.* 2012). Different human cells were reported to be susceptible to CHIKV infection and allowed viral production, including human epithelial and endothelial cells, primary fibroblasts and, to a lesser extent, monocyte-derived macrophages (Sourisseau *et al.* 2007). The African Green Monkey

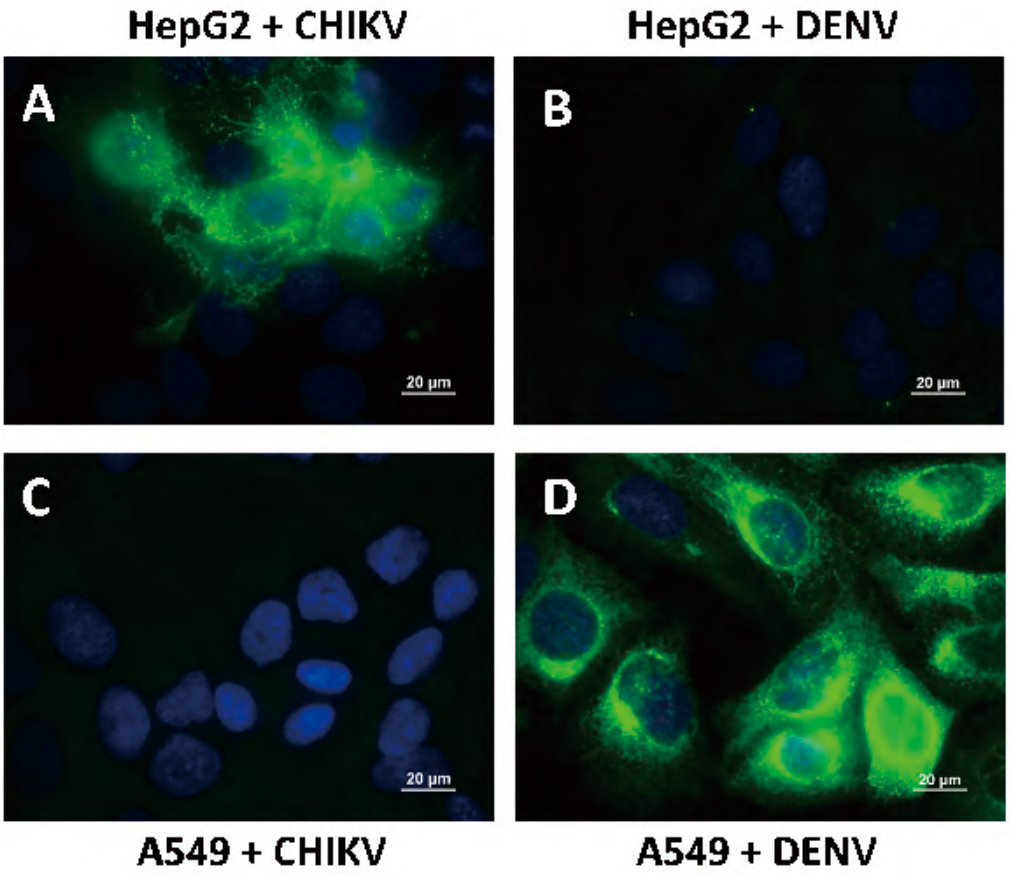

Fig 4 Immunofluorescence assay of CHIKV and DENV infection in HepG2 and A549 cell lines, visualized at 48-hours post infection. CHIKV (A and C) and DENV (B and D) was detected by FITC-labelled antibody complex (Green) with nucleus DAPI counter-staining (blue).

Vero cell line has been used as model cell line for propagation of CHIKV in non-human mammalian cell line (Kumar *et al.* 2014).

Several human cell lines have been used in arbovirus studies. Previous report suggested that A549 cell line has better cell tropism for arbovirus infection especially DENV (Fink *et al.* 2007). The A549 cell line is reported to be susceptible to DENV infection (Yohan *et al.* 2014) and have been used in the antiviral study of DENV (Kato *et al.* 2016). However, our result demonstrated that unlike DENV, CHIKV was suboptimal in infecting and replicating within A549 cell line. Instead, our results on cell viability and growth kinetic assay confirmed that human hepatocytes HepG2 has better susceptibility for CHIKV infection than A549 cells.

The replication of CHIKV in target cells is cytopathic and associated with apoptosis induction in infected cells (Sourisseau *et al.* 2007). Based on cell viability assay, in our study, the hepatocytes HepG2 cell was the only human cell line which showed susceptibility to CHIKV infection (Fig 1). Contrasting results were observed for Huh7, also a human hepatocytes cell line, and A549 alveolar epithelial cell line, that has been widely used in arboviruses study including DENV and Zika virus (ZIKV) (Frumence *et al.* 2016; Rückert *et al.* 2017). Susceptibility of HepG2 to CHIKV infection was further confirmed by growth kinetic assay and infection rate analysis. Significantly higher virus titer was observed in HepG2 cells compared to other cell lines (Fig 2). In contrast, when infected with DENV-1, HepG2 showed lower infection rate than A549 cells, as has been described in our previous findings (Yohan *et al.* 2014). The high infection rate of CHIKV to HepG2 cells has been observed in previous study using CHIKV isolate with East, Central and South African (ECSA) Genotype (Sourisseau *et al.* 2007; Wikan and Smith 2016). Thus, our results support their findings and provide additional data that HepG2 cell line is also susceptible to Asian Genotype of CHIKV.

Additional confirmation of HepG2 susceptibility to CHIKV was performed using immunofluorescence assay, in which CHIKV was clearly detected within the cytoplasm of the HepG2 cells (Fig 4). Altogether, our results demonstrated the susceptibility of HepG2 cells against infection of different genotypes of CHIKV and support the application of this cell line in CHIKV research.

It is interesting to note the contrasting cells susceptibility of A549 and HepG2 in CHIKV infection compared to DENV. With regard to HepG2 as liver cell line, CHIKV infection cases with liver involvement has been reported (Chua *et al.* 2010). Several CHIKV infection with liver as target organ have been reported to associate with severe clinical manifestation such as impaired liver function, acute hepatitis and multiple organ failures (Chua *et al.* 2010; Hoz *et al.* 2015; Chan and Leung 2017). These reports support our finding of HepG2 susceptibility to CHIKV infection. In regards to probable pathogenesis mechanism, several receptors have been reported to be involved in alphavirus infection including Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN), Liver/lymph node-specific intercellular adhesion molecule-3-grabbing non-integrin (L-SIGN), heparan sulfate, prohibitin, T-cell immunoglobulin and mucin domain (TIM) 1, and ATP synthase(Smit *et al.* 2002; Klimstra *et al.* 2003; Moller-Tank *et al.* 2013; Fongsaran *et al.* 2014; Wintachai *et al.* 2012). In addition, the host cell receptor Sphingosine Kinase 2 (SK-2) has been linked to CHIKV infection. Tested on HepG2 hepatocyte cell system, Sk-2 was proposed to be the host cell factor in CHIKV infection (Reid *et al.* 2015). These receptors may be a host cell factor that causes CHIKV infection in HepG2 cell compared to other cells in this study.

In conclusion, the human hepatocytes HepG2 cell line was susceptible to Asian Genotype of CHIKV and showed infection profiles suitable to be proposed as an alternative cell in *in vitro* CHIKV studies to the commonly used A549 and Vero cells.

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