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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 and also 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.

Keywords : Chikungunya virus, Genetic variability, Central African Republic

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, Laganier, and Nangouma, 2010).

Phylogenetic studies of the complete viral genome show the existence of three region-specific CHIKV lineages: the West 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, McGee, and Higgs, 2011; Tsetsarkin et al., 2009), and has been followed by the acquisition and selection of novel mutations that also impacts the adaptation to *A. albopictus*, like the neighbouring E2-*L210Q* mutation (Niyas et al., 2010; Tsetsarkin, McGee, and Higgs, 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, Gonzalez, and Georges, 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, Laganier, and Nangouma, 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*.

Material and Methods

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 four 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) (Figure 1). Nine strains were isolated from mosquitoes (four from *A. africanus,* three from *A. 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).

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).

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 (908bp, from genomic position 10193 to 11100) and E2 (691bp, 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 CLC Main Workbench, to quantify the divergence and to analyze non-synonymous mutations.

E2 and E1 sequences were concatenated for each sample, generating 1599 bp E2+E1 sequences that were aligned with the same regions from full genome sequences of several previously published strains (supplementary Table 1). This allowed the construction of phylogenetic trees encompassing all CHIKV lineages (global analysis). Another phylogenetic analysis was conducted on 776 bp segments of E1 sequences from the 15 strains sequenced in this study, and from previously published full-genome and partial E1-3'UTR sequences of central African CHIKV strains (Central African analysis). All previously published sequences were retrieved from GenBank and are listed in supplementary Table 1. The sequences were aligned, and the absence of substitution saturation was tested with the DAMBE program version 4.2.13 (Xia and Xie, 2001). The Modeltest program (version 3.6) (Posada, 2006) was used to select the best generalized time-reversible evolution model (according to the Akaike

information criterion) for the phylogenetic analysis. These parameters were used in PAUP program version 4.0b10 (Swofford, 2011) to infer trees according to the neighbor-joining method, and 1000 bootstrap replicates were performed. Phylogenetic analyses were also performed with MrBayes V.3.2 software, using the default chain with the GTR+G+I nucleotide substitution model. Trees were edited with TreeGraph 2 (Stover and Muller, 2010). Scale bar indicates nucleotide substitutions per site.

Results

Nucleotide and protein sequences

Nucleotide sequences of the E1 and E2 genes were generated from 15 strains of CHIKV isolated between 1975 and 1984, originating from six localities in the CAR. The length of the retrieved sequence varied from 918 to 954 nucleotides for E1 and from 707 to 718 nucleotides for E2. Overlap of the fragments spanned 908 nucleotides for E1 (positions 93–1001 of the E1 reference sequence from S27 isolate) and 691 for E2 (positions 43–733 of the E2 reference sequence), without any deletion or insertion. Several E1 sequences from the CAR were identical in different strains (Table 2: ArB10262, ArB10238, HB78P613 and previously published strains HB78 (a); HB81P18 and HB84P119 (b); ArB20599, ArB20636, HB84P07 and HB84P127 (c)). Overall, the 15 E1 sequences displayed paired identity at the nucleotide level ranging from 98.35% to 100%. The paired identity with the S27-prototype strain isolated in Tanzania in 1953 ranged from 97.14% to 98.13%. In the E2 region, several strains also showed 100% identity at the nucleotide level (Table 2: ArB20599, ArB20636 and HB84P127 (d); ArB10262, ArB10238, HB78P613 and previously published strain HB78 (e)). E2 sequences had paired identity at the nucleotide level ranging from 97.68% to 100% between CAR strains and 96.24% to 96.82% when compared to the S27-prototype.

Strains from the four CHIKV lineages (ESCA, West African, Asian, epidemic) were used to compare sequences at the amino-acid level (Table 2). Nineteen non-synonymous substitutions were observed in the E2 and E1 sequences. Four were unique to one of the CAR strains studied (E2 G55R in A6508; E2 D117G in HB84P93; E1 S77N in ArB18945; E1 V163A in ArB6445), while others were shared in two to four of these strains but were not observed in the reference sequences (E2 S1G in strains ArB16753 and HB84P07; E2 V113A in ArB18816 and ArB18945; E2 S118G in ArB18816, ArB18945 and ArB20636; E2 S118I in HB82P07 and HB84P93; E1 I278V in ArB18816 and ArB18945; E1 V367A) (Table 2). Of note, the 113–118 amino acids in the E2 protein of the CAR strains appeared to be highly variable. None of the strains studied here had mutations associated with better transmissibility by *A. albopictus*, as they exhibited the ancestral genotype at positions E1-A226, E2-L210 and E2-I211.

Phylogenetic analysis

Partial E1 and E2 sequences were concatenated into a 1599 pb E2+E1 sequence for each strain and used to generate a phylogenetic tree to assess the relations between CAR CHIKV strains and strains from all the lineages (Figure 2). The percentage of successful bootstrap replicates (out of 1000) was always above 80% at important nodes. Several strains shared an identical E2+E1 sequence: ArB20599, ArB20636 and HB81P127; and ArB10238, HB78P163 and ArB10262. The same results were obtained through Bayesian analyses (supplementary Figure S1), also with high bootstrap values in support of most nodes. West African strains formed the most distant monophyletic group (Figure 2, purple box), as described previously, which was therefore used as outgroup. Strains from the Asian lineage formed another monophyletic group, clearly separated from the others (Figure 2, yellow box).

The strains sequenced in this study form a monophyletic group within the ECSA lineage (blue box), with other CAR strains and one Ugandan strain from the same period (Figure 2 and supplementary Figure S1). Within this group, there is no clear geographical or temporal segregation of viral strains. Sequences from southern Africa (South Africa or Tanzania) belong to a significantly different group inside the ECSA lineage. Strains from the novel epidemic lineage (Figure 2, green box) did not fall inside the group of CAR sequences, although they were closely related to these strains. In the CAR and in the whole ECSA lineage, sequences of human origin (normal type) were not separated from those originating from sylvan mosquitoes (italic type), and most of these sequences were more closely related to sylvan than to other human sequences.

To gain further insight into the history of CHIKV circulation in central Africa, other phylogenetic trees were built from partial 776 bp E1 sequences from the 15 strains studied here as well as additional sequences from the DRC, Cameroon and Gabon isolated in 2000, 2006 and 2007, respectively (supplementary Table 1), using neighbor-joining (Figure 3) and Bayesian methods (supplementary Figure S2). All the samples isolated in Central Africa during the past 35 years clustered in the same monophyletic group, with high bootstrap values (Figure 3, supplementary Figure S2). A strong temporal pattern was observed among these strains: sequences isolated in the CAR and Uganda in the 1970s and 1980s clustered together, while sequences from DRC, Cameroon and Gabon isolated in the 2000s formed another monophyletic group, with longer branches for the more recently isolated strains. Interestingly, although none of the CAR strains exhibited the *A226V* mutation characteristic of better adaptation to *A. albopictus*, this mutation appeared independently (Figure 3, red stars) in two different phylogenetic groups flanking the CAR–Uganda group, the epidemic lineage (green) and the Cameroon–Gabon sequences (pink and purple).

Discussion

To gain further insight into the genetic diversity and background of local CHIKV strains, we sequenced and analyzed partial E1 and E2 sequences from 15 strains isolated from human and arthropod hosts between 1975 and 1984. Nucleotide and protein sequence analysis of the structural polyprotein gene coding for the E2 and E1 envelope glycoproteins showed that the E2 region is more variable than E1, although sequence comparisons revealed high stability of CHIKV over nine years. Similar stability was shown in strains from the DRC, the CAR, Cameroon and Gabon between 1996 and 2007 (Pastorino et al., 2004; Peyrefitte et al., 2008; Peyrefitte et al., 2007). Several sequences of human origin are identical to those retrieved from sylvan mosquitoes, and phylogenetic analysis showed that the CAR CHIKV strains are monophyletic, suggesting that most of the human infections were due to spill-over of sylvan viruses, even in urban or peri-urban area of Bangui. One of the few non-synonymous mutations observed in the CAR strains, the *S118G* mutation, had already been observed in Indian strains of the Asian lineage and probably represents a frequent polymorphism (Shrinet et al., 2012). Interestingly, all the CAR strains exhibited the ancestral genotype at positions known to influence adaptation to the domestic vectors *A. aegypti* and *A. albopictus*, especially I211 in E2 and A226 in E1 (Table 2) (Tsetsarkin et al., 2009). Accordingly, second-step mutations in E2 that are observed in sub-lineages of the epidemic lineage and result in further increased transmissibility by *A. albopictus*, i. e. R198Q and L210Q, are not observed in the CAR strains studied here (Tsetsarkin et al., 2011, Tsetsarkin et al., 2014). Another second-step E2 mutation, K252Q, is outside the region of E2 sequenced here and could not be analyzed (Tsetsarkin et al., 2014). This observation suggests that local strains would have to acquire at least two mutations (*E2-I211T* and *E1-A226V*) to gain an increased transmissibility by *A. albopictus* as observed in the epidemic lineage. The strains studied here also displayed

the ancestral genotypes E1-D284 and E1-V269, which are often replaced by *D284E* and *V269M* in the epidemic lineage, independently of the *A226V* mutation (Singh et al., 2012).

Both Bayesian and neighbor-joining phylogenetic analysis shows that strains originating from South Africa and Tanzania belong to a significantly different group, while all strains from the central African region form a monophyletic group (Figures 2 and 3, supplementary Figures S1 and S2). This confirms previous data suggesting extended geographical circulation of CHIKV in central Africa but separated from southern Africa (Peyrefitte et al., 2008; Peyrefitte et al., 2007; Volk et al., 2010). The strains from the epidemic lineage did not fall within the group of CAR sequences, although it was closely related to these strains. The tree topology in Figure 3 and supplementary Figure S2 also suggests that strains from the DRC outbreak evolved from the virus pool circulating in the CAR in the 1980s. Our data also confirm that the outbreaks in Cameroon and Gabon in 2006 and 2007 were linked to the previous outbreak in the DRC (de Lamballerie et al., 2008). Importantly, the E1-*A226V* mutation, together with CHIKV adaptation to *A. albopictus*, appeared independently in two monophyletic groups flanking the group of CAR strains (Figure 3, red stars). This finding, with our analysis of the partial E1 and E2 protein sequences, suggests that viruses in this area are likely to acquire this mutation again.

As the CHIKV strains so far isolated in the CAR are different from those that were responsible for the recent epidemics in numerous central African countries and as *A. albopictus*, which was identified as a major vector in those outbreaks, is now widely distributed in the CAR (Kamgang et al., 2013), further research is needed to assess the vector competence of *A. albopictus* for the ancient CHIKV strains described in this study. This will provide important information on the adaptability of the local strains and their ability to acquire the *A226V* mutation.

Conclusion

The genetic background of ancient strains from the CAR is compatible with rapid adaptation to *A. albopictus*, as observed in other central African countries. It is therefore of the utmost importance to develop a control program based on destruction of breeding sites, evaluation of the susceptibility of invasive populations of *A. albopictus* to insecticides and increased epidemiological surveillance of the population in order to prevent the emergence of a massive, *A. albopictus*-vectored CHIKF epidemic in the CAR.

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Figures legends

Figure 1. Localization of collected samples in the Central African Republic.

A. Sampling sites for virus isolation in Central African Republic between 1975-1984 are indicated by red triangles. B. Localization of CAR in the central African region and of other countries where CHIKV from ECSA lineage is known to circulate. Name of countries from which CHIKV strains used for phylogenetic analysis originate are in upper case.

Figure 2. Global phylogenetic analysis of CAR strains and strains of the four lineages of CHIKV.

1599 bp sequences of concatenated E1+E2 partial segments were used to build a phylogenetic tree with the neighbor-joining method. Bootstrap values above 70% are given just above or beside their node. Strains from the Western African lineage were used as outgroup. For each strain, the year and country of isolation is indicated, with the lineage in colored boxes (West African, purple; ECSA, blue; Asian, yellow; epidemic, green). Strains isolated from an arthropod host are shown in italics. The names of the sequences generated in this work are in bold, and the precise location of sampling in the CAR is indicated between brackets, when available.

Figure 3. Phylogenetic analysis of CHIKV strains and evolution in central Africa.

776 bp sequences coding for a partial segment of E1 were used to build a phylogenetic tree with the neighbor-joining method. A strain of the Asian lineage, isolated in India, was used as outgroup. Bootstrap values are given just above or beside their node when > 70%. For each strain, the year and country of isolation is indicated. Colors highlight the different regions and dates of sampling among the ECSA lineage (blue: CAR between 1978 and 1996; purple: DRC

in 2000; pink: Cameroon in 2006; orange: Gabon in 2006) and the epidemic lineage (green). Strains harboring the *A226V* mutation in E1 are marked with a red star.

Supporting information legends

Table S1. Previously sequenced CHIKV strains used for phylogenetic analysis.

Figure S1. 1599 bp sequences of concatenated E1+E2 partial segments were used to build a Bayesian phylogenetic tree using MrBayes software. Bootstrap values above 70% are given just above or beside their node. The PM2951 strain from Senegal, belonging to the Western African lineage, was used as outgroup. For each strain, the year and country of isolation is indicated. Strains isolated from an arthropod host are shown in italics. The names of the sequences generated in this work are in bold, and the precise location of sampling in the CAR is indicated between brackets, when available.

Figure S2. 776 bp sequences coding for a partial segment of E1 were used to build a Bayesian phylogenetic tree using MrBayes software. A strain of the Asian lineage, P0731460, isolated in India, was used as outgroup. Bootstrap values are given just above or beside their node when > 70%. For each strain, the year and country of isolation is indicated.

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.

Table 2. Protein sequences and amino-acid substitutions in E2 and E1.

Reference			Position of substitutions in E2								Position of substitutions in E1						
Lineage	Accession no. (full genome)		1	55	60	113	117	118	210	211	77	163	226	269	278	284	367
ECSA, S27	AF369024		**S**	**G**	**G**	**V**	**D**	**S**	**L**	**I**	**S**	**V**	**A**	**M**	**I**	**D**	**V**
ECSA, CAR	HM045784, HM045793, HM045822		–	–	D	–	–	–/G	–	–	–	–	–	V	–	–	–
ECSA, other	HM045805		–	–	D	–	–	–	-	T	–	–	–	–	–	–	–
Asian	HM045788, HM045796		–	–	D	–	–	–	-	T	–	–	–	–	–	–	–
West African	HM045818, HM045815		–	–	D	–	–	–	-	T	–	–	–	V	–	–	–
Epidemic	EU244823, HQ456255, AM258995		–	–	D	–	–	–	-	T	–	–	–/V	–	–	E	–
CAR samples			Position of substitutions in E2								Position of substitutions in E1						
Strain	E1 accession no.	E2 accession no.	1	55	60	113	117	118	210	211	77	163	226	269	278	284	367
ArB6445	KF925286	KF925301	–	–	D	–	–	–	–	–	–	A	–	V	–	–	–
A6508	KF925287	KF925302	–	R	D	–	–	–	–	–	–	–	–	V	–	–	–
ArB10262[a,e]	KF925288	KF925303	–	–	D	–	–	–	–	–	–	–	–	V	–	–	–
ArB10238[a,e]	KF925289	KF925304	–	–	D	–	–	–	–	–	–	–	–	V	–	–	–
ArB18816	KF925290	KF925305	–	–	D	A	–	G	–	–	–	–	–	V	V	–	–
ArB18945	KF925291	KF925306	–	–	D	A	–	G	–	–	N	–	–	V	V	–	–
ArB16753	KF925292	KF925307	G	–	D	–	–	–	–	–	–	–	–	V	–	–	–
ArB20599[c,d]	KF925293	KF925308	–	–	D	–	–	–	–	–	–	–	–	V	–	–	A
ArB20636[c,d]	KF925294	KF925309	–	–	D	–	–	G	–	–	–	–	–	V	–	–	A
HB82P18[b]	KF925295	KF925310	–	–	D	–	–	I	–	–	–	–	–	V	–	–	–
HB84P07[c]	KF925296	KF925311	G	–	D	–	–	–	–	–	–	–	–	V	–	–	A
HB78P613[a,e]	KF925297	KF925312	–	–	D	–	–	–	–	–	–	–	–	V	–	–	–
HB84P93	KF925298	KF925313	–	–	D	–	G	I	–	–	–	–	–	V	–	–	–
HB84P119[b]	KF925299	KF925314	–	–	D	–	–	–	–	–	–	–	–	V	–	–	–
HB84P127[c,d]	KF925300	KF925315	–	–	D	–	–	–	–	–	–	–	–	V	–	–	A

[a, b, c], strains with identical E1 sequences; [d,e], strains with identical E2 sequences.

Figure 1

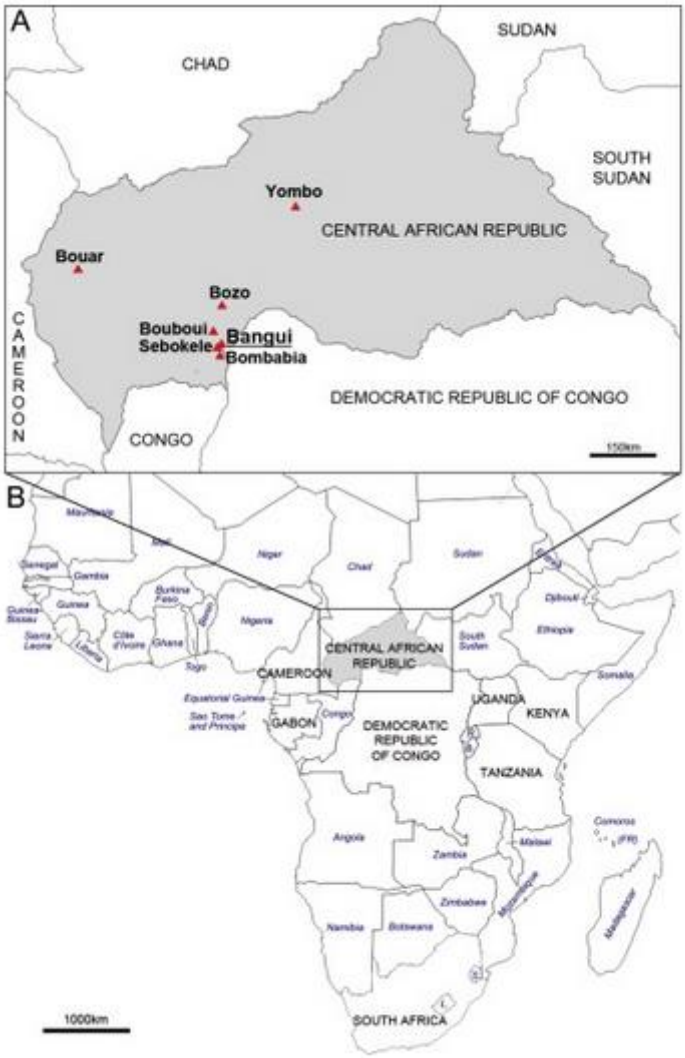
A
SUDAN
CHAD
SOUTH SUDAN
Yombo
CENTRAL AFRICAN REPUBLIC
Bouar
Bozo
Bouboui
Sebokele
Bangui
Bombabia
CAMEROON
DEMOCRATIC REPUBLIC OF CONGO
CONGO
150km
B
CENTRAL AFRICAN REPUBLIC
CAMEROON
GABON
DEMOCRATIC REPUBLIC OF CONGO
UGANDA
KENYA
TANZANIA
SOUTH AFRICA
1000km

Figure 2

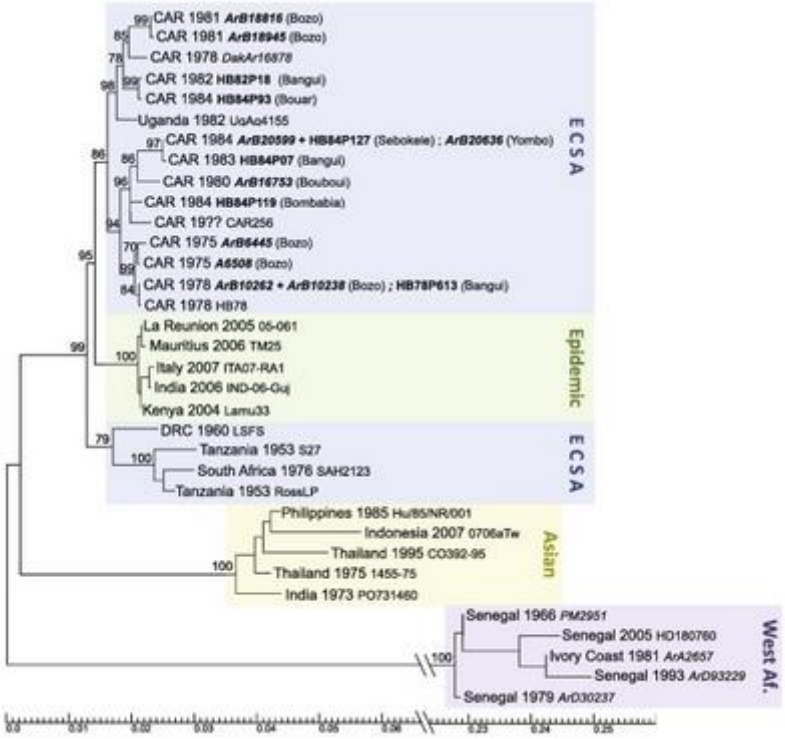

Figure 3