New reverse genetics and transfection methods to rescue arboviruses in mosquito cells

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Supplementary information

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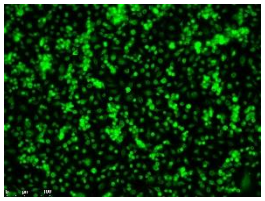
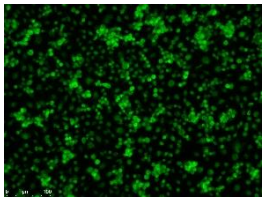
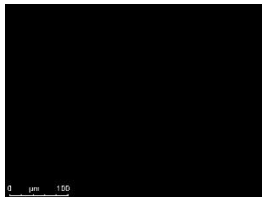
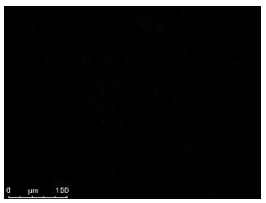
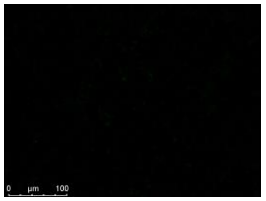
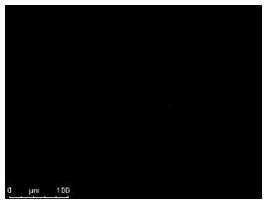
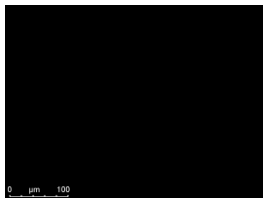
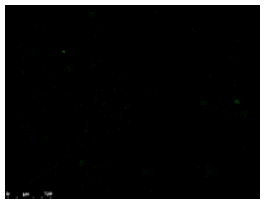
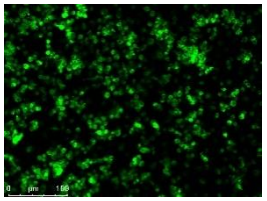
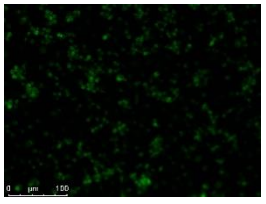
Supplemental Figures:

		M glycoprotein antibody	Human serum
a)	Amplicons I + II + III		
b)	Amplicons I alone		
c)	Amplicons II alone		
d)	Amplicons III alone		
e)	Amplicons I + III		
f)	C-prM-E encoding plasmid	ND	
g)	NS5 encoding plasmid	ND	

Supplementary Figure S1: Immunofluorescence staining for viral antigens of West Nile Virus rescued by the ISA method. The C6/36 cells transfected with all the three overlapping subgenomic DNA amplicons encompassing the entire viral genome of West Nile Virus (fragments I, II and III) (a), the first amplicon alone (b), the second amplicon alone (c), the third amplicon alone (d), the first and third amplicons that do not overlap (e), or a plasmid encoding the C-prM-E polyprotein (f) or the NS5 protein (g) of West-Nile virus under the control of the CMV promoter were probed with a specific M glycoprotein antibody (left panel) or a human serum containing anti-West-Nile virus-specific antibodies (right panel) and a FITC-conjugated secondary antibody. No fluorescence was observed with untransfected cells and with transfected cells stained only with the FITC-conjugated secondary antibody.

Supplemental Tables:

A

Temperature of transfection	Samples	Quantity of transfected plasmid (ng)			
		2.5	5	10	50
37°C	a	4.95 +/- 0.18	4.94 +/- 0.17	4.69 +/- 0.20	4.96 +/- 0.16
	b	<1.8¶	4.70 +/- 0.18	5.49 +/- 0.12	4.95 +/- 0.18
	c	<1.8¶	4.69 +/- 0.19	5.42 +/- 0.00	5.15 +/- 0.11
	d	5.15 +/- 0.11	5.37 +/- 0.07	5.24 +/- 0.11	5.19 +/- 0.04
28°C	a	<1.8¶	<1.8¶	4.70 +/- 0.20	4.95 +/- 0.18
	b	<1.8¶	4.96 +/- 0.16	4.94 +/- 0.18	4.69 +/- 0.19
	c	<1.8¶	<1.8¶	4.96 +/- 0.19	5.02 +/- 0.28
	d	<1.8¶	<1.8¶	5.94 +/- 0.16	5.24 +/- 0.11

B

Temperature of transfection	Samples	Quantity of transfected DNA(ng)		
		100	500	1000
37°C	a	<1.8¶	<1.8¶	6.63 +/- 0.08
	b	<1.8¶	<1.8¶	6.39 +/- 0.24
	c	<1.8¶	<1.8¶	6.16 +/- 0.10
	d	<1.8¶	<1.8¶	7.45 +/- 0.53
28°C	a	<1.8¶	<1.8¶	6.14 +/- 0.09
	b	<1.8¶	<1.8¶	<1.8¶
	c	<1.8¶	<1.8¶	<1.8¶
	d	<1.8¶	<1.8¶	<1.8¶

¶ Detection threshold of the assay = 1.8 $\log_{10}$ $TCID_{50}$/

Table S1: Infectious titers of the rescued viruses in C6/36 cell supernatant described in Figure 1.

A: Infectious titers of rescued viruses after transfection of 2.5ng, 5ng, 10ng and 50ng of West Nile Virus infectious clone in C6/36 cells under two different temperature 37°C and 28°C.

B: Infectious titers of rescued viruses by ISA procedure after transfection of 100ng, 500ng and 1000ng of the three DNA overlapping fragments in C6/36 cells at 28°C or 37°C.

Infectious titers are expressed as mean +/- SD of Log10 values. Each experiment was performed in quadruplicate.

Virus	cDNA fragments		Primer forward	Position*	Primer reverse	Position*	DpnI enzymatic treatment	Fragment used as negative control
	number of transfected fragments	length						
YFV Asibi	3	4818 bp	¶ CACCCAACTGATCTTCAGCATCT	¶	CTATGATAACCACGGTACAAAAGAG	4012-4037	No	
		3041 bp	AGCATCAAATACCATCTTGCCCCT	3936-3960	CCGGCCAACTCCAGGGTGAAGC	6955-6977	No	
		4199 bp	GGCAGCCAACGAGCTAGGCAT	6879-6900	CTCAGGGTCAATGCCAGCGCTT	£	No	
YFV 17D	3	4800bp	CACCCAACTGATCTTCAGCATCT	¶	AACATTCTTTGGATCCTGCACG	2718-2740	No	
		4717 bp	ACTCCCTTGAGCATGAGATGT	2633-2654	ACTGTTGGATTCCCATCAACC	7329-7350	No	
		3800 bp	ACTGGTCTCTCATTTTACCTGGA	7241-7264	CTCAGGGTCAATGCCAGCGCTT	£	No	
JEV	3	4815 bp	CACCCAACTGATCTTCAGCATCT	¶	GAAGAATGATTCTGTAAGTGTCCAG	4032-4057	No	
		3003 bp	CGTTGCCATGCCAATCTTAGCG	3980-4002	GGTGCTTGCGTCCTTCCACCAA	6961-6983	No	
		4247 bp	CAAATGAGTATGGAATGCTGGAAAA	6910-6935	CTCAGGGTCAATGCCAGCGCTT	£	No	
	4	2394 bp	CACCCAACTGATCTTCAGCATCT	¶	CATGGAACCATTCCCTATGGACT	1613-1636	Yes	x
		2519 bp	ACTGGATTGTGAACCAAGGAGTG	1538-1561	GAAGAATGATTCTGTAAGTGTCCAG	4032-4057	Yes	
		3003 bp	CGTTGCCATGCCAATCTTAGCG	3980-4002	GGTGCTTGCGTCCTTCCACCAA	6961-6983	No	x
		4247 bp	CAAATGAGTATGGAATGCTGGAAAA	6910-6935	CTCAGGGTCAATGCCAGCGCTT	£	No	x
	5	2394 bp	CACCCAACTGATCTTCAGCATCT	¶	CATGGAACCATTCCCTATGGACT	1613-1636	Yes	x
		2519 bp	ACTGGATTGTGAACCAAGGAGTG	1538-1561	GAAGAATGATTCTGTAAGTGTCCAG	4032-4057	Yes	
		1531 bp	CGTTGCCATGCCAATCTTAGCG	3980-4002	CAGTTCCACCTTGGTCGCAATG	5507-5529	Yes	x
		1565 bp	ATGTCACCAAACAGGGTGCCCAA	5418-5441	GGTGCTTGCGTCCTTCCACCAA	6961-6983	Yes	
		4247 bp	CAAATGAGTATGGAATGCTGGAAAA	6910-6935	CTCAGGGTCAATGCCAGCGCTT £	£	No	x
	6	2394 bp	CACCCAACTGATCTTCAGCATCT	¶	CATGGAACCATTCCCTATGGACT	1613-1636	Yes	x
		2519 bp	ACTGGATTGTGAACCAAGGAGTG	1538-1561	GAAGAATGATTCTGTAAGTGTCCAG	4032-4057	Yes	
		1531 bp	CGTTGCCATGCCAATCTTAGCG	3980-4002	CAGTTCCACCTTGGTCGCAATG	5507-5529	Yes	x
		1565 bp	ATGTCACCAAACAGGGTGCCCAA	5418-5441	GGTGCTTGCGTCCTTCCACCAA	6961-6983	Yes	
		2040 bp	CAAATGAGTATGGAATGCTGGAAAA	6910-6935	GCGCCGTGCTCCATTGATTCTG	8928-8950	Yes	x
		2336 bp	GGGAGAAACGACCCCGCTTGTG	8842-8864	CTCAGGGTCAATGCCAGCGCTT	£	Yes	
	8	1958 bp	CACCCAACTGATCTTCAGCATCT	¶	GCATCGAGCTACCGTTGAAATGT	1177-1200	Yes	x
		1270 bp	AGGAGACAGCTGTTTGACAATC	1055-1077	GAATGCACCGCCAAATACTTGGT	2302-2325	Yes	

	1876 bp	ACGCTGGGCAAAGCTTTCTCA	2181-2202	GAAGAATGATTCTGTAAGTGTCCAG	4032-4057	Yes	
	1022 bp	CGTTGCCATGCCAATCTTAGCG	3980-4002	GTGGGTAGTCCAAGCTGACTGCT	4979-5002	Yes	x
	1193 bp	ACAGATGATGTGCAAGTGATTGTG	4881-4905	CAGTGGGCTAAGTTGCTGTCAT	6052-6074	Yes	
	1058 bp	AGGTAGAGTCATTCTTGGAAAC	5924-5946	GGTGCTTGCGTCCTTCCACCAA	6961-6983	Yes	
	2765 bp	CAAATGAGTATGGAATGCTGGAAAA	6910-6935	ACTGATCGCCATCCTAGTCACT	9653-9675	Yes	x
	1654 bp	CTACGCTCTCAACACATTCACG	9503-9525	CTCAGGGTCAATGCCAGCGCTT	£	Yes	
	750bp	CACCCAACTGATCTTCAGCATCT	¶	AAGCCAAGAAGTTCACACAGATAAACT TCTCGGTTCACTAAACGAGCTCTGC		Yes	x
	1200 bp	AGAAGTTTATCTGTGTGAACTTCT	0-24	GCATCGAGCTACCGTTGAAATGT	1177-1200	Yes	x
	1270 bp	AGGAGACAGCTGTTTGACAATC	1055-1077	GAATGCACCGCCAAATACTTGGT	2302-2325	Yes	
	1876 bp	ACGCTGGGCAAAGCTTTCTCA	2181-2202	GAAGAATGATTCTGTAAGTGTCCAG	4032-4057	Yes	
10	1022 bp	CGTTGCCATGCCAATCTTAGCG	3980-4002	GTGGGTAGTCCAAGCTGACTGCT	4979-5002	Yes	x
	1193 bp	ACAGATGATGTGCAAGTGATTGTG	4881-4905	CAGTGGGCTAAGTTGCTGTCAT	6052-6074	Yes	
	1058 bp	AGGTAGAGTCATTCTTGGAAAC	5924-5946	GGTGCTTGCGTCCTTCCACCAA	6961-6983	Yes	
	1038 bp	CAAATGAGTATGGAATGCTGGAAAA	6910-6935	CGTAGTAGCTCCAGCCTCCG	7928-7948	Yes	x
	1899 bp	GAGGTGGACCGCACTGAAGCA	7776-7797	ACTGATCGCCATCCTAGTCACT	9653-9675	Yes	
	1654 bp	CTACGCTCTCAACACATTCACG	9503-9525	CTCAGGGTCAATGCCAGCGCTT	£	Yes	

Table S2: Primers used to obtain cDNA fragments of the different viruses used for transfection.

I, II and III designate respectively the first, the second and the third cDNA fragments obtained by PCR. bp: base pair. Primers located respectively at the 5' and 3' terminus of the pCMV (¶) and the HDR/SV40pA (£).

The primers described previously by Aubry *et al* and Atieh *et al* were used to amplified the three overlapped fragments for CHIKV, WNV and ZIKA viruses.

*All positions based on complete viral strain sequence

Virus	Position	Length	Primer Forward	Probe	Primer Reverse
YFV Asibi	6474-6579	105	CCCTGATTTCCTGGCTAAAAAA	FAM-TTCTCCACTCTGAGGAAGGCTCT-TAMRA	ATCATTGATAGTGCATTGCGGT
YFV 17D	8278-8354	76	TCCACTCATGAAATGTACTACGTGTCT	FAM-AGCCCGCAGCAATGTCACATTTACTGT-TAMRA	GGAGGCGGGATGTTTGGT

Table S3: Primers and probes used for the Real-time RT-qPCR assays.

The primers and probes described previously by Aubry *et al.* and Atieh *et al.* were used to amplified the three overlapped fragments for CHIKV, WNV and ZIKA (H/PF/2013 and A.taylori-tc/SEN/1984/41662-DAK).

cDNA fragments					
number	**length**	**Forward**	**position**	**Reverse**	**position**
I	2026	AGAAGTTTATCTGTGAACTTCT	1-20	GGGTCATGTCGTTTAAACTCGC	2004-2026
II	2137	GCGGACACAGGCCATGGAA	1920-1939	GAAGAATGATTCTGTAAGTGTCCAG	4032-4057
III	3003	CGTTGCCATGCCAATCTTAGCG	3980-4002	GGTGCTTGCGTCCTTCCACCAA	6961-6983
IV	2207	CAAATGAGTATGGAATGCTGGAAAA	6910-6935	CCACATGAACCAAATAGCCCTAC	9094-9117
V	1993	CAATGGAGCACGGCGCGGG	8934-8953	GGCGCTCTGTGCCTAGTAGC	10907-10927

Table S4. Primers used for sequencing of JEV full genome.

Sequencing of JEV full genome was performed by dividing the genome into five overlapped fragments. This table showed the set of specific primers used during RT-PCR assays to generate the overlapped fragments.

Supplemental Methods:

Immuno-fluorescence assay

Immunofluorescence staining assay was performed on C6/36 cells transfected for 12 hours at 37°C with the subgenomic amplicons used to rescue the West Nile virus through the ISA method [1]. In this model, three overlapping subgenomic DNA amplicons encompassing the entire viral genome (fragments I, II and III) are used to recover an infectious virus. One, two or the three subgenomic amplicons of the West Nile virus model were transfected in C6/36 cells. One day post-transfection cells were seeded on slide and incubated for 24h at 28°C. After then, cells were washed gently three times with PBS, and fixed in 4% paraformaldehyde for 20 min at room temperature. After three washes with PBS, cells were permeabilized by 0.2% Triton X-100 for 5 min at room temperature. Cells were washed once with PBS, and then incubated 1 hour in blocking solution containing 0.5% bovine serum albumin (BSA). Then, cells were probed overnight at 4°C with a specific M glycoprotein antibody (Abcam) diluted to 1/50 in PBS-1% BSA or a human serum containing anti-West-Nile virus-specific antibodies [2] diluted to 1/100 in PBS-1% BSA. After three washes with blocking solution, cells were incubated for 1hour at room temperature with the second antibody an anti-rabbit or an anti-human diluted at 1/100 in PBS-1% BSA. Both secondary antibodies are conjugated with fluorescein isothiocyanate (FITC). Slides were washed three times with PBS, and then visualized with a DMI8 Leica microscope and an FITC excitation and emission filter set. As controls for the presence of specific antibodies in our human immune serum, C6/36 cells were transfected at 37°C with in-house plasmids encoding the C-prM-E polyprotein or the NS5 protein of West-Nile virus under the control of the CMV promoter.

Supplemental References:

1 Aubry, F. *et al.* Single-stranded positive-sense RNA viruses generated in days using infectious subgenomic amplicons. *J Gen Virol* **95**, 2462-2467, doi:10.1099/vir.0.068023-0 (2014).

2 Gake, B. *et al.* Low seroprevalence of Zika virus in Cameroonian blood donors. *Braz J Infect Dis* **21**, 481-483, doi:10.1016/j.bjid.2017.03.018 (2017).