Kinetics of Asian and African Zika Virus Lineages over Single-cycle and Multi-cycle Growth in Culture:

gene expression, cell killing, virus production, and mathematical modeling

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Abstract

Since 2014, an Asian lineage of Zika virus has caused outbreaks, and it has been associated with neurological disorders in adults and congenital defects in newborns. The resulting threat of the Zika virus to human health has prompted the development of new vaccines, which have yet to be approved for human use. Vaccines based on the attenuated or chemically inactivated virus will require large-scale production of the intact virus to meet potential global demands. Intact viruses are produced by infecting cultures of susceptible cells, a dynamic process that spans from hours to days and has yet to be optimized. Here, we infected Vero cells adhesively cultured in well-plates with two Zika virus strains: a recently isolated strain from the Asian lineage, and a cell-culture-adapted strain from the African lineage. At different time points post-infection, virus particles in the supernatant were quantified; further, microscopy images were used to quantify cell density and the proportion of cells expressing viral protein. These measurements were performed across multiple replicate samples of one-step infections every four hours over 60 hours and for multi-step infections every four to 24 hours over 144 hours, generating a rich dataset. For each set of data, mathematical models were developed to estimate parameters associated with cell infection and virus production. The African-lineage strain was found to produce a 14-fold higher yield than the Asian-lineage strain in one-step growth and a 7-fold higher titer in multi-step growth, suggesting a benefit of cell-culture adaptation for developing a vaccine strain. We found that image-based measurements were critical for discriminating among different models, and different parameters for the two strains could account for the experimentally observed differences. An exponential-distributed delay model performed best in accounting for multi-step infection of the Asian strain, and it highlighted the significant sensitivity of virus titer to the rate of viral degradation, with implications for optimization of vaccine production. More broadly, this work highlights how image-based measurements can contribute to discrimination of virus-culture models for the optimal production of inactivated and attenuated whole-virus vaccines.

1 Introduction

On February 1st, 2016, the World Health Organization (WHO) declared the Zika virus (ZIKV) a public health emergency of international concern because of the epidemic of ZIKV infection spreading from Brazil to other American nations and the world (Teixeira et al., 2016; *WHO | Zika Virus and Complications: 2016 Public Health Emergency of International Concern*, 2017). It was noticed during the epidemic that ZIKV infection during pregnancy caused a broad spectrum of nervous system developmental defects including microcephaly termed congenital Zika syndrome (CZS) (Velho Barreto De Araújo et al., 2016; Mlakar et al., 2016; De Barros Miranda-Filho et al., 2016; França et al., 2016; Brasil et al., 2016), and miscarriages (van der Eijk et al., 2016). ZIKV infection is also associated with Guillain–Barre Syndrome, an autoimmune polyneuropathy that may develop into paralysis or death (*WHO | Zika Virus and Complications: 2016 Public Health Emergency of International Concern*, 2017; Mier-Y-Teran-Romero et al., 2018). So far, all the confirmed cases of neurological diseases have been associated with the Asian lineage of ZIKV, not the other major lineage, originating from Africa (Rossi et al., 2018; Liu et al., 2019). Future outbreaks of ZIKV infection and CZS are quite probable owing to three distinctive features of ZIKV infection. First, 50-80% of ZIKV infections are asymptomatic (Duffy et al., 2009; Aubry et al., 2017), so most cases may go undetected and the prevalence of the disease is challenging to assess. Second, ZIKV can persist in human hosts for up to 6 months and remain transmissible for over a month after the onset of symptoms (Turmel et al., 2016; Nicastri et al., 2016; Medina et al., 2018). Third, ZIKV infections are readily spread by mosquito vectors and sexual activity (Hills et al., 2016; Kindhauser et al., 2016; Moreira et al., 2017; Azar et al., 2017; Benelli & Romano, 2017), necessitating extreme vector control and human behavioral changes to contain the spread.

Because of the devastating consequences, challenging detection, and the outbreak potential of ZIKV infection, research efforts have been devoted to the development of Zika vaccines. So far, almost all the vaccine platforms, including ones based on DNA, RNA, purified inactivated virus, live attenuated virus, subunit, and viral vectored, have yielded vaccine candidates that have shown potential in animal studies (Butantan et al., 2018; Diamond et al., 2018). The most promising candidates include ZPIV, the purified inactivated ZIKV strain PRVABC59 developed by Walter Reed Army Institute of Research and Beth Israel

Deaconess Medical Center, which has shown efficacy in animals and completed phase I clinical trials (Modjarrad et al., 2018). However, since the infection by the African lineage protects nonhuman primates from the Asian lineage virus (Aliota et al., 2016), and no neuronal malfunctions associated with the African lineage has been reported, there is an argument for using the African lineage as a basis for a purified inactivated virus vaccine. Infection kinetics of the two lineages need to be studied *in vitro* to compare their potential in vaccine production. An *in vitro* comparison of the two lineages may also be of particular interest in revealing differences associated with their different neuropathology. Currently, there is a lack of consensus on whether the African lineage has higher titers, or whether its titers peak faster, even when compared in the same cell type (Willard et al., 2017; Anfasa et al., 2017; Moser et al., 2018; Ramos da Silva et al., 2019). A better quantitative understanding of virus production in cell culture by different lineages may be useful for establishing a bench-scale model of vaccine production as well as elucidating fundamental drivers of pathogenesis.

Such quantitative analysis and comparisons of virus growth have long been facilitated by mathematical models. Concepts and approaches adapted from ecology and epidemiology have been used to model virus growth, from the kinetics of bacteriophage in chemostats to *in vivo* loads of HIV-1 and other viruses in patients (Campbell, 1961; Levin et al., 1977; Perelson et al., 1996; Herz et al., 1996; Bonhoeffer et al., 1997; Grossman et al., 1998; Nowak & May, 2000; Perelson, 2002; Nelson & Perelson, 2002; Gilchrist et al., 2004; Baccam et al., 2006). In the culture of viruses for vaccines, Möhler et al. presented an unstructured model that neglects intracellular events while describing the multi-step kinetics of Influenza A virus production in a microcarrier culture of Madin-Darby canine kidney (MDCK) cells (Möhler et al., 2005). With a multiplicity of infection (MOI) of 0.025, one would expect a maximum of 2-3 percent of the total cells would be initially infected. The virus growth data were used to estimate model parameters, and their simulations indicated how the maximum yield of virus could be optimized, in principle, by increasing the specific replication rate of the virus and reducing the specific rate of cell death due to infection. By expanding experiments to characterize virus production from both high-MOI single-step infections and low-MOI multi-step infections, Pinilla et al. compared a wild-type and drug-resistant mutant of influenza A; their modeling of the data enabled a full quantitative characterization of the virus replication cycles, highlighting a 50-percent increase in the eclipse phase and 7-fold drop in the viral burst size for the

mutant relative to the wild-type (Pinilla et al., 2012). Recently, Bernhauerová et al. applied this modeling approach to multi-step infection of ZIKV, but only of one strain of the African lineage (MR766); their characterization of virus decay was more consistent with a gamma-distributed delay model, where particles appear initially stable, than the more commonly observed exponential decay (Bernhauerová et al., 2020).

In this work, we compared both the one-step and multi-step growth of two strains of different ZIKV lineages. The multi-step growth of the Asian lineage strain, PRVABC59, was performed with low multiplicities of infection (MOI) in Vero cells, similar to the virus growth process for vaccine production. The infections of cell culture were characterized experimentally with total and viral protein-expressing cell counts from fluorescent microscopic images in addition to plaque titers of infectious virus over time, and computationally with differential equations fitted to the virus growth curves. Three methods to model the delay between infection and production of progeny viruses, exponential distribution, gamma distribution, and fixed distribution, were tested, and some of the model variants conflicted with measurements from fluorescent microscopic images, highlighting opportunities for model validation using data other than virus titers. Finally, Ten Rules for credible practice of modeling and simulation have been applied in the modelling practices for better reproducibility and easier reuse (Erdemir et al., 2020). This first quantitative comparison of the kinetics of *in vitro* infection of different lineages of ZIKV provides an approach to understand the differences between the growth kinetics and pathogenesis of the two lineages, and it takes a step toward mathematical optimization of virus growth for vaccine production.

2 Materials and methods

2.1 Cells

Vero cells were obtained from American Type Culture Collection (ATCC® CCL-81™, ATCC, Manassas, VA) and cultured in Minimal Essential Media (MEM, Corning™ 15010CV, Corning, NY) supplemented with 10% fetal bovine serum (FBS, Atlanta Biologicals, Norcross, GA) at 37°C in a 5% CO2 atmosphere. Cells from Passage 10 to Passage 50 were used in the experiments and were tested to be free of mycoplasma contamination at Passage 9 and 50 using the Universal Mycoplasma Detection Kit (ATCC® 30-1012K™).

2.2 Viruses

Zika MR 788 obtained from ATCC (ATCC® VR-1838™) originated from the first isolate of Zika in 1947 by intracerebral passaged 146 times in adult Swiss mice and 1 time in Suckling mice, then adapted to cell culture by passaging 5 times in Vero cells. This strain was used to represent strains belonging to the African lineage. Zika PRVABC59 was a generous gift from Dr. Matthew Aliota (University of Minnesota); it was isolated from a patient sample, passaged 4 times in Vero cells, and it represents pandemic strains from the Asian lineage. Both strains were amplified twice in Vero cells and stored at -80°C.

2.3 Plaque assay

Virus titers were measured by plaque assay. Vero cells were seeded into 6-well plates (Corning™ 3506) and cultured till 90% confluency was achieved. Virus samples were serially diluted in MEM with 2% FBS and 400 µL of each dilution were added to 2 parallel wells. After incubation at 37°C for an hour, the supernatant was aspirated, and cells were rinsed with phosphate-buffered saline (PBS). 2 mL of 0.8% (w/v) agarose media was added to each well and topped with 2 mL MEM with 2% FBS. Plaques were visualized after about 90 hours by fixation with 4% (w/v) paraformaldehyde (PFA) in PBS and staining with 1:20 (v/v) crystal violet. Plaques were counted to calculate the plaque forming units (PFU) in the samples.

2.4 Staining and microscopic imaging

To distinguish live cells and dead cells, cells were sequentially stained with 5 µM Calcein AM and 2.5 µM SYTOX™ Orange (Thermo Fisher Scientific) and immediately imaged. Immunocytochemical staining was done with Anti-Flavivirus Group Antigen Antibody clone D1-4G2-4-15 (MAB10216, Sigma-Aldrich, St. Louis, MO) and/or Zika virus NS2B protein antibody (GTX133308, GeneTex, Irvine, CA). Cells were fixed in 4% PFA for 10 minutes, rinsed with PBS, permeabilized in PBS with 10% donkey serum (D9663 Sigma-Aldrich, St. Louis, MO) and 0.25% Triton X-100 for 20 minutes, and incubated with 1:500 dilution of primary antibodies overnight at 4°C. Unbound antibodies were rinsed away by PBS with donkey serum and Triton X-100. Then cells were stained by 1:500 dilution of Alexa Fluor® 594 conjugated donkey anti-mouse IgG (H+L) (ab150108, Abcam, Cambridge, United Kingdom) and/or FITC conjugated donkey anti-

rabbit IgG (H+L) (A16024, Invitrogen, Carlsbad, CA) for at least 4 hours, then rinsed with PBS prior to imaging. Nuclear staining was done by 5 μg/mL Hoechst 33324 in PBS. Images were taken using a Nikon Eclipse TE300 inverted epi-fluorescent microscope equipped with a QImaging ExiAqua charge-coupled device (CCD) camera (Surrey, BC, Canada). The objective used was a Nikon Plan Apo 10X, 0.45 NA objective. Illumination was provided by a Chroma PhotoFluor light source (89 North, Burlington, VT) and controlled with a Lambda 10-2 optical filter changer (Sutter Instrument, Novato, CA). The stage automation was enabled with a Prior ProScanII. These devices were controlled and images were taken by custom-written journals in MetaMorph v.7.7.8. All the image processing, including background subtraction, cell segmentation, cell counting, fluorescent intensity quantification, was performed with the open-source software JEX(Warrick & Berthier).

2.5 One-step infection

Vero cells were seeded into 12 well plates and cultured overnight. Cells in 2 wells were sacrificed for cell counting by a Bio-Rad® TC10™ Automated Cell Counter. Cells in other wells were rinsed by PBS and infected by 150 μL dilution of Zika African or Asian strain in MEM with 2% FBS so that the estimated ratio of virus particles to cells (multiplicity of infection [MOI]) was 10, or mock-infected. The inoculum was removed after 1 hour of incubation at 37 °C, followed by PBS rinse three times. Each well was replenished with 1 mL of MEM with 2% FBS. The plates were incubated at 37°C in a 5% CO_2 atmosphere. Every 4 hours, supernatant from replicate wells infected by either strain was collected into separate tubes and centrifuged at 2,000 g for 5 minutes. A mock-infected well was stained by Calcein AM and SYTOX™ Orange as described above side by side with emptied wells at each time point.

2.6 Multi-step infection

Vero cells in 12 well plates were counted. The stock of the Zika African or Asian strain was diluted in MEM with 2% FBS to infect the cells at low MOI. The cells were incubated with 150 μL inoculum for 1 hour, then supplemented with 850 μL of media. Supernatant from triplicate wells infected by either strain were collected for virus titers every 4 hours till 72 hours post-infection (h.p.i.) and at 80, 90, 96, 102, 120 and 144 h.p.i.. Cells sampled before 64 h.p.i. were immediately fixed in 4% PFA and

immunocytochemically stained. The remaining cells were stained by Hoechst 33342 for cell counting. Infections were performed in triplicate wells.

2.7 Mathematical model

Species in the infection process, the susceptible cells (*S*), the infected cells (*I*), and virus particles (*V*), are modeled with differential equations (see Supplemental Material). The susceptible cells were modeled with logistic growth; their infection was treated as a second-order reaction with virus particles *V*. After infection, cells need time to produce virus particles, so *I wa*s comparted into variables of early and the late infection stages. The delay between the infection of *S* the production of *V* as modeled as: (i) an exponentially distributed, (ii) a fixed, or (iii) a gamma-distributed delay. We assumed that the death rate of the non-producing cells was negligible (Best & Perelson, 2018; Holder & Beauchemin, 2011). Virus production and degradation were treated as first-order reactions.

For one-step infection, it was assumed that all the cells were initially infected, simplifying the model by omitting the equation for $\frac{dS}{dt}$ and the k_2SV term in the equation for $\frac{dV}{dt}$.

The above models were fitted to the virus titer data using the packages deSolve and minpack.lm in R (version 3.6.3) to obtain the parameter value estimates (Soetaert et al., 2010; Elzhov et al., 2015). The best fit was obtained by minimizing objective functions modified from the sum of squared residuals (SSR) because the virus titer spanned about six orders of magnitude. When fitting the models of one-step growth to the data, we used the objective function:

$$OF = \sum_{i=1}^{n}(\frac{\overline{exp}_i}{pre_i} - 1)^2 ,$$

where n is the number of experimental data points. $\overline{exp}_i$ and pre_i are the average of titer from the replicates and the model prediction at the *ith* sampling time (Shin et al., 2019). For multi-step model fitting, we used the following objective function:

$$OF = \sum_{i=1}^{n}(\frac{\overline{exp}_i - pre_i}{sd_i})^2 ,$$

where sd_i is the standard deviation of the virus titer in the triplicates. To compare different models, the Akaike information criterion corrected for small sample size (AIC_C) of each fit was calculated (Akaike, 1974; Beauchemin et al., 2008).

$$AIC_c = nln\left(\frac{OF}{n}\right) + \frac{2n(N_{par}+1)}{n-N_{par}-2},$$

where N_{par} is the number of the parameters in the model; models with lower AIC_C were considered better.

All data acquired in this study and R scripts are available at https://github.com/hshi44/ZikaKinetics.

3 Results

3.1 One-step virus infection

The African and the Asian strains of the Zika virus exhibited different behaviors in one-step infection. The increase of infectious virus was detected in both cases within 8 hours post-infection (h.p.i.), as in Figure 1A. The Asian strain grew by 104 fold in 8 hours, higher than that of the African strain (7.5 fold, p=0.19), indicating that the Asian strain replicated faster; this was consistent with the result of immunocytochemical staining at 8 h.p.i., where cells infected by the Asian strain had a higher positive rate and higher average staining intensity (Figure 1D). At 20 h.p.i, the titer of the Asian strain leveled off, while the titer of the African strain exceeded that of the Asian strain and continued to increase. The maximum cell specific yield of the Asian strain was 30 ± 2 PFU/cell at 24 h.p.i. and the maximum of the African lineage strain was 467 ± 6 PFU/cell at 36 h.p.i. (Figure 1A). In general, the Asian strain replicated faster during the early stage, but the African strain had a longer growth phase and higher titer.

3.2 Multi-step virus growth

The growth of the African and Asian strains shared similarities during multi-step growth. For both strains, a few cells expressing viral protein were detected after 12 h.p.i., and production of progeny infectious particles was observed at 20 h.p.i. (Figure 2A). In Vero cells infected by either strain, about 95% stained positive for viral protein at 64 h.p.i., around the same time decreases in cell density were seen (Figure 2C and D). Both strains reached their peak titers at 96 h.p.i., but the African strain had a higher titer, which resulted in a higher cell-specific yield (Figure 2A).

3.3 Virus and cell kinetics

The kinetics of virus degradation or host cell growth alone were examined by incubating virus or cells separately at the conditions used in the virus growth studies. The changes in virus infectivity followed the first-order degradation (Laude, 1981). The degradation rate constant k_6 for the African strain was $5.09 \pm 0.07 \times 10^{-2}\ /h$, and the Asian strain was less stable, with a larger degradation rate constant $7.05 \pm 0.10 \times 10^{-2}\ /h$. The growth of the Vero cells was fitted to a logistic growth model, where the cell growth rate constant was determined to be $3.11 \times 10^{-2}\ /h$ (95% confidence interval $2.29 - 3.92 \times 10^{-2}\ /h$), and the value of the capacity of cells was 1.94×10^6 cells/mL (95% confidence interval $1.62 - 2.26 \times 10^6\ cell/mL$). These rate constants were used in the kinetic models.

3.4 Models of the one-step infection

Both the gamma-distributed delay model and the fixed delay model provided close fits to experimental data, but the gamma-distributed delay model predicted unrealistically fast cell death for cells infected by the African strain. The initial conditions were assumed to be early-stage infected cells I_1 or $E_1 = 4.28 \times 10^5\ cell/mL$, the cell counts from a parallel plate, and free virus particle $V = 0.1$ because the initial virus titer should be low after multiple rinses. The best-fit curves using three modes of delay are shown in Figure 3B. For both strains, the exponentially distributed delay model fit poorly due to its failure to account for the delay. The parameter k_3, which describes the transition from the early stage to the virus-producing late stage, is forced to be so small that less than 0.5% of cells were predicted to produce progeny virus by the end of the experiment. The gamma-distributed delay model had the smallest prediction error and the lowest AIC_C. For the African strain, the best fit was achieved when $n_E = 22$ and $n_I \to \infty$ (Figure S1), meaning all the producing cells I have the same lifespan, predicting a peak titer of 1.3×10^8 PFU/mL around 37 h.p.i., close to the experimentally observed peak. However, this set of parameters predicts a fast death of producing cells, contradicting the observation in the experiment. In the contrast, the gamma-distributed delay model for the Asian strain has the smallest prediction error when $n_i = 1$, assuming the lifespan of producing cells follows the exponential distribution, and it recapitulates the decreasing trend of cell density observed in the experiment. The predicted peak titer is 1.1×10^7 PFU/mL, appearing around 28 h.p.i.. The fixed delay model captures the slow decrease of cells infected by the African strain better

than the gamma-distributed delay model. It predicts a peak titer of 7.5×10^7 PFU/mL around 53 h.p.i. for the African strain and 1.1×10^7 PFU/mL around 28 h.p.i. for the Asian strain. The predicted peak titer for the Asian strain is similar to the prediction from the gamma-distributed delay model. In addition, the fitted parameters are also close to those of the gamma-distributed delay model (Table 1). Taken together, the fixed delay model performed well for both strains. When comparing the two strains, the fixed delay model predicts a longer delay, a slower cell death, and a higher virus production rate for the African strain than the Asian strain in good alignments with the experimental observations.

3.5 Models of the multi-step growth

Three models performed differently when comparing the behavior of the two strains over multi-step growth. The initial conditions were set as the cell count of the parallel wells (4.3×10^5 cells/mL) and the titers of input virus (5.3×10^3 PFU/mL of the African strain or 6.8×10^2 PFU/mL of the Asian strain) at $t = -1$. The exponential-distributed delay model did not perform well on the African strain. It had the highest AIC_C for the African strain and predicted an unrealistically low number of producing cells with no cell death. But the exponential-distributed delay model had the lowest AIC_C for the Asian strain. Being infected, expressing the viral proteins, and producing progeny virus occur in chronological order, so the number of cells expressing viral protein should be smaller than the total number of infected cells, but larger than the number of producing cells. The exponential-distributed delay model also recapitulated this relationship for the Asian strain (Figure 4C). The gamma-distributed delay model had the lowest AIC_C for the African strain ($n_E = 31$ and $n_I = 145$) and captured the peak of the growth curve well but predicted a fast cell death that deviated from the experimental observation (Figure 4B); this model also had the highest AIC_C for the Asian strain ($n_E = 50$ and $n_I = 3$). The fixed delay model fit the growth curve and replicated the trend of cell death but predicted a deep dip in the virus titer at early time points and a slightly higher ratio of virus-producing cells for the Asian strain. The peak titer of the African strain was $1.6\pm0.4\times10^8$ PFU/mL at 90 h.p.i., and the gamma-distributed delay model predicted a peak of 2.1×10^8 PFU/mL at 90 h.p.i., while the fixed delay model predicted 6.8×10^7 PFU/mL at 105 h.p.i.. The Asian strain peaked at 90 h.p.i. in the experiment as well, with a titer of $2.1\pm0.5\times10^7$ PFU/mL, and the predicted peaks were 7.7×10^6 PFU/mL at 104 h.p.i., 7.2×10^6 PFU/mL at 92 h.p.i. and 7.1×10^6 PFU/mL at 77 h.p.i.

by the exponential-distributed delay, the gamma-distributed delay, and the fixed delay models, respectively. In summary, for the African strain, the fixed delay model worked best, and the exponential-distributed delay model had the best performance for the Asian strain.

3.6 Sensitivity analyses

To examine the sensitivity of the output of models to changes in different steps in virus replication, simulations of multi-step virus growth were run with each rate constant or delay set at 0.8, 0.9, 1.1, and 1.2 times the estimated values, using the fixed delay model for the African strain and the exponential-distributed delay model for the Asian strain. For the African strain, k_5 affected the peak titer most, followed by k_6 and k_1, while the timing of the peak was the most sensitive to k_6, k_5 and k_2, as in Figure 5. The highest and the lowest peaks of the Asian strain, increased by 21% and decreased by 15%, achieved with the smallest and the largest k_6, and the peak titer was also sensitive to k_1 and k_5. The earliest peak appeared with the largest k_3 and the latest peak was associated with the smallest k_5. Principal component analysis (PCA) on the peak titer was conducted when all the parameters changed simultaneously, and the results were in Table 3. Parameters k_5, k_6, and k_1 had the highest sensitive indices for the African strain, and k_6, k_1, and k_5 had the highest sensitive indices for the Asian strain, in good agreement with the result when parameters were changed individually. PCAs on the virus titer time course revealed different sensitivity, where τ, k_5, and k_1 were the most sensitive for the African strain and k_5, k_6, and k_3 for the Asian strain. The models had different sensitivity to different parameters, and further analysis would direct efforts to manipulate Zika virus infection.

4 Discussion

We measured Zika virus growth curves of both one-step and multi-step with short time intervals, with simultaneous measurements on cell growth/death and viral protein expression. These data provide rich information for the modeling of the Zika virus. The models revealed that the African strain had a larger production rate in both one-step and multi-step infection, consistent with experimental observations. In addition, one-step and multi-step infections had different kinetics. For both strains, the production rate of progeny virus was lower in multi-step infection than in one-step infection.

The approach we took to compare the one-step infection kinetics of the two strains can be extended to other strains of ZIKV. When modeling the one-step growth of ZIKV, we chose the initial virus titer to be zero, different from the experimental measurements because by allowing the estimate of the initial virus titer to vary, the estimated parameters in the best-performing fixed delay model remained mostly constant. In addition, the initial cell counts I_0only affected the viral production rate constant $k_5$in an inversely proportional manner for both strains. These observations indicate that the model can be used to estimate most parameters even if the initial conditions are missing or imprecisely estimated. For example, we analyzed the one-step growth of three other ZIKV strains in Moser et al., 2018, using the fixed delay model and found that the strains have similar delay times, and the isolate from Panama had the highest production rate, as in Table 4. The model identified a similar cell death rate (k_4) and virus production rate ($I_0 \times k_5$) for the Puerto Rican isolate PRVABC59 in comparison to our results. This example demonstrates how our model can be applied to compare the growth phenomena of different strains of ZIKV from literature reports.

We have observed differences in the kinetic parameters of the African strain and the Asian strain, and multiple variations in the genome of the two lineages have been identified previously (Beaver et al., 2018). However, it is not fully known how the kinetic differences might reflect genetic differences between the two lineages. The titer of our African strain in Vero cells is among the highest of reported values (Willard et al., 2017; Moser et al., 2018; Ramos da Silva et al., 2019), and it may be attributed to the 12 nucleotides in the region encoding the envelope (E) protein since the encoded amino acid residues have been reported as important to the fitness of the virus (Aliota et al., 2016). Despite the absence of links between genetics and function, particle degradation or stability are readily measured and often included in comparisons between strains; for example, a 2013 Zika virus isolate (H/PF/2013) and a 2015 isolate (Paraiba/2015) were found to exhibit similar or lower stability than the African lineage strain (Goo et al., 2016). In our study, the Asian strain isolated in 2015 (PRVABC59) was also less stable than the African strain. Whether the Zika virus gained mutations after 2013 that lowered its stability, and how the genetic variations affect virus fitness or stability remain open questions; methods of deep mutational scanning may well elucidate such questions (Setoh et al., 2019). There is a major and key genetic difference between the African lineage and the Asian lineage in the RNA-dependent RNA polymerase (RdRP) NS5,

specifically the mutation M2634V (Pettersson et al., 2016; Zhao et al., 2018). Since RdRP plays a central role in the intracellular replication of the virus, may be worth studying if this substitution can be associated with the shorter lag between infection and virus production or the lower production rate constant of the Asian strain. These linkages between phylogenetics and kinetic parameters are one direction for future studies because the knowledge may enable a better understanding of differences in strain-associated pathogenesis and design of higher yield vaccine strains.

Another future direction would be the refinement of the multi-step infection models, which had significant prediction errors from the virus titer data. This may be because the models were built on assumptions that deviated from the experimental reality. First, our models treated the system as spatially homogeneous or well-mixed, but the infection of surface-adsorbed cells in well-plates will not be spatially homogeneous, especially when a small fraction of the cells are initially infected by a low MOI. Second, the infection was considered as the interaction of a single cell with a single virus particle, and it was assumed that all the infected cells had similar outcomes; in reality, individual cells in the second and subsequent rounds of virus growth can be infected by multiple virus particles, demonstrating behaviors similar to the cells from one-step growth (high MOI) experiments, which exhibit higher virus production rates. To account for different kinetics associated with varying virus-to-cell ratios in multi-step infections, future models may need to include additional MOI- or time-dependent growth parameters. The solution to the mixing problem may depend on the scenario where the models are applied. In modeling infection *in vivo* or in tissue cultures, where transport is limited, the spatial spread of the virus particles can be incorporated (Yin & McCaskill, 1992; You & Yin, 1999; Haseltine et al., 2008). The models may further account for spread owing to fluid flows or employ rule-based cellular automata to model the coupling of virus growth with the spatial transport of virus particles (Anekal et al., 2009; Mitchell et al., 2011; Akpinar et al., 2016). In other scenarios of vaccine production, the microcarrier culture of Vero cells may be closer to well-mixed systems (Sugawara et al., 2002; Souza et al., 2009; Jiang et al., 2019), so the present simpler models might in such cases provide a starting point for modeling.

Our model on the multi-step infection of the Asian lineage strain made predictions applicable to the optimization of the vaccine production process. As the sensitivity analysis showed, the biological stability

of virus particles, as typically characterized by the rate of degradation of their infectious titer, has the largest effect on the peak titer and is potentially important for vaccine production. As with most traits, virus particle stability (or degradation) will depend not only on strain genetics, but also on the environment; genetically similar or identical strains can exhibit different stabilities under different culture conditions (Kostyuchenko et al., 2016; Goo et al., 2016; Bernhauerová et al., 2020). Considering that the African lineage strains used by us and others differ by two amino acids in the NS5 protein (Goo et al., 2016), which likely have little effect on thermal stability, observed differences in stability may reflect different media and incubation conditions. In fact, we measured a more than 5-fold increase in degradation rate if the virus was incubated in sealed microcentrifuge tubes (Figure S1), likely owing to higher resulting pH. This emphasizes the importance of media formulation and pH control in the large-scale production of the Zika virus for vaccines since they might slow degradation and increase titers. Other optimization strategies might include the adaptation of the vaccine strain to cell culture; the culture-adapted African strain exhibited a higher titer, and our modeling predicts that the titer can be increased by higher k_5, and the culture time can be decreased by higher k_3; both could in principle be achieved by adaptation to culture conditions. Finally, our simulations showed how higher cell densities at the time of virus infection can further increase peak titers, and higher MOI gives rise to earlier peaks. The resulting reduction in the overall duration of cultures could be beneficial for vaccine production, but such actions would need to be balanced against the need for higher inoculum concentrations.

To facilitate the application of our models by the community, we have complied with the Ten Simple Rules of credible practice of modeling and simulation (Erdemir et al., 2020). We have addressed the context and limitations of our models (Rules 1 and 4). To enable review, training, and reuse of our data and models, we have made raw and processed data available on GitHub along with the scripts (Rules 2, 7, and 8). The models have been evaluated within context; specifically, their ability to account for the data has been verified and validated by extensive testing, the uncertainty of parameters has been reported, and sensitivity analysis has been implemented with heatmaps and the Multivariate sensitivity Analysis package multisensi (Rule 3). The current models and comments are under version control using GitHub; further development and documentation will be synchronized in the GitHub repository (Rule 5, 6, and 7). The models have been reviewed within the research group, and third-party reviewers of the manuscript

and models are welcome (Rule 8). Our current work tested three different ways of modeling the delay between infection and virus production, and models with exponential cell growth and other variations will be compared against our model in the future (Rule 9).

While previous works combined quantitative measures and computational models to analyze the ZIKV infection *in vitro* or *in vivo* (Best & Perelson, 2018; Bernhauerová et al., 2020), our work has compared Zika virus one-step and multi-step growth of different strains, introduced measurements on cell density and viral protein expression as additional validation of the mathematical models, and provided a framework for understanding how controllable conditions, including cell density, MOI, inoculum size, and virus particle stabilities contribute to culture outcomes. Additional experimental data, including those from more ZIKV strains, purposefully designed mutants, intracellular replication, and microcarrier cultures, will enable further development of the kinetic models. The models have the potential of revealing how ZIKV causes disease and how virus titer for vaccine production can be optimized, facilitating our combat against the virus.

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