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Introduction

To monitor the spread of *Phlebotomus*-Borne (*PhB*) viruses in Europe, a field sand flies collection was carried out by *PhBD*-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 an 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 *PhB* viruses distribution in Europe.



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

Materials and Methods

During 2011-2013, sand flies were collected from 7 partners (from west to east: IHMT-Portugal, ISCHII-Spain, IRD-France, ISS-Italy, EUMS-Turkey, HUESRL-Turkey and NCDC-Georgia) (Figure 1). Collections included 4 monospecific sites for *P. perniciosus*, *P. perfliewi*, *P. ariasi* and *P. papatasi*, and 3 multispecific 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 was 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 (Sanchez-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).

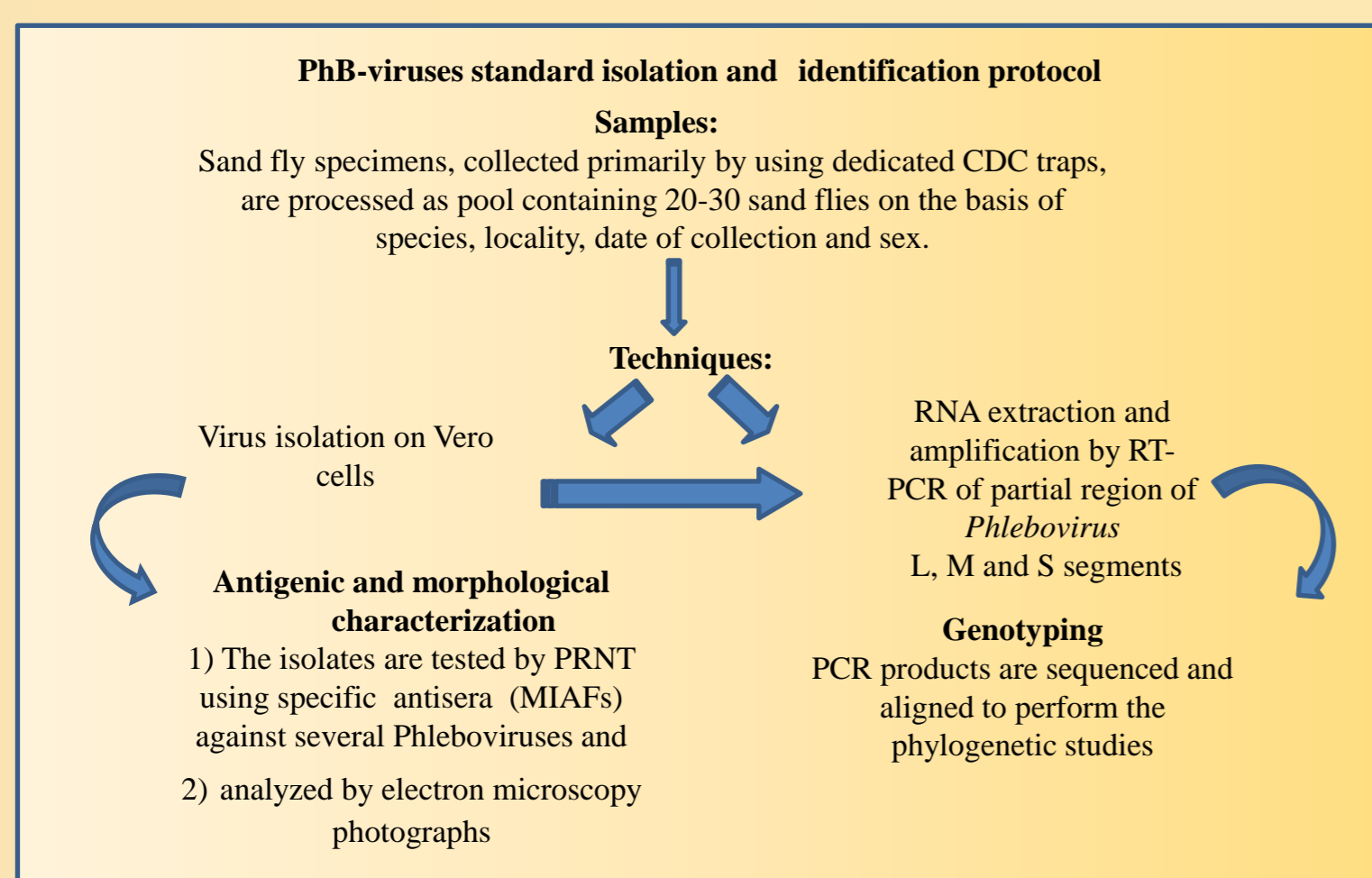


Figure 2. Serological and molecular methods for virus isolation and identification

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* spp.). 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. perfliewi*) respectively were captured. No virus detection was obtained from these collections.
- in 2012, from 900 *P. perfliewi* collected in Fermo Commune, Marche region, Italy, 7 *PhB*-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 *PhB*-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 *PhB*-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* spp.) 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* spp.) and 597 by Spain (*P. perniciosus*). Two *PhB*-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 monospecific 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. <i>Phlebotomus</i> sp. collected			<i>Phlebotomus</i> sp.	N° Phleboviruses / N° Phl.sp.tested (MFIR)*
		2011	2012	2013		
NCDC - Georgia	Tblisi (Vera area)	140	-	220	<i>P. kandelakii</i> 65% <i>P. sergenti</i> 80%	0/388
	Tblisi (Lotkini area)	-	28	-		
IHMT - Portugal	3 Faro, 1 Setubal	64	-	306	<i>Phlebotomus</i> sp.	0/370
	3 Faro	-	-	-		
ISCHII -Spain	4 Fuenlabrada	-	366	597	<i>P. perniciosus</i>	6/963 (0.62)
IRD - France	Roquedour	-	150	-	<i>P. ariasi</i>	0/150
EUMS - Turkey	Urla	-	560	-	<i>Phlebotomus</i> sp. Sites not monospecific	0/560
HUESRL - Turkey	4 Cukurova plain	-	244	-	<i>P. tobbi</i> 49% / <i>Larroussius</i> 26% Sites not monospecific	2/244 (0.82)
ISS - Italy	Borgata Finocchio, Rome province	162	961	-	<i>P. perniciosus</i>	0/162
ISS - Italy	Castel di Guido, Rome province	136	412	-	<i>P. perfliewi</i>	0/136
ISS - Italy	Fermo site, Marche	-	2,980	-	<i>P. perfliewi</i>	7/900 (0.77)
ISS-Italy	2 Ficarazzi (CT), Sicily 1 Ficarazzi (CT), Sicily 1 Pennisi (CT), Sicily 2 Catania città, Sicily	-	-	86	<i>P. papatasi</i> <i>P. perniciosus</i> <i>P. perniciosus</i> <i>P. perniciosus</i>	To be processed
		-	-	97		
		-	-	77		
		-	-	20		
TOTAL		502	5,701	1,403		15/3,873 (0.38)
			7,606			

*MFIR=Minimum Field Infection Rate /100 sandflies

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

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)	

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)

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	-	-	-

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

Discussion

In order to produce a map of *PhB* 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 *PhB* 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 *PhBD*-EDENext group, our findings allowed us to draw a comprehensive distribution map of *PhB* 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.

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