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ORIGINAL RESEARCH

Dengue viruses exhibit strain-specific infectivity and entry requirements in vitro

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Background: Globally, dengue viruses (DENV) are the most common human arboviral infection and the most important public health threat from mosquito-borne viral pathogens. Recent work has indicated that DENV affinity for cell lines is serotype-specific, which raises challenging questions regarding DENV-host interactions as well as the development of therapeutics and control programs.

Methods: We evaluated the infectious capacity of 11 strains of DENV for serotypes 1–4 in 17 distinct cell lines. The cell lines were evaluated for virus susceptibility via immunohistochemistry and quantitative reverse transcription polymerase chain reaction.

Results: Both methods demonstrated the ability of DENV to replicate in all cell lines, with viral titers ranging from 1×10^2 to 1×10^7 infectious units per mL. Cell line susceptibility to DENV infection was strain-specific, with DENV-4 strains infecting more cell lines than the other serotypes. DENV-2 New Guinea C and DENV-4 H241 were detected in more cell lines than any other strains. Viral fusion assays indicated that DENV requirements for fusion are strain-specific.

Conclusion: These data indicate that several cell lines can be used to culture and study DENV. The strain-specific susceptibility for certain cell lines may provide a tool for characterizing specific DENV strains and an in vitro platform for the development and optimization of therapeutics, the study of host-pathogen interactions, and ecological studies on the cocirculation of DENV strains in a specific region or individual.

Keywords: dengue virus, cell culture, host, fusion

Introduction

Globally, dengue viruses (DENV) are major re-emerging pathogens that are the most common and important public health threat from mosquito-borne viruses. Although a vaccine for DENV is in Phase III clinical trials, the development of interventions and therapeutics remains a high priority because mounting evidence indicates that understanding variation in the ecology and epidemiology of DENV serotypes and strains is important for the development of effective control programs.[1–4] Recent work has indicated that the affinity of DENV for cell lines is serotype-specific.[5] Variations in host range or infection requirements for DENV serotypes would have significant implications for the development of treatments and the control of the virus. We sought to determine if DENV infectivity was strain-specific in cell culture by evaluating the host range of 11 unique DENV strains in 17 distinct cell lines.

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Materials and methods

Cells and viruses

All cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured in Dulbecco's Modified Eagle's Medium 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, and 100 µg/mL streptomycin, and housed in a 37°C incubator with 5% CO_2. Table 1 describes the cell lines used. Eleven strains of DENV from the four serotypes were obtained from the World Reference Center for Emerging Viruses and Arboviruses, the Walter Reed Army Institute of Research, and BEI Resources. All strains were isolated from patients clinically diagnosed with dengue fever or dengue hemorrhagic fever. This panel of viruses included the four dengue prototype strains, DENV-1 Hawaii, DENV-2 New Guinea C, DENV-3 H87, and DENV-4 H241, which are used in most in vitro and basic research of DENV. The panel also included four prototype strains from the Walter Reed Army Institute of Research, ie, DENV-1 WestPac-74, DENV-2 s16803, DENV-3 CH5548904500, and DENV-4 941750. The final three strains of the panel were field isolates from South America and Malaysia. Table 2 provides a more complete description of the viruses used in this research.

Infection of cells with dengue viruses

All infections were performed using 24-well standard cell culture plates seeded with cells to reach 90% confluency upon infection. Individual wells were inoculated with 10,000 infectious units (IU) of a DENV strain in Dulbecco's Modified Eagle's Medium and then rocked at 37°C for one hour, after which the inoculum was removed, the cells were rinsed twice with sterile phosphate-buffered solution, then overlaid with 1 mL of Dulbecco's Modified Eagle's Medium (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% CO_2. The culture supernatant was collected at one hour and at 5 days post-infection.

Detection of DENV

DENV was detected in cell culture supernatants via quantitative real-time polymerase chain reaction, as described previously.[5] Virus in the culture supernatants was measured because DENV is not mature or infectious until its membrane protein is cleaved during exocytosis. The primers used to detect DENV were from Chutinimitkul et al,[6] ie, Den_F (TTAGAGGAGACCCCTCCC) and Den_R (TCTCCTCTAACCTCTAGTCC). These primers amplify the 3′-UTR region, which has been shown to be the most conserved region of the genome of the four serotypes.[6] Three independent assays were amplified in duplicate. Sample averages were normalized to the noninfected control, and those that were quantified with a relative titer of at least 100 IU/mL were considered to be positive for DENV infection. DENV infection was verified in cells via immunostaining of infected cell lines, as described elsewhere.[7] Here, virus foci were detected using specific mouse monoclonal antibodies from hybridoma 2H2 (Millipore, Bedford, MA, catalog #MAB8705) and D1-11(3) (Thermo Fisher Scientific Inc,

Table 1 Cell lines evaluated for susceptibility to dengue virus infection

Cell line	Common name	Species	Morphology	References
TB 1 Lu	Free-tailed bat	*Tadarida brasiliensis*	Lung epithelial	50
DF-1	Chicken	*Gallus gallus*	Embryonic fibroblast	51
Sf 1 Ep	Cottontail rabbit	*Sylvilagus floridanus*	Epidermis epithelial	52
EA.hy.926	Human	*Homo sapiens*	Vascular endothelial	53
CRFK	Domestic cat	*Felis catus*	Kidney epithelial	54
E.Derm	Horse	*Equus caballus*	Dermis fibroblast	55
FoLu	Grey fox	*Urocyon cineroargenteus*	Lung fibroblast	56
Pl 1 ut	Raccoon	*Procyon lotor*	Uterus fibroblast	57
OHH1.K	North American mule deer	*Odocoileus hemionus hemionus*	Kidney fibroblast	58
OK	Virginia opossum	*Didelphis marsupialis virginiana*	Kidney epithelial	59
MDOK	Sheep	*Ovis aries*	Kidney epithelial	60
DN 1 Tr	Nine-banded armadillo	*Dasypus novemcinctus*	Trachea fibroblast	61
PK(15)	Domestic pig	*Sus scrofa*	Kidney epithelial	62
LLC-MK2	Rhesus monkey	*Macaca mulatta*	Kidney epithelial	63
BT	Cow	*Bos taurus*	Turbinate	64
MDCK	Domestic dog	*Canis familiaris*	Kidney epithelial	65
WCH-17	Eastern woodchuck	*Marmota monax*	Liver epithelial	66
Mv 1 Lu	American mink	*Neovison vison*	Lung epithelial	67

Table 2 Dengue virus strains used to determine if infectivity is strain-specific in cell culture

Strain	Year isolated	Location	Notes	Provider	References
DENV-1 Hawaii	1944	Hawaii	Human	WRCEVA	68
DENV-2 New Guinea C	1944	New Guinea	Human	WRCEVA	68
DENV-3 H87	1956	Philippines	Human-DHF	WRCEVA	69
DENV-4 H241	1956	Philippines	Human-DHF	WRCEVA	69
DENV-1 BC-89/94	1994	Costa Rica	Human	BEI resources	D. Gubler
DENV-2 BC-100/98	1998	Bolivia	Human	BEI resources	D. Gubler
DENV-3 BC-14-97	1997	Malaysia	Human	BEI resources	D. Gubler
DENV-1 WestPac-74	1974	Nauru Island	Human	WRAIR	70
DENV-2 s16803	1974	Thailand	Human-DHF	WRAIR	70,71
DENV-3 CH5548904500	1973	Thailand	Human	WRAIR	
DENV-4 341750	1982	Columbia	Human	WRAIR	70,71

Waltham, MA, USA, catalog # MA1-27093) because not all strains could be detected with the 2H2 monoclonal alone or the D1-11 monoclonal alone. These monoclonal antibodies were chosen because they are commercially available and detect all four DENV serotypes without any cross-reactivity with other flaviviruses.

Fusion inhibition assay

To determine if DENV fusion was pH-dependent, we blocked the pH drop that occurs in the cellular endosome during viral fusion, as described elsewhere.[6,7] Briefly, LLC-MK2 cells were pretreated with blocking medium (Dulbecco's Modified Eagle's Medium, 0.2% bovine serum albumin, 10 mM HEPES, 20 mM NH_4Cl, pH 8) for 2 hours at 37°C, and the cells were then infected with 10,000 IU of virus in the presence of 50 mM NH_4Cl and incubated for one hour at 37°C. The cultures were then rinsed with phosphate-buffered solution and incubated in blocking medium at 37°C. Control treatments were incubated in Dulbecco's Modified Eagle's Medium with 10% fetal bovine serum at 37°C. Cell culture supernatants were harvested 48 hours post-infection to determine extracellular virus yields by quantitative real-time polymerase chain reaction. Results are expressed as the average of three independent trials amplified in duplicate. DENV infection was also detected via immunostaining the cell monolayers, as described above.

Results

Dengue virus infectivity is strain-specific

Eleven distinct DENV strains from three collections were selected to investigate if DENV infectivity is strain-specific. Cell lines with a normalized log titer of 2.0 (100 IU/mL) or greater were considered to be positive for DENV infection. All 17 cell lines tested for DENV infection showed positive cycle threshold values for at least one DENV strain based upon quantitative real-time polymerase chain reaction data at 5 days post-infection (Table 2). This was based upon a standard curve analysis using known virus concentrations, as well as confirmation of viable virus via immunostaining of the infected cell monolayers. Relative titers of positive samples were found to be in the exponential range of 1×10^1 to 1×10^7 IU/mL; however, we gated the results of positive samples at a minimum virus output of 2 logs because an output of 10 IU/mL is not really useful for most virological experiments.

Of the 11 DENV strains, DENV-2 NGC and DENV-4 H241 had the highest infectious capacity and showed positive cycle threshold values in all but two of the cell lines tested (Table 2). DENV-2 s16803 and DENV-3 H87 showed the lowest infectious capacity and were found to replicate in five cell lines each. The CRFK cell line was the only cell line to show positive cycle threshold values for all 11 strains, although the OHH1.K, Mv 1 Lu, PK(15), and E.Derm cell lines could support most DENV strains, the SF 1 ep, WCH-17, and EA.hy.926 cell lines were able to support no more than three DENV strains, and the MDCK and DF-1 cell lines were the least competent cell lines, being susceptible to only one DENV strain and producing roughly half as much output virus as any other cell line (Table 3, $P < 0.001$).

Replication potential of DENV is host cell-dependent

The amount of output virus that a DENV strain was capable of producing was dependent on the host cell. DENV-1 HI was detected in seven cell lines, with an output range from 100 IU/mL (OHH1.K) to 10,000 IU/mL (E.Derm), as shown in Figure 1. DENV-1 West-Pac produced the most virus overall compared with DENV-1 HI and DENV-1 BC89/94. This strain had the greatest output in PK(15) cells, whereas DENV-1 BC 89/94 had the lowest output in PK(15) cells (Figure 1).

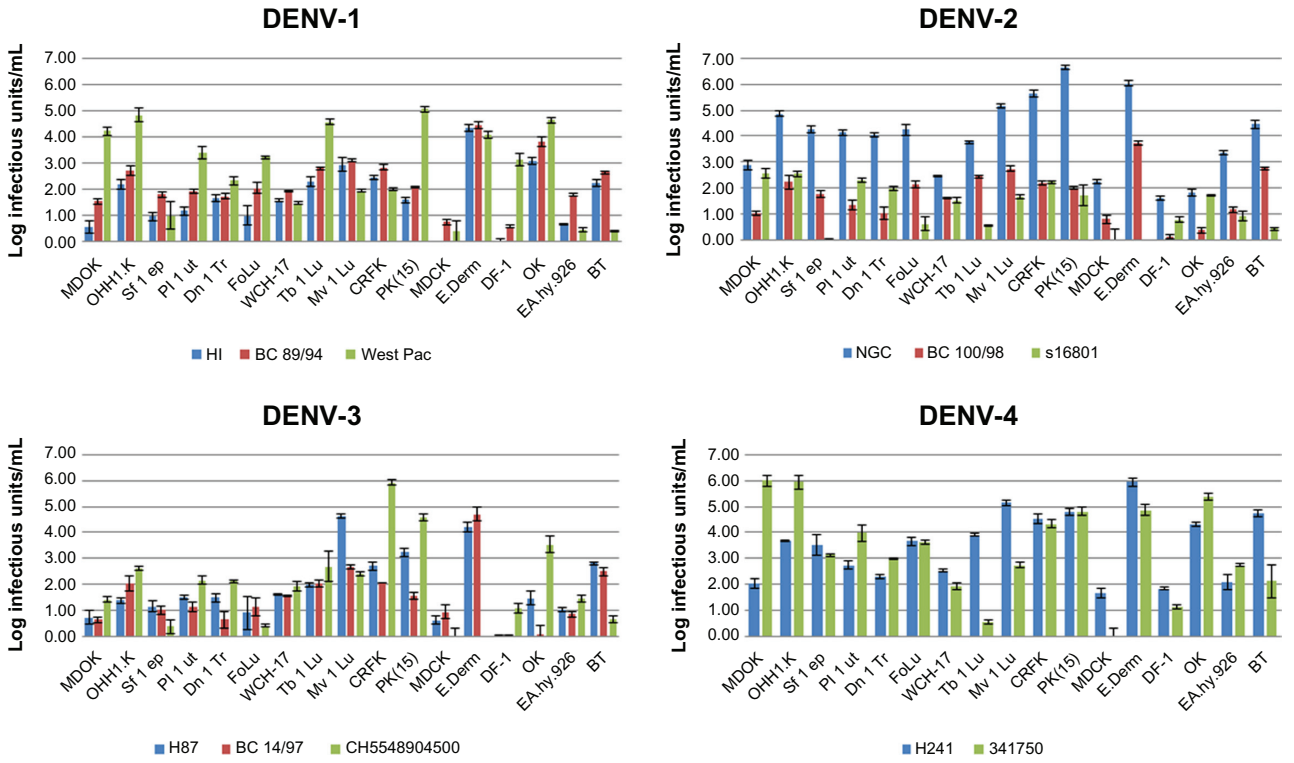

Figure 1 Normalized relative titers of DENV (mean ± standard error of the mean) produced from cell culture supernatants of 17 cell lines collected 5 days post-infection.
Note: Relative viral titers of infected cell lines were calculated according to a standard curve created using a serial dilution technique of known viral concentrations.

DENV-2 New Guinea C had the highest replication potential of all strains tested (Figure 1). When compared with the two other DENV-2 strains, DENV-2 New Guinea C produced twice as much output virus as DENV-2 BC 100/98 and three times that of DENV-2 s16801. DENV-2 New Guinea C produced the highest titers in PK(15) cells and the lowest titers in MDCK cells. DENV-2 BC 100/98 had the lowest titers in PK(15) cells and the highest in E.Derm cells (Figure 1). DENV-2 s16801 did not replicate above 1000 IU/mL in any cell line tested, and showed the greatest output in MDOK cells (Figure 1). DENV-3 H87 had five hosts in cell culture and produced the most IU in E.Derm and Mv 1 Lu cells. DENV-3 BC 14/97 also produced the most output virus in E.Derm cells. DENV-3 CH5548904500 produced the most output virus in CRFK and PK(15) cells (Figure 1). DENV-4 341750 produced the most output virus in MDOK, OHH1.K, and OK cells, whereas DENV-4 H241 produced the most output virus in E. Derm, MV 1 Lu, BT, and PK(15) cells (Figure 1). In respect to the host cells, E.Derm cells accommodated the most output virus of all strains considered ($P = 0.027$).

DENV fusion pathways are strain-specific

Studies have shown that DENV can exploit two main pathways for internalization into the cytoplasm after receptor binding, ie, membrane fusion within intracellular vesicles, which is pH-dependent, or membrane fusion with the cell surface, which is pH-independent.[8,10,11] Ammonium chloride was used to raise the pH of the intracellular acidic vesicle and inhibit pH-dependent fusion pathways (Figure 2). The data show that five of the 11 strains tested were not inhibited

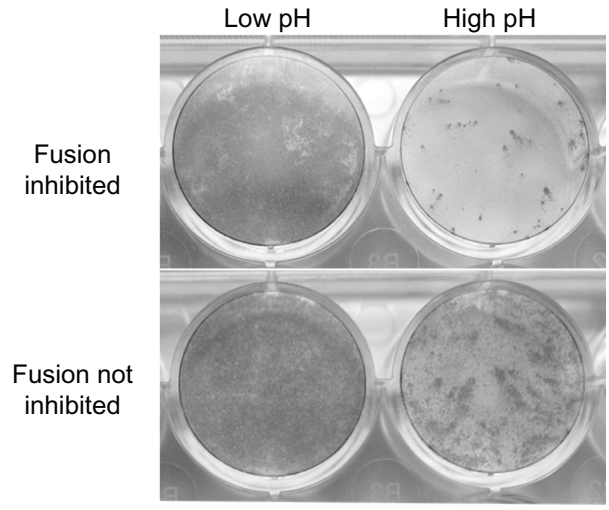

Figure 2 Not all DENV strains require an acidic endosome to infect a target cell as determined by a viral fusion inhibition assay.
Note: The figure illustrates the infectious capacity of a DENV strain that requires an acidic endosome and a DENV strain that does not.

by lack of an acidic environment in the endosome (Table 4). We compared features of origin ($P = 0.807546$), serotype ($P = 0.538369$), age of strain ($P = 0.464023$), current location ($P = 0.4784$), and host number ($P = 0.232245$), and found no significant differences between the strains that require pH-dependent fusion and those that do not.

Discussion

Susceptibility of cell lines to DENV is strain-specific

Our data show that DENV infects numerous cell lines and that infectivity is strain-specific in cell culture. Although in vitro work does not always reflect in vivo systems, our results raise some challenging questions regarding the basic biology of DENV and the definition of viral host. The strain-specific host susceptibility we observed may provide insights into the basic ecology of DENV, that include cocirculation of DENV strains as well as the establishment and displacement of DENV strains in a geographic area. The in vitro susceptibility we observed may help define the search for potential reservoirs. Furthermore, persistence of DENV in a location during interepidemic periods might be explained by the susceptibility of nonprimate animals to a specific strain of DENV. For instance, the data here show that Tb 1 Lu (bat) cells are susceptible to eight of the 11 DENV strains. This is supported by studies that have shown that bats are susceptible to DENV in vivo[12] and serve as reservoirs for DENV circulating in the human population.[13–15]

Production potential of DENV is host-dependent

For this experiment, although all cell lines treated were infected with the same amount of virus, the magnitude of output virus varied by individual cell line (Figure 2). Furthermore, the production potential of DENV strains within a serotype differed in response to the host cell. For instance, E.Derm cells produced roughly 1×10^6 IU of DENV-2 New Guinea C, 3.6×10^3 IU of DENV-2 BC100/98, and no DENV-2 s16801. The same trend was observed for these DENV-2 strains in PK(15), FoLu, BT, Tb 1 Lu, SF-1, and Mv 1 Lu cells. Pl 1 ut, OHH1.K, MDOK, and OK cells produced more DENV-2 s16801 than DENV-2 BC100/98. OK cells produced more DENV-1 West-Pac and DENV-4 341750 than any other strain. Only DF-1 cells produced detectable quantities of DENV-1 West-Pac, and MDCK cells produced only DENV-2 New Guinea C. These features suggest that there may be cell-specific factors that impact virus entry, exit, or replication.

A major requirement of a suitable DENV host is the presence of appropriate cell receptors to initiate binding and uptake of virus into the cell. The attachment requirements for DENV are serotype-specific and differ depending on the target cell.[16,17] In mammalian cells, there are many molecules that may be involved in receptor-mediated binding of DENV to the host cell, including DC-SIGN,[18] laminin receptor,[19] mannose receptor,[20] GRP78,[21] heat shock proteins,[22] and αvβ3 integrin.[23] Furthermore, there are residues on the DENV envelope that are involved with cell surface binding.[24] Because of inconsistent entry routes of DENV into mosquito and mammalian cells combined with inconsistent or divergent entry mechanisms among the serotypes, serotype-specific exploitation of alternative receptors has been hypothesized.[24] This host-dependent viral output could be useful in identifying receptors and characterizing attachment of DENV to cells.

Host-dependent factors for DENV replication also play a significant role in defining a competent host. After attachment and entry into the host cell, the DENV RNA that has positive polarity is translated at the rough endoplasmic reticulum where the viral polyprotein is formed. Host-dependent factors that impact DENV replication include the endoplasmic reticulum-associated degradation pathway, the ubiquitin-proteasome system, the focal adhesion complex, the unfolded protein response, and the subunits of vacuolar ATPase.[25–29] Other host-dependent factors, such as lipids, lipid metabolism, autophagy, and immune response, have been shown to impact DENV replication.[30–34] Although genome-wide screens have identified hundreds of host factors that impact DENV replication,[26,27,35] the mechanisms of how these host-dependent factors function are largely unknown. Host-dependent DENV output could be useful in defining mechanisms involved in DENV replication.

The assembly of DENV virions occurs in the membrane of the rough endoplasmic reticulum, where immature virions bud into the lumen of the endoplasmic reticulum and are transported out of the cytoplasm through the secretory pathway.[36–39] During exit in the trans-Golgi apparatus, the membrane protein is cleaved by furin or furin-like protease which results in the formation of mature virions.[40–44] Relatively little is known about DENV or host factors that are involved in the assembly and exit processes although recent studies indicate that the envelope and membrane proteins may play a role.[45–47]

DENV fusion requirements are strain-specific

There are four well-known entry routes that are available for flaviviruses, ie, phagocytosis, macropinocytosis, and

Table 3 Cell lines positive for DENV infection as determined by quantitative real-time polymerase chain reaction of cell culture supernatant and immunostaining of infected cells

	DENV-1			DENV-2			DENV-3			DENV-4	
	HI	West Pac	BC89/94	NGC	s16803	BC100/98	H87	CH5548904500	BC14/97	H241	341750
MDOK	–	+	–	+	+	–	–	–	–	+	+
OHH1.K	+	+	+	+	+	+	–	+	+	+	+
SF 1 ep	–	–	–	+	–	–	–	–	–	+	+
Pl 1 ut	–	+	–	+	+	–	–	+	–	+	+
Dn 1 Tr	–	+	–	+	–	–	–	+	–	+	+
FoLu	–	+	+	+	–	+	–	–	–	+	+
WCH-17	–	–	–	+	–	–	–	–	–	+	–
Tb 1 Lu	+	+	+	+	–	+	–	+	+	+	–
Mv 1 Lu	+	–	+	+	–	+	+	+	+	+	+
CRFK	+	+	+	+	+	+	+	+	+	+	+
PK(15)	–	+	+	+	+	+	+	+	–	+	+
MDCK	–	–	–	+	–	–	–	–	–	–	–
E.Derm	+	+	+	+	–	+	+	–	+	+	+
DF-1	–	+	–	–	–	–	–	–	–	–	–
OK	+	+	+	–	–	–	–	+	–	+	+
EA.hy.926	–	–	–	+	–	–	–	–	–	+	+
BT	+	–	+	+	–	+	+	–	+	+	+

Note: Cell lines with a mean normalized titer of ≥100 IU/mL were considered to be positive for DENV infection.
Abbreviations: DHF, dengue hemorrhagic fever; WRAIR, Walter Reed Army Institute of Research; WRCEVA, World Reference Center for Emerging Viruses and Arboviruses.

clathrin-mediated and caveolae-mediated endocytosis. Phagocytosis and macropinocytosis are pH-independent whereas endocytosis via clathrin or caveolae is not. The entry of DENV into a host cell is usually dependent upon clathrin-mediated endocytosis.[11,48] Furthermore, fusion of the viral membrane with the host cell requires conformational changes to the viral envelope glycoprotein that are induced by low pH.[49,50] Although alternative infectious pathways for DENV have been described,[8,51] it is agreed that an acidic pH is necessary for successful DENV fusion with the host cell.[8,9,48,49,52] There are very few reports that address the entry mechanisms utilized by strains other than DENV-2 New Guinea C and DENV-1 HI. In addition, the events, pathways, and requirements that lead up to and result in DENV internalization into a host cell are incongruent and not well studied. The data here show that five of the 11 DENV strains were able to fuse with a host cell in the absence of low pH (Table 3). This suggests that individual DENV strains may be using phagocytosis, macropinocytosis, direct penetration into the cytoplasm, or some other uncharacterized pathway to enter the target cell.

The mechanism of entry used by DENV to fuse with a target cell is a function of the tertiary protein structure and glycosylation sites of the envelope glycoprotein which is based on the nucleic acid sequence of the glycoprotein gene. It has been shown that differences in the amino acid sequence of the envelope glycoprotein are associated with significant changes in pathogenicity, clinical presentation, resistance, and hydrophobic/hydrophilic properties.[53–56] It could be that minor conformational differences between the glycoproteins of the DENV strains are contributing to strain-specific cell fusion requirements.

Table 4 Viral output of cells, expressed in log titers (mean ± standard error of the mean), after treatment with ammonium chloride to block low pH-mediated viral fusion pathways

	DENV-1			DENV-2			DENV-3			DENV-4	
	HI	West Pac	BC89/94	NGC	S16803	BC100/98	H87	CH5548904500	BC14/97	H241	341750
Fusion blocked	3.2 ± 0.1	3.7 ± 0.06	3.78 ± 0.03	5.7 ± 0.04	4.9 ± 0.04	3.2 ± 0.5	2.5 ± 0.09	4.1 ± 0.08	3.0 ± 0.2	4.3 ± 0.08	5.74 ± 0.2
Untreated	3.46 ± 0.7	6.0 ± 0.22	4.98 ± 0.5	6.9 ± 0.05	5.4 ± 0.12	3.78 ± 0.2	4.3 ± 0.05	4.8 ± 0.12	2.95 ± 0.3	5.3 ± 0.05	6.7 ± 0.02
P	0.437654*	0.000175	0.043922	3.14E-07	0.00619	0.120598*	1.616E-05	0.002327	0.794825*	3.59E-05	0.001351

Note: *Fusion of the DENV strain with the target cell was not inhibited in the absence of an acidic endosome, as determined by the Student's *t*-test.

Conclusion

DENV grow well in various animal cell lines, suggesting that animals may play a larger role than we previously believe in the preservation and spread of DENV in the environment. Our data support other reports showing that bats may serve as hosts for DENV and that DENV serotypes exploit unique cell entry pathways. Perhaps there is much more to learn about DENV entry into host cells and alternative animal hosts. Although these experiments raise some challenging questions, there are limitations to this study, which should be addressed. For instance, the strain-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.

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Disclosure

The authors report no conflicts of interest in this work.

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