











ORIGINAL ARTICLE

Exposure to *Phlebotomus perniciosus* sandfly vectors is positively associated with Toscana virus and *Leishmania infantum* infection in human blood donors in Murcia Region, southeast Spain

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Abstract

Antibodies against *Phlebotomus perniciosus* sandfly salivary gland homogenate (SGH) and recombinant protein rSP03B, sandfly-borne Toscana virus (TOSV), Sandfly Fever Sicilian virus (SFSV) and *Leishmania*, as well as DNA of the latter parasite, were investigated in 670 blood samples from 575 human donors in Murcia Region, southeast Spain, in 2017 and 2018. The estimated SGH and rSP03B seroprevalences were 69% and 88%, respectively, although correlation between test results was relatively low ($\rho = 0.39$). Similarly, TOSV, SFSV and *Leishmania* seroprevalences were 26%, 0% and 1%, respectively, and *Leishmania* PCR prevalence was 2%. Prevalences were significantly greater in 2017, overdispersed and not spatially related to each other although both were positively associated with SGH but not to rSP03B antibody optical densities, questioning the value of the latter as a diagnostic marker for these infections in humans.

KEYWORDS

anti-saliva antibodies, blood donors, *Leishmania infantum*, sandflies, sandfly fever sicilian virus, toscana virus

1 | INTRODUCTION

Human leishmaniasis (HumL) and phlebovirus infections are vector-borne diseases transmitted by phlebotomine sandflies that are endemic in Mediterranean countries. In Spain, HumL is caused by the

protozoan *Leishmania infantum* (Trypanosomatidae), and the most common clinical presentations include visceral leishmaniasis (VL) and cutaneous leishmaniasis (CL) (WHO, 2021). While subclinical infections by *L. infantum* are common in immunocompetent people in endemic areas (Acedo Sánchez et al., 1996), an unusual outbreak of human

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leishmaniosis recently occurred in a residential area in Madrid affecting more than one thousand mostly immunocompetent adults between 2010 and 2017 (Servicio de Epid, 2018, p. 17). Phlebovirus (genus *Phlebovirus*, family *Phenuiviridae*) infections and/or antibodies reported in Spain include Toscana virus (TOSV, *Toscana phlebovirus*), Sandfly Fever Sicilian virus (SFSV, *Sicilian phlebovirus*), Sandfly Fever Naples virus (SFNV, *Naples phlebovirus*), Granada virus (GRV, *Naples phlebovirus*), Arbia virus (ARBV, *Salehabad phlebovirus*) and Arrabida virus (ARRV, *Naples phlebovirus*) (Mendoza-Montero et al., 1998; Navarro-Marí et al., 2013; Remoli et al., 2016), although clinical phleboviroses have only been described for TOSV, namely meningitis and meningoencephalitis (Cardeñosa et al., 2013; Echevarría et al., 2003).

Diagnosing subclinical, sandfly-borne infections is challenging. Immunocompetent and asymptotically *L. infantum*-infected humans develop a predominantly cellular immune response which typically leads to parasite clearance, and antibodies may not be detectable (Tripathi et al., 2007). The estimated *L. infantum* antibody and PCR prevalences in blood samples from healthy humans in Spain range from 0% to 56% and 0% to 22%, respectively (Aliaga et al., 2019; Chitimia et al., 2011; Pérez-Cutillas et al., 2015; Riera et al., 2004). Phlebovirus infections have a short viraemic period and prevalence estimations rely on serological tests (Charrel & de Lamballerie, 2013). TOSV and SFSV seroprevalences in Spain range from 5% to 61% and 0% to 5%, respectively (Cardeñosa et al., 2013; de Ory et al., 2009; Echevarría et al., 2003; Mendoza-Montero et al., 1998; Sanbonmatsu-Gámez et al., 2005) depending on the region and the serological assay used, with the virus neutralization test being the gold standard (Ayhan & Charrel, 2020).

The risk of infection with these pathogens is likely related to sandfly biting rates which may be assessed by investigating circulating antibodies against sandfly salivary antigens (Drahota et al., 2014; Vlkova et al., 2012). Among *Phlebotomus* species present in Spain, *Phlebotomus perniciosus* is the most abundant and widely distributed *L. infantum* vector (Lucientes et al., 2005; Martín-Sánchez et al., 2000; Pesson et al., 2004), and a major phlebovirus vector (Dehghani et al., 2021). Sandfly salivary gland homogenates (SGH) from *P. perniciosus* have been successfully used as the antigen source in antibody enzyme-linked immunosorbent assay (ELISA) tests in dogs, cats, mice and leporids (Drahota et al., 2014; Kostalova et al., 2017; Martín-Martín et al., 2014; Pereira et al., 2019) but not in humans. However, SGHs are cumbersome to obtain as they require dissection of individual sandflies from laboratory colonies. Moreover, while initial studies reported no cross-reactivity between *P. perniciosus* and *Phlebotomus papatasi* SGH (Volf & Rohoušová, 2001), this cannot be totally ruled out between sympatric sandfly species (Lestina et al., 2017). To overcome this, recombinant salivary gland proteins provided a good alternative to SGH in studies in dogs, mice and leporids, particularly the 43-kDa yellow-related protein rSP03B (Drahota et al., 2014; Kostalova et al., 2015; Martín-Martín et al., 2014). No studies have investigated antibodies in humans against *P. perniciosus* salivary proteins.

Understanding the prevalence, distribution and risk factors of sandfly biting activity and its relationship with *L. infantum* and phlebovirus infection is important from a prevention and control perspective. Here we investigated human exposure to *P. perniciosus* for the first time, by

analysing the IgG antibody response to SGH and rSP03B proteins, and the prevalence of *L. infantum*, TOSV and SFSV.

2 | MATERIALS AND METHODS

2.1 | Study population

The study included 670 human blood samples taken in January to March 2017 and September to November 2018 from 575 voluntary healthy donors (95 donors provided samples both years), from 13 rural villages and periurban towns of Murcia Region, in southeast Spain (37°59'10"N 1°07'49"W) (Figure 1), where previous studies had detected subclinical *L. infantum* infections (Chitimia et al., 2011; Pérez-Cutillas et al., 2015). An informed consent was signed by each participant and a questionnaire survey was conducted to gather information on donor's demographical data, residence, work, dog ownership, use of insecticides and knowledge about leishmaniosis.

2.2 | Salivary gland homogenate (SGH) and recombinant protein rSP03B

Four- to six-day-old female *P. perniciosus* sandflies originating from Murcia region and kept in laboratory colonies at Charles University, Prague, for more than 50 generations, were dissected for salivary gland extraction (Volf & Volfova, 2011). Salivary glands were pooled in groups of 20 together with 20 μ l of Tris-NaCl buffer (20 mm Tris, 150 mm NaCl; pH 7.4) and submitted to three consecutive freeze-thaw cycles to obtain SGH, that were then stored at -20°C until used (Kostalova et al., 2015).

The recombinant *P. perniciosus* 43-kDa yellow protein (rSP03B, GenBank accession no. DQ150622), expressed in mammalian cells, was cloned, produced and purified according to the protocol described in Willen et al. (2019). A nanospectrophotometer at 280 nm was used for measuring the UV absorbance value of the protein and its theoretical molar extinction coefficient served for quantifying protein concentration.

2.3 | Detection of IgG anti-SGH and anti-rSP03B antibodies

The ELISA protocol described by Kostalova et al. (2015) was used for analysing specific IgG anti-saliva antibodies, with slight modifications. Sandfly salivary gland pools were mixed, and a single homogenate was used for antigen coating all microtitre ELISA plates, to limit between-plate differences. Microtitre plate antigen coating was performed with either SGH (40 ng per well, equivalent to 0.2 gland) or rSP03B (0.2 μ g per well). The ELISA/ELISPOT Diluent 1x (eBioscience™) was employed as the blocking solution. Human sera were diluted to 1:100 in 1x ELISA diluent. Secondary antibodies (goat anti-human IgG antibody, HRP conjugate, Sigma-Aldrich) were diluted to 1:5000 (for SGH) and 1:2500 (for rSP03B) in PBS-Tween. An ELx800 Absorbance Microplate Reader (BioTek, USA) was used to measure optical densities (ODs) at 490 nm. Each serum sample was tested in duplicate, and each plate

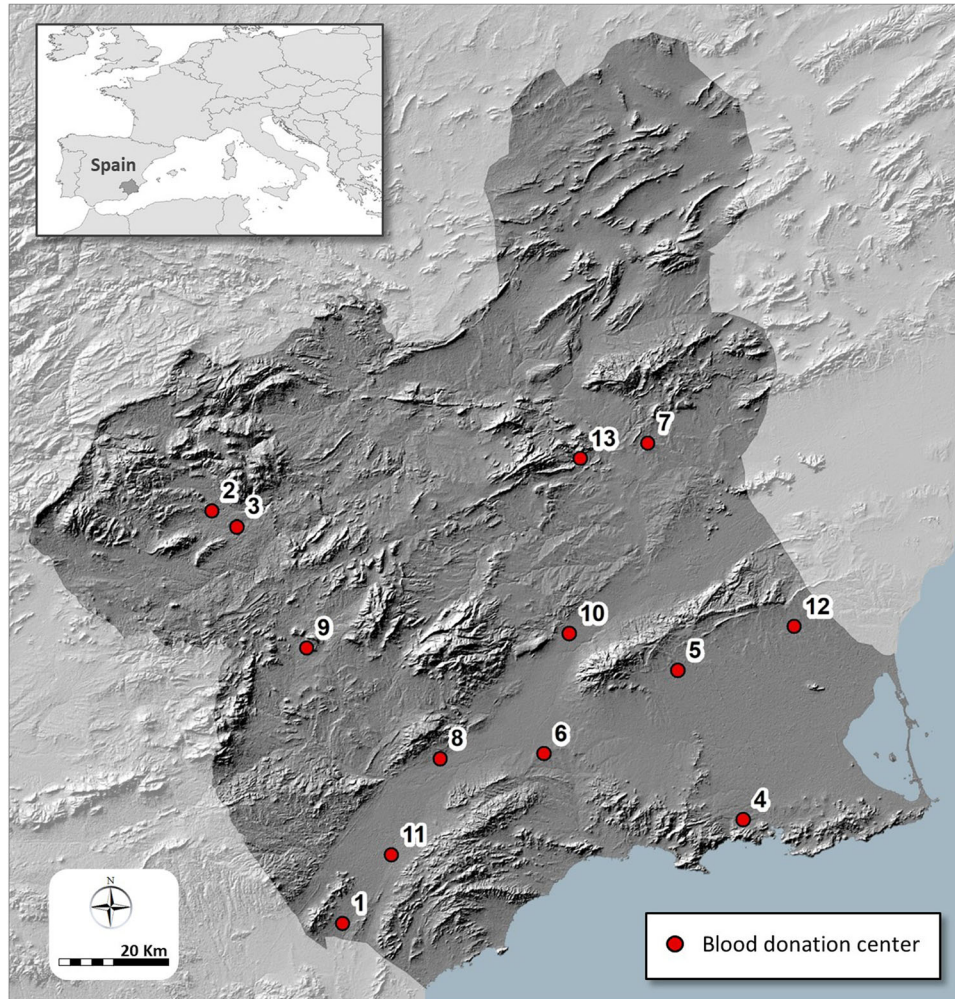


FIGURE 1 Towns in Murcia Region (southern Spain) where the study was performed. 1: Almendricos; 2: Archivel; 3: Barranda; 4: Canteras; 5: Corvera; 6: El Paretón; 7: Fenazar; 8: La Hoya; 9: La Paca; 10: Librilla; 11: Purias; 12: Sucina; 13: Ulea

contained four methodical controls (MC) and a positive control from rabbits exposed to *P. perniciosus* sandflies; for the latter, goat anti-rabbit IgG antibody, peroxidase conjugate (Sigma Aldrich), was used at 1:2500. Due to the unavailability of human positive and negative controls, a preliminary 96-well plate with a random set of samples was analysed and those with the highest and lowest ODs were selected as human positive and negative controls, respectively, in subsequent plates. All serum samples were processed by the same analyst in the same laboratory in 2 days in 2019 for SGH and 2020 for rSP03B.

Standardized optical densities (SOD) of ELISA antibodies against salivary gland proteins across plates were calculated using the following formula (Sanchez et al., 2002): $SOD = (OD_S - OD_{NC}) / (OD_{PC} - OD_{NC})$ (S: sample; NC: negative control; PC: positive control). After approximating negative values to zero, SODs + 1 were decimal log-transformed, multiplied by 100 and named LODs. A sample LOD was obtained by calculating the mean LOD of both readings. Human's serological status (seropositive or seronegative) was estimated as an indication of considerable antibody production. Since no cut-off has been established for these antigens in humans, we chose the mean OD_{NC} plus two standard deviations for both SGH and rSP03B.

2.4 | Detection of anti-TOSV and anti-SFSV neutralizing antibodies

The virus microneutralization assay (VNT) was performed to assess TOSV and SFSV neutralizing antibody titres (Sakhria et al., 2013) in plasma samples collected in 2017 ($n = 350$) and only for TOSV in those from 2018 ($n = 320$), since no SFSV-positive samples were detected in 2017. Briefly, assays were performed in 96-well plates, 50 μ l of serial plasma dilutions (preheated at 56°C for 30 min) were added to the wells to obtain final plasma dilutions ranging from 1:10 to 1:160, followed by 50 μ l of infectious virus at 100 TCID₅₀ and plates were incubated at 37°C with 5% CO₂ for 1 h. Subsequently, a 100 μ l suspension of Vero cells (ATCC CCL81) incorporating approximately 2×10^5 cells/ml, 5% foetal bovine serum, 1% penicillin-streptomycin, 1% L-glutamine and 1% kanamycin-enriched MEM medium (200 mM) was added to each well. The presence or absence of cytopathic effect was evaluated in an inverted microscope after incubating plates at 37°C in a 5% CO₂ atmosphere for 5 days for TOSV and for 6 days for SFSV. Control wells, containing virus + Vero cells, Vero cells only and plasma samples + Vero cells, were included in each plate. Samples showing

titres ≥ 40 were considered as positive as described (Masse et al., 2019).

2.5 | Detection of IgG and IgM anti-*Leishmania*

The 'LEISHMANIA ELISA IgG+IgM' kit (Vircell®) was used to detect *Leishmania*-specific serum antibodies. According to the protocol, the antibody-index ($I = \text{sample OD}/\text{cut-off OD}$) was calculated for all samples and samples were considered positive when $I > 1.1$, doubtful if $I = 0.9\text{--}1.1$ and negative if $I < 0.9$.

2.6 | DNA extraction and *Leishmania*-PCR amplification

Whole blood DNA was extracted using the Blood DNA Purification Kit in the Maxwell® 16 semi-automated nucleic acid purification robot (Promega). DNA was extracted individually for 550 donors and in pools of four samples each for 120 individuals. The latter included samples from five towns taken in 2017. DNA concentration and purity were analysed using NanoDrop 2000® spectrophotometer (Thermo Fischer Scientific). *Leishmania* DNA was investigated by real-time PCR with a TaqMan probe (rtPCR) that amplified an approximately 120 base pair (bp) fragment of the kinetoplast DNA (kDNA) minicircle (Francino et al., 2006), following Dantas-Torres et al. (2017). The rtPCR threshold cycle (Ct) was considered as a semi-quantitative measure of parasite DNA amplified. Samples with Ct ≥ 40 were considered negative.

2.7 | Data mapping and statistical analysis

Blood donors' residence addresses were mapped and spatial autocorrelation among *L. infantum* and TOSV positives and negatives was evaluated using Moran's I Index. Spearman's rank test was used to assess the correlation between SGH and rSP03B antibody levels (Kirkwood & Sterne, 2003; Prion & Haerling, 2014). Cohen's kappa coefficient (k) was employed to measure the degree of agreement between donors SGH and rSP03B serological status (seropositive or seronegative). This test takes into consideration the possibility of agreement by chance and k interpretations were as follows: $k < 0$: less than chance agreement, $k = .01\text{--}.20$: slight agreement, $k = .21\text{--}.40$: fair agreement, $k = .41\text{--}.60$: moderate agreement, $k = .61\text{--}.80$: substantial agreement and $k = .81\text{--}.99$: almost perfect agreement (Thrusfield, 2018). Bivariate analysis between tests results and questionnaire explanatory variables were carried out using Fisher's exact and Kruskal-Wallis statistics for proportions and medians, respectively. Multivariable negative binomial and logistic regression models were then developed to analyse the independent contribution of explanatory variables to SGH and rSP03B LODs in the first case, and to TOSV serological status and *Leishmania* antibody and/or PCR status in the second case. Initial models incorporated all explanatory variables associated with outcome variables in the binary analyses with $p < .20$, except that *Leishmania* and

TOSV infection variables were not included as explanatory variables in SGH and rSP03B LODs models, and year was excluded from TOSV and *Leishmania* infection models due to strong correlation with SGH and rSP03B. A backward modelling approach was used, and final models retained variables significantly ($p < .05$) or marginally significantly ($p < .10$) associated with the outcome, for a two-tailed test. The R statistical software (<http://cran.r-project.org/>) was used for all except for the autocorrelation analysis, which was performed in ArcGISv.10 (ESRI) geographical information system.

3 | RESULTS

3.1 | ELISA antibody LODs and seroprevalence against *P. perniciosus* SGH and rSP03B salivary proteins

Antibody LODs > 0 against salivary proteins SGH and rSP03B were detected in 86% and 97% of the blood donors, and 98% had antibodies against one or both preparations. Median LODs were 18 (range: 0–67) for SGH and 18 (range: 0–90) for rSP03B *P. perniciosus* salivary gland proteins (Table S1), and they were higher in 2017 compared to 2018 and varied significantly between towns. Correlation between SGH and rSP03B LODs was weak overall ($\rho = 0.39$, $p < .01$) (Figure 2), and moderate in 2018 ($\rho = 0.50$, $p < .01$). However, it was relatively strong when comparing towns medians in 2018 ($\rho = 0.66$, $p < .05$). Among the 95 donors sampled twice, in 2017 and 2018, SGH LODs decreased in 93% and increased in 7% of donors. Similar percentages for rSP03B were 66% and 34%, respectively.

Cut-off values for the SGH and rSP03B ELISA ODs were 0.396 and 0.454, respectively, and the resulting percentage of seropositives was 69% for SGH and 88% for rSP03B. Both assays coincided in

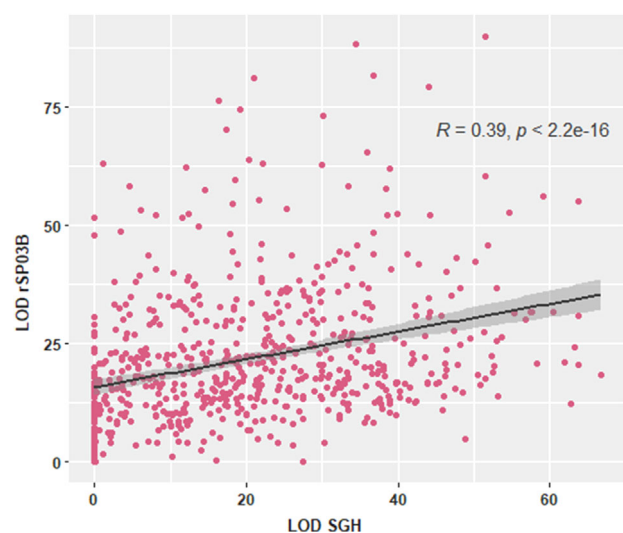


FIGURE 2 Spearman's correlation between individual human antibody log-transformed optical densities (LOD) against *Phlebotomus perniciosus* salivary gland homogenate (SGH) and rSP03B salivary protein

TABLE 1 Incidence rate ratios (RRs) from a negative binomial model of the relationship between anti-*P. perniciosus* SGH LODs in blood donors and sex, age, year and town

Variable	Level	RR	95% CI		p Value
Sex	Male	1.00	-	-	
	Female	1.24	1.09	1.42	.0012
Age	18-34	1.00	-	-	
	35-50	0.95	0.81	1.11	.5277
	51-65	0.81	0.67	0.97	.0227
Year	2017	1.00	-	-	
	2018	0.28	0.25	0.32	.0000
Town	Barranda	1.00	-	-	
	Almendricos	1.02	0.74	1.40	.8931
	Archivel	0.85	0.60	1.20	.3532
	Canteras	3.15	2.31	4.29	.0000
	Corvera	2.14	1.58	2.92	.0000
	El Paretón	0.75	0.54	1.04	.0830
	Fenazar	0.90	0.57	1.43	.6672
	La Hoya	2.67	1.96	3.62	.0000
	La Paca	1.04	0.75	1.44	.8060
	Librilla	1.48	1.08	2.02	.0141
	Purias	1.25	0.88	1.76	.2107
	Sucina	0.97	0.68	1.38	.8568
	Ulea	2.06	1.51	2.79	.0000

73% (488/670) of samples, although kappa coefficient (95% CI) was $k = .23$ (.16-.30) indicating only fair agreement between tests. The bivariate relationship between salivary antibodies and questionnaire variables is presented as supplementary material in Table S1. Results from regression models indicated that SGH LOD was independently and positively associated with women, decreased with age and was greatest in 2017 and in the towns of Canteras, La Hoya, Corvera, Ulea and Librilla ($p < .05$, Table 1). In contrast, SGH seropositivity was not associated to gender and age; it was highest only in Corvera and Ulea and in donors with dogs tested for *Leishmania* (Table 2). rSP03B LOD was similarly associated with gender and age and was lowest in people living in country houses and in the towns of Canteras, El Paretón and La Paca compared to Barranda ($p < .05$, Table 3). Likewise, rSP03B seropositivity was positively associated with women and year 2017; it was marginally lower in the oldest age group and lowest in the towns of El Paretón and La Paca (Table 4). The town of Purias, where every donor was seropositive, was excluded from this analysis to avoid model convergence failure.

3.2 | *Phlebovirus* neutralizing antibody seroprevalence

Antibodies against SFSV were not detected in any serum sample. Instead, the percentages of serum samples with 1/10, 1/20, 1/40,

TABLE 2 Odds ratios (ORs) from a logistic regression model of the relationship between SGH seropositivity and sex, age, year and town

Variable	Level	OR	95% CI		p Value	
Year	2017	1.00	-	-		
	2018	0.03	0.02	0.07	.0000	
Town	Barranda	1.00	-	-		
	Almendricos	1.43	0.32	6.47	.6390	
	Archivel	1.56	0.27	9.06	.6221	
	Canteras	1.30	0.27	6.31	.7486	
	Corvera	68.79	6.53	724.85	.0004	
	El Paretón	1.06	0.25	4.56	.9401	
	Fenazar	0.58	0.08	4.31	.5965	
	La Hoya	4.34	0.99	19.09	.0520	
	La Paca	2.67	0.56	12.71	.2185	
	Librilla	1.09	0.23	5.04	.9170	
	Purias	4.03	0.78	20.85	.0963	
	Sucina	0.42	0.07	2.75	.3685	
	Ulea	9.33	1.93	45.21	.0055	
	Dog tested for <i>Leishmania</i>	No	1.00	-	-	
		Yes	2.47	1.18	5.14	.0159

TABLE 3 Incidence rate ratios (RRs) from a negative binomial model of the relationship between anti-*P. perniciosus* rSP03B LODs in blood donors and sex, age, home type and town

Variable	Level	RR	95% CI		p Value
Sex	Male	1.00	-	-	
	Female	1.12	1.01	1.23	.0298
Age	18-34	1.00	-	-	
	35-50	0.86	0.76	0.97	.0117
	51-65	0.74	0.64	0.85	.0000
Home type	Semi-detached	1.00	-	-	
	Country house	0.75	0.60	0.94	.0112
	Detached	1.10	0.84	1.44	.4950
	Flat	1.05	0.92	1.20	.4542
Town	Barranda	1.00	-	-	
	Almendricos	0.81	0.64	1.03	.0853
	Archivel	0.85	0.66	1.09	.2025
	Canteras	0.76	0.60	0.96	.0198
	Corvera	1.03	0.82	1.30	.7984
	El Paretón	0.78	0.61	0.99	.0394
	Fenazar	0.74	0.52	1.04	.0862
	La Hoya	0.88	0.70	1.12	.2965
	La Paca	0.60	0.47	0.76	.0000
	Librilla	0.79	0.63	1.00	.0500
	Purias	0.95	0.73	1.24	.7225
	Sucina	0.87	0.67	1.13	.3053
	Ulea	1.25	1.00	1.57	.0541

TABLE 4 Odds ratios (ORs) from a logistic regression model of the relationship between rSP03B seropositivity and sex, age, year and town

Variable	Level	OR	95% CI		p Value
Sex	Male	1.00	-	-	
	Female	1.74	1.03	2.93	.0373
Age	18-34	1.00	-	-	
	35-50	0.66	0.34	1.26	.2059
	51-65	0.50	0.24	1.04	.0636
Year	2017	1.00	-	-	
	2018	0.45	0.27	0.75	.0024
Town	Barranda	1.00	-	-	
	Almendricos	1.11	0.34	3.67	.8646
	Archivel	1.81	0.42	7.90	.4284
	Canteras	1.99	0.52	7.68	.3176
	Corvera	1.91	0.50	7.28	.3449
	El Paretón	0.34	0.12	0.99	.0473
	Fenazar	0.39	0.08	1.88	.2406
	La Hoya	0.99	0.30	3.20	.9836
	La Paca	0.34	0.12	0.98	.0464
	Librilla	0.89	0.28	2.84	.8498
	Sucina	0.53	0.16	1.77	.3036
	Ulea	7.31	0.84	63.37	.0709

1/80 and 1/160 VNT titres were 7%, 5%, 9%, 13%, and 4%, respectively. Based on a 1/40 cut-off titre, TOSV seroprevalence was 26% (173/659), and the median titre among TOSV seropositive individuals was 1/80. TOSV seroprevalence was 38% in 2017 and 14% in 2018 ($p < .05$) and positives came from every town, ranging between 10% and 44%. There was some evidence of spatial autocorrelation in donor's TOSV status ($I = 0.15$, $p < .05$). Among the 95 donors sampled for TOSV in 2017 and in 2018, the percentage of donors seronegative both times was 56%, seropositive both times was 18%, seronegative in 2017 and seropositive in 2018 was 3% and seropositive in 2017 and seronegative in 2018 was 22%.

Table S2 shows the bivariate relationship between TOSV and explanatory variables. The most parsimonious logistic regression model indicated that TOSV seropositivity was positively associated with increasing SGH but not with rSP03B and was greatest in donors in the oldest age group, working outdoors and having a working dog (Table 5).

3.3 | *Leishmania* rtPCR and ELISA prevalences

Fifteen DNA samples were positive to *Leishmania*-rtPCR, including 13/550 (2.4%) donors analysed individually and 2/32 (6.3%) pools. The median (range) Ct in individually tested samples was 38.9 (range 36.9-39.5). Positives came from seven (54%) towns, with prevalence ranging between 1.7% and 10.3%, and all except one positive sample were col-

TABLE 5 Odds ratios (ORs) from a logistic regression model of the relationship between TOSV seropositivity and age, town, working environment, dog occupation and LODs against SGH

Variable	Level	OR	95% CI		p Value
SGH LODs	SGH LODs	1.03	1.01	1.05	.00085
Age	18-34	1.00	-	-	
	35-50	0.63	0.32	1.25	.18456
	51-65	2.53	1.18	5.42	.01665
Working environment	Outdoors	1.00	-	-	
	Indoors	0.44	0.24	0.79	.00616
Dog occupation	Companion	1.00	-	-	
	Work	2.02	1.08	3.78	.02766
Town	Almendricos	1.00	-	-	
	Archivel	6.97	1.59	30.54	.01004
	Barranda	0.60	0.09	4.05	.60273
	Canteras	0.92	0.20	4.14	.91066
	Corvera	2.71	0.74	9.93	.13143
	El Paretón	2.75	0.75	10.10	.12755
	Fenazar	1.00	0.14	6.99	.9993
	La Hoya	0.63	0.16	2.58	.52447
	La Paca	0.85	0.19	3.80	.83393
	Librilla	1.62	0.40	6.54	.49526
	Purias	4.42	1.00	19.60	.05043
Sucina	1.69	0.33	8.66	.52886	
Ulea	2.98	0.83	10.74	.09561	

TABLE 6 Odds ratios (ORs) from a logistic regression model of the relationship between *Leishmania* ELISA and/or PCR positivity and sex, leishmaniosis awareness and LODs against SGH

Variable	Level	OR	95% CI		p Value
SGH LODs	SGH LODs	1.04	1.01	1.06	.00604
Sex	Male	1.00	-	-	
	Female	0.43	0.16	1.17	.09866
Leishmaniosis awareness	No	1.00	-	-	
	Yes	0.44	0.17	1.12	.08577

lected in 2017 ($p < .05$). Overall *Leishmania* seroprevalence was 1.3% (9/670) and was not associated to any explanatory variable. Only one individual was positive to both ELISA and rtPCR. Six donors were coinfecting with *L. infantum* and TOSV, representing 5% and 32% of TOSV and *L. infantum*-positive individuals, respectively.

Twenty out of the 550 (3.6%) individuals analysed by ELISA and PCR were positive to either of those techniques. No explanatory variable was associated to *Leishmania* ELISA and/or rtPCR positivity (Table S2). The most parsimonious logistic regression model indicated increasing risk of being *Leishmania*-positive with increasing SGH LODs ($p < .05$) but not associated with rSP03B, and a marginally lower risk for women and for donors aware of leishmaniosis ($p < .10$, Table 6).

4 | DISCUSSION

This study provides first time population-based evidence that people living in rural and periurban areas in Murcia Region, southeast Spain, are widely exposed and develop antibodies to *P. perniciosus* sandfly salivary proteins. We also found that TOSV but not SFSV infections are widespread, with significant differences between years and areas and greatest prevalence among older donors that work outdoors and own working dogs. In contrast, relatively few donors were *L. infantum* positive but, in common with TOSV, infection was positively associated with SGH LODs. These results suggest that the risk of these sandfly-borne infections is positively associated to sandfly biting rates, and that SGH is a suitable diagnostic marker. Possible cross-reactions between antigens in SGH from *P. perniciosus* and closely related sandfly species (Lestinova et al., 2017), such as *Phlebotomus ariasi* and *Phlebotomus langeroni*, also belonging to the *Larrossius* subgenus, are possible and may be regarded as desirable given that all three species are implicated in *Leishmania* transmission and *P. perniciosus* and *P. ariasi* in phlebotomus transmission (ECDC, 2020; Izri et al., 2008; Remoli et al., 2016). *Phlebotomus langeroni* has only been occasionally described in Spain (Díaz-Sáez et al., 2018; Lucientes et al., 2001; Martínez Ortega et al., 1996) and not in Murcia, where *P. perniciosus* is the predominant species and *P. ariasi* is minoritarian (Martínez Ortega & Conesa Gallego, 1987; Muñoz et al., 2018; Risueño et al., 2017).

The diagnostic performance of rSP03B in humans was investigated for the first time, and correlation with SGH was only moderate. It appears that human anti-*P. perniciosus* saliva antibodies do not recognize rSP03B as strongly as total SGH in contrast to what was reported in dogs, mice and leporids (Drahota et al., 2014; Kostalova et al., 2015; Kostalova et al., 2017; Martín-Martín et al., 2014; Risueño et al., 2019). However, other studies have similarly reported negligible to moderate correlations between SGH and other recombinant proteins such as PpSP32 from *P. papatasi* in Tunisian people (Marzouki et al., 2015), the anti-clotting protein from *Lutzomyia longipalpis* in humans from Brazil (Barral et al., 2000) and the D7-protein SP04 from *P. perniciosus* in experimental mice (Martín-Martín et al., 2015). Rohoušová et al. (2005) highlighted differences between the intensity of mice and humans antibody responses against certain salivary protein in immunoblot assays. Variation in sialome composition between sandfly populations from different origins could also contribute to the low correlation of rSP03B with SGH (Rohoušová et al., 2012), although Martín-Martín et al. (2012) reported similar sialome composition between *P. perniciosus* colonies from different parts of Spain. Further investigations should be developed to explore the diagnostic use of other recombinant proteins to assess *P. perniciosus* exposure in humans.

Studies in dogs have shown that antibodies against salivary proteins are strongly seasonal, peaking in summer and decreasing thereafter to minimum levels in winter (Kostalova et al., 2015; Vlkova et al., 2011). In experimental trials, anti-*P. perniciosus* IgG antibody levels in dogs, mice and rabbits persisted for at least 5 weeks, 3 months and 7 months, respectively (Martín-Martín et al., 2015; Vlkova et al., 2011). In our study LODs to *P. perniciosus* salivary antigens were higher in donors sampled in January to March 2017 compared to those in September

to November 2018. Presumably, sandfly exposure was much greater in the summer and autumn 2016 compared to the same time in 2018, as interannual variation in sandfly abundance maybe common (Alten et al., 2016). This would also explain the lower TOSV and *L. infantum* prevalence in 2018 than in 2017, and the lower *L. infantum* prevalence in the present compared to a similar study in the same areas in 2008 and 2010 (Pérez-Cutillas et al., 2015). Longitudinal serological and entomological studies are needed to characterize the annual antibody dynamics to these infections and salivary antigens in humans from endemic areas. In contrast to anti-salivary protein antibodies, previous studies in humans showed that TOSV VNT IgG antibodies may last for up to 20 months (Pierro et al., 2017).

The reasons why SGH and rSP03B LODs were greater in women than in men and lower among older people are not evident. Gender- and age-associated behavioural differences could affect the degree of exposure to sandflies. Haematophagous insects are attracted to host's carbon dioxide emission and other volatile extracts, but the precise mechanisms for host selection remain poorly understood (Martínez et al., 2021). Antibody responses to *Aedes albopictus* salivary gland protein extracts and recombinant culicine-specific 34k2 salivary protein in humans in Italy similarly decreased with age and it was attributed to immunotolerance and progressive desensitization to the insect's salivary proteins (Buezo Montero et al., 2020). However, the relationship between age and antibodies against individual salivary proteins was antigen-dependent (Buezo Montero et al., 2020). Immunotolerance and desensitization might also explain the negative association between rSP03B antibodies and donors living in a country house compared to flats and other town homes.

The seroprevalence of TOSV in people from Murcia is on the high end of the 2–37% and 0–26% range seroprevalences with ELISA or IFAT and VNT techniques, respectively, reported in other studies of Spain, Portugal, Italy and France (Amaro et al., 2011; Amodio et al., 2011; de Ory et al., 2009; Echevarría et al., 2003; Maia et al., 2021; Mendoza-Montero et al., 1998), and lower than the 46% VNT positives in North African countries (Alkan et al., 2015) and the 77% ELISA positives reported among forestry workers in some areas in Italy (Valassina et al., 2003). Sandfly vectors are abundant in rural areas in Murcia (Muñoz et al., 2018; Risueño et al., 2017) and increasing TOSV seroprevalence with age in this study is compatible with increasing cumulative risk of infection, and the same relationship was reported in other studies (Cardeñosa et al., 2013; de Ory-Manchón et al., 2007; Pundapolíc et al., 2012; Sanbonmatsu-Gámez et al., 2005; Terrosi et al., 2009). Higher TOSV seroprevalences in donors working outdoors (and owning working dogs) would be related to increased risk of exposure to potentially TOSV-infected sandflies (Valassina et al., 2003). As previously mentioned, TOSV-associated meningitis has been reported in Murcia on several occasions (Echevarría et al., 2003; Martínez-García et al., 2007; Mendoza-Montero et al., 1998) but given the high seroprevalence, its real clinical impact may be underestimated. TOSV awareness should be raised as this pathogen is a prime candidate in the differential diagnosis of meningitis.

The absence of SFSV seropositive individuals suggests that the virus is not common in Murcia region. Its transmission has been

traditionally associated to *P. papatasi* (Alkan et al., 2013), and this species represented 10% of sandflies collected in a regionwide study in Murcia (Risueño et al., 2017) and 52% of those in a dog kennel outside Murcia city (Muñoz et al., 2021). Investigations in other parts of Spain reported a 1–5% SFSV VNT seroprevalence (Mendoza-Montero et al., 1998). Seroprevalence in other Mediterranean countries using ELISA or IFAT ranged between 1% and 9% using ELISA and between 0% and 32% with the VNT (Bichaud et al., 2011a; Calamusa et al., 2012; Cusi et al., 2013; Eitrem et al., 1991; Tesh et al., 1976).

The study reflects spatial heterogeneity TOSV and *L. infantum* infections but they did not always coincide, in contrast to a previous study in France (Bichaud et al., 2011b). This may be partly due to the low prevalence of *L. infantum* compared to TOSV in the present study. Presumably, in Murcia region, sandfly infection rates are higher for TOSV compared to *L. infantum*, but this has not been investigated. Studies on *L. infantum* infection rates in sandflies in Spain report widely variable results ranging from 0% to 59% depending on the area surveyed, the diagnostic technique used and the sandfly species analysed (Bravo-Barriga et al., 2016; Díaz-Sáez et al., 2018, 2021; González et al., 2017; Jiménez et al., 2013; Morillas et al., 1996; Muñoz et al., 2019). Two studies investigating TOSV infection in sandflies in Spain reported infection rates lower than 1% (Remoli et al., 2016; Sanbonmatsu-Gámez et al., 2005). The epidemiology of these infections is distinctly different. Dogs are the primary reservoir of *L. infantum* but not for TOSV, and the epidemiological cycle and reservoir hosts of TOSV have not been determined (Muñoz et al., 2020). Finally, it is important to consider that the observed prevalence of *L. infantum* in blood donors may be an underestimation of the true prevalence in Murcia. *Leishmania infantum* typically infects tissue macrophages and subclinical infections in dogs are common and often detectable by PCR in lymphoid tissue and not in blood samples (Baneth et al., 2008; Chitimia et al., 2011). The possibility that this may also occur in humans requires further investigation.

5 | CONCLUSION

Phlebotomus perniciosus exposure in human blood donors from periurban and rural areas of Murcia Region in southeast Spain is widespread and associated to TOSV and *L. infantum* infection. The high TOSV seroprevalence found reinforces its consideration as an important pathogen in the differential diagnosis of summer meningitis in this part of the country. The SGH provides a reliable marker to assess sandfly exposure but further studies are required to assess the diagnostic validity of rSPO3B and other potential sandfly salivary recombinant proteins in humans.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interests.

ETHICS APPROVAL

The study was approved by the University of Murcia Animal Ethics Committee (<http://www.um.es/web/vic-investigacion/contenido/vicerectorado/estructura/comisiones/etica-investigacion>) and Murcia's Regional Government.

CONSENT TO PARTICIPATE AND CONSENT FOR PUBLICATION

Informed consent for participation and data publication was obtained from all individual participants included in the study.

AUTHOR CONTRIBUTIONS

Conceptualization: MO, CM, EB. Methodology: MAI, TS, PS, NA, RC, PV, EB. Formal analysis and investigation: MO, CM, MAI, PPC, NA. Writing – original draft preparation: MO, EB. Writing – review and editing: MO, CM, TS, PS, MAI, PPC, NA, RC, PV, EB. Funding acquisition: MO, CM. Resources: TS, PS, MAI, RC, PV, EB. Supervision: TS, PS, RC, PV, EB.

DATA AVAILABILITY STATEMENT

The datasets supporting the conclusions of this article are included within the article. Raw data are available from the corresponding author on reasonable request.

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