Serological evidence of Zika virus infection in non-human primates in Zambia

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Abstract

Zika virus (ZIKV) circulation occurs between non-human primates (NHPs) in a sylvatic transmission cycle. To investigate evidence of flavivirus infection in NHPs in Zambia, we performed plaque reduction neutralization tests (PRNT) to quantify neutralizing antibodies. PRNT revealed that sera from NHPs (African green monkeys and baboons) exhibited neutralizing activity against ZIKV (34.4%; 33/96), whereas PRNT for yellow fever virus from NHP sera showed no neutralization activity. ZIKV genomic RNA was not detected in splenic tissues from NHPs suggesting that the presence of anti-ZIKV neutralizing antibodies represented resolved infections. Our evidence suggests that ZIKV is maintained in NHP reservoirs in Zambia.

Zika virus (ZIKV) is an enveloped, single-stranded RNA virus, belonging to the family *Flaviviridae*, genus *Flavivirus*. ZIKV was serendipitously isolated from a rhesus monkey during sentinel screening for yellow fever virus in Uganda in 1947 [5]. ZIKV infection was likely underreported in the intervening decades and considered a benign infection until a large epidemic in 2007 on Yap Island in Micronesia [13]. Thereafter, the Asian lineage of ZIKV infection expanded to French Polynesia and the Americas and was associated with severe neurological disease *in utero* and in adults [20].

ZIKV is mainly transmitted by *Aedes* mosquito vectors; however, human-to-human infections have also been reported. In 2008, a scientist, who had recently returned to the United States from Senegal, sexually transmitted ZIKV to his wife [15]. Maternofetal transmission of ZIKV was first reported in Brazil and was associated with cases of microcephaly, *in utero* growth restriction and placental insufficiency [22]. Cases of ZIKV-associated microcephaly were subsequently documented in Colombia [27], Thailand and Vietnam [24], and ZIKV-related Guillain–Barré syndrome (GBS), a severe autoimmune disease, was reported in French Polynesia [6] and Colombia [31].

ZIKV infection experiments in rhesus macaque (*Macaca mulata*) and cynomolgus macaque (*Macaca fascicularis*) exhibit similar symptoms with those observed in human cases [9, 19, 30], suggesting that some NHPs are susceptible to ZIKV. Previous serological studies in humans demonstrated antibodies to ZIKV and yellow fever virus (YFV) in Zambia [2, 3]. Anti-ZIKV antibody has also been detected from baboons in Tanzania and African green monkeys from Gambia but not from baboons in Zambia using an enzyme-linked immunosorbent assay (ELISA) [4]. Hitherto, little prior knowledge exists for ZIKV infection in NHPs in Zambia. Further investigation is important to ascertain the sero-status of ZIKV and YFV in Zambian NHPs. In the present study, we have examined sera from NHPs in Zambia for the presence of neutralization antibodies against ZIKV and YFV using a plaque reduction neutralization test (PRNT).

Blood and splenic tissues were collected from Zambian malbrouck monkeys (*Chlorocebus cynosuros*; n=48), chacma baboons (*Papio ursinus*; n=25) and yellow baboons (*Papio cynocephalus*; n=23) in the Livingstone (Southern) and Mfuwe (Eastern) districts between 2009 and 2010, as previously described [33] and stored at -80°C prior to examination. NHPs were speciated by mitochondrial cytochrome b (*cytb*) gene sequencing as described by Sasaki et al. (2013) [33]. Ethical approval was obtained from the then Zambia Wildlife Authority (ZAWA), now the Department of National Parks and Wildlife, Ministry of Tourism and Arts (certificate no. 2604) [7]. PRNT was conducted using ZIKV MR-766, YFV 17D and tick-borne encephalitis virus (TBEV) Oshima 5-10 reference strains as positive controls for detection of virus-specific antibody. PRNT

against TBEV was conducted to examine cross-reactivity between flaviviruses. Prior to the experiments, all sera were thawed at 37°C, then diluted 1:5, 1:20 and 1:80 in Dulbecco's Modified Essential Medium (DMEM) for ZIKV and YFV or Minimum Essential Medium (MEM) for TBEV. The diluted sera were inactivated at 56°C for 30 minutes and thereafter incubated 1:1 with ZIKV (25 PFU/well), YFV (750 PFU/well) or TBEV (50 PFU/well) at 37°C for 30 minutes. The diluted, inactivated sera were then inoculated onto Vero cells (ZIKV and YFV) and BHK cells (TBEV) and incubated at 37°C for 1 hour. After incubation, the cells were covered by DMEM supplemented with 1.25% methylcellulose and 2% fetal bovine serum (ZIKV and YFV) or MEM supplemented with 1.5% carboxymethyl cellulose and 2% fetal bovine serum (TBEV) and incubated for 5 days (ZIKV), 4 days (TBEV) or 8 days (YFV) at 37°C. Finally, the cells were fixed using 3.7% buffered formaldehyde for 30 minutes at room temperature and stained using 0.1% (TBEV) or 1% (ZIKV and YFV) crystal violet. The plaque number was counted, and neutralizing activity was determined by a 50% reduction number of viral plaques compared to the no serum control (PRNT50). Positive serum samples were judged by a 4-fold difference between flaviviruses or that, at least, showed PRNT50 ≥1:40 at the final dilution. For TBEV PRNT, serum samples, which showed PRNT50 ≥1:20, were considered to be positive for neutralization activity [35].

Total RNA was extracted from splenic tissues as described by Sasaki et al. (2013) [33] and employed to screen for the presence of ZIKV and YFV viral RNA. Detection of ZIKV and YFV genome were examined by real-time reverse transcriptase (RT)-PCR with an Express One-Step Super Script qRT-PCR kit (Invitrogen, Carlsbad, CA) using the StepOnePlus instrument (Applied Biosystems, Foster City, CA). The oligonucleotide primers and hydrolysis probe and final concentrations employed for detection of ZIKV were as follows: 500 nM ZIKA2 1176F (5'-GAC ATG GCT TCG GAC AG-3'), 500 nM ZIKA2 1308R (5'-CTT TGC CAA AAA GTC CAC A-3'), and 250 nM ZIKA2 1209 probe (5'-FAM-GGT GAA GCC TAC CTT GAC AAG CA-MGB-3'). Our ZIKV primers and probe were designed based on conserved regions of several ZIKV strains, MR-766 (East African, Uganda, LC002520), FSS13025 (Asian, Cambodia, KU955593), DaKAR 41525 (West African, Senegal, KU955591), and Brazil/PE243/2015 (American, Brazil, KX197192), by modifying a widely used ZIKV qRT-PCR system described by Lanciotti et al. [23] (Supplementary Figure 1). This ZIKA2 1209 qRT-PCR system detected ZIKV RNA at approximately one PFU of DaKAR 41525, MR-766, and FSS 13025 reference strains at C_T values of 32 (Supplementary Table 1). The primers sets and probe and final concentrations employed for detection of YFV genome were from a previous report by Domingo et al. (2012) [12]: 500 nM 15F (5'-GCT AAT TGA GGT GYA TTG GTC TGC-3'), 500 nM 103R (5'-CTG CTA ATC GCT

CAA MGA ACG-3') and 250 nM YFV Probe FAM41 (5'-FAM-ATC GAG TTG CTA GGC AAT AAA CAC-MGB-3'). This primer set detected YFV RNA at 1 PFU with C_T values around 33. Thermocycling conditions were 50°C for 15 minutes, 95°C for 2 minutes followed by 45 cycles 95°C for 15 seconds and 60°C for 1 minute for both ZIKV and YFV.

The PRNT revealed that 34.4% (33/96) of sera of NHPs had neutralizing antibodies against ZIKV with comparable levels of seropositivity in Zambian malbrouck monkeys in Livingstone (34.8%; 8/23) and in Mfuwe (32.0%; 8/25). Sera from chacma baboons in Livingstone had a relatively higher seroprevalence (48.0%; 12/25) compared to yellow baboons in Mfuwe (21.7%; 5/23) (Table 1, Table 2 and Supplementary Table 2). In addition, we did not detect any neuralization activity against TBEV in the NHP samples (Table 1). Although we could identify specific neutralization antibodies for ZIKV, we found one sample had both neutralization activity against both ZIKV and YFV with sera titer 160 and 40, respectively (Table 2). This phenomenon may have occurred because of either co-infection of ZIKV and YFV or because of cross-reactivity of antibodies. Flaviviruses possess similar structures and epitopes within their envelope proteins that could potentially be recognized by cross-neutralizing antibodies [34] and cross-neutralization activities among flaviviruses have been reported previously [21, 28]. Therefore, the one sample from a yellow baboon that has neutralization activities for both of ZIKV and YFV may have cross-neutralization activity. For genome screening, neither ZIKV nor YFV viral RNA were detected in splenic tissues from NHPs by real time-RT-PCR which suggested that no acute phase viremic samples were present and that the presence of anti-ZIKV neutralizing antibodies represented prior exposure to a resolved infection.

Serological studies in NHPs in Malaysia, Brazil and on the African continent revealed that some species of NHPs have been shown to have neutralization activities against ZIKV, including Bornean orangutans, African green monkeys, baboons, howler monkeys, marmosets and capuchin monkeys [4, 10, 14, 29, 37]. Along with previous reports, our study has shown seroprevalence of anti-ZIKV neutralization antibodies in malbrouck monkeys, chacma baboons and yellow baboons in Zambia. These studies suggest that NHPs play a role in ZIKV maintenance in nature has previously been proposed by Diallo et al. (2014) and Terzian et al. (2018) [11, 36]. Currently, neutralization assays are widely used as a method to differentiate arbovirus infections in human as reported by Fritzell et al. (2018) [16]. The viral neutralization test is the most frequently used method compared to other neutralization tests such as immunofluorescence assays. Several serological studies for ZIKV infection in animals are also used neutralization assay and, specifically PRNT [8, 10, 29, 37]. PRNT (using live viruses) was used for the determination of type-specific antibodies and differentiation of the infecting viruses. Even

though PRNT is the most specific serological diagnosis for flavivirus infection, this procedure requires tissue culture facilities and laboratories with Biosafety Level-3 (BSL-3) or BSL-4 using physical contaminant level 3 (P3) or P4 viruses) [25].

Serological studies of pathogens in NHPs can be applied as sentinel warning systems to assess the likelihood of human outbreaks of zoonotic disease. It has been reported that Almeida et al. succeeded in enhancing vaccine uptake prior to YFV outbreaks in humans by combined active and passive surveillance in NHPs, which resulted in vaccination being administered within a 2 km radius from the location where YFV in NHPs was detected [1]. Spillover events of flaviviruses from NHPs to humans are dependent on several factors, such as, reservoir hosts and their distribution, infectivity, pathogenicity/transmissibility of pathogens from infected hosts and survival rates of infected hosts [32]. Even though there is a paucity of information on precisely how sylvatic reservoirs with circulating ZIKV could infect humans, several urban transmissions, including mosquito-borne, sexually and maternofetal infection have been reported [17, 18, 26]. Our findings provide new insights into ZIKV circulation in Zambia and represent the first report of serologic detection of sylvatic ZIKV in Zambia. To fully understand the epidemiology and ecology of ZIKV in Zambia, further studies need to be conducted in sylvatic, urban and peri-urban settings in mosquito vectors and wildlife.

Acknowledgement

We are deeply grateful to the then Zambia Wildlife Authority (ZAWA), now the Department of National Parks and Wildlife, Ministry of Tourism and Arts, for assistance with sample collection in Zambia. ZIKV RNAs of DaKAR 41525 and FSS 13025 were kindly provided by Professor Scott C. Weaver, UTMB.

Compliance with Ethical Standards:

Funding: This study was supported by grants for Scientific Research (C), (B) from the Ministry of Education, Culture, Sports, Science and Technology, Japan (MEXT) /Japan Society for the Promotion of Science (JSPS) KAKENHI (JP15K07715, JP16H05805, JP17H03910); and grants for Scientific Research on Innovative Areas and International Group from the MEXT/JSPS KAKENHI (JP16H06431, JP16H06429, JP16K21723), and the Japan Initiative for Global Research Network of Infectious Diseases (J-GRID) from Japan Agency for Medical Research and Development (AMED) (JP18fm0108008).

Conflict of Interest: The authors declare that they have no conflicts of interest.

Ethical approval: All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Ethical approval: This article does not contain any studies with human participants performed by any of the authors.

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Table 1. Flavivirus seropositivity in Zambian non-human primates.

Collecting place	Non-human primate species	Number of sera	Not detected	PRNT (+) YFV	PRNT (+) ZIKV	PRNT (+) TBEV
Livingstone	Chacma baboons *(Papio ursinus)*	25	13	0	12 (12/25=48.0%)	0
	Zambian malbrouck monkeys (*Chlorocebus cynosuros*)	23	15	0	8 (8/23=34.8%)	0
Mfuwe	Yellow baboons (*Papio cynocephalus*)	23	18	0	5 (5/23=21.7%)	0
	Zambian malbrouck monkeys (*Chlorocebus cynosuros*)	25	17	0	8 (8/25=32.0%)	0
TOTAL		96	63	0	33 (33/96=34.4%)	0

Not detected: the antibody titer is ≤1:10 for ZIKV, YFV and TBEV; PRNT (+) YFV: the antibody titer >1:10 for YFV or 4-fold different to ZIKV and TBEV; PRNT (+) ZIKV: the antibody titer >1:10 for ZIKV or 4-fold different to YFV and TBEV; PRNT (+) TBEV: the antibody titer >1:10 for TBEV or 4-fold different to YFV and ZIKV.

Table 2. PRNT50 values for Zika virus (ZIKV), Yellow fever virus (YFV) and Tick-borne encephalitis virus (TBEV) from non-human primate sera collected in Livingstone, Zambia.

Sample No.	Species	PRNT 50			Serological interpretation
		ZIKV	YFV	TBEV	
1	*Papio ursinus*	<10	<10	ND*	
2	*Papio ursinus*	**160**	10	<10	ZIKV
3	*Papio ursinus*	**40**	10	<10	ZIKV
4	*Papio ursinus*	<10	<10	ND	
5	*Papio ursinus*	10	<10	ND	
6	*Papio ursinus*	10	<10	ND	
7	*Chlorocebus cynosuros*	<10	<10	ND	
8	*Papio ursinus*	**160**	<10	<10	ZIKV
9	*Papio ursinus*	<10	<10	ND	
10	*Papio ursinus*	**40**	<10	<10	ZIKV
11	*Papio ursinus*	**160**	**40**	<10	ZIKV
12	*Chlorocebus cynosuros*	10	<10	ND	
13	*Chlorocebus cynosuros*	**40**	<10	<10	ZIKV
14	*Chlorocebus cynosuros*	10	<10	ND	
15	*Papio ursinus*	**40**	<10	<10	ZIKV
16	*Papio ursinus*	<10	<10	ND	
17	*Papio ursinus*	**160**	<10	<10	ZIKV
18	*Chlorocebus cynosuros*	**40**	<10	<10	ZIKV
19	*Chlorocebus cynosuros*	<10	<10	ND	
22	*Chlorocebus cynosuros*	<10	<10	ND	
23	*Chlorocebus cynosuros*	<10	<10	ND	
24	*Chlorocebus cynosuros*	**40**	<10	<10	ZIKV
25	*Chlorocebus cynosuros*	<10	<10	ND	
26	*Chlorocebus cynosuros*	**40**	10	<10	ZIKV

27	*Chlorocebus cynosuros*	<10	<10	ND	
28	*Chlorocebus cynosuros*	10	<10	ND	
29	*Chlorocebus cynosuros*	**40**	<10	<10	ZIKV
30	*Chlorocebus cynosuros*	10	<10	ND	
31	*Papio ursinus*	<10	<10	ND	
32	*Papio ursinus*	10	10	ND	
33	*Papio ursinus*	<10	<10	ND	
34	*Papio ursinus*	**40**	<10	<10	ZIKV
35	*Papio ursinus*	**40**	10	<10	ZIKV
36	*Papio ursinus*	**40**	<10	<10	ZIKV
37	*Papio ursinus*	10	<10	ND	
38	*Papio ursinus*	<10	<10	ND	
39	*Papio ursinus*	**160**	<10	<10	ZIKV
40	*Papio ursinus*	<10	<10	ND	
41	*Papio ursinus*	<10	<10	ND	
42	*Chlorocebus cynosuros*	<10	<10	ND	
43	*Chlorocebus cynosuros*	<10	<10	ND	
44	*Chlorocebus cynosuros*	**40**	<10	<10	ZIKV
45	*Chlorocebus cynosuros*	<10	<10	ND	
46	*Chlorocebus cynosuros*	**160**	<10	ND	ZIKV
47	*Chlorocebus cynosuros*	**160**	10	<10	ZIKV
48	*Chlorocebus cynosuros*	<10	<10	ND	
49	*Chlorocebus cynosuros*	<10	10	ND	
50	*Papio ursinus*	**40**	<10	<10	ZIKV

Positives are shown in bold. *ND: not determined. ZIKV: Zika virus; YFV: Yellow fever virus; TBEV: Tick-borne encephalitis virus. The numbers of PRNT50 are represented as the greatest dilution with a positive result.

Supplementary Table 1.
Cycle thresholds (Cт) values of ZIKV qRT-PCR systems.

ZIKA1107 (Lanciotti 2008)

MR-766			**DaKAR 41525**				**FSS 13025**			
PFU	Cт mean	Cт SD	Dilution	PFU*	Cт mean	Cт SD	Dilution	PFU*	Cт mean	Cт SD
5000	19.94	0.06								
500	23.30	0.06	1.E-01	722	29.09	0.06	1.E-01	1088	21.51	0.17
50	26.83	0.06	1.E-02	87	32.41	0.21	1.E-02	132	24.63	0.05
5	30.64	0.12	1.E-03	11	35.29	0.36	1.E-03	15	27.97	0.08
0.5	33.95	0.29	1.E-04	0.9	ND		1.E-04	1.2	31.83	0.25
0.05	37.21	0.69	1.E-05	0.1	ND		1.E-05	0.1	35.30	0.42

ZIKA2 1209 (in this experiment)

MR-766			**DaKAR 41525**				**FSS 13025**			
PFU	Cт mean	Cт SD	Dilution	PFU*	Cт mean	Cт SD	Dilution	PFU*	Cт mean	Cт SD
5000	19.22	0.09								
500	22.70	0.08	1.E-01	722	22.17	0.12	1.E-01	1088	21.54	0.18
50	26.21	0.09	1.E-02	87	25.43	0.21	1.E-02	132	24.78	0.05
5	29.81	0.23	1.E-03	11	28.68	0.32	1.E-03	15	28.16	0.11
0.5	33.55	0.47	1.E-04	0.9	32.46	0.33	1.E-04	1.2	32.06	0.18
0.05	36.79	0.86	1.E-05	0.1	36.16	0.81	1.E-05	0.1	35.93	0.20

ZIKA1107 C_T, cycle number at threshold 0.13 ΔRn
ZIKA1209 C_T, cycle number at threshold 0.03 ΔRn
* Estimated PFU: calculated with ZIKA1209 based on PFU of MR-766

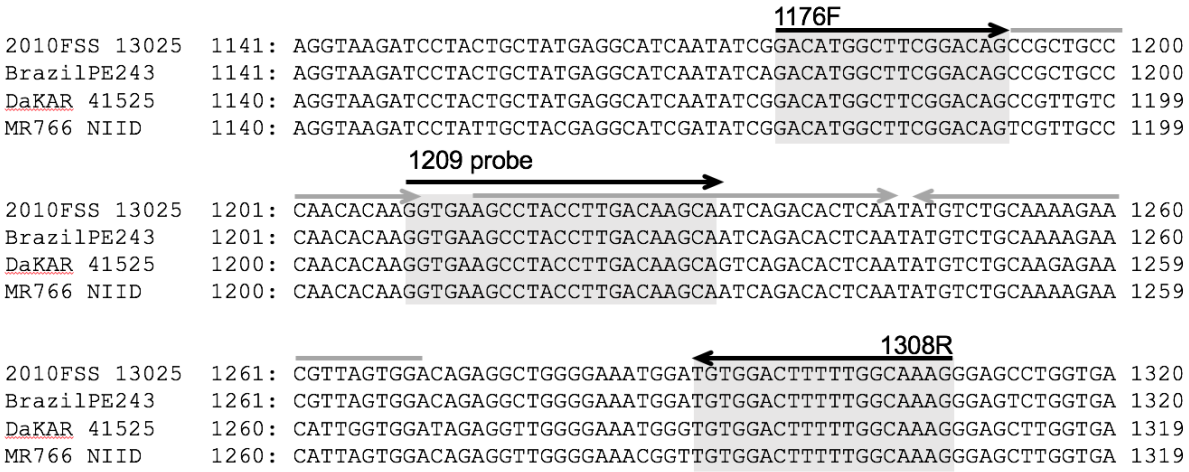
Supplementary table 2.
Nonhuman-primate's sera (collected from Mfuwe) PRNT 50 for Zika virus, Yellow Fever virus and Tick-Borne Encephalitis virus.

Sample No.	Species	PRNT 50			Interpretation
		ZIKV	YFV	TBEV	
1	*Papio kindae*	**40**	<10	<10	ZIKV
2	*Papio kindae*	<10	10	ND	
3	*Papio cynocephalus*	**160**	10	<10	ZIKV
4	*Papio cynocephalus*	<10	<10	ND	
5	*Papio cynocephalus*	**40**	10	<10	ZIKV
6	*Papio cynocephalus*	<10	10	ND	
7	*Papio cynocephalus*	<10	<10	ND	
8	*Papio cynocephalus*	<10	<10	ND	
10	*Papio cynocephalus*	10	<10	ND	
11	*Papio cynocephalus*	<10	<10	ND	
12	*Chlorocebus cynosuros*	10	<10	ND	
13	*Chlorocebus cynosuros*	<10	<10	ND	
14	*Chlorocebus cynosuros*	<10	<10	ND	
15	*Chlorocebus cynosuros*	10	<10	ND	
16	*Papio cynocephalus*	<10	<10	ND	
17	*Chlorocebus cynosuros*	**160**	<10	<10	ZIKV
18	*Papio cynocephalus*	10	<10	ND	
19	*Papio cynocephalus*	**40**	<10	<10	ZIKV
20	*Papio cynocephalus*	<10	<10	ND	
21	*Papio cynocephalus*	<10	<10	ND	
22	*Papio cynocephalus*	**160**	10	<10	ZIKV
23	*Papio cynocephalus*	<10	<10	ND	
24	*Papio cynocephalus*	<10	<10	ND	
25	*Papio cynocephalus*	<10	<10	ND	
26	*Chlorocebus cynosuros*	**160**	10	<10	ZIKV
27	*Chlorocebus cynosuros*	<10	<10	ND	
28	*Chlorocebus cynosuros*	10	<10	ND	
29	*Chlorocebus cynosuros*	<10	<10	ND	
30	*Chlorocebus cynosuros*	10	<10	ND	
31	*Chlorocebus cynosuros*	**40**	<10	<10	ZIKV
32	*Chlorocebus cynosuros*	**40**	<10	<10	ZIKV
33	*Papio cynocephalus*	<10	<10	ND	
34	*Papio cynocephalus*	<10	<10	ND	
35	*Chlorocebus cynosuros*	**160**	<10	<10	ZIKV
36	*Chlorocebus cynosuros*	<10	<10	ND	
37	*Chlorocebus cynosuros*	<10	<10	ND	

38	*Papio cynocephalus*	<10	<10	ND	
39	*Papio cynocephalus*	<10	<10	ND	
40	*Chlorocebus cynosuros*	**40**	<10	<10	ZIKV
41	*Chlorocebus cynosuros*	**40**	<10	<10	ZIKV
43	*Chlorocebus cynosuros*	<10	<10	ND	
44	*Chlorocebus cynosuros*	<10	<10	ND	
45	*Chlorocebus cynosuros*	10	<10	ND	
46	*Chlorocebus cynosuros*	10	<10	ND	
47	*Chlorocebus cynosuros*	<10	<10	ND	
48	*Chlorocebus cynosuros*	<10	<10	ND	
49	*Chlorocebus cynosuros*	<10	<10	ND	
50	*Chlorocebus cynosuros*	**160**	<10	10	ZIKV

*ND: not determined; ZIKV: Zika virus; YFV: Yellow Fever virus; TBEV: Tick-Borne Encephalitis virus. The numbers of PRNT50 are represented as the greatest dilution with a positive result.

Supplementary figure 1. Regions of the ZIKV qRT-PCR probe and primers on conserved regions of four ZIKV strains



2010FSS 13025: KU955593.1 Zika virus isolate Zika virus/H.sapiens-tc/KHM/2010/FSS13025
Brazil PE243: KX197192.1 Zika virus isolate ZIKV/H.sapiens/Brazil/PE243/2015
DaKAR 41525: KU955591.1 Zika virus isolate Zika virus/A.africanus-tc/SEN/1984/41525-DAK
MR766 NIID: LC002520.1 Zika virus genomic RNA, strain: MR766-NIID
Gray arrows show regions of ZIKA1107 primers and probe described by Lanciotti *et al* (2008).