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Prevalence and Factors Related to *Leishmania infantum* Infection in Healthy Horses (*Equus caballus*) from Eastern Spain

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Simple Summary: Leishmaniosis is a zoonotic disease transmitted by the sandflies. The main reservoir is the dog, although the number of species involved is increasing. The horses, due to high level contact with humans and its ability to control the disease, could be a silent reservoir. However, the data related to prevalence of *Leishmania* spp. infection in horses are scarce, especially in Europe. In this study, the prevalence and factors related *to L. infantum* infection in apparently healthy horses has been studied. Results indicate that the equine prevalence is elevated, and the main factors related to infection were equine breed, outdoor living, use and season. Horses with calm temperament and living outdoor conditions have more prevalence of infection, and the number of positive animals increase in spring, when the mean of temperature is higher. These results indicate that horses could be a silent reservoir of the parasite and that the increase in temperature due to climate change will probably increase the Leishmania spp. infections in all species, including humans, in the future. From a One Health perspective, to control this zoonosis it would be advisable to also incorporate measures in horses, such as the use of repellents.

Abstract: Leishmaniosis is a zoonoses caused by *Leishmania* spp., an intracellular protozoan parasite. This parasite is transmitted by sandflies and the disease is endemic in Mediterranean basin. In the last years, the number of species which could be reservoir of parasite is increased. One of the most relevant is the horses, due to contact with humans and its ability to control the disease, being a possible silent reservoir. In this study, we have analyzed the prevalence and factors related to *L. infantum* infection in healthy horses in Mediterranean region. Epidemiological data and serum samples were obtained to 167 apparently healthy horses and presence of *L. infantum* was evaluated by ELISA method and qPCR. The results show 27,5% of prevalence and the main factors related to infection are equine breed, outdoor living, use and season. In conclusion, the prevalence of L. infantum infection in apparently horses from Eastern Spain (Mediterranean basin) is elevated. To control this zoonosis, it would be advisable to carry out more studies on this and other species that could be silent reservoirs of the parasite, as well as carry out measures such as the use of repellents on a regular basis.

Keywords: equine; *Leishmania*; One Health; parasitic infection; zoonosis

1. Introduction

Leishmaniosis is a parasitic disease caused by *Leishmania* spp., which *includes L. infantum*, the most common causal agent of leishmaniosis in the Mediterranean basin. This protozoan parasite is

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transmitted by the phlebotomine sandflies from the Psychodidae family, being the genus *Phlebotomus* its principal vector in Europe [1–3]. Even though the domestic dog (*Canis lupus familiaris*) has always been considered the main reservoir of this parasite [4,5], *L. infantum* infection has been reported in different species in Europe, including cats [6], wild carnivores [7,8], wild rabbits [9,10], birds [11], reptiles [12] and horses [13].

In Europe, the number of clinical cases of equine leishmaniosis (EL) reported has been low, and clinical manifestations is mild without visceral involvement, and cutaneous lesions tend to self-recover without treatment [14–16]. *L. infantum* has been identified as the etiological agent of EL with cutaneous presentation in Germany [14] and Switzerland [17], as well as in countries where this zoonosis is considered endemic, such as Italy [18], Spain [16] and Portugal [15]. However, most data of EL have been recorded from South and Central America, being the main etiological agent *L. braziliensis* [19–21] or *L. infantum* [22–24].

Subclinical infections or *Leishmania* spp. infections in healthy horses has been reported around the world, and the prevalence in endemic areas depends on study and geographic area. In Venezuela and Brazil, the prevalence of *L. braziliensis* infection in healthy horses ranged between 7.1 to 28% [25,26], whereas the prevalence of *L. infantum* in Europe seems to be lower [27–30]. Given that both the techniques used, and the number of individuals evaluated differ greatly between studies, the observed prevalence varies between countries and in the same country or region, both in the Old and the New World.

The role of horses as a reservoir of the parasite has not been well studied, but undoubtedly, horses can play a very important role [31], not only as a source of food for sandflies [24] but also because their feces are a source of food for development of their larvae [32].

The aim of this work is to analyze the prevalence of *L. infantum* infection and related factors in apparently healthy horses in Eastern Spain, Mediterranean basin, an endemic region.

2. Materials and Methods

2.1. Animals and Epidemiological Data

A total of 167 horses without clinical signs living in Valencia Community (Eastern Spain, Mediterranean region) was studied and samples were recovered from December 2022 to June 2023. For all animals, epidemiological data were collected: sex (two categories: male or female), reproductive status in males (two categories: castrated or not), age (four categories: foal -less than five years old-, young -between five and twelve years old, adult -between thirteen and twenty-one years old-, and elder -more than twenty-one years old), breed (seventeen categories), crossbreed or purebred (two categories), use (six categories: teaching, breeding, dressage, hitch, walking, and leap), type of housing (two categories: outdoor or indoor), and living or not with dogs. Samples were recovered in two period of the year, winter (December 2022-January 2023) and spring (May 2023-June 2023). The mean of temperatures was annotated.

2.2. Sample Collection and DNA Extraction

Ten milliliters of whole blood were taken by jugular venipuncture using Vacutainer tubes with or without anticoagulant. Samples without anticoagulant were maintained at room temperature to obtain serum aliquots, which were stored at -80ºC until processing. Serological testing for *Leishmania* spp. detection of specific antibodies was performed using Enzyme-linked immunosorbent assay (ELISA) test for anti-Leishmania-specific immunoglobulin G (IgG) antibodies (*Leishmania* vet ELISA®, Demeditec Diagnostics GmbH, Germany), following the manufacturer's instructions. Sample with ELISA titer > cut-off was considered seropositive. Whole blood samples recovered with EDTA anticoagulant were used for DNA extraction before 24 h to recovery. Total DNA was isolated using Thermo Fisher Scientific DNA purification Kit following the manufacturer's protocol (Thermo Fisher Scientific, MA, USA). DNA was quantified using a Nanodrop spectrophotometer (Thermo Fisher Scientific), and only samples with A260/A280 > 1.8 were used. DNA samples were stored at -80ºC until use for PCR.

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2.3. Real-Time PCR Analysis

The primers and probes for *L. infantum* DNA detection were chosen in the constant region of the kinetoplast DNA minicircle, and fluorogenic probes were synthesized by FAM reported molecule attached to the 5′ end, a TAMRA quencher linked to the 3′ end, and ROX as the internal passive reference dye. Primers and probe sequence are: 5-GGCGTTCTGCGAAAACCG-3′ (forward), 5′-AAAATGGCATTTTCGGGCC-3′ (reverse), and 5′-AAAATGGCATTTTCGGGCC-3′ (probe) [33]. Real-time PCR was performed in a 20 µl of total volume of reaction, including 10 µl of qLUMEN MasterMix (Gquence®, Labbox Labware, SL, Barcelona, Spain), 300nM of each primer, 250 nM of the fluorogenic probe and 50-100 ng of DNA. Reactions were run at triplicate for all samples and were performed on the QuantStudio 5 Real-Time PCR System (Thermo Fisher Scientific, MA, USA). The thermal cycle conditions consisted of a 2 min initial incubation at 50ºC, followed by 10 min at 95ºC, and 40 cycles at 95ºC for 15 s and 60ºC for 1 min each. On each plate, a negative control (free RNA and DNA water) and positive control was included.

2.4. Statistical Analysis

Prevalence of parasitic infection was analysed using the GENMOD procedure of the statistical program SAS (North Carolina State University, USA). Binary (for sex, reproductive status in males, crossbreed or purebred, type of housing, living or not with dogs, and period of the year) or logistic regression model (for age, breed, and use) were used using each factor as a fixed effect in their respective statistical analysis. The statistical significance was set at p-value < 0.05.

3. Results

Of the 167 samples evaluated, 27.5% were positive in the ELISA test and qPCR (46/167). Presence of *L. infantum* DNA was confirmed in 40.7% of samples analyzed by qPCR (68/167). The total prevalence of *L. infantum* infection in animals evaluated was 27.5%. In terms of sex, 65.3% of animals evaluated were males (109/167), being 66.1% of them, castrated (72/109). Related to age, 21% of animals were foal (less than five years), 40% young animals (between five and twelve years old), 80% adults (between thirteen and twenty-one years old) and 26% old horses (more than twenty-one years old). The most of horses were purebred, including a total of eighteen different breeds, and 38.3% were crossbreed animals. In term of use, 44% of horses were used for dressage (74/167), whereas 19.2% (32/167) and 16.2% (27/167) were used for walking and teaching, respectively. Regarding type of housing, a total of 112 horses (67.1%) had outside access, and 159 (95.2%) living with dogs. A total of 106 samples were collected from December 2022 to January 2023 (63.5%) (Table 1).

Table 1. Epidemiological data of horses included in this study. KWPN: Koninklijk Warmbloed Paard Nederland; PRE: Purebred Spanish Horse.

Variable	Categories		Number of Horses (%)
Gender	Male	Castrated	72 (66.1%)
		No castrated	37 (33.9%)
	Female		58 (34.7%)
Age	Foal (<5 years)		21 (12.6%)
	Young (5-12 years)		40 (24.0%)
	Adult (13-21)		80 (47.9%)
	Elder (>21 years)		26 (9.6%)
Breed	Purebreed (125)	Anglo-Arabian	1 (0.6%)
		Belgian Warmblood	2 (1.2%)
		Spanish Sport Horse	7 (4.2%)
		Connemara	1 (0.6%)

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		Haflinger	1 (0.6%)
		Hannoverian	4 (2.4%)
		Hispano-Breton	8 (4.8%)
		Hispanic-Arabic	6 (3.6%)
		Holsteiner	1 (0.6%)
		Gypsy Cob	2 (1.2%)
		Jaca navarra	2 (1.2%)
		KWPN	5 (3.0%)
		PRE	74 (44.3%)
		Percheron	1 (0.6%)
		Pony	4 (2.4%)
		Frech Saddle Horse	5 (3.0%)
		Arabian	1 (0.6%)
	Crossbred		42 (25.1%)
Use	Teaching		27 (16.2%)
	Breeding		4 (2.4%)
	Dressage		74 (44.3%)
	Hitch		12 (7.2%)
	Walking		32 (19.2%)
	Leap		18 (10.8%)
Type of housing	Outdoor		112 (67.1%)
	Indoor		55 (32.9%)
Living with dogs	Yes		159 (95.2%)
	No		8 (4.8%)
Periodo of the year	Winter		106 (63.5%)
	Spring		61 (36.5%)

A total of 46 (27.5%) samples analyzed were positive for anti-leishmania antibodies and confirmed by qPCR. Related to factors evaluated, sex or castration in males, age, pure- or crossbred, and living of dogs were no effect in the *L. infantum* infection, whereas horse breed, life condition, use, period of recovery sample and location were effect. Breeds with most prevalence were Pony, Spanish Sport Horse (CDE), and Purebred Spanish Horse (PRE), with 75%, 57.1%, and 32.9% of prevalence, respectively. Only one Haflinger and one Percheron were included in this study, and two were positives, and one of two Irish cob horses was positive (Table 2).

Table 2. Prevalence of *L. infantum* infection according to horse breed. KWPN: Koninklijk Warmbloed Paard Nederland; PRE: Purebred Spanish Horse.

		Number of Positive Animals (%)
Purebred (125)	Anglo-Arabian	0
	Belgian Warmblood	0
	Spanish Sport Horse	4 (57.1%)
	Connemara	0
	Haflinger	1 (100%)

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Hannoverian	0
Hispano-Breton	0
Hispanic-Arabic	0
Holsteiner	0
Gypsy Cob	1 (50%)
Jaca navarra	0
KWPN	0
PRE	25 (32.9%)
Percheron	1 (100%)
Pony	3 (75%)
Frech Saddle Horse	0
Arabian	0

Outdoor horses present higher prevalence than indoor (38.9% and 5.5%, respectively, p<0.0001) (Figure 1a). The prevalence in horses used for teaching were higher than other uses, being the prevalence of 88.9% (p<0.0001) (Figure 1b). The number of positive samples recovered in spring (60.7%) were higher than in winter (8.5%) (p<0.0001). Mean of temperature were 13.1ºC and 24.1ºC for winter and spring, respectively (AEMET, Spanish Meteorology Statal Agency). Figure 1c shows the prevalence of positive animals according to period of recovered and mean of temperature for each location and period.

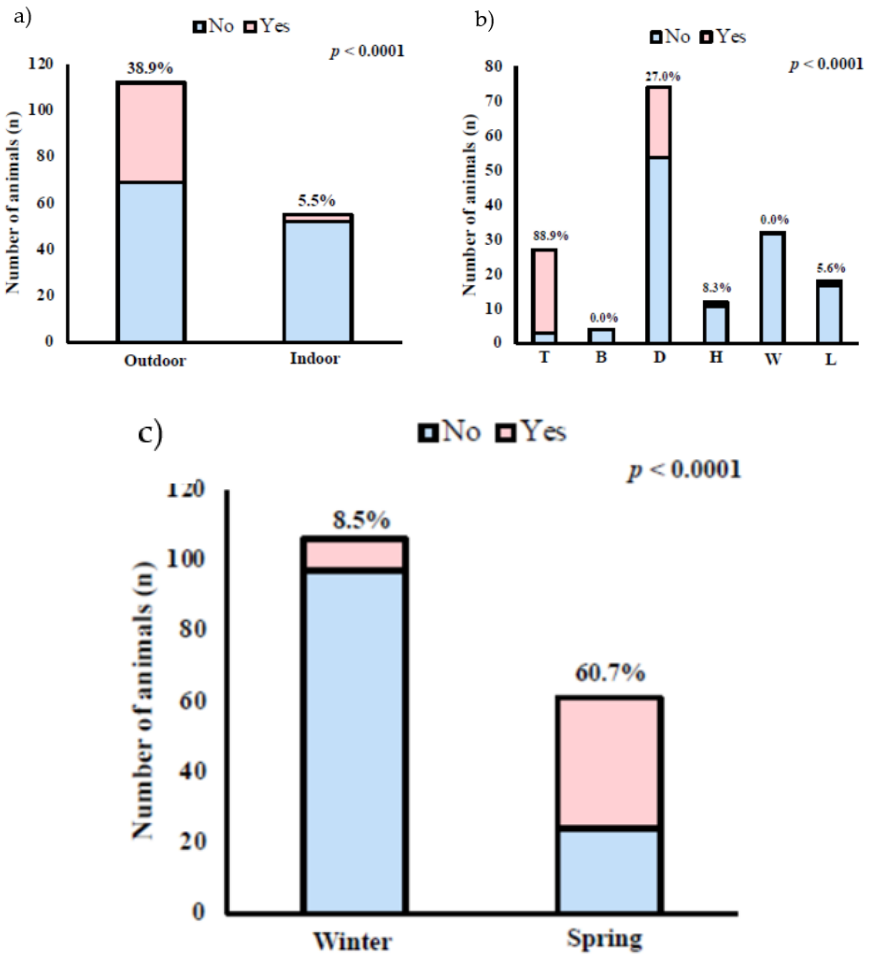

Figure 1. Number and percentage of positive horses by ELISA and qPCR regarding (**a**) type of housing (outdoor or indoor); (**b**) use (T: teaching; B: breeding; D: dressage; H: hitch; W: walking; L: leap), and (**c**) season (winter with mean temperature: 13.1ºC) and spring with mean temperature: 24.1ºC).

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4. Discussion

This study shows the prevalence and factors related to infection of *L. infantum* in horses from Eastern Spain, where this parasite is endemic. The results show a total prevalence of 27.5% by two methods (ELISA and qPCR) and 40.7% by qPCR, being the factors with effect of horse breed, if the animal had access to outside, the use of horse, and the period of recovery sample were effect.

This study has been realized in Eastern Spain, concretely Valencian region, where human leishmaniasis is endemic since 1982 [35]. Dog is consider the most common reservoir and its seroprevalence in dogs has been studied, and in Spain, depends on province, being between 57.1% for Balearic Islands (Mediterranean basin) to 0% for Vizcaya (North Spain) [36,37]. Inasmuch as other species could also serve as reservoirs, knowing the prevalence in these other species is very important to control this zoonosis. Concretely, horses have relevance, given that it is a species in direct contact with human and usually lives in urban arear or close to them.

Data of seroprevalence in horses is limited. The only study carried out in Spain shows a prevalence of 14.3% by ELISA [37], and similar results has been observed in Italy [13,24]. However, in other Mediterranean countries, seroprevalence seem to be more less. For example, studies realized by serological methods indicate a prevalence of 0.3% in Greece [29], 1.4% in Israel [30], and 4% in North Portugal [28], whereas Nardoni et al. (2019) observed 36.7% of donkeys evaluated positive. In South America, seroprevalence of *Leishmania* spp. in healthy horses has been estimated around 27% [39], reaching even 67.3% of horses from urban areas by qPCR [40]. These data are in agree with those observed in our study, confirming the sensitivity of the qPCR technique for the detection of *Leishmania* spp., especially in asymptomatic animals [41]. The evaluation of healthy horses in endemic areas of leishmaniasis is important, mostly in Europe, where equine leishmaniosis is produced by *L. infantum* and its clinical presentation is usually mild, appearing skin lesions that usually remit spontaneously, so the infection goes unnoticed in most cases [13].

No effect of sex, pure- or crossbred, and living with dogs has been observed, in accordance with other studies carried out on healthy horses and dogs [13,39,42]. Related to age, results of studies are contradictories. Our results indicate no effect on age, in according with Biral et al. (2021), whereas Gazzonis et al. (2020) show an increase of prevalence related to age [13,40]. In other species, such as dogs, seropositive has been related to age, so in this specie, the chronicity of the infection has an accumulative effect [43]. However, the immune response in horses is effective in controlling the infection [37], so there would not be this accumulative effect unlike what happens in dogs. Related to breed, equine breeds with more prevalence were CDE and PRE, in accordance with other studies, which meso-dolichomorphic horses present high prevalence than breeds with other morphotype [13]. Both equine breeds, CDE and PRE, have calm temperaments, as is the case with horses used for teaching, which could make it easier for them to serve as food for the sandflies and, therefore, increase the risk of *Leishmania* spp. infection. This also happens in outdoor horses, which the outdoor living increases the risk, factor that has been associated with this risk in other species, such as dogs [43,44].

Prevalence of *L. infantum* infection in spring was much higher than in winter when the average temperature recorded were lower (24.1ºC *versus* 13.1ºC in spring). The increase of temperature has been demonstrated as relevant factor in *Leishmania* spp. dissemination, facilitating the presence of the parasite and its vectors [45–47]. These data are more relevant considering that climate change will increase temperatures throughout the year and throughout the word. Some researchers are already carrying out studies with predictive models in this regard. Concretely, Daoudi et al. (2022) estimates an expansion of *L. tropica* and its vector using Ecological Niche Modeling [48]. This modelling could be extrapolated to the expansion of *L. infantum*, as well as to its vector. In fact, *L. infantum* has been detected not only in sandflies but also in dogs and horses of cold European countries [14,49,50], and the scientists already recommend including cutaneous leishmaniasis in the differential diagnosis in human patients [51].

5. Conclusions

In this study, high prevalence of *L. infantum* infection has been detected in healthy horses of Eastern Spain. The main related factors with increased the risk of infection are equine breed, outdoor

living, and use for teaching. The prevalence increases in the warm seasons, confirming that the climate change and the consequent increases in temperature suggest an increase in the spread of the parasite in all species. In horses, the high prevalence observed in this study shows that the horses could be an important reservoir for this zoonotic parasite, so it would be interesting to implement control measures, such as the use of repellents, mainly in horses living in urban areas.

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Institutional Review Board Statement: The animal study protocol was approved by the Ethics Committee of Universidad Cardenal Herrera CEU (protocol code VCS/2022/PEA/0279 and date approval 21/12/2022).

Informed Consent Statement: Informed consent was obtained from all owners of the animals involved in the study.

Data Availability Statement: The dataset of the current study is available from the corresponding author upon reasonable request.

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