Asymptomatic *Leishmania infantum* infections in humans living in endemic and non-endemic areas of Croatia, 2007 to 2009

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The prevalence of asymptomatic leishmaniasis in the general population of Croatia has not been studied to date. To assess the prevalence of *Leishmania infantum* specific IgG antibodies among immunocompetent residents of Croatia, sera from 2,035 persons (eastern coast of Adriatic Sea, n=1,186; Adriatic islands, n=653; mainland, n=196), were tested by an enzyme immunoassay. A total of 231 (11.4%) persons had anti-*Leishmania* antibodies. Multivariate analysis revealed that seropositivity was associated with geographic location and age. Residents of coastal areas and islands were significantly more seropositive than mainland residents (odds ratios (OR) 20.37 to 28.51). Moderate to high anti-*Leishmania* seroprevalence was found throughout the eastern Adriatic coast and islands (4.0% to 22.2%) including the sites previously considered non-endemic. A highly endemic focus was identified in central coastal Dalmatia (seroprevalence 22.2%; OR: 1.72; 95% confidence interval (CI): 1.33-2.22). Regarding age, children aged 0-9 years were the most vulnerable group for asymptomatic *Leishmania* infection (OR: 2.19; 95% CI: 1.16-4.14).

Introduction

Leishmaniasis is caused by *Leishmania* spp., hemoflagellate protozoa belonging to the family Trypanosomatidae. Infected phlebotomine sandflies serve as vectors for the transmission of all *Leishmania* species. Human leishmaniasis can be divided into three main disease manifestations: (i) visceral leishmaniasis (VL), (ii) cutaneous leishmaniasis (CL) and (iii) mucocutaneous leishmaniasis (ML). The strain of the infecting organism and the host's immunologic status greatly influence clinical manifestations.

Zoonotic VL caused by *Leishmania infantum* is endemic in Mediterranean countries of Europe and domestic dog is the main reservoir [1-3]. During the last decade, there have been reports of spreading leishmaniasis northward into previously non-endemic areas of central and northern Europe [1-3]. It is well known that, besides clinical cases of VL, asymptomatic infections are common in endemic areas [2,3].

Croatia encompasses 56,538 km2. According to the 2011 census, it had a population of 4,284,889 [4]. Geographically, Croatia is composed of three areas: the Adriatic coastal zone with islands in the south, the Pannonian Plain in the north, and the mountainous region in-between. The Croatian littoral comprises a relatively narrow land belt with islands along the eastern coast of the Adriatic Sea (Figure). The littoral is traditionally divided into two large areas based on geography, ecology and cultural heritage: the northern (Istria and Primorje counties) and the southern part (Dalmatia) which is further subdivided into northern, central and southern Dalmatia. The Adriatic islands and the coastal zone are characterised by Mediterranean climate which provides good living conditions for sandfly vectors of *Leishmania* [5,6]. Continental Croatia has temperate continental or continental climate and is usually considered free of phlebotomine vectors of *Leishmania*.

Human VL and canine VL has been reported in central and southern coastal and insular Dalmatia (from Split to Dubrovnik) since 1930 [7]. From 1931 to 1957, 398 human VL cases were diagnosed in this region [8]. A case of CL was firstly recorded in 1945 and 201 CL cases were recorded by 1957. After the late 1950s, the number of VL cases declined, probably because of mass spraying with antimalarial insecticides [8]. Since 1990, studies have identified re-emerging foci of both human and canine VL in central and southern Dalmatia [9-11].

In Croatia notification is compulsory for both VL and CL, although these diseases are not included in the decisions of the European Parliament for reporting communicable diseases [12, 13]. Medical practitioners are notifying human leishmaniasis cases through

Croatia's health information system or directly to the epidemiologist in the regional public health institutes. Cases are defined as *probable* which is a case clinically compatible in endemic region or with epidemiological connection to a *confirmed* case, and confirmed that is a case laboratory-confirmed by positive parasitological (microscopy or cultivation) and serological (IFA, ELISA) tests. Case definitions for CL are similar, excluding the serological tests [13]. According to data periodically published by the Croatian National Institute for Public Health there were between one and four new cases of VL reported each year in Croatia in the last decade [14]. The estimated mean annual incidence of human leishmaniasis is 0.4 per 100,000 population [5]. Leishmaniasis in Croatia is described as predominately paediatric disease: almost half of VL patients are children up to the age of 10 years and the disease is more often found in men [9,10]. Most of the reported cases occurred among inhabitants of the Croatian coast and islands [9,10]. Besides this, few VL cases were diagnosed in Austrian [15] and Hungarian [16] tourists after returning from the Dalmatian littoral.

Recently, three *Phlebotomus* species, known to serve as vectors for *L. infantum* were found in central and southern coastal and insular Dalmatia [5]. A veterinary seroepidemiologic survey conducted in central Dalmatia among apparently healthy dogs using dot-ELISA, found canine seroprevalence ranging from zero to 42.85%, which was in accordance with previous recognition of central and southern coastal and insular Dalmatia as a high endemic foci of *L. infantum* for dogs [11]. In contrast to high seroprevalence ratios in dogs, no information about the prevalence of the infection in otherwise healthy human inhabitants of different Croatian regions is available.

The aim of this study was to assess the prevalence of IgG antibodies to *L. infantum* among healthy people living in different regions of Croatia, and to compare the seroprevalence in endemic regions with that in non-endemic regions. This is the first investigation on asymptomatic leishmaniasis in residents of Croatia.

Methods

The target population for our study was the apparently healthy general population in previously recognised endemic and non-endemic areas for leishmaniasis in Croatia. The studied areas known as endemic areas: insular Croatia, and central and southern Dalmatia, as well as areas previously considered nonendemic but with favourable Medieranean climate: northern Dalmatia, Istria and Primorje. Two counties in northern Croatia with continental climate where sandflies were considered rare or absent: Brod-Posavina County (centre Slavonski Brod) and the most north-western Međimurje County (centre Čakovec), were also included.

Serological survey

Serum samples were collected from 2007 to 2009 to examine the prevalence of IgG antibodies to *L. infantum* in Croatia. We used 'residual' sera of apparently healthy, immunocompetent individuals who did not show any symptoms of leishmaniasis and came to hospitals or clinics for routine laboratory check-ups or for blood donation. Participating hospitals' or clinics' laboratories were selected according to their geographic location: 14 served population along the Adriatic coast and two were from non-endemic parts of Croatia. Laboratories provided data on age, sex and site of residence for the study participants.

Table 1

Sex distribution in the study population (n=2,035) compared with that of the total Croatian population

	Individuals tested	Male	Percentage (%)	(95% CI)		Census population	Male	Percentage (%)
All								
Adriatic coast	1,186	571	48.15	45.30%	50.99%	1,247,133	605,605	48.56
Adriatic islands	653	268	41.04	37.27%	44.81%	113,875	56,658	49.75
Croatian mainland[a]	196	137	69.90	63.48%	76.32%	2,923,881	1,404,072	48.02
Region/Site of residence								
Adriatic coast								
Istria and Primorje	117	47	40.20	(31.32%	49.08%)	467,678	226,115	48.35
Nothern Dalmatia	159	84	52.80	(45.04%	60.56%)	255,158	124,849	48.93
Central Dalmatia	571	293	51.30	(47.20%	55.40%)	419,131	203,513	48.56
Southern Dalmatia	339	147	43.40	(38.12%	48.68%)	105,166	51,128	48.62
Croatian mainland								
Brod-Posavina county[a]	107	96	89.70	(84.0%	95.5%)	158,575	77,115	48.60
Međimurje county	89	41	46.10	(35.7%	56.4%)	113,804	55,601	48.90

CI: confidence interval.

[a] Healthy donor effect bias present in Brod-Posavina county as blood donors constituted the majority of the sample.

To determine the target sample size for each region we used published statistical tables, which provide minimal sample sizes that are necessary for given combinations of precision, confidence levels, and variability.

A minimum of 1,100 individuals were selected as the target sample size for the coastal region with previously recognised endemic foci in order to reach the precision level of ±3% under assumption of the confidence level of 95%, p=0.5 and the size of population greater than 100,000 inhabitants. For islands, the targeted precision on population with more than 100,000 inhabitants was ±5% resulting in a minimum sample size of 400, whereas for the reference non-endemic continental region, under the assumption of maximum seroprevalence of 2%, the targeted sample size was 84 to reach the ±3% precision level.

Convenience sampling was used to select respondents in the most economically, technically, and operationally feasible method [17].

To estimate whether the study sample represents the research setting population, we compared the percentage of men estimated from the study sample with the 2011 census data (Table 1).

We classified sites based on their seroprevalence as 'high endemic focus' and 'moderate seroprevalence'. We derived these definitions combining the data from the literature with the results of this study. In particular, in a study by Federico et al. a seroprevalence of *L. infantum* infection of 4% in the Rome and Caltanissetta area was marked as 'moderate' [18] whereas Marty et al. detected a seroprevalence of 38% in the high endemic focus of Alpes-Maritimes, using Western blot technique, which is more sensitive than the technique used in this study [19]. The seroprevalence rate in the majority of our coastal areas was around 7% and defined as 'moderate seroprevalence'. The one area with a seroprevalence of 22% had a significantly higher seroprevalence in comparison with surrounding areas, all of which were classified as 'moderate seroprevalence'. Therefore, that site was defined as a 'high endemic focus' and the definition was further supported by epidemiological data since the majority of cases of VL in Croatia are reported from 'high endemic foci' [5,9-11].

Sera were tested for the presence of IgG antibodies to *L. infantum* by commercial enzyme-linked immunosorbent assay (NovaLisa *Leishmania infantum* IgG, NovaTec Immunodiagnostica GmbH, Dietzenbach, Germany), according the manufacturer's instructions. Declared specificity and sensitivity of the test were 85% and 91% respectively. A serum was considered positive when the ratio between the optical density (OD450) value of the serum and the cut-off was >1.1.

The study was approved by the ethics committees of the Split University School of Medicine and of the Split University Hospital. Individual informed consent was not required according to the ethical committee.

Statistical analyses

Data were analysed with statistical package SPSS 19.0 (SPSS; Chicago, Illinois, US). Associations of seropositivity with sex or geographic region were tested by Pearson's chi-square test whereas the significance of difference in median age of participants by seropositivity was tested by Mann-Whitney U test. The strength of association between seropositivity and the site of residence was estimated by odds ratio (OR), 95% CI and p-values. For each site observed seroprevalence was compared to unweighted average rate in the accompanying region (i.e. coastal or continental) and ORs were calculated from the standard 2x2 table.

A multiple logistic regression analysis was used to evaluate the potential risk factors associated with *Leishmania* infection, including age as categorical variable, sex, and region of residence. Significance level was set at 0.05. In case of multiple testing, we adjusted the p-values with the Bonferroni correction.

Results

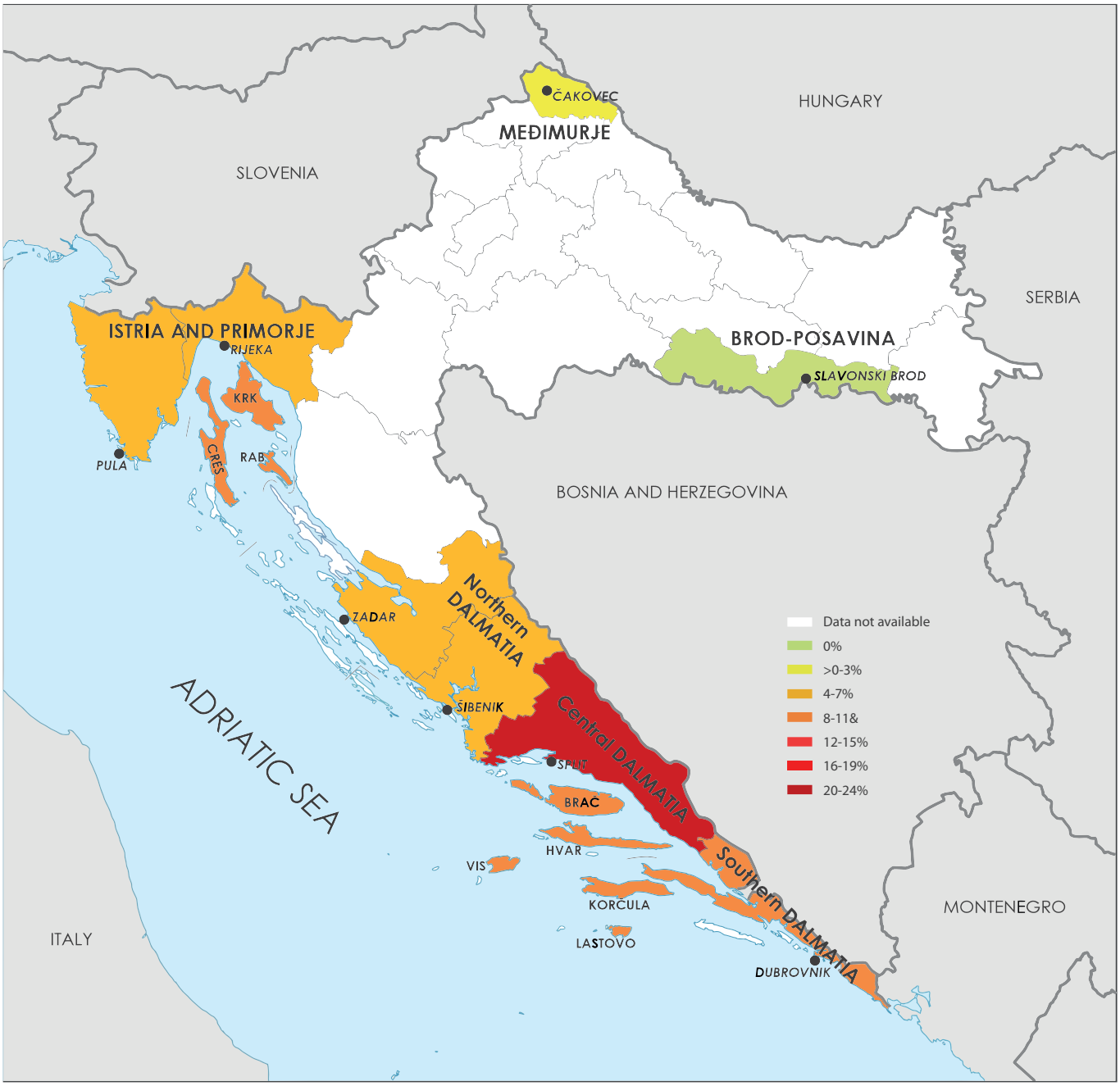
Blood specimens from 2,035 persons (975 men and 1,060 women) were collected. These included 1,186 persons in the coastal area from Istria to the Montenegro border, 653 on Adriatic islands and, for comparison, 196 residents of two northern continental Croatian counties (Table 2). The survey enrolled persons from all age groups, ranging from eight months to 88 years, with the median of 42 years (interquartile range (IQR): 21-59). The large deviation from the census data was observed only in the reference site in Brod-Posavina County, in which blood donors were overrepresented.

Of the total of 2,035 sera of healthy residents, anti-*L. infantum* IgG antibodies were found in 231 (11.4%). Seroprevalence differed significantly among Croatian sites ranging from 0.0% in Brod-Posavina County to 22.2% in central Dalmatia ($x2=112.24$; $df=13$; $P<0.001$) (Figure, Table 1). Overall, according to the place of residence, we found a strong association between seropositivity and residence in island, coast, or continental areas ($x2=35.41$; $df=2$; $P<0.001$). Post hoc chi-square analysis revealed that inhabitants of coastal areas had significantly higher seroprevalence than islanders ($x2=9.27$; $df=1$; $Pcorr=0.007$) or those from the two continental regions ($x2=29.43$; $df=1$; $Pcorr<0.001$). Furthermore, islanders had significantly higher seroprevalence than continental inhabitants ($x2=17.37$; $df=1$; $Pcorr<0.001$).

To analyse the spatial distribution pattern of *Leishmania* infection in more detail, we estimated strength of associations of seropositivity with the site of residence in particular regions: coastal, continental, or islands (Table 3). Only in Adriatic coastal counties seroprevalence of sites differed significantly from the average regional rate. While central Dalmatia inhabitants had

Figure

Geographic distribution of seroprevalence for anti-*Leishmania* IgG in asymptomatic healthy individuals, Croatia, 2007–2009 (n=2,035)



the highest prevalence of antibodies and the highest risk for *Leishmania* infection (OR: 1.72; 95% CI: 1.33 to 2.22; p<0.001), residents of the rest of coastal areas had lower risk (OR: 0.27 to 0.52; p-value: 0.002 to 0.006) as compared to the average rate in the coastal region. Although these results clearly indicated central Dalmatia as a high endemic focus for *Leishmania* infection, the observed seroprevalence in other coastal areas (ranging from 4.3% to 8.0%) also indicated these areas as endemic sites where the seroprevalence was higher than in continental Croatia.

Regarding sex and age as potential risk factors, we found no association of seroprevalence with sex (x2=0.11; df=1; p=0.739). Among 975 men, antibodies were found in 113 (11.6%) sera, while among 1,060 women, antibodies were found in 118 (11.1%) sera. This finding was further confirmed by the multivariate model with seropositivity as dependent, and age, sex and geographic location as independent variables. Multivariate logistic regression analysis of *Leishmania* seropositivity in association with age, sex and geographic location is shown in Table 4.

In contrast to sex, we found significant differences in the median age of seropositive and seronegative persons: 40 years (IQR: 16-58), and 42 years (IQR: 22-60), respectively (Mann-Whitney U test, p=0.039). This indicated age as a risk factor for *Leishmania* infection. Additionally, in the multivariate model with age, sex and geographical region as covariates, age was a significant predictor of seropositivity (overall significance p=0.022). The rates of seroprevalence for each age group of participants as well as the associated OR with 95% CI adjusted for covariates are shown in Table 4. Results show that *Leishmania* seropositives are most likely in 0 to 9 year-olds (17.5%; OR: 2.19; 95% CI: 1.16–4.14). It is noted that anti-*Leishmania* antibodies were found in nine of a subgroup of 71 children under the age of four, including one girl aged one year old. The data also show that *Leishmania* seropositivity does not continuously change with age. Instead, a bimodal distribution is indicated with comparable high risk of asymptomatic infection in those aged 0 to 9, 10 to 19 and 40 to 49 years (OR: 1.84 to 2.19; all p-values <0.05). In contrast, people of all other age groups had similar risk for *Leishmania* infection to those in the 30 to 39-year-old reference age group (OR: 1.00 to 1.33; p-values: 0.374 to 0.992).

Discussion

To our knowledge, this is the first study of the prevalence of asymptomatic *Leishmania* infection in the general population of Croatia. In order to determine the *Leishmania* infection distribution, residents of various geographical and ecological areas were included in a serological survey. The findings of this study reveal a strong association of seropositivity with geographic region (east Adriatic coastal and islands areas) and age group. The presence of seropositive people in northwestern coastal and island regions (Istria and

Table 2

Age and sex and seropositivity for anti-*Leishmania* IgG of study population by area of residence, Croatia, 2007–2009 (n=2,035)

	Number of sera	Participant age groups (years)							Information on age missing	Age range	Male (%)	Seropositive (%)
		0-9	10-19	20-29	30-39	40-49	50-59	60-69	>70				
Total	**2,035**	**177**	**272**	**250**	**246**	**242**	**315**	**205**	**290**	**38**	**0 - 88**	**975 (47.9)**	**231 (11.4)**
Region/Site of residence													
Adriatic coast	1,186	149	206	133	131	146	189	97	107	28	0 - 86	569 (48.0)	169 (14.2)
Istria and Primorje	117	12	13	11	27	17	17	10	10	0	0-83	47 (40.2)	5 (4.3)
Northern Dalmatia	159	10	29	19	15	21	36	15	14	0	3-84	84 (52.8)	10 (6.3)
Central Dalmatia	571	112	117	74	59	63	67	31	33	15	0-82	293 (51.3)	127 (22.2)
Southern Dalmatia	339	15	47	29	30	45	68	42	50	13	0-86	145 (42.8)	27 (8.0)
Adriatic islands	653	17	41	70	61	69	110	97	181	7	1-88	268 (41.0)	61 (9.3)
Croatian mainland	196	11	25	47	54	27	17	10	2	3	0 -72	137 (69.9)	1 (0.5)
Brod-Posavina county	107	0	12	25	34	19	10	4	0	3	18-62	96 (89.7)	0 (0.0)
Međimurje county	89	11	13	22	20	8	7	6	2	0	0 -72	41 (46.1)	1 (1.1)

Table 3

Seroprevalence of anti-*Leishmania* IgG by residence compared with unweighted average in the associated region, Croatia, 2007–2009, (n=2,035)

	p-value	OR	95% CI
Region/Site of residence			
Adriatic coast	Reference: seropositivity 14% (95% CI, 12-16%)		
Istria and Primorje	0.002[a]	0.27	0.11-0.67
Northern Dalmatia	0.006[a]	0.4	0.21-0.78
Central Dalmatia	<0.001[a]	1.72	1.33-2.22
Southern Dalmatia	0.002[a]	0.52	0.34-0.80
Adriatic islands	[b]seropositivity 9% (8-11%)		
Croatian mainland	Reference: seropositivity 0.2% (0.1-0.9%)		
Brod-Posavina county	0.758	0	NA
Međimurje county	0.849	2.22	0.14-35.83

CI: confidence interval; NA: not applicable; OR: odds ratio.

[a] Significant at level 0.01.

[b] Site samples were too small for reliable comparison.

Primorje) where cases of VL have not been reported yet, suggests that *Leishmania* transmission toward north may have occurred.

A highly endemic focus in central Dalmatia was confirmed in accordance with previous reports of both canine and human leishmaniasis in central and southern Dalmatia's coast and islands [5, 7-11]. However, the northwestern part of the Adriatic littoral, including Istria, Primorje, and northern Dalmatia, was considered a non-endemic region [5, 7-11]. In 2002, a case of VL was diagnosed in a patient who had no history of travel to known endemic regions and apparently had contracted the infection during his stay on the Velebit Mountain in northern Dalmatia [20]. Our results confirm this observation as they indicate that asymptomatic *Leishmania* infection is found throughout the eastern Adriatic coast and islands. Depending on the geographical location, a moderate to high (4%-22%) prevalence of asymptomatic infection has been observed.

Data from seroepidemiological studies conducted in other Mediterranean countries have also shown a variable prevalence (from 0.5 to 56%) of *Leishmania* infections depending on the geographic regions studied and on the test used for detection [3,21]. In our study we used a commercial ELISA as a relatively simple method for testing a large number of sera. Convenience sampling was used for selecting respondents. Despite some of its limitations, in a study by Kelly et al. it was shown that a convenience sample of sera from diagnostic laboratories was an appropriate sampling strategy to provide population immunity data to inform country's health policies [17]. Although the healthy donor effect bias was present in Brod-Posavina County, blood donors represented only a minority in other sites and are not expected to have influenced results.

The highest prevalence of 22.2%, significantly higher than in other coastal zones, was found in central Dalmatia. In comparison with other coastal areas, central Dalmatia has a high background of canine leishmaniasis and sandflies [5,6,11] which are likely to be associated with high prevalence of asymptomatic *Leishmania* infection among residents in this area. Central Dalmatia is also an active focus with the highest number of human VL cases in Croatia [9,10,14]. Most of VL cases diagnosed in Croatia during the study period from 2007 to 2009 occurred in central Dalmatia: seven cases were diagnosed with a mean annual incidence of 0.5 per 100,000 population (unpublished data). In southern Dalmatia, *Leishmania* exposure was higher than expected (8% seroprevalence), despite the lack or small number of clinical cases and apparently lower risk of infection than observed for central Dalmatia.

As expected, the seroprevalence was significantly lower in residents of continental areas of Croatia; in fact all but one person (a 40 year-old woman) were seronegative. As these regions are not considered to be endemic for sandflies, we cannot exclude the possibility that the one seropositive person could have been infected while traveling to the Croatian littoral where people from continental areas often spend their summer vacations.

The same possibility of travel-acquired infection with consecutive seroconversion has to be taken in account when the seemingly northward spread of asymptomatic leishmaniasis is interpreted. One could also speculate that autochthonous *Leishmania* infection may occur due to the observed spread of sandflies to

Table 4

Seroprevalence of anti-*Leishmania* IgG by age, sex and region of residence, Croatia, 2007–2009 (n=2,035)

Age group (years)	Sera tested	IgG positive (%)	p value	OR	95% CI
0-9	177	31 (17.5)	0.016[a]	2.19	(1.16-4.14)
10-19	272	39 (14.3)	0.048[a]	1.84	(1.01-3.38)
20-29	250	18 (7.2)	0.992	1.00	(0.50-2.01)
30-39	246	17 (6.9)		Reference	
40-49	242	37 (15.3)	0.015[a]	2.13	(1.16-3.93)
50-59	315	30 (9.5)	0.621	1.17	(0.63-2.19)
60-69	205	19 (9.3)	0.631	1.18	(0.60-2.36)
>70	290	30 (10.3)	0.374	1.33	(0.71-2.51)
Sex					
Men	975	113 (11.6)	0.61	1.08	(0.81-1.44)
Women	1,060	118 (11.1)		Reference	
Region					
Adriatic islands	653	61 (9.3)	0.003[a]	20.37	(2.78-149.2)
Adriatic coast	1,186	169 (14.2)	0.001[a]	28.51	(3.95-205.79)
Continental sites	196	1 (0.5)		Reference	

CI: confidence interval; OR: odds ratio.

[a] Significant at level p<0.05.

some regions at the North of Croatia [1,2], especially in light of the recent finding of *Phlebotomus* spp. in southern Hungary near the Croatian border [16].

In agreement with other reports [21-24] our study shows that there is no difference in the prevalence of asymptomatic *Leishmania* infection between men and women.

In respect to age, our prevalence results differ from findings in other studies. In the present study a bimodal distribution of *Leishmania* seropositivity by age, with peaks in young (0-19 years) and middle-aged (40-49 years) population, was suggested for asymptomatic population. Several studies reported the age distribution of seroprevalence to *Leishmania* in a healthy human population so far. The authors mainly noted a higher prevalence in older people suggesting that susceptibility to *L. infantum* infection increases with age [21-23] or they claimed no association with age [24,25]. Furthermore, Davies et al. suggested seroprevalence drops rapidly with age [26]. In some of these studies [21,23] age groups under 18 years were not included and/or samples were not equally distributed by age, resulting in different precision of seroprevalence estimate between different age groups. A study in Brazil, using a non-commercial ELISA found that 28.5% sera of 638 tested children aged between 0 and 5 years were positive, and concluded that infection is associated with the age of ≥2 years [27]. In our study, seropositives were most likely to be aged 0 to 9 years. Since in Croatia VL is still predominantly paediatric disease [6,7] it cannot be excluded that some of the seropositives in this age group might become symptomatic, therefore further differently designed studies with follow up of such participants are needed.

It can be concluded that compared to other parts of Croatia, seroprevalence is significantly higher in central and southern coastal as well as insular areas. This indicates the presence of asymptomatic *L. infantum* infection in humans and confirms the endemicity of these areas. This finding may be of particular importance in light of the increasing popularity of the Croatian coast and islands, from Istria to Dubrovnik, as a holiday destination for travellers from *Leishmania*-free areas or countries. Our data should alert physicians to consider leishmaniasis in the differential diagnosis of conditions such as unexplained febrile illness especially in immunocompromised subjects returning from these endemic areas. In addition, seropositivity observed in non-endemic areas and the higher seroprevalence in children should be investigated in the future. Therefore, further studies including clinical, parasitological, epidemiological and entomological investigation are required for elucidating the cycle of transmission, the maintenance and the role of *Leishmania* in human and animal health in different Croatian regions.

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