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Isolation, Genetic Characterization, and Seroprevalence of Adana Virus, a Novel Phlebovirus Belonging to the Salehabad Virus Complex, in Turkey

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ABSTRACT

A new phlebovirus, Adana virus, was isolated from a pool of *Phlebotomus* spp. (*Diptera*; *Psychodidae*) in the province of Adana, in the Mediterranean region of Turkey. Genetic analysis based on complete coding of genomic sequences indicated that Adana virus belongs to the *Salehabad virus* species of the genus *Phlebovirus* in the family *Bunyaviridae*. Adana virus is the third virus of the *Salehabad virus* species for which the complete sequence has been determined. To understand the epidemiology of Adana virus, a seroprevalence study using microneutralization assay was performed to detect the presence of specific antibodies in human and domestic animal sera collected in Adana as well as Mersin province, located 147 km west of Adana. The results demonstrate that the virus is present in both provinces. High seroprevalence rates in goats, sheep, and dogs support intensive exposure to Adana virus in the region, which has not been previously reported for any virus included in the Salehabad serocomplex; however, low seroprevalence rates in humans suggest that Adana virus is not likely to constitute an important public health problem in exposed human populations, but this deserves further studies.

IMPORTANCE

Until recently, in the genus *Phlebovirus*, the *Salehabad virus* species consisted of two viruses: Salehabad virus, isolated from sand flies in Iran, and Arbia virus, isolated from sand flies in Italy. Here we present the isolation and complete genome characterization of the Adana virus, which we propose to be included in the *Salehabad virus* species. To our knowledge, this is the first report of the isolation and complete genome characterization, from sand flies in Turkey, of a Salehabad virus-related phlebovirus with supporting seropositivity in the Mediterranean, Aegean, and Central Anatolia regions, where phleboviruses have been circulating and causing outbreaks. Salehabad species viruses have generally been considered to be a group of viruses with little medical or veterinary interest. This view deserves to be revisited according to our results, which indicate a high animal infection rate of Adana virus and recent evidence of human infection with Adria virus in Greece.

Sand fly-borne phleboviruses (genus *Phlebovirus*, family *Bunyaviridae*) may cause self-limiting febrile illness (sandfly fever) or neuroinvasive infections. The genus *Phlebovirus* contains 9 viral species (*Sandfly fever Naples virus*, *Salehabad virus*, *Rift Valley fever virus*, *Uukuniemi virus*, *Bujaru virus*, *Candiru virus*, *Chilibre virus*, *Frijoles virus*, and *Punta Toro virus*) and several tentative species as defined in the 9th Report of the International Committee for Taxonomy of Viruses (ICTV) (1). Of the 9 viral species recognized by the ICTV, *Sandfly fever Naples virus*, *Salehabad virus*, *Bujaru virus*, *Candiru virus*, *Chilibre virus*, *Frijoles virus*, and *Punta Toro virus* are exclusively or partially vectored by sand flies. In the Old World, there are two recognized species (*Sandfly fever Naples virus* [SFNV] and *Salehabad virus* [SALV]) and two tentative species (Sandfly fever Sicilian virus [SFSV] and Corfu virus [CFUV]) of sand fly-borne phleboviruses. In addition, many new phleboviruses have been recently isolated from phlebotomine flies (Fermo, Granada, and Punique viruses) (2, 3, 4), from ticks (Heartland and Hunter island group viruses) (5, 6), or from vertebrates (Malsoor and Salanga viruses) (7, 8). They remain to be recognized by the ICTV.

All members of the genus *Phlebovirus* have a trisegmented, single-stranded RNA genome. The L segment encodes the RNA-dependent RNA polymerase. The M segment encodes the viral

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envelope glycoproteins (Gn and Gc, formerly G1 and G2). The S segment encodes the viral nucleocapsid protein (N) and a nonstructural protein (NS_s) in an ambisense orientation (9, 10, 11). Sand fly-borne phleboviruses are transmitted mainly by sand flies belonging to the genus *Phlebotomus* in the Old World when females take a blood meal (May to October). Transovarial (vertical) transmission from female to offspring (12, 13, 14, 15, 16) and venereal (horizontal) transmission from infected males to uninfected females during mating have been recorded (12, 17). There is no defined reservoir of sand fly-borne phleboviruses. Therefore, their ecological distribution and evolutionary divergence seem to be highly dependent on their vectors.

Former seroprevalence studies indicated that Sicilian and Naples viruses are present in the Mediterranean and Aegean regions of Turkey (18, 19). Extensive investigations have been initiated during the last decade, especially in the regions where outbreaks have occurred (Mediterranean, Aegean, and Central Anatolia regions). Circulation of SFSV and a SFSV-like virus (Sandfly fever Cyprus virus [SFCV]) was detected serologically and Sandfly fever Turkey virus (SFTV) was isolated during the outbreaks (20). After the outbreaks in Kirikkale, a province 51 km from the outbreak region of Ankara (21), and in Kahramanmaras, 196 km northeast of Adana (22), antibodies were detected in convalescent patients. An acute hepatitis case due to SFSV in Kirikkale was recently reported (23). Toscana virus (TOSV) was serologically detected in several regions (24, 25, 26, 27, 28). Although there are extensive studies on seroprevalence of phleboviruses in Turkey, SFTV isolation from only one patient was reported (20). The sand fly-borne viruses appear to be widespread throughout the country. To understand the nature of the circulation of phleboviruses in Turkey, sand fly trapping campaigns were organized in the Central Anatolia, Mediterranean, and Aegean regions. Here we present the genetic and seroprevalence data on Adana virus (ADAV), a novel phlebovirus belonging to the Salehabad virus species. ADAV was isolated from sand flies trapped in the Mediterranean region of Turkey. Genetic and phylogenetic studies were performed on complete genomic sequence data. Seroprevalence studies using microneutralization (MN) assays were performed in 1,000 human sera and 289 animal sera from the same region.

MATERIALS AND METHODS

Sand fly trapping. Sand fly trapping campaigns were conducted from August 2012 to September 2012 in Adana (Mediterranean region, Turkey) using CDC miniature light traps as previously reported (29). Live sand flies were pooled based on sex, trapping site, and trapping day, with up to 30 individuals per pool, and placed in 1.5-ml tubes to be further stored at −80°C. No morphological identification of the captured sand flies was performed prior to viral testing. The rationale for this approach was to minimize manipulations to facilitate virus isolation. Adana is the 5th-most-densely populated province of Turkey, with a population of 2.1 million. It is located near the Seyhan River, 30 km inland from the Mediterranean Sea, in south-central Anatolia. Adana lies in the heart of Cukurova, a geographical, economical, cultural, and agricultural region that also covers the provinces of Mersin, Osmaniye, and Hatay. The region is agriculturally productive throughout the year.

Virus detection. Pools of sand flies were ground in 600 μl of Eagle minimal essential medium (EMEM) (supplemented with 7% fetal bovine serum, 1% penicillin-streptomycin, and 1% [200 mM] L-glutamine) in the presence of a 3-mm tungsten bead using a Mixer Mill MM300 (Qiagen, Courtaboeuf, France) (30). A 200-μl aliquot was used for viral nucleic acid (NA) extraction with the BioRobot EZ1-XL Advanced (Qiagen) using the Virus Extraction minikit (Qiagen) and eluted in 90 μl. Five microliters of this solution was used for reverse transcription-PCR (RT-PCR) and nested-PCR assays with primers targeting the polymerase gene and the nucleoprotein gene using protocols previously described (31, 32). PCR products of the expected size were column purified (Amicon ultracentrifugal filters; Millipore) and directly sequenced. Two real-time RT-PCR assays were designed for specific detection of the newly isolated Adana virus in the polymerase (ADAV-L) and nucleoprotein (ADAV-N) genes, respectively. The primers for the ADAV-L assay consisted of ADAV-L-FW (CACAGAT GTCTACTGAGCATGAG), ADAV-L-REV (ACTTATGAGAGGGTG AATATCTCT), and ADAV-L-Probe (6-carboxyfluorescein [6FAM]-TTAACTGGTCTGGATTATTCAACCC-6-carboxytetramethylrhodamine [TAMRA]). The primers for the ADAV-N assay consisted of ADAV-N-FW (GACCGATGATGCATCCTTGCTT), ADAV-N-REV (GCGGATTGATGGTCCTTGAGAA), and ADAV-N-Probe (6FAM-A TTGACAACACCCTTCCAGAGGA-TAMRA). The real-time RT-PCR was performed using the GoTaq probe 1-step quantitative RT-PCR (RT-qPCR) system (Promega) by following the manufacturer's protocol with the following incubation program on a CFX96 real-time system (Bio-Rad): (i) 50°C for 15 min, (ii) 95°C for 2 min; (iii) 40 cycles consisting of 95°C for 15 s and 60°C for 1 min.

Virus isolation and electron microscopy. A 50-μl volume of ground sand fly pools was inoculated onto 12.5-cm^2 flasks of Vero cells together with EMEM, enriched with 1% penicillin-streptomycin, 1% [200 mM] L-glutamine, 1% kanamycin, and 3% amphotericin B (Fungizone). After incubation at room temperature for 1 h, 5 ml of fresh EMEM containing 5% fetal bovine serum (FBS) was added. The flasks were incubated at 37°C in a 5% CO_2 atmosphere and examined daily for cytopathic effect (CPE). After detection of CPE during passage 1, the virus was passaged 4 times, and passage 4 (P4) was used for electron-microscopic examination. Negative-stained electron-microscopic specimens were prepared using infected cell supernatant mixed 1:1 with 2.5% paraformaldehyde, fixed onto Formvar/carbon-coated grids, and stained with 2% methylamine tungstate.

Complete genome sequencing. Adana virus (ADAV) passage 2 was used for complete genome characterization through next-generation sequencing (NGS). Briefly, 140 μl of cell culture supernatant was incubated at 37°C for 7 h with 30 U of Benzonase (Novagen; catalog no. 70664-3); then RNA was extracted using the Viral RNA minikit (Qiagen) onto the BioRobot EZ1-XL Advanced (Qiagen). Random amplification was performed using tagged random primers for reverse transcription (RT) and tag-specific and random primers for PCR amplification (Applied Biosystems). The PCR products were purified (Amicon ultracentrifugal filters; Millipore), and 200 ng was used for sequencing using the Ion PGM sequencer (Life Technologies SAS, Saint Aubin, France). Viral sequences were identified from the contigs based on the best BLAST similarity against reference databases. Sequence gaps were completed by PCR using primers based on NGS results and sequenced either by Sanger sequencing or by NGS. The 5′ and 3′ extremities of each segment were sequenced using a primer including the 8-nucleotide (nt) conserved sequence as previously described (33). For the confirmation of the final acquired sequences by NGS, specific primers were designed for Sanger sequencing of the complete genome.

Genetic distances and phylogenetic analysis. The sequences of S, M, and L segments were aligned with homologous sequences of other phleboviruses retrieved from GenBank until September 2014 using the CLUSTAL algorithm of the MEGA 5 software (34). Nucleotide and amino acid distances were calculated by the p-distance method. Neighbor-joining analysis (Kimura 2-parameter model) was done with amino acid sequences using MEGA version 5, with 1,000 bootstrap pseudoreplications. Amino acid sequences in the polymerase, Gn, Gc, N, and Ns proteins from all respective complete coding sequences retrieved from the GenBank

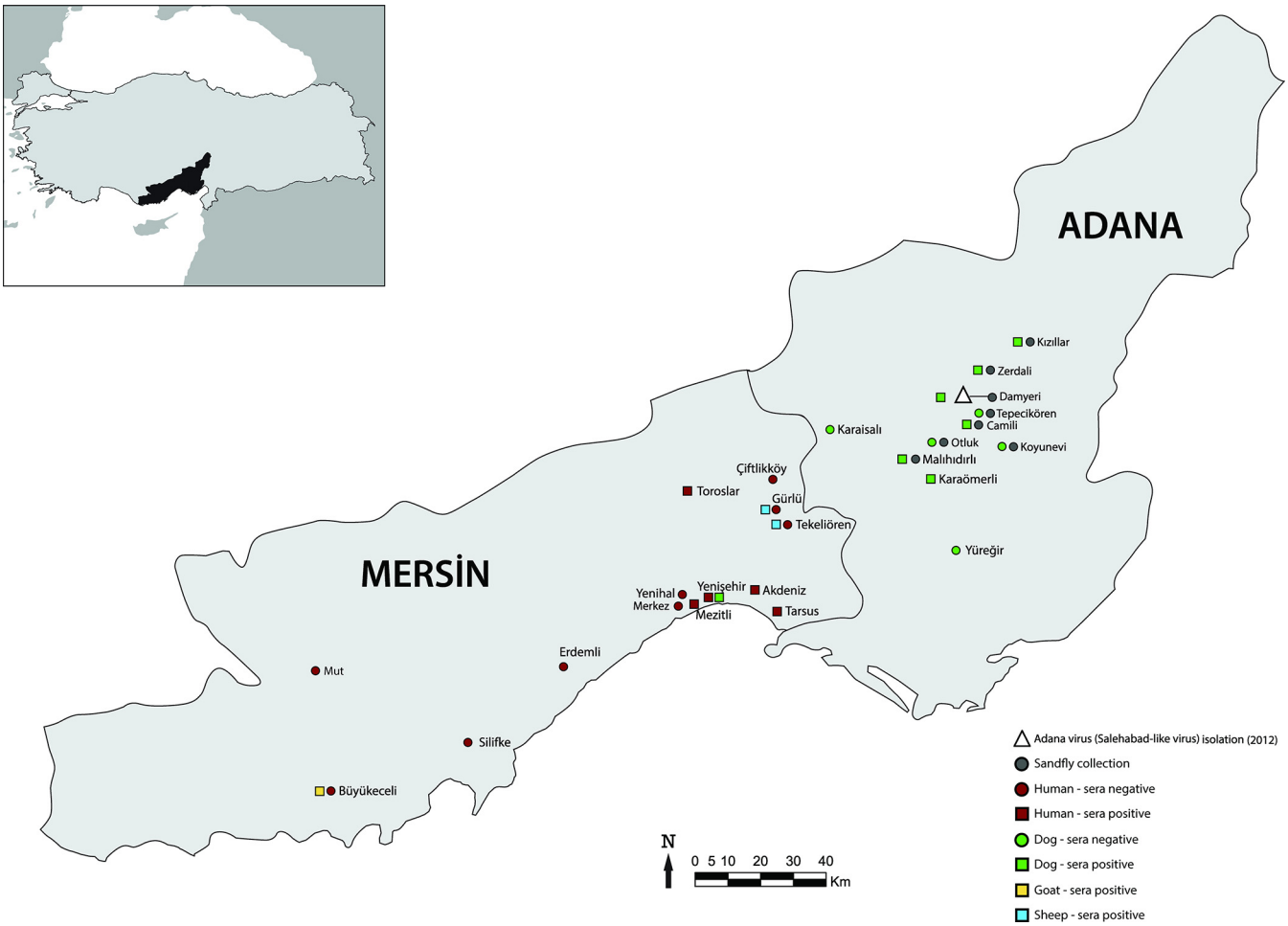


FIG 1 Geographic representation of the results.

database were used to study the distribution of evolutionary distances by pairwise comparison, as previously described (30, 35).

Microneutralization-based seroprevalence study. Human and domestic animal sera were collected in Adana and Mersin provinces after informed consent of the individuals and animal owners, according to the national regulations on the operation and procedure of animal experiment ethics committees (regulation no. 26220, date 7 September 2006). The study protocols were approved by the local ethics committees (MULEC/01.09.10 for human samples, AULEC/201-96-346 for animal samples) and by the Ege University Local Ethical Committee of Animal Experiment with the protocol number 2011-101. The virus microneutralization (MN) assay, previously described for phleboviruses (26, 35), was adapted with minor modifications using the ADAV strain. Briefly, 2-fold serial dilutions from 1:20 to 1:160 were prepared for each serum, and a volume of 50 μl of each dilution was transferred into 96-well plates. A volume of 50 μl containing 1,000 50% tissue culture infective doses ($TCID_{50}$) of virus was added to each well except for the controls, which contained phosphate-buffered saline (PBS). The plates were incubated at 37°C for 1 h. Then, a 100-μl suspension of Vero cells containing approximately 2×10^5 cells/ml of EMEM enriched with 5% fetal bovine serum, 1% penicillin-streptomycin, 1% [200 mM] L-glutamine, 1% kanamycin, and 3% amphotericin B was added to each well and incubated at 37°C in the presence of 5% CO_2. The first row of each plate contained control sera diluted 1:10 and Vero cells without virus. After 6 days the microplates were read under an inverted microscope, and the presence (neutralization titer of 20, 40, 80, or 160) or absence (no neutralization) of cytopathic effect was noted. To exclude the possibility that MN results observed with ADAV were due to cross-neutralizing antibodies raised against Arbia virus (ARBV), all sera were tested in parallel with a strain of Arbia virus.

Genotyping of sand flies in the virus-positive pool. To attempt identification of the sand fly species present in the Adana virus-positive pool, PCR was performed using 3-μl of nucleic acid extract of the pool to amplify the cytochrome *c* oxidase I (COI) gene, frequently used for biological bar coding (37). The PCR products were processed and sequenced through NGS as described above. NGS reads were compared with available sequences in GenBank using the CLC Genomic Workbench 6.5.

Nucleotide sequence accession numbers. The complete genome of Adana virus has been submitted to GenBank and assigned accession no. KJ939330, KJ939331, and KJ939332.

RESULTS

Sand fly trapping and virus detection. A total of 7,731 (3,524 females and 4,207 males) sand flies were collected in August and September 2012 from six villages (Fig. 1) located within the district of Adana province (Mediterranean Turkey). They were organized into 380 pools (including 179 female and 201 male pools). The numbers of sand flies and pools originating from individual villages are shown in Table 1. Pool 195, which consisted of 20 males trapped in Damyeri village (lat 36.50733357N, long 41.40570E; altitude, 194 m) was positive with primers N-phlebo1S and -1R (32). The resulting 505-nt sequence in the polymerase

TABLE 1 Distribution of sand fly specimens and pools according to the sampling locations in Adana, Mediterranean region of Turkey

Village	No. of collected sand flies		No. of pools	
	Female	Male	Male	Female
Damyeri	1,974	2,500	123	99
Zerdali	697	692	34	35
Camili	449	712	35	22
Otluk	202	139	7	9
Tepecikoren	112	111	5	5
Koyunevi	90	53	3	4
Total	3,524	4,207	207	174

gene was most closely related to the Salehabad virus (GenBank accession no JX472403) sequence (86% and 77% identity at the amino acid and nucleotide levels, respectively). Using the two real-time RT-PCR assays specifically designed to detect ADAV, only pool 195 was found to be positive (threshold cycle [C_T] values <26). Fourfold dilutions of pool 195 were tested and found positive until 1:4,096 dilution, with C_T values ranging from 36.2 to 38.2 for the 1:4,096 dilution. This is a convincing argument for the excellent sensitivity of these ADAV-specific real-time RT-PCR tests.

Virus isolation and electron microscopy. Vero cells that were inoculated with pool 195 showed a clear cytopathic effect after 4 days. Material corresponding to passage 3 was used for mass production and subsequent freeze-drying; these vials have been included in the collection of the European Virus Archive (www.european-virus-archive.com), where they are publicly available for academic research. The morphology of the virus was shown by electron-microscopic examination (Fig. 2). Electron-microcopic micrographs showed spherical or pleomorphic structures, with diameters of 80 to 120 nm and surface projections (5 to 10 nm long) that evenly covered the virions, and were compatible with images observed for other members of the *Bunyaviridae* family.

Complete genome sequencing. The reads obtained through using next-generation sequencing were processed by CLC Genomics Workbench 7.0.4. Reads, of minimum length 30 nucleotides, were trimmed using CLC Genomic Workbench 6.5, with a minimum of 99% quality per base, and mapped to reference sequences (Arbia virus; GenBank accession no. JX472400, JX472401, and JX472402 for the L, M, and S segments, respectively). Parameters were set such that each accepted read had to map to the reference sequence for at least 50% of its length, with a minimum of 80% identity to the reference. The complete genome of Adana virus consists of 6,405 nt, 4,229 nt and 1,758 nt for the L, M, and S segments, respectively (GenBank accession no. KJ939330, KJ939331, and KJ939332). The polymerase gene contains a 6,288-nt open reading frame (ORF) (2,096 amino acids [aa]), whereas the glycoprotein gene contains a 4,005-nt ORF (1,335 aa). The small segment contains 744-nt and 819-nt ORFs, which are translated to a nucleocapsid protein (248 aa) and a nonstructural protein (273 aa), respectively. Sequences obtained using

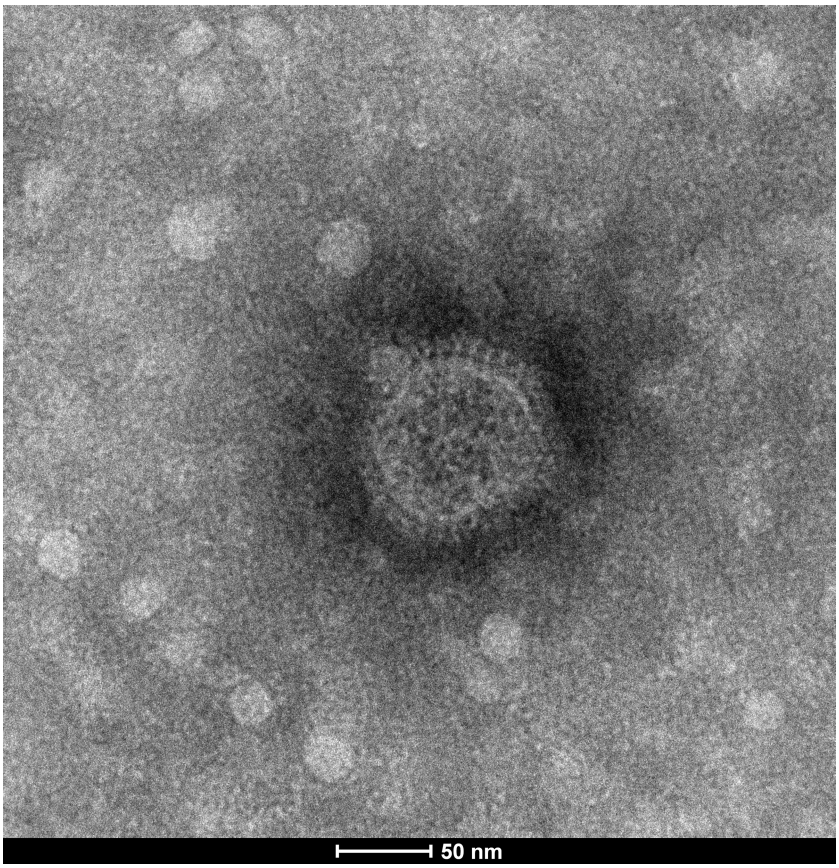


FIG 2 Negative staining electron microscopy of Vero cell culture supernatant medium at day five after infection with the Adana virus.

TABLE 2 Estimates of evolutionary divergence between sequences of the polymerase, Gn glycoprotein, Gc glycoprotein, nucleocapsid, and nonstructural genes and proteins of selected phleboviruses and Adana virus[a]

Protein and virus[b]	Divergence[c] (%) from sequence of:												
	ADAV	ARBV	SALV	ADRV*	AMTV	ODRV	SFTV	SFSV*	KARV	SFNV	THEV	MASV	TOSV
Polymerase													
ADAV		**15.0**	**14.4**	**11.5**	**37.2**	**36.3**	**45.6**	**40.5**	**42.9**	**44.5**	**44.4**	**44.7**	**44.9**
ARBV	**26.4**		10.4	16.7	36.9	36.1	45.1	39.5	42.0	44.7	45.3	45.1	45.6
SALV	**23.9**	22.2		3.8	36.3	35.5	44.7	37.8	41.9	44.9	44.7	45.1	45.0
ADRV*	**25.4**	23.4	18.8		34.6	34.6	46.2	46.2	41.0	43.6	39.7	43.6	42.3
AMTV	**38.2**	39.4	38.0	38.3		15.4	47.3	37.8	44.8	44.9	45.1	46.5	45.9
ODRV	**38.7**	38.6	34.6	37.8	26.1		47.9	37.8	44.6	45.6	45.0	46.1	45.8
SFTV	**42.7**	42.6	42.3	42.3	44.1	43.6		2.7	43.5	49.5	49.2	49.0	49.5
SFSV*	**37.1**	37.4	41.0	38.1	36.2	37.6	6.8		30.8	38.9	36.8	34.6	38.9
KARV	**40.8**	40.7	42.7	40.5	42.3	43.1	41.3	34.2		46.7	46.6	46.1	47.4
SFNV	**42.6**	42.3	42.7	42.3	43.3	43.3	44.9	39.9	43.0		15.8	19.2	16.5
THEV	**42.4**	42.9	37.6	42.1	42.8	42.9	45.2	39.6	42.3	26.1		16.6	11.9
MASV	**41.7**	43.0	41.0	42.8	43.1	43.4	44.5	36.0	42.5	27.6	26.1		17.6
TOSV	**42.3**	43.3	42.3	42.5	43.7	44.3	44.6	37.4	43.4	26.7	23.6	27.2	
Gn													
ADAV		**26.1**	**33.7**		**62.4**	**62.1**	**63.2**	**63.8**	**61.5**	**66.5**	**66.0**	**66.5**	**67.9**
ARBV	**31.2**		38.2		62.7	61.7	62.0	65.2	63.6	66.7	66.6	65.9	67.2
SALV	**36.1**	38.2			62.2	61.1	63.9	68.8	62.3	66.8	67.6	65.4	67.0
AMTV	**54.2**	54.1	54.1			38.7	69.7	72.2	67.0	68.7	68.9	67.2	69.1
ODRV	**52.7**	54.0	52.1		40.7		68.2	69.0	66.9	68.2	68.0	68.1	67.3
SFTV	**53.7**	52.7	52.7		56.9	56.6		24.6	58.6	67.5	68.6	67.7	67.2
SFSV*	**52.0**	53.8	54.4		58.9	57.1	29.3		57.2	66.7	65.9	67.6	67.0
KARV	**51.7**	52.8	51.4		55.3	55.3	50.3	50.5		64.3	66.2	65.5	65.7
SFNV	**55.0**	55.3	54.6		56.6	56.5	55.4	54.5	53.8		39.8	43.3	42.1
THEV	**55.0**	55.5	55.7		56.6	56.9	56.3	51.8	53.8	37.1		43.4	40.9
MASV	**54.9**	55.2	54.8		56.4	57.6	55.7	55.7	54.2	41.4	41.5		42.5
TOSV	**57.0**	56.0	56.2		58.0	56.1	55.9	53.8	54.3	40.6	40.3	41.8	
Gc													
ADAV		**15.4**	**25.1**		**51.6**	**50.2**	**53.6**	**54.8**	**51.1**	**55.0**	**52.6**	**54.0**	**55.8**
ARBV	**26.5**		26.9		52.0	50.0	51.0	52.7	52.3	55.8	53.0	54.4	55.4
SALV	**31.1**	32.7			51.8	48.2	53.6	54.8	50.9	57.1	55.0	52.6	53.4
AMTV	**46.8**	47.9	48.5			28.3	62.9	61.3	57.4	61.1	59.5	59.1	60.5
ODRV	**44.3**	48.0	45.9		32.7		61.3	61.3	57.0	59.1	57.9	59.7	57.7
SFTV	**47.1**	47.0	46.2		51.7	52.0		1.1	48.7	55.4	55.8	56.0	54.8
SFSV*	**48.9**	48.6	49.3		50.0	50.4	2.5		50.5	57.0	58.1	57.0	55.9
KARV	**45.9**	46.1	44.0		51.2	49.1	43.8	46.1		50.9	53.1	52.1	50.5
SFNV	**46.0**	48.1	47.5		50.7	49.2	47.3	48.6	45.5		26.5	30.7	28.9
THEV	**46.6**	47.2	48.5		50.4	49.2	47.7	48.9	46.2	29.8		27.5	27.7
MASV	**45.1**	48.7	47.8		49.9	52.2	47.2	51.4	44.7	32.9	32.0		25.1
TOSV	**49.7**	49.3	48.5		51.5	48.9	46.9	50.0	44.4	33.2	32.6	31.4	
Nucleocapsid													
ADAV		**21.4**	**21.8**		**53.7**	**52.0**	**56.6**	**56.6**	**45.4**	**54.3**	**53.0**	**54.7**	**53.4**
ARBV	**27.8**		13.3		51.2	48.0	55.5	55.5	48.1	53.8	53.8	52.2	53.8
SALV	**27.7**	23.3			54.1	51.6	55.9	56.3	46.9	54.7	54.7	54.3	54.3
AMTV	**46.2**	41.4	44.9			15.6	56.1	55.7	54.2	56.6	58.2	57.4	56.6
ODRV	**47.1**	43.4	44.6		26.5		56.1	55.3	53.3	58.6	58.2	57.0	57.4
SFTV	**46.0**	48.2	48.0		48.0	48.2		3.3	47.3	54.5	54.1	55.7	56.1
SFSV	**45.9**	47.3	47.8		47.4	48.0	14.6		47.3	54.9	54.5	55.7	56.1
KARV	**42.7**	43.6	43.4		47.2	48.5	43.0	44.7		54.8	53.5	53.9	53.1
SFNV	**46.4**	44.3	44.9		48.1	49.5	48.4	47.7	47.4		11.9	13.0	9.9
THEV	**45.2**	46.3	46.2		48.8	50.1	47.8	47.6	47.3	21.1		16.5	15.4
MASV	**46.0**	43.6	44.9		51.0	49.2	47.7	48.0	47.9	22.4	20.7		14.2
TOSV	**45.8**	45.2	46.0		47.3	49.9	47.2	48.0	46.7	21.3	22.5	23.2	
Nonstructural protein													
ADAV		**32.2**	**25.3**		**60.5**	**62.7**	**77.4**	**77.4**	**71.4**	**84.5**	**88.4**	**85.5**	**86.3**
ARBV	**32.1**		28.6		62.4	62.9	77.0	77.8	70.1	81.4	86.8	82.5	85.7

(Continued on following page)

TABLE 2 (Continued)

Protein and virus[b]	Divergence[c] (%) from sequence of:												
	ADAV	ARBV	SALV	ADRV*	AMTV	ODRV	SFTV	SFSV*	KARV	SFNV	THEV	MASV	TOSV
SALV	**32.6**	32.4			62.0	61.6	76.3	76.6	71.4	83.2	86.2	83.3	85.5
AMTV	**52.0**	50.8	50.7			39.1	79.7	80.0	77.0	84.8	85.7	83.6	83.8
ODRV	**50.3**	50.5	49.9		40.0		80.8	80.8	77.8	85.2	87.9	82.8	82.6
SFTV	**61.9**	63.1	62.1		65.3	65.0		6.1	70.4	82.4	89.1	86.5	85.1
SFSV	**63.4**	64.1	62.6		64.2	64.7	15.2		70.8	83.7	89.1	87.3	85.1
KARV	**56.5**	57.9	59.8		63.6	63.3	56.0	58.2		80.3	87.6	87.7	85.2
SFNV	**68.0**	67.5	67.4		68.6	68.1	63.1	62.3	67.7		56.7	58.0	51.0
THEV	**70.8**	69.0	71.7		69.0	67.7	67.2	67.2	66.1	50.4		56.3	43.7
MASV	**67.7**	65.5	66.8		65.7	67.0	67.7	67.2	67.1	50.5	49.8		54.8
TOSV	**68.2**	67.4	67.4		67.6	66.3	65.2	65.8	65.9	46.6	40.6	47.5	

[a] GenBank accession numbers for sequences (ADAV to TOSV, respectively) are as follows: polymerase gene, KJ939330, JX472400, JX472403, HM043726, HM566144, HM566174, NC_015412, EF095551, KF297909, HM566172, JF939846, EU725771, NC_006319; Gn and Gc glycoprotein genes, KJ939331, JX472401, JX472404, HM566143, HM566173, NC_015411, AY129740, KF297907, HM566171, JF939847, EU725772, EU003177; nucleocapsid and nonstructural protein genes, KJ939332, JX472402, JX472405, HM566145, HM566175, NC_015413, EF201827, KF297914, EF201829, JF939848, EU725773, NC_006318. *, partial sequence.

[b] AMTV, Arumowot virus; ODRV, Odrenisrou virus; KARV, Karimabad virus; THEV, Tehran virus; MASV, Massilia virus.

[c] For each protein, the upper-right matrix represents pairwise distances between amino acid alignments and the lower-left matrix represents pairwise distances between nucleotide alignments. Values for ADAV are in boldface.

NGS were confirmed by direct sequencing performed on overlapping PCR products using Sanger sequencing.

Genetic distances. Pairwise distances of the nucleotide and amino acid sequences among ADAV and viruses in the Salehabad virus complex as well as other phleboviruses are shown. in Table 2 Amino acid pairwise distances between ADAV and other Salehabad complex viruses were ≥21.4% (N), ≥25.3% (NS), ≥26.1% (Gn), ≥15.4% (Gc), and ≥11.5% (L), whereas, compared with other Old World phlebovirus species, they were ≥45.4% (N), 71.4% (NS), 57.7% (Gn), 51.1% (Gc), and 40.5% (L).

To determine if it was possible to distinguish the species using quantitative genetic data, the distribution of amino acid genetic distance was studied independently for each of the genes (L, Gn, Gc, N, and NS genes) (see Table S1 in the supplemental material) using only the complete sequences in the GenBank database. For each of the 9 species recognized by the ICTV, interspecies cutoff values and the highest distance observed between ADAV and other members of the *Salehabad virus* species were indicated on the histograms. The highest observed amino acid distances between ADAV and Salehabad virus species for the L, Gn, Gc, N, and NS genes are 15.3%, 35.6%, 28.3%, 21.8%, and 32.2%, respectively. Compared gene by gene, these distances are consistently lower than the lowest observed distances between ADAV and phleboviruses other than the Salehabad virus species, which are shown in Table S1 in the supplemental material as 40.0%, 58.1%, 50.6%, 44.4%, and 70.8% for the L, Gn, Gc, N, and NS genes, respectively. The lowest interspecific distances detected for the L, Gn, Gc, N, and NS genes, i.e., 40.0%, 46.2%, 33.6%, 35.8%, and 54.8%, respectively, among phlebovirus species groups were higher than the lowest distances observed between ADAV and Salehabad virus species compared gene by gene (species groups defined by the ICTV [1]). They are indicated in different colors in Table S1 in the supplemental material). This suggests that ADAV may be included in the Salehabad virus species group.

Phylogenetic analysis. ADAV belongs to the cluster that comprises viruses belonging to the Salehabad virus species, regardless of the viral gene used for analysis. The monophylety of the 3 viruses (SALV, ARBV, and ADAV) is supported with bootstrap values ≥99% for the 4 ORFs (Fig. 3). In phylogenetic analysis (Fig. 3), the major nodes enable identification of the virus species and confirm previously reported topologies (30, 33, 38, 39). For comparison, we also performed maximum-likelihood analysis, which showed the same phylogenetic relationships for all the gene segments (data not shown).

MN-based seroprevalence study. Detailed results of an MN-based seroprevalence study are presented in Table 3 and Fig. 1. A total of 124 dog sera were collected from the Adana region, of which 17 (13.7%) contained neutralizing antibodies against ADAV. These 124 sera consisted of 2 batches of 35 and 89 sera, respectively. Detailed information (village, sex, age) and the nature of dog usage (hunting, guard, sheep dog, pet, and village dog) were available for the 89-serum batch only. There was no correlation between these parameters and the presence/absence of neutralizing antibodies against ADAV. They were all negative when tested with Arbia virus.

A total of 1,000 human sera were collected from individuals living in the Mersin region, as well as 51, 48, and 66 sera from goats, sheep, and dogs, respectively. Of the 1,000 human sera, only 7 had neutralizing antibodies against ADAV (0.7%). In contrast, 39 of 165 (23.6%) animal sera collected in Mersin were positive. All, except one human serum, were negative when tested with Arbia virus.

Genotyping of sand flies in the virus-positive pool. The analysis of NGS reads indicated that pool 195 contained *Phlebotomus tobbi* (675 reads), *Phlebotomus perfiliewi* (65 reads), and *Phlebotomus papatasi* (58 reads) corresponding to the cytochrome *c* gene.

DISCUSSION

The first evidence for the presence of sand fly-borne phleboviruses in Turkey was reported in 1976 in a neutralization-based seroprevalence study (19). Recently, widespread circulation of these viruses was revealed via seroprevalence studies, clinical case reports, and a series of human cases (20, 21, 22, 23, 24, 25, 27, 28). Sandfly fever occurs commonly among local populations in three regions of Turkey (Mediterranean, Aegean, and Central Anatolia) as recorded in several outbreaks reported since 2004 (20, 21, 22). The presence of Sandfly fever Turkey virus (SFTV) and Toscana

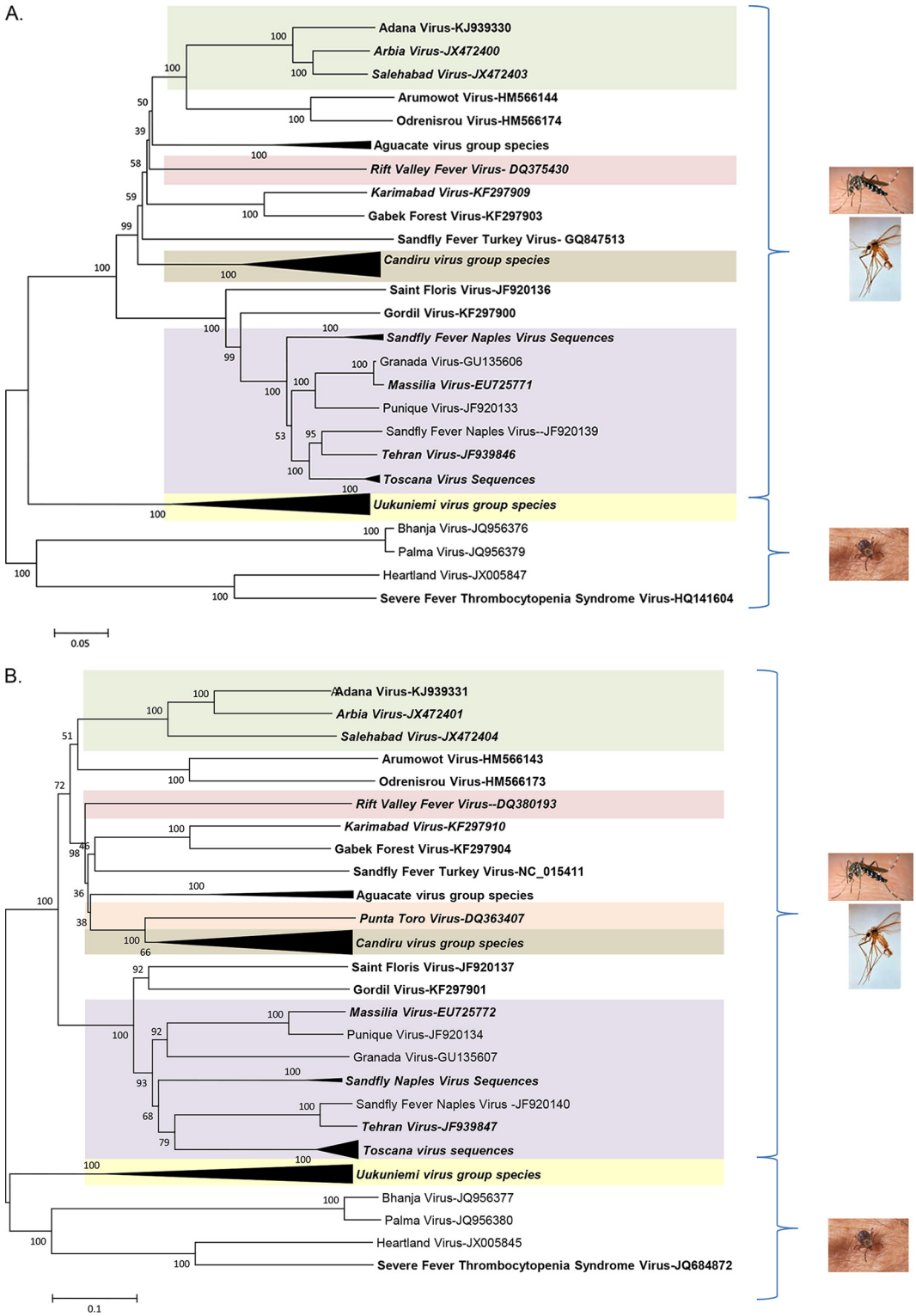
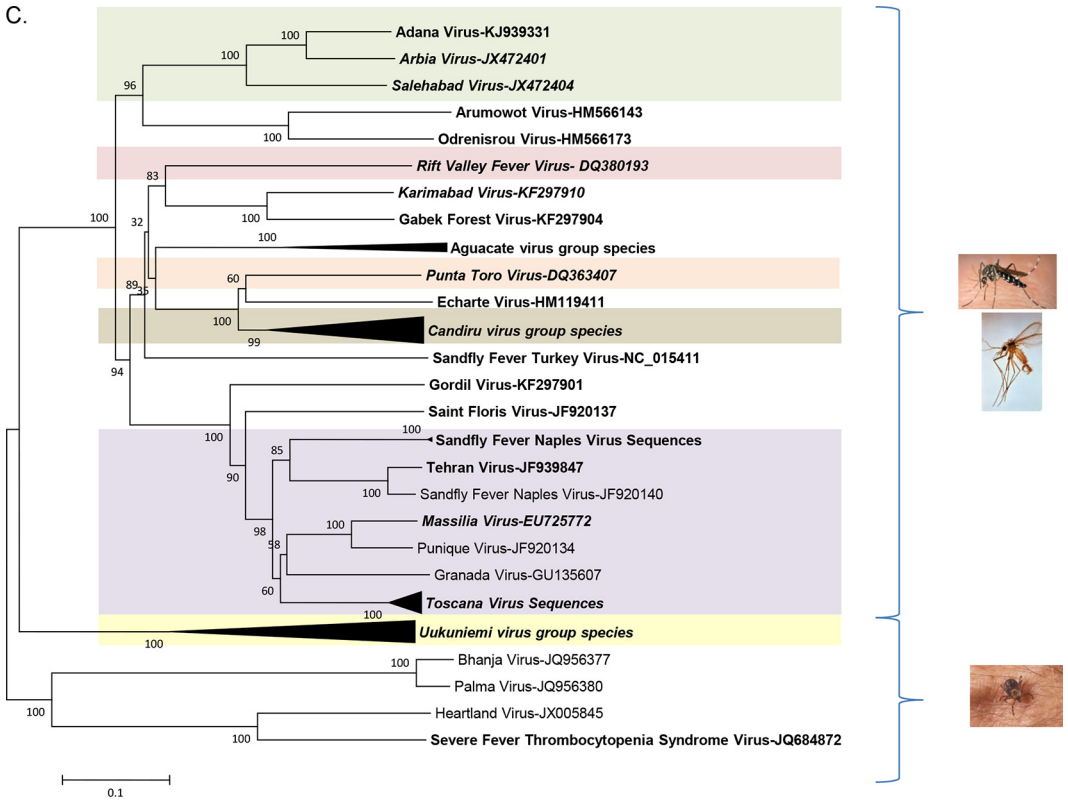


FIG 3 Phylogenetic analysis of the phlebovirus amino acid sequences. (A) L protein; (B) Gn protein; (C) Gc protein; (D) nucleocapsid protein; (E) nonstructural protein. The species recognized by ICTV are indicated in boldface and italics, and the tentative species are indicated in boldface roman. The GenBank accession numbers of all the phleboviruses included in the analysis can be found in Table S1 in the supplemental material.

virus (TOSV) was established in Turkey through virus isolation and molecular detection, respectively (20, 24, 25, 26, 27, 28). However, most field-based studies that combined entomological and virological aspects to understand the distribution of phleboviruses and their vectors have been inadequately conducted in the past. One noticeable exception was a study which identified *Phlebotomus major sensu lato* as a vector of SFTV in Central Anatolia although the virus was not isolated from sand



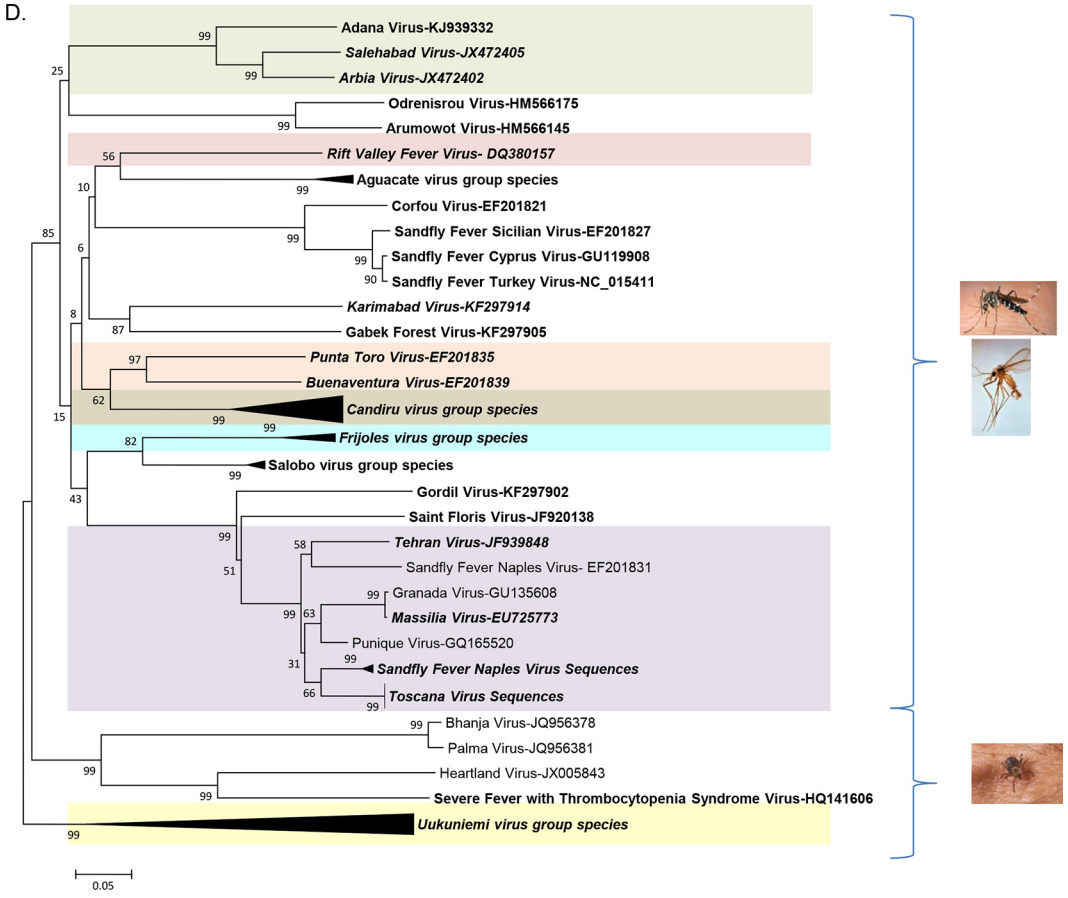


FIG 3 continued

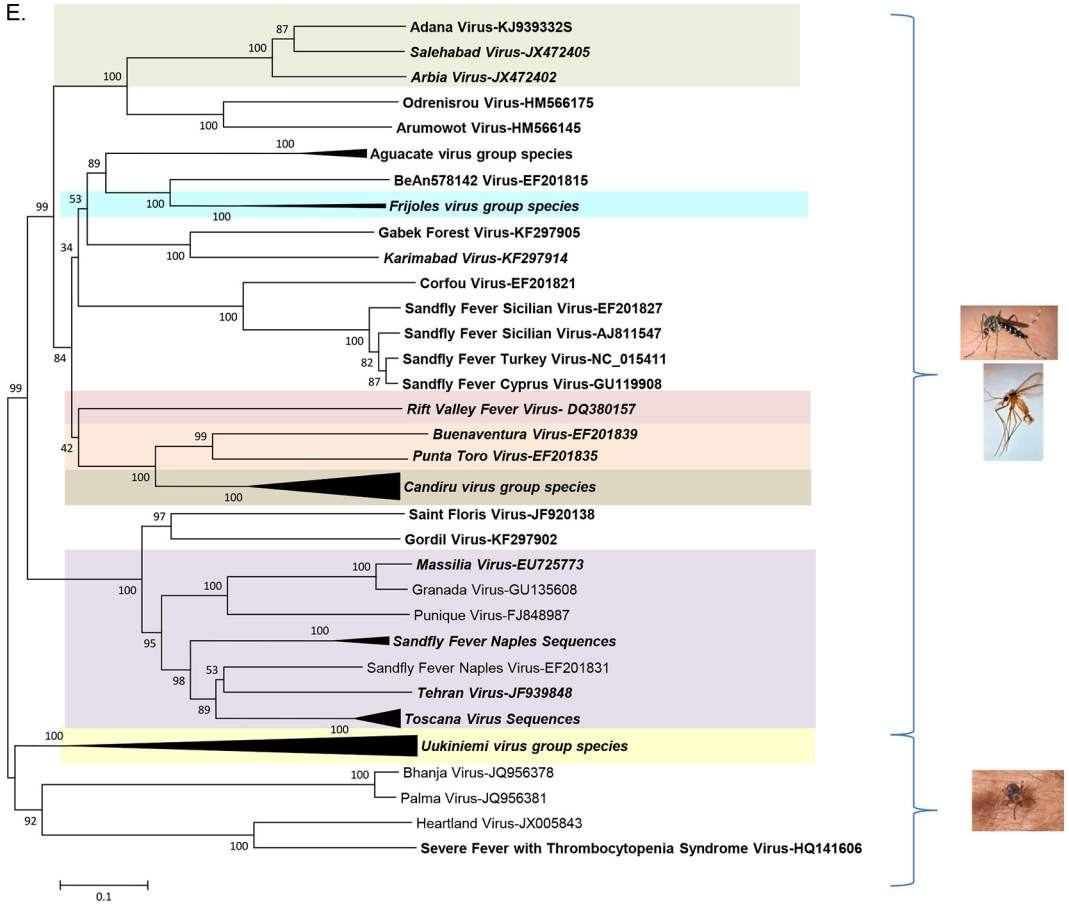


FIG 3 continued

flies. At the outset of this study, STFV was the only phlebovirus isolated in Turkey (20).

In this study, from 7,731 sand flies organized in 380 pools, we isolated a novel phlebovirus, tentatively named Adana virus (ADAV) from the eponymous name of the studied province (Fig. 1). The complete sequence of ADAV consists of 3 segments of 6,405, 4,229, and 1,758 nucleotides for the L, M, and S segments, respectively. Genetic and phylogenetic analyses showed that the SALV-ARBV-ADAV cluster is supported by high bootstrap values (≥99%) regardless of the gene segment used for the analysis. As previously reported by Palacios et al. (33) for other Old World sand fly-borne phleboviruses, the consistent grouping of ADAV together with viruses belonging to the *Salehabad virus* species may exclude the mechanism of recombination in the generation of ADAV.

According to ICTV *Salehabad virus* consists of two viruses: Salehabad virus isolated in 1959 from sand flies in Iran (40), and Arbia virus, isolated in 1988 from sand flies in Italy (41).

The highest observed amino acid distances between ADAV and *Salehabad virus* species for the L, Gn, Gc, N, and NS genes are 15.3%, 35.6%, 28.3%, 21.8%, and 32.2%, respectively (Table 2). These distances are consistently lower than the lowest distances observed between ADAV and non-*Salehabad virus* phleboviruses (40.0%, 58.1%, 50.6%, 44.4%, and 70.8% for the L, Gn, Gc, N, and NS genes, respectively) (see Table S1 in the supplemental material). Thus, genetic data indicate that ADAV belongs to the *Sale-*

TABLE 3 Distribution of Adana virus neutralizing antibodies according to sampling locations and species

Region[a]	Serum source	No. of tested sera	No. of sera with neutralizing titer of:					Total no. (%) positive
			Negative	≥20	≥40	≥80	≥160	
Adana 1	Dog	35	34	1	0	0	0	1 (2.9)
Adana 2	Dog	89	73	11	5	0	0	16 (18.0)
Adana 1 + 2	Dog	124	107	12	5	0	0	17 (13.7)
Mersin	Human	1,000	993	6	1	0	0	7 (0.7)
	Goat	51	33	9	6	2	1	18 (35.3)
	Sheep	48	31	9	5	3	0	17 (35.4)
	Dog	66	62	3	0	0	1	4 (6.1)

[a] Adana 1, dog sera provided by K.E.; Adana 2, dog sera provided by Y.O.

habad virus species. This is also supported by the fact that the lowest interspecific distances among ICTV-recognized species (1) (40.0%, 46.2%, 33.6%, 35.8%, and 54.8% for the L, Gn, Gc, N, and NS genes, respectively) are higher than the highest observed distances between ADAV and *Salehabad* viruses.

Recently, molecular data (although not confirmed by virus isolation) support the existence of other viruses in the *Salehabad virus* group: (i) sequences of Adria virus (ADRV) were reported from sand flies in Albania (42); (ii) one case of meningitis was attributed to Adria virus in a Greek patient with no history of traveling abroad (43); (iii) in northwestern Turkey (Eastern Thrace), sequences related to but clearly distinct from Salehabad virus, Arbia virus, ADAV, and Adria virus were detected in sand flies (44).

For many years, the lack of genetic data for most phleboviruses has dictated that the species are defined by their serological relationships and are distinguishable by 4-fold differences in two-way neutralization tests (1). We could not perform these tests due to the lack of ADAV hyperimmune antisera. In a previous study, amino acid pairwise distances of Gc and L were deemed suitable for delineating species of the *Phlebovirus* genus. Cutoff values for intraspecies distances were <29% (Gc) and <21% (L), whereas distances >40% (Gc) and >31% (L) were observed at the interspecies level (30). The increased number of complete sequences available for phleboviruses has drastically modified the picture, and specific studies are needed to revisit the possible utilization of genetic distance for taxonomy (33, 39, 45).

The high rates of neutralizing antibodies in domestic animal sera (13.7% for dogs in the Adana region; 6.1%, 35.3%, and 35.4% for dogs, goats, and sheep, respectively, in the Mersin region) demonstrate that ADAV is present and circulates actively in these contiguous regions of Mediterranean Turkey. We considered the possibility that antigenic cross-reactivity with SFTV or TOSV might have biased our results. However, the following points contradict this argument: (i) neutralization assay is the most specific and discriminative technique for seroprevalence studies (36), (ii) we employed a stringent microneutralization assay by using 1,000 $TCID_{50}$ of virus (for both ADAV and Arbia virus), i.e., a dose that is 10 times higher than that used in other studies (25, 46), and (iii) none of the 289 animal sera possessed neutralizing antibodies against Arbia virus. These results constitute compelling evidence that the positive sera contained antibodies truly elicited against ADAV and not another virus of the Salehabad virus complex.

Salehabad species viruses were long considered a group of viruses with no medical or veterinary interest. This view deserves to be revisited according to our results and to recent evidence of human infection with Adria virus in Greece (43).

In this study, we found that 0.7% of the human sera from people living in Mersin (147 km from Adana) had neutralizing antibodies against ADAV. This very low prevalence suggests either that the local populations are not exposed to ADAV or that ADAV replicates poorly or does not replicate in humans. Since in the Adana region local populations commonly live in the vicinity of domestic animals, human exposure to ADAV is likely to be equivalent to that of domestic animals. Therefore, we favor the second hypothesis. The 0.7% seroprevalence rate may relate to repeated exposure to virus antigen through significant and repetitive contact with the virus. Similar results were recently observed in Tunisia with Punique virus, where seroprevalence rates in humans were 0.4% despite frequent detection in sand flies and high seroprevalence in dogs (4, 36, 47). The low seroprevalence in humans suggests that ADAV is not likely to be important for public health in exposed human populations. However, further studies must be conducted to investigate its capacity to cause febrile illness, neuroinvasive infections, or other clinical manifestations in humans.

Sand flies are present in almost all regions of Turkey due to favorable climatic and ecological conditions of temperature, humidity, microhabitat, and social dynamics. In the study region, the most abundant species is *P. tobbi* (49%), followed by *Larroussius* spp. (26%), *Phlebotomus papatasi* (8%), *Sergentomyia dentata* (6%), and *P. perfiliewi transcaucasicus* (9%), *Phlebotomus major sensu lato* (1%), and *Phlebotomus sergenti* (1%) (48). Our results showing that pool 195 contained *P. tobbi*, *P. perfiliewi*, and *P. papatasi* are consistent with the previously established species distribution in the Adana region (48). The region is also a well-known focus of cutaneous leishmaniasis due to *Leishmania infantum* transmitted by *P. tobbi*, which feeds on cattle (70%) and humans (10%) according to blood meal identification (49, 50, 51). It is therefore likely that ADAV is transmitted by *Larroussius* sand flies, most probably *P. tobbi.* However, further studies using individual sand flies are required for indisputable identification of the vector of ADAV.

ADAV rates of infection in sand flies (0.01%) are lower than rates reported with other phleboviruses in other countries. This rate was calculated using two RT-nested-PCR assays that are commonly used in such studies (31, 32). It was confirmed by using two real-time RT-PCR assays specifically designed for ADAV. First, although lower than in other studies, the ADAV infection rate is in the same order of magnitude as that of Toscana virus in Tunisia (0.03%) and in Spain (0.05%) (52, 53). Second, this study is the first one to calculate a rate of infection for a phlebovirus belonging to the *Salehabad virus* species.

Despite studies searching for phleboviruses in sand flies in Turkey using the same molecular tools, ADAV was not previously identified (29, 44). In this regard, there are three important points. First, this is the only study screening field-caught sand flies for the presence of phleboviruses in Adana and Mersin. Second, a possible reason for these observations in a cross-sectional surveillance effort is the typically limited activity range of sand flies (54). Third, similar findings were observed in Central Anatolia and Eastern Thrace regions, where novel strains seem to be confined to relatively few sampling locations in rural areas (29, 44). Collecting sand flies over longer periods may help in understanding the circulation of Adana virus in the same region and also in the neighboring city Mersin, where seropositivities were detected.

Our discovery of ADAV, together with recent data (44), demonstrate that the *Salehabad virus* species have a much greater genetic diversity and may exhibit a much wider geographical distribution than initially believed. Future studies are required to address these points and to confirm whether or not specific members of the *Salehabad virus* species cause human or animal disease.

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