Supplementary appendix

Supplementary Information: Materials and Methods

Serum samples of individuals with convalescence Oropouche virus infection

Blood samples from individuals with previous Oropouche virus (OROV) infection were obtained by venipuncture in May 2016 from residents of Coari municipality, Amazonas State, Brazil. The previous OROV infection was confirmed by plaque reduction neutralization test value 50 ($PRNT_{50}$) [1]. All the samples used in this study were negative by real-time quantitative Reverse Transcription-Polymerase Chain Reaction (RT-qPCR) for OROV, Mayaro (MAYV), chikungunya (CHIKV), and dengue viruses (DENV), as described (Table S5, p. 7). All samples were stored at –80°C.

Real-time Quantitative Reverse Transcription-Polymerase Chain Reaction for Oropouche, *Chikungunya, Dengue, and Mayaro Viruses*

Viral RNA was extracted from the serum samples using the Maxwell HT Viral TNA Kit (Cat no. AX2340, Promega, USA) with the KingFisher Flex Purification System robot (Thermo Fisher Scientific, USA), following the manufacturer's instructions. The extracted RNA was then tested by real-time RT-qPCR targeting OROV[2,3], CHIKV[4], DENV serotypes 1 to 4[5,6], and MAYV[7] using the qPCR BIO Probe 1-Step Go Lo - ROX Kit (Cat no. PB25.11-03, PCR Biosystems, UK). Reactions were performed on QuantStudio 3 (Applied Biosystems, USA). The primers and probes used for the viral detection are described (Table S5, p. 7).

Oropouche virus isolation in cell culture

OROV isolation in culture cells was performed by inoculating Vero CCL-81 cells with ten serum samples that tested positive for OROV RNA using RT-qPCR (Table S1, p. 4). Briefly, Vero CCL-81 cells were plated in 24-well plates at a concentration of $2{\cdot}5\times10^5$ cells per mL ($1{\cdot}25\times10^5$ cells per well) in Minimum Essential Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), and 1% of penicillin of 10,000 units and 10,000 µg/mL streptomycin solution. Subsequently, the serum samples (n=10) were diluted 1:10 in DMEM, treated with 2% penicillin and streptomycin, and added to the monolayer. After a one-hour incubation at 37ºC for adsorption, DMEM supplemented with 5% FBS and treated with 1% penicillin and streptomycin was added to the monolayer for maintenance. The cells were kept at 37ºC with 5% CO_2 and monitored for 30 hours until the cytopathic effect (CPE) became visible on an optical microscope. Next, the supernatant was collected and subjected to an RT-qPCR assay[3] to confirm viral isolation, indicated by a decrease in the Ct-value.

Focus forming assay for Oropouche virus

A focus formation assay was performed for the titration of OROV-positive serum samples and isolates, as previously described elsewhere[8]. Briefly, the samples were serially diluted in an 8-fold series in DMEM treated with 1% penicillin and streptomycin solution. Next, 100 µL of the dilutions were transferred to 96-well plates containing Vero CCL-81 cells (5×10^4 cells per well) with 80% confluence, which were incubated for 1 hour at 37°C with 5% CO_2 for viral adsorption. Subsequently, 125 µL of DMEM containing 0·75% carboxymethylcellulose and 5% FBS was added to the wells, and the plates were incubated at 37°C with 5% CO_2 for 48 hours. Next, the cells were fixed with 70 µL of 8% paraformaldehyde solution (PFA) and incubated for 1 hour at room temperature. After removing the PFA, the cells were washed with phosphate-buffered saline (PBS). The cell monolayer was then blocked for 30 minutes with 150 µL of blotto. After blocking, the monolayers were washed with Perm/Wash Buffer (PBS supplemented with 0·1% BSA and 0·1% Triton X-100) and incubated with the polyclonal anti-OROV antibody (Cat no. VR-1228AF,

ATTC, USA). After a second wash with PermWash, the monolayers were incubated with an anti-mouse IgG secondary antibody (Cat no. AP124P, Sigma-Aldrich, USA). Finally, after a final wash with Perm/Wash Buffer, the assay was revealed using the True-Blue Peroxidase substrate (Cat no. 5510-0030, KPL, USA) for 30 minutes.

Oropouche virus genome sequencing and analysis

OROV genome sequencing was performed with two viral isolates using the Rapid SMART-9N protocol with the MinION platform (Oxford Nanopore Technologies, UK), as previously described[9]. The generated raw FAST5 files were then basecalled, demultiplexed, and trimmed using Guppy version 9·4·1 (Oxford Nanopore Technologies, UK). The barcoded files were aligned to the OROV reference genome (GenBank accession no. KP691612, KP691622, and KP691623) using minimap2 v·2·17·r941[10] and converted into BAM files using SAMtools[11]. Medaka_variants were employed for variant calling, followed by medaka_consensus (Oxford Nanopore Technologies, UK) for consensus sequence building. Genome regions with coverage below 20× were represented by the letter "N". NanoStat version 1·6·0[12], Samtools stats, and Samtools depth [12] were applied to compute the genome statistics.

Phylogenetic analysis

The two novel OROV genomes with >90% coverage were generated and aligned with the non-redundant OROV strains with complete coding sequences available in the GenBank database as of June 30, 2024. Then, we built a multiple sequence alignment (MSA) for each segment using MAFFT version 7·450[13], and manual adjustment was conducted using Geneious Prime 2023·0·4. A maximum likelihood (ML) phylogeny trees were performed using IQ-TREE version 2 under a GTR+I+γ model determined by ModelFinder[14,15]. The ultrafast-bootstrap approach with 1,000 replicates was used to determine the statistical support for nodes for the ML phylogenies. The phylogenetic trees were visualized using Figtree 1·4·2 (http://tree.bio.ed.ac.uk/software/figtree/). Additionally, we contacted the three segments of genomes, and we screened for reassortment events using all available methods in RDP version 4[16].

Plaque reduction neutralization test for Oropouche virus

To compare the neutralizing antibody capacity of serum from individuals previously infected with OROV against OROV strains BeAn 19991 (prototype) and AM0088 strain (2023-2024 OROV reassortment), we performed a $PRNT_{50}$ as described elsewhere[1]. Briefly, we inactivated the complement system by heating serum samples at 56ºC degrees, then we performed serial dilutions of each serum sample and incubated with a solution containing 2×10^3 PFU/mL for BeAn 19991 isolate, or 80 PFU/mL of the AM0088 isolate, both for 1 hour at 37°C. Subsequently, the virus-serum mixtures were added to pre-formed Vero CCL-81 cell monolayers and incubated for 1 hour at 37°C in a 5% CO_2 atmosphere. Next, we removed the inoculum and added 1 mL of DMEM containing 0·75% carboxymethylcellulose and 5% FBS was gently added to each well, and the plates were incubated at 37°C in a 5% CO_2 atmosphere for 3 days. Finally, the cells were fixed with 500 µL of 8% paraformaldehyde solution for 1 hour and stained with 1% methylene blue (Cat no. PHR3838, Sigma-Aldrich, USA) for 5 minutes. Plaque reduction was calculated as the average of values from two technical duplicates, corresponding to the percentages of the number of plaques counted compared to the positive control. These values were transformed to Log_2 for better visualization in the graph and subjected to a three-parameter nonlinear dose-response inhibition regression test.

Virus replication curves for Oropouche virus

To compare the viral fitness OROV strains BeAn 19991 (prototype) or AM0088 (2023-2024 OROV reassortment), we performed virus replication curves using Vero CCL-81 cells (African green monkey kidney), Huh7 cells (human liver carcinoma), and U-251 cells (Human glioblastoma astrocytoma). In summary, the cells were infected with OROV strains BeAn 19991 or AM0088 at a MOI of 0·1 for 1 hour at 37°C in a 5% CO_2 atmosphere. Then, we removed the inoculum, washed the cell monolayer three times using PBS, and added Minimum Essential Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), and 1% of penicillin of 10,000 units and 10,000 µg/mL streptomycin solution. At 3-, 6-, 12-, and 24-hours post-infection (hpi), we collected the cell culture supernatant and determined the infectious virus using FFA as described above[8]. All the experiments were conducted in triplicate.

Assessment of plaque phenotypes of Oropouche virus

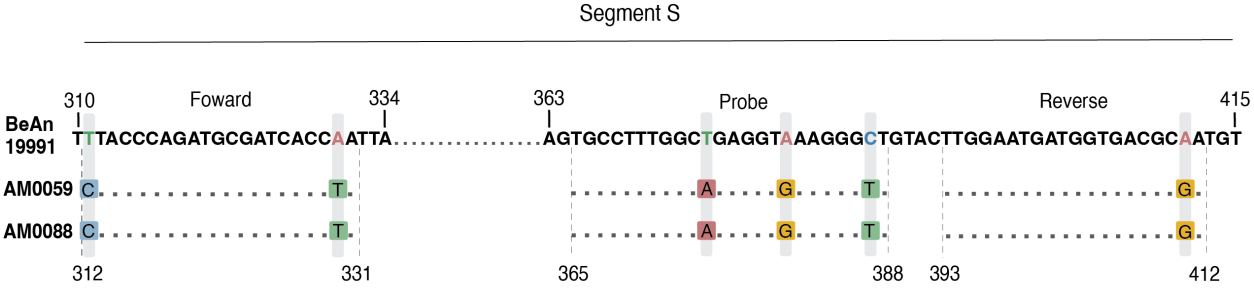
To evaluate the plaque phenotypes generated by OROV strains BeAn 19991 (prototype) or AM0088 (2023-2024 OROV reassortment), we counted the number of plaques and measured the size of plaques at 36-, 48-, and 72-hours post-infection (hpi) for both OROV isolate in Vero CCL-81 cells. The number of plaques produced by each strain at each time point was determined by visual observation and counting. To measure the plaques, we illuminated the plates with a white bottom light and took photos using a Canon EOS Rebel T7i at a focal distance of 54 mm. All assay images were then imported into Fiji software version 2·15·1 for further analysis. We pre-processed and filtered the images to obtain a clearer resolution of the plaques. Next, we used the "Analyze Particles" feature to identify and measure the plaques, using the well diameter as the reference scale. Finally, any identified noise was manually removed from the images.

Cross-neutralization antibody test for Oropouche virus

To investigate antigenic differences between the OROV BeAn 19991 and AM0088 isolates, we conducted cross-neutralization assays. Briefly, two groups of four-week-old C57BL/6 mice were intraperitoneally (IP) inoculated with 1×10^6 PFU using a final volume of 100 µl. One group was inoculated with the BeAn 19991 isolate, and the other with AM0088 isolate. The mice were then kept under pathogen-free conditions at the biosafety level 2 animal facility of the Institute of Biology at the University of Campinas. Next, serum from these animals was collected 28 days post-infection. All animals did not show any signs of disease. Then, we performed the $PRNT_{50}$ to evaluate the capacity of neutralizing antibodies against the same OROV isolate (homologous) or different OROV isolate (heterologous).

Supplementary Figure

Fig. S1. Mismatches between the segment S of 2023-2024 OROV reassortment strains and the primers and probes previously described[2].



Supplementary Table

Table S1. Anonymized patient information data for all PCR-positive samples of Oropouche virus.

Group	ID	Sample collection	RT-PCR-SegS[3]	RT-PCR-SegM[3]	RT-PCR-SegS[2]	Viral isolate	Sequencing
Febrile illnesses	0004	12-Jan-2024	36·4	32·1	ND	yes	N/P
	0044	16-Jan-2024	37·0	ND	ND	no	N/P
	0045	19-Jan-2024	37·3	29·7	ND	yes	N/P
	0046	21-Jan-2024	35·2	28·0	ND	yes	N/P
	0059	21-Jan-2024	34·5	28·6	ND	yes	yes
	0063	20-Jan-2024	36·9	35·0	ND	yes	N/P
	0064	21-Jan-2024	36·5	30·0	ND	yes	N/P
	0078	28-Jan-2024	34·2	26·0	ND	no	N/P
	0088	30-Jan-2024	36·1	28·7	ND	yes	yes
	0093	22-Jan-2024	36·3	not detected	ND	no	N/P
CNS manifestations	2051	20-Jan-2024	N/P	32·8	ND	N/P	N/P
	2062	31-Jan-2024	N/P	33·9	ND	N/P	N/P
	2134	16-Apr-24	N/P	36·9	ND	N/P	N/P

Seg S. segment S. SegM, segment M. CNS, central nervous system. N/P, not performed. ND, not detected.

Table S2. Amino acids change comparing OROV strain BeAn19991 to 2023-2024 OROV reassortment strains (AM0059 and AM0088).

Gene	Position	BeAn19991 strain	AM0059 strain	AM0088 strain
M	12	G	S	S
	22	S	S	N
	66	K	R	R
	71	del	T	T
	72	del	T	T
	247	F	F	S
	276	F	I	I
	394	R	S	S
	395	I	T	T
	430	D	N	N
	448	V	I	I
	516	S	N	N
	523	F	S	S
	616	N	K	K
	645	M	I	I
	734	L	V	V
	752	G	D	D
	766	N	S	S
	814	S	S	P
	824	A	T	T
	848	I	I	V
	959	I	I	V
	983	Q	K	K
	984	S	G	G
	1085	V	A	A
	1205	I	T	T
	1270	P	S	S
	1311	T	I	I
	1325	I	V	V
	1344	R	K	K
	1362	I	V	V
L	135	T	A	A
	144	V	M	M
	201	T	A	A

210	S	N	N
215	A	S	S
258	H	Q	Q
263	T	A	A
284	N	S	S
303	M	I	I
309	K	Q	Q
313	N	S	S
338	V	I	I
339	N	S	S
354	V	I	I
372	I	V	V
382	I	V	V
415	L	F	F
442	N	D	D
458	I	T	T
464	I	V	V
558	M	I	I
565	A	T	T
580	A	T	T
663	R	K	K
677	S	A	A
786	A	V	A
788	R	Q	Q
789	L	T	T
790	S	V	V
791	X	N	N
794	V	I	I
799	L	I	I
800	Q	A	A
801	E	R	R
802	X	N	N
850	R	K	K
853	L	T	T
854	R	K	K
855	M	N	N
856	I	D	D
857	Q	A	A
921	N	S	S
940	H	Y	Y
1035	N	S	S
1114	L	V	V
1159	I	T	T
1192	I	V	V
1314	S	N	N
1375	K	R	R
1436	D	N	N
1439	A	T	T
1505	V	I	I
1693	V	I	I
1758	V	I	I
1778	I	V	V
1911	R	Q	Q
1934	V	I	I
1942	V	I	I
1948	S	N	N
1961	I	V	V
1976	R	K	K
2056	G	E	E
2057	D	N	N
2171	K	R	R
2187	I	V	V

The positions refer to the competing coding sequences of OROV strain BeAn 19991 for the M segment (GenBank accession number NC_005775) and the L segment (GenBank accession number NC_005776).

Table S3. Neutralizing capacity of serum previously infected with OROV in Coari municipality, Amazonas State, Brazil against the BeAn 19991 and the AM0088 isolates.

ID	BeAn 19991	AM0088
A 19	640	<20
A 20	640	<20
A 24	160	<20
A 33	640	<20
A 35	160	<20
A 77	320	<20
A 91	640	<20
A 111	320	<20
A 193	640	<20
A 206	640	<20
A 216	640	<20
A 304	320	<20
A 324	640	<20
A 336	640	<20
A 340	320	<20
A 351	640	<20
A 372	160	<20
A 375	320	<20
A 385	640	<20
A 389	320	<20
A 392	320	<20
A 369	640	<20

Table S4. Genome sequences used in the phylogenetic analyses.

Isolate	Country	State	Host	Collection year	Accession GenBank numbers		
TRVL-9760	Trinidad and Tobago	-	Homo sapiens	1955	KC759124	KC759123	KC759122
BeH759620	Brazil	Amapá	Homo sapiens	2009	KP691623	KP691622	KP691621
TVP-19250/GML-444839	Panama	-	Homo sapiens	1989	KP795080	KP795079	KP795078
TVP-19256/IQE-7894	Peru	-	Homo sapiens	2008	KP795086	KP795085	KP795084
TVP-19257/IQT-1690	Peru	-	Homo sapiens	1995	KP795089	KP795088	KP795087
TVP-19258/IQT-4083	Peru	-	Homo sapiens	1997	KP795092	KP795091	KP795090
TVP-19259/IQT-7085	Peru	-	Homo sapiens	1998	KP795095	KP795094	KP795093
TVP-19260/MD-203	Peru	-	Homo sapiens	1994	KP795098	KP795097	KP795096
TVP-19264/PAN-481126	Panama	-	Homo sapiens	1999	KP795104	KP795103	KP795102
087/2016	Ecuador	-	Homo sapiens	2016	MF926352	MF926353	MF926354
BeH 389865	Brazil	Amazonas	Homo sapiens	1980	MG747506	MG747507	MG747508
BeH 390242	Brazil	Amazonas	Homo sapiens	1980	MG747509	MG747510	MG747511
BeH 472433	Brazil	Maranhão	Homo sapiens	1988	MG747512	MG747513	MG747514
BeH 472435	Brazil	Maranhão	Homo sapiens	1988	MG747515	MG747516	MG747517
BeH 421086	Brazil	Maranhão	Homo sapiens	1993	MG747518	MG747519	MG747520
BeAn 626990	Brazil	Minas Gerais	Callithrix sp.	2000	MG747521	MG747522	MG747523
BeAr 19886	Brazil	Pará	Ochlerotatus serratus	1960	MG747524	MG747525	MG747526
BeH 29086	Brazil	Pará	Homo sapiens	1961	MG747527	MG747528	MG747529
BeH 121923	Brazil	Pará	Homo sapiens	1967	MG747533	MG747534	MG747535
BeAr 136921	Brazil	Pará	Culex quinquefasciatus	1968	MG747536	MG747537	MG747538
BeAn 206119	Brazil	Pará	Bradypus tridactylus	1971	MG747539	MG747540	MG747541
BeH 355173	Brazil	Pará	Homo sapiens	1978	MG747548	MG747549	MG747550
BeAr 366927	Brazil	Pará	Culicoides paraensis	1979	MG747551	MG747552	MG747553

BeH 385591	Brazil	Pará	Homo sapiens	1980	MG747554	MG747555	MG747556
BeH 532314	Brazil	Pará	Homo sapiens	1994	MG747557	MG747558	MG747559
BeH 532490	Brazil	Pará	Homo sapiens	1994	MG747563	MG747564	MG747565
BeH 541140	Brazil	Pará	Homo sapiens	1994	MG747569	MG747570	MG747571
BeH 543857	Brazil	Pará	Homo sapiens	1996	MG747578	MG747579	MG747580
PPS 522 H 669314	Brazil	Pará	Homo sapiens	2003	MG747581	MG747582	MG747583
PMOH 682426	Brazil	Pará	Homo sapiens	2004	MG747587	MG747588	MG747589
PMOH 682431	Brazil	Pará	Homo sapiens	2004	MG747590	MG747591	MG747592
BeH 498913	Brazil	Rondonia	Homo sapiens	1990	MG747602	MG747603	MG747604
BeH 505768	Brazil	Rondonia	Homo sapiens	1991	MG747605	MG747606	MG747607
057/2016	Ecuador	-	Homo sapiens	2016	MK506818	MK506823	MK506828
Haiti-1/2014	Haiti	-	Homo sapiens	2014	MN264269	MN264268	MN264267
Bel90435/H853382	Brazil	Pará	Homo sapiens	2018	MT879230	MT879229	MT879228
ZDC388	Brazil	Rondonia	Homo sapiens	2023	PP153945	PP153946	PP153947
0628MJG	Brazil	Roraima	Homo sapiens	2022	PP153981	PP153982	PP153983
0026TSS	Brazil	Roraima	Homo sapiens	2023	PP154011	PP154012	PP154013
3896ERA	Brazil	Amazonas	Homo sapiens	2023	PP154038	PP154042	PP154043
0545	Brazil	Acre	Homo sapiens	2023	PP154152	PP154153	PP154154
ILMD_TF29	Brazil	Amazonas	Homo sapiens	2015	PP154170	PP154171	PP154172
BeH505764	Brazil	Rondonia	Homo sapiens	1991	PP357049	PP357049	PP357048
BeAn 19991	Brazil	Pará	Bradypus tridactylus	1955	KP052852	KP052851	KP052850
BeH 532314	Brazil	Pará	Homo sapiens	1994	MG747559	MG747558	MG747557
BeH 498913	Brazil	Rondonia	Homo sapiens	1990	MG747602	MG747603	MG747604
BeH 390242	Brazil	Amapá	Homo sapiens	1980	MG747509	MG747510	MG74751
BeH 385591	Brazil	Pará	Homo sapiens		MG747554	MG747555	MG747556
BeAr 19886	Brazil	Pará	Ochlerotatus serratus	1960	MG747524	MG7475215	MG747526
BeAn 423380	Brazil	Pará	Nasua nasua	1984	NC_043576	NC_043577	NC_043578

Table S5. Primers and probes used for the detection of arboviruses in this study.

Virus	Sequences (5'→3')	Primers and probes	Target	Genome position	Ref.
OROV	TCCGGAGGCAGCATATGTG	Forward	S	98-116	[3]
	ACAACACCAGCATTGAGCACTT	Reverse	S	160-139	
	ATTTGAAGCTAGATACGG	Probe		118-136	
	TACCCAGATGCGATCACCAA	Forward	S	356-375	[2]
	TTGCGTCACCATCATTCCAA	Reverse	S	437-456	
	TGCCTTTGGCTGAGGTAAAGGGCT	Probe		409-433	
	TCGTCAACAAAACTCAACCACTTT	Forward	M	428-451	[2]
	GACCACAATTTACGGTTACATGCT	Reverse	M	525-548	
	TCGGGACAACTTCGACATCAGGCTG	Probe		459-483	
DENV1	GACACCACACCCTTTGGACAA	Forward	NS5	8586-8606	[6]
	CACCTGGGCTGTCACCTCCAT	Reverse	NS5	8692-8673	
	AGAGGGTGTTTAAAGAGAAAGTTGACACGCG	Probe		8608-8638	
DENV2	CAGGTTATGGCACTGTCACGAT	Forward	M	1605	[5]
	CCATCTGCAGCAACACCATCTC	Reverse	M	1583	
	CTCCGAGAACAGGCCTCGACTTCAA	Probe		1008	
DENV3	GGGAAAACCGTCTATCAATA	Forward	C	118-221	[6]
	CGCCATAACCAATTTCATTGG	Reverse	C	241-221	
	CACAGTTGGCGAAGAGATCTCAAGAGGA	Probe		174-202	
DENV4	TGAAGAGATTCTCAACCGGAC	Forward	C	187-207	[6]
	AATCCCTGCTGTTGGTGGC	Reverse	C	293-275	

	TCATCACGTTTTTGCGAGTCCTTTCCA	Probe		247-273	
CHIKV	AAAGGGCAAACTCAGCTTCAC	Forward	NSP1	874-894	[4]
	GCCTGGGCTCATCGTTATTC	Reverse	NSP1	961-942	
	CGCTGTGATACAGTGGTTTCGTGTG	Probe		899-923	
MAYV	AAGCTCTTCCTCTGCATTGC	Forward	NSP1	51-70	[7]
	TGCTGGAAACGCTCTCTGTA	Reverse1	NSP1	141-160	
	TGCTGGAAATGCTCTTTGTA	Reverse2		141-160	
	GCCGAGAGCCCGTTTTTAAAATCA	Probe		116-140	

Legend: OROV, Oropouche virus. DENV, dengue virus. CHIKV, chikungunya virus. MAYV, Mayaro virus. S, S segment. M, M segment. NS5, non-structural protein 5. M, matrix protein. C, capsid protein. NSP1, non-structural protein 1.

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