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Original Article

Canine leishmaniosis in the Italian northeastern Alps: A survey to assess serological prevalence in dogs and distribution of phlebotomine sand flies in the Autonomous Province of Bolzano - South Tyrol, Italy

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ABSTRACT

The Autonomous Province of Bolzano-South Tyrol (APB), located in the northernmost territory of the Italian eastern Alps, is still considered non-endemic for canine leishmaniosis (CanL) despite clinical cases being observed and a competent *Leishmania infantum* vector (*Phlebotomus perniciosus*) having been recorded since 2008. A serological survey of leishmaniosis among a randomly-selected subpopulation of registered owned dogs was carried in 2018, followed by entomological investigations performed in 2019 and driven by canine survey results. A total of 457 resident dogs from all over the APB territory were examined through IFAT for antibodies against *L.infantum,* of which 63 (13.8%) tested positive. Thirty-five seropositive cases (7.7%) were considered autochthonous to APB, i.e. dogs born and lived in the province, or imported dogs with no travel history in the past 5 years. Most of these animals showed an antibody titre at the threshold level of 1:40, suggesting a low degree of parasite transmission/contacts. In 2 autochthonous cases with moderately high IFAT titre, the infection was confirmed by nested-PCR in peripheral blood. Thirty-one georeferenced sites were monitored for sand flies by means of interception (sticky papers) and attraction (CDC miniature light traps) collection devices. Traps were set during summer approximately on monthly basis, and extended up to October for positive sites. Only 2 sites were found positive for a total of 317 phlebotomine specimens collected by sticky traps, which included a previously known *P. perniciosus*-endemic site near Bolzano town. *Sergentomyia minuta* was by far the most prevalent (98.1%) and the only recorded sand fly species in the most northerly Italian site ever investigated (Coldrano municipality in Venosta valley). For the first time, *Leishmania* serology and n-PCR positive dogs autochthonous to APB were identified, however the spread of sand flies competent for L. *infantum* transmission could not be demonstrated in several places where endemic seropositive cases were recorded. APB can be considered a territory of low CanL endemicity, however awareness and continuous monitoring are needed to detect changes in the epidemiological status of the zoonosis.

1. Introduction

Canine leishmaniosis (CanL) caused by the protozoan parasite *Leishmania infantum* (Kinetoplastida: Trypanosomatidae) and transmitted in the Old World mainly by sand flies of the subgenus *Phlebotomus* (*Larroussius*) (Diptera: Psychodidae), is a zoonotic disease of emerging importance (Alten et al., 2016). In Europe the disease is endemic in southern countries, but several factors associated with elevated densities of phlebotomine vectors (e.g. climate changes) (Hemmer et al., 2007; Fischer et al., 2010) and infected canine hosts (e.g. mobility of dogs within a country and across borders) (Trotz-Williams and Trees, 2003; Naucke et al., 2008; Poeppl et al., 2013), may contribute to the spread and occurrence of this disease in other areas (Baneth et al., 2008). In particular, travelling with or translocation of dogs from endemic to non-endemic regions is currently a phenomenon that needs to be monitored for the risk of a shift in disease

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transmission from the European Mediterranean region to the unprotected naïve dog population of non-endemic regions of Central-Northern Europe (Menn et al., 2010; EFSA AHAW Panel, 2015).

At present, endemic regions or countries are defined as such based on the presence of dogs infected locally and phlebotomine competent vectors, whereas non-endemic regions or countries are those where vectors are absent and the confirmed cases represent *L. infantum* infections acquired abroad or by means of non-vectorial modes of transmission (Mettler et al., 2005; Maia and Cardoso, 2015; EFSA AHAW Panel, 2015).

In northern Italy, the occurrence of autochthonous CanL and human cases is associated with a 30-year northward expansion and increase in density of the phlebotomine vectors *Phlebotomus perniciosus* and *P. neglectus* (Maroli et al., 2008, 2013). However, information regarding the Autonomous Province of Bolzano-South Tyrol (APB) has been so far incomplete. APB, a populous province located in the northernmost territory of the Italian eastern Alps, is still considered non-endemic for CanL, despite *P. perniciosus* presence was recorded for the first time in 2008 at low densities (Morosetti et al., 2009). In 2014, an entomological reassessment confirmed again the presence of this vector species in a discontinuous distribution at sites in hilly areas (300–480 m asl) and at densities similar to those of 2008 (Morosetti et al., 2016). At the same time of the first positive entomological survey, stray and free-roaming dogs hosted at the public shelter were examined serologically for CanL but they tested negative. In 2009, a second survey among owned dogs showed 2 *Leishmania* seropositive in a sample of 88 dogs, however both animals had visited endemic areas in the recent past. In 2015, a retrospective case finding was carried out in dogs from the Bolzano Health District (Morosetti et al., 2016). Compared to 2009, the CanL seroprevalence has more than doubled, but still all seropositive dogs had a past history of travel to endemic areas.

As regards human leishmaniasis, in 2014 and 2015 two visceral cases were recorded. Interestingly, the case reported by Gallina et al. (2014) was initially described as acquired abroad, but after accurate epidemiological assessment, it was found that the patient had no history of travel to endemic areas and his habitual residence was in a Bolzano area where *P. perniciosus* was collected. Thus, it cannot be excluded that this was the first autochthonous infection detected in APB.

The canine population of APB has shown a steady increase in recent years (Fig. 1), which includes stray dogs collected by private welfare associations from central and southern Italian territories highly endemic for leishmaniosis (Fig. 2). This phenomenon may have modified the local risk of indigenous transmission of CanL. To investigate on the current status of CanL in APB and evaluate the risk of leishmaniosis becoming endemic in the region, a general survey in dogs was carried in 2018 followed by new entomological investigations performed in 2019, which were driven by canine survey results.

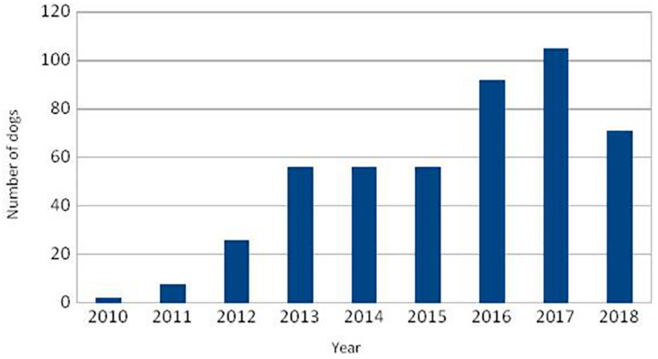

Fig. 2. Introduction of dogs from 3 regions of southern Italy to the Autonomous Province of Bolzano, 2010–2018 (courtesy of Centro Servizi Nazionale Anagrafi degli Animali, Istituto Zooprofilattico Sperimentale, Teramo).

2. Methods

2.1. Study site

APB is the largest Italian province, with a surface area of 7400 Km^2 including 116 municipalities and a total population of 528,918 inhabitants (ASTAT, 2018). It is the northernmost territory of Italy and is bordered by Austria to northeast and by Switzerland to the west. The Italian provinces of Belluno, Trento and Sondrio, belonging to 3 different regions, border to the southeast, south and southwest respectively. Most APB settlements are between 300 and 1200 m asl and a large proportion of the territory between 800 and 1800 m asl is covered by forest and highland pastures. The lower valleys are mostly cultivated (predominantly vineyards and fruit trees) with different types of shrubs and mixed forests. There are five distinct climate zones: humid subtropical, *oceanic*, humid continental, sub-arctic and alpine tundra (permanent frost above 3000 m) (Climate, 2008). Being on the southern side of the Alpine mountain chain, the climate is mild compared to the Austrian and Swiss border areas, and since the beginning of 2000s a steady increase in temperatures has been recorded (Fig. 3).

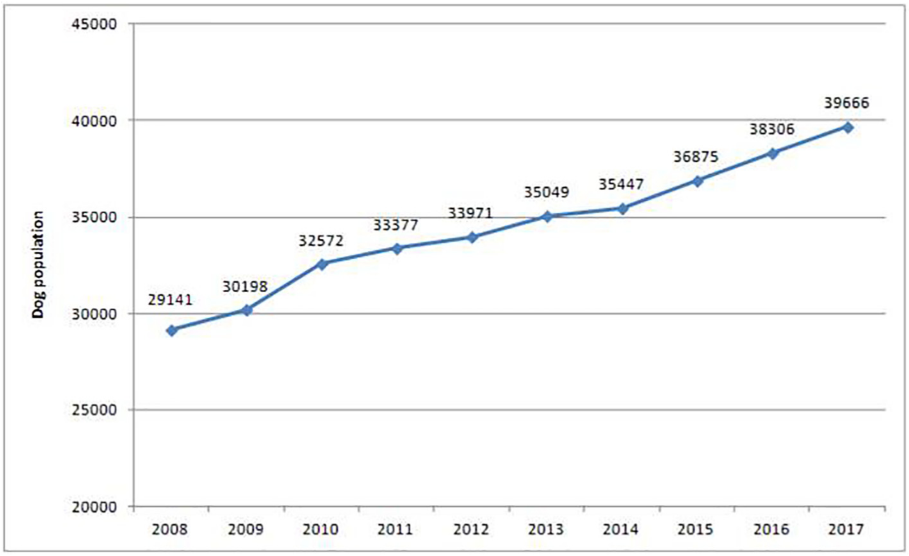

Fig. 1. Registered dog population in Autonomous Province of Bolzano, 2008–2017.

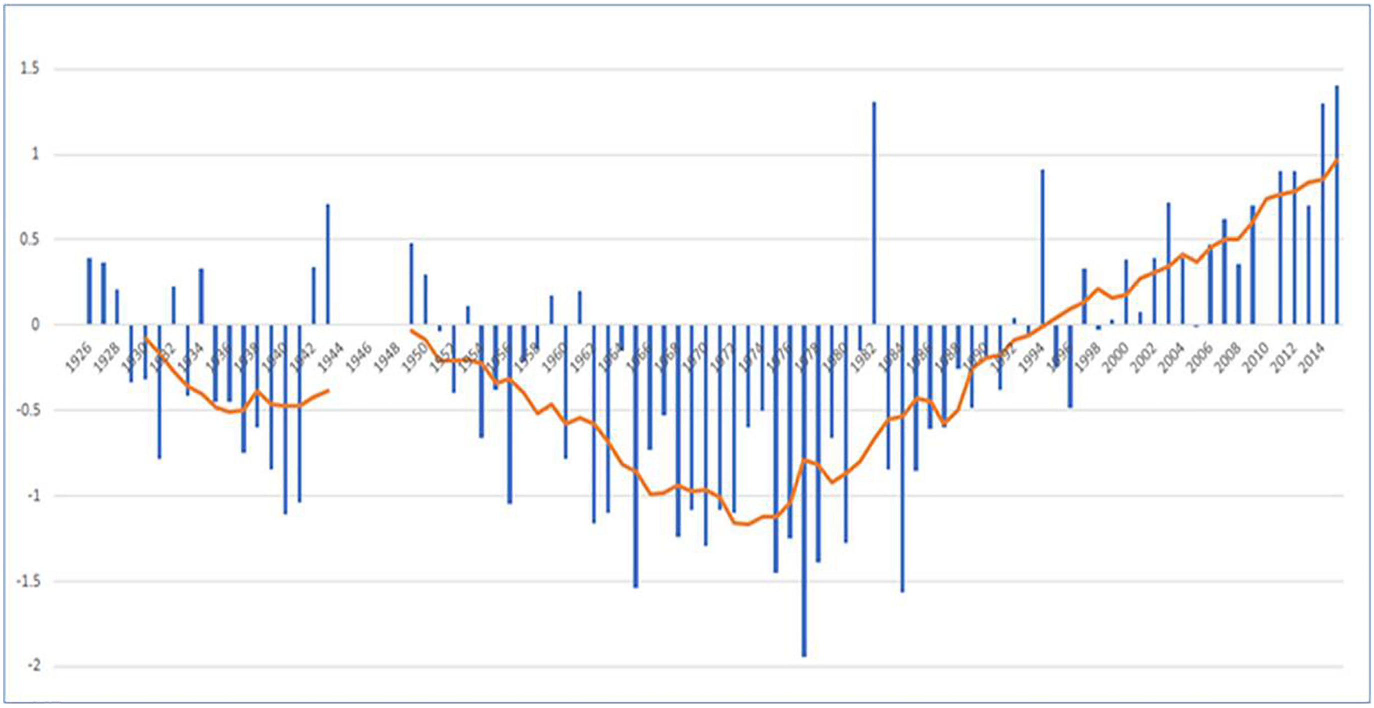

Fig. 3. Bolzano, Italy: trend of annual temperatures based on differences from mean value (°C) for the 1926–2015 period.

2.2. Canine survey design

About 40,000 owned dogs are registered in APB – a relatively dense canine population of 7.5 dogs/100 people. Stray or free-roaming uncontrolled dogs are very rare. The survey was carried out from February to May 2018, a period when adult sand flies are not emerged in northern Italy.

Eligible dogs were defined as resident in APB, of medium-large size, and over 4 years of age to increase the chance of detecting autochthonous infections assuming a low circulation of parasites in the area in each sand fly season. The sample size was determined assuming an expected prevalence of 1%, a 90% confidence interval and 0.5% desired precision, by performing exact binomial method. Eligible dogs were randomly selected from the APB register in proportion to canine population of each municipality. For municipalities with a total number of dogs below 10, all dogs were selected. For the other municipalities a minimum of 5 dogs was selected. Dog owners received a formal invitation to contact one among a group of participating private veterinarians. They were first invited by letter and then contacted by telephone to provide further information, answer questions and increase motivation. Testing their dogs was free of charge, not compulsory and was not compensated. When owners of more than a dog requested that all their animals were tested, thereby including the non-selected ones, they were satisfied regardless eligibility criteria of dogs' size and age. For each dog, a form was filled out including individual data, residence, usual management (i.e. places where the dog was taken for walks, hunting activity, shelter during day and night, and coexistence with other dogs), clinical status and travel history, and later analysed along with CanL diagnosis results.

Autochthonous dogs were primarily defined as those born and lived in APB. However, based on the current knowledge of the canine *Leishmania* infection, dogs imported to APB with no further travel history in the past 5 years, were also included in this category. It was assumed that events of local *Leishmania* transmission have most probably occurred if a positive diagnosis was made in autochthonous dogs. To increase reliability of serodiagnosis results and provide more robust evidence of local transmission, serology was complemented by molecular *Leishmania* demonstration in some representative cases.

2.3. CanL diagnosis

For the serosurvey, peripheral blood samples were obtained by the participating veterinarians, the sera collected and coded at the local section of the Istituto Zooprofilattico Sperimentale delle Venezie (IZS-VE), and sent to the reference serology laboratory of IZS-VE in Legnaro, Padua. Sera were tested by an in-house IFAT using L. *infantum* promastigotes as the antigen source. Considering an expected low seroprevalence in the study area, a serum dilution 1:40 was chosen as positive threshold antibody titre (Gradoni and Gramiccia, 2014).

Leishmania nested (n)-PCR was performed in samples of peripheral blood collected in EDTA-coated tubes and sent refrigerated to Istituto Superiore di Sanità in Rome. DNA extraction and conditions of n-PCR were those reported by Gramiccia et al. (2010). This sensitive technique targeting the small-subunit rRNA gene sequences of *Leishmania* sp. was considered the most suitable one for the predicted low-endemic status of APB.

2.4. Entomological survey

Search for potential sand fly collection sites was performed in the northwestern and northeastern valleys of APB, proceeding northwards along the upper Adige valley from Merano city into the upper Venosta valley towards the Swiss border, and along the Isarco valley into the Pusteria valley towards the Austrian border. The inspected municipalities were those with *Leishmania* seropositive records in autochthonous dogs. Following a preliminary evaluation based on climatic and environmental maps and, above all, visual inspection of biotopes and landscape, sand fly trapping has actually focused to the northwestern territories, Merano area and Val Venosta, as they showed general features such as temperature, humidity, aspect and land cover more suitable for sand fly presence than the northeastern part of APB (Bongiorno et al., 2008; Rossi et al., 2007; Rioux et al., 1984). Here, suitable sites were chosen using detailed local maps (Tobacco, 1:25.000) and visiting the areas with the help of local personnel of the veterinary service. Entomological collections were performed in three periods of the warm season 2019, based on previous knowledge on the seasonal activity of phlebotomines in mountainous areas of central Italy or in hilly territories of northern Italy (Bongiorno et al., 2003; Ferroglio et al., 2005). Trapping was carried out using 20 × 20 cm sticky papers coated with castor oil (Rioux et al., 1967), mainly set in wall holes along side roads and recovered after 48 h. CDC miniature light traps equipped with a fine net cage (John W. Hock Co., Gainesville, Florida, USA) were placed overnight outside or inside animal shelters. Sand fly specimens were identified by morphological characteristics to species level according to Theodor (1958) and Léger et al. (1983).

2.5. Statistical analysis

PROC POWER and PROC SURVEYSELECT procedures of statistical software SAS 9.4 were used for the determination of sample size and selection of samples. Epidemiological/anamnestic data of each tested dog were registered and analysed in Epiinfo and Stata 12.1. Geographical analysis was carried out using GIS software. Association between a positive diagnosis of L. *infantum* and categorical variables was evaluated by the Pearson's Chi-squared test and two-sample test of proportion, *p*-value ≤.05 being considered statistically significant. For quantitative variables, descriptive statistics was performed.

3. Results

3.1. Examined canine population

A representative, randomly-selected subpopulation of 1125 dogs was identified from a population of 10,371 eligible dogs and invited to participate in the serosurvey. An additional number of non-selected dogs (no = 26) was also included because belonging to the selected owners; for them, eligibility criteria of size and age could not be taken into account. Among the invited dogs, 129 were cancelled from the list either because the owner was unknown/unreachable, or because the dog had died or was untestable for various reasons. Hence, a sample of 997 dogs was actually invited for enrolment. The response rate by the dogs' owners was pretty high (45.8%) and a total of 457 resident dogs, coming from all over the APB territory, had their peripheral blood collected and tested for the presence of specific antibodies against *L. infantum.*

The examined population consisted of 290 dogs belonging to 66 pure breeds, and 167 to mixed breeds. Two hundred and seventeen dogs (47.5%) were male. The mean age was 7.0 yrs. (median 7 yrs.; min = 6 months; max = 18 yrs.; SD 2.3468), with a mean weight of 27.5 kg (median 27 kg; min = 1 kg; max = 80 kg, SD 11.3765). Rural dogs were the majority (278, 60.8%), whereas 95 (20.8%) and 84 (18.4%) dogs lived in suburban or urban contexts, respectively.

3.2. Seroprevalence and nested-PCR results

Sixty-three out of 457 dogs (13.8%) (95% CI: 10.8%–17.4%) tested positive by IFAT (Table 1). Twenty-five were males (39.7%), with no statistical difference between the proportion of positive animals for gender (Pearson Chi^2 = 1.7833, p = .182). Most of the positive dogs (56/63) were so at the threshold antibody titre of 1:40, however in 4 dogs with infection putatively acquired in APB the titre was higher (1:80–1:160). Three dogs with a travel history to leishmaniosis-endemic territories of central and southern Italy had a high IFAT titre of 1:1280. Among the seropositive dogs, only 5 were imported from abroad - 2 from Austria and 1 each from Bosnia, Germany and Spain. The large majority had Italian origin (58/63, 92%), and 35/63 (55.5%) were autochthonous to APB.

Leishmania n-PCR analysis could only be made in 3 dogs, whose owners consented to perform a second blood sampling after the serology results were made available. Dog 1 was an imported case with 1:1280 IFAT titre and clinical signs compatible with CanL. Both dogs 2 and 3, coming from Bolzano Health District, were clinically healthy, had an IFAT titre of 1:80 and their history was indicative of a locally-acquired infection. The molecular test was found positive in all three.

Table 1
Leishmania seroprevalence in dogs from four Health Districts of Autonomous Province of Bolzano-South Tyrol.

Health District	Total dogs tested by IFAT	Total positive (%)	Autochthonous positive (%)
Bolzano	207	35 (16.9)	20 (9.7)
Merano	124	17 (13.7)	6 (4.8)
Bressanone	54	9 (16.7)	7 (13.0)
Brunico	72	2 (2.8)	2 (2.8)
Total	457	63 (13.8)	35 (7.7)

The greatest number of tested and positive dogs were from the Health District of Bolzano, however their seroprevalence was similar to those of Bressanone and Merano Health Districts (a range of 13.7–16.9%; p = .6). In the District of Brunico only two dogs tested positive, although neither had moved from the place of residence in the past 5 years. In this District the seroprevalence (2.8%) was significantly lower than in the above Districts (e.g. vs 13.7% of Merano, p = .01).

3.3. Mapping seropositive dogs and analysis of risk factors

APB has an alpine topography, with 81/116 municipalities well over 600 m asl. An analysis was made by ranking municipality altitudes into two broad ranges, 200–600 and over 600 m asl (Table 2). Of the 63 *Leishmania* seropositive cases, 42 where resident in places belonging to the lower range, representing a seroprevalence of 16.9% among the 249 examined dogs from that range. This rate was found significantly higher than that recorded among dogs living at altitudes of over 600 m (21/208; 10.1%) (p = .037). Autochthonous seropositive dogs were from 17 municipalities (Fig. 4), belonging to Health Districts of Bolzano (4 municipalities), Merano (8), Bressanone (4) and Brunico (1). Twenty of these cases (57.1%) lived in municipalities at the altitude range of 200–600 m asl. The highest elevation at which an autochthonous seropositive dog was recorded, was 1256 m asl (Dobbiaco, Brunico Health District).

Regarding developed human settlements, there was a significantly lower proportion of seropositive subjects among dogs living in rural environments (11.1%) compared to dogs living in urban or semi-urban areas (17.9%) (p = .042).

As per study design, age classes under 4 years were excluded from the analysis. The highest number of positive cases was observed in the age group 8–9 years, and the highest proportion among the examined dogs was in the age group 13–14 years. This proportion, however, was not statistically different from the proportions calculated for the younger age groups (p = .27) (Fig. 5).

Among the following variables related to dog management that

Table 2
Altitude ranges and places of residence of examined and positive dogs.

Altitude range (meters asl)	Distribution of municipalities	Distribution of examined dogs	Municipalities with at least a positive dog	Number of positive dogs (%)
200–600	35	249	16	42 (16.9)
Over 600	81	208	19	21 (10.1)
Total	116	457	35	63 (13.8)

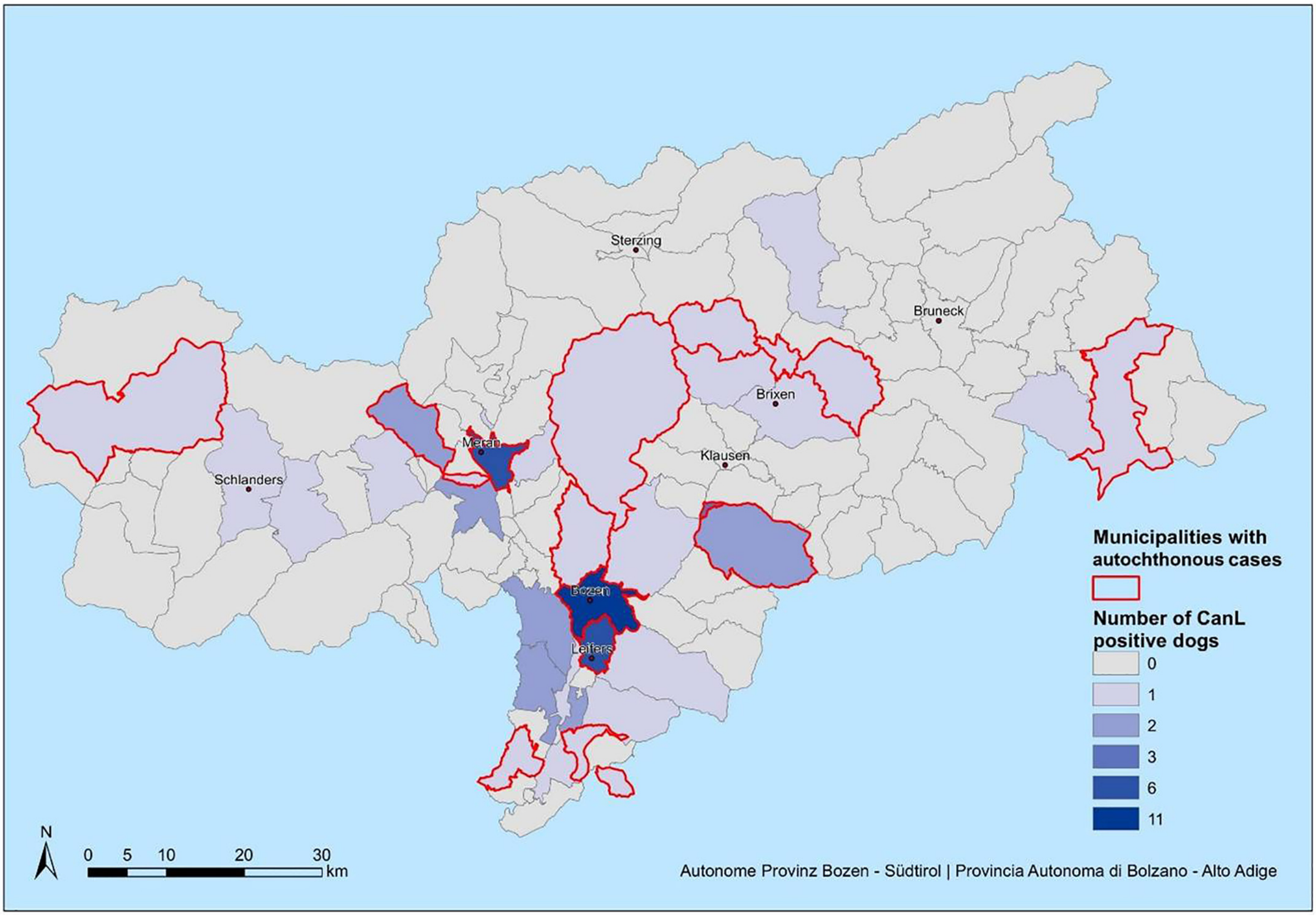

Fig. 4. Map of the Autonomous Province of Bolzano-South Tyrol showing the number of positive cases per municipality. Areas with autochthonous positive dogs are marked in red. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

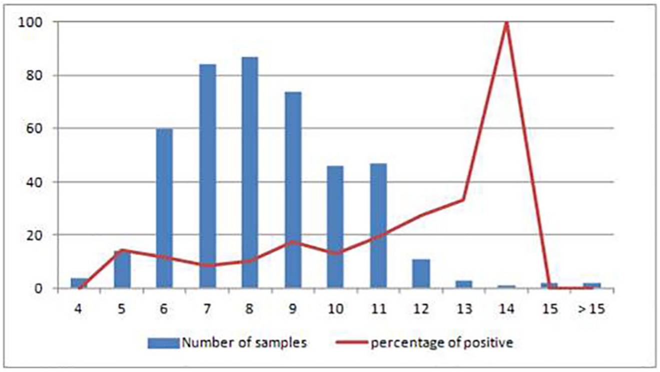

Fig. 5. Age distribution of examined dogs and respective proportion of *Leishmania* seropositives.

could lead to a higher risk of exposure to sand flies: "hunting", "living outside the house (-during the day-; -during the night-; -during day and night)", "being regularly walked in nature areas at dusk", no significant association was found with seropositive condition (data not shown).

The risk of being seropositive was evaluated for any pure breeds versus mixed breeds. Among 290 pure-breed dogs 47 were seropositive, a proportion (16.2%) significantly higher than among mixed-breed subjects (16/167; 9.6%) ($p = .048$). Of the 66 pure breeds tested, the most numerous and apparently at-risk were German shepherd (8 seropositives/33 examined; 24.2%), followed by Golden retriever (7/35; 20.0%) and Labrador retriever (15/35; 14.3%). Despite being represented by only 5 dogs, the shorthaired Segugio italiano could be considered the most at-risk breed, with 4 seropositives (80.0%). Finally, the coat type seemed not to be associated with a higher risk of infection when comparing short haired and long haired dogs ($p = .91$).

3.4. *Sand fly collections*

Thirty-one georeferenced sites were monitored for sand fly presence, whose geographical and environmental characteristics are shown in Table 3. Of them, the site of Guncina, Bolzano (no. 24) was taken as sand fly (*P. perniciosus*) positive control based on collections performed over the past decade (Morosetti et al., 2009, 2016). A total of 864 sticky traps were set starting from mid June and continuing in early August and early September (Table 4). One to 3 CDC light traps were set for 1 or 2 nights in different periods, in 6 sites. Not necessarily each site was monitored in all three periods. Furthermore, the sites found positive in summer trappings were surveyed again at the end of the sand fly season in early October. An average of 28 (5–116) traps were set per site, with a recovery rate of 97.2%.

Only 2/31 sites were found positive for phlebotomines, including the positive control site. A total of 317 specimens (23.7% males) were collected by 507 sticky traps, whereas none of the CDC light traps set in different types of animal shelters from different municipalities gave positive results. The altitudes of positive sites were 456 and 650 m asl, respectively. The specimens identified belonged to 2 species, of which *Sergentomyia minuta* was by far the most prevalent (98.1%) (Table 4). The proven competent vector of L. *infantum*, *P. perniciosus*, was only collected in Guncina. For the first time, phlebotomine specimens have

Table 3
Sites monitored for sand fly presence.

No	Site name	Altitude[a]	Latitude	Longitude	Location reference	General environment	Method[b]
1	Schlachthof Toblach	1095	46°44′05″ N	12°12′44″ E	Slaughterhouse	Rural, alpine forest	ST
2	Silvesterstr	1128	46°44′03″ N	12°13′06″ E	Hof 11	Rural, alpine forest	ST
3	Oberolang St. Franziskus	1050	46°45′17″ N	12°03′15″ E	Dog shelter	Rural, forest	LT
4	Natz Vallazza	730	46°46′34″ N	11°38′30″ E	Dog shelter	Rural, hills, fruit-tree plantations	ST
5	Aicha	870	46°46′38″ N	11°38′48″ E	Village entrance	Rural village	ST
6	Schabs Gasser	588	46°44′02″ N	11°38′35″ E	Cow farm	Rural, fruit-tree plantations	LT
7	Töll	507	46°40′58″ N	11°05′41″ E	Parking area	Rural, near main road	ST
8	Partschins-Vertigen	645	46°41′15″ N	11°04′28″ E	Village entrance	Rural, alpine forest	ST
9	Tierheim Naturns Plaus	478	46°39′42″ N	11°02′44″ E	Dog shelter	Rural, plain, apple plantations	LT
10	Burgeis/Burgisio	1236	46°42′34″ N	10°31′28″ E	Main road curve	Rural village	ST
11	Schmiedhof Mals/Malles	970	46°41′32″ N	10°32′36″ E	Cow farm	Rural	ST + LT
12	Mals/Malles a	1084	46°41′32″ N	10°32′37″ E	Planol side entrance	Rural village	ST
13	Mals/Malles b	1066	46°41′24″ N	10°32′22″ E	Carolingian Church	Rural village	ST
14	Schloss Tirol/Castel Tirolo	628	46°41′38″ N	11°08′40″ E	Hen-house	Rural, old castle	ST + LT
15	Dorf Tirol -Tirolo	610	46°41′45″ N	11°08′46″ E	Road, after a tunnel	Rural, Mediterranean forest	ST
16	Goldrain/Coldrano	650	46°37′27″ N	10°49′37″ E	Road inside village	Rural village	ST
17	Partschins/Parcines a	614	46°41′45″ N	11°08′47″ E	Romani's streat	Rural	ST
18	Partschins/Parcines b	744	46°41′10″ N	11°03′59″ E	Mill waterfall	Rural village	ST
19	St.Peter	601	46°41′39″ N	11°08′27″ E	Birds of prey	Rural, Mediterranean forest	ST
20	St. Peter - Thurnstein	566	46°41′31″ N	11°08′03″ E	Along side road	Rural, Mediterranean forest	ST
21	St.Peter a	528	46°41′31″ N	11°08′07″ E	Along side road	Rural, Mediterranean forest	ST
22	St. Peter b	474	46°41′29″ N	11°08′18″ E	Along side road	Peri-urban, cypresses	ST
23	Schloß	479	46°40′01″ N	11°11′20″ E	Labers castle	Rural, vineyards	ST
24	Guntschna/Guncina	456	46°30′40″ N	11°20′25″ E	Paved side road	Peri-urban, Mediterranean bush	ST
25	Lana Katzenthaler Hof	326	46°36′27″ N	11°09′08″ E	Road inside village	Rural village, fruit-tree	ST
26	Lana, Palade	337	46°36′46″ N	11°08′39″ E	Side road, pathway	Rural village, fruit-tree, vineyard	ST
27	Tscherms/Cermes	429	46°37′34″ N	11°08′18″ E	Road inside village	Rural village, fruit-tree, vineyard	ST
28	Kuens/Caines Hilberhof	548	46°41′49″ N	11°10′17″ E	Road inside village	Rural village, fruit-tree, vineyard	ST
29	Kuens/Caines	588	46°41′58″ N	11°10′22″ E	Road inside village	Rural village, fruit-tree, vineyard	ST
30	Maso Tarfusser	450	46°38′05″ N	11°08′19″ E	Animal[c] shelters	Rural village	LT
31	Lebenburg	508	46°38′16″ N	11°08′07″ E	Castle	Rural, hills, fruit-tree, vineyard	ST

[a] meters above sea level; [b] ST = sticky trap, LT = CDC miniature light trap; [c] pigs, donkeys, sheep, goats, hens, dogs.

been captured in the most northerly Italian site ever investigated (Coldrano, Latsch municipality, site no. 16), which was so because of a number of seropositive dogs recorded in the surrounding territory of Venosta Valley and the presence of environmental characteristics suitable for sampling (Fig. 6).

4. Discussion

The main objective of this study was the assessment of CanL seroprevalence in dogs resident in APB and the identification of locally-transmitted cases. On the one hand, for the first time we identified by serology and *Leishmania* n-PCR positive dogs native/autochthonous to APB, on the other hand we failed to demonstrate the spread, at least at high densities, of sand fly species competent for L. *infantum* transmission in places where putative endemic CanL infections have been recorded.

Both dog owners' motivation and cooperative attitude of veterinarians allowed satisfactory coverage of the study area despite the difficulty of testing a great number of rural dogs, often large-sized, not used to being transported or handled and sometimes living in remote mountain areas. This is the first serological survey for CanL in a randomised sample of dogs involving the whole APB territory, since previous monitoring activities were limited in territorial coverage and had different study design (Morosetti et al., 2009, 2016). By comparing with other endemic settings of southern Europe, the overall CanL seroprevalence of 13.8% can be considered as moderately high although it drops by half when only autochthonous dogs are considered. Importantly, this rate seems to have increased rapidly over a decade as it was estimated to be around 2.2% in 2009 based on a limited sample of APB dogs (Morosetti et al., 2016). In a review of data from 377 CanL surveys involving 424,000 Italian dogs mostly from central and southern territories, a median *Leishmania* seroprevalence of 17.7% (with peaks above 80%) was calculated over 4 decades (Franco et al., 2011). CanL seroprevalence in APB is certainly not comparable with recent figures from highly endemic regions of southern Italy (e.g. > 50% reported by Foglia Manzillo et al., 2018), however it still indicates that a certain number of dogs can be considered exposed locally to *Leishmania* (Paltrinieri et al., 2010). A seroprevalence rate is not sufficient per se to represent the endemic status of CanL in a territory, as also the level of specific antibodies measured by a quantitative

Table 4
Periods and density of sand fly species collected by sticky traps in sites detected positive by summer trappings.

N°	Site name	Date	*P. perniciosus*		*S. minuta*		Total
		(days/month)	(% M)	density (m^2 trap)	(% M)	density (m^2 trap)	(% M)
24	Guntschna/Guncina	2–4/08	4 (75.0)	1.0	110 (30.0)	27.8	114 (31.6)
24	Guntschna/Guncina	4–6/09	2 (50.0)	0.5	196 (19.4)	49.5	198 (19.7)
16	Goldrain/Coldrano	4–6/09	0 (0.0)	0.0	4 (0.0)	1.0	4 (0.0)
24	Guntschna/Guncina	1–3/10	0 (0.0)	0.0	1 (0.0)	0.2	1 (0.0)
16	Goldrain/Coldrano	1–3/10	0 (0.0)	0.0	0 (0.0)	0.0	0 (0.0)
	Overtotal		6 (66.7)	0.3	311 (22.8)	15.3	317 (23.7)

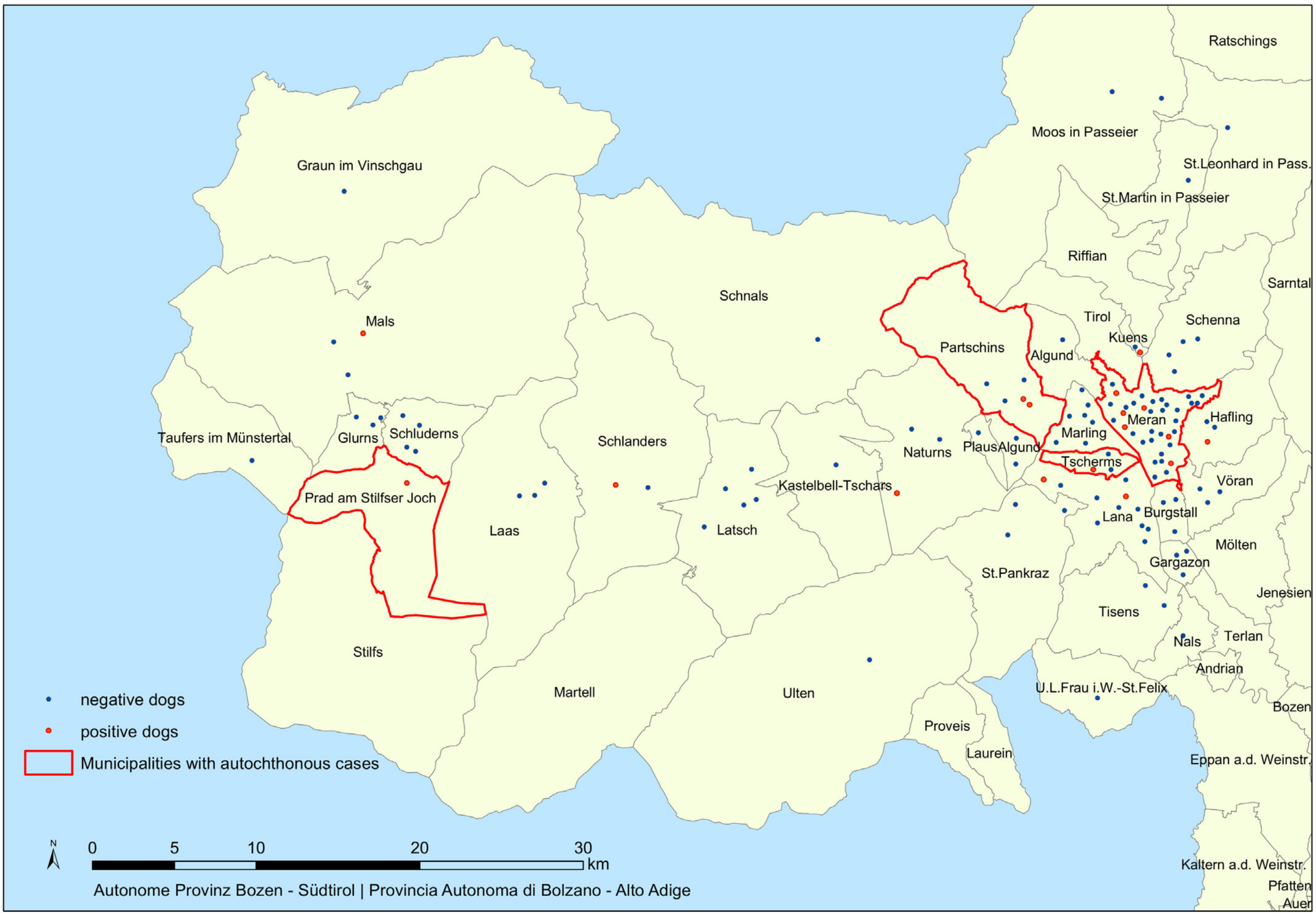

Fig. 6. Distribution of dogs tested serologically for *Leishmania* and positives recorded in the northwestern part of the Autonomous Province of Bolzano-South Tyrol (Merano area and Venosta Valley).

method such as IFAT represents an important parameter. Most of the APB positive dogs showed an antibody titre at the threshold level of 1:40, suggesting a low degree of transmission/contacts. Indeed, when dogs are exposed to elevated forces of *Leishmania* infection (namely, frequent infective sand fly bites) IFAT titres tend to be elevated too in a high proportion of dogs (Oliva et al., 2006; Kostalova et al., 2015). Several autochthonous cases were from locations where neither CanL nor sand fly presence had ever been suspected or documented. On the other hand, there were 3 autochthonous dogs with moderate IFAT titre (1:80–1:160) living in the Bolzano Health District, where imported clinical cases of CanL have become common and entomological surveys shown the presence of *P. perniciosus* (Morosetti et al., 2009, 2016). A fourth dog with moderate IFAT titre originated from the small municipality of Parcines (626 m asl) in the Merano Health District, suggesting the possibility of a local focus of CanL transmission despite sand fly trapping returned no results.

Because of the northward spread and increase in density of competent phlebotomine vectors, the neighbouring Italian regions in the south and east of APB have all reported autochthonous CanL, with moderate seroprevalence among clinically-healthy canine populations (Cassini et al., 2007; Maroli et al., 2008). Hence, APB is geographically at the very limit of this northeastern expansion which at present seems to be interrupted by the Alpine borders. This endemic situation, incidentally, is quite different from northwestern Italy at the borders with France, where Ferroglio et al. (2018) found an overall Western-blot seroprevalence of 42.2% among autochthonous dogs. Northern neighbours of APB, Austria and Switzerland, are still considered non-endemic for CanL, with infections acquired abroad or, in rare cases, by vertical transmission. The epidemiological situation might change rapidly, however, since phlebotomine sand fly presence has been recorded, infected dogs have been introduced and individual canine and human cases have occasionally been described in this part of continental Europe, including Germany (Mencke, 2011). *P. perniciosus* was described in southern Switzerland, Canton of Ticino (Knechtli and Jenni, 1990) and in southern Germany (Naucke et al., 2008). In Austria only *P. mascittii,* a *Phlebotomus* (*Transphlebotomus*) species unproven to transmit *Leishmania*, has been detected in several sites (Poeppl et al., 2013).

The increasing numbers of travelling dogs from *Leishmania*-endemic to free areas has brought changes in the epidemiology of CanL, with rising incidence of the infection in non-endemic countries (Mettler et al., 2005; Mencke, 2011; Maia and Cardoso, 2015). This is of particular concern for the EU authorities, whose objective is to mitigate the probability of introduction of the infection into free territories in the EU through movements of infected dogs (Espejo et al., 2015; EFSA AHAW Panel, 2015). It should be noted that although sand flies are the main mode of CanL transmission, alternative vertical and horizontal routes should not be excluded (Karkamo et al., 2014; EFSA AHAW Panel, 2015; Naucke et al., 2016; Svobodova et al., 2017).

The relatively low number of infected dogs and clinical cases does not allow any hypothesis regarding genetic susceptibility to *Leishmania* and dog breeds prevalent in APB (Solano-Gallego et al., 2000; Baneth et al., 2008; De Vasconcelos et al., 2019). We were able to confirm, however, that pure breed dogs showed a significantly higher seroprevalence than mixed breeds (França-Silva et al., 2003). Other risk factors related to intrinsic (e.g. age, sex or hair length) and behavioural characteristics (e.g. daily dog's management) were analysed, however none of them was found correlated positively or negatively with seroprevalence. On the other hand, the canine residence in human settlements was statistically significant in discriminating between high-rate seropositive dogs resident in urban and peri-urban settlements, and low-rate seropositive dogs living in rural/natural environments. A similar difference was recently reported by Ferroglio et al. (2018) between a context of urbanised and agricultural hilly areas, and natural mountainous areas in Piedmont. Dog population density in APB urbanised areas is usually high; for example, at the time of the survey in Bolzano city there were 6983 dogs for 107,542 inhabitants (6.5 dogs per 100 persons) in an area of 52.3 km^2 (Official Provincial Dog Register; ASTAT, 2018), so that in presence of an active CanL focus, a greater proportion of healthy dogs might be exposed to potential infection transmission. It was also found that altitude of the place of residence correlated negatively with seroprevalence, higher rates being recorded in the lower range of 200–600 m asl. In APB the overall seroprevalence value of 13.7% was very similar to that found by Santi et al. (2014) in the Emilia-Romagna region of northern Italy, in an altitude range of 200–600 m a.s.l. (13.6%). Mollicone et al. (2003) also found in a survey in Bologna province that the majority of positive dogs lived in hilly environments, as compared with mountainous or plain areas. It is long established that the reasons for this eco-epidemiological feature are to be found in the higher density of phlebotomine sand flies at the hill-level altitudes (Rioux et al., 1980).

It is very likely that infected dogs are the main cause of the establishment of CanL in APB; dogs with overt clinical disease and, to a lesser extent, sub-clinically infected dogs are considered the main reservoir of *L.infantum* (Quinnell and Courtenay, 2009). Other domestic or wild animals, however, were demonstrated or may have potential role as reservoir for the parasite (Millan et al., 2014; Maia et al., 2018). Besides cats, synantropic rodents (e.g. *Rattus rattus*) and foxes (*Vulpes vulpes*) for which infectiousness to competent vectors and L. *infantum* transmissibility was demonstrated, other mammals common in APB natural environments such as leporids (*Lepus timidus* and *Lepus europaeus*), martens (*Martes* spp.), badgers (*Meles meles*), weasels (*Mustela* sp.) and wolves (*Canis lupus*) could all potentially represent permanent parasite sources for sand flies. The role of these wild animals in the epidemiology of the disease in the study area and elsewhere must however be further clarified.

The entomological survey confirmed the stable presence of the competent *Leishmania* vector *P. perniciosus*, which was demonstrated to be endemic in the Bolzano area since 2008 (Morosetti et al., 2009). *S. minuta*, however, was the most abundant sand fly species recorded; though this species was never demonstrated to be a competent vector of *L.infantum*, it could be considered as a good ecological indicator for the identification of habitats suitable to phlebotomine sand flies establishment and breeding. By analyzing the total number of specimens collected in APB in the past decade, an almost tenfold increase in sand fly density was evident, ranging from 0.016 specimens/m^2 of sticky trap in 2008, to 0.092 in 2019. A contribution to this increase was given mostly by *S. minuta* in the present survey. It should be noted, however, that at the investigated latitudes, the seasonal dynamic of *P. perniciosus* should result in density peaking during July (Bulent et al., 2016), but the control site of Guncina was not monitored during this month. For the first time the presence phlebotomine sand flies has been recorded in a site of Venosta Valley. Overall, results from this study indicate that natural *Leishmania* transmission by sand flies cannot be excluded in APB and medical professionals should be aware of the local epidemiological situation, include zoonotic visceral leishmaniasis in their differential diagnosis and work closely with veterinarians when collecting information on suspected human cases. Furthermore, early diagnosis and treatment of CanL cases, both imported and autochthonous, are important to prevent expansion of the disease. The potential spread of the infection to CanL-free areas with recorded sand fly presence should be reduced by mitigation measures, principally by the combined use of canine topical pyrethroids and *Leishmania* vaccines (Bongiorno et al., 2013; EFSA AHAW Panel, 2015; Mirò et al., 2017).

5. Conclusions

To our knowledge, the present study gives the first evidence of *Leishmania* infections in dogs with no recent history of travel outside APB. It also documented the northernmost collection of phlebotomines (*S. minuta*) in Italy. Hence, APB could be considered a territory of low endemicity, however the zoonotic situation needs continuous monitoring to ascertain any changes in the epidemiological status. Raising awareness in the population concerning prevention of the disease appears important at this stage.

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Ethical statement

Dog owners participated voluntarily to the screening of their dogs. Blood sampling of dogs was not an invasive procedure and it was performed by veterinary practitioners.

Declaration of Competing Interest

None.

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