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Genetic characterization of Chikungunya virus in the Central African Republic

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ABSTRACT

Chikungunya virus (CHIKV) is an alphavirus transmitted by the bite of mosquito vectors. Over the past 10 years, the virus has gained mutations that enhance its transmissibility by the *Aedes albopictus* vector, resulting in massive outbreaks in the Indian Ocean, Asia and Central Africa. Recent introduction of competent *A. albopictus* vectors into the Central African Republic (CAR) pose a threat of a Chikungunya fever (CHIKF) epidemic in this region. We undertook this study to assess the genetic diversity and background of CHIKV strains isolated in the CAR between 1975 and 1984 was studied to estimate the ability of local strains to adapt to *A. albopictus*. Our results suggest that, local CHIKV strains have a genetic background compatible with quick adaptation to *A. albopictus*, as previously observed in other Central African countries.

Intense surveillance of the human and vector populations is necessary to prevent or anticipate the emergence of a massive CHIKF epidemic in the CAR.

1. Introduction

Chikungunya virus (CHIKV), first isolated from human serum in 1953 during an epidemic in Tanzania (Robinson, 1955), is an arbovirus belonging to the genus *Alphavirus* (*Togaviridae* family). In humans, it is responsible for Chikungunya fever (CHIKF), an acute fever characterized by arthralgia and myalgia that can evolve into chronic arthropathy. CHIKV is present in tropical Africa and Asia, where it is transmitted to vertebrate hosts through the bites of mosquito vectors of the *Aedes* genus (Powers and Logue, 2007). Two distinct transmission ecological cycles have been documented. In an enzootic cycle, described in West Africa in forested habitats, sylvan mosquitoes, mainly *Aedes furcifer*, *Aedes taylori*, *Aedes africanus* and *Aedes luteocephalus*, serve as vectors (Diallo et al., 1999). Non-human primates are the most likely reservoirs and amplification hosts, and human cases have been found sporadically (Diallo et al., 1999). In Asia, CHIKV displays a mainly urban epidemic cycle essentially involving the *Aedes aegypti* and *Aedes albopictus* anthropophilic vectors, with humans as its only amplification host (Powers and Logue, 2007). In the recent past, CHIKF was described primarily in rural areas of sub-Saharan Africa and urban areas in Southeast Asia (Powers et al., 2000; Robinson, 1955; Thonnon et al., 1999). Since 2005, however, massive epidemics indicate emergence or re-emergence of the virus in the Indian Ocean, including the island of La Réunion (Paquet et al., 2006), in India (Ravi, 2006), in urban areas of Central Africa (Kelvin, 2011; Leroy et al., 2009; Peyrefitte et al., 2007), in the Caribbean (Fischer and Staples, 2014; Van Bortel et al., 2014), in South America (Albuquerque et al., 2014) and even in Europe (Depoortere and Coulombier, 2006; Depoortere et al., 2008). The major vector in almost all these outbreaks was *A. albopictus* except for some area of India and Southeast Asia where *A. aegypti* was identified as main vector. *A. albopictus* a mosquito originating in Asian forests has invaded the five continents during the past three decades (Benedict et al., 2007). This mosquito was first reported in Central Africa in 2000, in Cameroon (Fontenille and Toto, 2001), and has since invaded almost all the countries in the region, including the Central African Republic (CAR) (Diallo et al., 2010).

Phylogenetic studies of the complete viral genome show the existence of three region-specific CHIKV lineages: the West

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African (WA), East-Central-South African (ECSA) and Asian lineages (Arankalle et al., 2007; Volk et al., 2010). A new lineage, the "epidemic lineage", derived from ECSA, emerged in Kenya in 2004 and spread across the Indian Ocean, switching its main mosquito vector to *A. albopictus* (Ng and Hapuarachchi, 2010; Schuffenecker et al., 2006). Indeed, in the Indian Ocean in 2005, CHIKV has acquired a critical mutation at position 226 in the E1 envelope protein, which enhanced its transmissibility by *A. albopictus* (Schuffenecker et al., 2006; Vazeille et al., 2007). This E1-*A226V* mutation was possible because of the presence of epistatic mutations in E2 in the ECSA lineage, especially the E2-*I211T* mutation (Tsetsarkin et al., 2011, 2009), and has been followed by the acquisition and selection of novel mutations that also impacts the adaptation to *A. albopictus*, like the neighboring E2-*L210Q* mutation (Niyas et al., 2010; Tsetsarkin et al., 2011) or other mutations in E2 (K252Q, R198Q) (Tsetsarkin et al., 2014). The *A. albopictus*-adapted epidemic lineage has since spread to India, Southeast Asia and Europe (Ng and Hapuarachchi, 2010; Ng and Ojcius, 2009) with expansion of the distribution of its vector. Furthermore, the *A226V* mutation in E1 has now appeared several times independently, as in Cameroon in 2006 and in Gabon in 2007 and 2010, where local CHIKV strains from the ECSA lineage have acquired the mutation, resulting in urban outbreaks mediated by *A. albopictus* (de Lamballerie et al., 2008; Gonzalez et al., 1989; Ng and Hapuarachchi, 2010).

CHIKV isolated in the CAR belongs to the ECSA lineage and is thought to follow an enzootic, sylvan cycle, but its ecology is poorly known. No epidemics have been reported in the CAR during the past 20 years, despite the fact that the virus has been isolated repeatedly from human sera (Gonzalez et al., 1989; Mathiot et al., 1988; Meunier et al., 1987; Saluzzo et al., 1980) and from mosquito pools collected throughout the country (Saluzzo et al., 1980). The recent introduction of *A. albopictus* (Diallo et al., 2010), associated with its rapid spread and good adaptation (Kamgang et al., 2013) in the CAR could lead to the emergence of CHIKF epidemics mediated by this new vector, due to introduction of foreign CHIKV belonging to the epidemic lineage or to enhanced circulation of local CHIKV.

We undertook a study to assess the genetic background and diversity of local strains isolated in the 1970s and 1980s to gain further insight into the ecology of CHIKV in the CAR and in the Central African region and to estimate the ability of these strains to become better adapted to *A. albopictus*.

2. Materials and methods

2.1. Origin of samples

The virus strains used in this study were isolated from human sera and mosquitoes collected between 1975 and 1984 in the CAR. These were selected for molecular analysis on the basis of sample date, location and host (Table 1). The human sera were collected from febrile patients presenting to the Institut Pasteur in Bangui with arthralgia and myalgia for diagnostic procedures. The mosquitoes specimen were collected in sylvan environments, identified and grouped into pools of 30 individuals per species per site, stored at −20 °C during 4 days maximum in the field and transported at the Institut Pasteur at stored at −80 °C until virus isolation. Viruses were isolated and amplified by four serial passages in suckling mice brain, as described by Saluzzo et al. (1980). The brain suspensions were then lyophilized and stored in sealed glass vials at room temperature between the year of isolation and 2011.

The strains originated from six locations in the CAR separated by a maximum distance of 500 km (Bouar–Yombo) (Fig. 1). Nine strains were isolated from mosquitoes (four from *A. africanus*, three from *Aedes opok* and two from *Mansonia africana*) in forest areas (Bozo, Bouboui, Yombo, Bombabia and Sebokele) and six from human sera in rural and peri-urban areas (Bouar, Bangui).

Table 1
Characteristics of CAR samples used for molecular analysis.

Strain	Sampling site	Sampling date	Host
ArB6445	Bozo	June 1975	*Aedes opok*
A6508	Bozo	August 1975	*A. africanus*
ArB10262	Bozo	August 1978	*A. africanus*
ArB10238	Bozo	August 1978	*A. opok*
ArB18816	Bozo	August 1981	*A. africanus*
ArB18945	Bozo	July 1981	*A. opok*
ArB16753	Bouboui	November 1980	*A. africanus*
ArB20599	Sebokele	March 1984	*Mansonia africana*
ArB20636	Yombo	April 1984	*M. africana*
HB82P18	Bangui	February 1982	*Homo sapiens*
HB84P07	Bangui	December 1983	*H. sapiens*
HB78P613	Bangui	September 1978	*H. sapiens*
HB84P93	Bouar	April 1985	*H. sapiens*
HB84P119	Bombabia	April 1984	*H. sapiens*
HB84P127	Sebokele	April 1984	*H. sapiens*

All passaged four times through suckling mouse brain; ArB16753 also passaged through mosquito cells in culture.

2.2. E1 and E2 amplification and sequencing

Lyophilized samples were suspended in 2 mL phosphate-buffered saline (PBS), and 140 µL of each sample were used to extract viral RNA with the RNeasy mini kit (Qiagen, Courtaboeuf, France) following the manufacturer's protocol.

Reverse transcription was performed on 8 µL of RNA template with a high-capacity reverse transcription kit (Applied Biosystems) and random hexamer primers, according to the manufacturers' instructions. Two sets of primers, E1-10145F and E1-11158R and E2-8458F and E2-9240R (Schuffenecker et al., 2006), were used to amplify partial sequences of the structural polyprotein gene in the E1 and E2 coding region, respectively. Polymerase chain reaction (PCR) amplification was performed in a GeneAmp 9600 thermocycler (PerkinElmer) with Platinum Taq DNA Polymerase (Invitrogen), following the manufacturer's instructions, with 2 mmol/L dNTP, 2.5 mmol/L $MgCl_2$ and 0.3 µmol/L of each primer. The temperature program was as follows: initial denaturation, 5 min at 94 °C, 35 cycles of denaturation for 30 s at 94 °C, annealing for 30 s at 54 °C, elongation for 70 s at 72 °C and final elongation for 10 min at 72 °C. PCR products were detected by 2% agarose gel electrophoresis in Tris–borate–EDTA buffer, stained with 0.5 µg/mL ethidium bromide and visualized under ultraviolet light. Each sample showed a unique band of the expected size, which was cut and purified with a QIAquick Gel extraction kit (Qiagen, Courtaboeuf, France). PCR products were directly sequenced by GATC Biotech (Konstanz, Germany).

2.3. Sequences and phylogenetic analysis

E1 and E2 nucleotide sequences were visualized chromatographically, read manually when necessary and aligned with CLC Main Workbench (CLCbio, Aarhus, Denmark). The resulting E1 (908 bp, from genomic position 10193 to 11100) and E2 (691 bp, from genomic position 8507 to 9198) sequences were numbered according to the reference sequence of the full genome from the S27-prototype strain (GenBank accession No. AF369024). They were also compared with sequences from other CHIKV strains previously published in GenBank, originating from several African countries, including the CAR, and from Asia and the Indian Ocean (Supplementary Table 1). Multiple alignments were generated for nucleotide and for in silico-translated protein sequences with

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