

# Working with Zika and Usutu Viruses in Vitro

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## Abstract

Usutu and Zika viruses are emerging arboviruses of significant medical and veterinary importance. These viruses have not been studied as well as other medically important arboviruses such as West Nile, dengue, or chikungunya viruses. As such, information regarding the behavior of Zika and Usutu viruses in the laboratory is dated. Usutu virus re-emerged in Austria in 2001 and has since spread throughout the European and Asian continents causing significant mortality among birds. Zika virus has recently appeared in the Americas and has exhibited unique characteristics of pathogenesis, including birth defects, and transmission. Information about the characteristics of Usutu and Zika viruses are needed to better understand the transmission, dispersal, and adaptation of these viruses in new environments. Since their initial characterization in the middle of last century, technologies and reagents have been developed that could enhance our abilities to study these pathogens. Currently, standard laboratory methods for these viruses are limited to 2-3 cell lines and many assays take several days to generate meaningful data. The goal of this study was to characterize these viruses in cell culture to provide some basic parameters to further their study. Cell lines from 17 species were permissive to both Zika and Usutu viruses. These viruses were able to replicate to significant titers in most of the cell lines tested. Moreover, cytopathic effects were observed in 8 of the cell lines tested. The data show that, unlike other flaviviruses, neither Zika nor Usutu viruses require an acidic environment to fuse with a host cell. This may provide a tool to help characterize events or components in the flavivirus fusion process. These data indicate that a variety of cell lines can be used to study Zika and Usutu viruses and may provide an updated foundation for the study of host-pathogen interactions, model development, and the development of therapeutics.

**Keywords:** Usutu virus; Zika virus; flavivirus; host range; fusion; cytopathic effects

## 32 **Author Summary**

33 Usutu and Zika viruses are arboviruses of identified in significant medical and veterinary  
34 outbreaks in recent years. Currently, standard laboratory methods for these viruses are limited to  
35 2-3 cell lines and basic viral characterization has not been performed since the mid-20<sup>th</sup> century.  
36 Zika and Usutu viruses were characterized in cell culture. The data show that a variety of cell  
37 lines can be used to study the viruses. Neither Zika nor Usutu viruses require an acidic  
38 environment for host cell infection.

39

# **Introduction**

Usutu virus (USUV), first identified in South Africa in 1959, is a flavivirus belonging to the Japanese encephalitis complex [1,2]. In 2001, USUV emerged in Austria and spread throughout the European and Asian continents [3-10]. Unlike USUV circulating in Africa, the new emergent strains caused significant mortality among Old World blackbirds, owls, and other wild and captive birds [3,11].

The host range of USUV includes primarily *Culex* mosquitoes, birds, and humans [1] and is most often transmitted between avian reservoir hosts and mosquitoes in a sylvatic transmission cycle. Infections with USUV are usually non-pathogenic in humans. Other than birds, evidence for USUV infection has been found in humans and horses [12-14]. Several human cases have been identified in Europe and Croatia [15-17]. Recently, USUS has been linked to neuroinvasive infections in 3 patients from Croatia [10] and has been detected in horses in Tunisia [14].

Zika virus (ZIKA) is an emerging, medically important arbovirus. It is classified as a flavivirus and is descendent from Yellow fever virus [18]. Like many other tropical arboviruses, human infection with ZIKA typically presents as an acute febrile illness with fever, rash, headache, and myalgia. The flavivirus, Dengue virus (DENV) and the alphavirus, chikungunya virus (CHIK) produce similar symptoms to ZIKA but are more commonly diagnosed. The high seroprevalence of ZIKA antibodies in human populations in Africa and Asia suggests the misdiagnosis of ZIKA for other arboviral illnesses is an ongoing problem [19]. There are two geographically distinct lineages of circulating ZIKA; African and Asian [19]. The Asian lineage has recently emerged in Micronesia where it was the cause of a large outbreak in 2007 [20] and currently in the Americas [21].

The natural hosts of ZIKA include humans, primates, and *Aedes* mosquitos [22-25]. Though no solid evidence exists of non-primate reservoirs of ZIKA [26], antibodies to ZIKA have been detected in elephants, goats, lions, sheep, zebra, wildebeests, hippopotamuses, rodents, and other African ruminants [27,28].

There are several characteristics of ZIKA that distinguish it from other medically important arboviruses. In recent outbreaks in French Polynesia, ZIKA exhibited increased pathogenicity and atypical symptoms including respiratory involvement and conjunctivitis [20,29]. A ZIKA strain acquired in Senegal during 2008 exhibited the ability to spread from human to human through sexual transmission [30]. Zika virus has been detected in cell nuclei, unlike other flaviviruses that are confined to the cellular cytoplasm [31]. During the current outbreak in the Americas, ZIKA has been linked to serious medical conditions. Maternal-fetal transmission of ZIKA has resulted in microcephaly and other brain abnormalities [32, 33]. In five of 49 cases, ZIKA was detected in the brain of the children [33]. Guillain-Barré (GB) syndrome is also being associated with these outbreak isolates [34], however GB is one of the most common differential diagnoses for West Nile virus.

Due to their lack of apparent clinical importance in humans and animals, USUV and ZIKA have not been studied to the same degree as other, wide spread flaviviruses such as West Nile virus, DENV or CHIK. While research in serology and genetic characterization are underway [19,20, 34], the recent changes in biology and distribution of these viruses warrant further investigation as many questions regarding the basic biology and ecology of ZIKA and USUV remain unanswered. To better understand the characteristics of USUV and ZIKA *in vitro*, we investigated the permissiveness of several cell lines and determined the basic fusion requirements of these viruses in cell culture.

## Materials and Methods

### Cells and viruses

Seventeen cell lines were obtained from the ATCC (Manassas, VA) and included TB 1 Lu, DF-1, Sf 1 Ep, EA.hy.926, CRFK, E.Derm, FoLu, Pl 1 Ut, OHH1.K, OK, DN1.Tr, PK(15), LLC-MK2, BT, MDCK, WCH-17, Mv1 Lu (Table 1). These lines were selected to include representatives of species found only in the Americas; specifically, North America. All cell lines were cultured in Dulbecco's modified eagle medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 1% (v/v) L-glutamine, 1% (v/v) non-essential amino acids (NEAA), 1% (v/v) sodium pyruvate, 100 U/ml penicillin, 100 µg/ml streptomycin, and housed in a 37°C incubator with 5% CO<sub>2</sub>.

**Table 1.** Cell lines used for characterization of Usutu and Zika viruses.

Cell Line	Common Name	Species	Tissue	Reference
TB 1 Lu	Free-tailed bat	<i>Tadarida brasiliensis</i> *	Lung epithelial	[31]
DF-1	Chicken	<i>Gallus gallus</i>	Embryonic fibroblast	[32]
Sf 1 Ep	Cottontail rabbit	<i>Sylvilagus floridanus</i> *	Epidermis epithelial	[33]
EA.hy.926	Human	<i>Homo sapiens</i>	Vascular endothelial	[34]
CRFK	Domestic cat	<i>Felis catus</i>	Kidney epithelial	[35]
E.Derm	Horse	<i>Equus caballus</i>	Dermis fibroblast	[36]
FoLu	Grey fox	<i>Urocyon cinereoargenteus</i> *	Lung fibroblast	[37]

PI 1 Ut	Raccoon	<i>Procyon lotor</i>	Uterus fibroblast	[38]
OHH1.K	Mule deer*	<i>Odocoileus hemionus</i> *	Kidney fibroblast	[39]
OK	Virginia opossum	<i>Didelphis virginiana</i> *	Kidney epithelial	[40]
DNI.Tr	Nine-banded armadillo	<i>Dasypus novemcinctus</i> *	Trachea fibroblast	[41]
PK(15)	Domestic pig	<i>Sus scrofa</i>	Kidney epithelial	[42]
LLC-MK2	Rhesus monkey	<i>Macaca mulatta</i>	Kidney epithelial	[43]
BT	Cow	<i>Bos taurus</i>	Turbinate	[44]
MDCK	Domestic dog	<i>Canis familiaris</i>	Kidney epithelial	[45]
WCH-17	Eastern woodchuck	<i>Marmota monax</i> *	Liver epithelial	[46]
Mv1 Lu	American mink	<i>Neovison vison</i> *	Lung epithelial	[47]

\*Indicates that species is native to the New World.

Usutu virus (SAAR-1776), ZIKA (MR766), Yellow Fever virus (17D), Sindbis virus (EgAr 339), CHIK (181/25), DENV-1 (H87), DENV-2 (NGC), DENV-3 (HI), and DENV-4 (H241) were obtained from the World Reference Center for Emerging Viruses and Arboviruses (Robert Tesh, UTMB, Galveston, TX). West Nile virus (NY99) was obtained from the University of Florida (Maureen Long).

### *Infection of cells with viruses*

All infections were performed using 12 or 24-well standard cell culture plates seeded with cells which had reached a 90% confluence upon infection. Individual wells were inoculated with 1,000 infectious units (IU) of virus in MEM and then rocked at 37°C for one hour after which the

inoculum was removed, rinsed twice with sterile PBS, overlaid with 1 ml of DMEM (10% FBS, 1% glutamine, 1% NEAA, 100mg/ml penicillin/streptomycin, 1% sodium pyruvate) and incubated at 37°C incubator with 5% CO<sub>2</sub>. Culture supernatants were collected at 1 and 72 hours post-inoculation (PI).

# *Visualization of cytopathic effects*

Cells were examined daily for cytopathic effects (CPE). All cell lines were allowed to develop CPE for 7 days post infection. Cells were stained using 70% ethanol containing 1% wt/vol crystal violet. Plates were incubated for 15 minutes at 22°C after which the fixative was decanted. The plates were rinsed in cold tap water and dried overnight at room temperature. Images were obtained using Micron imaging software (Westover Scientific) and an inverted microscope at 40X magnification.

# *Primer design for qRT-PCR*

Primers for USUV were designed against the USU181 sequence (Genbank accession: JN257984) and amplify a 104 base pair fragment of the envelope protein gene starting at nucleotide position 239 and ending at position 342. Primers for ZIKA were designed against the MR766 strain (Genbank accession: AY632535) and amplify a 128 base pair fragment of the envelope glycoprotein starting at nucleotide position 1398 and ending at position 1525. Blasts for these primer sequences showed sequence homology to multiple strains of the reference virus but no homology to other viruses. The USUV primer set could detect as few as 10 IU per mL and the ZIKA primer set was able to detect as few as 100 IU/mL. Both primer sets did not amplify other arboviruses tested including: West Nile virus, Sindbis virus, Yellow fever virus, DENV serotypes 1-4, and CHIK. Sequences for the primer sets are listed below:



128 Usutu\_Foward (5'-AGCTCTGACACTCACGGCAACTAT-3')

129 Usutu\_Reverse (5'-TCACCCATCTTCACAGTGATGGCT-3')

130 Zika\_Foward (5'-TATCAGTGCATGGCTCCCAGCATA-3')

131 Zika\_Reverse (5'-TCCTAAGCTTCCAAAGCCTCCCAA-3')

132 *Virus detection via real-time RT-PCR*

133 Viral RNA was extracted from cell culture supernatant using the Ambion MagMax-96 extraction  
 134 kit (Life Technologies: Grand Island, NY) per manufacturer's instructions. Quantitative, real  
 135 time, reverse transcriptase polymerase chain reaction (qRT-PCR) was conducted with BioRad  
 136 Superscript One Step SYBR Green qRT-PCR kit (Winooski, VT). The following cycling  
 137 conditions were employed: reverse transcription at 50°C for 10 min, denaturation at 95°C for 5  
 138 min, followed by 40 cycles of denaturation and amplification at 95°C for 10 sec and 55°C for 30  
 139 sec. Cycle threshold (Ct) values were used to estimate relative viral titers of infected cell lines  
 140 according to a standard curve created using a serial dilution of known viral concentrations.  
 141 Results are expressed as the average of 3 independent trials amplified in duplicate.

142 A series of controls were performed for each cell line in order to identify true positives not  
 143 related to background. A no-template control and a no-primer control were performed to verify  
 144 that the reagents and equipment were working as expected. A positive virus control was used to  
 145 verify that the PCR primers were functioning as expected. A non-infected cell culture  
 146 supernatant control was included to verify that there was no increase in non-specific binding  
 147 from the PCR primers that could cause a higher background signal. Finally, the cell culture

supernatant collected 1 hour PI to ensure that qRT-PCR results, 72 hours PI, were not convoluted by input virus.

# *Fusion inhibition assay*

To determine if virus infectivity was pH dependent, the pH drop that occurs in the cellular endosome during viral fusion was inhibited as previously described [52]. Briefly, LLC-MK2 cells were pre-treated with blocking media (DMEM, 0.2% BSA, 10 mM Hepes, 50 mM NH<sub>4</sub>Cl pH8) for two hours at 37°C, and the cells were then infected with 10,000 IU virus in the presence of 50 mM NH<sub>4</sub>Cl and incubated for 1 hour at 37°C. The cultures were then rinsed with PBS and incubated for an additional two hours at 37 °C in blocking media after which the media was replaced with DMEM with 10% FBS. Cell culture supernatants were harvested 48 hours PI to determine extracellular virus yields by qRT-PCR. RNA extractions and qRT-PCR were performed on the cell monolayers. Results are expressed as the average of three independent trials amplified in duplicate.

# *Virus binding assay*

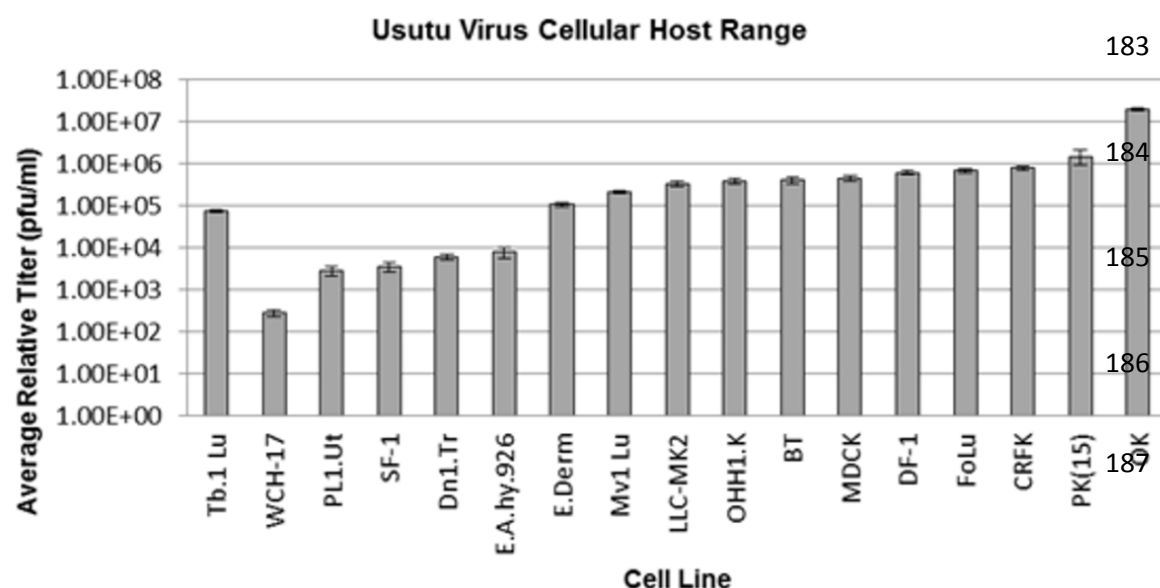
To determine if cell resistance to USUV or ZIKA was binding dependent, a virus: cell binding assay was performed as previously described [52] with RNA extractions and qRT-PCR performed on the cell monolayers. Results are expressed as the average of 3 independent trials amplified in duplicate.

## Results

### *USUV and ZIKA replicate in multiple cell lines*

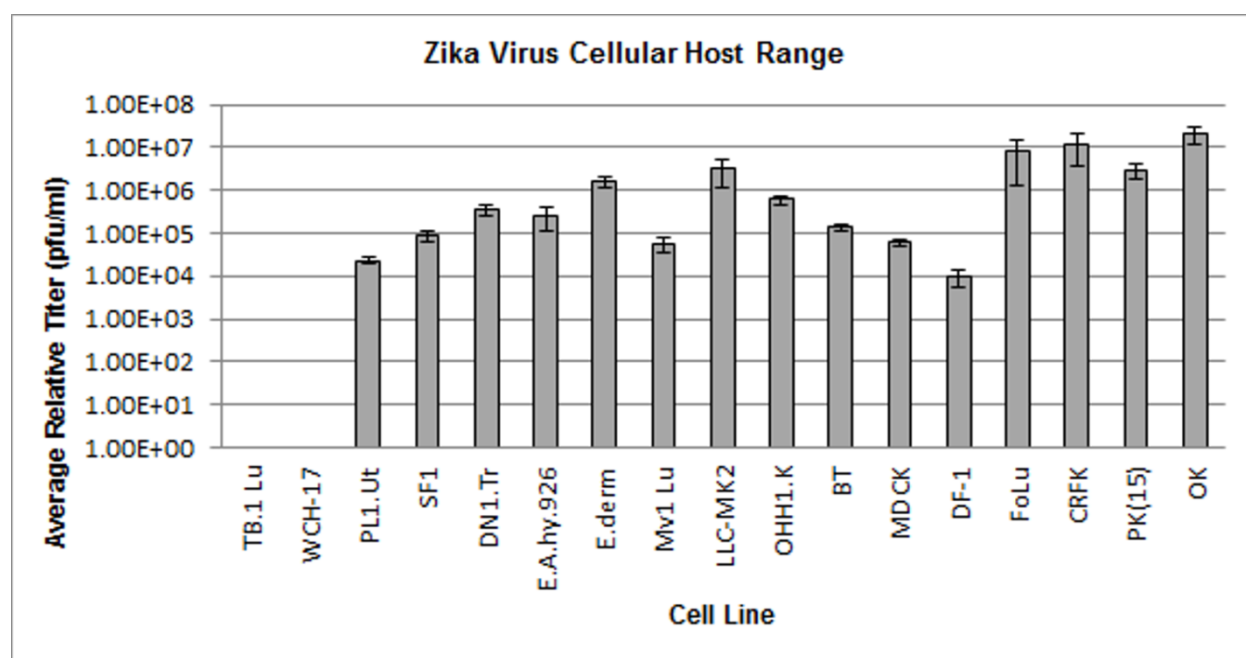
Of the 17 cell lines tested for USUV infection, 16 showed quantifiable Ct values based upon qRT-PCR data at 72 hours PI (Figure 1). All cell lines except WHC-17 (*Marmota monax*) produced at least  $10^3$  relative infectious units. The cell lines BT (*Bos Taurus*), PK(15) (*Sus scrofa*), FoLu (*Urocyon cinereoargenteus*), CRFK (*Felis catus*), OHH1.K (*Odocoileus hemionus hemionus*), DF-1 (*Gallus gallus*), MDCK (*Canis familiaris*), and OK (*Didelphis marsupialis virginiana*) were able to replicate USUV as well as or better than the LLC-MK2 cell line (Figure 1). The OK cells were able to produce over  $10^7$  relative infectious units; over one and a half logs more USUV than LLC-MK2 cells (Figure 1), indicating their potential use for virus culture .

**Figure 1. The host range of USUV in cell culture.** Mean relative titers of USUV  $\pm$  SEM produced from cell culture supernatants from 17 cell lines collected at 72 hours post-infection. Relative viral titers of infected cell lines were calculated according to a standard curve created using a serial dilution technique of known viral concentrations.



Of the 17 cell lines tested for ZIKA infection, 15 showed quantifiable Ct values based upon qRT-PCR data at 72 hours PI (Figure 2). All cell lines except WHC-17 (*Marmota monax*) and TB 1 Lu (*Tadarida brasiliensis*) produced at least  $10^4$  relative infectious units. The cell lines E.Derm (*Equus caballus*), PK(15), FoLu, CRFK, and OK were able to replicate ZIKA as well as or better than the LLC-MK2 cell line (Figure 2). The OK cells were able to produce over  $10^6$  relative infectious units; up to a log more ZIKA than LLC-MK2 cells (Figure 2), indicating their potential use for virus culture.

**Figure 2. The host range of ZIKA in cell culture.** Mean relative titers of ZIKA  $\pm$  SEM produced from cell culture supernatants from 17 cell lines collected at 72 hours post-infection. Relative viral titers of infected cell lines were calculated according to a standard curve created using a serial dilution technique of known viral concentrations.



WCH-17 and Tb 1 Lu cells are not competent hosts

Usutu was not detected in low quantities in WCH-17 (*Marmota monax*) cells and ZIKA was not detected in Tb 1 Lu (*Tadarida brasiliensis*) or WCH-17 cells via qRT-PCR nor was CPE

evident. A virus: cell binding assay was performed in order to determine if cell receptors were present that would allow ZIKA or USUV to attach to the Tb 1 Lu or WCH-17 cell surface. The Ct values for all treatments express the amount of virus present in the sample. The statistical similarity of the data suggests that both ZIKA and USUV bind to WCH-17 cells and ZIKA binds to Tb 1 Lu cells as efficiently as they bind to the LLC-MK2 control cells (Table 2).

**Table 2. Ct values as determined by qRT-PCR of ZIKA and USUV after binding to LLC-MK2, Tb 1 Lu, and WCH-17 cells.** The lack of significant difference between Ct values of the three cell lines indicate that both Zika and Usutu viruses bind to WHC-17 and/or Tb1. Lu cells as efficiently as they bind to the LLC-MK2 control cells.

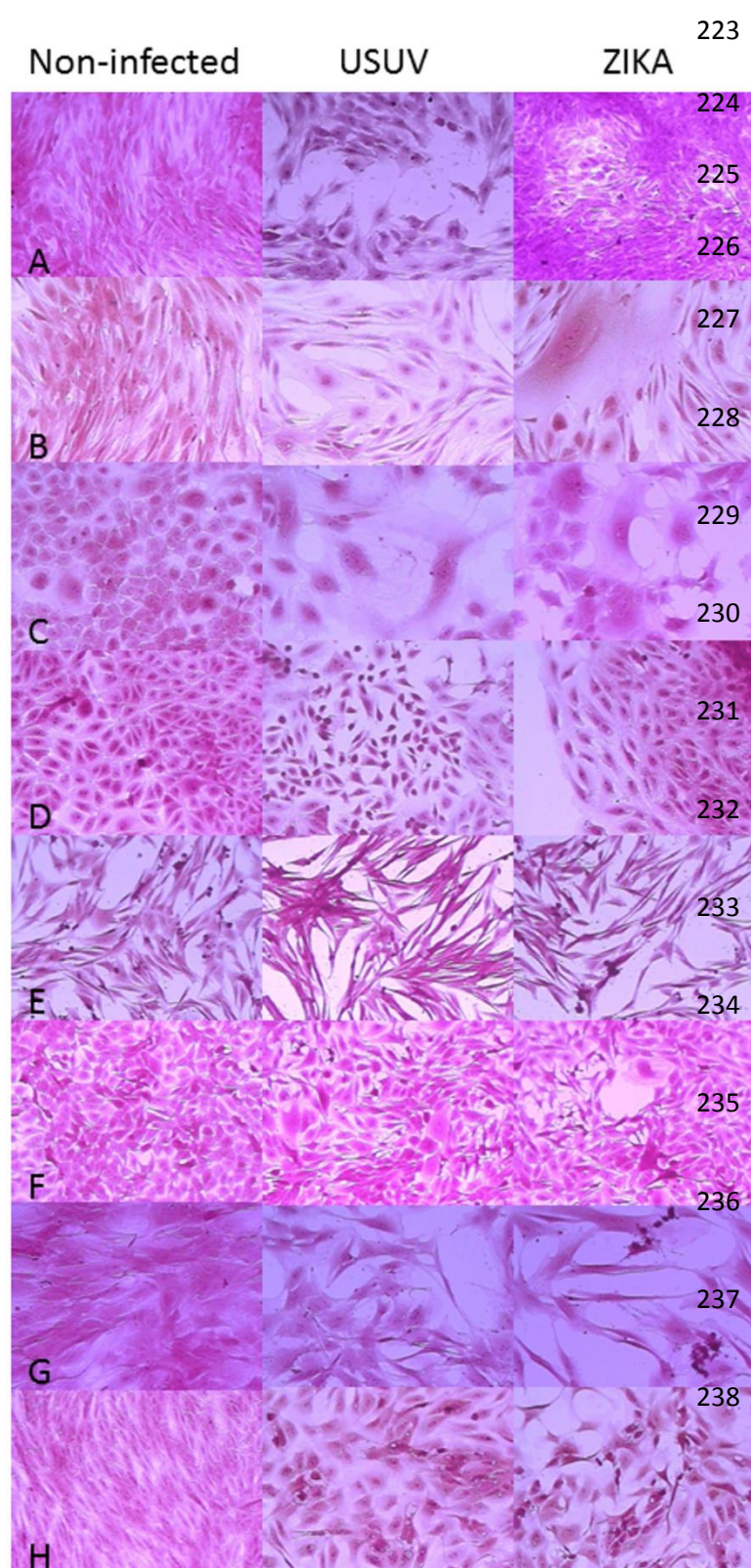
	LLC-MK2	WCH-17	Tb1. Lu
<b>ZIKA</b>	20.09 ( $\pm 0.095$ )	20.19 ( $\pm 0.26$ )	20.57 ( $\pm 0.08$ )
<b>USUV</b>	20.95 ( $\pm 0.095$ )	20.99 ( $\pm 0.095$ )	NA

*USUV and ZIKA produce cytopathic effects in multiple cell lines*

Cytopathic effects were observed in CRFK, Dn1.Tr (*Dasypus novemcinctus*), Sf 1 Ep (*Sylvilagus floridanus*), PK(15), FoLu, Mv 1 Lu (*Neovison vison*), OHH1.K, and OK cell lines from both ZIKA and USUV infection. Forms of CPE caused by USUV included the formation of koilocytes (Figure 3a, 3b, 3c), cellular enlargement (Figure 3d, 3f), rounding (Figure 3f, 3h), focal degeneration (Figure 3g), and pyknosis (Figure 3d, 3e, 3h). Forms of CPE caused by ZIKA included the formation of koilocytes (Figure 3b, 3f, 3h), cellular enlargement (Figure 3b, 3h), focal degeneration (Figure 3c, 3d, 3f, 3g), and pyknosis (Figure 3a, 3d, 3e).



**Figure 3. The cytopathic effects of USUV and ZIKA.** Cytopathic effects of Zika and Usutu viruses were visualized a 40X magnification on an inverted microscope. Cytopathic effects were



observed for both viruses in (a) CRFK, (b) Dn1.Tr, (c) Sf 1 Ep, (d) PK(15), (e) FoLu (f) Mv 1 Lu, (g) OHH1.K, and (h) OK cells.

FoLu, *USUV and ZIKA do not require pH mediated fusion*

The ability of USUV and ZIKA to fuse with a host cell was evaluated by blocking the drop in pH that occurs in the cellular endosome which has been shown to induce conformational changes in the viral envelope necessary for fusion. The performance of DENV-2 was evaluated in tandem as a control as it has been shown that DENV, and other flaviviruses, require an acidic pH to fuse with a host cell [52, 54]. The data show that USUV and ZIKA were able to fuse with the target cells in the absence of acidic pH as well as the control (acidic) treatment (Table 3). The Ct values for USUV and ZIKA were statistically similar despite pH level. The Ct values for DENV (the control virus) were significantly lower for cells treated with and acidic pH. This indicates that more virus was present in the cells than what was detected in the cells infected with DENV in an environment of basic pH.

**Table 3. Ct values as determined by qRT-PCR of USUV, ZIKA, and DENV fusion with LLC-MK2 cells in the presence and absence of and acidic endosome.** Usutu and Zika viruses did not exhibit inhibited host cell fusion in the presences of a basic pH whereas the fusion of dengue virus, the control, was significantly inhibited in the presence of a basic pH.

	Acidic pH	Basic pH
<b>USUV</b>	13.34 ( $\pm 1.11$ )	14.58 ( $\pm 0.44$ )
<b>ZIKA</b>	7.8 ( $\pm 0.2$ )	8.87 ( $\pm 0.54$ )
<b>DENV</b>	17.32 ( $\pm 0.3$ )	22.18 ( $\pm 0.15$ )

## Discussion

Though evidence of ZIKA infection has been found in non-primate species, the host range for ZIKA, both *in vitro* and *in vivo*, has not yet been explored. Preliminary studies using the 2001 USUV emergent strain indicated that the virus could infect several species in cell culture [55]. For this experiment, we sought to examine the host range of the prototype ZIKA and USUV isolates in cell culture. Eighteen distinct cell lines were selected from the inventory at the American Type Culture Collection (ATCC) (Manassas, VA) and included species that were only found in the Americas. Cell lines were selected based on the susceptibility of the host species to flaviviral infection and utility of the cell line in virus research. ZIKA and USUV are Old World viruses and as such have not encountered New World species like opossum, armadillo, North American mule deer, raccoon, gray fox, and cotton-tail rabbit. Most of these animals are peridomestic and inhabit the same environment as the mosquito vectors. It is of particular interest that both USUV and ZIKA replicate well in cells from many domestic and peridomestic animals. These animals may be susceptible to clinical disease and if viremia is high enough, they may serve as reservoirs or hosts. Viral transmission and encroachment may reflect that of the West Nile introduction to the United States in 1999. The data agree with other work that shows USUV can infect PK (15), MDCK, and primate cells [55] and suggest that the USUV prototype strain may behave similarly in cell culture to the emergent strains of the virus. In addition, This work agrees with recent work showing that DENV can replicate in a variety of cell lines [56, 57]

The data show that ZIKA and USUV bind to WCH-17 cells and ZIKA binds to Tb 1 Lu cells as efficiently as the LLC-MK2 control cells. This suggests that USUV or ZIKA infection of WCH-17 and Tb 1 Lu cells may be inhibited during the virus: cell fusion or viral replication process.



Notably, WCH-17 cells are infected with hepatitis B virus which may be a contributing factor to the inability of these viruses to establish an infection in this cell line.

In addition to replicating in various cell lines, USUV induced cytopathic effects (CPE) in 8 of the 16 positive cell lines. The characteristics of CPE caused by a flavivirus vary in accordance to the host cell [58] and are dependent on various factors including host genetics, viral receptors, immune-response, and defective virus particles [58]. Previous studies on the 2001 USUV emergent strain indicated that CPE was induced in PK (15), Vero, and GEF (goose embryo fibroblast) cells [55]. The range and extent of CPE observed suggests that these cell lines may be useful for virus culture and viral titer studies such as TCID<sub>50</sub> and plaque reduction neutralization tests.

The entry of a flavivirus into a host cell is dependent upon clathrin-mediated endocytosis [59-62]. Fusion of the viral membrane with the host cell requires conformational changes to the viral envelope glycoprotein that are induced by a low-pH [63-66]. Though, alternative infectious pathways for flaviviruses have been described [reviewed by Smit et al. [52,67], it is agreed that an acidic pH is necessary for successful flaviviral fusion with the host cell [45-50]. The ability of a flavivirus to fuse with a target cell is a function of the tertiary protein structure of the envelope glycoprotein. The conformation of the glycoprotein is based on the nucleic acid sequence of the glycoprotein gene. It has been shown that differences in the amino acid sequences of the envelope glycoprotein are associated with significant changes in pathogenicity, clinical presentation, resistance, and hydrophobic/hydrophilic properties [68-71]. The data for DENV agree with other research as its ability to infect a target cell was significantly inhibited in the absence of an acidic environment within the endosome [52]. It may be that unique features

are present on the envelope glycoprotein of USUV and ZIKA that are involved in the fusion process that are permitting these viruses to fuse in a non-acidic environment.

Research has shown that USUV is genetically distinct from other flaviviruses [72]. Different strains of USUV have been shown to differ by as much as 5% in amino acid sequence [8]. These amino acid substitutions may influence virulence and other characteristics of USUV [8,72,73]. ZIKA too, is genetically distinct from other flaviviruses, and different strains of ZIKA have been shown to differ by as much as 11.7% in nucleotide sequence [19]. Moreover, significant amino acid deletions have been identified at glycosylation sites of the envelope glycoprotein in some strains of ZIKA [19], which may influence virulence or other characteristics of the virus [74].

USUV and ZIKA may achieve their broad host range by exploiting alternative infectious entry pathways. Cellular membrane components such as clathrin, dynamin, actin, and lipids have been shown to be involved with viral entry into the host cell cytoplasm [52,75-79]. The impact of these various components on virus entry is has been shown to be host specific for DENV [52] and may be contributing to the ability of USUV or ZIKA to establish infection in a wide variety of cell lines.

## Conclusions

The data herein indicate that several cell lines can be used to culture and study USUV and ZIKA and that an acidic environment is not required in the cellular endosome to achieve successful fusion with a host cell. The susceptibility for certain cell lines to USUV and ZIKA may provide a tool for characterizing these viruses and may provide an *in vitro* platform for the study of host-pathogen interactions, model development, and the development of therapeutics. The unique fusion requirements of USUV and ZIKA may be useful in understanding flaviviral infection and

may identify novel targets for the development of interventions. Though these experiments raised some provocative questions, there were some limitations to this study, which should be addressed. For instance, the broad host infectivity observed may be a function of the virus strains that were used for the experiments. These strains may not accurately reflect the characteristics of USUV or ZIKA currently circulating, or that of other laboratory-adapted strains. Finally, the behavior of USUV and ZIKA in the laboratory does not reflect the behavior of these viruses in their natural environment.

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