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Characterization of the infectivity of an Indonesian Zika virus strain in mammalian cell lines

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ABSTRACT

Objective: To characterize the infection patterns and growth characteristics of the Zika virus (ZIKV) strain JMB-185 from Indonesia in various mammalian cell lines.

Methods: ZIKV was grown in human (A549, HEK293, HepG2, Huh7, Jurkat, and THP-1) and non-human mammalian (RAW264.7, Vero, and Vero76) cell lines. Viral replication kinetics were measured using plaque assay, while intra- and extracellular viral RNA concentrations were assessed using RT-PCR. Flow cytometry was used to quantify the infected cells and cell viability was measured using an MTT assay. The ability of ZIKV to infect cell lines was visualized using a fluorescence immunostaining assay.

Results: This ZIKV strain preferentially infected the lung, kidney, and liver cell lines A549, HEK293, Huh7, Vero, and Vero76, but not the immune cells Jurkat, RAW264.7, and THP-1. By contrast, the ZIKV showed no sign of infection in HepG2 cells, while maintaining viral titer over 3 days post-infection, with no infection recorded in immunostaining, no increase in viral RNA, and no indication of cell deterioration.

Conclusions: The Indonesian ZIKV strain has a similar infection profile as other strains, except for its poor infectivity on HepG2 cells. Information on the growth characteristics of Indonesia ZIKV will help expand our understanding of the biology of ZIKV which will be useful for various applications including antiviral discovery.

KEYWORDS: Zika virus; Infectivity; Replication; Cell lines; *In vitro*

1. Introduction

Zika virus (ZIKV) is a *Flavivirus* (*Flaviviridae* family) transmitted

by *Aedes* mosquitoes first isolated from a rhesus monkey in 1947[1]. Its genome is a ~11kb single-stranded positive-sense RNA virus consisting of one long open reading frame, encoding a polyprotein that is cleaved into 3 structural (N, prM, E) and 7 non-structural (NS) proteins[2]. *Aedes* mosquitoes acquire the virus from a blood meal from an infected human or non-human primate and they replicate in their salivary glands[3] until they pass it to a mammalian host, where they replicate in a wide variety of tissues including the kidney, liver, brain, and sexual organs[4,5]. Infection in healthy adults is frequently asymptomatic or otherwise limited to a mild transient fever, leading to the disease being largely neglected for decades following its discovery[6,7]. This remained the case until ZIKV strains that had

Significance

Previous studies have established the tropism of ZIKV for specific cell types; however, the infection patterns of the Indonesian ZIKV strain JMB-185 remain underexplored. This study elucidates the preferential infectivity of JMB-185 for lung, kidney, and liver cell lines, with notable resistance in HepG2 cells. These findings expand our understanding of ZIKV biology, providing important information for antiviral development and other future ZIKV studies.

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mutated to replicate far better in humans[8] spread throughout the Pacific Islands, which began with an outbreak on Yap Island in 2007[6] and culminated in the Zika epidemic in the Americas in 2015–2016[9]. In this epidemic, the more worrying disease progressions of Zika came into focus, namely the development of Guillain-Barré syndrome, with debilitating muscular weakness and possible paralysis[10], and the risk to fetuses of pregnant women. *In-utero* infections carry a significant risk of leading to a range of neurological symptoms in the child, primarily microcephaly, with this risk being highest (5%–10%) in the first trimester[11]. The severity of the condition varies, with some cases of intrauterine death[12], while in milder cases, the children may survive, but suffer neurological symptoms like epilepsy and seizures[13], as well as disabilities in learning and motor skills[14]. During a ZIKV epidemic situation, where the incidence of microcephaly in a country may rise 15-fold[15], this represents a significant public health burden.

While ZIKV is endemic in Indonesia, where its symptoms may often be misdiagnosed as mild infections of other circulating arboviruses, such as dengue or chikungunya[16], the risk to naïve populations from arboviruses spreading to new areas is substantial, as demonstrated by both the Zika epidemic in the Americas and the Chikungunya outbreaks in Italy and France[17–19]. A scenario such as this is increasingly likely due to the expansion of *Aedes* mosquito habitats driven by urbanization and climate change, and the ease of international travel, as ZIKV persists in other bodily fluids long after it is undetectable in serum[20], allowing long-term sexual and urine-to-mosquito larva spread[21]. All these factors make continued monitoring of ZIKV of high importance, in which evaluating changes in cell tropism plays a part.

In the current study, we aimed to characterize the infectivity patterns and the growth characteristics of the only ZIKV strain from Indonesia, JMB-185, which was isolated from a febrile patient in Sumatra, Indonesia in 2014[22,23] and has not been previously assessed for its biological characteristics. In order to get a complete profile of the infection, we measured total viral titer, intra- and extracellular viral RNA, and cell viability, quantified infected cell number, and took qualitative measurements of infection. This evaluation included a range of human (A549, HEK293, HepG2, Huh7, Jurkat, and THP-1) and non-human mammalian (RAW264.7, Vero CCL-81, and Vero76) cell lines that are infected by ZIKV in previous studies in order to maximize information garnered.

2. Materials and methods

2.1. Cell lines and virus

Human lung carcinoma (A549, CCL-185), embryonic kidney (HEK293, CRL-1573), hepatocellular carcinoma (HepG2, HB-8065;

Huh7, JCRB0403), lymphoblast (Jurkat, TIB-152) and monocyte (THP1, TIB-202), *Mesocricetus auratus* kidney (BHK-21, CCL-10), murine macrophage (RAW 264.7, TIB-71), *Cercopithecus aethiops* kidney (Vero, CCL-81; Vero E6 Clone 76, CRL-1587) and *Aedes albopictus* larval cells (C6/36, CRL-1660) were acquired from the American Type Culture Collection (ATCC, Manassas, VA, USA). Vero cells were maintained in Minimum Essential Medium (MEM) (Gibco, Waltham, MA, USA) supplemented with 2 mM *L*-glutamine (Gibco), 1% antibiotic-antimycotic (Gibco), and 5% fetal bovine serum (FBS, Gibco). Other cell types were cultured in RPMI 1640 medium (Gibco), supplemented with 1% antibiotic-antimycotic and 10% FBS. All cell lines were tested for mycoplasma contamination before use using the LookOut Mycoplasma qPCR detection kit (Sigma-Aldrich, St. Louis, MO, USA).

Zika virus JMB-185 (GenBank accession no. KU179098.2) was isolated from a febrile patient in Jambi, Indonesia, in 2014[22] and has been genetically characterized previously, showing it to be part of the Asian lineage[23]. The virus was propagated in C6/36 cells for 6 passages then concentrated 10-fold using 10 KDa centrifugal filter units (Amicon/Merck-Millipore, Burlington, MA, USA) and stored at -80°C . Virus titer was measured by standard plaque assay using BHK-21, adapted from an assay for Chikungunya virus[24]. Then 1.5×10^5 cells were seeded in 24-well plates for 2 d, after which 10-fold serial dilutions of inoculant (200 μL /well) were added for 1 h at 37°C . The inoculant was aspirated and replaced with an overlay medium containing 1% Aquacide II (Millipore-Merck) supplemented with 2% FBS, 1% antibiotic-antimycotic, and 2 mM of *L*-glutamine, and incubated at 37°C for 5 d. The cells were fixed with 3.7% formaldehyde (Sigma-Aldrich) and stained with 2% crystal violet (Sigma-Aldrich).

2.2. Time-series infection

Mammalian adherent cell lines (all except Jurkat and THP-1) were seeded in 24-well plates at 4×10^5 cells/well. Following overnight (approximately 16 h) incubation, cells were infected with ZIKV at a multiplicity of infection (MOI) of 1 in inoculation media (containing only 2% FBS) and incubated at 37°C , 5% CO_2 for 1 h. For each time point, two negative controls and six infected wells were assigned. Supernatant was then removed and replaced with fresh inoculation media and incubated at 37°C , 5% CO_2 . At 1, 2, and 3 days post-infection (dpi), supernatant was combined from two identical wells. Supernatant from each well was aliquoted in 1.5 mL tubes for use in plaque assay or RNA extraction, with a total of one control and three infected samples to analyze in each case. Quickly, 300 μL of Buffer RLT (Qiagen, Hilden, Germany) supplemented with 1% β -mercaptoethanol (Bio-Rad, Hercules, CA, USA) was added to the wells and vigorously mixed over the remaining cell monolayer. In the same pattern as for the supernatant, a lysate from identical

wells was combined and aliquoted in 1.5 mL tubes. Supernatant and cell lysates were stored at -80°C . At a later date, viral titer in the supernatant was assessed using plaque assay as described above.

Mammalian suspension cell lines (Jurkat and THP-1) were similarly seeded in 24-well plates, but infected immediately after seeding. Cells were moved to 1.5 mL tubes and centrifuged at $100 \times g$ to aspirate supernatant, after which cells were resuspended and transferred back to their wells. At 1, 2, and 3 dpi, supernatant was collected following centrifugation at $2500 \times g$ for 10 min, and lysis of the remaining cells was carried out in the 1.5 mL tubes.

2.3. Intra- and extracellular viral RNA extraction and quantitative RT-PCR

Extracellular viral RNA was extracted from supernatant samples using the MagNA Pure LC Total Nucleic Acid Isolation Kit (Roche, Basel, Switzerland) on the MagNA Pure LC 2.0 Instrument (Roche), following the external lysis protocol, in which 200 μL of sample was mixed with 300 μL of a lysis buffer before processing. Extracted nucleic acids were stored at -80°C . Intracellular viral RNA was extracted from a cell lysate using the RNEasy Mini Kit (Qiagen) following the manufacturer's instructions, with 50 μL of RNase-free water being used in the final step. Eluted RNA was stored at -80°C .

Measurement of viral RNA concentration was done using the SuperScript III Platinum SYBR Green One-Step qRT-PCR Kit (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions on a 7500 Fast Real-Time PCR System (Applied Biosystems, Waltham, MA, USA). Forward primer (5'-CCGCTGCCCAACACAAG-3') and reverse primer (5'-CCACTAACGTTCTTTTGCAGACAT-3') were taken from a previous study[25] checked to fit the sequence of ZIKV JMB-185, with a ~ 77 bp product in the *E* gene region. All positive samples were confirmed using gel electrophoresis to eliminate unspecific replication. For extracellular samples, C_T values of samples were plotted against a standard curve of RNA from known PFU/mL concentrations of ZIKV. For intracellular samples, the standard curve was constructed from known concentrations of ZIKV JMB-185 complete *E* gene cDNA.

2.4. Fluorescence-activated cell sorting (FACS)

Cells were seeded in 6-well plates at 1.5×10^6 cells/well. Suspension cells were then infected with ZIKV at MOI 1 for 1 h, while mammalian adherent cells were infected following overnight incubation. For each time point, three controls and three infected wells were assigned. Supernatant was removed and replaced with fresh inoculation media and incubated at 37°C , 5% CO_2 . At 1, 2, and 3 dpi, supernatant was removed from designated wells. As described previously[26], following DPBS washing (Invitrogen) and

trypsinization (Gibco), cells were collected and washed with DPBS, followed by simultaneous fixation and permeabilization for 30 min using BD Cytotfix/Cytoperm (Becton Dickinson, Franklin Lakes, NJ, USA). Following washing using BD Perm/Wash Buffer (Becton Dickinson), 1×10^6 cells were counted out and incubated with a protein-blocking buffer [0.5% BSA (Himedia, Mumbai, India) and 2% FBS in PBS (Sigma-Aldrich)], after which the infected samples were incubated with mouse monoclonal primary antibody clone 4G2 targeting a section of the *E* gene of flaviviruses (Genetex, Irvine, CA, USA), diluted 1:1 000. Following washing, infected samples were incubated with fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse IgG secondary antibody (Invitrogen) diluted 1:400, after which cells were washed again and stored in FACS buffer [0.5% BSA and 0.05% sodium azide (Sigma-Aldrich) in PBS], at 4°C until use. Control samples were either unstained, stained with secondary antibody only, or stained with both antibodies. All samples were read in a BD Accuri C6 Plus Cell Analyzer (Becton Dickson), with 100 000 cells sampled. Results were gated for singlets and infected cell gating was done based on the double-stained control.

2.5. Immunofluorescence assay

Cells were seeded on sterile coverslips in 6-well plates at 1.5×10^6 cells/well as previously described[26]. Following overnight incubation, cells were infected with ZIKV at MOI 1 for 1 h. At 1, 2, and 3 dpi, depending on cell type, supernatant was removed, cells were washed with Dulbecco's Phosphate-Buffered Saline, followed by fixation (3.7% formaldehyde in PBS) and permeabilization [2% Triton-X 100 (Sigma-Aldrich) in PBS]. Next, cells were washed using a washing/blocking buffer [0.5% Tween-20 (Sigma-Aldrich) and 1% FBS in PBS] on a rocking shaker and incubated with the buffer for blocking. Samples were then stained with 4G2 anti-flavivirus primary antibody diluted 1:1 000, followed by staining with FITC-conjugated secondary antibody diluted 1:400. The coverslips were then mounted on a microscope slide and counterstained with ProLong Gold Antifade Mountant with DAPI (Invitrogen). Slides were viewed under a Nikon 80i fluorescence microscope. Captured images were combined using ImageJ (v.1.53k, National Institutes of Health, USA).

2.6. MTT assay

A standard MTT assay was carried out using the CyQUANT MTT Cell Viability Assay (Thermo Fischer Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Cells were seeded in a 96 well-plate at 1×10^4 cells/well and incubated at 37°C for 72 h. Cells were then incubated with ZIKV at MOI 1 for 1 h, after which cells were washed with DPBS, replenished with new media, and incubated once again. Plates containing suspended

cell lines were centrifuged at $96 \times g$ for 5 min before supernatant removal in all cases and resuspended using pipette mixing. At the appropriate time point, supernatant was removed and replaced with 100 μ L fresh media per well, after which 10 μ L of 12 mM MTT in PBS was added, and cells were incubated at 37°C for 4 h. Following incubation, all except 25 μ L of supernatant were removed from wells, after which 50 μ L of dimethyl sulfoxide (DMSO) was added and mixed with vigorous pipette mixing. Following another 10-minute incubation at 37°C, the absorbance was read at 540 nm using a Multiskan FC microplate reader (Thermo Fisher Scientific).

2.7. Statistical analysis

Statistical tests (two-way ANOVA and Tukey's test) and figures were created using GraphPad Prism (v.8.2, GraphPad Software Inc., San Diego, CA, USA) and Microsoft Office Excel (Microsoft, Redmond, WA, USA). FACS data was analyzed and graphed using BD CSampler Plus Software (Becton Dickinson, Franklin Lakes, NJ, USA) and FlowJo (v.10.8, Becton Dickinson).

3. Results

3.1. Viable virus production

The time-course analysis of viral titers in the supernatant shows significant differences between cell lines and over time ($P < 0.01$ in both cases). Out of all tested cell lines, the highest viral production was observed from Vero CCL-81 at 2 dpi (9.67×10^7 PFU/mL, Figure 1A). This same cell line had a significantly ($P < 0.05$) higher viral titer at both 2 dpi and 3 dpi than all other cell lines, with A549 viral titer also being significantly higher than the remaining cells

at 3 dpi. The data shows increasing viral titers across all three days from A549, Huh7, and HEK293, while HepG2 virus titer remained stable at a lower level. The three immune cell lines (THP-1, Jurkat and RAW264.7) showed low and declining viral titers from 1 dpi, suggesting that they were not suitable hosts for ZIKV infection.

3.2. Infected cell proportion

The FACS analysis also showed significant differences between cell lines and over time ($P < 0.01$). The infected Vero76 and A549 cells reached a peak at 1 dpi and then decreased at 2 and 3 dpi, while Huh7 and Vero CCL-81 peaked at 2 dpi, with HEK293's peak likely occurring beyond 3 dpi (Figure 1B). The other four cell lines, including HepG2, showed immediate drops to 0% infected cells, suggesting that ZIKV is not able to replicate in these cell lines.

3.3. Viral RNA production

Both extra- and intracellular ZIKV RNA production were significantly different between cell lines and over time ($P < 0.01$). A549, Huh7, and Vero CCL-81 cell lines showed consistent significant increases in extracellular ZIKV RNA across all three days ($P < 0.05$, Figure 2A, D and I), while HEK293 and Vero76 saw non-significant increases (Figure 3B and H), possibly due to large variance in the samples. Huh7 and Vero76 showed the same pattern in intracellular RNA, with significant increases at 2 dpi, followed by a significant drop at 3 dpi, while A549 only showed a significant increase at 3 dpi. Vero CCL-81 extracellular ZIKV RNA levels were significantly higher than all other cell lines on all three days, while A549 and Huh7 levels were significantly higher than the remaining cells at 2 dpi as the second and third highest, respectively, with them switching places at 3 dpi. The remaining cells were not significantly

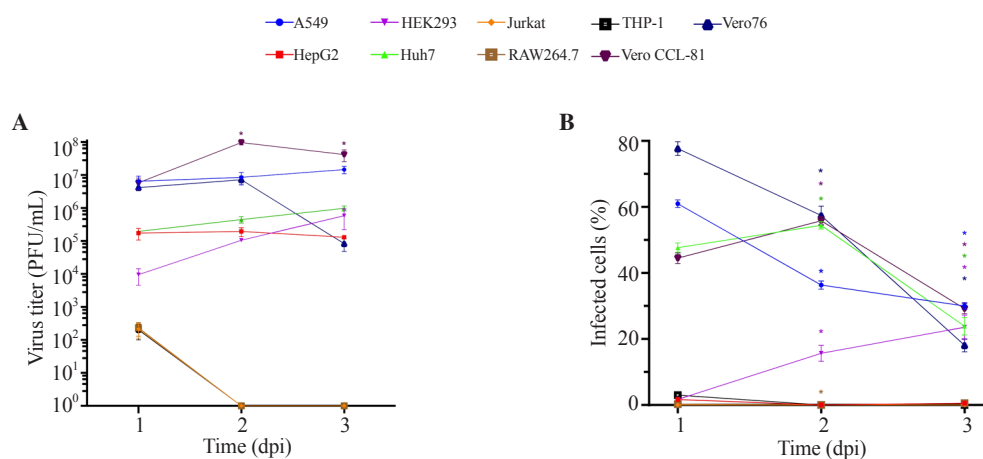


Figure 1. (A) Zika virus (ZIKV) titer levels determined by plaque assay and (B) infected cell proportion determined by fluorescence-activated cell sorting analysis. Experiments are done in triplicates and error bars represent standard deviation (SD). * $P < 0.05$ compared to the previous day. dpi: days post-infection. PFU: plaque forming unit.

different from each other, with unexpectedly low levels in Vero76 and large variance in HEK293 samples (Figure 2B and H). On the side of intracellular ZIKV RNA levels, A549 and Vero CCL-81 cells once again clustered at the top at 1 dpi, significantly higher than the remaining cell lines with Huh7 and both Vero cell lines being the highest at 2 dpi. A549 and Vero CCL-81 cell lines again clustered at the top at 3 dpi, although Huh7 and Vero76 saw a significant drop in intracellular RNA concentration at this timepoint. There were no significant differences between the other cell lines at any time point.

3.4. Immunofluorescence assay

The results of the immunofluorescence assay clearly showed the presence of ZIKV in A549, HEK293, Huh7, and both Vero cell lines (Figure 3A, B, D, H, and I), but not in any of the remaining cell lines.

3.5. Cell condition and cytopathic effect

The observation of cells under a light microscope showed the

development of cytopathic effect in some cell lines, while others were not markedly different from the uninfected control. A549, Huh7 and both Vero cell lines (Figure 4A, D, H, and I) showed marked and highly visible cytopathic effect at 3 dpi with significant cell death and lysis compared to control. HEK293 cells also began showing signs of cytopathic effect (Figure 4B) at 3 dpi, but were mostly in good condition, suggesting ongoing but slower infection. All other cell lines showed no appreciable differences between infected and uninfected cells in this comparison.

3.6. Cell viability

The MTT assay showed significant differences in cell viability between cells and over time, with an interaction effect ($P < 0.01$). The cell lines that demonstrated obvious cytopathic effects under the microscope all showed more or less clear cell viability decreases with ongoing infection, with all cell lines dropping below 70% viability at 4 dpi (Figure 5A). By contrast, cell lines that showed no cytopathic effect did not show consistent patterns in cell viability. THP-1 viability dropped to a comparable level to infected cell lines

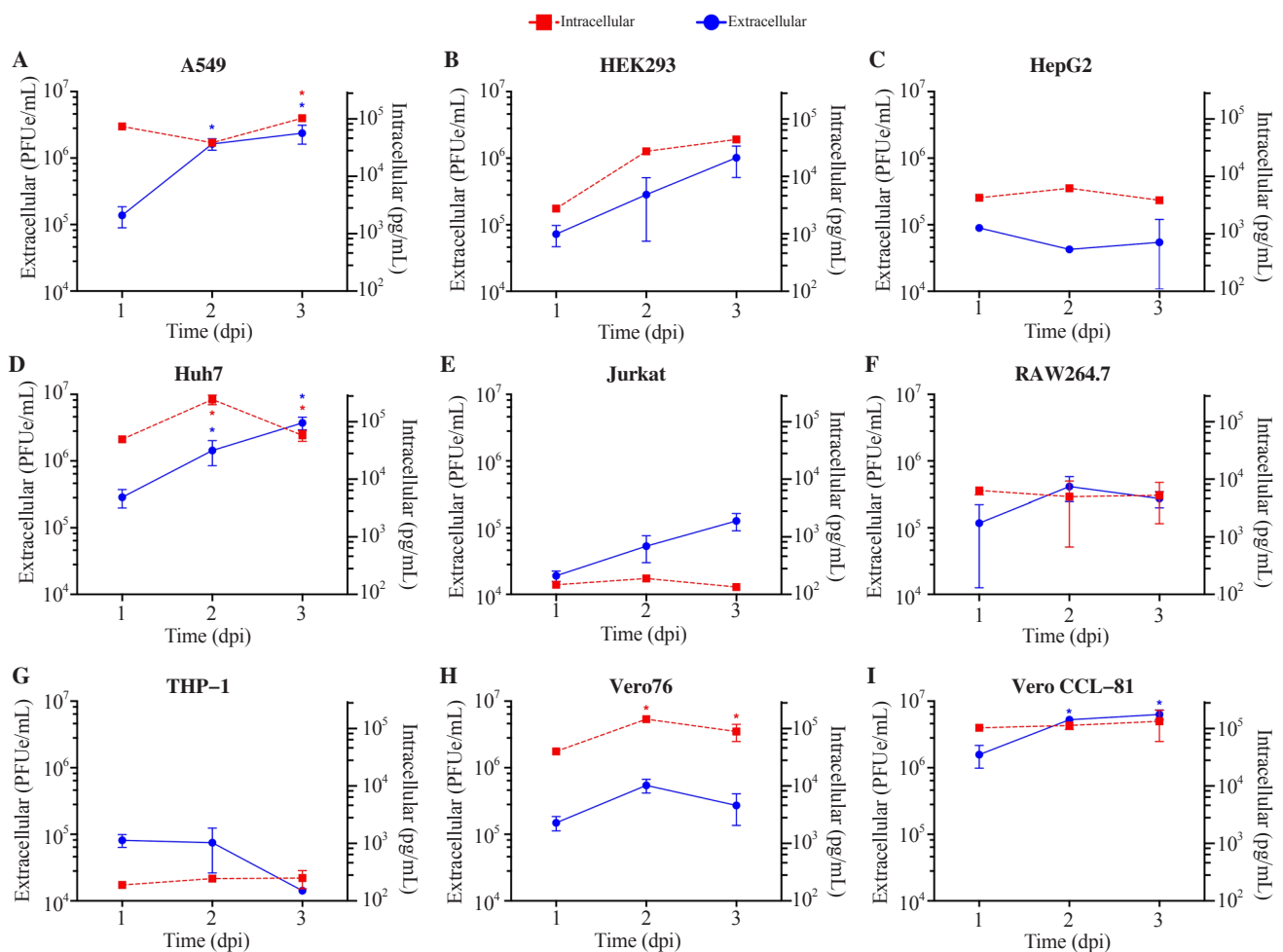


Figure 2. Intra- and extracellular ZIKV RNA production determined using qRT-PCR. Data are from triplicate experiments and error bars show SD. * $P < 0.05$ compared to the previous day. PFUe: plaque forming unit equivalents.

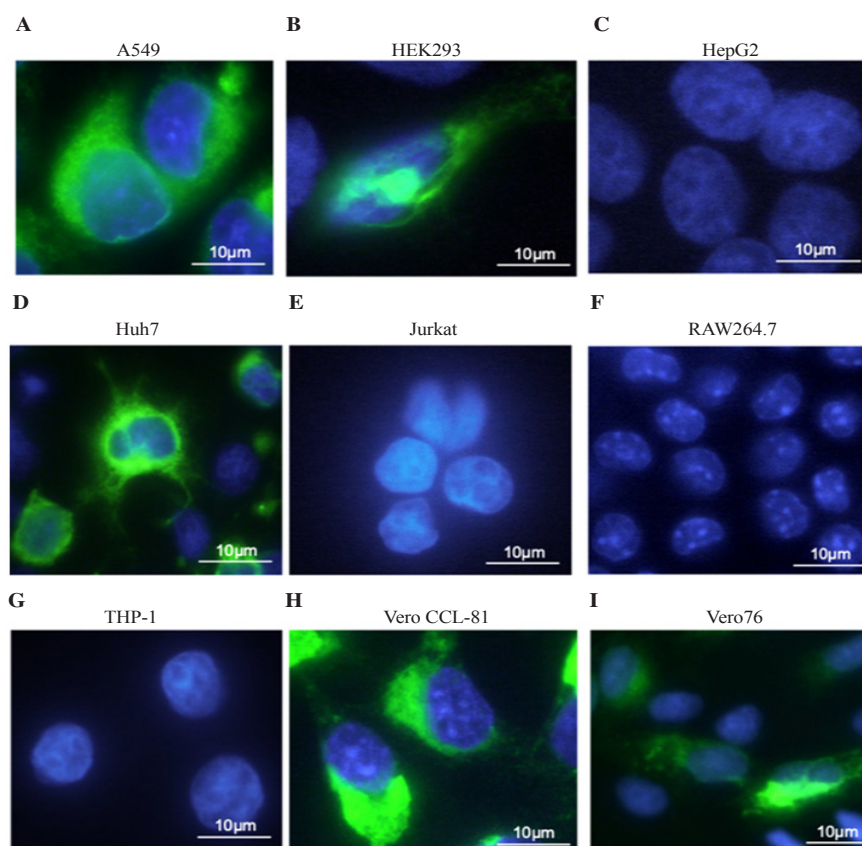


Figure 3. ZIKV E protein production visualized using an immunofluorescence assay. FITC-labelled secondary antibody in green; DAPI-stained cell nuclei in blue.

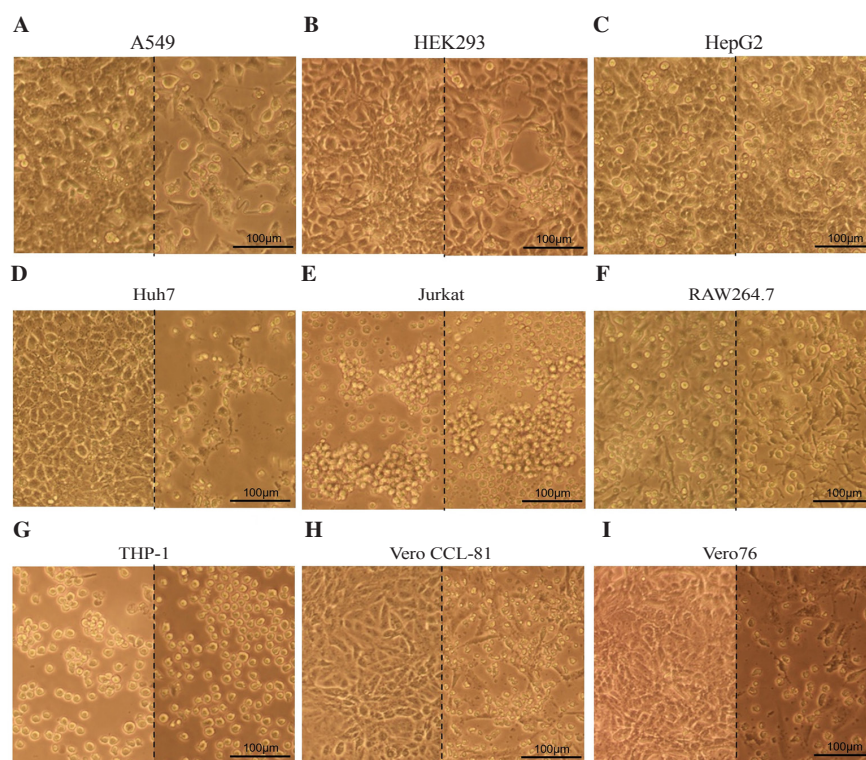


Figure 4. Cell condition in response to ZIKV infection at 3 dpi. In each picture, the left panel is the uninfected control while the right panel is ZIKV infected.

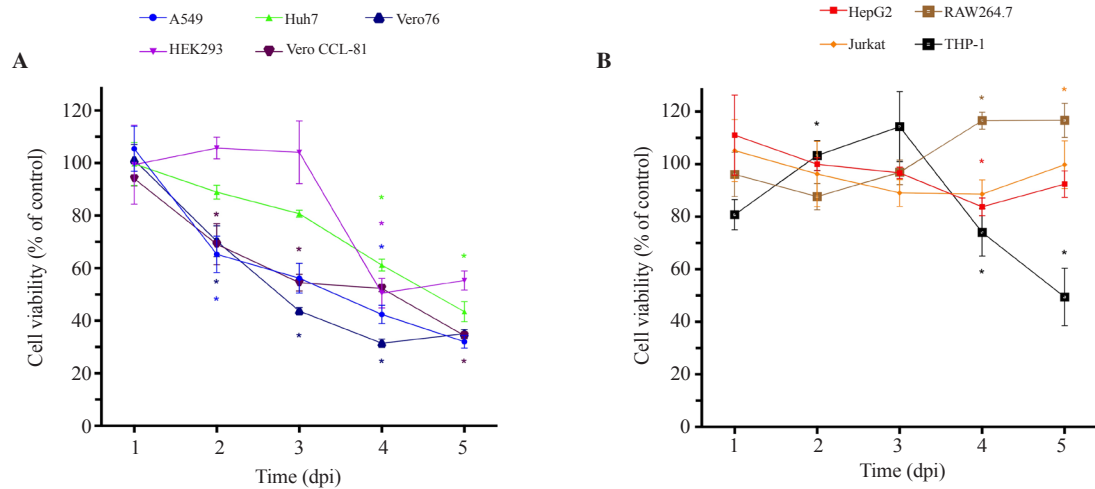


Figure 5. Cell viability following ZIKV infection measured using MTT assay. (A) Cell lines showing cytopathic effects and (B) those not showing cytopathic effects. Error bars show SD. * $P < 0.05$ compared to the previous day.

at 5 dpi, this was only after a significant rise in relative viability at 2 dpi (Figure 5B).

4. Discussion

The tissue tropism of ZIKV in mammals seems to be fairly non-specific, with the virus having been detected in a large range of tissues and organs throughout the body. Previous analyses have found kidney, liver, lung, brain, and sexual organ cells to be permissive to infection[27–32], with some immune cells showing replication *in vitro*[29,33,34]. Our study has identified similar patterns of infection, though with some marked differences for a few cell lines.

As with other flaviviruses, the high susceptibility of Vero cells (*Cercopithecus aethiops* kidney) to ZIKV infection has made them widely used in studies examining the virus[27,35]. This was no different in our case, as Vero cell lines showed high levels of infected cells and high virus RNA production, and produced among the highest viral titers of the tested cells, coinciding with a rapid and strong decline in cell viability and strongly visible cytopathic effects. The human kidney cell line HEK293, by comparison, was also vulnerable to infection but this infection progressed slowly, with all assessed markers of infection rising from 1–3 dpi, which is consistent with the lower ZIKV titers observed in other studies using these cells[28,29]. Human lung carcinoma A549 cells were readily infected with ZIKV, with infection peaking quickly and high levels of RNA production and viral titer, in line with observations from other studies[28,29].

The human hepatocyte Huh7 cell line was similarly easily infected with a peak at 2 dpi and showed high virus titer, which is in line with previous research[27–29]. By contrast, HepG2 cells, also human

hepatocytes, did not seem to be readily infected by the Indonesian ZIKV strain, with viral titer and RNA remaining unchanged across all 3 days and no indication of infection using immunostaining or cytopathic effect. This is in contrast to previous studies using other ZIKV strains[27,28,36], although the viral titers recorded in these studies were in fact similar to the ones presented here. While it has been demonstrated that ZIKV does not degrade exponentially in media outside cells and virion decay has a delay of ~40 hours per-infection[37], the complete lack of change in viral titer may suggest an extremely low level of replication, which allows replenishment of degraded virions while remaining below the level of detection of immunostaining. This, however, should coincide with an increase in extracellular ZIKV RNA, which we did not observe. The tested immune cell lines (Jurkat, RAW264.7, and THP-1) were also not amenable to infection by the Indonesian ZIKV strain, apparent in the immediate drop of viral titer at 2 dpi to effectively 0 PFU/mL, which is in contrast to previous studies using these cell lines[29,33,34], though some have reported RAW264.7 and THP-1 not being infected[29,38,39].

No one major factor has been identified to explain differential susceptibility to infection of different cell lines. An inability for ZIKV to replicate is unlikely the cause in the present study, as all the tested cells contain furin, the key protease required for infection[40]. Another possible explanation is the differential expression of factors required for ZIKV cell entry. ZIKV entry into cells may occur through a large variety of mechanisms[41], but it has been observed that the expression levels of attachment and entry receptors for ZIKV do not necessarily correlate with susceptibility[29], and our data suggests that while intracellular ZIKV RNA levels in uninfected cells are low, they are not absent. Differential infectivity of ZIKV strains is observed frequently[29,42,43] and is likely due to mutations that promote replication in cells, as happened in the case of the

American epidemic strain[8].

This study has some limitations. While this study could identify some differences in infectivity between the Indonesian strain and others observed in other studies namely about replication in HepG2 cells, the limited scope of this study led us to be unable to state the exact causes. For such conclusions, a direct analysis of which mutations have impacted our results would be needed. There is also some possibility that these differences are simply due to the uncertainty of replication of *in vitro* research, frequently impacted by cell quality, unwanted differentiation of cells, or statistical variance[44]. Additionally, as all the cells used in this study were cancerous, we are limited in our ability to extrapolate these results to viral replication in healthy cells in a whole organism. While neuronal cell lines were not available to us for this experiment, ZIKV's strong tropism for these cell lines and their possible involvement in the disease progression of Zika congenital syndrome[45] should make them a major focus of future research.

In conclusion, this study characterized the infectivity of the Indonesian ZIKV strain JMB-185 in mammalian cell lines and found that the patterns of cellular susceptibility to infection for this strain are largely the same as for other ZIKV strains. Like others, this strain replicates well in A549, HEK293, Huh7, and Vero cell lines and shows no replication in Jurkat, RAW264.7, and THP-1. The major difference in other strains we observed is an apparent lack of replication in HepG2 cells, though viral titer did not decline over time as it did for the other cell lines. Overall, this study enhances our understanding of ZIKV biology by characterizing the infection patterns and growth characteristics of the Indonesian ZIKV strain JMB-185 in mammalian cell lines. These findings contribute valuable data for future research such as in drug discovery and antiviral therapies.

Conflict of interest statement

All authors declare that there are no conflicts of interest.

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Data availability statement

The data supporting the findings of this study are available from the corresponding authors upon request.

Authors' contributions

JTOS conducted the experimental procedures, analyzed the data, and wrote the manuscript, which was edited and amended by RTS. RTS designed the study. AB and RTS provided the funding and supervised the study.

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