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# Circulation of Chikungunya Virus in Gabon, 2006–2007

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This study reports the first isolation and partial genetic characterization of Chikungunya virus (CHIKV) from patients during a 2006–2007 dengue-like syndrome outbreak in Gabon. The isolated viruses were phylogenetically close to strains isolated in the Democratic Republic of the Congo 7 years ago and to strains isolated more recently in Cameroon. These results indicate a continuing circulation of a genetically stable CHIKV population during 7 years in Central Africa. ***J. Med. Virol. 80:430–433, 2008.***



**KEY WORDS:** alphavirus; arbovirus; Chikungunya virus; Dengue virus; Gabon

## INTRODUCTION

Chikungunya virus (CHIKV) has been identified as the cause of large outbreaks of febrile illness in Africa and Asia since the early 1950's [Ross, 1956]. Since the end of 2004, numerous countries faced the emergence or re-emergence of CHIKV [Bessaud et al., 2006; Schuffenecker et al., 2006; Yergolkar et al., 2006; Peyrefitte et al., 2007]. Recently recognized clinical and epidemiological features of CHIKV have generated extensive research programs in both developing and industrialized countries. Particular attention has been focused on the molecular epidemiology and genetic polymorphisms of the various viral strains [Pastorino et al., 2004; Bessaud et al., 2006; Schuffenecker et al., 2006; Peyrefitte et al., 2007].

Interesting data were reported recently in Asia where Indian Ocean emerging CHIKV strains spread to India in 2006 [Yergolkar et al., 2006] and created a zone where Asian and Indian Ocean isolates overlapped. However, the remergence of CHIKV in Malaysia provided evidence of co-circulation of different genotypes in the same geographic area [AbuBakar et al., 2007]. Recently, CHIKV was isolated from French soldiers in Douala Cameroon [Peyrefitte et al., 2007], which suggests that CHIKV is endemic in Central Africa.

Serological surveys conducted in Gabon during the late 1970's revealed a 20% of seroprevalence CHIKV exposure in the general population [Jan et al., 1978; Saluzzo et al., 1982].

To our knowledge, CHIKV reference sequences from Gabon are not available in open access database.

This study reports the first characterization of CHIKV strains isolated from the outset of the 2007 outbreak in Libreville, Gabon. An investigation for arboviruses revealed some evidence of co-circulation of CHIKV and Dengue virus (DENV) in late 2006 mimicking the Cameroonian episode that same year [Peyrefitte et al., 2007].

## MATERIALS AND METHODS

### Patients

From January 1st 2006 through May 2nd 2007, 35 patients were examined at the infirmary of the French camp in Gabon. All patients were members of the French military stationed at the camp or civilian employees supporting camp operations. They had dengue-like syndromes and intense joint pain. All patients lived in Libreville and had no history of travel

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outside of Gabon in the previous 2 months. Blood samples from the patients were sent to the French Military Tropical Medicine Institute (IMTSSA) in Marseille for arbovirus diagnosis.

### Serological Diagnosis and Virus Isolation

Samples were tested by ELISA for the presence of DENV, West Nile virus (WNV), and CHIKV antibodies [Peyrefitte et al., 2007] and tested by RT-PCR for the presence of DENV and CHIKV genomic RNA [Pastorino et al., 2005]. Virus isolation on C6/36 and Vero cells was attempted on positive RT-PCR samples as described previously [Peyrefitte et al., 2005].

### RNA Preparation, cDNA Synthesis, DNA Sequencing

Viral RNAs were extracted from 140 μL of infected cell supernatant using the High Pure Viral RNA kit (Roche Diagnostics, Meylan, France). Viral cDNAs were generated by reverse transcription using Superscript II reverse transcriptase (Invitrogen, Cergy Pontoise, France) according to the manufacturer's protocols. CHIKV-specific primers OP16 and OP17 [Pastorino et al., 2004] were used for PCR amplification using AmpliTaq DNA Gold (Applera SA, Courtabœuf, France). PCR products were separated on 1.5% agarose gels and the 1.2 kb band corresponding to the desired PCR product was purified using the QIAquick Gel extraction kit (Qiagen, Courtabœuf, France). The purified PCR product was sequenced using the OP16 and OP17 primers and the GenomeLab DTCS quick start sequencing kit (Beckman Coulter, Roissy, France). Sequencing was carried out using an automatic sequence analyzer (CEQ 8000, Beckman Coulter) following the manufacturer's protocol.

## RESULTS AND DISCUSSION

Since mid-April 2007, the incidence of febrile syndromes associated with arthralgia in both the general population of suburban Libreville and in the French military camp located nearby increased. The Gabonese Ministry of Public Health estimated the number of new cases around 300 per day [personal communication]. Thirty-five cases were investigated among military and civilian employees at the French military camp located near Libreville. Most of the patients consulted a physician within the first 7 days of symptom onset; this is most likely due to the rapid and intense onset of symptoms experienced by the individuals. The patient demographics are presented in Table I. All patients had fever >38.5°C, and most also experienced arthralgia, cephalalgia, and myalgia. Retro orbital pain, skin rash, and gastritis were reported in some cases; however, no neurological signs were recorded. Because malaria is endemic in Gabon, the patients were tested for *P. falciparum*; all the samples were found negative using the rapid detection Core Malaria (Core Diagnostics Ltd., Birmingham, UK).

The serological investigation revealed the presence of anti-CHIKV IgM in three samples (7559, 8541, 8877) and anti-CHIKV IgM and IgG in one patient (8659; Table I). A fourth sample (8497) was positive for anti-CHIKV IgG alone. Among all sera, seven samples were positive by TaqMan RT-PCR for CHIKV. All seven were culture positive for CHIKV when inoculated onto Vero E6 and C6/36 cell lines.

TABLE I. Characteristics of Patients Having Febrile Acute Dengue-Like Syndrome

| Serum no. | Sex/age (years) | Sampling date | Delay after symptoms onset (days) | IgM[b] | IgG[b] | RT-PCR |
|---|---|---|---|---|---|---|
| 7469 | M/28 | 30/08/2006 | <3 | Neg | Pos DENV | Neg |
| 7559 | M/31 | 04/10/2006 | >5 | Pos CHIKV | Neg | Neg |
| 8480 | M/37 | 30/11/2006 | >5 | Pos DENV | Neg | Neg |
| 8497 | M/47 | 08/12/2006 | >5 | Neg | Pos CHIKV | Neg |
| 8498 | M/26 | 05/12/2006 | >5 | Neg | Pos DENV | Neg |
| 8510 | M/33 | 14/12/2006 | <3 | Neg | Neg | Pos DENV |
| 8526 | M/45 | 03/01/2007 | >5 | Neg | Pos DENV | Neg |
| 8541 | M/21 | 19/01/2007 | >5 | Pos CHIKV | Neg | Neg |
| 8583 | M/40 | 09/02/2007 | <3 | Neg | Pos DENV | Neg |
| 8584 | M/45 | 09/02/2007 | >5 | Neg | Pos DENV | Neg |
| 8659 | M/28 | 09/03/2007 | 40 | Pos CHIKV | Pos CHIKV | Neg |
| 8660[a] | M/28 | 08/03/2007 | <3 | Neg | Neg | Pos CHIKV |
| 8833 | M/26 | 16/04/2007 | 38 | Neg | Pos DENV | Neg |
| 8847[a] | M/9 | 16/04/2007 | <3 | Neg | Neg | Pos CHIKV |
| 8848[a] | M/50 | 17/04/2007 | <3 | Neg | Neg | Pos CHIKV |
| 8849[a] | F/9 | 17/04/2007 | <3 | Neg | Neg | Pos CHIKV |
| 8856[a] | F/9 | 20/04/2007 | <5 | Neg | Neg | Pos CHIKV |
| 8875[a] | F/16 | 01/05/2007 | <5 | Neg | Neg | Pos CHIKV |
| 8876[a] | M/27 | 30/04/2007 | 6 | Neg | Neg | Pos CHIKV |
| 8877 | M/26 | 30/04/2007 | 7 | Pos CHIKV | Neg | Neg |
| 8878 | M/19 | 23/04/2007 | <5 | Neg | Pos DENV | Neg |

[a]CHIKV isolation successful.
[b]DENV, WNV, and CHIKV antibodies tested.

Evidence of primary dengue infection was found in two patients (8480, presence of anti-DENV IgM; 8510, DENV genomic detection by real time RT-PCR for DENV). Seven samples displayed anti-DENV IgG. None of these seven sera was found positive by real time RT-PCR for DENV. Secondary dengue infection could be suspected in this context but no definitive conclusion could be drawn in the absence of patient follow up. Most of the patients who tested positive for CHIKV (n = 7; Table I) developed symptoms from April 16th through April 24th 2007. However, two patients were observed in March and one in January 2007. As shown in Table I, the first positive patient for anti-CHIKV IgM was found in October 2006 whereas the number of RT-PCR positive patients for CHIKV significantly increased since mid-April. These results suggested a low level of CHIKV circulation in Gabon before the onset of the outbreak in late April 2007. The remaining patients whose samples were negative for CHIKV as well as DENV were diagnosed: "fever with unknown origin."

No attempt has been made to direct sequence the CHIKV RT-PCR product obtained from patient sera. Partial genetic characterization was performed on three of the seven CHIKV isolates recovered from patients by sequencing the E1-3′UTR junction (GenBank accession number EF613342, EF613343, EF613344). The 1.1 kb sequence did not feature any codon deletion or insertion when compared to other African CHIKV sequences available in the GenBank database [Powers et al., 2000; Pastorino et al., 2004; Bessaud et al., 2006; Schuffenecker et al., 2006; Yergolkar et al., 2006; Peyrefitte et al., 2007]. The three Gabon CHIKV partial genomic sequences displayed paired identity that ranged from 99.4 to 99.6% at the nucleotide level and from 99.7 to 99.9% at the deduced amino-acid level.

A high degree of identity was observed when the sequence data were compared to the Cameroonian sequence data obtained from isolated during 2006 [Peyrefitte et al., 2007] (paired identity ranging from 98.5 to 99% at the nucleotide level and from 99.3 to 99.6% at the amino-acid level). When the sequence data from our current isolates were compared to sequences obtained from strains isolated in the Democratic Republic of the Congo (DRC) in 2000 [Pastorino et al., 2004], a lower level of identity was found (paired identity ranged from 97.2 to 98.4% at the nucleotide level and 98.7 to 99.5% at the amino-acid level). The Gabon isolates showed a higher nucleotide divergence when compared with the 2006 Reunion island strains (paired identity ranging from 95.9 to 96.4%). The comparison of Gabon isolates with the short E1 Indian sequences [Yergolkar et al., 2006] showed a paired identity ranging from 94 to 98.6% at the nucleotide level. Collectively, the sequence data suggest that the 2006 Indian isolates are most divergent from Gabon CHIKV. Our results clearly demonstrate that the Gabonese epidemic originated from the emergence of a local strain and not from the westward spread of the Indian Ocean variant to Central Africa. The sequence identity among the West African isolates reinforces the hypothesis of a common origin and emphasizes the genetic stability of CHIKV despite the 7 years and geographic distance from the DRC outbreaks. As shown in the phylogenetic tree (Fig. 1), the CHIKV Gabon isolates clustered with the Cameroon CHIKV isolate and DRC CHIKV strains with a high bootstrap value of 97. The close genetic relationship suggests a continuous circulation of a closely related CHIKV population in Central Africa with a high degree of genetic stability.

However, Gabonese and Cameroonian isolates are significantly different from CHIKV from the Central African Republic and the 1982 Uganda isolate [Powers et al., 2000; Pastorino et al., 2004]. The cumulative genetic data obtained from Cameroon and Gabon

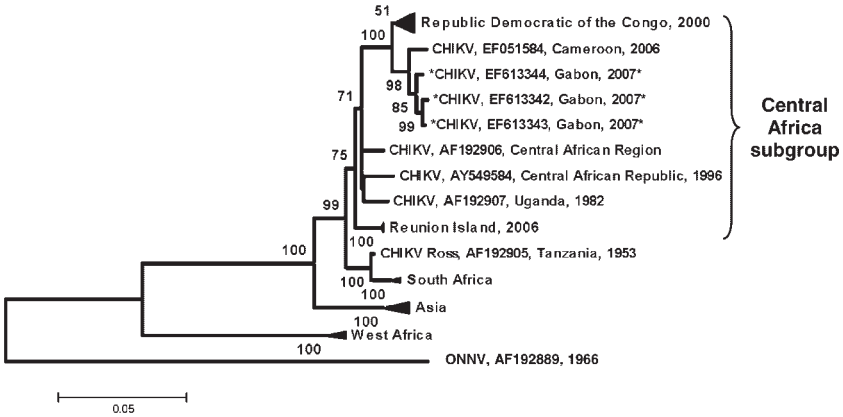


Fig. 1. Phylogenetic tree of CHIKV based on partial nucleotide sequences (3′ extremity of E1/3′-UTR). Phylogram was constructed with MEGA 2 program and tree drawing used the Jukes-Cantor algorithm for genetic distance determination and the Neighbor Joining method. The percentage of successful bootstrap replicates (500 bootstrap replications, confidence probability higher than 90%) is indicated at nodes. The length of branches is proportional to the number of nucleotide changes (% of divergence). Asterisk (*) indicates the strains isolated in this work. The dark triangles correspond to viruses clustering together. O'nyong-nyong virus (ONNV) sequence has been introduced for correct rooting of the tree. West Africa subgroup includes AF192891-93 CHIKV genomes, Asia AF192896-902 and L37661, South Africa AF192903-4, Reunion island DQ462746-50 and AM258990-95, Republic Democratic of the Congo AY549575-84.

isolates obtained in less than 1 year raised the question of the apparition of a new genetic variant of CHIKV in the Occidental part of Central Africa.

## CONCLUSIONS

Our 18-month survey of CHIKV in Gabon revealed the circulation of both CHIKV and DENV at low level since October 2006 followed by the mid-April CHIKV outbreak. Partial sequencing grouped the Gabonese CHIKV strains in the Central African cluster. The high level of genetic identity with DRC and Cameroon strains leads to the hypothesis that a new genetic variant of CHIKV exists in the western part of Central Africa.

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