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Viral Exploration of Negative Acute Febrile Cases Observed during Chikungunya Outbreaks in Gabon

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Keywords
Gabon · Virus isolation · Emerging viruses · Next-generation sequencing · Chikungunya virus

Abstract
Non-malarial febrile illness outbreaks were documented in 2007 and 2010 in Gabon. After investigation, these outbreaks were attributed to the chikungunya and dengue viruses (CHIKV and DENV). However, for more than half of the samples analyzed, the causative agent was not identified. Given the geographical and ecological position of Gabon, where there is a great animal and microbial diversity, the circulation of other emerging viruses was suspected in these samples lacking aetiology. A total of 436 undiagnosed samples, collected between 2007 and 2013, and originating from 14 urban, suburban, and rural Gabonese locations were selected. These samples were used for viral isolation on newborn mice and VERO cells. In samples with signs of viral replication, cell supernatants and brain suspensions were used to extract nucleic acids and perform real-time RT-PCR targeting specific arboviruses, i.e., CHIKV, DENV, yellow fever, Rift Valley fever, and West Nile and Zika viruses. Virus isolation was conclusive for 43 samples either on newborn mice or by cell culture. Virus identification by RT-PCR led to the identification of CHIKV in 37 isolates. A total of 18 complete genomes and 19 partial sequences containing the E2 and E1 genes of CHIKV were sequenced using next-generation sequencing technology or the Sanger method. Phylogenetic analysis of the complete genomes showed that all the sequences belong to the East Central South Africa lineage. Furthermore, we identified 2 distinct clusters. The first cluster was made up of sequences from the western part of Gabon, whereas the second cluster was made up of sequences from the southern regions, reflecting the way CHIKV spread across the country following its initial introduction in 2007. Similar results were obtained when analyzing the CHIKV genes of the E2 and E1 structural proteins. Moreover, study of the mutations found in the E2 and E1 structural proteins revealed the presence of several mutations that facilitate the adaptation to the *Aedes albopictus* mosquito, such as E2 I211T and E1 A226V, in all the Gabonese CHIKV strains. Finally, sequencing of 6 additional viral isolates failed to lead to any conclusive identification.

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Introduction

In recent years, emerging viral diseases (EVDs) are widely relayed to the public, frightening populations, and overwhelming mainstream media worldwide. The current extension of the Zika virus to Latin American countries and the Pacific islands, and its new epidemiological features [1–4] have fostered increased scientific interest in emerging viruses. An emerging infectious disease can be defined as an infection that has newly appeared in a population or has existed previously but is rapidly increasing in incidence or geographic range [5]. Examples of EVDs include the Middle East respiratory syndrome coronavirus (MERS-coV), which was first identified in 2012 [6], or the invasion of the chikungunya virus (CHIKV) in the Americas [7].

Emerging infectious diseases impose a significant burden on global economies and public health in general. The emergence of infectious diseases is driven by numerous factors inherent to the microbial agent, the human host, and the environment [8, 9]. Due to the multifactorial nature of these drivers, it is difficult, at best, to predict which pathogens will develop in human or animal populations, or both [8]. RNA viruses, in particular, can evolve (and adapt) quickly because their genome-replicating RNA polymerases have high error rates. Therefore, unsurprisingly, several recent prominent examples of emerging or re-emerging diseases have been caused by RNA viruses during these last decades.

Sub-Saharan Africa is known to be endemic to various EVDs. For instance, sero-surveillance studies have demonstrated the circulation of some viruses of major public health interest which are known to be circulating in Africa [10–15]. These EVDs cause illnesses that range from mild febrile syndromes to more serious life-threatening conditions affecting the nervous or circulatory system, accompanied by haemorrhage. Most of these viruses are zoonotic pathogens and some can be transmitted to or between vertebrate hosts through hematophagous arthropods. Once confined to specific geographical areas, several of these viruses have spread beyond their historically endemic regions to become major threats for public health worldwide [7, 16]. Sub-Saharan Africa is likely the site of origin of many of these viruses: the human immunodeficiency virus (HIV), Ebola virus, Marburg virus, and Zika virus (ZIKV) emerged years ago. Although some EVDs are of minor significance, others represent serious afflictions for humans, livestock, and wild animals. The unprecedented explosive epidemics of the Ebola virus in West Africa [17] and Central Africa [18–20], yellow fever in Angola [21, 22] and ZIKV in Brazil [2] highlight the threat posed by these viruses.

Until the last century, no large-scale epidemic activity of CHIKV has been reported in Africa, probably due to herd immunity [23]. However, an outbreak hit coastal Kenya in 2004, subsequently invading Indian Ocean islands and uncovering a new lineage, the so-called Indian Ocean lineage (IOL). This new lineage spread across Indian Ocean islands, Asia, and many African countries, with some changes in its epidemiological patterns [23, 24]. CHIKV reached Gabon in 2007, having originated from neighbouring Cameroon [25, 26]. Epidemics were initially concentrated in the north-western regions of the country in 2007, and then in the south-eastern regions in 2010, following a north-west to south-east route. During this period, >20,000 suspected cases were identified [27]. In about 4,000 *Plasmodium falciparum*-free samples, 9 and 36% were positive for dengue virus (DENV) and CHIKV using specific qRT-PCRs, respectively [28]. However, the aetiology of 52% of cases could not be identified.

The aim of this study was to assess the potential circulation of other vector-borne viruses in the Gabonese population during these 2 CHIKV outbreaks. Virus isolation has been historically used and remains the gold standard for virus identification, so we attempted this approach either on newborn mice or on cell culture. For all viral isolates obtained, the virus was identified by specific real-time RT-PCR and next-generation sequencing (NGS). Indeed, NGS has been widely used for virus surveys [29] because both known and unknown viruses can be characterised by this method [30–32].

Materials and Methods

Patients

The serum samples used in this study were randomly selected from those collected between 2007 and 2013 as part of an active surveillance program of acute febrile syndromes. Inclusion criteria were fever (a temperature >38.5 °C) and at least 2 of the following symptoms: arthralgia, myalgia, headache, rash, asthenia, nausea, vomiting, diarrhoea, jaundice, or bleeding. In addition, thick and thin blood smears were negative for *P. falciparum*, the causative agent of malaria. We randomly selected 436 samples originating from 14 urban, suburban, and rural locations in 7 of the 9 Gabonese provinces (Fig. 1). These samples had all previously tested negative for CHIKV, ZIKV, yellow fever virus (YFV), West Nile virus (WNV), Rift Valley fever virus (RVFV), and the 4 serotypes of DENV based on a qRT-PCR assay [28].

Virus Isolation

Selected serum samples were first used to inoculate newborn mice (24–72 h old). Thus, 0.2 mL of a 1:10 diluted PBS serum was intracranially inoculated in all pups in a litter of 8 newborn mice.

Fig. 1. The Republic of Gabon and its 9 provinces. Except for the Nyanga and Oogoué-Ivindo provinces (in white), samples were collected in all other 7 provinces (in grey). Numbers of positive samples out of the total of samples collected appear in parentheses. LBV, Libreville; LAMB, Lambaréné; LTV, Lastourville; KLT, Koulamoutou; HO, Haut Oogoué; FCV, Franceville.

The mice were monitored daily for 7 days to record any morbidity or mortality. Thereafter, they were sacrificed when clinical symptoms or death were observed; specimens were then frozen and thawed once. The brain was harvested, ground, suspended in 1× PBS pH 7.4, and then used to inoculate another litter of suckling mice for virus propagation. Concurrently, serum samples, 1:10 diluted in PBS (1×, pH 7.4) and filtered (Millipore, 0.22 µm), were used to infect confluent fresh VERO E6 cells culture in Dulbecco's modified Eagle's medium (Gibco®, Life Technologies) supplemented with 2% FCS (Gibco) and a 1% v/v antimicrobial solution (10,000 units/mL of penicillin, 10,000 µg/mL of streptomycin and 25 µg/mL of amphotericin B, Gibco). The cell cultures were then screened for 7 days for cytopathic effects using an optical inverted microscope. Once a cytopathic effect was noticed or on day 7 post-infection, the supernatants were harvested and used for a second passage on VERO E6 cells [32]. The cell culture supernatant and brain suspensions were stored at –80 °C.

Nucleic Acid Extraction and Real-Time RT-PCR

Total RNA was extracted from the brain suspensions and cell culture supernatants using the QiAamp® Viral RNA mini kit (Qiagen, Germany) as per the manufacturer's protocol. Total RNA was

quantified by fluorescence using the Qubit RNA HS assay (Invitrogen, Life Technologies, USA). The first-strand cDNA was generated using random hexamers and M-MLV reverse transcriptase (Invitrogen). The cDNA samples were then tested by quantitative real-time RT-PCR (qRT-PCR) targeting several arboviruses including CHIKV, the 4 serotypes of DENV, ZIKV, RVFV, YFV, and WNV using published primers and probes [33–37]. The reaction was carried out in a volume of 25 µL containing 5 µL of template cDNA, 400 nM each of the forward and reverse primers and probe, and 12.5 µL ready-to-use TaqMan universal PCR master mix 2× buffer (Applied Biosystems, USA) on the 7500 fast real-time PCR system thermocycler. PCR cycling conditions were as follows: 50 °C for 2 min, activation of the Taq polymerase and template denaturation at 95 °C for 10 min, and amplification of the target cDNA for 45 cycles, repeating the denaturation of DNA at 95 °C for 15 s and the combined annealing and elongation at 60 °C for 60 s (except for ZIKV, YFV, and RVFV, for which the annealing temperature was 57 °C).

Sanger Sequencing of CHIKV E1 and E2 Genes and the Whole CHIKV Genome

Extracted total RNA was reverse-transcribed and amplified in a single tube with SuperScript III One Step RT-PCR system with Platinum Taq DNA polymerase (Invitrogen, USA) and specific primers targeting different regions of the CHIKV genome [38]. Briefly, 2 µL of RNA was amplified in 25 µL of reaction mix containing 400 nM of each forward and reverse primer. Reverse transcription was done at 50 °C for 30 min, followed by denaturation at 94 °C for 2 min. The amplification steps were done during 45 cycles of 94 °C for 15 s, 55 °C for 45 s, and 68 °C for 1 min. The annealing temperature was specific to the primer set employed. The final amplification was done at 68 °C for 5 min. After visualization on 1.5% w/v agarose gel, enzymatic purification was carried out on PCR products and these were sequenced using the Big Dye terminator kit (Applied Biosystems, USA).

Sequencing of Whole Viral Genomes with a High-Throughput Sequencing Approach

The DNA libraries were prepared from total RNA using the NEBNext Ultra RNA Library Prep kit for Illumina (New England BioLabs, UK) as per the manufacturer's instructions. In brief, RNAs were quantified and thermally fragmented. Then, first- and second-strand cDNAs were constructed with ProtoScript II reverse transcriptase and large-fragment DNA polymerase, respectively. After end repair and adaptor ligation, size selection purification was carried out using Agencourt AMpure XP beads (Beckmann Coulter, The Netherlands) to obtain inserts of about 300–400 bp (total library size 400–500 bp). Library amplification was then performed using the i5 and i7 index primers from NEBNext Multiplex Oligos for Illumina (New England BioLabs). The final library quality and size were assessed on an Agilent BioAnalyser 2100 (Agilent Technologies, USA) and run on the Illumina Miseq Instrument using MiSeq V2 Reagents to obtain 2 × 150 bp paired-end reads. The quality of Illumina Miseq output raw data was checked using the FastQC tool (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Reads with a Phred score <30, ambiguous bases, and small reads were removed from the dataset. The trimmed reads were mapped to a reference sequence using Bowtie2 software [39]. Depending on the reference used, mapped or unmapped reads were assembled using SPAdes Genome Assembler v3.11 [40]. The taxonomic assignment of the resulting contig was performed using BLASTn (https://blast.ncbi.nlm.nih.gov/Blast.cgi#).

Phylogenetic Analysis

Freshly generated CHIKV genomic sequences as well as additional CHIKV sequences from other countries representing all known genotypes available in GenBank and the O'nyong nyong virus strain SG650 were aligned using the ClustalW algorithm and manually edited. The Bayesian Markov chain method in MrBayes (v3.2) was used to construct the phylogenetic trees using 2 runs of 4 chains with 1 million generations, with a burn-in rate of 25% and the GTR + G + I nucleotide substitution model [41]. Reliability of the analysis was evaluated using a bootstrap test with 1,000 iterations. Phylogenetic analysis was performed using Geneious R11 v11.04 (Biomatters).

Ethical Considerations

The samples used in this study were collected as part of a febrile illnesses surveillance network established in 2007 in a partnership involving the Gabonese Ministry of Health (MoH) and the Centre International de Recherches Médicales de Franceville (CIRMF). In the absence of an ethics committee in Gabon at this time, the study was authorized by the Gabonese MoH (authorization No. 000006 DRSSE) and approved by an institutional review board (the Scientific Advisory Board of the CIRMF). Moreover, written informed consent was obtained from all Libreville patients included in this study. However, in the context of the urgent need to collect samples during outbreaks and due to the technical difficulties of obtaining written informed consent, and in agreement with the regional health authorities (authorization No. 000189 MSP/SG), only verbal consent was obtained for the collection of samples from patients outside the city of Libreville. Viral isolation on mice was authorized by the Institut Pasteur de Bangui which has an animal house dedicated to this purpose. All the experiments were conducted in a way that minimized animal suffering as recommended when using laboratory animals.

Nucleotide Sequence Accession Numbers

All sequences are available in the DDBJ/EMBL/GenBank database under accession numbers (submissions in progress).

Results

Viral Isolation and Quantitative RT-PCR

A total of 436 serum samples collected between 2007 and 2013 were analysed in this study. Viral isolates were obtained from 43 samples (9.86%) on suckling mice and/or cell culture. Cytopathic effects were observed predominantly on day 3 post-infection on cell culture and suckling mice (Table 1). The qRT-PCR analysis on the cell culture supernatants or brain suspensions led to the identification of CHIKV in 37 isolates (86.00%). None of the other tested viruses (YFV, WNV, ZIKV, RVFV, and DENV) were detected in any sample; and no virus could be identified for the remaining 6 virus-positive isolates

Table 1. Positive viral isolates by location and by year

Location	Year	Sample	Age	Sex	Post-infection day of isolation		CHIKV cycle threshold	
					VERO cells	mice	isolate	primary serum[b]
LBV	2007	Libreville_2007_17	35	M	D3	D3	17.3	n.d.
LBV	2007	Libreville_2007_23	31	F	D3	D3	24.3	n.d.
LBV	2007	Libreville_2007_05	27	F	D3	D3	13.76	28
LBV	2007	Libreville_2007_07		F	D5	D2	21.85	28
LBV	2007	Libreville_2007_49	29	F	D5	D5	13.97[a]	n.d.
LBV	2007	Libreville_2007_47	37	F	D5	D3	25.85	n.d.
LBV	2007	Libreville_2007_128	34	F	D5	D3	12.35[a]	26
LBV	2007	Libreville_2007_130	4	F	D5	D3	n.d.	
LBV	2007	Libreville_2007_231	–	F	D5	D3	10.9	n.d.
LBV	2007	Libreville_2007_25	13	M	D4	D4	13.24[a]	n.d.
LBV	2007	Libreville_2007_42	50	F	D4	D4	13.42[a]	30
LBV	2007	Libreville_2007_105	33	F	D4	D3	11.22	n.d.
LBV	2007	Libreville_2007_232	–	M	D5	D2	12.89[a]	n.d.
LBV	2007	Libreville_2007_40	46	F	D4	D2	12.52[a]	n.d.
LBV	2007	Libreville_2007_64	–	M	D3	D2	12.67	n.d.
LBV	2007	Libreville_2007_233	–	F	D3	D2	12.05[a]	n.d.
LBV	2007	Libreville_2007_68	–	M	D3	D2	12.72[a]	n.d.
LBV	2007	Libreville_2007_82	47	F	D5	D5	10.95	n.d.
LBV	2007	Libreville_2007_106	35	F	D3	D2	14.07[a]	n.d.
LBV	2007	Libreville_2007_126	55	F	D3	D2	13.24[a]	n.d.
LBV	2007	Libreville_2007_072	17	F	D3	D5	12.45[a]	n.d.
LBV	2007	Libreville_2007_91	20	F	D5	D5	13.70	n.d.
LBV	2007	Libreville_2007_03	40	F	D4		14.11	n.d.
LBV	2007	Libreville_2007_65	43	M	D5		11.91	n.d.
LBV	2007	Libreville_2007_127	26	F	D3		12.73	n.d.
KANGO	2007	Kango_2007_89	–	F	D5	D3	13.61	n.d.
LTV	2007	LTV_2007_235	60	F	D3	D3	12.4	23
NDJOLE	2008	Ndjolé_2008_14	19	M	D3	D3	15.53	n.d.
LBV	2010	Libreville_2010_10	14	F	D3		14.33	n.d.
KLT	2010	KLT_2010_395	10	F	D2		12.89	n.d.
KLT	2010	KLT_2010_21	4	F	D2		12.93	m.d.
KLT	2010	KLT_2010_30	37	F	D2		12.72	m.d.
KLT	2010	KLT_2010_25	50	M	D2		12.51	27
KLT	2010	KLT_2010_27	1	M	D2		13.05	m.d.
LTV	2010	LTV_2010_10	–	F	D2	D3	13.17[a]	23
NB	2010	Ndakaba_2010_8	72	M	D5	D3	14.31	m.d.
NB	2010	Ndakaba_2010_3	3	F	D5	D3	15.15	n.d.
NB	2010	Ndakaba_2010_7	39	F	D5	D3	14.27	n.d.
KLT	2010	KLT_2010_12	6	F	–	D2	n.d.	
LAMB	2010	LAMB_2010_6469	56	F	–	D2	n.d.	
KLT	2010	KLT_2010_302	23	M	–	D2	n.d.	
KLT	2010	KLT_2010_36	1	F	–	D4	n.d.	
KLT	2010	KLT_2010_16	4	F	–	D3	n.d.	

CHIKV qRT-PCR was performed using 2 different systems. Cycle thresholds shown here are for samples extracted from brain suspensions or from VERO cell supernatants. D, post-infection day; CHIKV, chikungunya virus; KLT, Koulamoutou; NB, Ndakaba Balandja; LAMB, Lambaréné; LTV, Lastourville; n.d., not determined; m.d., missing data (samples were not tested because of lack of serum).

[a] Results obtained with the CDC protocol [56]. [b] qRT-PCR was done only for isolates which were positive to CHIKV after virus isolation.

Table 2. Amino acid changes identified in the partial sequences of the structural polyprotein in Gabonese chikungunya virus isolates relative to the S27 prototype strain

	Structural protein																								
	E3	E2															6K		E1						
Nucleotide position[a]	284	382	399	404	443	474	485	489	506	519	536	547	571	592	624	669	740	802	808	818	846	1,035	1,059	1,078	1,131
Amino acid position[a]	23	57	74	79	118	149	160	164	181	194	211	222	216	267	299	344	415	54	60	9	37	226	250	269	322
Tanzania/S27	I	G	I	G	S	K	N	A	L	S	I	V	A	M	S	A	I	I	S	N	T	A	S	M	V
GABOPY1	T	K	T	E	G	R	T	T	M	G	T	I	A	R	N	T	L	V	N	S	I	V	P	V	A
BRAZZA_MRS1	T	K	T	E	G	R	T	T	M	G	T	I	D	R	N	T	L	V	N	S	I	V	P	V	A
New Gabonese isolates	T	K	T	E	G	R	T	T	M	G	T	T	D	R	N	T	L	V	N	S	I	V	P	V	A

Accession Nos.: Tanzania/S27 NC_004162.2; GABOPY1 KP 003812.1; BRAZZA_MRS1 KP003813.2. [a] Based on S27 numbering.

(Table 1). CHIKV isolates obtained through viral isolation came from samples collected in 2007, 2008, and 2010 in the Estuaire, Moyen-Oogoué, and Oogoué-Lolo provinces. Viral isolation attempts on samples from the Oogoué-Maritime, Ngounié, Haut-Oogoué, and Woleu-Ntem provinces gave negative results. Unidentified viral isolates were obtained from 1 sample collected in Libreville in 2007, 1 from Lambaréné in 2010, and 4 from Koulamoutou in 2010, from the Estuaire, Moyen-Oogoué, and Oogoué-Lolo provinces, respectively. Because all initial samples were assumed to be CHIKV-negative by qRT-PCR, total RNAs were extracted from initial serum samples and screened once more. These initial samples remained negative, except for 7 samples showing a cycle threshold (Ct) of 23–30 (Table 2). Finally, the mean age of the patients from whom samples were collected was 28 years (range: 4 months to 82 years) with a male-to-female sex ratio of 0.65. Viruses were isolated predominantly from samples collected from women (75.00%); this frequency is consistent with the samples' sex ratio.

Phylogenetic Analyses

Chikungunya whole-genome sequencing was performed on 18 samples obtained through virus isolation either on newborn mice or cell culture (Table 1) and 5 serum samples (online suppl. Table S1; for all online suppl. material, see www.karger.com/doi/10.159/000495136). All Gabonese isolates belonged to the East Central South Africa (ECSA) lineage of CHIKV. The obtained genome sequences were distributed across years, with 11 from the 2007 epidemics (7 of which were from Libreville, including the previously published strain GABOPY1 [accession No. KP003812.1]), 1 from 2008, and 1 from 2009 (Fig. 2). The remaining 10 sequences were obtained in 2010, with only 1 from Libreville. Except for the sequence isolated in 2007 in Minvoul, all the other Gabonese strains were different from the previously identified 2006 Cameroonian strain [25, 26]. The town of Minvoul is located near the border between Gabon and Cameroon. The sequence obtained in this town was closer to the Cameroonian strain than to other Gabonese strains. The 7 sequences from 2007 coming from Libreville were all different (Fig. 2), either found at the root of all other Gabonese sequences or appearing to be the origin of 2 subgroups. The first subgroup appears to indicate the spread of this variant (LBV 2007-82) from Libreville towards eastern Gabon from the Estuaire province to the Oogoué-Lolo province, via the Moyen-Oogoué province (Fig. 1). The second subgroup appears to show the spread between 2007 and 2010 towards the Haut-Oogoué province, located in south-east

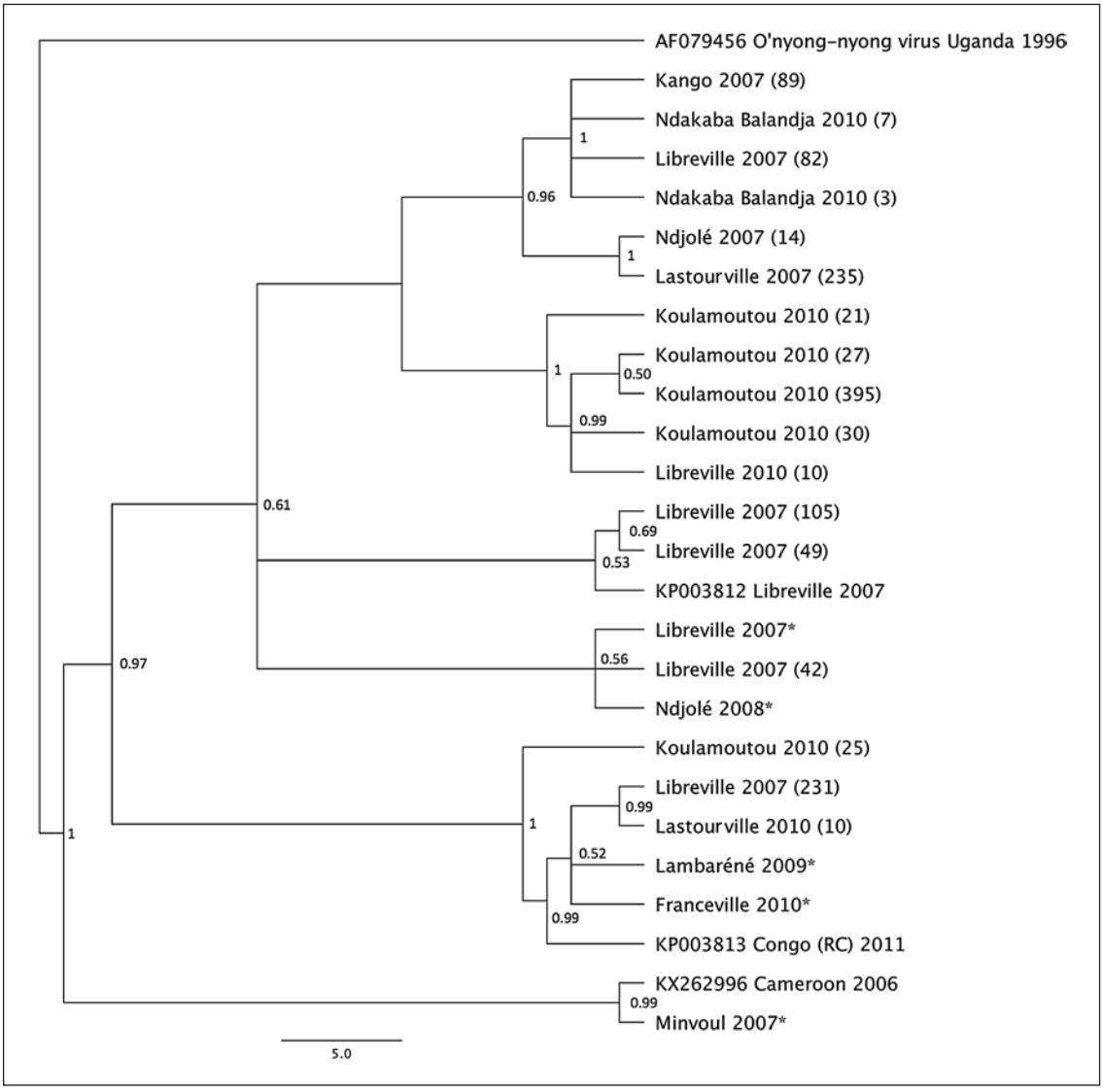

Fig. 2. Phylogenetic tree based on the whole-genome sequences of the chikungunya virus (CHIKV). Asterisks denote the sequences diagnosed CHIKV-positive after re-testing the serum sample using RT-PCR; all other sequences (n = 18) from this study were obtained after inoculations of mice or cells with serum samples that were initially declared CHIKV-negative.

Gabon. This subgroup contained sequences from the cities of Libreville (2007), Lastourville (2010), Koulamoutou (2010), and Franceville (2010). However, the Koulamoutou 2010 sequence was different from the others isolated in the same year in the same geographical area, illustrating that several variants of the virus can circulate simultaneously in any one city of Gabon. Finally, the CHIKV sequence isolated in the Republic of the Congo in 2011 (retrieved from GenBank) also clustered in this second subgroup (Fig. 2).

In addition to sequencing these whole genomes, we sequenced the 3′end of the chikungunya genome, including the envelope glycoprotein E1 and E2 genes for the 19 remaining isolated viruses. Phylogenetic analysis of these 3,200 bp sequences confirmed the results obtained using the whole CHIKV genomes. Sequences clustered either in the 2 above-described subgroups or were positioned at the root of the Gabonese isolates. These additional sequences confirmed the high genetic diversity of the isolates identified in Libreville in 2007 (data not shown).

Table 3. Amino acid changes identified in the partial sequences of the structural polyprotein in Gabonese chikungunya virus isolates relative to the S27 prototype strain

	Structural protein																								
	E3	E2																6K		E1					
Nucleotide position[a]	284	382	399	404	443	474	485	489	506	519	536	547	571	592	624	669	740	802	808	818	846	1,035	1,059	1,078	1,131
Amino acid position[a]	23	57	74	79	118	149	160	164	181	194	211	222	216	267	299	344	415	54	60	9	37	226	250	269	322
Tanzania/S27	I	G	I	G	S	K	N	A	L	S	I	V	A	M	S	A	I	I	S	N	T	A	S	M	V
Gabonese isolates	T	K	T	E	G	R	T	T	M	G	T	T	D	R	N	T	L	V	N	S	I	V	P	V	A

Accession numbers of the isolates are S27/NC_004162.2. [a] Based on S27 numbering.

Furthermore, the predicted amino acid sequences were aligned to follow CHIKV microevolution over time relative to the African prototype S27 strain (accession No. NC_004162.2), used as a reference. Most of the mutations observed in our virus isolates were in other Central African strains such as Congolese BRAZZA_MRS1 and Gabonese GABOPY1 (accession No. KP 003813.2 and KP 003812.1), which were obtained without any virus amplification in a living system. Regarding these structural proteins, 16 and 6 amino acid changes were present in the predicted E2 and E1 proteins, respectively (Table 3), in all the described Gabonese isolates. These mutations are common mutations present in recently isolated CHIKV strains within the ESCA genotype. The E1 A226V mutation associated with adaptation to and transmission by the tiger mosquito *A. albopictus* was present in all the isolates as previously described.

Discussion

The aim of this study was to identify the potential circulation of other vector-borne viruses that were circulating in Gabonese population simultaneously to the chikungunya outbreaks. In this study, we therefore targeted samples from febrile patients already screened for several pathogens. Accordingly, *Plasmodium*-, CHIKV- and DENV-positive samples were excluded from this study. The selected samples were used to isolate potentially emerging viruses, resulting in 43 virus-positive samples, 37 of which turned out to be positive for CHIKV. The remaining 6 samples harboured unknown, yet-to-be-identified viruses.

Here, all samples used were allegedly CHIKV-negative as reported by Caron et al. [28]. However, the identification in our study of 37 CHIKV strains including 7 that could have been diagnosed early can be attributed to diagnostic failure arising from a variation in the primer- and probe-targeted sites, or, alternatively, to a low initial viral load in the allegedly negative samples. The hypothesis of the variation in the primer and probe targets was tested by aligning the obtained sequences with those of the primers and the probe. No variation or modifications were observed. However, although no mutations were detected, this does not exclude the possibility that there were mutations in the initial samples. For instance, it has been demonstrated in the CHIKV Asian lineage that the overall diversity of the CHIKV population drops dramatically after a single passage in cell lines [42].

Overall, our data tends to support the hypothesis of low viral loads. In the case of very low viral loads, the virus may escape detection by real-time RT-PCR using standard amplification cycles. Clinical signs of chikungunya infection appear 2–6 days post-infection. It is generally accepted that the viremic phase lasts for 7–12 days after the onset of symptoms, but high viremia is more likely to be detected in newborn and elderly chikungunya patients [43]. Furthermore, 1 study showed that in a sample of 48 CHIKV-positive cases, only 86% could be detected by RT-PCR using blood collected within the first week after the onset of symptoms [44]. Therefore, samples must be collected as soon as possible, and the age of patient can impede CHIKV diagnosis, especially in samples collected long after onset. Therefore, knowing that the mean age for CHIKV-positive patient in this study was 31 years, it is possible than the samples from which we isolated CHIKV were collected after the viremic peak. In Africa, a febrile person usually does not seek medical care straightaway, preferring to begin a self-medication treatment [45]. Only after this medication has failed does the patient seek medical help. By this time, the virus is probably low in concentration. Moreover, there is increasing evidence that arboviruses persist longer in urine than in blood [46, 47], which is further supported by the presence of ZIKV

in urine almost 21 days after the onset of symptoms [48, 49]. However, for the CHIKV positives detected after inoculation, no other type of sample would have given better results, because blood remains the sample matrix of choice for CHIKV detection [44]. Finally, only 7 CHIKV-positive samples were retrospectively detected. This can be explained by the amount of sample that must be simultaneously and analysed daily during outbreaks and was most likely due to human error. Unfortunately, detection of the virus is critical for the containment of a disease, particularly for communicable infections.

All the CHIKV nucleotide sequences obtained in this study belonged to the ECSA lineage. This is in agreement with previous studies in Gabon and its neighbouring countries where this genotype has already been described [25, 26]. Moreover, the sequences obtained via virus isolation on negative serum samples were not significantly different from those obtained directly from serum samples. Although the number of isolates identified in Libreville was greater than that of other cities in Gabon, they all clustered either in the 2 previously described subgroups or were found at the root of all Gabonese isolates, illustrating high genetic diversity in the Libreville isolates. Moreover, our data confirms that the propagation of CHIKV strains through Gabon spread from Libreville in successive waves from different strains that subsequently evolved over time [28]. Given the large population movements between countries in this African sub-region, CHIKV strains regularly cross borders; therefore, unsurprisingly, the CHIKV strain from the Republic of Congo (KP0038131), a neighbouring country, clustered with our Gabonese isolates, especially that of Franceville in 2010. This strain therefore likely originated from Gabon, particularly as the first outbreak of CHIKV in Republic of Congo was reported in 2011, almost 5 years after the initial Gabonese outbreak [26, 50].

The analysis of partial gene sequences of the structural polyprotein (encompassing E2 and E1) from 42 Gabonese isolates showed the presence of several mutations common to all isolates relative to the 1953 Tanzania S27 strain, although some mutations were present in just a few sequences. In protein E3, at position 23, parental isoleucine is replaced by threonine in all strains. This mutation has also been described in Indian Ocean strains [51]. Many substitutions in the E2 protein were detected (Table 3), accompanying a major adaptive mutation in E1. It has been demonstrated that the E1 A226V substitution enhances the transmission of CHIKV by *A. albopictus* by significantly accentuating virus dissemination through the salivary glands and secondary organs. The E2 I211T mutation is widely distributed among CHIKV isolates obtained after 2005 and has a synergistic effect on CHIKV infectivity for *A. albopictus* when expressed in combination with the E1 A226V substitution [52]. The constant presence of the E1 V322A mutation in all the isolates is consistent with the higher prevalence of *A. albopictus* in Gabon compared with *Aedes aegypti*. Moreover, this mosquito was identified as the main vector involved in the 2007–2010 epidemics in the country [28, 53–55]. All these amino acid differences reveal changes in the genomic diversity of CHIKV isolates.

Finally, deep sequencing of 6 viral isolates failed to lead to conclusive identification. These isolates were obtained only after inoculation in newborn mice, but not in VERO cell cultures (except 1, LBV 2007-130). These experiments were repeated on mice several times with the same results for all samples. Given that just a few viral particles can be responsible for the death of newborn mice, and that culture on VERO cells was not conducive to amplification, it is plausible that the viral load was not high enough to be detected using our NGS methodology. In fact, although RNA was quantified at the beginning of the assay, it does not give an indication of the actual amount of viral nucleic acids. Regardless of the library preparation methods used, the procedure was always followed by a loss in the number of viral genome copies (data not shown). Thus, if the starting material already contains a low amount of the nucleic acid of interest, it is highly probable that this cannot be ultimately recovered.

Conclusion

In this study, a viral isolate was obtained in only 9.8% (43/436) of the tested negatives, and CHIKV was identified in 86% (37/43) of these cases. All detected CHIKV samples obtained belonged to the ECSA genotype, and they carried the E1 A226V substitution which confers better transmissibility by *A. albopictus.* Unfortunately, we did not succeed in demonstrating the circulation of other emerging viruses, but 6 virus isolates remain to be identified. Finally, our study showed the importance of associating classic but robust viral isolation methods with various contemporary sequencing methods for the investigation of febrile syndromes for which the aetiology remains to be determined.

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Statement of Ethics

The study was authorized by the Gabonese MoH (authorization No. 000006 DRSSE) and approved by an institutional review board (the Scientific Advisory Board of the CIRMF). Written informed consent was obtained from all Libreville patients included in this study.

Disclosure Statement

No competing financial interests exist.

Author Contributions

E.N. and N.B. conceived and designed the study. H.S.T., R.S.O., and B.S. performed viral cultures and inoculation in newborn mice. H.S.T. and R.S.O. performed all the molecular biology assays. H.S.T., I.L., and A.A.N.-M. constructed all the libraries, and carried out the sequencing and bioinformatics analyses, respectively. All authors analysed the data. H.S.T. and N.B. wrote the paper. All authors read and approved the final manuscript.

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