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Dengue Infection in Neotropical Forest Mammals

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Abstract

In South America, dengue is the arbovirus-transmitted disease with the highest incidence. Unlike other arboviruses, wild mammals have no confirmed role in the cycle of dengue in the neotropics, although serological studies have suggested a possible secondary amplification cycle involving mammals other than nonhuman primates. In French Guiana, where all four serotypes (DENV-1, DENV-2, DENV-3, DENV-4) are present, the disease is endemic with outbreak events. To determine whether wild mammals can be infected by DENV, rodents, marsupials, and bats were captured over several periods, from 2001 to 2007, at two sites. The first location is a secondary forest surrounded by an urban area where dengue is endemic. The second location is a forest edge site where the disease has not yet emerged. A total of 10,000 trap-nights were performed and 616 mammals were captured. RNAs representing the four DENV serotypes were detected at both sites by reverse-transcriptase polymerase chain reaction in the livers and/or sera of 92 mammals belonging to 14 out of 32 species distributed among all the orders investigated: Rodentia (33 positive/146 tested), Marsupialia (40/318), and Chiroptera (19/152). Sequence analyses of a portion of the capsid and premembrane junction revealed that mammal strains of DENV-1, DENV-2, DENV-3, and DENV-4 had only 92.6%, 89%, 95%, and 95.8% identity, respectively, with strains circulating in the human population during the same periods. Regarding DENV-2, strains related (99% identity) to those responsible for an epidemic event in humans in French Guiana concurrent to the capture sessions were also evidenced, suggesting that wild mammals in edge habitats can be infected by circulating human strains. Our results demonstrate, for the first time, that neotropical wild mammals can be infected with dengue virus. The question of whether mammals maintain DENV in enzootic cycles and can play a role in its reemergence in human populations remains to be answered.

Key Words: Dengue virus; wild mammals; South America; periurban areas.

Introduction

Dengue virus (DENV) belongs to the *Flaviviridae* family and has been responsible for major epidemics in the tropics since the 17th century (Gubler 2001). Its associated disease manifestations (dengue fever, dengue hemorrhagic fever, and dengue shock syndrome) are caused by four viral serotypes designated DENV-1, DENV-2, DENV-3, and DENV-4 that are widespread in the tropics throughout the world with significant public health and economic impacts. Indeed, with respect to both morbidity and mortality, dengue is the arbovirus with the highest incidence in humans (an estimated 80–100 million infections are reported each year), with exponentially increasing occurrence (Weaver and Barrett 2004) and expanding distribution (Solomon and Mallewa 2001). The extent of epidemic events has been increasing due to several factors: the increase in human population densities and movements worldwide,

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poorly managed urbanization, and less attention paid to the eradication of domestic and peridomestic *Aedes* (*Stegomyia*) spp. mosquitoes. Up until now, factors regulating dengue outbreaks have mainly been related to the ecology of vectors, including seasonal variation of densities, relative abundance of infective females, daily survival rates (Kuno 1997), immune status of human hosts (Halstead 2007), and possibly their genetic background (Rico-Hesse 2003).

Unlike most of the other arboviruses, dengue virus possesses a unique human-to-human cycle, without the need for an intermediary wild mammalian reservoir (Rodhain 1991, Monath 1994). In Africa, Asia, and possibly in the Philippines, the virus nevertheless has, together with the human urban cycle, a sylvatic cycle, most likely involving nonhuman primates as reservoirs (Fagbami et al. 1977, Inoue et al. 2003). Serological investigations in mammals suggest that the circulation of dengue follows epidemiological patterns, without detectable clinical signs in hosts (Saluzzo et al. 1986a, de Silva et al. 1999). The oscillating nature of cycles (Diallo et al. 2003) can suggest that herd immunity of nonhuman primates and/or possibly other mammalian hosts regulates viral amplifications, but so far, wild mammals have no demonstrated role in the dengue virus emergence processes in humans. Indeed, sylvatic dengue strains are genetically and ecologically distinct from urban ones. Nevertheless, though epidemic events are induced by nonsylvatic strains (Rico-Hesse 1990), sylvatic strains can induce characteristic febrile syndromes in West Africa (Carey et al. 1971, Saluzzo et al. 1986b) and limited epidemics in urban settings (Vasilakis et al. 2008).

Sylvatic cycles have not been recognized in South America (Hayes et al. 1996, Scott 2001), in agreement with the supposed recent introduction of dengue in the New World due to human population flows during the colonization and slave trade periods, a scheme supported by both genetic variability (Gaunt et al. 2001) and historical records (Christie 1881, Steadman 1828). Two serological studies in remote forest Amerindian communities nevertheless led to divergent results with regard to the potential sylvatic circulation of DENV (Roberts et al. 1984, Valero et al. 2004). These discrepancies can be explained by virus divergence that may have led to technical difficulties in the detection of dengue viral infection by commonly used serological markers. Investigations on dengue sylvatic strains in wild mammals have also led to contradictory conclusions. Following the negative results obtained in serological investigations (Rosen 1958, Karesh et al. 1998, Contigiani et al. 2000), other works have shown indirect evidence of dengue virus infection in mammals. In Central America, antibodies that neutralized DENV-1 and DENV-2 were detected in 20% of tested bats, while in Ecuador 30% of bats had neutralizing antibodies for DENV-2 and/or DENV-3 (Platt et al. 2000). In French Guiana, neutralizing antibodies for DENV-2 virus were also detected in the sera of rodents, bats, marsupials, ungulates, and xenarthres (de Thoisy et al. 2004). But, because these neutralizing titers were generally low, they could have been induced by other flaviviruses. Nevertheless, DENV is presumed to make its own antigenic complex within the *Flaviviridae* (Calisher et al. 1989). On the basis of these studies, a strictly sylvatic dengue circulation can therefore not be excluded.

To resolve these questions, we investigated the presence of dengue virus in wild mammal fauna in areas of French Guiana, using molecular tools, where dengue is endemic, with outbreaks of DENV-1, DENV-2, and DENV-3 reported since 1991. Only DENV-4 has been detected sporadically (Dussart et al. 2006). Two distinct sites were targeted. The first was near the city of Cayenne, where all four dengue serotypes circulate. There, we monitored fauna over a 6-year period, during and between dengue epidemics, to gain insights into the susceptibility of free-ranging mammals present close to human settlements to infection with DENV and the possibility that they can maintain a continuous transmission cycle in the wild. A second site was chosen in a rural location where DENV is practically absent. This area is undergoing rapid development, with the construction of roads and the establishment of new human settlements. It was thus of twofold interest. First, it would allow us to investigate potential occurrence of sylvatic circulation. Second, as dengue emergence events are expected to be related to environmental disturbances, a long-term study site would be valuable to evaluate potential movements of DENV from humans to mammals and conversely.

Materials and Methods

Sites and trapping

French Guiana is a French overseas department on the northeast coast of the South American continent, between Brazil and Suriname (4°N 53°W). Ninety percent of its surface area of 89,000 km^2 FD is tropical rain forest; the remaining 10%, situated in the northern part of the country, is coastal plain, where 90% of the 200,000 inhabitants live. The main urban center is Cayenne, with a total of 100,000 inhabitants. The diurnal temperature in the country varies from 28°C to 30°C. The dry season occurs from August to November with a short dry period in March (50 to 150 mm rain/month) and the rainy season lasts throughout the remainder of the year (200 to 600 mm rain/month).

Animals (bats, terrestrial and arboreal rodents, and marsupials) were captured at two different sites. The first, known as "le Camp du Tigre" (CT), is a secondary forest fragment of about 100 hectares located in a periurban area near Cayenne, where dengue is endemic. Seven trapping periods were implemented: in March 2001, and in the months of May (rainy season) and October (dry season) 2005, 2006, and 2007. The site is surrounded by urban areas and has a low diversity of mammalian fauna, including small monkeys (squirrel monkeys, golden-handed tamarins), sloths, rodents, marsupials, and bats. The second site, "Saint Georges de l'Oyapock" (SG), is located near the Brazilian border and has a low human population. This trapping site is located between the edge of a primary forest and a rural area. Three trapping periods were carried out there in November 2006 and in June and November 2007.

At each site, 20 to 30 trap stations were used to capture nonflying mammals. Each station included one Tomahawk trap (50 × 18 × 18 cm; Tomahawk Live Trap Co., Tomahawk, WI) on the ground and 2 BTTm traps (33 × 11 × 10 cm; BTTm, Besançon Trap Service mécanique, France) or one BTTm and one Sherman trap (23 × 9 × 8 cm; Sherman Trap Co., Tallahassee, FL), with one trap being placed on the ground and the other in trees between 1 and 3 m in height. Traps were baited with apples and/or peanut butter for 15 to 21 consecutive nights and checked every morning. A to-

tal effort of 7257 trap-nights (a trap-night is one trap set for one night) was carried out between March 2001 and October 2007 at CT and 2822 trap-nights at SG between November 2006 and November 2007. A total of 418 mammals of the orders Rodentia and Marsupialia were captured at CT, and 46 were captured at SG (Table 1). In addition, 125 and 27 bats representing 14 species were captured at CT and SG, respectively, during the same periods on 2 to 4 consecutive nights using capture mistnets (12 × 2.40 m, 16-mm mesh; Lavorazione Reti, Monte Isola, BS Italy).

Captured animals were brought back to the laboratory, identified using external morphological characteristics (Emmons and Feer 1997), and their age and sex were determined. They were euthanized with pentobarbital (Dolethal©, Vétoquinol, Lure, France) after chemical anesthesia (ketamine 10 mg/kg + xylazine 1 mg/kg) under veterinary supervision. Blood and liver samples were collected aseptically and preserved at −80°C for later use.

Detection of dengue viral RNA in sera and liver samples by reverse-transcriptate polymerase chain reaction

RNA was extracted from sera and liver tissues using Trizol reagent (Invitrogen Life Technologies, Paisley, Refrewshire, UK) following the manufacturer's protocol. The RNA was reverse transcribed into cDNA using random hexamer oligonucleotides (Roche, Mannheim, Germany) and moloney murine leukemia virus reverse transcriptase (RT; Promega, Madison, WI). Primers and polymerase chain reaction (PCR) conditions were used following Lanciotti et al. (1992). cDNA (4 μL) was then used for the seminested PCR: During the first run of PCR, primers D1 and D2 were used to amplify a fragment of 511 bp corresponding to a portion of the capsid and premembrane (C/prM) genes. The second amplification run corresponds to a seminested PCR using primers D1 and TS1-TS2-TS3-TS4. Each of the last four primers is serotype specific, generating PCR products of different sizes (482 bp for DENV-1, 119 bp for DENV-2, 290 bp for DENV-3, and 392 bp for DENV-4). For each experiment and at every step, all the necessary controls were carried out to confirm that contamination did not occur.

After serotype identification of positive animals, the first RT-PCR products were then used for another seminested PCR amplification using D1 and one of the newly designed primers to obtain longer amplification products (Table 2). The amplification was carried out as follows: an initial denaturation at 94°C for 5 min, then 25 cycles at 94°C for 30 s, 52°C for 90 s, and 72°C for 60 s, followed by a final incubation at 72°C for 10 min. The obtained PCR products were then directly sequenced using the amplification primers. Sequencing was carried out with an automatic sequence analyzer (ABI PRISM 3700, Applera, Courtaboeuf, France) following the manufacturer's protocol. DENV PCR products obtained from humans (CNR des Arbovirus et Virus Influenza, Institut Pasteur de la Guyane) during contemporary periods were sequenced using the same primer pairs. Sequences were then aligned with other previously published sequences of dengue virus using MEGA software and alignments were checked manually. Database searches using the BLAST web server (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) were performed to identify the most closely related strains to those obtained from wild mammals.

Phylogenetic analyses were performed using PAUP*4.0b8 (Swofford 1998). The MODELTEST 3.7 program (Posada and Crandall 1998) was used to determine the optimal model of nucleotide evolution for the dataset, which was then applied for distance and maximum likelihood analyses. The resultant topology was examined by bootstrapping. One thousand iterations were performed for distance analyses and 100 replicates for maximum likelihood analyses. In addition, a Bayesian analysis was performed with MrBayes 3.1.0 (Ronquist and Huelsenbeck 2003) to infer phylogenetic relationships. Markov Chain Monte Carlo simulations were run for 1,000,000 generations, with six simultaneous chains, using a sample frequency of 100 and a burn-in of 100,000. Default settings for the prior probabilities of the model parameters (GTR+Γ4) were used.

Results

Detection of dengue virus RNA in mammals

A total of 569 livers and 560 sera collected from 616 captured mammals were tested for the presence of viral RNA by RT-PCR. Dengue viral RNA representing the four serotypes was detected in samples from both collecting sites for a total of 92 mammals (Table 1). At the CT site, 16% (87/543) of the animals were infected; the distribution of DENV-1, -2, -3, and -4 at this site was 41% (36/87), 20%, 33%, and 6% respectively. The distribution of DENV positive mammals by order was: Chiroptera 4% (19/543), Rodentia 5%, and Marsupialia 7%. At the SG site, only 7% (5/72) of the mammals, representing a single rodent species (*Proechimys cuvieri*) and trapped during a single session, were positive; they represented the DENV-3 serotype: 4% (3/72) and the DENV-4 serotype: 3% (2/72).

Sequence analyses and phylogenetic investigations

At least one C/prM sequence was obtained for each serotype from mammals collected in 2006 and 2007. These sequences were deposited in GenBank under the accession numbers EU518594–EU518605, EU522110, and EU642553.

One sequence was obtained from DENV-1 RNA detected in one bat (*Carollia perspicillata*) captured in May 2007 at CT (accession number: EU518594). BLAST database searches of the obtained sequence revealed that the DENV-1 Mochizuki strain detected in Japan in 1943 is the most closely related strain with 97.3% identity (419 bp analyzed). Furthermore, the DENV-1 strain circulating in humans in French Guiana (EU518605) during the same period presents a lesser degree of identity (92.6%; Table 3). Phylogenetic analyses show that the major genotypes of DENV-1 (genotypes IV and V) are supported with high bootstrap values with the exception of genotype I (Fig. 1). The phylogenetic reconstruction confirms that the D1 C0558 bat French Guiana 2007 sequence is closely related to the Japanese DENV-1 Mochizuchi strain with high bootstrap values (80% in distance, 64% in maximum likelihood [ML], 76% in Bayesian analysis) and belongs to genotype I. In contrast, the DENV-1 strain identified in French Guiana in the human population at a contemporary period belongs to genotype V composed of American and Asian strains.

Four sequences were obtained from DENV-2 RNA detected in four mammals captured in 2006 at CT. These sequences reveal two distinct groups. The first two sequences (EU518601 and EU518602) were detected with DENV-2 RNA

Table 1. Prevalence of DENV RNA Obtained by RT-PCR for Each Serotype in Mammals Captured in the Site of Le Camp du Tigre (CT) and in the Site of Saint Georges de l'Oyapock (SG)

	March 2001 CT				*May 2005 CT*			*Oct 2005 CT*		*May 2006 CT*					*Oct 2006 CT*					*Nov 2006 SG*			*May 2007 CT*			*June 2007 SG*	
		RT-PCR				*RT-PCR*		*RT-PCR*		*RT-PCR*					*RT-PCR*					*RT-PCR*			*RT-PCR*			*RT-PCR*	
	n	*DENV-1*	*DENV-2*	*DENV-3*	n	*DENV-1*	*DENV-2*	n	*DENV-3*	n	*DENV-1*	*DENV-2*	*DENV-3*	*DENV-4*	n	*DENV-1*	*DENV-2*	*DENV-3*	*DENV-4*	n	*DENV-3*	*DENV-4*	n	*DENV-1*	*DENV-3*	n	*DENV-1/4*
Rodentia																											
Dasyprocta leporina										1	—	—	—	—													
Holochilus sciureus	2	—	—	—																							
Makalata didelphoides	3	—	—	—	1	—	—			1	—	—	—	—									1	—	—		
Mesomys hispidus	3	—	—	—																			1	—	1	1	—
Neacomys paracou																				1	—	—					
Oecomys spp	18	12	1	—	1	—	—	2	—	2	—	—	—	—	1	—	—	—	—				4	—	—		
Oryzomys megacephalus	5	1	—	1						1	—	—	—	—	1	—	—	—	—				1	—	—		
Proechimys cayennensis	16	—	—	—	5	1	—	6	—	4	—	—	2	—	6	—	1	2	1	1	—	—	7	—	—		
Proechimys cuvieri																				10	3	2				5	—
Rattus rattus																				1	—	—					
Rhipidomys nitela																										2	—
Zygodontomys brevicaudata	16	5	—	—																							
Marsupialia																											
Caluromys philander	13	1	—	—																1	—	—					
Micoureus demerarae	17	1	—	—	3	—	—	3	—	6	—	1	—	1	4	—	—	1	—	4	—	—	7	—	—	1	—
Didelphis marsupialis	6	—	1	—	15	—	—	19	—	13	—	—	1	—	18	—	3	4	3				16	—	—	1	—
Marmosops parvidens										5	—	—	1	—	1	—	—	1	—				1	—	1		
Marmosa murina	6	—	1	—	17	—	2	15	—	13	1	2	1	—	21	—	—	7	—	2	—	—	17	—	2	5	—
Philander opossum	26	1	2	—	1	—	—	2	—	6	—	—	—	—	2	—	—	—	—							1	—
Chiroptera																											
Artibeus planirostris	20	6	—	1	14	3	3	6	1	2	—	—	—	—													
Carollia perspicillata	18	1	—	1	4	—	—	9	—	11	1	—	—	—	7	1	—	—	—				11	1	—	3	—
Cormura brevirostris															1	—	—	—	—	1	—	—					
Eptesicus chiriquinus								1	—																		
Eumops maurus																				1	—	—					
Lasiurus blossevillii																				1	—	—					
Lonchophylla thomasi																				1	—	—				1	—
Molossus molossus																				7	—	—					
Platyrrhins brachicephalus								1	—																		
Platyrrhinus helleri					3	—	—																				
Rhinolophus sp																										1	—
Sturnira lilium					4	—	—	2	—	4	—	—	—	—														
Uroderma bilobatum					2	—	—	3	—	1	—	—	—	—														
Vampyressa brocki					1	—	—																				
Total	169	28	5	3	71	4	5	69	1	70	2	3	5	1	62	1	4	15	4	31	3	2	66	1	4	21	0

n corresponds to the total of animals captured.
Dash indicates negative result.

Table 2. Oligonucleotide Primers Used to Amplify and Sequence the 4 Different Serotypes of Dengue Viruses

Primer	*Sequence*	*Genome position*	*Size in bp of amplified DNA product*
D1	5′-TCAATATGCTGAAACGCGCGAGAAACCG-3′	[a]	
D1L	5′-TAGGTCATTGTGTCCTCACATAACTCTCC-3′	560–588	457 bp (D1/D1L)
D2L	5′-CTTGTACGTGATTGTATCTTCACACA-3′	569–594	461 bp (D1/D2L)
D3L	5′-TTGTAAGTGACCGTGTCATCACACAT-3′	566–594	460 bp (D1/D3L)
D4L	5′-TCCATGGCAATGAGAGTGCATTTGTTGA-3′	533–560	424 bp (D1/D4L)

[a]The priming position of primer D1 in each genome was as follows: type 1: 132; type 2: 134; type 3: 132; type 4: 137.

The position of each primer (D1L to D4L) is given according to its position in reference sequences (DENV-1: AF226685; DENV-2: NC_001474; DENV-3: NC_001475; DENV-4: NC_002640)

The size of the amplified RT-PCR product for each serotype using D1 and each specific primer (D1L to D4L) corresponds to the length observed between D1 and each primer on its respective genome.

identified in two marsupials (*Didelphis marsupialis*). BLAST database searches revealed that they share approximately 95% identity (390 bp analyzed) with a strain isolated in India in 2001 (Table 3). Comparison with a human sequence obtained in French Guiana during the same period reveals approximately 89% identity (Table 2). The two other sequences (EU518603 and EU518604) were obtained from DENV-2 RNA detected in two *Marmosa murina* (Marsupialia) and are most closely related to a sequence detected in the Dominican Republic in 2001 with which identities vary between 99.2 and 99.7%. They are also closely related to the DENV human sequence obtained in French Guiana at the same period (98.7 and 99.2% similarity; Table 2).

Phylogenetic analyses performed on these sequences with other representative strains enabled the five major DENV-2 lineages to be identified on the basis of consistent topological associations. Nevertheless, although the Asian, American-Asian, American, and sylvatic lineages are supported with high bootstrap values, the cosmopolitan lineage is less supported and divided into two clades that are supported with high bootstrap values (Fig. 2). The DENV-2 sequences identified in mammals segregated into two distinct clusters. Two strains (D2 B1004 Marsupial French Guiana 2006 and D2 B1010 Marsupial French Guiana 2006) were related with high bootstrap values (81% in distance, 78% in ML, 100% in Bayesian analysis) to an urban strain circulating in French Guiana during the same period, and to strains identified in the Dominican Republic in 2001 (Fig. 2). These sequences are included in the American-Asian genotype that is well supported (93% in distance, 96% in ML, 100% in Bayesian analysis). The two other sequences (D2 B1015 Marsupial French Guiana 2006 and D2 B1032 Marsupial French Guiana 2006) are located at the base of the Native American lineage. This association is also well supported (87% in distance, 69% in ML, 98% in Bayesian analysis).

Three sequences were obtained from DENV-3 RNA extracted from three mammals: two in *Marmosa murina* (Marsupialia) captured at CT in October 2006 and 2007 (EU518599 and EU518598, respectively) and one in *Procchimys cuvieri* (Rodentia) captured at SG in November 2006 (EU518600). These three sequences are closely related to each other and to a strain identified in Brazil in 2002 as well as to the H87 strain isolated in the Philippines in 1956. They show from 99.7 to 100% identity (on a 364 bp fragment) with these sequences. Comparison of the three nucleotide sequences with the nucleotide sequence representing a human DENV-3 isolated in French Guiana during the same period shows approximately 95% identity (Table 3).

Phylogenetic reconstruction reveals that the three sequences detected in mammals (D3 B1018 Marsupial French Guiana 2006, D3 C1034 Marsupial French Guiana 2007, and D3 B1106 Rodent French Guiana 2006) are related with high bootstrap values (99% in distance, 97% in ML, 100% in Bayesian analysis) to strains isolated in the Philippines in 1956, in China in 1980, and in Brazil in 2002. These sequences cluster together and belong to genotype V (Fig. 3). The human strain circulating in French Guiana at a contemporary period belongs to genotype III composed of strains from Central and South America, Africa, Asia, and the West Indies (Fig. 3).

Two sequences were obtained from DENV-4 RNA detected in a marsupial *Didelphis marsupialis* (EU518596) and a rodent *Procchimys cuvieri* (EU518595), which were trapped in 2006. The C/prM sequence is identical to a strain isolated in 2001 in Peru and is closely related to the H241 strain isolated in 1956 in the Philippines (AY947539) with 99.5% identity (360 bp analyzed; Table 2). Finally, they share 95.8% identity with a strain observed in French Guiana (EU522110) in 2005. Phylogenetic reconstructions reveal that the mammalian strains (D4 B1008 Marsupial French Guiana 2006 and D4 B1106 Rodent French Guiana 2006) are associated with strains isolated in the Philippines in 1956 and 1995, in Peru (2001), and in Brazil (2006). This group of sequences belongs to genotype I, which is not well supported (Fig. 4). In contrast, the only DENV-4 strain isolated in humans in French Guiana in 2005 belongs, together with other South American and West Indian strains, to genotype II.

Discussion

In the New World, unlike in the Old World, the commonly held belief is that dengue infection is absent in the fauna. After recent evidences of positive seroneutralization in wild fauna, we identified viral RNA in many species of South American bats, rodents, and marsupials, and we provide the first C/prM sequences of strains of DENV-1, DENV-2, DENV-3, and DENV-4 circulating in animal communities. These results were found repeatedly both in an endemic area where all four serotypes circulate in the adjacent human populations (Fig. 5) and in an area where the disease is nearly

Table 3. Accession Numbers of the Different DENV Sequences Detected in Mammals and in Humans in French Guiana

	Sample		*DENV reference strain*			*% of nucleotide identity with human isolates circulating in French Guiana (GenBank accession number)*
	Identification number	*GenBank accession number*	*% nucleotide identity*	*Year/origin*	*GenBank accession number*	
DENV-1	D1 C0558 Bat French Guiana 2007	EU518594	97.3	1943/Japan	AB074760	92.6 (**EU518605**)
DENV-2	D2 B1032 Marsupial French Guiana 2006	EU518601	94.6	2001/India	DQ448238	89.2 (**EU642553**)
	D2 B1015 Marsupial French Guiana 2006	EU518602	95.1	2001/India	DQ448238	89.7 (**EU642553**)
	D2 B1010 Marsupial French Guiana 2006	EU518603	99.7	2001/Dom. Republic	AB122021	99.2 (**EU642553**)
	D2 B1004 Marsupial French Guiana 2006	EU518604	99.2	2001/Dom. Republic	AB122021	98.7 (**EU642553**)
DENV-3	D3 C1034 Marsupial French Guiana 2007	EU518598	99.7	2002/Brazil	EF629370	95.0 (**EU518598**)
			99.7	1956/Phillipines	M93130	
	D3 B1018 Marsupial French Guiana 2006	EU5185599	100	2002/Brazil	EF629370	95.0 (**EU518598**)
			100	1956/Phillipines	M93130	
	D3 B1106 Rodent French Guiana 2006	EU518600	99.7	2002/Brazil	EF629370	95.0 (**EU518598**)
			99.7	1956/Phillipines	M93130	
DENV-4	D4 B1106 Rodent French Guiana 2006	EU518595	100	2001/Peru	AY079176	95.8 (**EU522110**)
	D4 B1008 Marsupial French Guiana 2006	EU518596	100	2001/Peru	AY079176	95.8 (**EU522110**)

The percentage of nucleotide identity of the strains detected in mammals are given with (i) the most closely related strains and their GenBank accession number; and (ii) the strains detected in human in French Guiana at contemporary periods.

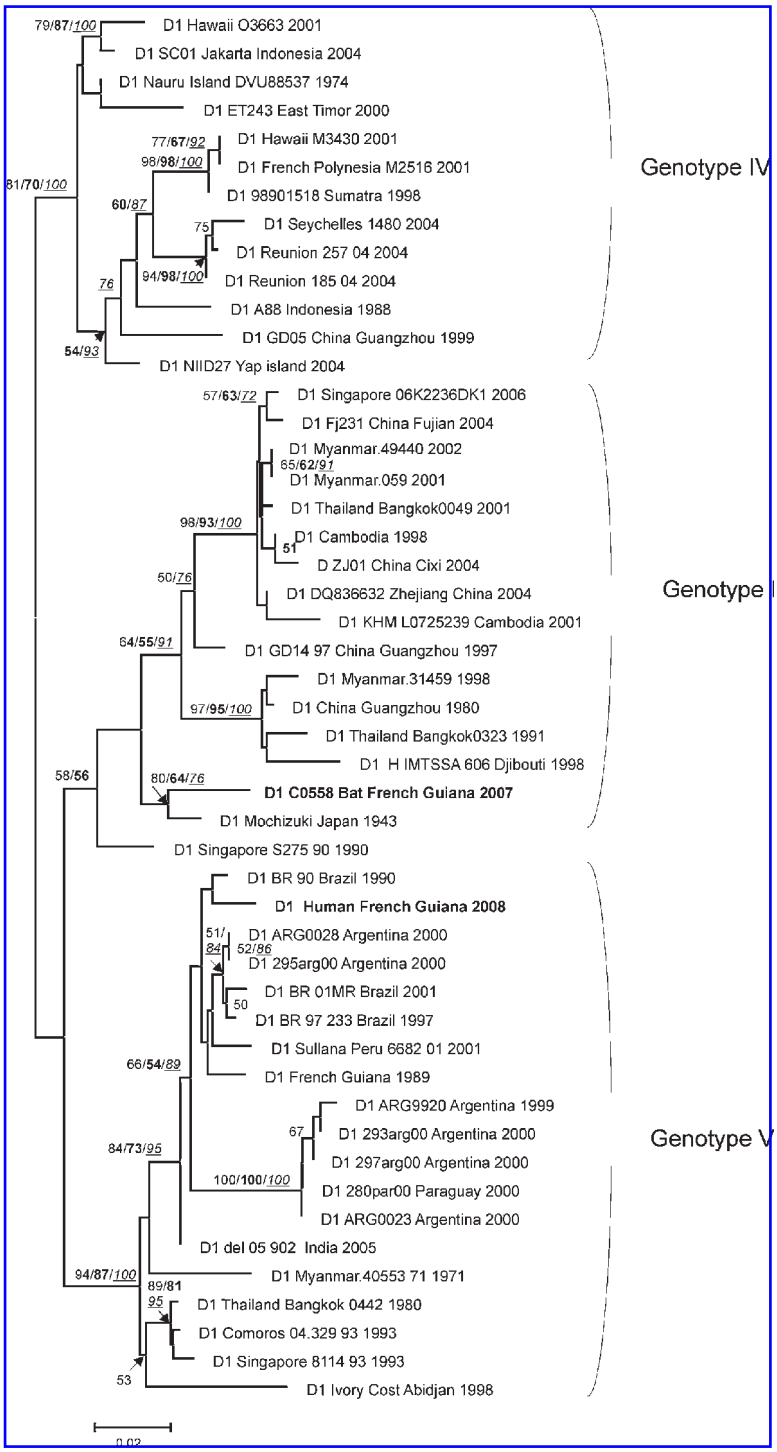

FIG. 1. Phylogenetic Analysis of DENV-1 Isolates. The phylogenetic tree was derived from the partial nucleotide sequences of the C/prM genes (419 bp excluding primers) of representative strains belonging to the major lineages of DENV-1 (as designated by Goncalvez et al., 2002) using Distances, Maximum Likelihood (ML) (PAUP, version 4.10), and Bayesian analyses (1,000,000 replicates, MrBayes, version 3.1b). The following ML parameters corresponding to the TrN + G model were used: Nucleotide frequencies were estimated to be as follows: A = 0.3098, C = 0.1997, G = 0.2389, T = 0.2515. The –ln*L* value was 1816.49. The shape parameter of the gamma distribution was estimated to be 0.2005. The tree was rooted on the strains belonging to genotype IV. Support for nodes was provided by bootstrapping and the posterior probabilities of the corresponding clades (*i.e.* 1000 and 100 replicates under distances and ML (in normal and boldface type respectively) and under Bayesian analyses (in italics and underlined). DENV strains sequenced in this study are shown in boldface.

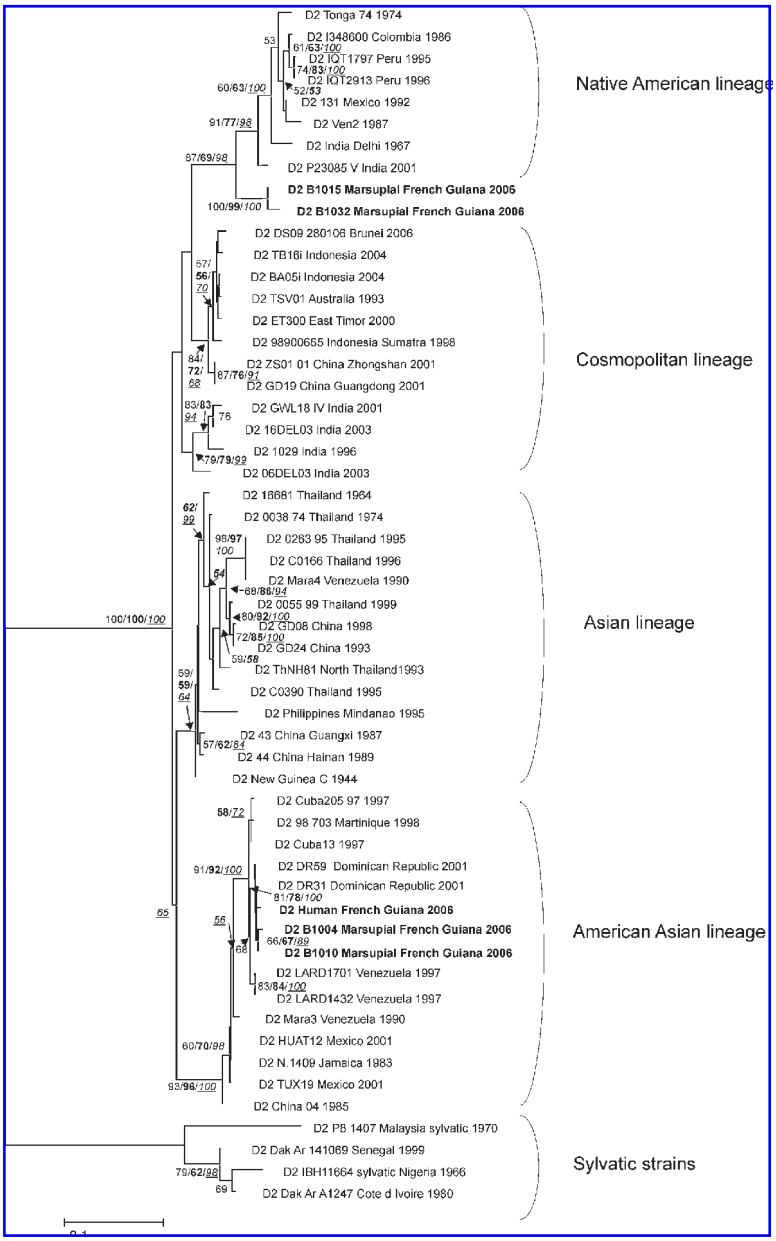

FIG. 2. Phylogenetic Analysis of DENV-2 Isolates. The phylogenetic tree was derived from the partial nucleotide sequences of the C/prM genes (390bp excluding primers) of sylvatic and representative endemic DENV-2 (genotypes designated by Twiddy et al., 2002) using Distances, Maximum Likelihood (ML) (PAUP, version 4.10), and Bayesian analyses (1,000,000 replicates, MrBayes, version 3.1b). The following ML parameters corresponding to the GTR + G model were used: Nucleotide frequencies were estimated to be as follows: A = 0.3249, C = 0.2254, G = 0.2467, T = 0.2030. The –ln*L* value was 2082.37. The shape parameter of the gamma distribution was estimated to be 0.2596. The tree was rooted on sylvatic strain sequences. Support for nodes was provided by bootstrapping and the posterior probabilities of the corresponding clades (*i.e.* 1000 and 100 replicates under distances and ML (in normal and boldface type respectively) and under Bayesian analyses (in italics and underlined). DENV strains sequenced in this study are shown in boldface.

absent. These findings suggest either spillover infections from humans to mammals or a possible enzootic cycle involving mammals. DENV sequences obtained from mammals led to the development of two plausible scenarios.

First, on the basis of C/prM sequences, most of the DENV-1, -2, -3, and -4 strains obtained from wild mammals were divergent from those circulating in the surrounding human populations during the same periods. The strains identified in the wild mammalian fauna were found, at different periods, in periurban areas and in forest areas, where human contacts are limited. This suggests that DENV infection of wild mammals and its circulation in mammal communities are widespread, which eliminates the possibility that our positive results could be related to incidental, local, and/or time-restricted epiphenomena. Nevertheless, some authors have already recorded such divergent strains and attributed them to laboratory contamination (Rico-Hesse 2003). Although laboratory contamination can-

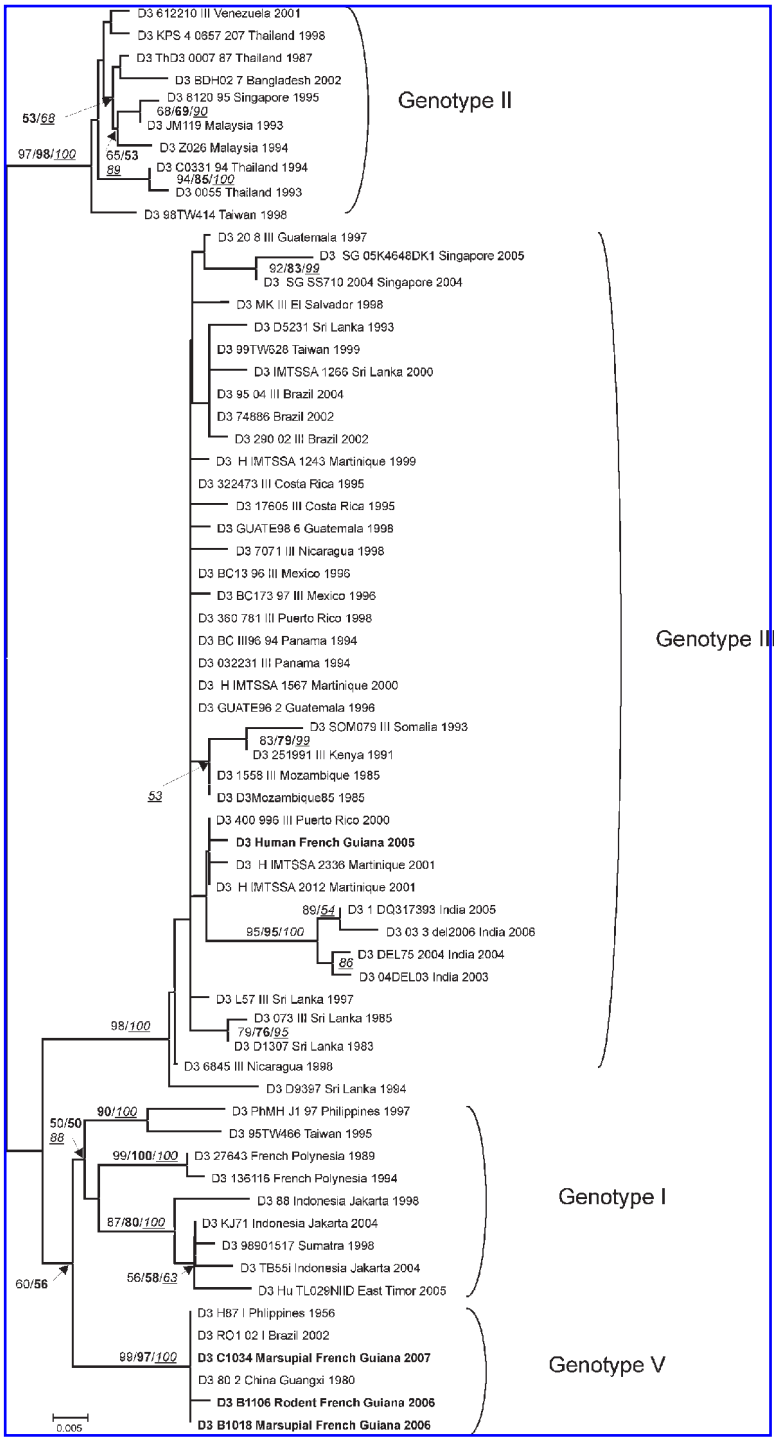

FIG. 3. Phylogenetic Analysis of DENV-3 Isolates. The phylogenetic tree was derived from the partial nucleotide sequences of the C/prM genes (364 bp excluding primers) of representative strains belonging to the major lineages of DENV-3 (designated by Wittke et al. 2002) using Distances, Maximum Likelihood (ML) (PAUP, version 4.10), and Bayesian analyses (1,000,000 replicates, MrBayes, version 3.1b). The following ML parameters corresponding to the TIM + G model were used: Nucleotide frequencies were estimated to be as follows: A = 0.3134, C = 0.2206, G = 0.2354, T = 0.2306. The –ln*L* value was 1279.91. The shape parameter of the gamma distribution was estimated to be 0.2177. The tree was rooted on the strains belonging to genotype II. Support for nodes was provided by bootstrapping and the posterior probabilities of the corresponding clades (*i.e.* 1000 and 100 replicates under distances and ML (in normal and boldface type respectively) and under Bayesian analyses (in italics and underlined). DENV strains sequenced in this study are shown in boldface.

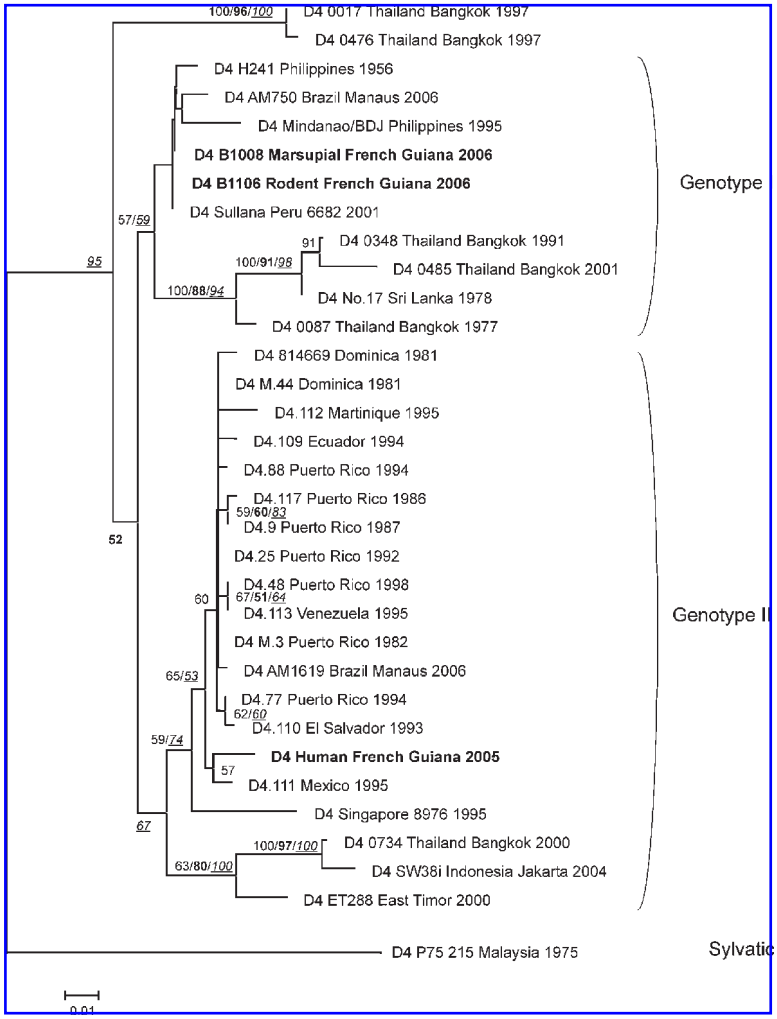

FIG. 4. Phylogenetic Analysis of DENV-4 Isolates. The phylogenetic tree was derived from the partial nucleotide sequences of the C/prM genes (360 bp excluding primers) of sylvatic and representative endemic DENV-4 (genotypes designated by Lanciotti et al. 1997) using Distances, Maximum Likelihood (ML) (PAUP, version 4.10), and Bayesian analyses (1,000,000 replicates, MrBayes, version 3.1b). The following ML parameters corresponding to the TIM + G model were used: Nucleotide frequencies were estimated to be as follows: A = 0.3071, C = 0.2188, G = 0.2431, T = 0.2310. The –ln*L* value was 1251.68. The shape parameter of the gamma distribution was estimated to be 0.3141. The tree was rooted on sylvatic strain sequences. Support for nodes was provided by bootstrapping and the posterior probabilities of the corresponding clades (*i.e.* 1000 and 100 replicates under distances and ML (in normal and boldface type respectively) and under Bayesian analyses (in italics and underlined). DENV strains sequenced in this study are shown in boldface.

not be excluded, we considered that this hypothesis bore little relevance to our work. Indeed, none of the strains closely related to the ones we identified in the fauna, with the exception of the H87 DENV-3 strain, have been handled in our institution. Furthermore, viral RNAs were extracted and amplified right after the collection of the samples, and the different mammal dengue strains were identified repeatedly. Our data thus strongly suggest a forest circulation of all four serotypes of DENV, but conclusive evidence will only be supported by isolating DENV from forest-dwelling mosquitoes.

Moreover, although the relatedness of the mammalian strains to the human ones is only based on a 400 bp analysis, DENV-1, DENV-3, and DENV-4 strains are related to Asian strains that were isolated decades ago. The origin of these strains in South American fauna thus remains highly questionable. It could be explained either by the history of dengue dispersal during the 17th and 18th centuries (Duenas 1909), or by its introduction by Asian migrants in the first half of the 20th century (Rico-Hesse 2003). Then, mammals could have maintained these strains and, under the right conditions (vector pullulation, environmental disturbances), these strains could have escaped from mammals and circulated at low levels among human populations. For instance, recent DENV-4 strains isolated in humans in Brazil in 2005 and 2006 cluster with the mammalian strains we describe here (Pinto de Figueiredo et al. 2008). In the same way, some DENV-3 strains detected in Brazil in 2002 (EF629370) and during epidemics that occurred there from 2002 to 2004 (Figueiredo et al. 2008) belong to genotype I and are closely related to the ones we detected in mammals in French Guiana. Nevertheless, considering the lim-

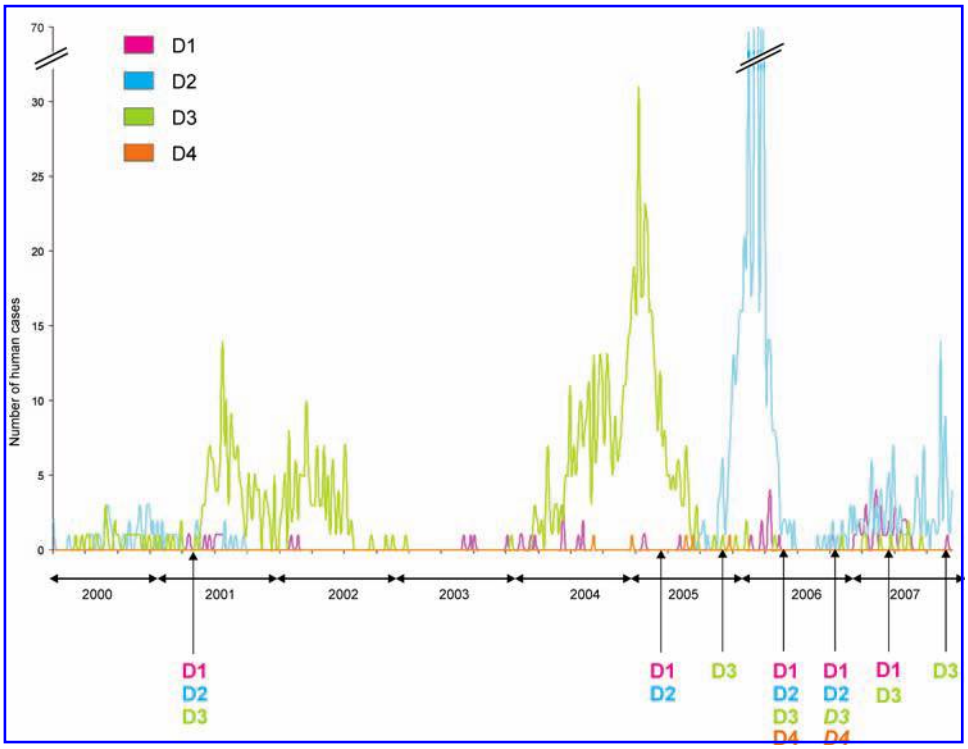

FIG. 5. Prevalence of the Different Serotypes of DENV in the Human Population from January 2000 to December 2007 in French Guiana as registered by the Centre National de Référence des Arbovirus et Virus Influenza. Time is indicated in years using horizontal black arrows. Vertical black arrows indicate the period during which mammals were trapped and samples were collected. DENV serotypes identified in mammals during the different sampling periods are presented below each arrow. Dengue serotypes in boldface correspond to those identified in the area of le Camp du Tigre (CT) and serotypes in boldface and italics correspond to those identified at CT and at the Saint Georges de l'Oyapock (SG) site.

ited size of the sequences analyzed and, despite the fact that the phylogenetic picture obtained is relevant and congruent, hypotheses on the origins of these mammalian strains and the possible infection of humans by them have to be considered with caution. Generating the complete envelope gene sequences should enable us to support or refute this scenario.

Secondly, the D2 B1004 20066 and D2 B1010 2006 sequences support the notion that fauna can also be infected by strains circulating in surrounding human populations. In fact, DENV-2 caused an epidemic in French Guiana from January to September 2006, and the two strains identified in two *Marmosa murina* (marsupialia) in October 2006 are related to the ones responsible for that epidemic. This strongly suggests that under the pressure of a strong epidemic event, urban strains could enter the forest and then infect the fauna. Such transmission, facilitated by hunting, logging, or tourism, has already been suspected with *Plasmodium falciparum* (Volney et al. 2002). Moreover, the vector *Aedes* (*Stegomyia*) *aegypti*, though mainly anthropophilic, can be found at the edge of forest habitats (Fouque and Carinci 1996) and could therefore provide a mechanism for virus introduction in forest animal communities. It thus seems to be of major importance to consider wildlife species not only as potential reservoirs for DENV, but also as potential hosts, sensitive to infection by "human" infectious agents.

On the basis of these results, it is suggested that wild animals could maintain the four DENV serotypes in South America. Further investigations will have to be conducted to confirm their potential role as reservoirs and/or as secondary hosts (Haydon et al. 2002). Actually, all these mammalian species can be either an epidemic dead end or can play a role in maintaining the virus during interepidemic periods or even in virus amplification. Experimental infections will therefore be necessary to assess the efficiency of wild mammals as reservoirs. The ecological dynamics of the mammalian species in relation to that of the virus will also need to be explored. Lastly, extensive research on vectors will be required. *Aedes* (*Stegomyia*) *aegypti* is the vector of DENV in French Guiana, since *Aedes* (*Stegomyia*) *albopictus* is not recorded in the department. Alhough it is associated with human settlements, it has also been found in the rainforest (Fouque et al. 2004) and could therefore be involved in DENV transmission between humans and mammals and between mammals. Our preliminary mosquito surveys at CT revealed relatively low population densities of *Aedes* spp., which raises the question of whether other mosquito species identified in the area (e.g., *Coquilletidia, Culex*, etc.) might be involved in the circulation of the virus among mammals. Experimental infections have suggested that the role of *Culex* mosquitoes may be negligible in DENV transmission (Vazeille-Falcoz et al. 1999), but extensive vectorial capacity studies on locally captured mosquitoes will be required. Once the questions raised above will have been explored, the results obtained could profoundly modify our vision of the epidemiology of dengue virus in South America.

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Disclosure Statement

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References

Calisher, CH, Karabatsos, N, Dalrymple, JS, Westaway, EG, et al. Antigenic relationships between flaviviruses as determined by cross-neutralization with polyclonal antisera. J Gen Virol 1989; 70:37–43.

Carey, DE, Causey, OR, Reddy, S, Cooke, AR. Dengue viruses from febrile patients in Nigeria, 1964–68. Lancet 1971; 1:105–106.

Contigiani, MS, Fernandez, C, Spinsanti, LI, Diaz, GE. Prevalencia de anticuerpos en el primate *Alouatta caraya* autoctono de la Argentina. Medicina 2000; 60:348–350.

Christie, J. On epidemics of dengue fever: their diffusion and etiology. Glasgow Medical Journal 1881; 16:161–176.

Diallo, M, Ba, Y, Sall, AA, Diop, OM, et al. Amplification of the sylvatic cycle of dengue virus type 2, Senegal, 1999–2000: entomologic findings and epidemiologic considerations. Emerg Infect Dis 2003; 9:362–367.

de Silva, AM, Dittus, WP, Amerasinghe, PH, Amerasinghe, FP. Serologic evidence for an epizootic dengue virus infecting toque macaques (*Macaca sinica*) at Polonnaruwa, Sri Lanka. Am J Trop Med Hyg 1999; 60:300–306.

de Thoisy, B, Dussart, P, Kazanji, M. Wild terrestrial rainforest mammals as potential reservoirs for flaviviruses (yellow fever, dengue 2 and St Louis encephalitis viruses) in French Guiana. Trans R Soc Trop Med Hyg 2004; 98:409–412.

Duenas, DJ. Dengue Muermo. In: Pfaundler, M, Schlossmann A, eds. *Tratado Enciclopedico de Pediatria*. San Augustin, Barcelona, Spain, 1909:5–42.

Dussart, P, Lavergne, A, Lagathu, G, Lacoste, V, et al. Reemergence of dengue virus type 4, French Antilles and French Guiana, 2004-2005. Emerg Infect Dis 2006; 12:1748–1751.

Emmons, L, Feer, F. *Neotropical rainforest mammals: a field guide*. Chicago, University of Chicago Press, 1997:307.

Fagbami, AH, Monath, TP, Fabiyi, A. Dengue virus infections in Nigeria: a survey for antibodies in monkeys and humans. Trans R Soc Trop Med Hyg 1977; 71:60–65.

Figueiredo, LB, Cecilio, AB, Ferreira, GP, Drumond, BP et al. Dengue virus 3 genotype 1 associated with dengue fever and dengue hemorrhagic fever, Brazil. Emerg Infect Dis 2008; 14: 314–316.

Fouque, F, Carinci, R. *Aedes aegypti* in French Guiana. Some aspects of history, general ecology and vertical transmission of the dengue virus. Bull Soc Pathol Exot 1996; 89:115–119.

Fouque, F, Garinci, R, Gaborit, P. Epidemiological and entomological surveillance of the co-circulation of DEN-1, DEN-2 and DEN-4 viruses in French Guiana. Trop Med Int Health 2004; 9:41–46.

Gaunt, MW, Sall, AA, de Lamballerie, X, Falconar, AK, et al. Phylogenetic relationships of flaviviruses correlate with their epidemiology, disease association and biogeography. J Gen Virol 2001; 82:1867–1876.

Goncalvez, AP, Escalante, AA, Pujol, FH, Ludert, JE, et al. Diversity and evolution of the envelope gene of dengue virus type 1. Virology 2002; 303:110–119.

Gubler, DJ. Human arbovirus infections worldwide. Ann N Y Acad Sci 2001; 951:13–24.

Halstead, SB. Dengue. Lancet 2007; 370:1644–1652.

Haydon, DT, Cleaveland, S, Taylor, LH, Laurenson, MK. Identifying reservoirs of infection: a conceptual and practical challenge. Emerg Infect Dis 2002; 8:1468–1473.

Hayes, CG, Phillips, IA, Callahan, JD, Griebenow, WF, et al. The epidemiology of dengue virus infection among urban, jungle, and rural populations in the Amazon region of Peru. Am J Trop Med Hyg 1996; 55:459–463.

Inoue S, Morita K, Matias RR, Tuplano JV, et al. Distribution of three arbovirus antibodies among monkeys (*Macaca fascicularis*) in the Philippines. J Med Primatol 2003; 32:89–94.

Karesh, WB, Wallace, RB, Painter, RLE, Rumiz, D, et al. Immobilization and health assessment of free-ranging black spider monkeys (*Ateles paniscus chamek*). Am J Primatol 1998; 44:107–123.

Kuno, G. Factors influencing the transmission of dengue viruses. In Gubler, D, Kuno, G, eds. *Dengue and dengue hemorrhagic fever*. Wallingford, England, CAB international, 1997:61–88.

Lanciotti, RS, Calisher, CH, Gubler, DJ, Chang, GJ, et al. Rapid detection and typing of dengue viruses from clinical samples by using reverse transcriptase-polymerase chain reaction. J Clin Microbiol 1992; 30:545–551.

Lanciotti, RS, Gublern, DJ, Trent, DW. Molecular evolution and phylogeny of dengue-4 viruses. J Gen Virol 1997; 78:2279–2284.

Monath, TP. Yellow fever and dengue—the interactions of virus, vector and host in the re-emergence of epidemic disease. Semin Virol 1994; 5:133–145.

Pinto de Figueiredo, RM, Gomes Naveca, F, de Souza Bastos, M, dos Nascimento Melo, M, et al. Dengue virus type 4, Manaus, Brazil. Emerg Infect Dis 2008; 14:667–669.

Platt, KB, Mangiafico, JA, Rocha, OJ, Zaldivar, ME, et al. Detection of dengue virus neutralizing antibodies in bats from Costa Rica and Ecuador. J Med Entomol 2000; 37:965–967.

Posada, D, Crandall, KA. Modeltest: Testing the model of DNA substitution. Bioinformatics 1998; 14:817–818.

Rico-Hesse, R. Molecular evolution and distribution of Dengue viruses type 1 and 2 in nature. Virology 1990; 174:479–483.

Rico-Hesse, R. Microevolution and virulence of dengue viruses. Adv Virus Res 2003; 59:315–341.

Roberts, DR, Peyton, EL, Pinheiro, FP, Balderrama, F, et al. Associations of arbovirus vectors with gallery forests and domestic environments in southeastern Bolivia. Bull Pan Am Health Organ 1984; 18:337–350.

Rodhain, F. The role of monkeys in the biology of dengue and yellow fever. Comp Immunol Microbiol 1991; 14:9–19.

Ronquist, F, Huelsenbeck, JP. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 2003; 19:1572–1574.

Rosen, L. Observations on the epidemiology of Dengue in Panama. Am J Hygiene 1958; 68:45–58.

Saluzzo, JF, Cornet, M, Adam, C, Eyraud, M, et al. Dengue 2 in eastern Senegal: serologic survey in simian and human populations. 1974–85. Bull Soc Pathol Exot Filiales 1986a; 79:313–322.

Saluzzo, JF, Cornet, M, Castagnet, P, Rey, C, et al. Isolation of Dengue 2 and Dengue 4 viruses from patients in Senegal. Trans R Soc Trop Med Hyg 1986b; 80:5.

Scott, TW. Are bats really involved in dengue virus transmission? J Med Entomol 2001; 38:771–772.

Solomon, T, Mallewa, M. Dengue and other emerging flaviviruses. J Infect 2001; 42:104–115.

Steadman, GW. Some account of an anomalous disease which raged in the islands of v St. Thomas and Santa Cruz, in the West Indies, during the months of September, October, November, December, and January 1827–8. Edinb. Med. and Surg. Journ. 1828; 30:227–248.

Swofford, DL. PAUP*. Phylogenetic Analysis Using Parsimony (and Other Methods) 1998; Version 4. Sinauer, Sunderland, Mass.

Twiddy, SS, Farrar JJ, Vinh Chau, N, Wills, B, et al. Phylogenetic relationships and differential selection pressures among genotypes of dengue-2 virus. Virology 2002; 298:63–72.

Valero, N, Espina, LM, Estevez, J, Melean, E, et al. Immunity to flavivirus in Amerindian population of the Sierra de Perija, Zulia state, Venezuela. Invest Clin 2004; 45:337–345.

Vasilakis, N, Tesh, RB, Weaver, SC. Sylvatic Dengue virus type 2 activity in humans, Nigeria, 1966. Emerg Infect Dis 2008; 14:502–504.

Vazeille-Falcoz, M, Rosen, L, Mousson, L, Rodhain, F. Replication of dengue type 2 virus in *Culex quinquefasciatus* (Diptera : Culicidae). Am J Trop Med Hyg 1999; 60:319–321.

Volney, B, Pouliquen, JF, de Thoisy, B, Fandeur, T. A sero-epidemiological study of malaria in human and monkey populations in French Guiana. Acta Trop 2002; 82:11–23.

Weaver, SC, Barrett, AD. Transmission cycles, host range, evolution and emergence of arboviral disease. Nat Rev Microbiol 2004; 2:789–801.

Wittke V, Robb TE, Thu HM, Nisalak, A, et al. Extinction and rapid emergence of strains of dengue 3 virus during an interepidemic period. Virology 2002; 301:148–156.

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