Co-Infections from Zika and Chikungunya Virus in Bahia, Brazil Identified by Metagenomic Next-Generation Sequencing

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Keywords: chikungunya virus, Bahia, Brazil, Zika virus, flaviviruses, co-infection, mosquito-borne infections, emerging outbreaks, metagenomic next-generation sequencing (mNGS), whole-genome assembly

ABSTRACT

Metagenomic next-generation sequencing (mNGS) of 15 patients with documented ZIKV infection in Bahia, Brazil from April 2015 to January 2016 identified co-infections with chikungunya virus (CHIKV) in 2 of 15 ZIKV PCR-positive cases (13.3%). While generally non-specific, the clinical presentation corresponding to these two CHIKV/ZIKV co-infections reflected infection by the virus present at higher titer. Aside from CHIKV and ZIKV, co-infections from other viral pathogens were not detected. The mNGS approach is promsiing for differential diagnosis of acute febrile illness and identification of co-infections, although targeted arbovirus screening may be sufficient in the current ZIKV outbreak setting.

INTRODUCTION

Zika virus (ZIKV), a flavivirus, and chikungunya virus (CHIKV), an alphavirus, are infectious RNA arboviruses transmitted to humans by the bite of *Aedes* spp. mosquitoes. Both viruses have only recently emerged in the Western Hemisphere (1, 2), and along with dengue virus (DENV), another flavivirus, now circulate widely in Brazil. The acute illness caused by these viruses, characterized by fever, rash, myalgia, arthralgia, and conjunctivitis, is non-specific, and differential diagnosis on the basis of clinical findings alone is challenging. Later infectious sequelae include chronic arthritis for CHIKV (2) and encephalitis, immune-mediated syndromes, and stroke for DENV (3). Recently, the association between ZIKV infection and severe fetal complications such as microcephaly in pregnant women has been established (4), and the virus has also been linked to neurological complications such as Guillain-Barré syndrome (5). Thus, broad-based assays are needed for differential diagnosis of vector-borne febrile illnesses and to

identify potential co-infections. Here we report the utility of metagenomic next-generation sequencing (mNGS) as a screening tool to identify co-infections and for genome recovery and phylogenetic analyses directly from patient serum samples in the context of the ongoing ZIKV outbreak. We also show that the clinical presentation of arboviral co-infections seems to favor the virus present at higher titer in acutely infected individuals.

MATERIALS AND METHODS

ZIKV serum sample collection, ZIKV RT-PCR, and DENV antibody testing. Written patient consent and ethics committee approval for this study were obtained under CAAEV 45483115.0.0000.0046, number 1159.184, Brazil. Serum samples were obtained from 15 patients seen at Aliança Hospital in Salvador, Bahia, Brazil from April 2015 to January 2016 who were given a presumptive diagnosis of an acute viral illness by emergency department physicians and were found to be positive by qualitative RT-PCR testing for ZIKV. Serum samples Bahia01 – Bahia15 were subjected to RNA extraction using the QIAamp Viral RNA Mini Kit (Qiagen), and RNA was reverse transcribed using the Superscript II Reverse Transcription Kit (Invitrogen), followed by qualitative RT-PCR testing for ZIKV using primers targeting the NS5 gene (6). Serum samples were also tested by DENV infection using an ELISA specific for the NS1 antigen and anti-DENV IgG/IgM according to the manufacturer's instructions (Dengue Duo Test, Bioeasy Diagnostica, Brazil).

Metagenomic next-generation sequencing. A separate serum aliquot was extracted for total nucleic acid using the Qiagen Viral RNA Mini Kit (Qiagen), followed by DNase treatment using a cocktail of Turbo DNase (Thermo-Fischer Scientific and Baseline-ZERO DNase Epicentre), followed by NGS construction using the NexteraXT kit (Illumina) as previously described (7, 8). Two independent runs of single-end, 160

base pair (bp) dual-indexed barcoded mNGS libraries were performed on an Illumina MiSeq instrument. To minimize flow cell cross-contamination during sequencing, a known high-titer ZIKV PCR-positive sample was sequenced independently from the other samples. The metagenomic data were scanned for any reads corresponding to known pathogens using the SURPI (sequence-based ultra-rapid pathogen identification) computational pipeline (9).

Confirmatory CHIKV RT-PCR testing. Confirmatory RT-PCR testing for chikungunya virus was performed using a qualitative nested RT-PCR assay targeting the E2 gene as previously described (10, 11). PCR primers and assay conditions were identical to those outlined in (11) except for the substitution of 25 µL master mix taken from the Qiagen One-Step RT-PCR Kit (Qiagen). A presumptive ZIKV/CHIKV co-infection identified by mNGS was considered established only if confirmed by positive CHIKV RT-PCR testing from the original extract (10, 11).

Determination of ZIKV titers. To quantify ZIKV viremia, a standard curve was established and repeat ZIKV PCR testing of the 15 patient serum samples performed using a SYBR-Green quantitative RT-PCR (qRT-PCR) assay with primers targeting the envelope gene (ZIKV-1086/ZIKV-1162) (12).

DENV RT-PCR testing. RNA testing for DENV of the 15 patient serum samples was performed using a previously published nested RT-PCR assay (13). Both first-round and second-round PCR amplicons were visualized by 2% agarose gel electrophoresis, and bands of expected size were extracted from the gel and sequenced by Sanger sequencing. DENV RT-PCR testing of the 15 serum samples in this study yielded only one band in a single sample that was sequenced and found to correspond to *Aedes aegyptii* mosquito genome.

Capture probe enrichment. To aid genome recovery of sample Bahia08, we enriched the mNGS library for ZIKV sequences using a set of 299 XGen biotinylated

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lockdown capture probes (IDT Technologies) targeting all ZIKV genomes in the National Center for Biotechnology Information (NCBI) GenBank database, as previously described (14). Enrichment was performed using the XGen lockdown protocol and SeqCap EZ Hybridization and Wash Kit (Roche Molecular Systems) according to the manufacturer's instructions.

Phylogenetic analysis. Using the MAFFT program in Geneious, all 43 ZIKV genome sequences available in NCBI GenBank as of March 2016 and 13 CHIKV sequences from the East / Central / South African (ECSA) clade were aligned together with 3 ZIKV and 2 CHIKV complete or partial genomes recovered in the current study. Phylogenetic trees were constructed using the neighbor joining algorithm with 1,000 bootstrap replicates, followed by refinement using the MrBayes algorithm at default settings in the Geneious software package (Biomatters, Inc.).

Accession Numbers. NGS reads with human sequences removed have been deposited in the Sequence Read Archive (accession number SRP072069). The 3 ZIKV and 2 CHIKV genome sequences have been deposited in NCBI GenBank (accession numbers KU940224, KU940227, and KU940228 for the ZIKV genomes and KU940225 and KU940226 for the CHIKV genomes).

RESULTS

Metagenomic next-generation sequencing of ZIKV serum samples

Serum samples were collected from 15 patients within 5 days of symptom onset and at the first visit seen during an ongoing ZIKV outbreak at Aliança Hospital in Salvador, Bahia, Brazil from April 2015 to January 2016 (15). All 15 patients tested positive for ZIKV by RT-PCR and negative for DENV by serology. From 24,063 to 6,903,397 million mNGS reads were generated per sample, and reads ailgning to ZIKV

were identified in 13 of 15 (86.7%) ZIKV PCR-positive samples (Table 1). Two ZIKV PCR-positive samples (BR13 and BR14) were negative for ZIKV reads by mNGS, and both exhibited low viral titers by qRT-PCR (<30 copies / mL and 517 copies / mL, respectively) (Table 1). A log-log plot of ZIKV mNGS reads (in reads per million, or RPM) against viral titer revealed a moderate correlation, with a log R-squared value of 0.73255 (Figure 1).

Reads aligning to CHIKV were detected in 6 of 15 (40.0%) ZIKV-positive samples. Given the possibility of cross-contamination from a previously unknown high-titer CHIKV sample (Bahia08), the mNGS run was repeated after removing this sample library. However, the repeat run still resulted in detection of CHIKV reads in 5 of 15 (33.3%) samples. Since we could not reliably distinguish between mNGS library cross-contamination versus low-level metagenomic detection near the limits of detection for RT-PCR, a co-infection with CHIKV was only considered established if independently confirmed by orthogonal testing using a CHIKV nested RT-PCR (10, 11). Using this criterion (both mNGS and RT-PCR positivity for CHIKV), 2 of 15 (13.3%) ZIKV-positive samples (Bahia08 and Bahia09) were designated as CHIKV/ZIKV co-infections. Aside from ZIKV and CHIKV, apparent co-infections from other viral pathogens associated with acute febrile illness were not detected. Additional viral reads detected in the mNGS data were sparse and were attributed to known commensals (e.g. human pegivirus 1 / GBV-C, papillomaviruses), viruses with non-human hosts (e.g. phage, insect viruses), or laboratory contamination due to detection in unrelated mNGS datasets (e.g. adenovirus, rotavirus, polyomavirus) (Table 2). Notably, no mNGS reads aligning to DENV were detected, and DENV RT-PCR testing of all 15 samples was also negative (Table 1).

Clinical presentation of patients with CHIKV/ZIKV co-infection

The first patient of two found to be co-infected with ZIKV and CHIKV (Bahia08) was a 48 year-old woman from Salvador, Brazil, seen in the hospital emergency room (ER) on July 15th, 2015 with 2 days of joint pain involving the elbows, hands, knees, and ankles associated with fever, myalgia, nausea, and headache. She also complained of dysuria, but denied rash or conjunctivitis. Vital signs in the ER revealed a low-grade fever (37.9°C), and physical exam showed diffuse joint pain that made it difficult for her to lift her arms or grasp objects with her hands. Laboratory tests were remarkable only for leukopenia (white blood cell (WBC) count = 1,900 cells/μL [normal range 4,500 - 10,000]) and thrombocytopenia (platelets = 124,000 cells/μL; [normal range 150,000 - 400,000)]; hemoglobin was 13.4 [normal range 12.0 - 15.5 g/dL]. Dengue IgG, IgM, and NS1 serologies were unreactive. A urinalysis showed 36,500 RBCs and 11,500 WBCs per mL; leukocyte esterase was negative, as was urine culture. The patient was treated with pain medications and discharged home. She returned to the ER 15 and 21 days after the initial visit with persistent neck pain and arthralgias and was discharged from the ER both times on pain medications.

The second patient (Bahia09) was a 40 year-old woman presenting April 15th, 2015 with a 2-day history of fever, conjunctivitis, myalgia, and pruritic rash. Exam revealed a diffuse rash, conjunctival hyperemia, and a painful posterior cervical lymph node measuring 5 mm. Vital signs were normal. Laboratory tests were remarkable for mild leukopenia (WBC count = 3,930 cells/μL, with 39% neutrophils and 43% lymphocytes); hemoglobin and platelet counts were normal at 13.0 g/L and 227,000 cells/μL, respectively. Dengue serologies were negative. The patient fully recovered 7 days after symptom onset.

Genome Assembly and Phylogenetic Analysis of ZIKV and CHIKV

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We assembled 100% and 71% of the CHIKV genome corresponding to the two co-infected patients Bahia08 and Bahia09, respectively, by mapping the CHIKV mNGS reads to the most closely matched reference genome in NCBI GenBank identified using SURPI. Similarly, 99% of the ZIKV genome from Bahia09 and 100% of the ZIKV genome from third ZIKV patient who was not co-infected (Bahia07) were assembled directly from mNGS reads. To recover 88% of the ZIKV Bahia08 genome, we boosted the number of mNGS reads using ZIKV capture probe enrichment of the metagenomic libraries (16). Bayesian phylogenetic analysis, including all of the 43 publicly available ZIKV genomes in the NCBI GenBank database as of March 2016, positioned the 3 newly sequenced ZIKV genomes in a Brazilian subclade corresponding to all of the sequenced strains to date from the ongoing 2015-2016 ZIKV outbreak (Figure 2C). Similarly, the 2 CHIKV genomes were placed within a previously described Brazilian subclade (17) that is an offshoot of the East / Central / South African lineage (2) (Figure 2A and B). The 3 ZIKV genomes from Bahia, Brazil, as well as the 2 CHIKV genomes, grouped together into local clusters by phylogenetic analysis.

DISCUSSION

We report mosquito-borne ZIKV / CHIKV co-infections in 2 of 15 (13.3%) acutely symptomatic individuals with established ZIKV infection in Bahia state, Brazil. These data suggest that the incidence of arboviral co-infections in an ongoing ZIKV outbreak setting (1) may be higher than previously thought. There have been only three cases of ZIKV co-infections described to date, 2 patients with ZIKV and DENV co-infection in New Caledonia (18) and 1 patient with ZIKV, CHIKV, and DENV co-infection from Colombia (19). Similar to these prior reports, the co-infections in the current study did not appear to result in more severe or fulminant disease requiring hospitalization. However, it is notable that infection by the virus present at higher titer was reflected in the clinical

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presentation of the two co-infected patients. The first patient (strain Bahia08), with a high serum titer of CHIKV, presented with a prolonged "CHIKV-like" illness characterized by urinary inflammation (20) and prominent arthralgias (2) that persisted for weeks, resulting in repeated ER visits, whereas the second patient (strain Bahia09), with a higher titer of ZIKV, presented with a classic "ZIKV-like" presentation consisting of fever, rash, myalgia, and conjunctivitis (1).

An emerging diagnostic approach, mNGS, enables detection of all potential pathogens in clinical samples on the basis of uniquely identifying sequence information (9, 10). As the number of viral reads appears to be positively correlated with viral titer (Figure 1), quantitative or at least semi-quantitative information can potentially be extracted from mNGS data. In addition, the genetic information obtained by sequencing is useful for tracking of viral evolution (21), monitoring the geographic and temporal spread of the outbreak (22), and discovery of new viral lineages circulating in the region (16). As a surveillance tool, mNGS also has the potential to elucidate the spectrum of infection in a local geographic area, and thus guide the development of targeted diagnostics, antimicrobial drugs, and vaccines. Traditionally, barriers to NGS implementation have included high costs, complex instrumentation, and lack of dedicated bioinformatics tools. These barriers are being overcome with the development of rapid computational pipelines for analysis of mNGS data (9, 23, 24), emergence of portable sequencers that can be used in field laboratories and other point-of-care settings (25, 26). The establishment of robust cutoff thresholds for calling positives will also be needed before mNGS can be used routinely for infectious disease diagnosis. In particular, our results show that the concordance between PCR and mNGS or between different PCR assays at borderline titers near the limits of detection, while very good, is not perfect (Table 1). Specifically, mNGS was likely more sensitive for detection of CHIKV than the CHIKV PCR used in the current study, given that 8.6% and

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9.8% of the viral genome was recovered by mNGS from two CHIKV PCR negative samples, while mNGS was less sensitive than or comparable in sensitivity to the ZIKV PCR assays (Table 1). Such discrepancies between mNGS and PCR at very low viral titers have been previously reported in the other metagenomic studies (27, 28), and can potentially be addressed by formal clinical validation of mNGS assay performance and the use of rigorous negative and positive controls (29).

It is now established that ZIKV is the cause of severe fetal complications in pregnancy such as in utero demise and microcephaly (4). In addition, current co-circulation of all 3 mosquito-borne arboviruses in Latin America makes diagnosis based solely on clinical or epidemiological criteria unreliable. In our study, CHIKV co-infection was detected incidentally by mNGS of ZIKV-infected patients, underscoring the potential utility of untargeted mNGS sequencing for differential diagnosis of vector-borne febrile illness and identification of co-infections. The failure to detect other pathogens, such as malaria, by comprehensive mNGS suggests that a multiplex assay confined to arboviral infections (ZIKV, DENV, and CHIKV) may be sufficient for diagnosis and surveillance during the ongoing ZIKV outbreak (30).

Acknowledgements

We thank multiple researchers worldwide for permission to include their unpublished ZIKV genomes in our analysis. This study was supported in part by National Institutes of Health (NIH) grants R01-HL105704 and R21-AI120977 (CYC), and an award from Abbott Laboratories, Inc. (CYC).

Conflict of Interest

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258 C.Y.C. is the director of the UCSF-Abbott Viral Diagnostics and Discovery Center and
259 receives research support from Abbott Laboratories, Inc. The other authors disclose no
260 conflict of interest.
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Table 1. ZIKV and CHIKV testing of 15 PCR-positive ZIKV cases in Bahia, Brazil from April 2015 to January 2016.

Table 2. Reads in the mNGS data corresponding to other viruses aside from CHIKV and ZIKV.

Figure 1. Log-log plot of detected ZIKV reads against viral titer. The number of mNGS reads is normalized to reads per million (RPM) of raw reads sequenced. A power trendline is fitted to the data, showing an R^2 correlation of 0.73255.

Figure 2. Whole-genome phylogeny of CHIKV and ZIKV. (A) Phylogeny of all 314 CHIKV genomes available in NCBI GenBank as of March 2016 and 2 new complete or partial genomes from the current study. The 3 major lineages of CHIKV are shown in different colors. (**B)** Phylogeny of 14 genomes corresponding to a local cluster within the ECSA (East / Central /South African) clade outlined with a dotted box in **(A)**. An ECSA CHIKV isolate located outside of the cluster, HM045809, is included as an outgroup. **(C)** Phylogeny of all 44 ZIKV genomes available in NCBI GenBank as of March 2016 and 3 new complete or partial genomes from the current study. Genomes corresponding to the 2015-2016 ZIKV outbreak in Latin America are highlighted with a light orange background. The asterisks denote genomes corresponding to ZIKV cases in returning travelers. New CHIKV and ZIKV genomes sequenced in the current study are highlighted in boldface red, with the percent genome recovery provided in parentheses. Branch lengths are drawn proportionally to the number of nucleotide substitutions per position, and support values are shown for each node.

	Testing before mNGS		Metagenomic next-generation sequencing (mNGS)									Testing after mNGS			
Sample	**ZIKV RT-PCR***	**DENV Ab**	**# of raw reads**	**# of ZIKV reads**	**% ZIKV reads**	**ZIKV mNGS (run1/run2)**	**% ZIKV coverage**	**# of CHIKV reads**	**% CHIKV reads**	**CHIKV mNGS (run1/run2)**	**% CHIKV coverage**	**CHIKV RT-PCR**	**ZIKV qRT-PCR****	**ZIKV viral load (copies/mL)**	**DENV RT-PCR**
Bahia01	+	-	3,507,376	103	0.003	+ / +	34.1%	0	0	- / -	0.0%	-	+	1,042	-
Bahia02	+	-	3,668,673	129	0.003	+ / +	39.5%	0	0	- / -	0.0%	-	+	4,086	-
Bahia03	+	-	24,063	2	0.008	+ / +	1.3%	0	0	- / -	0.0%	-	+	3,272	-
Bahia04	+	-	3,060,229	14	0.0005	+ / +	9.6%	0	0	- / -	0.0%	-	+	1,464	-
Bahia05	+	-	3,501,316	19	0.0005	+ / +	11.8%	0	0	- / -	0.0%	-	+	1,091	-
Bahia06	+	-	2,576,002	11	0.0004	+ / +	5.4%	4	0.0002	+ / +	1.1%	-	-	<31	-
Bahia07	+	-	6,903,397	281,099	4.1	+	100.0%	0	0	- / -	0.0%	-	+	9.00E+08	-
Bahia08	+	-	1,094,355	55	0.005	+	35.1%	252,649	23.1	+ / +	100.0%	+	+	2,470	-
Bahia09	+	-	743,266	719	0.1	+	97.6%	84	0.01	+ / +	49.1%	+	+	23,121	-
Bahia10	+	-	2,482,665	22	0.0009	+ / +	5.7%	0	0	- / -	0.0%	-	-	<31	-
Bahia11	+	-	2,384,416	234	0.01	+ / +	40.9%	37	0.002	+ / +	8.6%	-	+	3,981	-
Bahia12	+	-	3,712,405	44	0.001	+ / +	21.3%	23	0.0006	+ / +	9.8%	-	+	1,327	-
Bahia13	+	-	2,556,556	0	0	- / -	0.0%	1	0	+/-	1.4%	-	-	<31	-
Bahia14	+	-	3,658,143	0	0	- / -	0.0%	0	0	- / -	0.0%	-	+	517	-
Bahia15	+	-	2,848,486	17	0.001	+ / +	7.9%	0	0	- / -	0.0%	-	-	<31	-

*RT-PCR assay using primers and conditions from Balm, et al., 2012

**SYBR-Green qRT-PCR assay using PCR primers targeting the envelope gene (ZIKV-1086/ZIKV-1162) and conditions from Lanciotti, et al., 2008

Viral species or genus	# of reads	# of samples	Explanation
Human mastadenovirus A	1	1	suspected lab contaminant**
Human pegivirus 1 (GBV-C)	1,710	1	viral blood commensal
Papillomavirus	1-12	5	viral skin commensal
Merkel cell polyomavirus	1	3	unclear clinical significance; suspected lab contaminant**
WU polyomavirus	3	1	unclear clinical significance; suspected lab contaminant**
Rotavirus	1-4	5	suspected lab contaminant**
Enterovirus D68	1-3	2	suspected lab contaminant (Greninger, et al., 2014, *Lancet Infectious Diseases*)**
Molluscum contagiosum virus	1	1	suspected lab contaminant**

*viruses with nonhuman hosts (e.g. insect viruses, phages, mouse gammaretroviruses) are not reported

**detected in other unrelated sequencing datasets processed in the research laboratory at the same time

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