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Article

Cross-Reactivity of Zika and Other Flaviviruses in Serological Diagnostics

Kelli L. Barr[1]*, Erika R. Schwarz[2], Dhani Prakoso[2], Kehkashan Imtiaz[3], Ruiyu Pu[2] and J. Glenn Morris, Jr.[4], Erum Khan[3], Maureen T. Long[2]

1. Department of Biology, Baylor University, Waco, Texas, USA; kelli_barr@baylor.edu;
2. Department of Comparative Diagnostic and Population Medicine, College of Veterinary Medicine, University of Florida, Gainesville, USA; eschwarz@ufl.edu; dprakoso@ufl.edu; pur@ufl.edu; longm@ufl.edu
3. Department of Pathology and Laboratory Medicine, Aga Khan University, Karachi, Pakistan; kehkashan.imtiaz@aku.edu; erum.khan@aku.edu
4. Emerging Pathogens Institute, University of Florida, Gainesville, Florida, USA; jgmorris@epi.ufl.edu

*Corresponding Author: Kelli L Barr PhD
E-mail: kelli_barr@baylor.edu

Abstract: Zika virus (ZIKV) co-circulates with several closely related flaviviruses which exhibit similar clinical manifestations thus, clinicians rely on molecular and serological techniques for diagnosis. Cross-reactivity of patient specimens to flaviviruses is a significant impediment to serological diagnosis in areas where multiple flaviviruses co-circulate. Furthermore, patient exposure history to any of these viruses could complicate serological response patterns which could result in over and/or underdiagnosis of ZIKV infection. Three strains of ZIKV, dengue serotypes 1-4, West Nile virus, Japanese Encephalitis virus, and Yellow Fever virus were evaluated for neutralizing properties against 3 monoclonal antibodies, 4 ZIKV-naïve patients with flavivirus exposure history, 5 patients with verified ZIKV exposure and unknown flavivirus exposure history, and 5 flavivirus-naive patients with ZIKV-only exposure. Patients naïve for ZIKV exposure effectively neutralized multiple strains of ZIKV. Overall, the prototype ZIKV isolate MR-766 did not behave like the other ZIKV isolated used in this study. MR-766 was neutralized more completely by polyclonal patient serum than recent ZIKV isolates. MR-766 was neutralized better than dengue virus in ZIKV-naïve patients with prior dengue exposure. MR-766 was neutralized significantly less than recent ZIKV isolates when treated with monoclonal antibodies. The data herein show that without RT-PCR, serological diagnosis may not be possible in areas where multiple flaviviruses are endemic.

Keywords: Zika virus; flavivirus; cross-reactivity; neutralization; diagnostics; serology; plaque reduction neutralization test; flavivirus exposure

1. Introduction

Shortly after the identification of Zika virus (ZIKV) as the causative agent of microcephaly, researchers had released reagents and assays to assist in research and diagnostics. ZIKV can be detected by RT-PCR in urine for 10-14 days after symptom onset but only for about 3 days from serum (1-4). Unfortunately, urine testing or paired testing of serum and urine is rarely performed on patients presenting to healthcare with a dengue-like illness. For most patients with ZIKV or other flaviviral infection, the short duration of detectable viremia leads to the reliance on serological testing (1, 5). When undetected by RT-PCR, diagnosis is made following guidelines that require the presence of both virus-specific IgM antibodies and virus-specific neutralizing antibodies (5, 6). While clinicians use IgM ELISA to screen for infection, the plaque reduction neutralization test (PRNT) is

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recommended by CDC to confirm diagnosis when RT-PCR is negative, and IgM ELISA is "not negative" (5).

ZIKV co-circulates with other flaviviruses, including dengue virus (DENV), West Nile Virus (WNV), Japanese Encephalitis Virus (JEV) and Yellow Fever Virus (YFV). Antibody-based assays can be problematic as serological cross-reactivity can confound results (7, 8). Furthermore, previous exposures and co-infections can further complicate diagnostic tests (9, 10). Additional difficulties arise when diagnosing clinical samples in different locations as reference virus and antigen sources can produce divergent results in response to locally circulating viral isolates (11). The data herein illustrate the variable manner in which ZIKV and other flaviviruses are neutralized by patient serum and monoclonal antibodies (mAb) and that ZIKV neutralization may not indicate ZIKV exposure.

2. Results

2.1. RT-PCR sensitivity is strain dependent

When equal quantities of infectious particles were amplified via RT-PCR using CDC diagnostic primers and probes, cycle threshold (Ct) values varied significantly between ZIKV strains. Ct values for PRVABC59 were consistently higher than the other isolates, differing by as many as 5 cycles translating to at least a log difference in relative titer as determined via plaque assay (Table 1). This may be a result of infectious:non-infectious virus particle ratios as both R103451 and PRVABC59 have genetically identical target sequences.

Table 1. Average Ct ± standard deviation of three ZIKV strains detected with CDC ZIKV general primers and probe.

Est PFU/mL	MR-766	PRVABC59	R103451
1000	22.26 ±0.07	25.99±0.11	21.19±0.06
100	25.86±0.02	29.79±0.03	24.71±0.02
10	29.56±0.09	33.10±0.04	28.33±0.08
1	33.13±0.06	36.43±0.14	31.96±0.07

2.2. Monoclonal antibody neutralization of ZIKV and DENV is strain/serotype dependent

The mAbs ZKA185 and 753(3) C10 neutralized ZIKV in a dose-dependent manner (Figure 1). Inhibitory concentrations for ZKA185 were 90-100% at 5 μg/μL, ~ 80% at 0.5 μg/μL, and ~60% at 0.05 μg/μL for both ZIKV PRVABC59 and R103451 (Figure 1A). ZIKV MR-766 was significantly less neutralized than the other 2 strains with ~65% neutralization at 5 μg/μL then approximately 45% neutralization at concentrations of 0.5 to 0.05 μg/μL (Figure 1A). mAb 753(3) C10 also neutralized ZIKV PRVABC59 and R103451 better than ZIKV MR-766 (Figure 1B). Both ZIKV PRVABC59 and R103451 were neutralized over 90% at 5.0 and 0.5 μg/μL with neutralization rapidly decreasing for the following concentrations (Figure 1B). Conversely, MR-766 did not achieve the same level of neutralization with this mAb and was neutralized at 76% at 5.0 μg/μL then neutralization activity hovered in the 50%-60% range for concentrations of 0.5, 0.05, 0.005, and 0.0005 μg/μL (Figure 1B). When treated with 4G2 antibody, infection of PRVABC59 and R103451 were enhanced in a dose-dependent manner with significant viral enhancement occurring at mAb concentrations of 0.005 μg/μL for R103451 and 0.0005 μg/μL for PRVABC59 (Figure 1C). MR-766 was neutralized approximately 30% at 5 μg/μl but was not neutralized at any of the lesser dilutions (Figure 1C).

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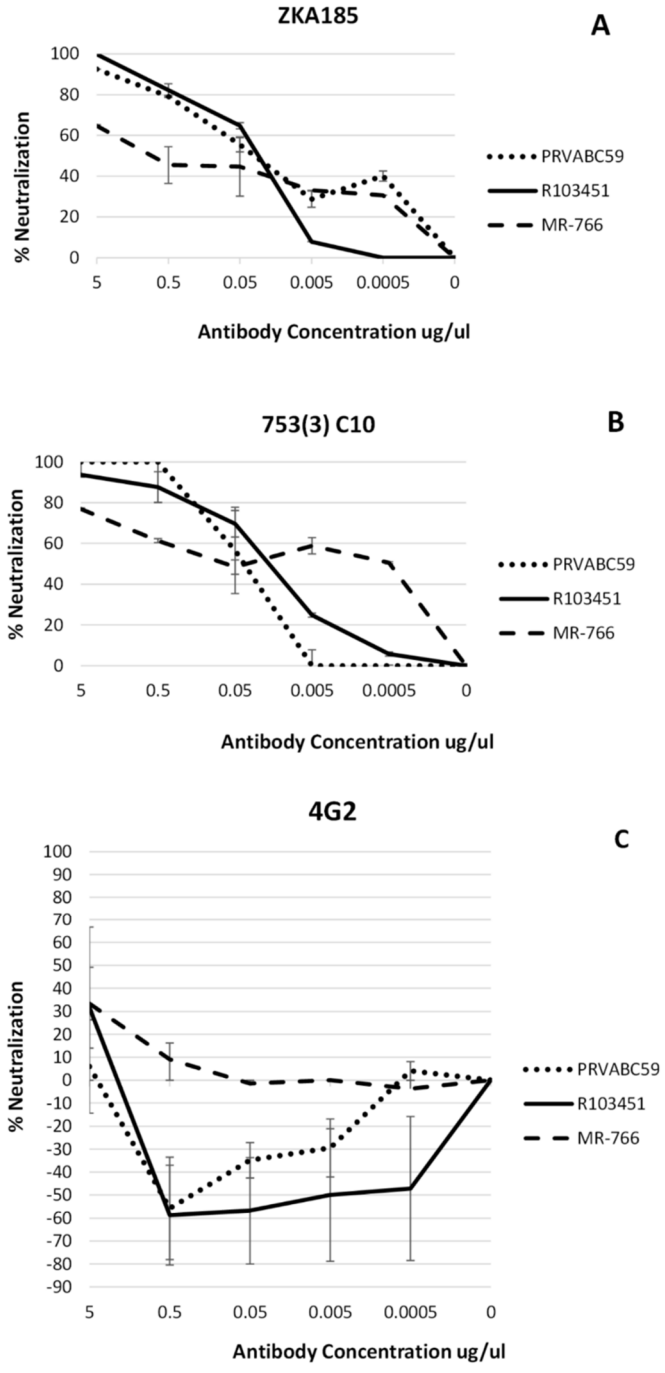
DENV 1-4 exhibited a serotype-dependent neutralization by both mAb 753(3) C10 and ZKA185 (Figure 2). ZKA185 weakly neutralized DENV-3, did not neutralize DENV-1 or DENV-2, but enhanced DENV-4 with significant increases in viral titer at concentrations of 0.5u µg/µL and 0.05 µg/µL (Figure 2A). For mAb 753(3) C10, DENV-1 and DENV-2 were neutralized as effectively as ZIKV R013451 and significantly more than ZIKV MR-766 (Figure 3B). DENV-3 was inhibited significantly less than all ZIKV strains at 53.5% at 5 µg/µL and 16.5% at 0.5 µg/µL (Figure 3B). DENV-4 was not neutralized at any concentration of 753(3) C10 (Figure 2B). The 4G2 antibody neutralized DENV-2 100% at 5 µg/µl but not at any other concentration. DENV-3 was neutralized 62% at 5 µg/µl, 54% at 0.5 ug/ug, 39% at 0.05 µg/µl, 20% at 0.005 µg/µl and 12% at 0.0005 µg/µl (Figure 2C).

Figure 1. Neutralization and Enhancement of Zika by 3 monoclonal antibodies.



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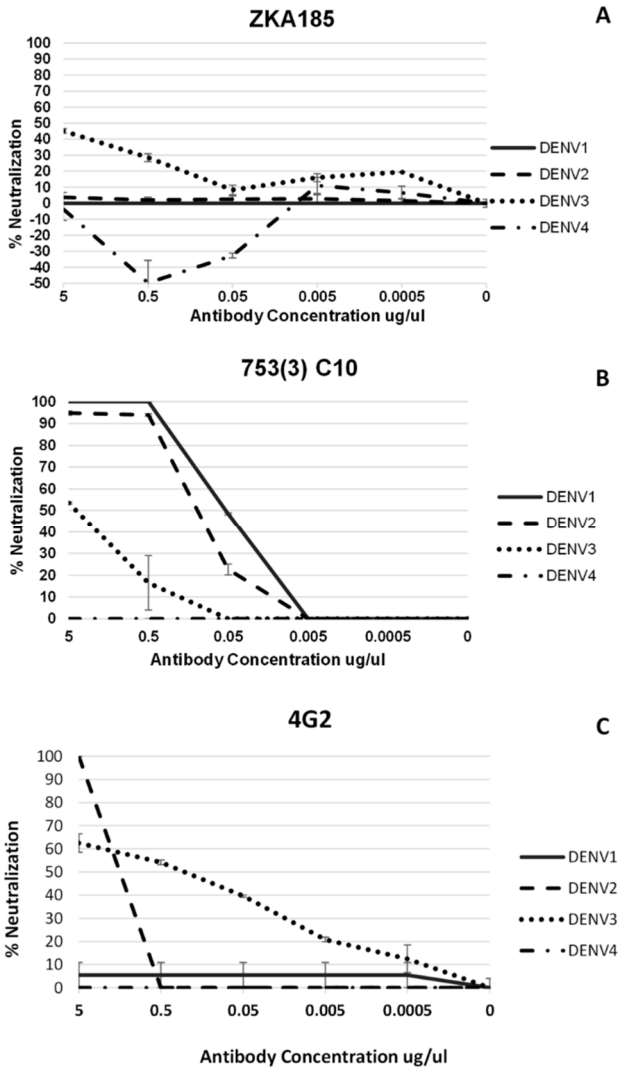
91 **Figure 2.** Neutralization and Enhancement of Dengue viruses by 3 monoclonal antibodies. .



92

93 **Figure 3.** Neutralization of Zika viruses by patient serum with and without ZIKV exposure.

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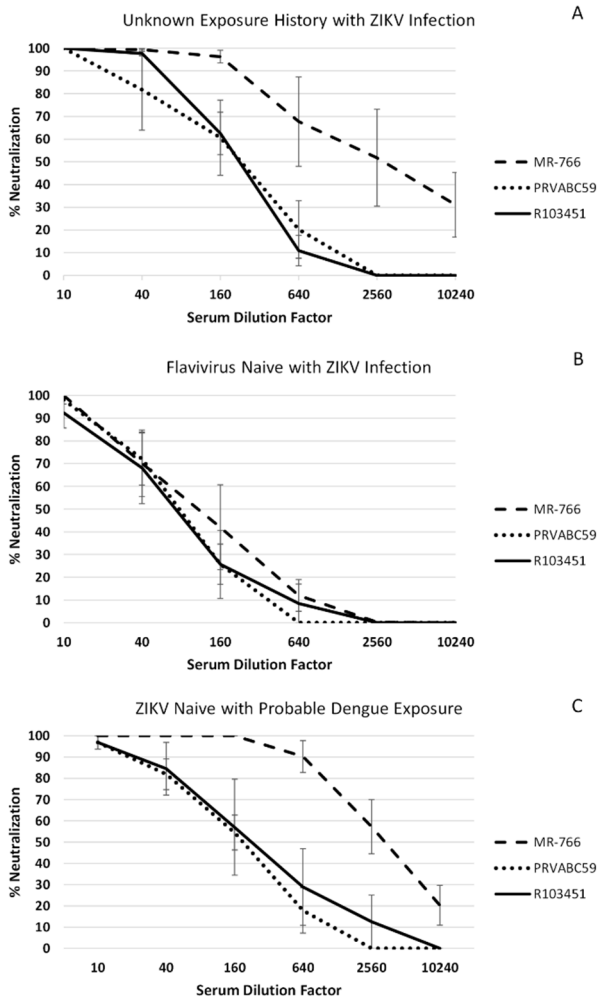

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95 *2.3. Patients with confirmed ZIKV exposure with unknown flaviviral exposure history exhibit strain-specific*
96 *neutralization of ZIKV*

97 5 patients with RT-PCR confirmed ZIKV exposure were evaluated for their ability to neutralize
98 multiple, geographically distinct isolates of ZIKV. The profile of neutralization was patient specific
99 though the data described here is the averaged response of all patients in the group. The data indicate
100 that ZIKV MR-766 was neutralized best by all patients with significantly greater neutralization
101 present over all other ZIKV strains (Figure 3A). An average neutralization of 80% was observed for
102 MR-766 at the 1:400 serum dilution which indicates a robust immune response (Figure 3A). ZIKV
103 PRVABC56 and R103451 were neutralized significantly less than MR-766 by all patients with
104 neutralization dropping below 80% at the 1:160 serum dilution (Figure 3A).

105 *2.4. Flavivirus naïve patients with ZIKV only exposure exhibit similar neutralization of ZIKV strains*

106 4 female sheep, naïve for flaviviral exposure were infected with ZIKV R013451 and serum
107 collected 4 weeks post infection. The data show that all ZIKV strains were neutralized in a similar
108 manner (Figure 3B). The less robust neutralization observed, compared with the other 2 exposure
109 groups is likely due to collection being performed 4 weeks post infection prior to the complete rise in
110 neutralizing antibodies.
111 ZIKV naïve patients with flaviviral exposure history neutralize ZIKV in a strain dependent
112 manner

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For all ZIKV naïve patients with a history of flaviviral exposure, ZIKV strains were neutralized in a strain-dependent manner. ZIKV MR-766 was neutralized significantly more than either PRVABC59 or R013451 (Figure 3C). Over 80% neutralization of all ZIKV strains was observed up to the 1:40 dilution with MR-766 neutralizing at least 80% out to the 1:160 dilution (Figure 3C). Even at the 1:10240 dilution, MR-766 was neutralized at an average of 20% and nearly 60% at the 1:2560 dilution (Figure 3C). ZIKV PRVABC59 and R103451 exhibited approximately 80% neutralization at the 1:40 serum dilution and at least 50% neutralization at the 1:160 serum dilution (Figure 3C). These elevated neutralization profiles suggested potential ZIKV circulation and exposure and thus, all patient specimens from this study were tested via RT-PCR, using the above primers. All patients (n=991) were negative for ZIKV nucleic acids (data not shown).

Patients with confirmed ZIKV exposure exhibit cross-neutralization of other flaviviruses

For patients with RT-PCR confirmed ZIKV infection, cross neutralization of other flaviviruses occurred for 3 out of 5 patients at the 1:10 serum dilution (Table 2). Patient 50620 exhibited cross neutralization to DENV-3 and JEV (Table 2). Patient 50622 exhibited cross-neutralization of DENV-3, JEV, WNV, and YFV (Table 2). Patient 88 possessed neutralizing antibodies for DENV-2, DENV-3, and YFV (Table 2). Flaviviral naive patients also neutralized other flaviviruses at the 1:10 dilution including DENV-2, JEV, and YFV 4 weeks following experimental inoculation with ZIKV R013451 (Table 2).

Table 2. Cross neutralization of flaviviruses by patients with and without ZIKV exposure. Data denotes average neutralization ± standard deviation.

Patient ID	Virus						
	DENV 1	DENV 2	DENV 3	DENV 4	YFV	WNV	JEV
ZIKV Exposure with Unknown Exposure History							
50616	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
50620	0 ± 0	0 ± 0	99.5 ± 0.7	0 ± 0	0 ± 0	0 ± 0	67 ± 0.7
50622	0 ± 0	0 ± 0	99.5 ± 0.7	0 ± 0	97 ± 0.7	0 ± 0	61 ± 2.1
125	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
88	0 ± 0	100 ± 0	99.5 ± 0.7	0 ± 0	92 ± 1.4	0 ± 0	13 ± 3.5
Flavivirus Naive with ZIKV Infection							
4155	0 ± 0	100 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	79 ± 0.7
4158	0 ± 0	0 ± 0	0 ± 0	0 ± 0	97 ± 0.7	0 ± 0	78 ± 5.6
4072	0 ± 0	78 ± 1.4	0 ± 0	0 ± 0	0 ± 0	0 ± 0	82 ± 0
4171	0 ± 0	0 ± 0	0 ± 0	0 ± 0	100 ± 0	0 ± 0	0 ± 0
ZIKV Naïve with Unknown Flavivirus Exposure							
315	100 ± 0	0 ± 0	100 ± 0	0 ± 0	86.6 ± 1.4	85.3 ± 4.2	93.2 ± 2.8
070	100 ± 0	100 ± 0	0 ± 0	0 ± 0	96.6 ± 1.4	47 ± 9.9	0 ± 0
200	100 ± 0	100 ± 0	100 ± 0	100 ± 0	55 ± 6.3	0 ± 0	92.2
252	98.1 ± 2.5	100 ± 0	100 ± 0	100 ± 0	0 ± 0	58.5 ± 1.4	91.2

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2.5. ZIKV-naïve patients with previous flavivirus exposure effectively neutralize multiple flaviviruses

For all ZIKV naïve patients with flaviviral exposure history, significant neutralization of multiple flaviviruses was observed at the 1:10 dilution. Patient K-252 and K-200 completely neutralized all DENV serotypes as well as ZIKV while patients K-315 and K-070 neutralized just 2 DENV serotypes (Table 2). Even though YFV does not circulate in Pakistan, patients K-315 and K-070 neutralized YFV 87% and 97% at the 1:10 serum dilution, respectively (Table 2). Patient K-315 also neutralized WNV 85% and JEV 95% at the 1:10 serum dilution (Table 2). Patient K-070, K-200 and K-252 did not significantly neutralize WNV but patient K-200 significantly neutralized JEV (Table 2). For these patients, neutralization for DENV and other flaviviruses was significantly less than what was observed for ZIKV MR-766 up through the 1:640 serum dilution and for one patient, MR-766 was neutralized significantly more than DENV 1-4 at all serum dilutions (data not shown). Conversely, ZIKV R103451 and PRVABC59 were neutralized less that DENV for patients in this group at higher serum dilutions (data not shown).

3. Discussion

The use of monoclonal antibodies for ZIKV therapeutics and vaccine development is a prominent focus of ZIKV research with most neutralizing mAbs mapped to the fusion loop on the envelope protein (12). The monoclonal antibodies used in this study neutralize and/or enhance ZIKV and DENV in a strain dependent manner. Similar results have been reported for other mAbs which has been a complicating factor for developing useful therapeutics (13). Many commercially available ZIKV mAbs have been isolated from human donors in regions where multiple flavivirus co-circulate and for which there is no available exposure history (13, 14). It is of interest to note that the ZIKV isolates in this study displayed much higher sensitivity to mAb 753(3) C10 (a DENV mAb) than DENV which may indicate that the source donor had a prior exposure to ZIKV (15).

ZIKV is described as a single serotype although there are two genetically distinct lineages and multiple unique viral clades circulating. When vector-borne flaviviruses are introduced into new systems, there can be a rapid development of viral lineages, genotypes, and serotypes (16, 17). ZIKV is no exception with new genetic changes and syndromic presentations being reported in recent outbreaks (18). Strain and serotype-specific performance of flaviviruses is well described (16, 19, 20) and the different inhibition properties of ZIKV shown here and described in the literature may be a function of conformational differences due to changes in amino acid sequences (13, 21). ZIKV MR-766 and other African isolates have been shown to possess significant differences in amino acid sequence from Asian and American isolates (22-24).

The data here indicate that when used for serological diagnostics, ZIKV MR-766 is neutralized in a different manner than ZIKV isolated from the Western Hemisphere, which could lead to the misdiagnosis of patient specimens. Here, all human patients neutralized MR-766 more effectively than R103451 and PRVABC59. Even in ZIKV naïve patients, MR-766 was neutralized better than DENV and other known viruses in circulation in the region. CDC diagnostic criteria for "non-negative" IgM ELISA with neutralizing antibodies may not allow for accurate diagnosis of ZIKV or other flaviviruses in areas endemic to multiple flaviviruses. The use of MR-766 as a diagnostic reagent is of significant concern especially if used in the context of pre-natal care or serosurveillance.

We were unable to determine whether there might be a potential lineage/serotype behavior present as recent African strains are not readily available for making a reasonable comparison. This issue warrants an in depth investigation especially since the designation of ZIKV as a single serotype was made based on the convalescent serum of 2 European travelers and mice (25).

In areas where flaviviruses co-circulate, patient samples are diagnosed via RT-PCR, IgM ELISA, or by PRNT. Though in most endemic areas, RT-PCR is the most common diagnostic method employed as facilities and expertise are not often available for serological assays. The data here and in the literature show that detection of ZIKV nucleic acids in patient serum is not only time limited but also strain dependent (1, 22, 26). RT-PCR and antigen preparations using locally circulating strains should be employed in areas of cocirculating arboviruses. Strain-dependent RT-PCR sensitivity should be recognized as a potential means for misdiagnosis for blood bank and clinical

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specimens collected prior to IgM production. For diagnostic purposes, this highlights the need to use locally relevant strains for optimizing diagnostic assays.

4. Methods

4.1. Viruses

All experiments were performed using the initial expansion of virus from Vero cells. ZIKV and DENV were obtained from BEI Resources. ZIKV PRVABC59 (Cat. # NR-50240), was isolated from a human patient in Puerto Rico during 2015 and is of Asian lineage. ZIKV R103451 (Cat. # NR-50355) is also of Asian lineage and was isolated from a human placenta during 2016 from a patient who had traveled to Honduras the previous year. ZIKV MR-766 (Cat. # NR-50065) is a prototype isolate of African lineage that was isolated from a rhesus monkey in the Zika forest of Uganda during 1947 [6].

A DENV 1-4 diagnostic reference panel was obtained from BEI Resources and included: DENV-1 TS-SMAN (Cat #: NR-83), DENV-2 New Guinea C (Cat #: NR-84), DENV-3 Philippines/H87/1956 (Cat #: NR-80), and DENV-4 H241 (Cat #: NR-86). Other reference flaviviruses were obtained from the World Reference Center for Emerging Viruses and Arboviruses and included: Japanese Encephalitis virus (JEV SA-14-14-2), and Yellow Fever virus (YFV 17D). West Nile virus (WNV) strain New York 99 was kindly provided by Dr. Long and was from the second passage of virus isolated from a crow during the 1999 WNV outbreak in New York.

4.2. Monoclonal antibodies

4G2 antibody was purified from D1-4G2-4-15 hybridoma cells (ATCC #HB-112) cultured in RPMI medium with 10% FBS. This mAb targets a highly conserved portion of the flavivirus envelope glycoprotein and has been shown to react with most flaviviruses (27). The mAb ZKA185 was produced from B cells derived from ZIKV-infected but DENV naïve donor (13). This antibody is reported to neutralize ZIKV but not DENV (13). The mAb 753(3) C10 binds to the envelope dimer region and was produced from a human patient hospitalized with DENV hemorrhagic fever with RT-PCR confirmed infection with DENV1(15). Both 753(3) C10 and ZKA185 were obtained commercially (Absolute Antibody, Oxford, UK).

4.3. Patient specimens

ZIKV naïve clinical human specimens with flaviviral exposure history were obtained through an ongoing study enrolling patients with symptoms of arboviral disease in Pakstan (6, 28). Specimens were collected at presentation to health care with symptoms of acute febrile illness. Informed consent and study procedures were reviewed and approved by the Ethics Review Committee at Aga Khan University (#3183-PAT-ERC-14). All enrolled subjects gave written consent in accordance with the Declaration of Helsinki. De-identified, curated human specimens of verified ZIKV exposure but unknown flavivirus exposure were obtained from BEI Resources. Serum from 4 adult female specific pathogen-free Polypay sheep (New England Ovis) was included as a flavivirus naïve background. These sheep were part of a separate ongoing study to evaluate the consequences of congenital ZIKV infection. Sheep were infected with 10^8 infectious units of ZIKV R103451 i.v. Specimens for this study were collected 4 weeks post inoculation following guidelines approved by the University of Florida IACUC protocol #201609345. Sheep were housed under BSL2+ containment and husbandry conditions.

4.4. Serologic and molecular assays

PRNT of patient specimens was performed in Vero cells as described elsewhere (28, 29). RT-PCR for ZIKV isolates was performed using the CDC diagnostic one-step RT-PCR protocol with ZIKV general primers and probe (22, 26). A modified probe with an internal Nova quencher, 5′ FAM-AGCCTACCT[Nova]TGACAAGCAGTCAGACACTCAA BHQ-1 3′ was used to improve sensitivity (4).

Virus inhibition assays using monoclonal antibodies were performed using serial dilutions of mAb diluted in PBS as described elsewhere (30, 31). Briefly, 100 infectious units of virus were incubated with mAb in PBS for 1 hour at 37°C after which Vero cells were inoculated with the mixture and incubated for 1 hour at 37°C. The inoculum was then removed, and the cells rinsed with PBS to remove any residual mAb and then the cells were covered with a methylcellulose overlay and allowed to incubate at 37°C until viral plaques formed (3-10 days depending on virus). Results are expressed as the average of at least 2 independent trials with 2 technical replicates for each dilution. Assay controls included 2 wells each for mock infection, virus only, and mAb with a known neutralized virus. Percent neutralization, inhibition, and enhancement were calculated using the virus only well as the baseline value.

5. Conclusions

When exposure history is unknown, ZIKV diagnostics are complicated and ZIKV MR-766 should not be used as a reagent. The current testing algorithm for pregnant women may not be appropriate in areas with multiple circulating flaviviruses due to serologic cross-reactivity. Perhaps this population should be assessed more frequently by RT-PCR in ZIKV endemic regions. Serologic diagnosis of ZIKV, or any other flaviviruses cannot be consistently or reliably achieved under CDC guidelines where arboviruses co-circulate. While ZIKV was the focus of this paper, it deserves mention that neutralization of YFV and JEV was observed in patients inhabiting non-endemic regions and without vaccination history. The inconsistent activity of individual ZIKV isolates highlights the necessity of characterizing virus and reagents for diagnostic use.

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Authors' contributions: KLB, EK, and MTL conceived and designed the experiments. KLB, KI, RP, ERS, DP, EK, JGM, and MTL performed experiments and analyzed data. KLB drafted the manuscript and all other authors edited and approved the text.

Conflicts of Interest: The sponsors had no role in the design, execution, interpretation, or writing of the study

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