Massilia Virus, A Novel *Phlebovirus* (*Bunyaviridae*) Isolated from Sandflies in the Mediterranean

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Abstract

A new virus was isolated from three independent pools of *Phlebotomus perniciosus* sandflies (*Diptera*; *Psychodidae*) trapped in two regions of southeastern France, located 90 miles apart. Microscopic, antigenic and genetic analyses indicate that this novel virus belongs to the genus *Phlebovirus* in the family *Bunyaviridae*. The new virus is designated Massilia virus since the first isolate was obtained from sandflies collected in the suburban area of Marseille. The complete genome sequence was determined and used to compare the genetic and phylogenetic relationships of Massilia virus with other phleboviruses. Genetic and antigenic properties were employed to address whether or not Massilia virus should be considered a new species within the genus, or a member of a previously recognized species. Cerebrospinal fluid specimens, collected from local patients with central nervous system infections during the previous four-year period were tested for the presence of Massilia virus RNA, but gave negative results. In conclusion, Massilia virus is proposed as a member of the *Sandfly fever Naples virus* complex; its public health importance has yet to be determined.

Key Words: Aedes—Arbovirus—Culex—Chikungunya—Dengue—Rovent-borne—Sand fly—Vector-borne—Mosquito.

Introduction

THE GENUS *PHLEBOVIRUS* currently includes 37 viruses grouped into 9 species with 16 additional viruses registered as tentative species (Nichol et al. 2005). Based on serologic (antigenic) relationships, the *Phlebovirus* genus is further subdivided into the sandfly fever and Uukuniemi groups. Phleboviruses are arthropod-borne RNA viruses with a genomic organization consisting of three segments of single stranded RNA, designated S (small), M (medium), and L (large), respectively, encoding nucleoprotein and NS, envelope glycoproteins, and the viral polymerase. Most of the sandfly fever group viruses are associated with and presumably transmitted by sandflies (*Diptera*; *Psychodidae*). Sandflies are tiny insects living in periurban or rural environments, often close to domestic animals and humans. Sandflies are silent when flying; their bite causes pain, and usually occurs at night. Thus, they are often unnoticed or ignored by humans. In temperate regions, peak sandfly ac-

tivity occurs during summertime. Sandflies are widely distributed in all countries around the Mediterranean. Therefore, human populations in this area are exposed to sandfly-transmitted diseases, including those caused by phleboviruses. Toscana virus is a prominent cause of aseptic acute meningitis in the Mediterranean Region (Charrel et al 2005; Hemmersbach-Miller et al 2004), but seroprevalence data suggest that asymptomatic or mild febrile illnesses due to Toscana virus are significantly more prevalent than was previously believed. Sandfly fever Sicilian and Naples viruses also frequently cause epidemics of febrile illness during the summer. Sandflies trapped in two different regions of southeastern France, located 90 miles apart, were recently tested for the presence of phleboviruses. A new phlebovirus was detected and isolated from pools of *Phlebotomus perniciosus* from both sources. This report describes the morphologic, genetic and antigenic characterization of the new virus, provisionally called Massilia virus.

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Materials and Methods

Capture of phlebotomine sandflies and description of study sites

Sandflies were trapped in July 2005 over a four-day period in two cities in southern France (Marseille and Nice). CDC Miniature Light Traps (John W Hock Company, Gainesville, FL) were modified using an ultra-fine mesh placed on a 20 cm by 20 cm cubic steel frame and hung 1–2 m above the ground in quiet shaded places, as previously described (Izri et al. 1990; Charrel et al. 2006). In Marseille, traps were placed in animal housing facilities, such as horse stalls, rabbit hutches and henhouses in the northwestern and eastern areas of the town. In Nice, traps were placed in the suburban neighborhoods of the city, in the vicinity of rabbit hutches and in sites previously known as endemic areas for canine visceral leishmaniasis (also transmitted by *Phlebotomus* sandflies) (Marty et al. 1994). All traps were placed at

dusk (around 6:00 P.M.) for the night, during four successive nights. Geographic coordinates were recorded using a Global Positioning System. Each morning, sandflies were collected, and identified morphologically, by dissecting genital organs, according to morphologic taxonomic keys (Leger et al. 1983). Sandflies were pooled with a maximum of 30 individuals per pool, based on trapping origin, species and gender, and were placed in 1.5 mL tubes and stored at -80°C .

Detection of phleboviruses in sandfly pools by RT-PCR

Pools of entire sandflies were ground in 20% fetal bovine serum-enriched phosphate buffered saline, using a Mixer Mill MM300 (Qiagen, Courtaboeuf, France) with one 3-mm tungsten bead and 24 cycles/sec frequency for 30–60 sec, depending on the homogeneity of the material. The resulting mixture was clarified by centrifugation at 5800 g for 5 min, and the supernatant fluid was aliquoted (3 aliquots of 200 μL for each pool) and stored at -80°C . One 200 μL aliquot was

FIG. 1. Phylogeny of Massilia virus and other phleboviruses—Phylogenetic trees based on multiple alignment of the six virus genes of Massilia virus together with homologous sequences of other phleboviruses for which data were available in the Genbank database. Viruses which are listed in the 8th Report of the International Committee for Taxonomy of Viruses [ICTV] (Nichol et al. 2005) are indicated by the Genbank protein acc no and the acronym. Viruses which are not listed in the Report are indicated by the Genbank protein acc no and the strain reference. Recognized and tentative species, as recognized by the ICTV, are indicated by color codes: green, *Punta Toro virus*; yellow, *Sandfly fever Naples virus*; blue, *Sandfly fever Sicilian virus* and *Corfou virus*; grey, *Frijoles virus*; brown, *Rift valley fever virus*; black, *Uukuniemi virus*; pink, *Salehebad virus*. Massilia virus is indicated in red since its taxonomic status is not yet defined.

FIG. 1. (Continued).

used for viral RNA purification with the BioRobot EZ1 (Viral RNA Mini Kit, Qiagen, Courtaboeuf, France) and directly stored at -80°C . A total of $10\ \mu\text{L}$ of RNA suspension was used for RT-PCR. A variety of primers targeting different genes was used in independent reactions: (i) phlebovirus consensus primers targeting the polymerase gene in the L RNA segment (Sanchez-Seco et al 2003), and (ii) primers specific for phleboviruses within the *Sandfly fever Naples virus* complex and targeting the nucleoprotein gene in the S RNA segment (Charrel et al 2007). The RT-PCR was performed using the Access RT-PCR kit (Promega, Charbonnières-les-

Bains, France), according to the manufacturer's recommendations. L and S RNA primers were used at $0.8\ \mu\text{M}$ and $0.4\ \mu\text{M}$ per reaction, respectively. The cycling program of the RT-PCR reaction consisted of 48°C for 45 min and 94°C for 2 min, followed by 40 cycles at 94°C for 30 sec, the annealing temperature for 1 min, and 68°C for 45 sec, with a final elongation step at 68°C for 7 min. Nested PCRs were performed using the same conditions with 1,25UI Taq DNA polymerase (Invitrogen, Cergy Pontoise, France). PCR products were analyzed in a 2% TAE agarose electrophoresis gel, and sequenced in both directions.

Sequence analysis and molecular characterization of phleboviruses

Consensus sequences were produced by overlapping the sequences obtained in both directions in the same gene using the Sequencher 4.5 software (Gene Codes Corporation, Ann Arbor, MI) and submitted to NCBI BLASTn and BLASTx programs (www.ncbi.nlm.nih.gov/BLAST/) for identification. Multiple sequence alignment was achieved with ClustalX 1.81 together with sequences from other phleboviruses retrieved from Genbank. Accession numbers of Genbank sequences used for genetic analyses are indicated in the phylogenetic trees (Fig. 1). Arbia virus was obtained from Loredana Nicoletti (Department of Infectious, Parasitic, and Immunomediated Diseases, Istituto Superiore di Sanita, Rome) and cultured in Vero cells. A clarified supernatant extract of infected cells was used for nucleic acid extraction and further PCR amplification and sequencing, as described above.

Genetic distances and phylogenetic analyses

Phylogenetic studies were conducted using MEGA version 2.1 (Kumar et al. 2001). Genetic distances were calculated with the p-distance method at the amino acid level. Phylogenetic trees were constructed using the neighbor-joining method. The robustness of the nodes was tested by 500 bootstrap replications. Nucleotide and amino acid sequences in the nucleoprotein gene of selected phleboviruses were used to study the distribution of evolutionary distances upon pairwise comparison, as previously described (Charrel et al. 2001).

Virus isolation, morphologic and antigenic studies

Sandfly homogenates from the PCR-positive pools W, BM and BP) were used to inoculate Vero-E6 cells. Briefly, 100 μ L

of each homogenate was used to seed Vero-E6 monolayers under a 12-well plate format in the presence of an equal volume of Basal Medium Eagle (BME) without fetal bovine serum (FBS), but enriched with antibiotics (100 U/mL penicillin G, 100 U/mL Streptomycin sulfate). After incubation at 37°C for one hour, the supernatant fluid was collected and mixed with 1.8 mL of BME enriched with 5% FBS; this 1:10 dilution was used to seed two wells of the 12-well plate (1 mL in each well). The first well was then filled with 1 mL of fresh 5% FBS enriched BME. The plate was incubated at 37°C in an atmosphere containing 5% CO₂. Plates were examined daily for the presence of cytopathic effect, and 100 μ L of each supernatant medium was tested by RT-PCR after total nucleic acid extraction with the EZ-1 biorobot (Qiagen) following the virus protocol. Tissue-culture samples showing cytopathic effect were also prepared for electron-microscopic examination. Negative-stained electron-microscopic specimens were prepared by drying culture supernatant medium, mixed 1:1 with 2.5 percent paraformaldehyde, onto formvarcarbon-coated grids and staining with 2 percent methylamine tungstate. Massilia virus infected cells were used to prepare slides for immunofluorescence microscopy after cytospin centrifugation. Slides were air dried and fixed with 100% cold acetone for 20 min. The immunofluorescence assay used a human serum, containing Toscana virus IgG (Hemmersbach-Miller et al., 2004), in conjunction with a fluorescein isothiocyanate-conjugated goat antihuman IgG (Fluoline G; BioMerieux, Marcy l'Etoile, France), as previously described (Fulhorst et al. 1997).

Serologic techniques

Hyperimmune mouse ascitic fluids (antibodies) were prepared against Massilia virus and each of the other phleboviruses was tested, as described previously (Beaty et al. 1989). Complement-fixation (CF) tests were performed ac-

FIG. 2. Morphological identification of Massilia virus—A–D. Negative-stain electron microscopy of Vero E6 culture supernatant medium at day six post infection (sixth passage). The bars represent 100 nm. E. Vero E6 cells infected with biological material from pool W are shown reacting with anti-TOSV human serum. F. Uninfected Vero E6 cells reacting with anti-TOSV human serum

according to a previously described microtechnique (Beatty et al. 1989). The immune ascitic fluids were inactivated at 60°C for 20 minutes and mixed with test antigen and 2 full units of complement. This first phase of the test was incubated overnight at 4°C; the following day, the haemolytic system (hemolysin + sheep cells) was added. After 30 minutes of incubation at 37°C and 60 minutes at 4°C, the plates were read visually. Titers were recorded as the highest dilutions giving 3+ or 4+ fixation of complement on a scale of 0 (negative = complete hemolysis) to 4+ (positive = no hemolysis). Hemagglutination-inhibition (HI) testing was done in microtiter plates, according to methods described by Shope (1963). Non-specific inhibitors of sera were extracted with acetone by the method of Clarke and Casals (1958). HI tests were performed with 4 units of antigen at the pre-determined optimal pH, against a 1:20 serum dilution used for screening. The tests were incubated for 15 minutes at room temperature before reading. Endpoints were determined on positive sera.

RT-PCR assays

A real-time RT-PCR assay was designed in the nucleoprotein gene specifically to target Massilia virus, not the other viruses in the *Sandfly fever Naples virus* complex. Sense and reverse primer sequences were CATGAACAGYCCATGTGCG and CATTGCTGGSACAACCTTATCC, respectively. The Taqman probe (AGCCAGCATAGCACCAGTRTTGT) was labeled with FAM and TAMRA. The PCR product was 118-bp long. The specificity of this assay was evaluated using Toscana virus (Italian and Spanish-French genotypes), and Arbia virus. A second PCR assay, using primers SFNV-S2 and SFNV-R2, in a SYBR Green real-time RT-PCR format, amplifying all phleboviruses within the *Sandfly fever Naples virus* complex was used to test the CSF samples (Charrel et al. 2007).

Results

Sandfly trapping and phlebovirus RNA detection

A total of 798 sandflies (675 *Phlebotomus* sp. and 123 *Sergentomyia minuta*) were collected, 622 in Marseille, and 176 in Nice (Table 1). A total of 76 *Phlebotomus* flies could not be identified at the species level due to morphologic damage during trapping and transportation. They were recorded as *Phlebotomus* sp. A total of 66 pools were prepared to be tested by PCR for the presence of phlebovirus RNA (Tables 1 and 2). Seven sandfly pools were found positive, and these positive pools were sequenced for genetic characterization. Four pools contained Toscana virus sequences. This work has previously been reported (Charrel et al. 2006; Charrel et al. 2007). Phlebovirus sequences obtained from the three other pools of sandflies collected in Marseille (pool W) and Nice (pools BM and BP) showed that nucleoprotein sequences on the one hand, and polymerase sequences on the other, were closely related to each other (0–2.9% and 0–4.2%, respectively), but clearly distinct from the French, Spanish, and Italian strains of Toscana virus and of sandfly fever Naples virus-Sabin (22.5–28.6%), and distantly related to sandfly fever Sicilian virus, Rift valley fever virus, and Uukuniemi virus (41.2–50.5%) (Tables 3 and 4). Comparisons in the S and L RNA showed that Massilia virus was clearly distinct (50.5% and 44% distance for N and polymerase proteins) from Arbia virus (acc nos EU266620 and DQ862467) which was sequenced de novo for this study. Partial sequence data, gathered from two independent genes and genome segments, demonstrated that viral RNA corresponding to a unique novel phlebovirus was present in these three sandfly specimens.

Massilia virus isolation and electron microscopy

Vero cells inoculated with the sandfly homogenate of pool W (male *Ph. perniciosus* trapped in Marseille) showed a cy-

TABLE 1. SPECIES, GENDER AND COLLECTION LOCALITY OF SANDFLIES PROCESSED FOR VIRUSES

Trapping locality	Species	Gender	Number of sandflies	Number of pools
Marseille	<i>Phlebotomus perniciosus</i>	M	291	17
		F	185	15
		Unknown	7	3
	<i>Phlebotomus ariasi</i>	M	2	2
		F	1	1
	<i>Phlebotomus mascitii</i>	M	1	1
		F	3	3
	Phlebotomus sp.	M	3	3
		F	2	1
		Unknown	4	4
Nice	<i>Sergentomyia minuta</i>	nd	123	5
	TOTAL Marseille		622	55
	<i>Phlebotomus perniciosus</i>	M	78	5
		F	32	3
	Phlebotomus sp.	M	43	2
		F	23	1
TOTAL	TOTAL Nice		176	11
	TOTAL Marseille + Nice		798	66

Note: nd, not determined.

TABLE 2. PRIMERS USED FOR DETECTION OF TOSCANA VIRUS AND OTHER PHLEBOVIRUSES IN THIS STUDY

Primer	PCR reaction	Gene, RNA segment	Sequence	Annealing T°C	PCR product size	Reference
NPhlebo 1+	First round	polymerase, L RNA	5'ATGGARGGTTTGTIWSIICC 3'	45°C	554 bp	Sánchez Seco et al. 2003
NPhlebo 1-	First round	polymerase, L RNA	5'AARTTRCTIGWIGCYTTIARIGTIGC 3'	45°C		
NPhlebo 2+	Second round	polymerase, L RNA	5'WTICCIAAICCIYMSAARATG 3'	45°C	245 bp	
NPhlebo 2-	Second round	polymerase, L RNA	5'TCYTCYTTRITTYTTRARRTARCC 3'	45°C		
SFNV S1	First round	nucleoprotein, S RNA	5'CTTYTTRTCYTCYCTRTGAAGAA 3'	59°C	438 bp	Charrel et al. 2007
SFNV S1	First round	nucleoprotein, S RNA	5'ATGATGAAGAARATGTCAGAGAA 3'	59°C		
SFNV S2	Second round	nucleoprotein, S RNA	5'GCRGCCATRTKGGYTTTCAAA 3'	59°C	323 bp	
SFNV S2	Second round	nucleoprotein, S RNA	5'CCTGGCAGRGACACATCAC 3'	59°C		

TABLE 3. CHARACTERISTICS OF SANDFLY POOLS THAT TESTED PCR POSITIVE FOR TOSCANA VIRUS AND THE NEWLY DESCRIBED MASSILIA VIRUS

Pool	Place of capture	GPS coordinates	Species	Gender	PCR system	Segment	Virus identification	Genbank acc no
W	Marseille, Allauch	43°18'27"N, 5°32'06"E	<i>Phlebotomus perniciosus</i>	Male	NPhlebo 1+ / 1- SFNV S2/R2	L RNA S RNA	Massilia virus	EU725771 EU725773
BM	Nice, La Turbie	43°43'54"N, 7°24'42"E	<i>Phlebotomus perniciosus</i>	Female	NPhlebo 1+ / 1- SFNV S2/R2	Complete S Complete M Complete L L RNA S RNA	Massilia virus	EU725773 EU725772 EU725771 DQ656072 DQ656077
BP	Nice, La Turbie	43°43'54"N, 7°24'42"E	<i>Phlebotomus perniciosus</i>	Male	NPhlebo 2+ / 2- SFNV S2/R2	L RNA S RNA	Massilia virus	DQ656073 DQ656078

topathic effect after five days that was reproduced during six serial passages. The presence of the virus was confirmed by positive RT-PCR at each passage. Virus titers progressively increased as demonstrated by analysis of sequential assays of quantitative PCR (data not shown). Supernatant medium and cells (passage 6) were prepared for electron microscopic (EM) analysis (Fig. 2A–D). EM photographs showed spherical or pleomorphic structures, with a size of 80–120 nm in diameter. Surface projections (5–10 nm long) that evenly covered the surface were clearly visualized. Together these characteristics were compatible with viruses belonging to the genus *Phlebovirus* within the family *Bunyaviridae*. In Fig. 2E, Massilia virus-infected Vero cells are shown reacting in an indirect fluorescence antibody assay with the serum of a convalescent patient who experienced past infection with Toscana virus. This positive reaction demonstrated antigenic cross-reaction between Toscana and Massilia viruses, and it was confirmed in serologic tests (see below).

Full-length sequence of Massilia virus and comparative genetic analysis

Supernatant from a culture of Vero cells infected with the W strain of Massilia virus was used to determine the complete sequence of the 3 RNA segments. Full length sequences of the S, M and L RNA genomic segments of Massilia virus were registered in the Genbank database with acc no EU725771–EU725773, respectively. Complete homologous genomic data were available for only 6, 5, and 4 distinct virus species or tentative species within the genus *Phlebovirus* for segments S, M and L, respectively. Therefore, phylogenetic analysis included comparison of incomplete amino acid sequences by using the pairwise deletion algorithm (Fig. 1). In each tree, sequences corresponding to a given taxonomic species are represented in a different color. The major nodes (representing the viruses belonging to the same species or species complex) were supported with bootstrap values higher or equal to 97%, except for G1 phylogram (73%). Surprisingly, in the N derived phylogram, Rift valley fever sequences did not group with Icoaraci, Salobo or Belterra viruses. In contrast, the latter grouped with Joa and Frijoles with the possible common ancestor supported by a 73% bootstrap in the N tree, and by a 99% bootstrap in the G2 tree. In the NS and GPC derived trees, Rift valley fever virus on

the one hand, and Icoaraci, Salobo and Belterra on the other were grouped together, but this was supported only by 66% and 56% bootstrap values, respectively. These discrepant results will need to be confirmed by analysis of longer sequences. However, the current data may not affect the relationships between Massilia virus and its closely related phleboviruses. Regardless of the virus protein used for analysis, Massilia virus sequences were most closely related to viruses in the *Sandfly fever Naples virus* complex.

Distribution of amino acid evolutionary distances

To determine whether or not it is possible to distinguish between species of the genus *Phlebovirus* by using quantitative genetic data, the distribution of genetic distance was studied for each of the 3 RNA segments. Genetic distances between sequences provided in this study and homologous sequences of selected phleboviruses are presented in Figure 3. To include a significant number of sequences, the pairwise deletion algorithm was used, thus allowing the inclusion of partial sequences. Intra- and interspecies cut-off values were indicated on the histograms when appropriate. Delineation of nucleotide distances within a species (<29%) from distances between species (>32%) was rather narrow. Similar results were observed with GPC (50–54%) and G1 (no possible interval). In contrast, a clear delineation was observed when using M and L RNA sequences. The most suited protein sequences for species delineation were NS, G2 and L, with respective distance distributions of 58–69%, 29–40%, and 21–32%. However, since few sequences were available for the L protein analysis, the results should be interpreted carefully.

Massilia virus genetic relationships

Each of the six phylograms (Fig. 1) showed that Massilia virus is more closely related to *Sandfly fever Naples virus* complex members than to other phleboviruses. Distances between Massilia virus and viruses in the *Sandfly fever Naples virus* complex were equal to or lower than 19%, 57%, 48%, 63%, 29%, and 18% for N, NS, GPC, G1, G2, and L proteins, respectively. These values are in the range of those observed between viruses belonging to the same species or species complex as reported in the preceding paragraph and in Figure 3. In contrast, distances observed between Massilia virus

TABLE 4. GENETIC DISTANCES OBSERVED BETWEEN SEQUENCES OF MASSILIA VIRUS AND OTHER PHLEBOVIRUSES FOR WHICH COMPLETE OR PARTIAL SEQUENCE DATA WAS AVAILABLE. DIVERSITY WAS CALCULATED BY THE PAIRWISE-DISTANCE ALGORITHM IMPLEMENTED IN THE MEGA SOFTWARE PROGRAM (KUMAR ET AL. 2004).

	Species or complex							
	<i>Sandfly fever</i>	<i>Sandfly fever</i>	<i>Corfou</i>	<i>Frijoles</i>	<i>Rift valley</i>	<i>Punta Toro</i>	<i>Salehebad</i>	<i>Uukuniemi</i>
Massilia								
N	12.7–18.7	55.7–60.2	58.9	44.1–	41.3–52.2	49.0–50.6	50.5	69.2
NS	54.1–55.7	83.3–87.2	85.5	nd	83.9–85.4	85.5–88.6	nd	84.0
GPC	41.0–48.1	66.3–67.0	67.7	61.0–	54.2–66.0	57.6–71.9	nd	78.0
G1	45.0–63.0	73.0–77.0	78.0	75.0–	69.0–74.0	68.0–82.0	nd	82
G2	16.0–29.0	52.0–54.0	52.0	40.0	48.0–53.0	46.0–56.0	nd	72
L	17.6–26.9	37.3–46.5	nd	nd	45.9–48.9	25.6–25.9	49.3	63.9

Note: nd, not determined.

and phleboviruses other than those belonging to the *Sandfly fever Naples virus* complex were equal to or higher than 41%, 83%, 55%, 69%, 40%, and 40% for N, NS, GPC, G1, G2, and L proteins, respectively. The combination of intra- and interspecies distances observed between Massilia virus and other phlebovirus represents convincing evidence, based on genetic data, that Massilia virus might be considered a member of the *Sandfly fever Naples virus* complex. This dataset has been submitted to The International Committee for Taxonomy of Viruses which will decide on the taxonomic status of Massilia virus within the genus *Phlebovirus*.

Antigenic relationships with other phleboviruses

The antigenic relationships of Massilia virus with other regional phleboviruses was tested by complement fixation (CF) and hemagglutination inhibition (HI) assays. Data are pre-

sented in Tables 5 and 6. Within the family *Bunyaviridae*, the CF test generally detects nucleoprotein antibodies, a marker for the S genomic segment; while the HI and neutralization tests measure antibodies to the glycoproteins, gene products of the M RNA segment. In CF tests, the closest antigenic relationships of Massilia virus were observed with viruses belonging to the *Sandfly fever Naples virus* complex (Table 5). No antigenic relationships were demonstrated between other regional viruses belonging to the *Salehebad* species complex or the tentative Sicilian/Corfou species complex. In HI tests, Massilia virus appeared to be distinct from the other four members of the *Sandfly fever Naples virus* complex. In both CF and HI tests, it was most closely related to the prototype Naples virus. These serological data argue for the inclusion of Massilia virus as a fifth member of the *Sandfly fever Naples virus* complex which currently consists of Naples, Toscana, Tehran, and Karimabad viruses.

FIG. 3. Distribution of evolutionary distances—The distances were calculated from amino acid sequence alignment by the p-distance method using the pairwise deletion algorithm. The genetic distance is reported on the x-axis. Frequency of genetic distances is recorded on the y-axis. The two dashed lines represent the possible boundaries for species delineation. The corresponding distances are indicated above the dashed lines. The cross in the G1 histogram means that it is impossible to define an interval between intra- and inter-species distances because of overlapping.

TABLE 5. RESULTS OF COMPLEMENT FIXATION TESTS COMPARING MASSILIA ANTIGEN WITH THE HOMOLOGOUS ANTIBODY AND ANTIBODIES AGAINST OTHER REGIONAL PHLEBOVIRUSES

Antigens	Complement fixation test*								
	Antibody								
	Phleboviruses								
	Naples species complex					Salehebad species complex		Tentative species in genus	
	Massilia	Karimabad	Naples	Tehran	Toscana	Arbia	Salehebad	Sicilian	Corfou
Massilia	1024/2048*	0	64/32	≥256/≥128	≥256/≥128	0	0	0	0
Control	0	0	0	0	0	0	0	0	0

Antigens	Complement fixation test*		
	Antibody		
	Massilia	Naples	Control
Massilia	1024/2048*	512/512	0
Naples	256/512	1024/512	0
Control	0	0	—

*antibody/antigen

Massilia virus and CNS disease

To investigate the possible association of Massilia virus with CNS illness (meningitis, encephalitis, and meningoencephalitis), CSFs collected for diagnostic purpose from local patients with these illnesses were tested retrospectively, using a Massilia virus specific real-time RT-PCR assay (primers and probes aforementioned, and full protocol available upon request to the corresponding author). The real-time PCR assay was designed to detect Massilia virus genomes, but not Toscana virus (Italian and Spanish-French genotypes) or Arbia virus, the two other phleboviruses for which circulation has been demonstrated in southeastern France (Charrel et al. 2007). Parallel testing was performed with the SFNV-S2 and SFNV-R2 primers in the format of SYBR green real-time PCR (Charrel et al. 2007; full protocol available upon request to the corresponding author). A total of 477 CSFs spanning years 2002 to 2006 were tested. None was found to contain

Massilia virus RNA. Meantime, four CSFs were found positive for Toscana virus RNA. Sequence data confirmed the Toscana virus identification. All these patients except one were diagnosed previously, based on immunofluorescence assays (IgM and/or seroconversion in sequential sera) (Charrel and de Lamballerie, personal data).

Discussion

At the outset of our work, Toscana virus (another member of the *Sandfly fever Naples virus* complex) was the only phlebovirus known to circulate in sandfly populations of southeastern France (Charrel et al. 2006; Charrel et al. 2007). In this study, we used sandfly specimens collected locally to investigate the presence of other phleboviruses. The aims of our study were to detect, isolate and characterize new phleboviruses in sandfly populations of southeastern France. Sandflies are small, nocturnally active insects with a short

TABLE 6. RESULTS OF HEMAGGLUTINATION-INHIBITION TESTS COMPARING MASSILIA VIRUS WITH OTHER KNOWN MEMBERS OF THE SANDFLY FEVER NAPLES VIRUS COMPLEX

Antibody	Hemagglutination inhibition test*				
	Antigens 4u.				
	Karimabad	Massilia	Naples	Theran	Toscana
Karimabad	1:320*	1:20	1:40	0	0
Massilia	1:20	1:1280	1:320	1:40	1:80
Naples	1:20	1:80	1:640	1:40	1:80
Theran	1:80	1:640	1:1280	1:1280	1:320
Toscana	1:320	1:80	1:160	1:40	≥1:2560

*0 = <1/20. Values indicate highest antibody dilution giving a positive reaction.

flight range (Lane 1996). Only the females are hematophagous, whereas both sexes obtain sugars for their energy requirements from various sources including honeydew and the vascular tissues of plants. Adults rest during the day in dark, protected, humid places and their eggs are laid in terrestrial microhabitats rich in organic matter which provides food for the larvae, including soils containing herbivorous animal feces. Many species are opportunistic human-biters, including highly but not exclusively, anthropophilic species. In southern France, as in temperate zones, sandflies are active during the summer and go into diapause during the winter. These parameters determined the time period and the sites that were chosen in this study, namely in the immediate proximity of proven or potential breeding sites as well as close to places where animal or human diseases transmitted by sandflies have occurred. Indeed southern France, and more broadly in southern Europe and the Mediterranean area, *P. perniciosus* is known as the main vector of visceral leishmaniasis in dogs, but also in children and AIDS patients (Dujardin et al. 2008). The methodology used for trapping, identification, preparation and storage of sandflies was previously used, and proved suitable for virus detection and isolation (Charrel et al. 2007).

Prevalence of phlebovirus RNA in sandflies

The number of sandflies trapped during this study was relatively small ($n = 798$), yet 7 phlebovirus-infected pools were detected. This is not unusual as assessed by four other studies conducted in France and Algeria, with respective infection rates of 6/798, 1/460, 2/471, and 1/314 (Charrel et al. 2007; Izri et al. 2008; Charrel and Izri personal data). These findings suggested that a relatively high proportion of sandflies are naturally infected. Assuming, that only one sandfly was positive in each pool (up to 30 individuals), then 7 infected-sandflies (4 containing Toscana virus, 3 containing Massilia virus) were identified, yielding a minimum pool infection rate of 9.1% (6/66) (Charrel et al. 2007; this study). In another study in Algeria, 460 sandflies were trapped and tested (aliquoted into 24 pools) Only one (a single female *Ph. ariasi*) was positive (Izri et al. 2008). The analysis of 4 distinct trapping campaigns provided rates of positivity between 0.22–0.75% of individual sandflies, and 4.2–9.1% of pools (containing up to 30 sandflies). Similarly high phlebovirus infection rates have been reported in studies of sandflies in other regions (Tesh et al. 1977; Verani et al. 1988). Our trap collection in southeastern France in this study yielded *Ph. perniciosus*, *Ph. ariasi*, *Ph. mascitii*, and *Sergentomyia minuta*, with a majority of *Ph. perniciosus*.

Sequence and phylogenetic analyses

Full-length genome sequencing was performed using a combination of PCR amplification with (i) degenerate primers designed from the amino acid alignment of available phlebovirus sequences, (ii) primers based on the conserved extremities of the genome, and (iii) primers reported in the literature (Sanchez-Seco et al. 2003; Liu et al. 2003; Charrel et al. 2007). Due to the genetic heterogeneity in the genus *Phlebovirus*, analyses were based on amino acid sequences. At the outset of this study, complete sequence was available for only four species (Punta Toro, Sandfly fever Naples, Rift valley fever, and Uukuniemi). There was a to-

tal lack of sequence (even partial) for four species (Bujaru, Chandiru, Chilibre and Salehebad). Of the 16 tentative species, partial sequence data were available for Sandfly fever Sicilian and Corfou viruses. Amongst the other 37 viruses classified into recognized species, sequence data were available for 12 of them. Due to the paucity of genetic data, phylogenetic analyses were performed by using the pairwise deletion algorithm to include the largest set of sequences. However, the results should be interpreted with caution, and merit further investigations for confirmation since such an analysis may compare genome regions which encode for different proteins that in turn may be under different types of host pressures.

Massilia virus, a new phlebovirus previously unrecognized

Arbia virus was isolated from sandflies in Italy in the 1970s. It has been reported to circulate in Italy in populations of *Ph. perniciosus* and *Ph. perfiliewi* at rates comparable to that of Toscana virus (Verani et al. 1988). Thus it seemed possible that Massilia virus could be Arbia virus. To address this point, we sequenced partial regions of the S and L RNA sequences to enable inclusion of Arbia virus in the genetic and phylogenetic analyses. The data demonstrated that Massilia virus is distinct from Arbia virus. In the phylogenetic tree based on M RNA sequences, only Karimabad virus was lacking as a member of the *Sandfly fever Naples* virus complex. Therefore, the possibility that Massilia and Karimabad viruses could be a unique virus had to be raised. However, the results of HI and CF tests showed little or no cross-reactivity between Massilia virus and Karimabad or Arbia virus. This precludes the possibility that Massilia and Karimabad or Arbia virus are the same.

Genetic criteria as a possible tool for species definition

According to the rules of the International Committee for Taxonomy of Viruses, there is a unique criterion for species definition within the genus *Phlebovirus*: "The lack of biochemical data for most phleboviruses dictates that the species are defined by the serological relationships, and are distinguishable by a 4-fold difference in 2-way neutralization tests". (Nichol et al. 2005). Because such tests are tedious and require the availability of all recognized phleboviruses as well as the corresponding mouse immune ascitic fluid, we investigated a possible alternative based on genetic criteria to determine the existing species of phleboviruses. A well-recognized and extensively used approach relying on the determination of the distribution of evolutionary distances upon pairwise comparison was adopted (Simmonds et al. 1993; Simmonds et al. 1994; Charrel et al. 2001). The independent analysis of the distribution of pairwise amino acid distances observed between phleboviruses for N, NS, GPC, G1, G2 and L proteins indicates that histograms reflecting the distance distribution of N, GPC and G1 proteins did not provide a clear delineation between intra- and inter-species distances. In contrast, the NS, G2 and L regions did discriminate species on the basis of genetic criteria. Indeed, the large gap observed between intra- and inter-species distances provides a rational alternative to the antigenic based criteria. The analysis of the shape of the histogram suggests that the G2 region, showing three groups of sequences corresponding to the genetic heterogeneity at the level of virus strain, intra-species and

interspecies, is the best suited for species delineation. However, these promising results need to be confirmed by a study that includes sequences of the four species as yet genetically uncharacterized, viz., Bujaru, Chandiru, Chilibre, and Salehebad.

Taxonomic status of Massilia virus

The identification of new sequences displaying a high level of heterogeneity with previously documented phleboviruses indicates that Massilia is a novel phlebovirus. According to the preceding genetic analyses, Massilia virus should be included in the *Sandfly fever Naples virus* complex with the status of a new genotype genetically distinct from other members of the complex. The results of serologic test support this conclusion.

Public health importance

The public health importance of Massilia virus is unknown at this time. However, the fact that it is a member of the *Sandfly fever Naples virus* complex and that it is associated with *Ph. Perniciosus*, a proven vector of visceral leishmaniasis and Toscana virus infection in the Mediterranean region (Dujardin et al. 2008), suggest that it could be a human pathogen. Also the fact that two of our three Massilia virus isolates were from male sandflies suggests that transovarial and/or venereal transmission may occur. The latter characteristics would tend to enhance and insure continued circulation of the virus in the endemic area (Tesh et al. 1992).

In conclusion, a novel phlebovirus, provisionally named Massilia virus, was discovered in sandflies (*Ph. perniciosus*) in southeastern France. The virus was isolated and characterized using full-length genome sequence data and antigenic cross-reactivity studies. Together these data permitted to propose to consider Massilia virus as a member of the species Sandfly fever Naples virus within the genus *Phlebovirus*. The definitive taxonomic status will be decided by the International Committee for Taxonomy of Viruses. Further studies are necessary to determine whether Massilia virus could infect humans and play a role in public health.

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Disclosure Statement

No competing interests exist.

References

- Beaty, BJ, Calisher, CH, Shope RE. Arboviruses. Schmidt NJ, Emmons RW, eds. *Diagnostic Procedures for Viral, Rickettsial and Chlamydial Infections*. Sixth Edition, Washington DC: American Public Health Association, 1989; 797–855.
- Charrel, RN, Gallian, P, Navarro-Mari, J-M, Nicoletti, L, et al. Emergence of Toscana virus in Europe. *Emerg Infect Dis* 2005; 11:1657–1663.
- Charrel RN, Izri A, de Lamballerie X, Parola P. Toscana virus RNA in *Sergentomyia minuta* flies. *Emerg Infect Dis* 2006; 12:1299–1300.
- Charrel, RN, Izri, A, Temmam, S, Delaunay, P, et al. Cocirculation of 2 genotypes of Toscana virus, southeastern France. *Emerg Infect Dis* 2007; 13:465–468.
- Charrel, RN, Zaki, AM, Attoui, H, Fakeeh, M, et al. Complete coding sequence of the Alkhurma virus, a tick-borne flavivirus causing severe hemorrhagic fever in humans in Saudi Arabia. *Biochem Biophys Res Commun* 2001; 287:455–461.
- Clarke, DH, Casals, J. Techniques for hemagglutination-inhibition with arthropod borne viruses. *Am J Trop Med Hyg* 1958; 7:561–573.
- Dujardin, J-C, Campino, L, Canavate, C, Dedet, J-P et al. Spread of vector-borne diseases and neglect of leishmaniasis, Europe. *Emerg Infect Dis* 2008; 14:1013–1018.
- Fullhorst, CF, Monroe, MC, Salas, RA, Duno, G, et al. Isolation, characterization and geographic distribution of Cano Delgado virus, a newly discovered South American hantavirus (family *Bunyaviridae*). *Virus Res* 1997; 51:159–171.
- Hemmersbach-Miller, M, Parola, P, Charrel, RN, Durand, JP, Brouqui, P. Sandfly fever due to Toscana virus: an emerging infection in southern France. *Eur J Intern Med* 2004; 15:316–317.
- Izri, A, Belazzoug, S, Boudjebla, Y, Dereure, J, et al. *Leishmania infantum* MON 1, isolée de *Phlebotomus perniciosus* en Kabylie, Algérie. *Ann Parasitol Hum Comp* 1990; 65:151.
- Izri A, Temmam S, Moureau G, Hamrioui B, de Lamballerie X, Charrel RN. Sandfly fever Sicilian virus, Algeria. *Emerg Infect Dis*. 2008; 14:795–797.
- Kumar, S, Tamura, K, Jakobsen, IB, Nei, M. MEGA2: molecular evolutionary genetics analysis software. *Bioinformatics* 2001; 17:1244–1245.
- Lane, RP. Sandflies (Phlebotominae). In Lane RB, Crosskey RW, eds. *Medical Insects and Arachnids*. London: Chapman Hall 1996; p. 78–119.
- Leger, N, Pesson, B, Madullo-Leblond, G, Abonnenc, E. Sur la différenciation des femelles du genre *Laroussius* Nitzelescu, 1931 (Diptera, Phlebotomidae) de la région méditerranéenne. *Ann Parasitol Hum Comp* 1983; 58: 611–623.
- Liu, DY, Tesh, RB, Travassos Da Rosa, AP, Peters, CJ, et al. Phylogenetic relationships among members of the genus *Phlebovirus* (*Bunyaviridae*) based on partial M segment sequence analyses. *J Gen Virol* 2003; 84: 465–473.
- Marty, P, Le Fichoux, Y, Pratlong, F, Gari-Toussaint, M. Human visceral leishmaniasis in Alpes-Maritimes, France: epidemiological characteristics for the period 1985–1992. *Trans Roy Soc Trop Med Hyg* 1994; 88:33–34.
- Nichol, ST, Beaty, BJ, Elliott, RM, Goldbach, R, et al. Genus *Phlebovirus*. In Fauquet, CM, Mayo, MH, Maniloff, J, Desselberger, U, Ball, LA. *Virus Taxonomy: Classification and Nomenclature of Viruses*. Eighth Report of the International Committee of the Taxonomy of Viruses. Elsevier Academic Press. 2005 pp 709–716.
- Sanchez Seco, MP, Echevarría, JM, Hernández, L, Estévez, D, et al. Detection and identification of Toscana and other Phle-

- boviruses by RT-PCR assays with degenerated primers. *J Med Virol* 2003; 71:140–149.
- Shope, RE. The use of a micro hemagglutination-inhibition test to follow antibody response after arthropod-borne virus infection in a community of forest animals. *An Microbiol (Rio de Janeiro)* 1963; 11:167–169.
- Simmonds, P, McOmish, F, Yap, PL, Chan, SW, et al. Sequence variability in the 5' non-coding region of hepatitis C virus: identification of a new virus type and restrictions on sequence diversity. *J Gen Virol*. 1993; 74:661–8.
- Simmonds, P, Smith, DB, McOmish, F, Yap, PL, et al. Identification of genotypes of hepatitis C virus by sequence comparisons in the core, E1 and NS-5 regions. *J Gen Virol* 1994; 75:1053–61.
- Tesh, RB, Lubroth, J, Guzman, H. Simulation of arbovirus overwintering: survival of Toscana virus (*Bunyaviridae:Phlebovirus*) in its natural sand fly vector *Phlebotomus perniciosus*. *Am J Trop Med Hyg* 1992; 47:574–581.
- Tesh, RB, Saidi, S, Javadian, E, Nadim, A. Studies on the epidemiology of sandfly fever in Iran. 1. Virus isolates obtained from *Phlebotomus*. *Am J Trop Med Hyg* 1977; 26:282–287.
- Verani, P, Ciufolini, MG, Caciolli, S, Renzi, A, et al. Ecology of viruses isolated from sand flies in Italy and characterized of a new Phlebovirus (Arabia virus). *Am J Trop Med Hyg* 1988; 38:433–439.

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