

Dengue serotypes 1–4 exhibit unique host specificity in vitro

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Background: Over 3000 cell lines from over 150 species are commercially available today from the American Type Culture Collection. These cell lines offer alternative approaches to investigating the interactions between arboviruses and other vertebrates at the cellular level. The various cell origins, types, and morphologies can be valuable resources for studying viral ecology and examining hypotheses regarding viral reservoirs. Dengue viruses (DENV) are major re-emerging pathogens that have been studied classically in only a few cell lines.

Methods: We evaluated the susceptibility of 19 distinct mammalian, avian, and reptilian cell lines to DENV infection. Cell lines were infected with DENV serotypes 1–4 and evaluated for susceptibility via focus-forming unit assays and quantitative reverse-transcription polymerase chain reaction.

Results: Both methods demonstrated the ability of DENV to replicate in 14 cell lines derived from various vertebrates with viral titers ranging from 1×10^3 to 1×10^7 infectious units per milliliter. Cell line susceptibility to DENV infection was serotype specific, with DENV-1 and DENV-4 infecting more cell lines than either DENV-2 or DENV-3. Cellular type also seemed to affect the infectivity of DENV. Human endothelial cells were only susceptible to DENV-4. Of six fibroblast lines, 100% were susceptible to at least one DENV serotype whereas only 62% of 13 epithelial lines were susceptible to DENV serotypes 1–4.

Conclusion: These data indicate that a variety of cell lines from human and animal species can be used to culture DENV. The serotype-specific susceptibility for certain cell lines may provide a tool to help characterize specific DENV serotypes as well as an in vitro platform for the study of host–pathogen interactions and the co-circulation of DENV serotypes in a specific region or individual.

Keywords: dengue virus, cell culture, host

Introduction

Dengue viruses (DENV) are major re-emerging pathogens that are endemic in all continents except Europe and Antarctica. Over half of the world's population is at risk of infection.¹ DENV is the most common human arboviral infection and the most important public health threat from mosquito-borne viral pathogens. It causes an estimated 50 million cases of dengue infection, half a million hospitalizations, and approximately 15,000 deaths each year.² Children are much more likely to die from DENV complications than adults.³ In the past 50 years, the incidence of dengue infection has increased 30-fold.⁴

During the 1950s and 1960s, DENV was found to propagate well in Vero, LLC-MK2, baby hamster kidney, and mosquito cell lines (Table 1).^{5–8} Other continuous

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Table 1 Cell lines traditionally used for dengue virus research

Cell line	Species	Tissue	Use
BHK	<i>Mesocricetus auratus</i>	Kidney fibroblast	Plaque assays and culture ^{6,45}
Vero	<i>Cercopithecus aethiops</i>	Kidney epithelial	Plaque assays and culture ^{7,22,43}
LLC-MK2	<i>Macaca mulatta</i>	Kidney epithelial	Plaque assays and culture ^{8,43}
C6/36	<i>Aedes albopictus</i>	Clone	Culture ^{5,44,45}
HeLa	<i>Homo sapiens</i>	Cervix epithelial	Culture ^{10,46}
THP-1	<i>Homo sapiens</i>	Peripheral blood monocyte	Antibody-dependent enhancement ^{13,47}
HepG2	<i>Homo sapiens</i>	Liver epithelial	Plaque assay ^{9,48}
U-937	<i>Homo sapiens</i>	Monocyte	Antibody-dependent enhancement ^{11,27}
K-562	<i>Homo sapiens</i>	Bone marrow lymphoblast	Antibody-dependent enhancement ^{12,43}

Abbreviations: BHK, baby hamster kidney; HepG2, hepatocellular carcinoma; THP-1, human acute monocytic leukemia.

human epithelial cell lines have been used to investigate DENV–host interactions,^{9,10} while human monocyte cell lines have been used to investigate antibody-dependent enhancement (Table 1).^{11–13} The early studies that discovered the utility of these cell lines were focused on determining whether DENV could replicate and plaque in a specific cell line. They also often examined the duration of persistent infection within the culture.^{5–10} Since then, DENV has been rarely studied in other cell lines (Table 1).

The Vero, LLC-MK2, baby hamster kidney, and human monocyte cell lines were sufficient during the twentieth century when research was focused on virus propagation, vaccines, and treatment strategies. However, the multitude of cell lines commercially available today offer alternative approaches to investigating the interactions between DENV and other organisms at the cellular level and can be valuable resources for evaluating vector ecology and alternative viral reservoirs.

Mounting evidence indicates that accounting for variation in the ecology and epidemiology of dengue serotypes and strains will be important for the development of more effective, locally adapted control programs.^{14–17} Advances in genomics, proteomics, host cell defense, and methods for genetic manipulation allow us to examine the complex interactions between viruses and their hosts at cellular and molecular levels. These processes can be studied in a variety of animal models or cell lines. Here, we report on the susceptibility of different vertebrate cell lines to DENV infection and the level of propagation in susceptible cells.

Methods

Cells

All cell lines were obtained from the American Type Culture Collection (Manassas, VA) and cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% (v/v) fetal bovine serum, 1% (v/v) L-glutamine, 1% (v/v)

nonessential amino acids, 1% (v/v) sodium pyruvate, 100 U/mL penicillin, 100 µg/mL streptomycin, and placed in a 37°C incubator with 5% CO₂. Table 2 describes the cell lines used in this work.

Virus

The following serotypes and strains were obtained from the Walter Reed Army Institute of Research and were used for all experiments. Strain identities are as follows: DENV-1: West Pak 74 (Western Pacific strain from Nauru Island 1974), DENV-2: s16803 (Southeast Asia), DENV-3: CH5548904500, DENV-4: 341750.

Infection of cells with DENV

All infections were performed using 12-well standard cell culture plates seeded with cells to reach 90% confluency upon infection. Individual wells were inoculated with 1000 infectious units (IU) of a DENV serotype in modified Eagle medium and then rocked at 37°C for 1 hour after which the inoculum was removed, rinsed twice with sterile phosphate-buffered saline, then overlaid with 1 mL of DMEM (10% fetal bovine serum, 1% glutamine, 1% nonessential amino acids, 100 mg/mL penicillin/streptomycin, 1% sodium pyruvate) and placed in a 37°C incubator with 5% CO₂. The culture supernatant was collected at 1 hour and at 72 hours postinfection (PI).

Virus detection via immunostaining

DENV infection was visualized in cells via immunostaining infected cell lines 72 hours PI cells infected with DENV were fixed and permeabilized using 1 mL of a 1:1 acetone/methanol solution with a 60-minute incubation at 4°C. Virus foci were detected using a specific mouse monoclonal antibody from hybridoma 2H2 (EMD Millipore, Billerica, MA), followed by a horseradish peroxidase-conjugated goat anti-mouse immunoglobulin (Millipore), and developed

Table 2 Cell lines evaluated for susceptibility to dengue virus infection

Cell line	Common name	Species	Tissue	Reference
TB I Lu	Free-tailed bat	<i>Tadarida brasiliensis</i>	Lung epithelial	49
DF-I	Chicken	<i>Gallus gallus</i>	Embryonic fibroblast	50
Sf I Ep	Cottontail rabbit	<i>Sylvilagus floridanus</i>	Epidermis epithelial	51
EA.hy926	Human	<i>Homo sapiens</i>	Vascular endothelial	52
CRFK	Domestic cat	<i>Felis catus</i>	Kidney epithelial	53
E.Derm	Horse	<i>Equus caballus</i>	Dermis fibroblast	54
FoLu	Grey fox	<i>Urocyon cinereoargenteus</i>	Lung fibroblast	55
PI I Ut	Raccoon	<i>Procyon lotor</i>	Uterus fibroblast	56
OHHI.K	North American mule deer	<i>Odocoileus hemionus hemionus</i>	Kidney fibroblast	57
OK	Virginia opossum	<i>Didelphis marsupialis virginiana</i>	Kidney epithelial	58
MDOK	Sheep	<i>Ovis aries</i>	Kidney epithelial	59
DNI.Tr	Nine-banded armadillo	<i>Dasybus novemcinctus</i>	Trachea fibroblast	60
PK(15)	Domestic pig	<i>Sus scrofa</i>	Kidney epithelial	61
LLC-MK2	Rhesus monkey	<i>Macaca mulatta</i>	Kidney epithelial	62
BT	Cow	<i>Bos taurus</i>	Turbinata	63
MDCK	Domestic dog	<i>Canis familiaris</i>	Kidney epithelial	64
WCH-17	Eastern woodchuck	<i>Marmota monax</i>	Liver epithelial	65
TH-I	Eastern box turtle	<i>Terrapene carolina</i>	Heart epithelial	66
MvI Lu	American mink	<i>Neovison vison</i>	Lung epithelial	67

Abbreviations: CRFK, Crandell-Rees feline kidney; MDCK, Madin–Darby canine kidney; MDOK, Madin–Darby ovine kidney.

using a 50 mg tablet of 3,3'-Diaminobenzadine tetrahydrochloride (Sigma-Aldrich, St Louis, MO) dissolved in 20 mL phosphate-buffered saline with 8 μ L 30% hydrogen peroxide.

Virus detection via real-time polymerase chain reaction (PCR)

Viral RNA was extracted from cultured supernatant using the Ambion MagMax-96 extraction kit (Life Technologies, Grand Island, NY) per the manufacturer's instructions. Quantitative real-time reverse-transcriptase PCR (qRT-PCR) was conducted utilizing the Bio-Rad iQ5 platform (Bio-Rad, Hercules, CA) with the Bio-Rad Superscript One Step SYBR Green qRT-PCR kit, using primers Den_F (TTAGAGGAGACCCCTCCC) and Den_R (TCTC-CTCTAACCTCTAGTCC) from Chutinimitkul et al⁴² and the following cycling conditions: reverse transcription at 50°C for 10 minutes and 95°C for 5 minutes, followed by 40 cycles of denaturation and amplification at 95°C for 10 seconds and 48°C for 30 seconds, respectively.

A series of controls was performed for each cell line to identify true positives not related to background. A no-template control and a no-primer control were performed to verify that the reagents and equipment were working as expected. A positive virus control of DENV-1 to DENV-4 cultured on LLC-MK2 cells collected from cell culture supernatants was performed to verify that the PCR primers were functioning as expected. A noninfected control

from both LLC-MK2 cells and the cell line being tested was included to verify that there was no increase in nonspecific binding from the PCR primers that could cause a higher background signal. Finally, the cell culture supernatant collected 1 hour PI with DENV from both LLC-MK2 cells and the cell line being tested was assayed to ensure that qRT-PCR results, 72 hours PI, were not convoluted by input virus. Cycle threshold (Ct) values were used to estimate relative viral titers of infected cell lines according to a standard curve created using a serial dilution technique of known viral concentrations derived from LLC-MK2 control cells. Each of the three independent assays was amplified in triplicate for a total of nine measurements per cell line tested.

Statistical analysis

Mean relative titers between a specific cell line and the LLC-MK2 cell line were compared with an unpaired *t*-test, with the Satterthwaite unequal variance method used where appropriate. Analyses were performed using SAS v 9.2 (SAS Institute, Inc, Cary, NC).

Results

A number of cell lines were susceptible to DENV

For these experiments, 19 distinct cell lines were selected from the inventory at the American Type Culture Collection. Cell lines were selected based on the susceptibility of the host species to flaviviral infection and utility of the cell line

in virus research. Table 2 describes the selected cell lines and references their use for in vitro virology studies. Of the 19 cell lines tested for DENV infection, 14 showed positive Ct values for at least one DENV serotype based upon qRT-PCR data at 72 hours PI (Table 3). Of these 14 cell lines, TB 1 Lu and DF1 cell lines showed positive Ct values for DENV-1 alone. SF1 and EA.hy.926 were positive for DENV-4 alone. Five cell lines were positive for both DENV-1 and DENV-4 (E.Derm, FoLu, Pl 1.Ut, OHH1.K, and OK). Crandell-Rees feline kidney (CRFK) and DNI.Tr were positive for three serotypes, DENV-1, DENV-3, and DENV-4. Only the Madin–Darby ovine kidney (MDOK) cell line was positive for DENV-1, DENV-2, and DENV-4. The LLC-MK2 and PK(15) cell lines were the only two to show positive Ct values for all four serotypes.

DENV can replicate to high titers in a number of cell lines

Real-time data allowed for the estimation of relative virus titers for each cell line with a positive Ct value. This was based upon a standard curve analysis using known virus concentrations derived from LLC-MK2 cells, as well as confirmation of viable virus via immunostaining. Relative titers of positive samples were found to be in the exponential range of 1×10^3 to 1×10^7 IU/mL, with the MDOK

Table 3 Quantitative real-time reverse-transcription polymerase chain reaction results for cell lines tested for dengue virus (DENV) infection across four virus serotypes (DENV-1 to DENV-4)

Source	Cell line	DENV-1	DENV-2	DENV-3	DENV-4
Bat	TB.1 Lu	+	–	–	–
Chicken	DF-1	+	–	–	–
Rabbit	SF 1 Ep	–	–	–	+
Human	EA.hy.926	–	–	–	+
Cat	CRFK	+	–	+	+
Horse	E.Derm	+	–	–	+
Fox	FoLu	+	–	–	+
Raccoon	PL 1 Ut	+	–	–	+
Deer	OHH1.K	+	–	–	+
Opossum	OK	+	–	–	+
Sheep	MDOK	+	+	–	+
Armadillo	DNI.Tr	+	–	+	+
Pig	PK(15)	+	+	+	+
Monkey	LLC-MK2	+	+	+	+
Cow	BT	–	–	–	–
Dog	MDCK	–	–	–	–
Woodchuck	WCH-17	–	–	–	–
Turtle	TH-1	–	–	–	–
Mink	Mv1 Lu	–	–	–	–

Note: +DENV detected.

Abbreviations: CRFK, Crandell-Rees feline kidney; MDCK, Madin–Darby canine kidney; MDOK, Madin–Darby ovine kidney.

and LLC-MK2 cell lines having the highest propagation potential (Figure 1).

Considering each serotype, one cell line yielded a significantly higher mean relative titer compared with the other cell lines ($P < 0.05$). The LLC-MK2 cell line yielded the highest mean relative titer for DENV-1, DENV-2, and DENV-3 serotypes; the MDOK cell line yielded the highest mean relative titer for DENV-4. For the only two cell lines to test positive for all four serotypes, LLC-MK2 had significantly higher mean relative titers for all four serotypes compared to PK(15) ($P < 0.05$).

Cell susceptibility to DENV infection is serotype dependent

DENV susceptibility based upon tissue type was evaluated. Of the 19 cell lines investigated, 13 (12 mammalian and one reptilian) were epithelial and six (five mammalian and one avian) were fibroblast. All six (100%) of the fibroblast cell lines and eight of 13 (62%) of the epithelial cell lines were susceptible to infection with at least one serotype of DENV. All fibroblast lines were susceptible to DENV-1, five (83%) to DENV-4, one (17%) to DENV-3, and none to DENV-2. Only LLC-MK2 and PK(15), both epithelial cell lines, were found to be susceptible to all four serotypes. Of the five kidney epithelial cell lines investigated, LLC-MK2 and PK(15) were susceptible to all four serotypes, MDOK and CRFK were susceptible to three serotypes, and Madin–Darby canine kidney (MDCK) was found not susceptible to any serotype.

Some cell lines had a similar performance to the LLC-MK2 cell line

Although the LLC-MK2 cell line yielded the highest mean relative titer for DENV-1, DENV-2, and DENV-3, other cell lines yielded sufficiently high relative titers to be candidates for further study (Figure 1). For DENV-4, the LLC-MK2 cell line yielded a mean relative titer lower than the MDOK cell line ($P = 0.02$), and was not statistically different from the cell lines OHH1.K, OK, FoLu, and CRFK (Figure 1).

Discussion

The data show that DENV infects numerous cells lines and infection is serotype specific in vitro. Although in vitro work does not always reflect in vivo systems, these results raise some challenging questions regarding DENV–host interactions. The literature has shown that livestock, companion, and peridomestic animals can serve as hosts and/or alternate reservoirs for flaviviruses that are closely related to

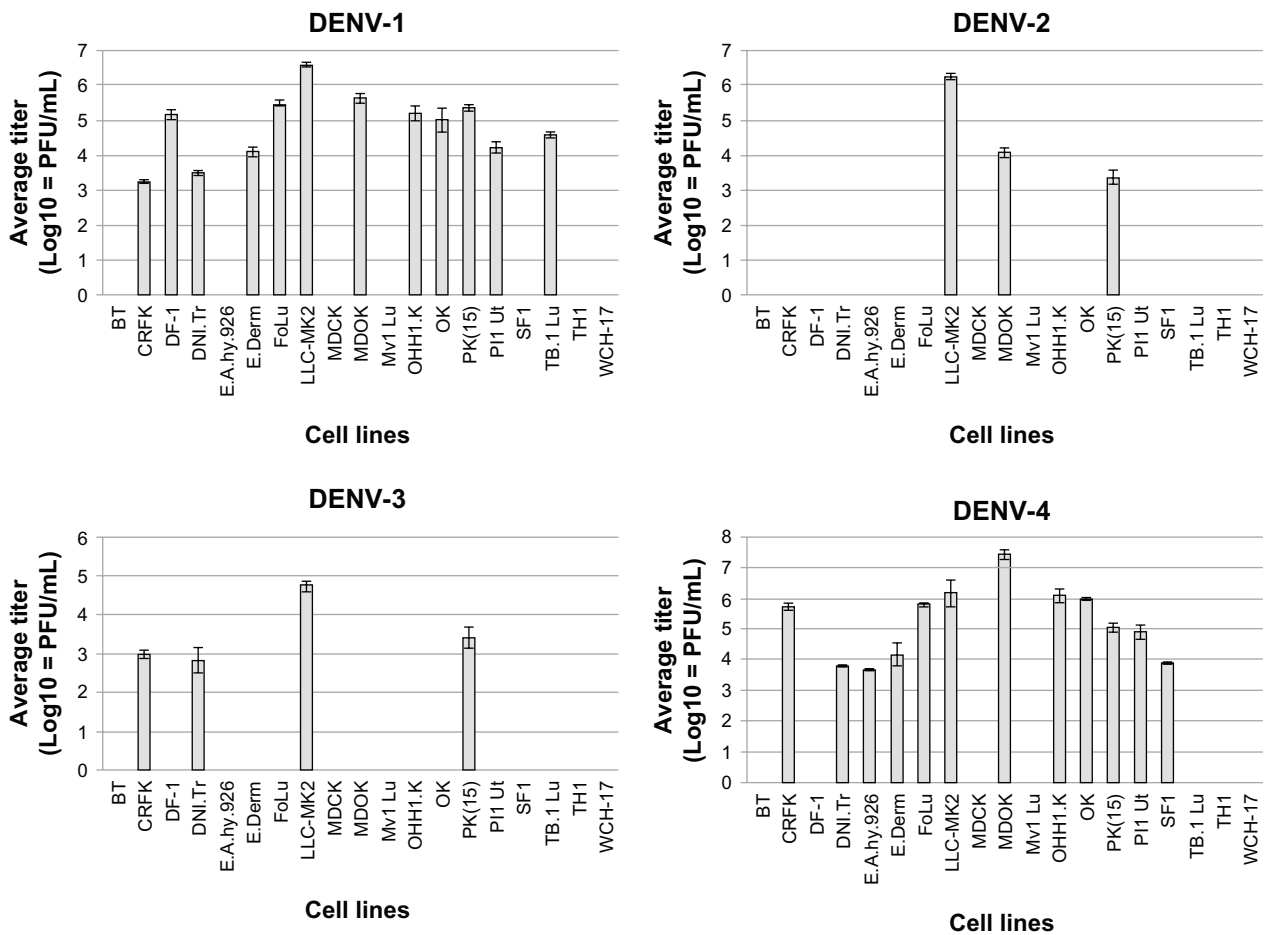


Figure 1 Mean relative titers of dengue virus (DENV) ± standard error of the mean produced from cell culture supernatants from 19 cell lines collected at 72 hours postinfection. **Note:** Relative viral titers of infected cell lines were calculated according to a standard curve created using a serial dilution technique of known viral concentrations. **Abbreviations:** CRFK, Crandell-Rees feline kidney; MDCK, Madin–Darby canine kidney; MDOK, Madin–Darby ovine kidney.

and morphologically similar to DENV (Table 4). For instance, chipmunks, alligators, raccoons, opossums, and squirrels are animals that have been shown to be susceptible to infection with St Louis encephalitis, West Nile virus, yellow fever virus, and Japanese encephalitis virus.^{18–24} Yellow fever virus, a flavivirus that is defined as having humans and other primates as natural hosts,^{25,26} has been shown to cause viremia and seroconversion in a variety of vertebrates.²⁷ The same has been observed for the St Louis encephalitis virus, which has a transmission cycle limited to humans and birds.^{20,28–30}

DENV infects vertebrates in vivo

It is accepted that the natural hosts for dengue are humans and other primates.³¹ However, the results of our experiments support previous studies that indicate that there may be alternate hosts or reservoirs for DENV. During a DENV outbreak in 1942 in Japan, sick animals were observed in Osaka and Kobe.³² Subsequent animal studies in Japan using the outbreak strains showed that guinea pigs (*Cavia porcellus*), rabbits, mice, rats (*Rattus rattus* and *Rattus norvegicus*), and striped squirrels (*Eutamias asiaticus*) were susceptible to

Table 4 Vertebrate animals that exhibit viremia and/or seroconversion to flaviviruses

Virus	Host
West Nile virus	Cat, ⁶⁸ dog, ⁶⁸ horse, ⁶⁹ alligator, ¹⁸ deer, ⁷⁰ primates, ^{71,72} rodents, ²⁰ rabbit, ^{71,73} reptiles, ⁷⁴ opossum, ¹⁹ birds, ^{75,73} raccoon, ²³ squirrels ²³
Japanese encephalitis virus	Birds, ^{76,77} pig, ^{78,79} cow, ⁷⁷ horse, ²⁴ monkeys, ²⁴ rodents, ²³ reptiles ⁸⁰
St Louis encephalitis virus	Birds, ²⁹ armadillo, ²⁹ rodents, ^{28,30} opossum, ^{20,30} raccoon, ²⁰ squirrel ^{76,77}
Yellow fever virus	Monkeys, ^{27,43,78,79} opossum, ^{21,77} rodents, ²² kinkajou, ²¹ bats, ^{24,45} hedgehog, ⁴⁶ wild dog, ³⁰ mongoose, ²⁹ wild birds, ⁴⁸ anteater, ²¹ squirrel ^{20,59}
Dengue virus	Bats, ^{34,81} chipmunk, ^{32,33} rabbits, ^{32,33} guinea pig, ^{32,33} mice, ^{32,33} Yucatan miniature pig, ⁸² horse ³⁵

DENV and exhibited morbidity, mortality, or both.^{32,33} DENV has been isolated from bats³⁴ and recent work has shown that horses infected with DENV develop an antigenic response.³⁵ These incidents of faunal infections were isolated and may be a function of the DENV strain, which was circulating in that specific location at that specific time. However, if DENV does infect other vertebrates *in vivo*, this may explain how the virus is maintained during inter-epidemic periods.

Cell line susceptibility is serotype specific

The results from the work presented here demonstrate that DENV affinity for cell lines may be serotype specific. This feature may be useful in the study of host–pathogen interactions *in vitro* including mechanisms of virus entry/exit, and virus replication. The data suggest that DENV-1 and DENV-4 may infect a broader diversity of cell lines than DENV-2 and DENV-3. Currently, most DENV research is performed using a few strains of DENV-2, usually DENV-2 New Guinea C or DENV-2 16681 whereas this study employed DENV-2 16803. Here, the data showed that DENV-2 had a very limited infectivity range, replicating only in MDOK, PK(15), and LLC-MK2 cells. Contrary to our results, which indicate that DENV-2 does not infect MDCK cells, infection of a different strain of DENV-2 was recently demonstrated in MDCK cells.³⁶

This strain-dependent infectivity may explain the co-circulation and maintenance of distinct DENV serotypes in one niche/biospace. The competitive exclusion principle states that no two species can permanently occupy the same niche: either the niches will differ or one will be excluded by the other. The co-circulation of DENV serotypes in the same biospace is contrary to this principle and has puzzled scientists for decades. The movement and evolution of DENV serotypes has been studied but not defined, and remains an area of active epidemiologic research.^{37,38} Furthermore, increases in replication fitness have not yet been detected for any given DENV genotype, thus the characteristics of a particular DENV serotype that allow it to disperse to other geographical areas have yet to be defined and/or measured.³⁹ The serotype-specific host susceptibility we observed might provide insights into the basic ecology of DENV that include: co-circulation of DENV serotypes as well as establishment and displacement of serotypes or strains in a geographic area.

Tissue type contributes to susceptibility

This work also evaluated the susceptibility of human EA.hy.926 cells, which originate from vascular

endothelial tissue. Due to the hemorrhagic nature of DENV and other work with DENV in endothelial cells,^{40,41} it was hoped that these cells might be useful in evaluating viral pathogenicity as well as exhibit obvious cytopathic effect and develop plaques more rapidly than Vero or LLC-MK2 cells. Study data showed that EA.hy.962 cells were only susceptible to DENV-4. This observation was unexpected, since this cell line has been shown to be susceptible to other DENV strains and serotypes.⁴¹ In addition, it was observed that all fibroblast cell lines were susceptible to DENV, whereas only 62% of the epithelial cell lines were susceptible. The ability for a DENV serotype to exploit unique tissues may contribute to understanding the phenomena of co-infections with multiple DENV serotypes and the co-circulation of DENV serotypes in the same biospace. However, the wide variety of species and the nonrandom selection of the cell lines limit the amount of meaningful quantitative analysis available. Perhaps the species from which the cell line was selected is far more important than tissue type, or perhaps the nonrandom selection of cell lines has skewed the results.

Although these experiments have raised some provocative questions, the study has some limitations that should be addressed. For instance, the serotype-specific infectivity we observed may be a function of the DENV strains that were used for the experiments. These strains may not accurately reflect the characteristics of DENV currently circulating or that of other laboratory-adapted strains. Finally, the behavior of DENV in the laboratory does not reflect the behavior of DENV in its natural environment.

Conclusion

The data showed that DENV is capable of infecting multiple and varied cell lines from a wide range of host species, which may provide researchers with new tools to study the virus. The data also showed that susceptibility to DENV is serotype specific. This specificity may shed light on the co-circulation of DENV serotypes in a specific region or individual.

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Authors' contributions

BA carried out the real-time PCR studies and assisted with the cell culture. DF and GG assisted with the drafting of the manuscript. GH participated in the design and coordination of the study. JF performed the statistical analysis. KB conceived of the study, participated in its design and coordination, performed all experiments, and drafted the manuscript. All authors read and approved the final manuscript.

Disclosure

The authors declare that no competing interests, financial or otherwise, exist in this work.

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