Epidemiological investigation on the spread of *Phebotomus*-Borne viruses in Europe

GERI 2015 21-22-23 April 2015 • Heraklion, Crete, Greece
Genes, Ecosystems and Risk of Infection

Maria Elena Remoli[1], Carla Maia[2], Maria Odete Afonso[2], Lenea Campino[2], Maribel Jimenez[3], Ricardo Molina[3], Anne Laure Banuls[4], Jorian Prudhomme[4], Denis Sereno[4], Claudia Fortuna[1], Antonella Marchi[1], Gioia Bongiorno[1], Luigi Gradoni[1], Mehmet Karakus[5], Suha K. Arserim[6], Yusuf Ozbel[5], Ozge Erisoz Kasap[7], Filiz Gunay[7], Gizem Oguz[7], Sinan Kaynas[8], Bulent Alten[7], Nikoloz Tsertsvadze[9], Lamzira Tskhvaradze[9], Ekaterina Giorgobiani[9], Marina Gramiccia[1], and Maria Grazia Ciufolini[1]

[1]Istituto Superiore di Sanità, MIPI Department, Rome, Italy; [2]Universidade Nova de Lisboa, Lisboa, Portugal; [3]Instituto de Salud Carlos III, Majadahonda- Madrid, Spain, [4] Institut de Recherche pour le Développement, MIVEGEC, Montpellier, France; [5]EGE University, Department of Parasitology, Bornova, Izmir, Turkey; [6]Celal Bayar University, Vocational School of Health Sciences, Manisa, Turkey; [7]Hacettepe University, Department of Biology, Ecology Division, Ankara, Turkey; [8]Mehmet Akif Ersoy University, Veterinary Faculty, Burdur, Turkey; [9]National Center for Disease Control and Public Health, Tbilisi, Georgia

Introduction

To monitor the spread of *Phebotomus*-Borne (*Ph*B) viruses in Europe, a field sand flies collection was carried out by *Ph*BD-EDENext partners. Phlebotomine sand flies are natural vectors of several human pathogens including viruses belonging to the genus *Phlebovirus* (family *Bunyaviridae*), *Vesiculovirus* (family *Rhabdoviridae*) and *Orbivirus* (family *Reoviridae*) relevant for human health. During the recent years, several surveillance studies on Phlebotomine sand flies activity have highlighted new Phleboviruses suggesting that many of these viruses remain to be discovered. Granada virus was identified in Spain in 2003 as a natural *Phlebovirus* reassortant of the Sand fly Fever Naples Serocomplex (Collao *et al.*, 2010); Adria virus was detected in a Albania region, close to Adriatic sea in 2005 (Papa *et al.*, 2011); Massilia virus was isolated in 2005 in France (Charrel *et al*, 2009); Punique virus was isolated in 2008 in Tunisia (Zhioua *et al.*, 2010); Fermo virus was identified in Marche region, Italy, during 2012 sandflies collection (Remoli *et al.*, 2014); Adana virus, a novel phlebovirus belonging to the Salehabad virus complex, was detected in Turkey (Alkan *et al.*, 2015). On the basis of these findings, an epidemiological investigation was carried out in several European countries, with the aim to develop a map of *Ph*B viruses distribution in Europe.

Figure 1. PhBD-EDENext partners involved in the PhB-viruses investigation

Materials and Methods

During 2011-2013, sand flies were collected from 7 partners (from west to east: IHMT-Portugal, ISCIII-Spain, IRD-France, ISS-Italy, EUMS-Turkey, HUESRL-Turkey and NCDC-Georgia) (Figure 1). Collections included 4 monospecic sites for *P.perniciosus*, *P.perliewi*, *P.ariasi* and *P.papatasi*, and 3 multispecic-sites for *P.kandelakii*, *P.sergenti*, *P.tobbi* and *P. (Larroussius)* spp. (Table 1). The specimens were sent to ISS-Italy and processed as pools of 20-30 sandflies/pool. The pools were homogenized, centrifuged and the supernatants used for virus isolation, and genotyping assays according to the procedure described in Figure 2. Briefly for viral isolation, all supernatants were inoculated in Vero cells and cytopathic effects were observed daily up to 14 days. The serological identification was obtained by plaque reduction neutralization test (PRNT) using home-made hyperimmune sera of several Phleboviruses. Genotyping analysis were simultaneously carried out. The supernatants of sand fly pools were used to extract viral RNA according to the manufacturer's recommendations (viral RNA kit, QIAGEN). *Phlebovirus* detection was performed using degenerated consensus primers able to amplify partial L and S genomic segments (Sànchez-Seco *et al*, 2003; Charrel *et al*, 2007; Remoli, data not shown). To amplify partial M genomic segment, two different degenerated primers were designed by alignment of several Naples group and Salehabad group viruses sequences respectively (Remoli, data not shown). Phylogenetic studies from sequence data of the isolates were carried out by Neighbour-joining using Mega version 5 software (Tamura *et al*, 2011).

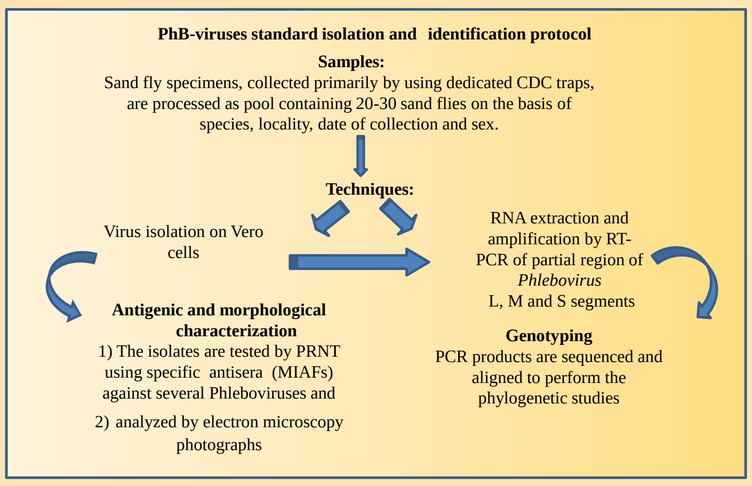

Figure 2. Serological and molecular methods for virus isolation and identification

Results

Seven thousand six hundred forty-six sand flies, collected from 21 sites by 7 European partners, were processed.
The results are summarized as follows:
- in 2011, ISS received 140 specimens by NCDC-Georgia (*P. kandelakii* 65%, *P. sergenti* 80%) and 64 sand flies by IHMT-Portugal (*Phlebotomus sp.).* In addition, a field collection was carried out by ISS in Rome Province (Italy): 162 and 136 specimens from Borgata Finocchio *(P. perniciosus)* and Castel di Guido (*P. perfiliewi*) respectively were captured. No virus detection was obtained from these collections.
- in 2012, from 900 *P. perfiliewi* collected in Fermo Commune, Marche region, Italy, 7 *Ph*B-viruses isolates were obtained (Table 1). By phylogenetic and serologic analysis, 6 of them appeared to be a novel *Phlebovirus*, named Fermo virus (belonging to Naples serocomplex), and one was identified as Toscana virus (TOSV)-lineage A. Minimum Field Infection Rates (MFIR) for the novel and TOSV were 0.67 and 0.10, respectively (Remoli et al, 2014) (Table 2A). Four *Ph*B-viruses were obtained from 366 *P. perniciosus* collected in Fuenlabrada, Madrid, Spain. The phylogenetic and serological identification of the Spanish isolates showed the presence of 3 Arbia virus (ARBV) strains, belonging to the Salehabad group, and one TOSV-lineage B known to circulate in Iberian Peninsula (Sanbonmatsu-Gamez et al, 2005; Sanchez-Seco *et al*, 2003). MFIR for the ARBV and TOSV were 0.82 and 0.28, respectively (Table 2B). Two *Ph*B-viruses were detected from 244 specimens [*P.tobbi* 49% and *Phlebotomus (Larroussius*) spp.] collected in Cukurova plain by HUESRL-Turkey (Table 1). The molecular and serological analysis of the 2 Turkish isolates identified the presence of ARBV in Cukurova plain, MFIR being 0.82 (Table 2C). Moreover ISS received 560 phlebotomine specimens (*Phlebotomus sp.*) by EUMS –Turkey (Urla area), 28 sandflies (*P. kandelakii* 65%, *P. sergenti* 80%) by NCDC – Georgia and 150 specimens (*P. ariasi*) by IRD – France. No virus detection was obtained from sandflies received by these countries.
- in 2013, ISS received 220 specimens by Georgia (*P. kandelakii*), 306 by Portugal (*Phlebotomus sp*.) and 597 by Spain (*P. perniciosus*). Two *Ph*B-viruses isolates were obtained from Spanish sand flies. Molecular and serological identification confirmed the presence of TOSV-lineage B in Fuenlabrada focus (Spain) and showed the detection of a putative novel *Phlebovirus* belonging to the Naples sero-complex, clustering with Granada, Massilia and Arrabida viruses. MFIR for both isolates was 0.16 (Table 2B). The characterization of this last isolate is in progress. We started to process 280 sand flies (86 *P. papatasi* and 194 *P. perniciosus*) collected in 2 monospecic sites of Catania province (Sicily, Italy). In particular, the analysis of these two sand flies species could provide additional information on the TOSV and Sicilian virus circulation in Sicily where seroprevalence and risk factors for TOSV and Sicilian virus infections in Sicilian human subjects were recently assessed by Calamusa *et al.* (Calamusa *et al.*, 2012).

Partners	Collection sites	N. *Phlebotomus* sp. collected			*Phlebotomus* sp.	N° Phleboviruses / N° *Phl.*sp.tested (MFIR)*
		2011	2012	2013		
NCDC - Georgia	Tblisi (Vera area) Tblisi (Lotkini area)	140 -	- 28	220 -	*P. kandelakii* 65% *P. sergenti 80%*	0/388
IHMT - Portugal	3 Faro, 1 Setubal 3 Faro	64 -	-	- 306	Phlebotomus sp.	0/370
ISCIII -Spain	4 Fuenlabrada	-	366	597	*P. perniciosus*	*6/963 (0.62)*
IRD - France	Roquedour	-	150	-	*P. ariasi*	0/150
EUMS – Turkey	Urla	-	560	-	*Phlebotomus sp.* Sites not monospecific	0/560
HUESRL-Turkey	4 Cukurova plain	-	244	-	*P. tobbi 49% / Larroussius 26%* Sites not monospecific	*2/244 (0.82)*
ISS - Italy	Borgata Finocchio, Rome province	162	961	-	*P. perniciosus*	0/162
ISS – Italy	Castel di Guido, Rome province	136	412	-	*P. perfiliewi*	0/136
ISS – Italy	Fermo site, Marche	-	2,980	-	*P. perfiliewi*	*7/900 (0.77)*
ISS-Italy	2 Ficarazzi (CT), Sicily 1 Ficarazzi (CT), Sicily 1 Pennisi (CT), Sicily 2 Catania città, Sicily	-	-	86 97 77 20	*P. papatasi* *P. perniciosus* *P. perniciosus* *P. perniciosus*	*To be processed*
TOTAL		502	5,701	1,403		*15/3,873 (0.38)*
			7,606			

*MFIR=Minimun Field Infection Rate /100 sandflies

Table 1. Sand flies collections and virus isolation during 2011-2013

A)

Virus isolation from Italy (Fermo and Rome Province)					
ITALY	Year of collection	Sand flies/pools	TOSV (MFIR)*	FerV (MFIR)*	Total (MFIR)*
Rome Province (Latium Region)	2011	18/298	-	-	-
Fermo area (Marche Region)	2012	30/900	1(0.11)	6(0.67)	7(0.77)

B)

	Virus isolation from Spain (Fuenlabrada, Madrid)					
SPAIN	Year of collection	Sand flies/pools	TOSV (MFIR)*	ARBV (MFIR)*	Arrabida-like (MFIR)*	Total (MFIR)*
Fuenlabrada area (Madrid)	2012	19/366	1(0.27)	3(0.82)	-	4(1.09)
	2013	30/597	1(0.17)	-	1(0.17)	2(0.34)
	Total	49/963	2(0.21)	3(0.31)	1(0.10)	6(0.62)

C)

Virus isolation from Turkey (Cukurova and Urla areas)					
TURKEY	Year of collection	Sand flies/pools	TOSV (MFIR)*	ARBV (MFIR)*	Total (MFIR)*
Cukurova plain	2012	15/244	-	2(0.82)	2(0.82)
Urla area	2012	20/560	-	-	-

*MFIR=Minimum field infection rate/100 *sand flies tested*

Table 2. Virus isolation and identification in A) Spain; B) Italy; C) Turkey.

Discussion

In order to produce a map of *Ph*B viruses distribution in Europe, the involvement and the contribution of the EDENext European partners is the important key to realize the goals of this project. An active field-based study that combine entomological and virological aspects is considered an important approach for providing early warning and predictive capability for viral epidemic. It is must take into account that the *Phlebovirus* genomic organization makes possible genetic molecular evolution by antigenic drift, antigenic shift (genetic reassortment), and genetic recombination (Holland *et al.*, 1998; Pringle *et al.*, 1996). These genomic properties make them good candidates as emerging human pathogens with the appearance of new variants both as reassortant virus and as novel putative virus. Indeed, as previously mentioned, recent investigations have indicated that *Phlebovirus* diversity in the Mediterranean basin is higher than initially suspected and novel viruses are yearly discovered (Charrel *et al.*, 2009; Zhioua *et al.*, 2010; Collao *et al.*, 2010; Papa *et al.*, 2010; Kocak Tufan *et al.*, 2011; Remoli *et al.*, 2014; Alkan *et al.*, 2015).
However even if the number of sand flies received from European Partners was, in some case, limited, several goals of the project were satisfactory completed.
The main results obtained by these studies can be summarized as follows:
1. characterization of new *Phlebovirus* foci such as Fuenlabrada (Madrid) where the circulation of several species of Phleboviruses belonging to different serological group have been demonstrated;
2. identification and characterization of novel putative *Phleboviruses* such as Fermo virus in Marche region and Arrabida-like in Fuenlabrada focus;
3. demonstration of the stability of the natural focus, Italian Fermo focus (Marche Region), already characterized 30 years ago, still active for the circulation of *Phlebovirus* as TOSV-lineage A (Ciufolini *et al.*, 1996; Remoli *et al.*, 2013);
4. detection of ARBV in Spain and Turkey where the presence of this virus had never been demonstrated, suggesting its wide diffusion in the Mediterranean basin.

Conclusion

A total of 15 *Ph*B viruses were isolated from 3913 sand flies, including 2 putative novel *Phleboviruses* (Fermo and Arrabida-like viruses). Due to the participation of the majority (7 partners) of *Ph*BD-EDENext group, our findings allowed us to draw a comprehensive distribution map of *Ph*B viruses in Europe. It should be highlighted that higher number of viral isolates were obtained when partners could provide a significant number of sand fly specimens increasing the chance of the virus isolation.

Reference

Alkan C, Alwassouf S, Piorkowski G, Bichaud L, Tezcan S, Dincer E, Ergunay K, Ozbel Y, Alten B, Lamballerie X, Charrel RN. Isolation, genetic characterization and seroprevalence of Adana virus a novel phlebovirus belonging to the Salehabad virus complex in Turkey. J Virol. 2015; 4. pii: JVI.03027-14.

Calamusa G, Valenti RM, Vitale F, Mammina C, Romano N, Goedert JJ, Gori-Savellini G, Cusi MG , and Amodio E. Seroprevalence of and risk factors for Toscana and Sicilian virus infection in a sample population of Sicily (Italy). J Infect. 2012; 64(2): 212–217. doi:10.1016/j.jinf.2011.11.012.

Charrel RN, Izri A, Temmam S, Delaunay P, Toga I, Dumon H, Marty P, de Lamballerie X, Parola P. Cocirculation of 2 genotypes of Toscana virus, southeastern France. Emerg Infect Dis. 2007;13(3):465-468

Charrel RN, Moureau G, Temmam S, Izri A, Marty P, Parola P, da Rosa AT, Tesh RB, de Lamballerie X. Massilia virus, a novel Phlebovirus (Bunyaviridae) isolated from sandflies in the Mediterranean. Vector Borne Zoonotic Dis. 2009; 9(5):519-530

Ciufolini MG, Maroli M, Miceli N, Nicoletti L, Cecchetti S, Amore R, Percopo S, Verani P, 1990. Monitoring of Phlebotominae sandflies transmitted viruses activity in Marche region(Italy). Arbo Info Exch 58–59.

Collao X, Palacios G, De Ory F, Sanbonmatsu S, Pérez-Ruiz M, Navarro JM, Molina R, Hutchison SK, Lipkin I, Tenorio A, Sancez-Seco MP. Granada Virus: a Natural Phlebovirus Reassortant of the Sand fly Fever Naples Serocomplex with Low Seroprevalence in Human. Am J Trop Med Hyg. 2010;83(4):760-765. doi: 10.4269/ajtmh.2010.09-0697.

Holland J. and Domingo E. Origin and evolution of viruses. Virus Genes. 1998; 16:13-21

Kocak Tufan Z, Weidmann M, Bulut C, Kinikli S, Hufert FT, Dobler G, Demiroz AP. Clinical and laboratory findings of a sandfly fever Turkey Virus outbreak in Ankara. J Infect. 2011;63(5):375-81. doi: 10.1016/j.jinf.2011.07.011

Papa A, Velo E, Bino S. A novel phlebovirus in Albanian sandflies. Clin Microbiol Infect. 2011;17(4):585-587. doi: 10.1111/j.1469-0691.2010.03371.x.

Pringle CR. Genetics and genome segment reassortment. In R. M. Elliott (ed.), The Bunyaviridae. Plenum Press, New York, N.Y. 1996; p. 189-226.

Remoli ME, Fortuna C, Marchi A, Bucci P, Argentini C, Bongiorno G, Maroli M, Gradoni L, Gramiccia M, Ciufolini MG. Viral isolates of a novel putative phlebovirus in the Marche Region of Italy. Am J Trop Med Hyg. 2014; 90(4):760-763. doi: 10.4269/ajtmh.13-0457.

Sanbonmatsu-Gamez, S., Perez-Ruitz, M., Collao, X., Sanchez-Seco, M.P., Tenorio, A., 2005. Toscana virus in Spain. Emerg. Infect. Dis. 11, 1701–1707

Sánchez-Seco MP, Echevarría JM, Hernández L, Estévez D, Navarro-Marí JM, Tenorio A. Detection and identification of Toscana and other phleboviruses by RT-nested-PCR assays with degenerated primers. J Med Virol. 2003;71(1):140-149.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol. 2011;28(10):2731-2739. doi: 10.1093/molbev/msr121. Epub 2011 May 4

Zhioua E, Moureau G, Chelbi I, Ninove L, Bichaud L, Derbali M, Champs M, Cherni S, Salez N, Cook S, de Lamballerie X, Charrel RN. Punique virus, a novel phlebovirus, related to sandfly fever Naples virus, isolated from sandflies collected in Tunisia. J Gen Virol. 2010; 91(Pt 5):1275-1283. doi: 10.1099/vir.0.019240-0.

This work was supported by EDENext, a collaborative project of the 7th FP (2011-2014) funded by the European Commission under the DG Health; Contract Number: 261504