SHORT COMMUNICATION

The recombinant protein rSP03B is a valid antigen for screening dog exposure to *Phlebotomus perniciosus* across foci of canine leishmaniasis

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Abstract. The frequency of sandfly–host contacts can be measured by host antibody levels against sandfly salivary proteins. Recombinant salivary proteins are suggested to represent a valid replacement for salivary gland homogenate (SGH); however, it is necessary to prove that such antigens are recognized by antibodies against various populations of the same species. *Phlebotomus perniciosus* (Diptera: Psychodidae) is the main vector of *Leishmania infantum* (Trypanosomatida: Trypanosomatidae) in southwest Europe and is widespread from Portugal to Italy. In this study, sera were sampled from naturally exposed dogs from distant regions, including Campania (southern Italy), Umbria (central Italy) and the metropolitan Lisbon region (Portugal), where *P. perniciosus* is the unique or principal vector species. Sera were screened for anti-*P. perniciosus* antibodies using SGH and 43-kDa yellow-related recombinant protein (rSP03B). A robust correlation between antibodies recognizing SGH and rSP03B was detected in all regions, suggesting substantial antigenic cross-reactivity among different *P. perniciosus* populations. No significant differences in this relationship were detected between regions. Moreover, rSP03B and the native yellow-related protein were shown to share similar antigenic epitopes, as canine immunoglobulin G (IgG) binding to the native protein was inhibited by pre-incubation with the recombinant form. These findings suggest that rSP03B should be regarded as a universal marker of sandfly exposure throughout the geographical distribution of *P. perniciosus*.

Key words. *Leishmania infantum*, *Phlebotomus* spp., antibody response, dog, markers of exposure, Mediterranean region, salivary proteins, sandflies.

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Leishmaniasis is a widely distributed disease caused by *Leishmania* protozoans and transmitted by phlebotomine sandfly vectors. During blood feeding, sandflies inoculate saliva into the host. Bitten hosts then develop a species-specific antibody response against salivary antigens that reflects the intensity of sandfly exposure and thus provides a useful marker of exposure to generate epidemiological data (Vlkova *et al.*, 2011; Martín-Martín *et al.*, 2014; Kostalova *et al.*, 2015).

Large-scale serological studies using total sandfly salivary gland homogenate (SGH) are currently impractical because it is difficult to dissect the high numbers of sandflies necessary to obtain sufficient amounts of SGH. Another potential complication refers to variability in the protein composition of sandfly saliva, which has been found to fluctuate depending on physiological factors such as sandfly age and diet (Volf *et al.*, 2000; Prates *et al.*, 2008). Studies in Old World sandfly species also revealed a certain degree of intra- and inter-population variability in protein and mRNA levels (Rohousova *et al.*, 2012; Ramalho-Ortigão *et al.*, 2015). Therefore, salivary recombinant proteins have been suggested to represent valid replacements for the whole salivary gland protein cocktail, and some have already been validated in the field (Drahota *et al.*, 2014; Martín-Martín *et al.*, 2014; Kostalova *et al.*, 2015). The use of specific recombinant salivary antigen circumvents the necessity for the laborious maintenance of sandfly colonies, and potentially provides a more refined way to minimize antigenic cross-reactivity with taxonomically close sandfly relatives. A useful recombinant salivary protein would demonstrate antigenicity comparable with that of SGH, share similar antigenic epitopes with the native proteins, and demonstrate similar antigenic patterns throughout the geographical distribution of a particular sandfly vector.

This study follows the canine longitudinal study conducted in southern Italy by Kostalova *et al.* (2015), which described the dynamics and diagnostic potential of antibodies recognizing *Phlebotomus perniciosus* (*Larroussius* subgenus) salivary recombinant proteins in dogs following natural exposure to sandflies over 2 years. Factors such as salivary antigens, age and expected sandfly dynamics were considered as variables and were therefore carefully evaluated. The most reactive and reproducible antigen was found to be the 43-kDa yellow-related recombinant protein (rSP03B) from *P. perniciosus* saliva. In view of these promising results, the rSP03B antigen was tested in canine sera samples collected cross-sectionally in canine leishmaniasis (CanL) endemic settings in Italy and Portugal. The study evaluated levels of individual canine antigenic responses to *P. perniciosus* rSP03B compared with *P. perniciosus* SGH, and the degree of similarity in these antigenic associations, across endemic canine populations in Portuguese and Italian foci in order to assess the universal use of rSP03B as a marker of natural sandfly exposure. Previous research had confirmed the presence of two native yellow-related proteins in *P. perniciosus* salivary gland transcriptome and proteome (Anderson *et al.*, 2006). Therefore, the antigenic similarity of rSP03B to its native form was studied and the specificity of the anti-rSP03B immunoglobulin G (IgG) antibody response was confirmed by the inclusion of 42-kDa yellow-related recombinant protein (rSP03).

Canine sera originated from three regions: (a) Campania ($n = 118$), a traditional high-risk area for CanL in southern continental Italy (Oliva *et al.*, 2006); (b) Umbria ($n = 96$), an inland area of central Italy recently recorded as a medium- to high-risk area for CanL (Di Muccio *et al.*, 2012), and (c) the metropolitan Lisbon region ($n = 341$), which is well known as a CanL endemic locality on the west coast of Portugal (Cortes *et al.*, 2012). In all three areas, *P. perniciosus* is the only or principal vector of CanL (Bongiorno *et al.*, 2003; Rossi *et al.*, 2007; Alten *et al.*, 2016). *Phlebotomus perfiliewi* (*Larroussius* subgenus), another vector of *Leishmania infantum*, was found to be abundant in some areas in Umbria (Maresca *et al.*, 2009). However, *P. perfiliewi* is found in association with large animals (cattle and equine species) in rural habitats (Bongiorno *et al.*, 2003). Dogs examined in Umbria included urban pets and animals hosted in kennels, but all lived in populated areas including residential zones surrounding urban centres, which represent typical habitats for *P. perniciosus* (Maroli *et al.*, 1994). Additionally, sampled dogs may have been exposed to sandflies from other subgenera occurring in study localities (Cortes *et al.*, 2007; Rossi *et al.*, 2007; Maresca *et al.*, 2009). A previous study by Volf & Rohousova (2001) suggested there was no cross-reaction of *Larroussius* species with other sandflies present in these study regions, namely *Phlebotomus papatasi* (*Phlebotomus* subgenus), *Phlebotomus sergenti* (*Paraphlebotomus* subgenus) and members of the genus *Sergentomyia*.

Single sera samples from Campania and Umbria were purposely selected from archived samples collected in 2007–2013 to represent the period from July (i.e. at least 2 months after the beginning of the sandfly season) to October (i.e. the end of the sandfly season). The selected sera were collected from dogs ranging in age from 1.5 to 13 years. The dogs from both Italian regions represented a mixture of hunting breeds and mongrels. Single sera samples from the metropolitan Lisbon region were randomly collected from kennelled dogs (mostly mongrel) at the beginning of the sandfly season in May 2012. These dogs ranged from young (6–12 months) to more senior (> 7 years) dogs.

Samples from Campania consisted of stored sera sent by veterinary clinics to the Istituto Superiore di Sanità for routine serological diagnosis of suspected CanL in owned dogs. Sera from Umbria were collected from healthy dogs that were enrolled on a voluntary basis in the Perugia University CanL surveillance programme. Blood sampling was performed in accordance with the Italian guidelines for animal welfare, following owners' consent, and did not include additional or unnecessary invasive procedures. The collection of sera in the metropolitan Lisbon region was ethically approved by the board of the Institute of Hygiene and Tropical Medicine, New University of Lisbon (IHMTUNL) (authorization no. 8 2011-PI) in compliance with Portuguese legislation for the protection of animals (Law 113/2013).

Anti-*Leishmania* IgG in canine sera from Campania and Umbria was detected with an in-house indirect fluorescent antibody test (IFAT) using *L. infantum* promastigotes as antigen, as described in Gradoni & Gramiccia (2008). Samples showing an IFAT titre of 1 : 40 or greater were considered to indicate exposure to *Leishmania*. Immunoglobulin G antibodies against *Leishmania* in canine sera from the metropolitan Lisbon region were detected using an enzyme-linked immunosorbent assay

(ELISA) kit (Bordier Affinity Products SA, Crissier, Switzerland) according to the manufacturer's guidelines (Maia *et al.*, 2010). The result was considered positive when the absorbance of the analysed sample was higher than the absorbance of the weak positive control serum provided with the kit.

A longterm established laboratory colony of *P. perniciosus* originating from Spain (Murcia) was reared under standard conditions as described in Volf & Volfova (2011). Salivary glands, rSP03B (GenBank accession no. DQ 150622) and rSP03 (GenBank accession no. DQ 150621) from *P. perniciosus* were obtained for this study as previously described (Kostalova *et al.*, 2015) and used as antigens for testing the canine sera.

Antibodies against *P. perniciosus* SGH and rSP03B protein were measured by ELISA as described by Kostalova *et al.* (2015). Each serum was tested in duplicate. Test absorbance values were reported as optical densities (ODs) with subtracted blanks (the ELISA plate background mean absorbance value measured in control wells).

Western blot analysis was used to confirm the similarity of antigenic epitopes between the native yellow-related protein found in *P. perniciosus* SGH and the corresponding recombinant protein rSP03B. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of SGH (equivalent to 4 μg total salivary proteins per lane) and rSP03B (2 μg per lane) was run on a 12% gel and blotted onto the nitrocellulose membrane using the iBLOT instrument (Invitrogen Corp., Carlsbad, CA, U.S.A.). Membrane with separated proteins was cut into strips and blocked in 5% milk diluted in Tris-buffered saline with 0.05% Tween 20 (Tris-Tw) overnight at 4 °C. For the inhibition test, three Italian canine sera possessing high levels of anti-*P. perniciosus* IgG against SGH and rSP03B were pooled. The positive serum pool was diluted 1 : 50 in Tris-Tw and split into halves. The first half was incubated for 2 h on a shaker with rSP03B (20 μg/mL) and the second half was incubated without rSP03B. Negative control sera (canine sera from a non-endemic locality) were diluted 1 : 50 in Tris-Tw and incubated without rSP03B on a shaker for 2 h. In the next step, part of the positive sera pool, incubated either with or without rSP03B protein, and part of the negative control sera was incubated with strips of separated *P. perniciosus* SGH. The same procedure was repeated for strips containing rSP03B, except that sera were diluted 1 : 100 in Tris-Tw. After 1 h, all strips were rinsed in Tris-Tw and subsequently incubated for 1 h with peroxidase-conjugated anti-dog IgG (1 : 3000) (Bethyl Laboratories, Inc., Montgomery, TX, U.S.A.). The colour reaction was developed by substrate solution containing 3,3′-diaminobenzidine (Sigma-Aldrich Corp., St Louis, MO, U.S.A.). Furthermore, in order to confirm the specificity of the western blot analysis, the same procedure was repeated for rSP03 protein.

Statistical analyses were carried out using R software (http://cran.r-project.org/) and STATA Version 13.1 (Stata Corp., College Station, TX, U.S.A.). Correlations were analysed using the Spearman rank correlation test and medians were compared between groups using a Wilcoxon rank sum test. Optical density values were logarithmized (natural logarithm) for better readability. Statistical analyses of the relationships between SGH and rSP03B OD values among the canine populations were statistically tested by fitting Poisson general linearized models (GLMs) with an ln link function as the right-skewed frequency

distributions were found not to follow a negative binomial distribution (deviance goodness-of-fit $\chi^2 > 56.2$; $P = 1$, d.f. $= 549$, for each antibody). The full Poisson GLMs included interaction terms to test differences between the regions, both in terms of baseline anti-rSP03B value (intercept where anti-SGH equals 0) and the relationship between antibodies against SGH and rSP03B (slopes). A P-value of < 0.05 was considered to indicate statistical significance.

The use of *P. perniciosus* rSP03B as an epidemiological tool was tested for investigations of canine exposure to sandfly bites in geographically distinct localities in which *P. perniciosus* is the prevalent phlebotomine vector. The recombinant protein rSP03B used in this study was obtained from the salivary glands of *P. perniciosus* in a laboratory-reared colony originating from Murcia in Spain, and was used as an antigen in the serology of dogs living in the Campania and Umbria regions of Italy and in the metropolitan Lisbon region in Portugal.

Levels of canine IgG antibodies reacting with SGH and rSP03B were measured by ELISA. Positive but variable correlations between antibody responses to SGH and rSP03B antigens were observed in sera from all three localities [Campania: $r = 0.73$, 95% confidence interval (CI) 0.62–0.82 ($P < 0.001$); Umbria: $r = 0.56$, 95% CI 0.38–0.71 ($P < 0.001$); metropolitan Lisbon: $r = 0.81$, 95% CI 0.76–0.84 ($P < 0.001$)] (Fig. 1). Table 1 summarizes the OD values for each region and indicates that OD frequency distributions were over-dispersed. To query possible differences in the relationships between SGH and rSP03B antibody responses between geographical regions, the equality of the population-specific regression slopes was tested by fitting a Poisson model. No significant differences were detected (population × antigen interaction terms: $Z > -0.85$, $P > 0.365$). Relative to the metropolitan Lisbon region, both the Campania and Umbria populations tended to produce higher baseline antibody responses against rSP03B, although these differences failed to reach significance at the 5% level (Campania: $Z = 1.66$, $P = 0.097$; Umbria: $Z = 1.95$, $P = 0.051$). One plausible explanation for the putative differences in baseline rSP03B antibody levels among populations is that the populations differ in their condition or past history of infections and that these differences affect general immunological responses to certain antigens, and/or that sandfly biting pressure differs across these populations. The seasonal exposure of dogs to sandflies has been found to lead to antibody response fluctuations related to the period of activity and abundance of vectors (Vlkova *et al.*, 2011; Kostalova *et al.*, 2015). Secondly, as age is a frequent covariate of cumulative exposure used to model cross-sectional age-related prevalence data of *Leishmania* infection (Courtenay *et al.*, 1994), the average older dog is expected to have experienced more sandfly seasons (Kostalova *et al.*, 2015). Dogs from Campania and Umbria were sampled from July (i.e. during the period of highest sandfly abundance in Italy). All of the animals tested from these two regions had experienced at least two consecutive transmission seasons. Sera from dogs in the metropolitan Lisbon region were sampled in May, which is the beginning of the sandfly season, and were sourced mainly from dogs aged > 1 year. Thus these dogs had experienced at least one transmission season. According to reactivity data shown by Kostalova *et al.* (2015), dogs will be reactive to saliva at the beginning of the transmission season if they have already been 'primed' in the
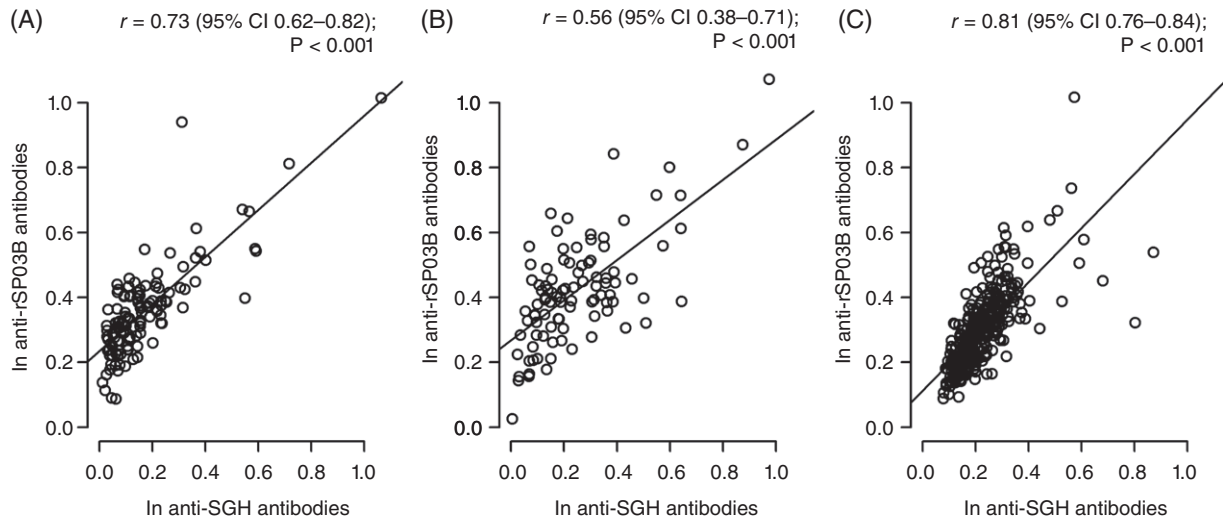
Fig. 1. Correlations between antibodies recognizing salivary gland homogenate (SGH) and rSP03B in dogs naturally bitten by *Phlebotomus perniciosus* in (A) Campania, (B) Umbria and (C) the metropolitan Lisbon region. Correlations were ascertained using Spearman rank correlation. *r*, correlation index; 95% CI, 95% confidence interval.

Table 1. Summary of optical density (OD) values recorded by enzyme-linked immunosorbent assay (ELISA) using *Phlebotomus perniciosus* salivary antigens.

Antigen	Region	Dogs, *n*	OD values	
			Median (IQR)	Min–max
SGH	Campania*	118	0.131 (0.073–0.241)	0.011–1.899
	Umbria†	96	0.218 (0.133–0.409)	0.005–1.652
	Lisbon‡	341	0.221 (0.165–0.311)	0.081–1.390
rSP03B	Campania*	118	0.407 (0.311–0.516)	0.091–1.761
	Umbria†	96	0.495 (0.386–0.649)	0.026–1.925
	Lisbon‡	341	0.323 (0.234–0.436)	0.092–1.766

*Southern Italy.
†Central Italy.
‡Metropolitan Lisbon region (Portugal).
IQR, interquartile range; SGH, salivary gland homogenate.

previous season. These results indicate substantial salivary antigen cross-reactivity amongst *P. perniciosus* populations from Campania, Umbria and the metropolitan Lisbon region. Strong antigenic cross-reactivity between populations of the same sandfly species was similarly observed between two geographically distant colonies of *Phlebotomus orientalis* (*Larroussius* subgenus) in Ethiopia (Vlkova *et al.*, 2014), and among colonies of *P. sergenti* originating from Israel and Turkey (Rohousova *et al.*, 2012).

The similarity of antigenic epitopes between native yellow-related proteins in Spanish *P. perniciosus* SGH and rSP03B was demonstrated by an inhibition test (Fig. 2). For this analysis, sera of dogs from Campania and Umbria with high levels of specific antibodies were selected and pooled. The inhibition test showed that all IgG antibodies specific for the native yellow-related protein bind to the recombinant form during pre-incubation of the sera, which resulted in the complete disappearance of the corresponding band on western blotting

(Fig. 2). This demonstrated that rSP03B shares antigenic epitopes with the native yellow-related protein contained within *P. perniciosus* saliva and presumably identifies the proportion of bitten dogs in a manner similar to the use of SGH. By contrast, when the inhibition test was performed with rSP03 protein, intended to confirm the specificity of the western blot analysis, no band appeared and no inhibition was observed (Fig. 2). Therefore, rSP03 is considered to be a non-immunogenic antigen. These results show that the band observed in western blotting with SGH as antigen corresponds to the native 43-kDa yellow-related protein and that the anti-SP03B IgG antibodies are highly specific for the tested rSP03B protein.

Italy and Portugal are generally assumed to show endemic CanL transmission (Oliva *et al.*, 2006; Cortes *et al.*, 2012; Di Muccio *et al.*, 2012). In this study, CanL seropositivity ranged from 5% to 30%, with the lowest prevalence in Umbria and the highest in Campania (Table 2). The use of antibodies against sandfly salivary proteins as risk markers of *L. infantum* infection has been tested earlier for SGH (Vlkova *et al.*, 2011), as well as for salivary recombinant proteins, among which rSP03B proved to be a powerful marker of host exposure to sandflies (Kostalova *et al.*, 2015). Therefore, the present study analysed the relationship between anti-*P. perniciosus* antibodies and *Leishmania* serological status. When using rSP03B antigen, significantly higher levels of specific IgG in *Leishmania*-seropositive dogs [median = 0.346, interquartile range (IQR) 0.257–0.536] than in *Leishmania*-seronegative dogs (median = 0.320, IQR 0.229–0.422) were found only in the metropolitan Lisbon region (Wilcoxon rank sum test, $W = 5391.5$, $P = 0.025$). In Campania, the differences in antibodies against rSP03B between *Leishmania*-seropositive (median = 0.457, IQR 0.357–0.550) and *Leishmania*-seronegative (median = 0.379, IQR 0.303–0.499) dogs were marginally significant (Wilcoxon rank sum test, $W = 1123.5$, $P = 0.053$). Previous studies on the relationship between anti-*P. perniciosus* antibodies and seropositivity to *L. infantum* show variable correlations. In Kostalova *et al.*
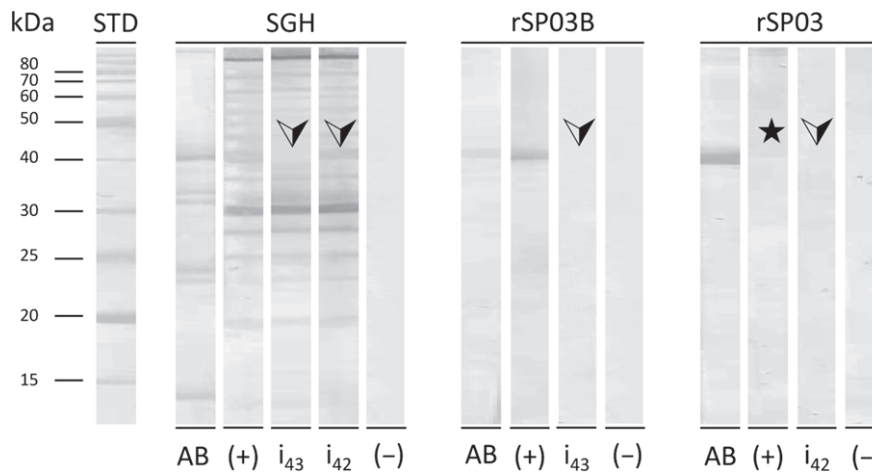
Fig. 2. Western blot analysis of salivary gland homogenate (SGH), rSP03B and rSP03 and inhibition test. A mixture of canine sera positive to *Phlebotomus perniciosus* SGH was pre-incubated with rSP03B or rSP03 and then tested in western blotting against SGH. Arrows indicate the points at which inhibition should take place. The star indicates the position of rSP03. STD, standard; AB, strip stained by Amido black; (+), positive control strip; i_{43}, inhibition strip for rSP03B; i_{42}, inhibition strip for rSP03; (−), negative control strip.

Table 2. Frequencies of *Leishmania* seropositivity and seronegativity in dogs from different regions.

| Diagnostic method | Cut-off | Serological status∗ | Anti-*L. infantum* IgG positive/total animals sampled, *n* (%) | | |
			Campania	Umbria	Lisbon
IFAT	1 : 40	Positive	35/118 (30%)	5/96 (5%)	—
		Negative	83/118 (70%)	91/96 (95%)	—
ELISA	0.26	Positive	—	—	46/341 (13%)
		Negative	—	—	295/341 (87%)

∗As determined by the IFAT titre or ELISA cut-off.
IgG, immunoglobulin G; IFAT, indirect fluorescent antibody test; ELISA, enzyme-linked immunosorbent assay.

(2015), a positive association was observed between levels of canine IgG antibodies against sandfly saliva and active CanL infection in dogs sampled longitudinally over 2 years. By contrast, the study by Vlkova *et al.* (2011) described a negative correlation between levels of specific IgG2 and risk for *Leishmania* infection. Comparisons between studies are difficult following observations that anti-saliva antibodies wax and wane with sandfly exposure and seasonality (Kostalova *et al.*, 2015). In actively infected dogs, anti-*Leishmania* antibodies tend to persist after an initial increase, whereas in exposed resistant animals they tend to fluctuate or convert to negative (Oliva *et al.*, 2006). As studies tend to be cross-sectional and use different approaches to determine *Leishmania* infection status, cross-study comparisons are difficult. Although longitudinal studies have already demonstrated the potential usefulness of the sandfly saliva antigenic response in dogs as a marker for *Leishmania* infection (Kostalova *et al.*, 2015; R. J. Qinnell, personal communication, 2016), the possibility of using sandfly salivary recombinant proteins in a similar way in cross-sectional surveys still needs to be validated.

In conclusion, this study showed that *P. perniciosus* rSP03B, the 43-kDa yellow-related recombinant protein, possesses the same antigenic epitopes as its native form in salivary glands, and binds similarly in canine sera from foci in Italy and Portugal. Therefore, it could serve as a universal marker of

sandfly exposure across the entire geographical distribution of *P. perniciosus*, even in dogs of various breeds and ages.

Acknowledgements

References

Alten, B., Maia, C., Afonso, M.O. *et al.* (2016) Seasonal dynamics of phlebotomine sand fly species proven vectors of Mediterranean leishmaniasis caused by *Leishmania infantum*. *PLoS Neglected Tropical Diseases*, **10**, e0004458.

Anderson, J.M., Oliveira, F., Kamhawi, S. *et al.* (2006) Comparative salivary gland transcriptomics of sandfly vectors of visceral leishmaniasis. *BMC Genomics*, **7**, 52.

Bongiorno, G., Habluetzel, A., Khoury, C. & Maroli, M. (2003) Host preferences of phlebotomine sand flies at a hypoendemic focus of canine leishmaniasis in central Italy. *Acta Tropica*, **88**, 109–116.

Cortes, S., Afonso, M.O., Alves-Pires, C. & Campino, L. (2007) Stray dogs and leishmaniasis in urban areas, Portugal. *Emerging Infectious Diseases*, **13**, 1431–1432.

Cortes, S., Vaz, Y., Neves, R., Maia, C., Cardoso, L. & Campino, L. (2012) Risk factors for canine leishmaniasis in an endemic Mediterranean region. *Veterinary Parasitology*, **189**, 189–196.

Courtenay, O., Macdonald, D.W., Lainson, R., Shaw, J.J. & Dye, C. (1994) Epidemiology of canine leishmaniasis: a comparative serological study of dogs and foxes in Amazon Brazil. *Parasitology*, **109**, 273–279.

Di Muccio, T., Veronesi, F., Antognoni, M.T., Onofri, A., Piergili Fioretti, D. & Gramiccia, M. (2012) Diagnostic value of conjunctival swab sampling associated with nested PCR for different categories of dogs naturally exposed to *Leishmania infantum* infection. *Journal of Clinical Microbiology*, **50**, 2651–2659.

Drahota, J., Martín-Martín, I., Sumova, P. *et al.* (2014) Recombinant antigens from *Phlebotomus perniciosus* saliva as markers of canine exposure to visceral leishmaniases vector. *PLoS Neglected Tropical Diseases*, **8**, 2597.

Gradoni, L. & Gramiccia, M. (2008) Leishmaniosis. *OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (Mammals, Birds and Bees)*, 6th edn, pp. 240–250. Office International des Epizooties, Paris.

Kostalova, T., Lestinova, T., Sumova, P. *et al.* (2015) Canine antibodies against salivary recombinant proteins of *Phlebotomus perniciosus*: a longitudinal study in an endemic focus of canine leishmaniasis. *PLoS Neglected Tropical Diseases*, **9**, e0003855.

Maia, C., Nunes, M., Cristóvão, J. & Campino, L. (2010) Experimental canine leishmaniasis: clinical, parasitological and serological follow-up. *Acta Tropica*, **116**, 193–199.

Maresca, C., Scoccia, E., Barizzone, F. *et al.* (2009) A survey on canine leishmaniasis and phlebotomine sand flies in central Italy. *Research in Veterinary Science*, **87**, 36–38.

Maroli, M., Bigliocchi, F. & Khoury, C. (1994) Sandflies in Italy: observations on their distribution and methods for control. *Parassitologia*, **36**, 251–264.

Martín-Martín, I., Molina, R., Rohoušová, I., Drahota, J., Volf, P. & Jiménez, M. (2014) High levels of anti-*Phlebotomus perniciosus* saliva antibodies in different vertebrate hosts from the re-emerging leishmaniasis focus in Madrid, Spain. *Veterinary Parasitology*, **202**, 207–216.

Oliva, G., Scalone, A., Foglia Manzillo, V. *et al.* (2006) Incidence and time course of *Leishmania infantum* infections examined by parasitological, serologic, and nested-PCR techniques in a cohort of naive dogs exposed to three consecutive transmission seasons. *Journal of Clinical Microbiology*, **44**, 1318–1322.

Prates, D.B., Santos, L.D., Miranda, J.C. *et al.* (2008) Changes in amounts of total salivary gland proteins of *Luzomyia longipalpis* (Diptera: Psychodidae) according to age and diet. *Journal of Medical Entomology*, **45**, 409–413.

Ramalho-Ortigão, M., Coutinho-Abreu, I.V., Balbino, V.Q. *et al.* (2015) *Phlebotomus papatasi* SP15: mRNA expression variability and amino acid sequence polymorphisms of field populations. *Parasites and Vectors*, **8**, 298.

Rohousova, I., Volfova, V., Nova, S. & Volf, P. (2012) Individual variability of salivary gland proteins in three *Phlebotomus* species. *Acta Tropica*, **122**, 80–86.

Rossi, E., Rinaldi, L., Musella, V. & Maroli, M. (2007) Mapping the main *Leishmania* phlebotomine vector in the endemic focus of the Mt. Vesuvius in southern Italy. *Geospatial Health*, **1**, 191–198.

Vlkova, M., Rohousova, I., Drahota, J. *et al.* (2011) Canine antibody response to *Phlebotomus perniciosus* bites negatively correlates with the risk of *Leishmania infantum* transmission. *PLoS Neglected Tropical Diseases*, **5**, e1344.

Vlkova, M., Sima, M., Rohousova, I. *et al.* (2014) Comparative analysis of salivary gland transcriptomes of *Phlebotomus orientalis* sand flies from endemic and non-endemic foci of visceral leishmaniasis. *PLoS Neglected Tropical Diseases*, **8**, e2709.

Volf, P. & Rohousova, I. (2001) Species-specific antigens in salivary glands of phlebotomine sandflies. *Parasitology*, **122**, 37–41.

Volf, P. & Volfova, V. (2011) Establishment and maintenance of sand fly colonies. *Journal of Vector Ecology*, **36**, S1–S9.

Volf, P., Tesarová, P. & Nohýnková, E.N. (2000) Salivary proteins and glycoproteins in phlebotomine sandflies of various species, sex and age. *Medical and Veterinary Entomology*, **14**, 251–256.