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Serosurvey of canine leishmaniasis in five departments near an identified human clinical case in Marseille (France)

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ABSTRACT

Leishmania infantum is a protozoan parasite of the family Trypanosomatidae, transmitted by the bite of phlebotomine sand flies (Diptera: Psychodidae). It is responsible for human and canine leishmaniasis in countries bordering the Mediterranean basin. Here we describe a clinical case of human cutaneous leishmaniasis in a 76-year-old female patient living in Marseille. Upon interrogation, the patient had no history of recent travel or contact with animals. The study involved clinical, serological, and molecular investigation of the occurrence of *Leishmania* infection in 718 dogs from five departments within a 130 km radius perimeter around Marseille. Five dogs showed signs of moderate leishmaniasis. Additionally, the serological survey of dogs revealed a global seroprevalence of 5.1%, with a significantly different prevalence in the Vaucluse department. Molecular analysis and phylogenetic studies highlighted the close relatedness of *Leishmania* strains between human and canine hosts with 99.6% of identity, indicating local transmission. The findings underscore the importance of serological surveillance in dogs and human. In a One Health approach, it is necessary to continue investigating *Leishmania* infection in all potential reservoirs, especially for zoonotic *L. infantum* in wildlife (red foxes, leporids, rodents, etc.) but also in dogs, cats, and equids.

1. Introduction

Canine leishmaniasis (CanL) is a major significant zoonotic disease caused by *Leishmania infantum*, a protozoan belonging to the Trypanosomatidae family, transmitted by phlebotomine sandflies (Diptera: Psychodidae) [1,2]. The dog is considered the main reservoir of *L. infantum* in the Mediterranean basin [3]. In France, CanL persists in the southeast of the country (Pyrénées-Orientales, Provence, Alpes-Maritimes, and Corsica) despite notable advances in the prevention and treatment of canine infection [4]. As with many other vector-borne-pathogens, the transmission is related to a complex episystem involving vector density and competence, the reservoir hosts, environmental conditions, and the pathogen itself. Due to climate change, parasite transmission period becomes broader, covering a period from March to November [5]. In addition, the environment-parasite relationship drives hybridisation and introgression in *Leishmania* parasites, resulting in uncommon leishmaniasis clinical forms, like cutaneous leishmaniasis caused by *Leishmania donovani* and *L. infantum* which are classical visceral leishmaniasis agents.

Regarding epidemiological surveillance, one-time investigations are particularly informative, as reporting of human and animal cases is not mandatory. Meanwhile, the Leishmaniasis Reference Center reported 380 clinical cases from 1998 to 2020 in the southeast of mainland France, distributed across several departments [6]. In the region of Nice (Alpes-Maritimes), a survey revealed that a total of 66.2% of asymptomatically infected humans ($n = 227$) and 25.7% of clinically healthy dogs ($n = 156$) tested positive through serological and/or PCR tests [7].

The high prevalence of asymptomatic people suggests that they may act as reservoir hosts for *Leishmania*. Further studies are needed to confirm this hypothesis. To assess the risks for human and canine leishmaniasis transmission, we conducted a prevalence survey of CanL during 2023 in five departments around Marseille, where an autochthonous case of human leishmaniasis was diagnosed at the beginning of that year. Using a One Health approach, we were able to compare the phylogeny of *Leishmania* strains that infect humans and dogs in this endemic region.

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2. Case report

A 76-year-old female patient was referred to the University Hospital Institute Mediterranean Infection (Marseille) for etiological assessment of cutaneous lesions located on the forehead. Her past medical history was notable for asthma, and the patient reported daily medication with antihistamines. Upon questioning, the patient complained about the appearance of three nodular erythematous lesions, with one located on the right forehead and two over the right temple. The lesions had been evolving for 3 months and were described as painless and slightly pruritic. Initially, they presented as nodules which evolved to ulceration within a few weeks. No other complaints were reported. The patient lived in a house with a garden in Marseille, France. Her house was located 50 m from an area with vegetation, suitable for sandfly development, where foxes may also dwell (Supplementary Fig. 2). She denied any recent travel in the past years, including to other regions of southern France. No contact with animals was reported, and the patient did not own any pets at the time of presentation. Upon examination, three superficial erythematous ulcerated nodules were observed on the patient's right forehead (Fig. 1A-B). Physical examination did not reveal any other abnormalities. In this context, a crust from one of the lesions was gently removed for molecular diagnosis. Additionally, a superficial dry swab was obtained from the same lesion also for molecular diagnosis. A biopsy specimen was also collected for histopathological diagnosis. Following DNA extraction, both the crust specimen and dry swab sample tested positive for the detection of *Leishmania* spp. through PCR analysis. Further DNA sequencing allowed the identification of *L. infantum* (LR697137, 99.8% identity). PCR analysis targeting *Leishmania* spp. performed on peripheral blood was negative. Moreover, the western-blot (WB) assay identified anti-*Leishmania* antibodies. Histopathological examination of the biopsy specimen revealed the presence of amastigotes of leishmania within histiocytes. Routine laboratory workup revealed parameters within normal ranges. All these results led to the diagnosis of cutaneous *L. infantum* leishmaniasis.

3. Materials and methods

3.1. Study area, sampling, and data collection

From May to October 2023, 718 dogs over 1 year of age, including military working dogs ($N = 167$) and shelters dogs ($N = 551$) were enrolled in the present study, according to their accessibility for sampling in the studied area. They belonged to 18 localities covering five department (i.e., departments of Hérault, Gard, Bouches-du-Rhône, Var, and Vaucluse) within a 130 km radius perimeter around Marseille. They were mainly shepherd dogs, staffs, and bull terriers. They all received veterinary care and 27 dogs had been vaccinated (LetiFend®, Laboratorios Leti, Madrid, Spain) against leishmaniasis. The LetiFend® vaccine yielding to specific antibodies that do no cross-react within the ELISA. Clinically, there were 11 treated cases of CanL and five were suspected cases.

Dogs were examined and sampled by veterinarians after obtaining the consent of their owners. The blood samples were drawn from radial vein by veterinarians in accordance with French veterinary code of ethics. No approval from any ethical committee was needed. A volume of 4–5 mL of blood was collected of each dog, into dry and EDTA tubes. Serum was recovered after centrifugation (10 min, 3000 *g*). Samples were stored at −20 °C until further analysis. Information related to age, gender and breed was collected from each animal.

3.2. Serological screening

Serological screening relied on the enzyme-linked immunosorbent assay (ELISA), using ID Screen® Leishmaniasis indirect test (Innovative Diagnostics, Grabels, France) following the manufacturer's recommendations. Briefly, the assay validation required a minimum accepted optical density for the positive control (ODPC) $\geq$0.35, with a ratio for the optical density of the ODPC to the negative control (ODNC) >3.0. Positive control ratio (S/P%), calculated as $SP\% = \frac{(ODN-ODNC)}{(ODPC-ODNC)} \times 100$, for each sample (ODN), was used to determine the sample positivity within 50% positivity threshold.

3.3. Molecular analysis and phylogeny

EDTA blood samples were individually subjected to DNA extraction: a total of 150 µL of EDTA blood was incubated for 2 h at 56 °C with 30 µL of proteinase K, then subjected to DNA extraction using Kingfisher automaton (London, UK), according to the manufacturer's recommendations. Samples were then screened for *Leishmania* spp. DNA using a *Leishmania* qPCR system targeting the 18S SSU rRNA gene as previously described [8]. Secondarily, a *Leishmania* standard PCR assay targeting the partial ITS2 gene was used for amplification and Sanger sequencing of all qPCR positive samples, as well as the DNA extracted from the patient skin lesion as previously described [8]. DNA amplicons were subjected to assembling, trimming and manual correction using Chromas 2.6.6 software. Resulting DNA sequences were aligned against the representative homologous gene from the genus *Leishmania* using MAFFT aligner [9]. Multisequence alignment (MSA) was trimmed

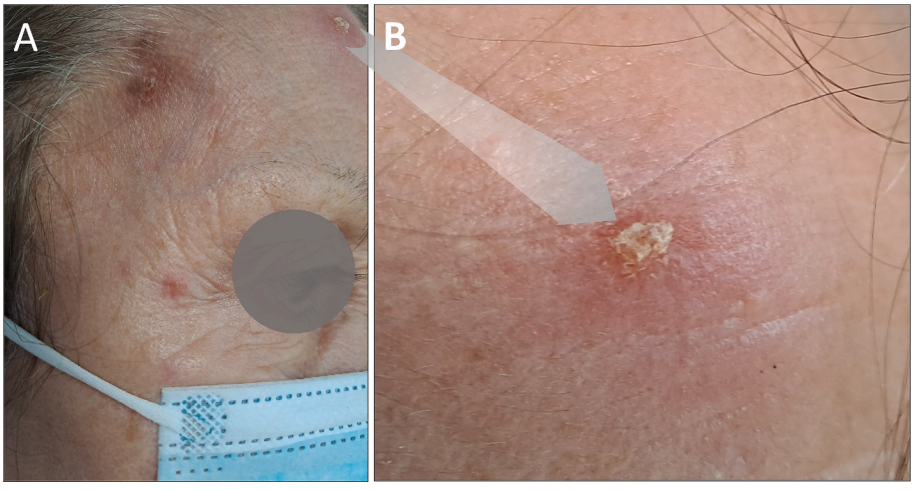

Fig. 1. Photograph of the patient showing the cutaneous lesions caused by *L. infantum*. A. Dermatological nodular erythematous lesions distributed through the right forehead and the temple. B. Pruritic aspect of the nodular erythematous ulceration on the temple.

manually using Bioedit software [10]. The maximum likelihood phylogeny was performed under 100 K bootstraps. The Best-fit model according to BIC was TN + F, which was selected using Model Finder, implemented as functionality within the IQ-Tree software [11]. Finally, phylogenetic tree was edited using iTOL v5 software [12].

3.4. Statistical analysis

The multivariable analysis of variance (MANOVA) model was used to compare the effect ELISA OD and qPCR Ct values on the clinical status. The statistical significance of seroprevalence among departments and localities were compared using the Kruskal-Wallis test, and Chi-square and Fisher's exact tests were used to compare the seroprevalences recorded in departments and localities. Statistical analysis was performed using Pandas, while Seaborn and Matplotlib libraries from Python 3.12 were used for plotting.

4. Results

The detailed results of serological and molecular assessment are presented in Table 1. Out of 718 dogs, the average seroprevalence was estimated 5.1% (37 positives), including: Hérault 2% (2/99), Bouches-du-Rhône 3.7% (13/345), Var 5.3% (4/75), Gard 7.4% (5/67) and Vaucluse 11.9% (13/109). The prevalence recorded in the Vaucluse department was significantly different compared to the Hérault and Bouches-du-Rhône departments (Fig. 2, Fig. 3). The prevalence of infection or exposure was 5.3%, representing a total of 38 dogs with at least one positive test (serology and/or qPCR). Based on clinical findings, positive dogs were distributed into three groups including 62.2% clinically healthy, 10.8% showing clinical signs of leishmaniasis and 27% clinically cured after a leishmaniasis episode. Statistical comparison revealed the absence of significant differences in OD and qPCR Ct values between the three groups (Fig. S1). The prevalence of *L. infantum* detection in the blood (qPCR positive) was 3.2% (23/718). Among seropositive dogs, 59% (22/37) had a positive blood qPCR. Only one dog tested positive for qPCR with a negative serology. On the other hand, 14 dogs tested negative in qPCR but positive for serology. Non-agreement was calculated to be 39% (15/38). Of the 11 sick dogs, treated and clinically cured, there were eight that tested positive in qPCR (75%). Among the five symptomatic dogs, three had a positive blood qPCR (60%). Five dogs showed signs of moderate leishmaniasis: non-pruritic exfoliative dermatitis (3 cases) and anterior uveitis (2 cases) (Fig. 3).

Despite several attempts, only three out of the 23 qPCR positive dogs were successfully amplified and sequenced. DNA sequences from canine strains from the present study were identical to each other and showed identity of 99.6% with the human isolate from the present study (Fig. 4). Blast analysis showed an identity of 98.2% and 98.7% of identity for canine and human strains respectively with *L. infantum*, previously isolated from a red fox from Marseille (MK67907). Accordingly, phylogenetic analysis highlighted the close relatedness of strains isolated in the present study and those previously identified in foxes' population of Marseille.

5. Discussion

Although not commonly observed, pure cutaneous clinical forms of *L. infantum* leishmaniasis are well known in endemic areas in southern France [13]. Several factors may explain the occurrence of cutaneous leishmaniasis caused by *L. infantum* such as its potential for long-lasting natural evolution of the disease, even in healthy and immunocompetent patients. While cutaneous leishmaniasis caused by *Leishmania major* or *Leishmania tropica* typically heals within several months, cases caused by *L. infantum* can persist for years. Chronic, non-healing cutaneous or mucocutaneous leishmaniasis caused by *L. infantum* is usually seen in patients with HIV or immunosuppressive treatments, or in cases with atypical clinical presentations. The parasite's ability to escape macrophage defense mechanisms and suppress cell-mediated immune responses contributes to its chronicity. Additionally, diagnostic molecular methods might have limitations for species differentiation, as evidenced by the identification of a natural hybrid strain between *L. infantum* and *L. donovani* [14].

The commercial multi-species ELISA, using soluble extracts of *L. infantum* as antigen, is reliable and easy to implement, providing a 95.3% sensitivity and a specificity of 100% [15]. The numerous CanL prevalence surveys carried out in Mediterranean countries have been carried out with molecular detections and/or various serological tests: indirect fluorescent antibody test (IFAT), ELISA, direct agglutination test (DAT), WB and rapid immunochromatographic assay. All these methods

Table 1
Result of molecular and serological screening of *Leishmania infantum* through the 718 dogs from the present study.

Departments (seroprevalence)	Localities	Number of dogs	Sick dogs diagnosed, treated, clinically cured	Sick dogs showing clinical signs	No. positive ELISA *Leishmania* (%)	Blood EDTA positive qPCR *Leishmania infantum*	Discordances ELISA positive and qPCR negative
Hérault (22/99 2%)	Montpellier	99			2 (2%)	0	2
	Nîmes	31			0	0	
Gard (5/67 7.46%)	Garons	8			0	0	
	Alès	28	4		5 (18.5%)	4	1
	Istres	47			1 (2%)	0	1
	Marseille	102	**1**	2 (Fig. 2)	7 (6.86%)	2	5
	Miramas	26			0	0	
	Fos-sur-Mer	10			0	0	
Bouches-du-Rhône (13/345 3.7%)	Cabriès	66	**2**		2 (3%)	2	
	Aix-en-Provence	42	**1**	1	3 (7%)	3	
	Saint-Paul-les-Lez	8			0	0	
	Salon-de-Provence	67			0	0	
	Draguignan	25		1 (Fig. 2)	1 (4%)	0	1
Var (4/75 5.3%)	Toulon	42			0	0	
	Hyères	8			3 (37.5%)	0	3
Vaucluse (13/109 11.9%)	Orange	29			5 (17.2%)	5	1
	Isle-sur-la-Sorgue	80	3	1	8 (10%)	7	1
TOTAL (5.1%)		**718**	**11 including 10 seropositives**	**5 all seropositives**	**37 (5.1%)**	**23**	**14**

Fig. 2. Map showing the geographical distribution of CanL cases through sampled dog populations diagnosed by the ELISA.

are not based on the same principles, thus the results obtained are difficultly comparable. Indeed, serological tests are designed with various antigens and different positivity cut-offs. Due to parasite location within infected hosts (mainly skin and spleen macrophages) PCR assays over blood samples are not suitable for epidemiological studies, and several discrepancies may appear compared to the other assays, as demonstrated in the present study. The highest mean parasite load is found in the bone marrow, followed by lymph nodes [3]. PCR prevalence in blood samples ranged from like below seroprevalence rates. The prevalence of PCR on blood was 2.7% ($N = 112$) in Turkey [16]. Another important statement concerns vaccinated dogs, which were ELISA-negative, except for one dog which was infected before vaccination. This statement highlights that the vaccine antibodies probably do not cross-react with the ELISA assay.

In Marseille, a 7% seropositivity level suggests ongoing *Leishmania* infection in dogs. According to a meta-analysis, the average canine seroprevalence is 23.2% (median: 10%) of 504,369 dogs in Southern Europe [17]. In Portugal, DAT seroprevalence was calculated at 5% in kennels ($N = 179$) [18]. In northern Italy, WB seroprevalence was estimated to 42% in 803 dogs and 17% in 815 humans. There were no correlations between dog and owner positivity's [19]. In Spain, the survey carried out on 5451 dogs showed an ELISA seroprevalence of 5.5% close to that highlighted in our study [20]. In the North-East of Spain, near the French border, the ELISA seroprevalence was 11.9% among 226 dogs [21]. The south-east of France is an endemic region for CanL with an occurrence of clinical cases estimated at 2–3% [22].

Accordingly, phylogenetic analysis highlights that *Leishmania* strains from both human and canine hosts are closely related in the present study. Due to the close similarity between human and canine (foxes and dogs) *Leishmania* strains, and despite the detection of a positive dog just a few kilometers from the patient's location, the clear-cut decision about the origin of *Leishmania* infection cannot be ruled out as the patient was living near a typical biotope for sandflies, with possible presence of foxes (Fig. S2). CanL is a well-known disease in Marseille province. In 1986, out of 557 dogs sampled in veterinary clinics in Marseille, IFAT seroprevalence was 12% [23]. Over the past 35 years, the seroprevalence of CanL in Marseille has declined significantly. On the other hand, this was not observed in the rest of southeastern France. In 1993, the IFAT seroprevalence was 5.6% (17/303) among military dogs in the same five departments investigated by our study, where the seroprevalence is now 5.4% (9/167) [24]. Endemic regions in the Cévennes and Vaucluse showed a confirmed 15% seroprevalence [25].

The abundance of sandfly vectors correlates with the presence of reservoir dogs. This has already been shown in the Cévennes with *Phlebotomus ariasi* and in the Vaucluse with *Phlebotomus perniciosus* [26]. In Marseille, in the same neighborhood where the patient diagnosed in 2023 lived, a study showed that *P. perniciosus* was present and even infected with *Leishmania* the minimum infection rate in pooled sand flies (88% *P. perniciosus*) was 0.6% [26]. At the same time, the detection of the Toscana virus was ruled out. In asymptomatic humans from Marseille, a serological survey (WB) was carried out from 2001 to 2010. There were 28% (132/472) positive [27]. The proportion of clinically healthy dogs likely acts as a driver for the spread of *L. infantum*. Vector borne transmission of *Leishmania* from an asymptomatic dog to another dog and even to human host has been demonstrated [28,29]. On the other hand, vector transmission of *L. infantum* from an asymptomatic man to another man has not yet been proved.

Even in the peri-urban environment, wildlife can also act as a reservoir for *Leishmania*, as has been shown in Madrid with Iberian hares and rabbits [30,31]. In the region studied, the red fox is a potential reservoir for parasites. We previously demonstrated this by applying qPCR assays for detection of *L. infantum* kinetoplast DNA in the spleens of 93 hunted red foxes [32]. The prevalence was 15% (5/57 red foxes near Marseille, 8/33 near Draguignan and 1/3 in Hyères). Furthermore, the *Leishmania* strains from the present study were genetically very close to the previously strains we characterized from red foxes in Marseille [32].

6. Conclusions

Considering the role of domestic (dogs, cats, equids) and wild dogs (red foxes, rodents, leporids) as sentinels for human leishmaniasis surveillance, it is essential to carry out regular serological surveys and to broaden their scope to ensure continuous monitoring. Moreover, supporting epidemiological studies using molecular typing tools may facilitate canine and human disease surveillance and contribute to

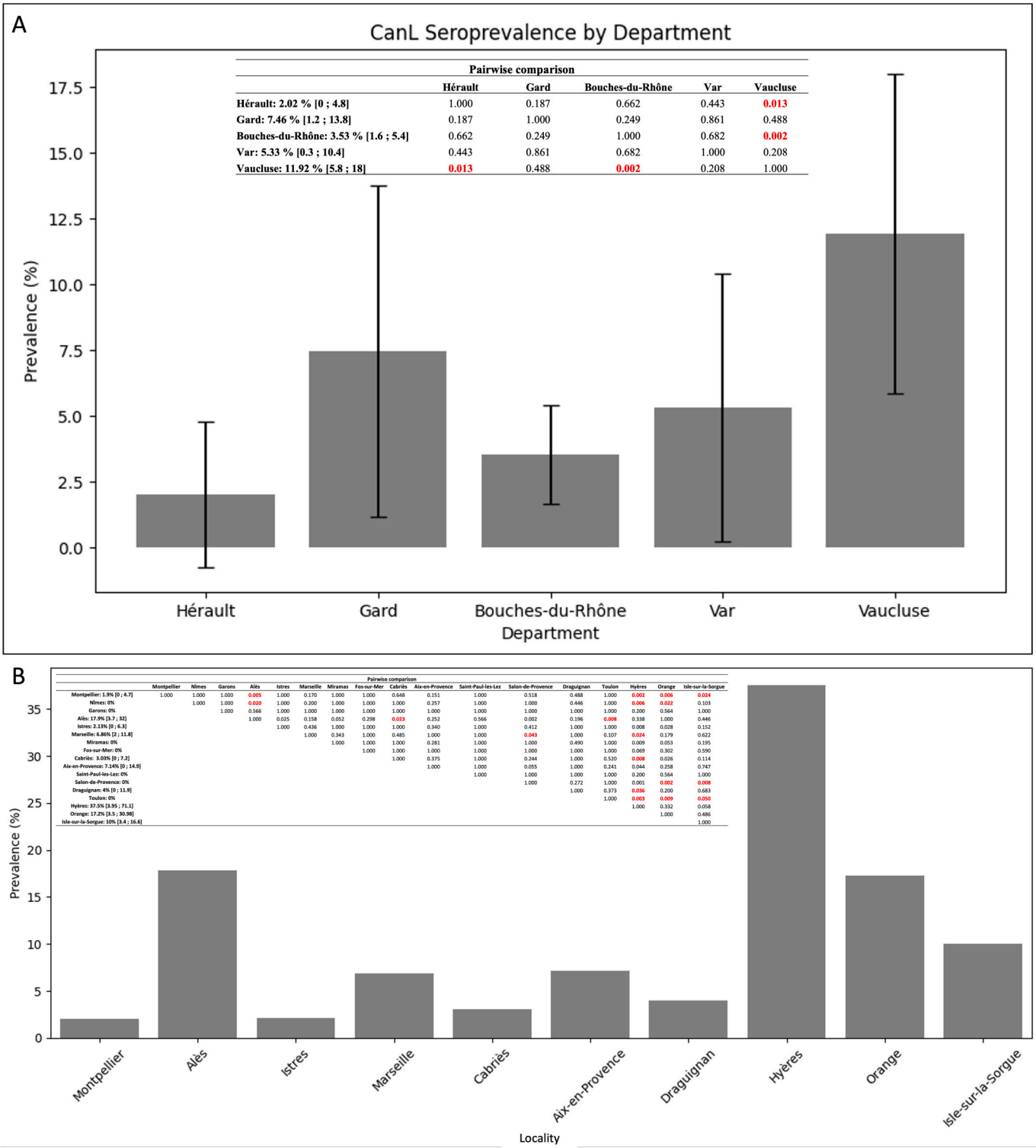

Fig. 3. Statistical plotting of CanL seroprevalence across departments (A) and localities (B). Red bolded values indicate *p*-value <0.05 from Chi-square and exact Fisher's test of the pairwise comparison of the seroprevalences across the geographical distributions. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

identify the hosts and reservoirs in each epidemiological context.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.onehlt.2024.100855.

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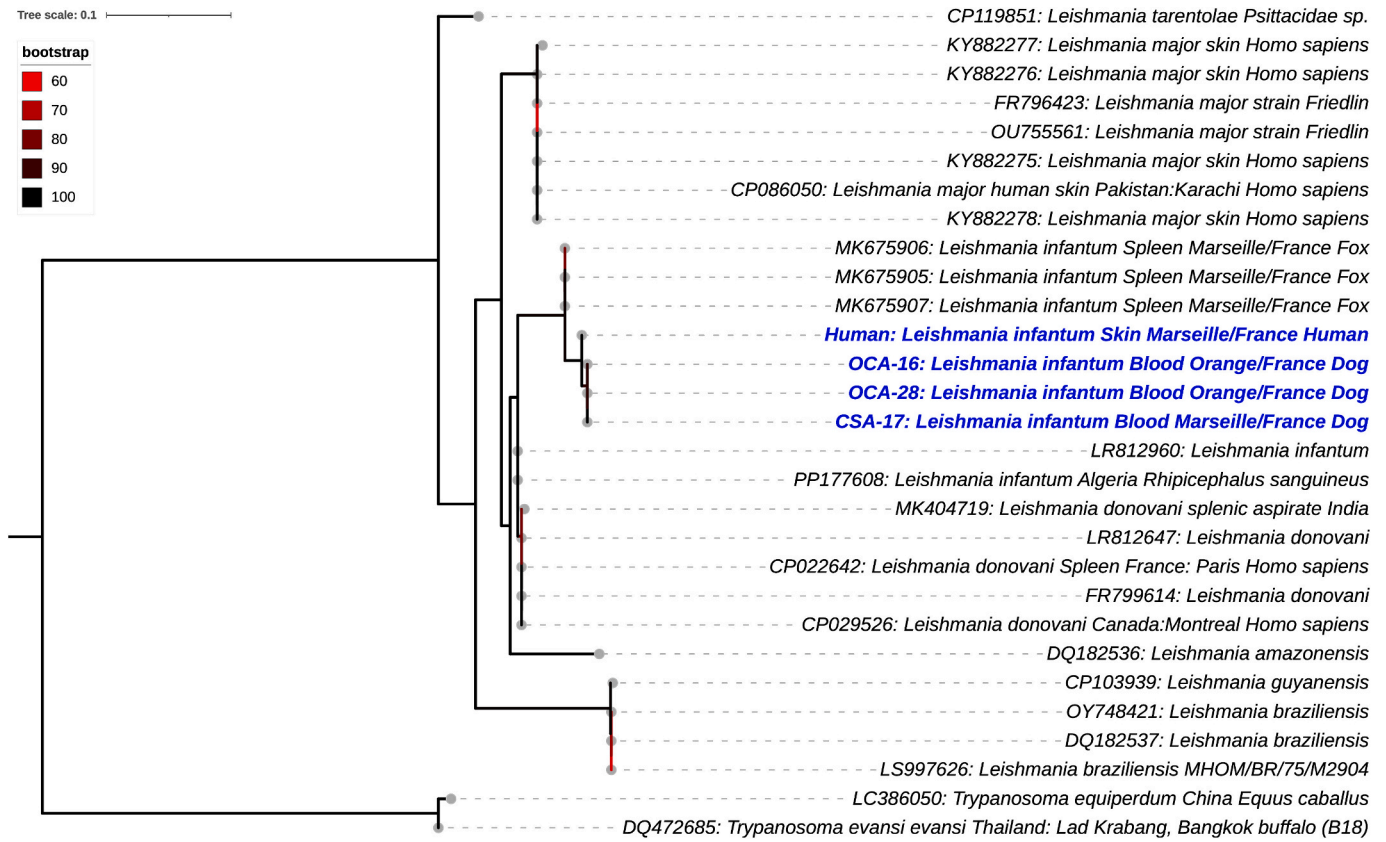

Fig. 4. Maximum likelihood phylogenetic representation of 27 *Leishmania* partial (530 nt) ITS2 gene with a total of 206 (38.9%) of parsimony informative sites. Sequence from *Trypanosoma evansi evansi* (accession number: DQ472685) and *Trypanosoma equiperdum* (accession number: LC386050) were used as outgroup to root the tree. The tree was generated using the TN + F + G4 as the best-fit model according to BIC and 100,000 replicates of ultrafast bootstrap using IQ-TREE software. Tree parameters are as follow: Total tree length of 1.0156 with a sum of internal branch lengths: 0.8774 (86.3919% of tree length), Bayesian (BIC) and Akaike (AIC) information criterion scores are respectively: 4119.0530 and 3918.2278. Number of free parameters is 47 with a log-likelihood −1912.1139 (s.e. 58.6374). Blue bolded labels represent ITS2 sequences generated within the present study. Species names, hosts, and geographical origin, if available, are showed at the tip of each taxon. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Consent to publish

Written informed consent was obtained from the patient for publication of her clinical case.

CRediT authorship contribution statement

Younes Laidoudi: Writing – review & editing, Writing – original draft, Visualization, Software, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Jacques Sevestre:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Samia Bedjaoui:** Writing – review & editing, Investigation, Formal analysis, Data curation. **Stéphanie Watier-Grillot:** Writing – review & editing, Project administration, Funding acquisition, Conceptualization. **Bernard Davoust:** Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare no conflict of interest.

Data availability

Data will be made available on request.

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