Journal of Medical Virology 74:277–282 (2004)

Epidemic Resurgence of Chikungunya Virus in Democratic Republic of the Congo: Identification of a New Central African Strain

B. Pastorino,[1,2] J.J. Muyembe-Tamfum,[3] M. Bessaud,[1,2] F. Tock,[1,2] H. Tolou,[1,2] J.P. Durand,[1,2] and C.N. Peyrefitte[1,2]*

[1]*Unité de Virologie, Laboratoire Associé au Centre National de Référence Pour les Arboviroses, Institut de Médecine Tropicale du Service de Santé des Armées, Marseille Armées, France*
[2]*EA 3292, IFR 48, Université de la Méditerranée, Marseille, France*
[3]*Institut de National de Recherche Bio-Médicale, Kinshasa, République Démocratique du Congo*

The resurgence of Chikungunya virus is described during an urban epidemic in Kinshasa Democratic Republic of the Congo, after 39 years without any isolation of the virus. Chikungunya virus was isolated in sera from nine patients with clinical symptoms. A 1,200 bp long partial sequence of the E1/3′UTR genomic region was determined for each isolate. All sequences clustered in the central African lineage. They constitute Chikungunya virus reference sequences for the Democratic Republic of the Congo. ***J. Med. Virol. 74:277–282, 2004.*** © 2004 Wiley-Liss, Inc.

KEY WORDS: *Alphavirus*; outbreak; molecular epidemiology; co-infection; Africa

INTRODUCTION

The resurgence of well-known epidemic arboviral diseases such as Chikungunya, thought to be actually controlled or anecdotal, is now considered as a worldwide public health problem [Gubler, 2002].

Chikungunya virus, an alphavirus of the Togaviridae family first isolated from a human serum in 1953 in Tanzania [Robinson, 1955], is a single stranded positive RNA-enveloped virus. Widely spread throughout Africa [Saluzzo et al., 1983], Chikungunya virus is maintained in a sylvatic cycle involving mosquitoes of the genus *Aedes*, wild forest primates, and eventually rodents [Diallo et al., 1999]. The virus is endemic mainly in rural tropical Africa [Diallo et al., 1999], but is penetrating Asian urban areas [Halstead et al., 1969; Thaikruea et al., 1997; Yadav et al., 2003], where its transmission cycle involves anthropophilic populations of *Aedes aegypti*. Known to produce a dengue-like illness in man, with fever, arthralgia, and maculopapular rash sometimes followed by persistent joint pain for several months [Brighton et al., 1983], it was also suspected to be the causative agent of haemorrhagic fever [Sarkar et al., 1964] in Asia.

Chikungunya virus was isolated in the Democratic Republic of the Congo for the first time in 1958 in a village called Doruma, Eastern Congo province [Osterrieth and Blanes-Ridaura, 1960] and was isolated again in the Democratic Republic of the Congo in 1960 [Osterrieth et al., 1961]. During the last 39 years, there has been no further report on Chikungunya virus isolation in the Democratic Republic of the Congo. However in February 1998, IgM antibodies against Chikungunya virus were found in 12 patients during an outbreak of West Nile fever among migrants in Kisangani (Democratic Republic of the Congo) [Nur et al., 1999]. In 1999 and 2000, two important outbreaks of febrile illness were reported following heavy rain falls in the Matete and Kingabwa quarters of the capital city Kinshasa (Democratic Republic of the Congo). An estimated 50,000 persons were infected and Chikungunya virus identified as the main causative agent [Muyembe-Tamfum et al., 2003]. During this period, blood samples collected at early and late stages of the infection were examined at our laboratory in Marseille. Chikungunya virus was isolated on C6/36 cells (*Aedes albopictus*). Partial genomic sequences were determined for each isolated virus, in order to evaluate the origin and diversity of this re-emerging virus. Our

Grant sponsor: The French Armed Medical Service.

*Correspondence to: C.N. Peyrefitte, Unité de Virologie, Laboratoire Associé Au Centre National de Référence Pour les Arboviroses, Institut de Médecine Tropicale du Service de Santé des Armées, BP 46, 13998 Marseille Armées, France; EA 3292, IFR 48, Université de la Méditerranée, Institut de National de Recherche Bio-Médicale, République Démocratique du Congo.
E-mail: publi.viro@laposte.net

Accepted 22 June 2004
DOI 10.1002/jmv.20168
Published online in Wiley InterScience (www.interscience.wiley.com)

approach should help the understanding of the geographic distribution of the isolates in the Democratic Republic of the Congo and the viral circulation and transmission to humans.

MATERIALS AND METHODS

Patients

The 76 patients examined in the Democratic Republic of the Congo presented with dengue-like syndromes and heavy joint pain. They were indistinguishable clinically.

Collection and Transport of Samples

Blood samples from all patients were collected in Democratic Republic of the Congo medical services, frozen, and transported to the laboratory in Marseille (France) at −20°C by air.

Serological Diagnosis

"In-house" IgM capture enzyme immunoassays (MAC-ELISA) were used for the detection of Chikungunya, Dengue, West Nile, and Bunyamwera viruses serum IgM antibodies. Briefly, IgM antibodies were captured with a rabbit anti-human IgM antibodies (Interchim, Montluçon, France). Chikungunya, Dengue, West Nile, and Bunyamwera antigens, prepared on Vero cells and inactivated by betapropiolactone (Sigma-Aldrich, St. Quentin Fallavier, France), were added. Specific binding was demonstrated by using a Chikungunya, Dengue, West Nile, and Bunyamwera mouse hyper immune ascitic fluid and a goat anti-mouse peroxidase-labelled conjugate (Interchim). Sera were considered positive if the optical density was >3 SD above the negative sera average.

Virus Isolation and Propagation

Work with infectious virus was carried out in a biosafety level 3 laboratory. The isolation was attempted when the sample-first onset delay was below 3 days and when there was enough serum for serological analysis. The Chikungunya virus strains (Chik007, Chik010, Chik027, Chik1718, Chik1719, Chik1720, Chik1725, Chik1728, Chik1730 referred as GenBank accession number from AY549575 to AY549584) were all isolated from the serum fraction of patients. Briefly, 200 µl of the serum samples were directly incubated with C6/36 cells (*Aedes albopictus*) grown at 28°C in Leibowitz's L15 medium (Biowhittaker, Europe sprl, B48 Verniers, Belgique) supplemented with 1% L-glutamin and 2% tryptose phosphate broth. Fetal bovine serum (5% final) was added 1 hr later. Supernatants were collected on day 5 post infection.

Indirect immunofluorescence was performed on C6/36 cells, using in-house developed Sindbis, Semliki Forest, Chikungunya virus-specific murine hyper-immune ascitic fluids.

RNA Preparation and cDNA Synthesis

Viral RNAs were extracted from 200 µl aliquots of infected cells supernatants, as previously described [Peyrefitte et al., 2003], using the High Pure Viral RNA kit® (Roche Diagnostics, 38242 Meylan cedex, France). Viral cDNAs were generated by reverse transcription using Superscript II reverse transcriptase® (Gibco BRL, Invitrogen sarl/Life Technologies, 95613 Cergy Pontoise cedex, France) according to the manufacturer protocols. Specific primers (direct: OP16 5′ AGCTGTAAGGTCTTCACCGG 3′ nucleotide numbers 10200–10219 and OP17 5′ GTATTTTGTTACTATTCAGG 3′ nucleotide numbers 11380–11399) designed from the nucleotide sequence of the reference S27 Chikungunya virus (GenBank accession number AF490259) [Khan et al., 2002] were used for PCR amplification using AmpliTaq® DNA Gold (Applera SA, 91943 Courtaboeuf cedex, France).

DNA Sequencing

The 1.2 kb PCR products were purified from 1.5% agarose gels, using the QIAquick Gel extraction kit® (Qiagen SA, 91974 Courtaboeuf, France) and cloned into the pGEMT TA cloning vector (Promega, Les Ulis, France). White bacterial colonies were screened for plasmids containing inserts of expected size. Selected clones were sequenced using the plasmid specific T7 promoter and SP6 reverse primers and the Big Dye Sequencing kit® (Applera SA, 91943 Courtaboeuf cedex, France). Sequencing was carried out using an automatic sequence analyser (ABI PRISM 3100, Applera SA, 91943 Courtaboeuf cedex, France) following the manufacturer protocol.

Sequence Analysis

Sequences of the Democratic Republic of the Congo Chikungunya virus isolates were compared to GenBank database Chikungunya viruses sequences (complete list in Table I). Alignments of nucleotide and amino acid sequences of the E1 protein/3′UTR were performed using the ClustalW1.7 software [Thompson et al., 1994]. Phylograms were constructed using the MEGA program [Kumar et al., 1993], and tree drawing used the Jukes Cantor algorithm for genetic distance determination and the Neighbor Joining method. The robustness of the resulting tree was tested by 1,000 bootstrap replications.

RESULTS

Serological Diagnosis and Virus Identification

In May 1999 and February 2000, after heavy rain falls, a total of 76 blood samples from dengue-like fever patients from the Matete and Kingabwa quarters of Kinshasa (Democratic Republic of the Congo) were examined at the laboratory in Marseille. Forty-four out of the 76 blood samples analysed were found positive

TABLE I. Origin of Chikungunya Virus Sequences

Strain	Pf	Location	Cluster	Date	Accession nb.	Ref.
		Nigeria	O'nyong-nyong	1966	AF192889	Powers et al. [2000]
		Senegal	West Africa	1966	AF192891	Powers et al. [2000]
		Senegal	West Africa	1983	AF192892	Powers et al. [2000]
		Nigeria	West Africa	1964	AF192893	Powers et al. [2000]
		Thailand	Asia	1988	AF192896	Powers et al., 2000
		Thailand	Asia	1995	AF192897	Powers et al. [2000]
		Thailand	Asia	1975	AF192898	Powers et al. [2000]
		Thailand	Asia	1978	AF192899	Powers et al. [2000]
		India	Asia	1973	AF192902	Powers et al. [2000]
		South African Republic	South Africa	1976	AF192903	Powers et al. [2000]
		South African Republic	South Africa	1976	AF192904	Powers et al. [2000]
Ross		Tanzania	Central/East Africa	1953	AF192905	Powers et al. [2000]
S27		Africa	Central/East Africa	?	AF369024	Powers et al. [2000]
		Uganda	Central Africa	1982	AF192907	Powers et al. [2000]
		Central African Region	Central Africa	?	AF192906	Powers et al. [2000]
ChikRCA		Central African Republic	Central Africa	1996	AY549584	This study
Chik007	+	Democratic Republic of the Congo	Central Africa	2000	AY549575	This study
Chik010		Democratic Republic of the Congo	Central Africa	2000	AY549576	This study
Chik027	+	Democratic Republic of the Congo	Central Africa	2000	AY549577	This study
Chik1718		Democratic Republic of the Congo	Central Africa	2000	AY549578	This study
Chik1719	+	Democratic Republic of the Congo	Central Africa	2000	AY549579	This study
Chik1720	+	Democratic Republic of the Congo	Central Africa	2000	AY549580	This study
Chik1725		Democratic Republic of the Congo	Central Africa	2000	AY549581	This study
Chik1728	+	Democratic Republic of the Congo	Central Africa	2000	AY549582	This study
Chik1730		Democratic Republic of the Congo	Central Africa	2000	AY549583	This study

Chikungunya isolates obtained from positive *Plasmodium falciparum* (Pf) patients are indicated by +.

for Chikungunya infection by the presence of IgM specific for Chikungunya virus and negative for other tested viruses (Dengue, West Nile, and Bunyamwera). Viruses were isolated in 9 out of 21 sera from patients from Kingabwa during the February 2000 outbreak. Chikungunya were detected by IFI using Chikungunya virus-specific murine hyper-immune ascitic fluid. No fluorescence was observed using Sinbis, Semliki Forest virus-specific murine hyper-immune ascitic fluid.

Plasmodium falciparum was found locally using blood thick smear in five out of the nine samples with positive virus isolation.

O'nyong nyong should be considered as a differential virus until PCR and genome sequencing because of the partial antigenic cross-reactivity of Chikungunya virus murine hyper-immune ascitic fluid used in IFI [Blackburn et al., 1995].

Sequence Analysis

Chikungunya virus was identified in all isolates by blasting the sequences obtained using BLAST-NCBI software (E value $7e^{-31}$).

Comparison of partial sequences revealed a high degree of identity between the strains isolated from the Democratic Republic of the Congo: paired identity at the nucleotide and amino acids level ranged from 98.1 to 100% and from 99 to 100% respectively. Considering the Ross original strain, the values ranged from 96 to 96.5% and from 99 to 99.7%, respectively. Considering the Chikungunya isolate from the Central African Republic paired identity at the nucleotide and amino acids level ranged from 96.6 to 97.3% and from 98.8 to 99.3%, respectively. Genetic comparisons including virus sequences from different origins available in the GenBank database showed a close relationship between Chikungunya viruses from the Democratic Republic of the Congo and viruses from the Central African Region and Uganda, despite an 18-year time gap in the isolation of the two strains. Moreover, Chikungunya nucleotide sequence from the Democratic Republic of the Congo was closely related to Chikungunya isolate from the Central African Republic (GenBank accession number AY549583) nucleotide sequence (96.6 to 97.3% paired identity). We had isolates of the latter virus strain in 1996 from a French soldier posted in Central African Republic.

Phylogenetic Analysis

A phylogenetic tree was constructed based on partial sequences of the 3′ extremity E1/3′UTR region (position 10238–11367) of the Chikungunya virus genomes, including all the strains from the Democratic Republic of the Congo and Central African Republic and several others from different origins previously characterised (Fig. 1) [Powers et al., 2000; Khan et al., 2002]. All Chikungunya virus strains isolated from the Democratic Republic of the Congo formed a new cluster among the Central/East African genotype proposed by Powers et al. [2000], the latter also containing strains from Central African Republic Tanzania, South Africa, Uganda, and Central African Region. The analysis of the deduced amino acid sequences generated a similar phylogram (data not shown). Overall, these results could indicate the existence of a

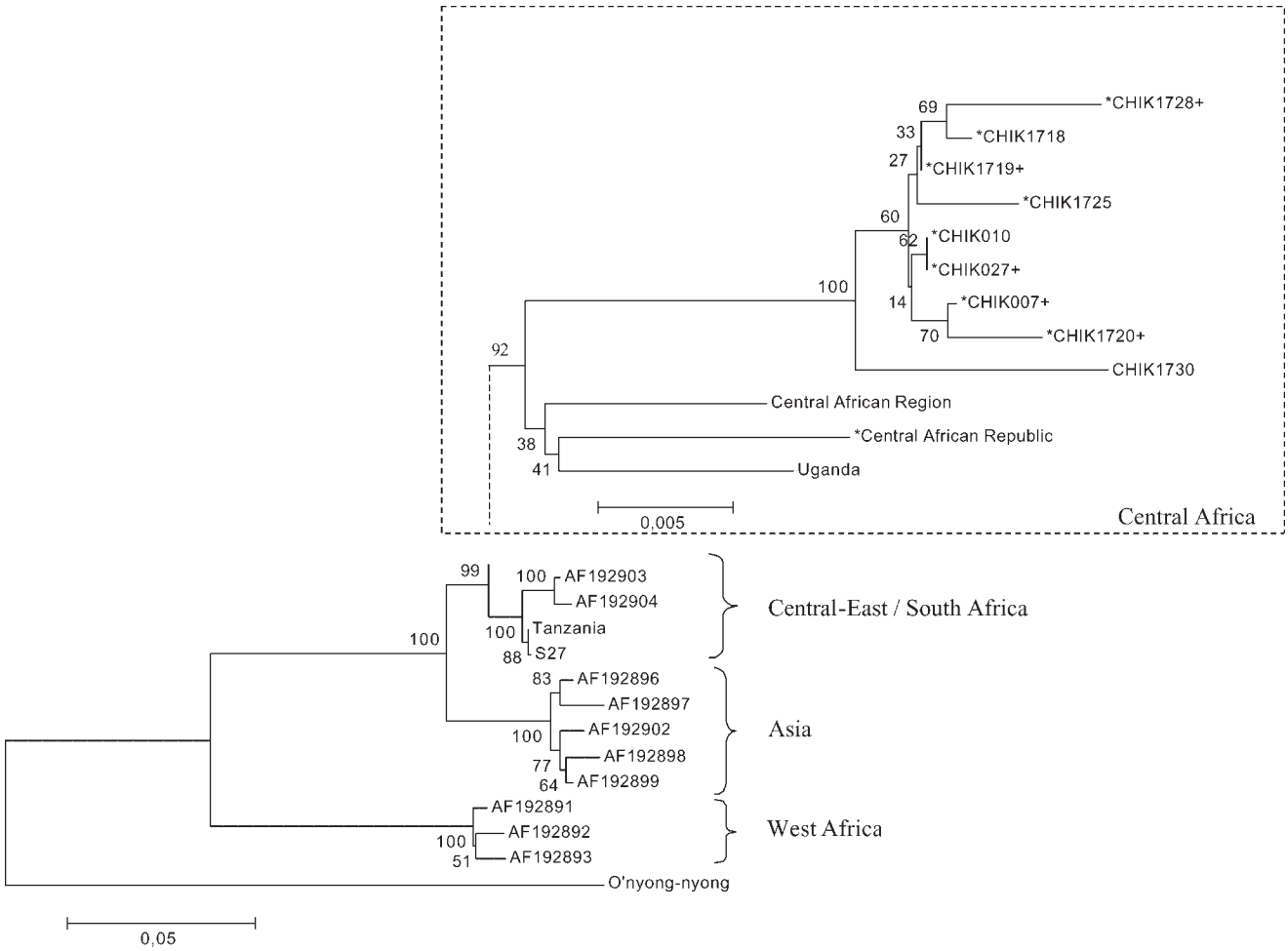

Fig. 1. Phylogenetic tree of Chikungunya virus based on partial nucleotide sequences: 3′ extremity E1/3'UTR region (position 10238–11367). Phylograms were constructed with the MEGA program [Kumar et al., 1993], using the Jukes Cantor algorithm and the Neighbor Joining method. The percentage of successful bootstrap replicates (1,000 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). The strains sequenced in this work are indicated by asterisks (*). Chikungunya isolates obtained from positive *Plasmodium falciparum* patients are indicated by +. Dots indicate a change in scale. Complete list of these viruses is given in Table I. O'nyong-nyong sequence has been introduced for correct rooting of the tree.

specific Democratic Republic of the Congo genotype, closer to Central African (Central African Republic and Uganda) isolates than to those from Tanzania and South Africa.

DISCUSSION

During the 2000 outbreak in the Democratic Republic of the Congo, we isolated nine Chikungunya virus strains from blood samples taken from patients suffering a dengue-like syndrome from the Kigabwa urban zone of Kinshasa. Chikungunya virus is known to be essentially endemic in rural areas of tropical Africa [Diallo et al., 1999]. It had not been found in the Democratic Republic of the Congo during the last 39 years, the epidemics of 1999 and 2000 being the first reported urban zones in the area to be implicated in such an outbreak [Muyembe-Tamfum et al., 2003], although urban outbreaks are a common trait of Chikungunya virus epidemiology in Asia [Halstead et al., 1969; Thaikruea et al., 1997; Yadav et al., 2003].

To identify the agent responsible for the outbreak, both an "in-house" developed IgM capture-ELISA were used on patient sera and the virus isolation on C6/36 cells. To characterise further the viral strains, RT-PCR products were cloned and sequenced. After serology and isolation, too little serum was available for diagnosis using RT-PCR. Among the sera analysed, 58% were found positive for Chikungunya specific IgM. In samples collected 24–48 hr after the onset of symptoms, 70.6% were negative, which was a close figure to the 75% previously reported by Hasebe et al. [2002]. Chikungunya viruses were isolated mainly from these early sampled sera, different from results reported in Kisangani [Nur et al., 1999]. RT-PCR for the detection of alphavirus species and Chikungunya virus have been developed by others [Pfeffer et al., 1997; Powers et al., 2000; Hasebe et al., 2002]. Nevertheless, we used

another set of primers in the E1 genome region, available in our laboratory. The RT-PCR used for this study was suitable to detect the Chikungunya virus RNA extracted from IFI positive C6/36 cell cultures inoculated with sera from patients of the Democratic Republic of the Congo.

The phylogenetic tree based on partial sequences of the Chikungunya virus genomes (position 10238–11367) indicated that all Chikungunya viruses isolated from the Democratic Republic of the Congo and Central African Republic belonged, with a high bootstrap value, in the Central/East African genotype proposed by Powers et al. [2000], indicating a common origin for all Chikungunya viruses within this cluster.

The nine isolates from the Democratic Republic of the Congo clustered together and constituted a homogenous group regarding the 10% of the Chikungunya virus genome sequenced. They may represent a Democratic Republic of the Congo genotype, closer to Central African (RCA and Uganda) isolates than those from Tanzania and South Africa. Moreover, the high paired identity values suggested some stability among these Central African isolates despite their geographical and temporal distances. Such a stability has been observed for the Asian genotype [Powers et al., 2000; Yadav et al., 2003]. It may result from both a low level virus maintenance of old strains and a viral circulation limited to the Central African region. The phylogenetic tree also highlighted a Central African genotype including the Democratic Republic of the Congo, Central African Republic, Central African Region, and Uganda isolates distinct from Central-East/South African genotype including Tanzania and South African Republic isolates. However, there is a need for expanding the sample of Chikungunya virus nucleotide sequences from Central-East Africa to help the understanding of the Chikungunya virus circulation in the region.

Interestingly, *Plasmodium falciparum* infection was found in five patients positive for Chikungunya virus. The question arose whether the co-infection with *Plasmodium falciparum* could be related to particular Chikungunya virus sequences. The weak bootstrap values (Fig. 1) did not allow any definitive conclusion, except that all Chikungunya virus sequences from *Plasmodium falciparum* positive patients were not grouped in a sub-cluster. Their distribution in the Democratic Republic of the Congo cluster seemed to be independent of their *Plasmodium falciparum* status.

Our report provides the first direct evidence of simultaneous infection, to our knowledge, and confirmed the possibility of Chikungunya virus and *Plasmodium falciparum* co-infection. Only indirect evidence of such a co-infection has been reported [McCarthy et al., 1996]. Hypothetically, co-infection may be due to a long term *Plasmodium falciparum* carrying, a common vector transmission of both pathogens, or different vector transmission during the same time period. In fact, Chikungunya virus can experimentally be transmitted by the urban *Anopheles stephensi*, a *Plasmodium falciparum* vector [Yadav et al., 2003].

Chikungunya, Dengue, and Yellow fever viruses could have the same *Aedes* vectors, indicating that the conditions were combined for a possible urban outbreak of these last two flaviviruses in the Democratic Republic of the Congo. Co-infection with Chikungunya and Dengue viruses [Halstead, 1966] and Chikungunya and Yellow fever viruses [Osterrieth et al., 1961] have been observed. Further characterisation of potential vectors present in the Democratic Republic of the Congo and their competence for transmitting Chikungunya virus could serve to anticipate the resurgence of Chikungunya virus in humans.

ACKNOWLEDGMENTS

The authors are indebted to H. Pugelli for primer synthesis, A. Buguet and M. Grandadam for manuscript revision.

REFERENCES

Blackburn NK, Besselaar TG, Gibson G. 1995. Antigenic relationship between Chikungunya virus strains and O'nyong nyong virus using monoclonal antibodies. Res Virol 146:69–73.

Brighton SW, Prozesky OW, de la Harpe AL. 1983. Chikungunya virus infection. A retrospective study of 107 cases. S Afr Med 63:313–315.

Diallo M, Thonnon J, Traore-Lamizana M, Fontenille D. 1999. Vectors of Chikungunya virus in Senegal: Current data and transmission cycles. Am J Trop Med Hyg 60:281–286.

Gubler DJ. 2002. The global emergence/resurgence of arboviral disseases as public health problems. Arch Med Res 33:330–342.

Halstead SB. 1966. Mosquito-borne haemorrhagic fevers of South and South-East Asia. Bull WHO 35:3–15.

Halstead SB, Scanlon JE, Umpaivit P, Udomsakdi S. 1969. Dengue and Chikungunya virus infection in man in Thailand, 1962–1964. IV. Epidemiologic studies in the Bangkok metropolitan area. Am J Trop Med Hyg 18:997–1021.

Hasebe F, Parquet MC, Pandey BD, Mathenge EGM, Morita K, Balasubramaniam V, Saat Z, Yusop A, Sinniah M, Natkunam S, Igarashi A. 2002. Combined detection and genotyping of Chikungunya virus by a specific reverse transcription-polymerase chain reaction. J Med Virol 67:370–374.

Khan AH, Morita K, del Carmen Parquet M, Hasebe F, Mathenge EGM, Igarashi A. 2002. Complete nucleotide sequence of chikungunya virus and evidence for an internal polyadenylation site. J Gen Virol 83:3075–3084.

Kumar S, Tamura K, Nei M. 1993. Molecular evolutionary genetics analysis, version 1.01, Pennsylvania State University.

McCarthy MC, Haberger RL, Salib AW, Soliman BA, El-Tigani A, Khalid IO, Watts DM. 1996. Evaluation of arthropod-borne viruses and other infectious disease pathogens as the cause of febrile illnesses in the Khartoum province of Sudan. J Med Virol 48:141–146.

Muyembe-Tamfum JJ, Peyrefitte CN, Yogolelo R, Mathina Basisya E, Koyange D, Pukuta E, Mashako M, Tolou H et Durand JP. 2003. Epidémies à virus Chikungunya en 1999 et 2000 en République Démocratique du Congo. Med Trop 63:637–638.

Nur Y, Groen J, Heuvelmans H, Tuynman W, Copra C, Osterhaus ADME. 1999. An outbreak of West Nile fever among migrants in Kisangani, Democratic Republic of Congo. Am J Trop Med Hyg 61:885–888.

Osterrieth P, Blanes-Ridaura G. 1960. Recherche sur le virus Chikungunya au Congo Belge. 1. Isolement du virus dans le Haut-Uélé. Ann Soc Belge Med Trop 4:199–203.

Osterrieth P, Rathe E, Deloplanque-Liégeois P. 1961. Isolement simultané des virus de la fièvre jaune et Chikungunya à Bili (Haut-Uélé, Congo Belge). Ann Soc Belge Med Trop 3:207–212.

Peyrefitte CN, Couissinier-Paris P, Mercier-Perennec V, Bessaud M, Kenane N, Martial J, Durand JPA, Tolou HJ. 2003. Genetic characterization of newly reintroduced dengue virus type 3 in Martinique (French West Indies). J Clin Microbiol 41:5195–5198.

Pfeffer M, Proebster B, Kinney RM, Kaaden OR. 1997. Genus-specific detection of alphavirus by semi-nested reverse transcription-polymerase chain reaction. Am J Trop Med Hyg 57:709–718.

Powers AM, Brault AC, Tesh RB, Weaver SC. 2000. Re-emergence of Chikungunya and O'nyong-nyong viruses: Evidence for distinct geographical lineages and distant evolutionary relationships. J Gen Virol 81:471–479.

Robinson MC. 1955. An epidemic of virus disease in Southern Province, Tanganyika territory, in 1952–1953. Clinical feature. Trans R Soc Trop Med Hyg 49:28–32.

Saluzzo JF, Adam C, Castagnet P, Chapelle P, Cornet M, Heme G, Digoutte JP. 1983. Une poussée épidémique due au virus Chikungunya dans l'ouest du Sénégal en 1982. Médecine d'Afrique Noire 30:427–430.

Sarkar JK, Chatterjee SN, Chakravarty SK. 1964. Haemorrhagic fever in Calcutta: Some epidemiological observations. Ind Jour Med Res 52:651–659.

Thaikruea L, Charearnsook O, Reanphumkarnkit, Dissomboon P, Phonjan R, Ratchbud S, Kounsang Y, Buranapiyawong D. 1997. Chikungunya in Thailand: A re-emerging disease ?. Southeast Asian J Trop Med Public Health 28:359–364.

Thompson JD, Higgins DG, Gibson TJ. 1994. CLUSTALW: Improving the sensitivity of progressive multiple sequence alignments through sequence weighting, position specific gap penalties, and weight matrix choice. Nucleic Acid Res 22:4673–4680.

Yadav P, Gokhale MD, Barde PV, Singh DK, Mishra AC, Mourya DT. 2003. Experimental transmission of chikungunya virus by *Anopheles stephensi* mosquitoes. Acta Virologica 47:45–47.

Yadav P, Shouche YS, Munot HP, Mishra AC, Mourya DT. 2003. Genotyping of Chikungunya virus isolates from India during 1963–2000 by reverse transcription-polymerase chain reaction. Acta Virol 47:125–127.