

# Phlebovirus detection on phlebotomine sandflies in Lampedusa Island (Italy)

Reale S., Anzà D., Bruno F. \*, Scibetta S., Oliveri E., Vitale F. and Castelli G.

Istituto Zooprofilattico Sperimentale della Sicilia "A Mirri"

\*Corresponding author at: Istituto Zooprofilattico Sperimentale della Sicilia "A. Mirri".  
E-mail: federica.bruno@izssicilia.it.

*Veterinaria Italiana* 2023, **59** (2), 171-177. doi: 10.12834/VetIt.2711.17825.2  
Accepted: 07.07.2022 | Available on line: 30.06.2023

## Keywords

Phlebotomine,  
Toscana Virus,  
Lampedusa,  
Surveillance.

## Summary

Phleboviruses are common human pathogens diffused on the Mediterranean area whose infection can cause the typical prodromal symptom of a mild three-days fever. In particular, Toscana Virus (TOSV) has a great concern since its capacity to provoke central nervous system disorders like meningoencephalitis. Furthermore, as the phlebotomine arthropod vectors represent the main carrier for pathogens of the genus *Leishmania* as well, the purpose of the study was to investigate the presence of TOSV in Lampedusa, Italy previously reported for leishmaniosis infection cases. The survey was carried out through an initial sampling phase of sand flies, by means of CDC light traps, and a second step of molecular analyses. The genomic S-segment of TOSV was targeted. The positive samples were sequenced and compared with those available in GenBank™ using Basic Local Alignments Tool (BLAST) analyses. The study revealed for the first time the presence of TOSV in Lampedusa, Italy. The entomological studies directed on vectors are currently widely used in sand fly surveillance, and new data on TOSV are of public health concern.

## Introduction

Pathogens transmitted to humans and animals by phlebotomine sand flies are relatively neglected, as they cause infectious diseases that are not on the priority list of national and international public health agencies in Europe (Alvar *et al.*, 2012; Moriconi *et al.*, 2017). Phlebotomine sand flies (Diptera: Psychodidae) are important vectors of various human and animal pathogens such as *Bartonella bacilliformis*, *Phlebovirus*, and parasitic protozoa of the genus *Leishmania*, causative agent of leishmaniases that account among most significant vector-borne diseases (Benallal *et al.*, 2022; Remoli *et al.*, 2016). Among these, in particular, the Phleboviruses, largely spread in the Mediterranean region (Alkan *et al.*, 2013). The Phlebovirus genus comprises nine viral species (Sandfly fever Naples, Salehabad, Rift valley fever, Uukuniemi, Bujaru, Candiru, Chilibre, Frijoles, Punta Toro), all enveloped and with a nucleocapsid containing a single-chain

RNA genome divided into three segments L, M and S, which encode for reverse transcriptase, two glycoproteins, capsid proteins and non-structural proteins (Alkan *et al.*, 2013; Wuerth and Weber, 2016). Furthermore, like other RNA viruses that possess a reverse transcriptase lacking a proofreading activity, mutations that can generate new and more virulent quasi-species are certainly not uncommon (Alkan *et al.*, 2013). The four most common serotypes associated with human infections include sandfly fever Naples virus (SFNV), sandfly fever Sicilian virus (SFSV), sandfly fever Cyprus virus (SFCV) and Toscana virus (TOSV) (Marchi *et al.*, 2017; Wuerth and Weber, 2016). Almost all of these may cause a mild three-days fever known as "Pappataci-fever", especially during the warm months and coinciding with the peak activity of sand fly species (Remoli *et al.*, 2016). Among all mentioned viruses, TOSV is the only one associated with severe inflammations of the central nervous system in humans, such as meningitis, encephalitis or meningoencephalitis (Dincer *et al.*,

2015). In details, the replication and vehiculation of TOSV depends exclusively to the disposability of specific arthropod carriers and their copiousness, such as *Ph. perfliewi* and *Ph. perniciosus*, thus allowing its prevalence in those regions where the vectors are present, such as in Italy, Algeria, Spain, Portugal, and Cyprus (Foglia Manzillo et al., 2018; Valassina et al., 2003). A further threatening agent for human health is *Leishmania* spp., which shares with TOSV the circulation in some Mediterranean localities, as well as the conveyance by means of the same carriers (Fares et al., 2020; Maia et al., 2017). This includes *Ph. ariasi* and *Ph. longicuspis* for *Leishmania infantum* (*L. infantum*) and SFSV, while *Ph. perniciosus* can transmit both *L. infantum* and TOSV (Es-Sette et al., 2014). *Phleboviruses* and *Leishmania* share the same principal vectors: sand flies. Sand flies are tiny hematophagous insects that often live close to domestic animals and humans. Only females bite, typically from dusk to dawn. They are poor fliers, flying silently for short distances, and they have activity peaks during summertime in non-tropical regions; sand flies are widely distributed in Mediterranean countries. The two species that are most abundant in the natural and rural environments of the Sicilia region are *Ph. perniciosus* and *Ph. perfliewi*; both species are proven vectors of *L. infantum* and TOSV in Italy (Calzolari et al., 2022, 2021; Maroli et al., 2013). With the purpose of a better surveillance, several researchers have suggested the creation of screening methods to control the spread of sand fly viruses, in particular over the areas where both the arthropod carriers and *Leishmania* are located (Calamusa et al., 2012; Mammina et al., 2012). Moreover, since 2018 the Italian National Health Service has included neuro invasive infections caused by TOSV in the special surveillance of human arboviral infections, with periodic bulletins (<https://www.epicentro.iss.it/arboviroso/bulletins>). Foglia Manzillo et al. (2018) confirmed high Canine Leishmaniosis (CanL) seroprevalence on Lampedusa

island. The high seropositive dogs proportion and the presence of competent vectors demonstrated that *L. infantum* abundantly circulated in the island and might constitute a serious risk for people, living or hosting there. Therefore, the aim of our study was to investigate the presence of TOSV on the island of Lampedusa in the Sicilia region (Italy), an endemic area for Leishmaniasis, through direct molecular analysis in sand flies collected during 2016.

## Materials and methods

### Sand flies collection and storage

The present study was carried out in Lampedusa, a small island (20,2 km<sup>2</sup>) in the Italian Pelagie archipelago (35°30'56"N 12°34'23"E). This archipelago is the southern-most territory of Italy (205 km from Sicily) and the near-est to North Africa (113 km). Sticky traps and CDC miniature light traps (Hausherr's Machine Works, Toms River, NJ, USA) were used in four urban (n = 1 trap), periurban (n = 1 trap) and rural (n = 2 traps) sites of the island during July 2016. Urban site consisted of 2 dog's owner private homes that were housing two and four dogs, respectively, while the periurban site was a house with courtyard housing six dogs. The rural sites consisted of two small farms where goats and sheep were farmed. In details, the trapping sites (ST), throughout light traps sited outside the entrances of houses and chalets as well as in the seaside and mountain stretch of the island, had the relative coordinates: ST-1 35°30'20.0"N, 12°36'35.4"E, ST-2 35°30'48.1"N, 12°37'05.3"E, ST-3 35°30'34.4"N, 12°37'23.6"E, and ST-4 35°30'08.4"N, 12°36'52.7"E. The entrapped insects were registered and morphologically examined to select only phlebotomine, eventually sorted to constitute 12 pools, each containing around 20 insects, locality, date of collection and sex. Subsequently, they were stored in 100% Ethanol and frozen at -20 °C. (Table I).

**Table I.** Geographical characteristics of sand fly sampling sites in Lampedusa Island and IDs pools included in the study.

Area	Stations	Latitude	Longitude	IDs Pools
Urban	ST-1	35°30'20.0"N	12°36'35.4"E	p-Phl-1, p-Phl-2, p-Phl-3;
Periurban	ST-2	35°30'48.1"N	12°37'05.3"E	p-Phl-4, p-Phl-5, p-Phl-6;
Rural	ST-3	35°30'34.4"N	12°37'23.6"E	p-Phl-7, p-Phl-8, p-Phl-9;
Rural	ST-4	35°30'08.4"N	12°36'52.7"E	p-Phl-10, p-Phl-11, p-Phl-12;

### Sand flies processing and RNA extraction

The investigation has been articulated in two parts, the first of which concerned a field campaign to trap sand flies through light traps in those neighbourhoods of Lampedusa previously reported

for leishmaniasis cases in domestic and farm animals (dogs and goats). On the other hand, the second section implicated a laboratory work through the design and use of a protocol divided into three fundamental steps: the extraction of the genetic material, the amplification of a portion of the N

gene within the viral genomic S segment by nested PCR and, finally, the genetic characterization of the fragments by capillary sequencing. Indeed, this kind of investigation about *Phleboviruses* are common worldwide to monitor the spread of these pathogens (Ayhan *et al.*, 2017). For what concerned the sand fly collection, it was focused on leishmaniosis endemic areas, as this would provide a greater chance of finding TOSV (Charrel *et al.*, 2007; Es-Sette *et al.*, 2014; Foglia Manzillo *et al.*, 2018; Maia *et al.*, 2017). Sand flies samples were transferred from tubes with ethanol into 2 milliliter (ml) tubes filled with sterile water. After washing, the samples were processed for the RNA extraction using the AllPrep DNA/RNA Kit (Qiagen, Hilden, Germany). Then, the samples were lysed with 600 microliter ( $\mu$ l) of RLT Plus Buffer and shaken by using a conventional rotor-stator homogenizer (FastPrep-FP120), for at least 1 minute (min). Subsequently, the lysates were centrifuged and the supernatants transferred on the RNeasy spin columns with one volume of 70% ethanol. Finally, after two steps of washing, using two washing buffers, and centrifugation, the RNA was eluted in 30  $\mu$ l of RNase-free water and immediately stored at  $-80^{\circ}\text{C}$ .

### Reverse-transcription of viral RNA

Reaction was performed employing the "High-capacity cDNA Reverse Transcription Kit" (Applied Biosystems Inc, CA, USA), according to the manufacturer's protocol.

In details, the reaction mix contained a 10X RT-Buffer, 2.5 U of MultiScribe™ Reverse Transcriptase, 10mM dNTPs mix and 10X RT Random Primers. Then, the tubes were placed into a SimplyAmp Thermal Cycler (Applied Biosystems) with the following program: 10 min at  $25^{\circ}\text{C}$ , 2 hours at  $37^{\circ}\text{C}$  and 5 min at  $85^{\circ}\text{C}$ . At the end of the process, the samples were stored at  $-20^{\circ}\text{C}$ .

### Amplification of the S segment

Subsequently, for the amplification of the TOSVS-segment, a set of two pairs of primers was used, chosen from the work of Valassina *et al.* (Valassina *et al.*, 1996). In particular, the outer primers TV1 (5'-CCAGAGGCCATGATGAAGAAGAT-3') and TV2 (5'-CCACTCCTATGAGCAGCTT CT-3'), forward and reverse respectively, were employed for the amplification of a larger fragment, while the inner primers TV3 (5'-AACCTGATTCAGTCTACCAGTT-3') and TV4 (5'-TTGTTCTCAGAGATGGAT TTATG-3') for the Nested-PCR reaction. The PCR was performed in a 50  $\mu$ l volume by using the AmpliTaq Gold® 360 DNA Polymerase Kit (Applied Biosystem Inc, CA, USA) according to the manufacturer's protocol, containing the

AmpliTaq Gold® 360 Buffer 10X, 2mM  $\text{MgCl}_2$ , 10mM dNTPs mix, 20pmol of each primer (TV1 and TV2), 1.25 U of DNA polymerase and 10  $\mu$ l of the cDNA. The thermocycling conditions were: an initial denaturation step at  $94^{\circ}\text{C}$  for 5 min, then 35 cycles of 1 min at  $94^{\circ}\text{C}$ , 30 seconds (sec) at  $56^{\circ}\text{C}$ , 30 sec at  $72^{\circ}\text{C}$  and a final elongation step at  $72^{\circ}\text{C}$  for 7 min. After this, a Nested-PCR was conducted on 1  $\mu$ l of amplicon, using the same mix components of the previous reaction, with the exception of the primers (TV3 and TV4). The mixtures were subjected to a cycle of denaturation at  $94^{\circ}\text{C}$  for 2 min, 30 cycles of 45 sec at  $94^{\circ}\text{C}$ , 25 sec at  $58^{\circ}\text{C}$  and 30 sec at  $72^{\circ}\text{C}$ , then a final step at  $72^{\circ}\text{C}$  for 5 min. The products were stored at  $-20^{\circ}\text{C}$ . The results of the Nested-PCR were detected by gel-electrophoresis, using a 2% agarose gel and a TBE buffer, with Gel Red (solution at 1:10,000). For each run a 1-Kilobase ladder was added. PCR was carried out according to the recommended guidelines, including negative control (NC) and one positive control (PC, kindly provided by Prof. Remoli, Istituto Superiore di Sanità) in assay.

### Sequencing and data analysis

Before sequencing, a purification step of the PCR products was performed, by using the GFX PCR-DNA and gel band purification kit (GE Healthcare, Buckinghamshire, UK).

Then, the sequencing reaction was carried out with the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, CA, USA) and the sequence products purified employing the illustra AutoSeq G-50 Dye (GE Healthcare, Buckinghamshire, UK).

A total of 2  $\mu$ l of purified products were loaded in a 96 wells plate with 11  $\mu$ l of formamide, the mixture was denatured at  $95^{\circ}\text{C}$  for 3 min and instantly cooled on ice for several minutes. Finally, the sequences were analysed by an ABI Prism model 3130 automated sequencer, and eventually matched with BLAST on the GeneBank Database.

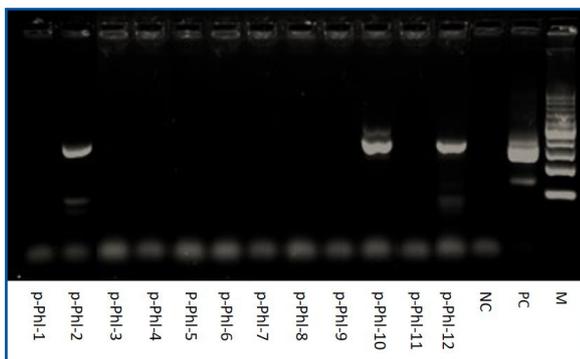
### Results

A total of 240 phlebotomine were captured in different sites of the island, as indicated in Table I. 240 sand flies specimens were collected and used for species identification. Among them, 4 species were identified: *Ph. perniciosus* (164/240, 67.9%), *Ph. papatasi* (69/240, 28.8%), *Ph. neglectus* (7/240, 2.9%) and *S. minuta* (1/240, 0.4%).

The set of samples were arranged in 12 pools, each pool containing 20 sand flies, mixing together the phlebotomine collected with the same trap. TOSV detection was successful in 3/12 (25 %) of

pools, although considering that the samples were collected in separate sites. The results for 3 of 12 phlebotomine pools (p-Phl) were positive by nested PCR with the TV3 and TV4 primers (Valassina *et al.*, 2003, 1996) as shown in Figure 1. A 309-bp fragment was amplified on the S RNA segment of the TOS virus in the samples p-Phl-2, p-Phl-10 and p-Phl-12.

Finally, the samples, which were positive for nested PCR, were processed by sequencing and the sequences aligned with BLAST confirmed the belonging of the sequenced fragments to the N gene of TOSV, as displayed in Fig. 2A, and 2B, with a genotype corresponding to the identification number KM275783.

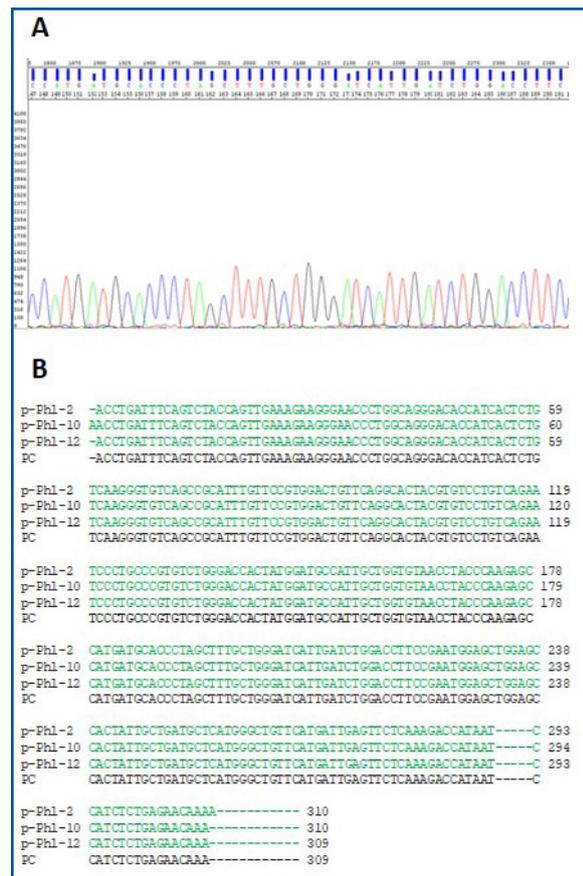


**Figure 1.** Agarose gel electrophoresis (2%) of the Nested-PCR products obtained after amplifying the total pools phlebotomine (P-phl) sandfly. Lane shows the amplicons of the samples from p-Phl-1 to p-Phl-12, with the band size of the samples p-Phl-2, p-Phl-10, and p-Phl-12 approximately ~309 base pair; NC, Negative control; PC, Positive control TOS virus; M, molecular weight standard.

### Discussion

The spread of pathogens like *Phlebovirus* and *Leishmania* has been constituting a public health issue since their discovery, especially in those countries where these pathogen species are endemic and, since they are both carried by the same arthropod vector, human coinfections events are not rare (Remoli *et al.*, 2016). In particular, novel researches have proved that new *Phleboviruses* are frequently found in the Mediterranean regions (Remoli *et al.*, 2015), overlapping the areas where their vectors are present. For these reasons, active surveillance systems constitute an important strategy to keep under control the spread of sand fly and their viruses (Remoli *et al.*, 2015).

Among these, intense entomological and virologic monitoring make a significant effort to put on guard in advance by the hazard of phleboviral infection outbreaks (Remoli *et al.*, 2015). In this regard, the present study was carried out to investigate TOSV, directly on sand flies, in Lampedusa, a Leishmaniasis endemic area.



**Figure 2.** A) Electropherogram shot of the p-Phl-2 fragment sequenced from nucleotide 182 to 226. B) Samples positive for nested PCR, were processed by sequencing and the sequences aligned with BLAST confirmed the belonging of the sequenced fragments to the N gene of TOSV with a genotype corresponding to the identification number KM275783.

On the island of Lampedusa more than 50% of dogs tested were seropositive to *Leishmania* (Foglia Manzillo *et al.*, 2018) and most dogs shared the same circumscribed environment as their owners, living in urban/periurban areas. Foglia Manzillo *et al.*, 2018) showed that *P. perniciosus*, the most competent vector of *L. infantum*, circulated abundantly both in the urban center (ST-1) and in the rural parts of the island (ST-4). The high presence of this sand fly species amplified the risk for human population, both resident and tourists.

In our investigation, approximately 240 sand flies were sorted into 12 pools, where each pool represented a vector population collected at different locations, of which 3 pools resulted positive for TOSV. Assuming that only one sand fly was positive in each pool (up to 20 individuals), at least 3 out of 240 infected sand flies were identified, with a minimum infection rate in the pool of 25% and a rate of positivity of 1.2% of infected sand flies out of the total. This is not unusual as data about the phleboviral presence in many Mediterranean countries are different.

For instance, the ratio of positives pools on total analysed sand flies are the following: 1/460 (Charrel *et al.*, 2007), 7/798 (Charrel *et al.*, 2009), 5/427 (Peyrefitte *et al.*, 2013), 4/896 (Amaro *et al.*, 2015), 5/1910 (Ergunay *et al.*, 2014), 7/900 (Remoli *et al.*, 2014), 10/1489 (Zhioua *et al.*, 2010). In this regard, it is postulated that these results approximately give information about the quantity of phleboviral spreading in a territory (Ayhan *et al.*, 2017). Effectively, most of the detections about novel *Phleboviruses* are performed by using molecular techniques, as the PCR tests (Alkan *et al.*, 2013, 2015; Baggieri *et al.*, 2015; Baklouti *et al.*, 2016; Charrel *et al.*, 2009; Peyrefitte *et al.*, 2013; Zhioua *et al.*, 2010). As a matter of fact, RT-PCR assays usually constitute a peculiar way of investigation to isolate and identify a ribovirus (Alkan *et al.*, 2015; Charrel *et al.*, 2009; Zhioua *et al.*, 2010), whereas, serological analysis mainly refer to epidemiologic studies on serologic samples. Moreover, the RT-PCR test alone would be not a reasonable procedure to identify the virus, mainly due to the low yield of the products and the low quantity of TOSV in phlebotomine samples. In our entomological investigation, the molecular techniques were applied directly on the vector samples, through a prior RNA extraction and quantification, followed by a reverse transcription and a nested PCR with a two set of primer. Following this first screening, a further confirmation of the positivity of the amplified samples was obtained by capillary sequencing.

This preliminary study highlight two aspects: I) entomological investigation, already widely used as a method of both screening and research, confirms the effectiveness of molecular techniques directly on vectors; II) the correlation of the role of phlebotomes in the spread of both *Leishmania* and *Phleboviruses* in the Mediterranean area is confirmed.

## Conclusions

In conclusion, the experiments were carried out respecting the criteria of previous studies, employing analogous protocols and approaches. Furthermore, the overall ratio between the number of positive pools on the total tested samples fits with the data of other researches, as previously stated.

Here we pointed out that there is very limited information on the biology and epidemiology of TOSV, its reservoirs and vectors. Because there is neither a vaccine nor a specific treatment, control of TOSV infections can only be achieved through sand fly control measures (indoor and outdoor residual spraying, attractive toxic baits, etc.) or personal protection against bites (skin repellents, impregnated bed nets, etc.)

In order to identify regions where these measures could be promoted, it is necessary to know the geographic distribution of populations at risk through seroepidemiological studies and surveillance of cases of neuroinvasive TOSV infection. The recent demonstration that TOSV circulates on an island that is endemic for leishmaniasis has provided strong evidence for the establishment of stable transmission cycles of both human pathogens at the study site. This work reports, for the first time, the co-circulation in the same geographic area, Lampedusa Island, of two pathogens transmitted by the same vectors, which could have an important impact on human and animal health. The results of this study should be considered by the country's health authorities, who should be aware of the danger of co-circulation of Toscana virus and *L. infantum*.

## Acknowledgement

This research was granted by Ministry of Health "RC IZS SI 01/19".

## References

- Alkan C., Alwassouf S., Piorkowski G., Bichaud L., Tezcan S., Dincer E., et al. 2015. Isolation, genetic characterization, and seroprevalence of Adana virus, a novel phlebovirus belonging to the Salehabad virus complex, in Turkey. *J Virol*, **89**, 4080–4091.
- Alkan C., Bichaud L., de Lamballerie X., Alten B., Gould E.A. & Charrel R.N. 2013. Sandfly-borne phleboviruses of Eurasia and Africa: epidemiology, genetic diversity, geographic range, control measures. *Antiviral Res*, **100**, 54–74.
- Alvar J., Vélez I.D., Bern C., Herrero M., Desjeux P., Cano J., et al. 2012. Leishmaniasis worldwide and global estimates of its incidence. *PLoS One*, **7**, e35671.
- Amaro F., Zé-Zé L., Alves M.J., Börstler J., Clos J., Lorenzen S., et al. 2015. Co-circulation of a novel phlebovirus and Massilia virus in sandflies, Portugal. *Virology Journal*, **12**, 174.
- Ayhan N., Baklouti A., Prudhomme J., Walder G., Amaro F., Alten B., et al. 2017. Practical Guidelines for Studies on Sandfly-Borne Phleboviruses: Part I: Important Points to Consider Ante Field Work. *Vector Borne Zoonotic Dis*, **17**, 73–80.
- Baggieri M., Marchi A., Bucci P., Nicoletti L. & Magurano F. 2015. Genetic variability of the S segment of Toscana virus. *Virus Res*, **200**, 35–44.
- Baklouti A., Leparç Goffard I., Piorkowski G., Coutard B., Papageorgiou N., De Lamballerie X., et al. 2016. Complete Coding Sequences of Three Toscana Virus Strains Isolated from Sandflies in France. *Genome Announc*, **4**, e01676-15.
- Benallal K.E., Garni R., Harrat Z., Volf P. & Dvorak V. 2022. Phlebotomine sand flies (Diptera: Psychodidae) of the Maghreb region: A systematic review of distribution, morphology, and role in the transmission of the pathogens. *PLOS Neglected Tropical Diseases*, **16**, e0009952.
- Calamusa G., Valenti R.M., Vitale F., Mammina C., Romano N., Goedert J.J., et al. 2012. Seroprevalence of and risk factors for Toscana and Sicilian virus infection in a sample population of Sicily (Italy). *J Infect*, **64**, 212–217.
- Calzolari M., Romeo G., Bergamini F., Dottori M., Rugna G. & Carra E. 2022. Host preference and Leishmania infantum natural infection of the sand fly Phlebotomus perfiliewi in northern Italy. *Acta Tropica*, **226**, 106246.
- Calzolari M., Romeo G., Callegari E., Bonilauri P., Chiapponi C., Carra E., et al. 2021. Co-Circulation of Phleboviruses and Leishmania Parasites in Sand Flies from a Single Site in Italy Monitored between 2017 and 2020. *Viruses*, **13**, 1660.
- Charrel R.N., Izri A., Temmam S., Delaunay P., Toga I., Dumon H., et al. 2007. Cocirculation of 2 Genotypes of Toscana Virus, Southeastern France. *Emerg Infect Dis*, **13**, 465–468.
- Charrel R.N., Moureau G., Temmam S., Izri A., Marty P., Parola P., et al. 2009. Massilia Virus, A Novel Phlebovirus (Bunyaviridae) Isolated from Sandflies in the Mediterranean. *Vector Borne Zoonotic Dis*, **9**, 519–530.
- Dincer E., Gargari S., Ozkul A. & Ergunay K. 2015. Potential animal reservoirs of Toscana virus and coinfections with Leishmania infantum in Turkey. *Am J Trop Med Hyg*, **92**, 690–697.
- Ergunay K., Kasap O.E., Orsten S., Oter K., Gunay F., Yoldar A.Z.A., et al. 2014. Phlebovirus and Leishmania detection in sandflies from eastern Thrace and northern Cyprus. *Parasites & Vectors*, **7**, 575.
- Es-Sette N., Ajaoud M., Bichaud L., Hamdi S., Mellouki F., Charrel R.N., et al. 2014. Phlebotomus sergenti a common vector of Leishmania tropica and Toscana virus in Morocco. *J Vector Borne Dis*, **51**, 86–90.
- Fares W., Dachraoui K., Barhoumi W., Cherni S., Chelbi I. & Zhioua E. 2020. Co-circulation of Toscana virus and Leishmania infantum in a focus of zoonotic visceral leishmaniasis from Central Tunisia. *Acta Tropica*, **204**, 105342.
- Foglia Manzillo V., Gizzarelli M., Vitale F., Montagnaro S., Torina A., Sotera S., et al. 2018. Serological and entomological survey of canine leishmaniasis in Lampedusa island, Italy. *BMC Veterinary Research*, **14**, 286.
- Maia C., Alwassouf S., Cristóvão J.M., Ayhan N., Pereira A., Charrel R.N., et al. 2017. Serological association between Leishmania infantum and sand fly fever Sicilian (but not Toscana) virus in sheltered dogs from southern Portugal. *Parasites Vectors*, **10**, 92.
- Mammina C., Calamusa G., Vitale F., Amodio E., Romano N., Valenti R.M., et al. 2012. Seroprevalence of and risk factors for Toscana and Sicilian virus infection in a sample population of Sicily (Italy). *The Journal of Infection*, **64**, 212–217.
- Marchi S., Trombetta C.M., Kistner O. & Montomoli E. 2017. Seroprevalence study of Toscana virus and viruses belonging to the Sandfly fever Naples antigenic complex in central and southern Italy. *Journal of Infection and Public Health*, **10**, 866–869.
- Maroli M., Feliciangeli M.D., Bichaud L., Charrel R.N. & Gradoni L. 2013. Phlebotomine sandflies and the spreading of leishmaniasis and other diseases

- of public health concern. *Med Vet Entomol*, **27**, 123–147.
- Moriconi M., Rugna G., Calzolari M., Bellini R., Albieri A., Angelini P., et al. 2017. Phlebotomine sand fly-borne pathogens in the Mediterranean Basin: Human leishmaniasis and phlebovirus infections. *PLoS Negl Trop Dis*, **11**, e0005660.
- Peyrefitte C.N., Grandadam M., Bessaud M., Andry P.-E., Fouque F., Caro V., et al. 2013. Diversity of *Phlebotomus perniciosus* in Provence, southeastern France: Detection of two putative new phlebovirus sequences. *Vector Borne Zoonotic Dis*, **13**, 630–636.
- Remoli M.E., Bongiorno G., Fortuna C., Marchi A., Bianchi R., Khoury C., et al. 2015. Experimental evaluation of sand fly collection and storage methods for the isolation and molecular detection of *Phlebotomus*-borne viruses. *Parasites & Vectors*, **8**, 576.
- Remoli M.E., Fortuna C., Marchi A., Bucci P., Argentini C., Bongiorno G., et al. 2014. Viral isolates of a novel putative phlebovirus in the Marche Region of Italy. *Am J Trop Med Hyg*, **90**, 760–763.
- Remoli M.E., Jiménez M., Fortuna C., Benedetti E., Marchi A., Genovese D., et al. 2016. Phleboviruses detection in *Phlebotomus perniciosus* from a human leishmaniasis focus in South-West Madrid region, Spain. *Parasites & Vectors*, **9**, 205.
- Valassina M., Cusi M.G. & Valensin P.E. 2003. A Mediterranean Arbovirus: The Toscana Virus. *Journal of Neurovirology*, **9**, 577–583.
- Valassina M., Cusi M.G. & Valensin P.E. 1996. Rapid identification of Toscana virus by nested PCR during an outbreak in the Siena area of Italy. *J Clin Microbiol*, **34**, 2500–2502.
- Wuerth J.D. & Weber F. 2016. Phleboviruses and the Type I Interferon Response. *Viruses*, **8**, 174.
- Zhioua E., Moureau G., Chelbi I., Ninove L., Bichaud L., Derbali M., et al. 2010. Punique virus, a novel phlebovirus, related to sandfly fever Naples virus, isolated from sandflies collected in Tunisia. *J Gen Virol*, **91**, 1275–1283.