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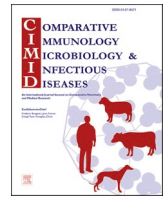
Full length article

New records of pathogenic bacteria in different species of fleas collected from domestic and peridomestic animals in Spain. A potential zoonotic threat?

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ABSTRACT

Climate change is causing many vectors of infectious diseases to expand their geographic distribution as well as the pathogens they transmit are also conditioned by temperature for their multiplication. Within this context, it is worth highlighting the significant role that fleas can play as vectors of important pathogenic bacteria. For this purpose, our efforts focused on detecting and identifying a total of 9 bacterial genera (*Rickettsia* sp.; *Bartonella* sp.; *Yersinia* sp.; *Wolbachia* sp., *Mycobacterium* sp., *Leishmania* sp., *Borrelia* sp., *Francisella* sp. and *Coxiella* sp.) within fleas isolated from domestic and peridomestic animals in the southwestern region of Spain (Andalusia). Over a 19-months period, we obtained flea samples from dogs, cats and hedgehogs. A total of 812 fleas was collected for this study. Five different species were morphologically identified, including *C. felis*, *C. canis*, *S. cuniculi*, *P. irritans*, and *A. erinacei*. *Wolbachia* sp. was detected in all five species identified in our study which a total prevalence of 86%. Within *Rickettsia* genus, two different species, *R. felis* and *R. asembonensis* were mainly identified in *C. felis* and *A. erinacei*, respectively. On the other hand, our results revealed a total of 131 fleas testing positive for the presence of *Bartonella* sp., representing a prevalence rate of 16% for this genus identifying two species *B. henselae* and *B. clarridgeiae*. Lastly, both *Y. pestis* and *L. infantum* were detected in DNA of *P. irritans* and *C. felis*, respectively isolated from dogs. With these data we update the list of bacterial zoonotic agents found in fleas in Spain, emphasizing the need to continue conducting future experimental studies to assess and confirm the potential vectorial role of certain synanthropic fleas.

1 Introduction

Climate change, coupled with the overall increase in temperatures, is causing many vectors of infectious diseases to expand their geographic distribution to areas where they historically had not been developed. It is important to consider that the majority of known vectors are arthropods, and they directly depend on the ambient temperature to regulate their vital functions. Climate change is leading to an average increase in annual temperatures, much more pronounced during winter temperatures. With shorter winters, the onset of their presence is advancing, and their entry into hibernation is delayed, significantly extending their period of activity, which makes it possible for them to have more generations during that time. Additionally, not only does temperature influence the vectors, but the pathogens they transmit are also conditioned by temperature for their multiplication. The higher temperatures

shorten the multiplication cycle of pathogens, facilitating the emergence of diseases in areas where previously the vectors were unable to transmit pathogens due to inadequate temperatures for pathogen multiplication within the vector [1–4].

Spain is one of the regions in the world where the effects of climate change and its close relationship with the expansion of significant vectors and the diseases they transmit can already be observed. In Spain, between 1970 and 2000, there has been a general increase in the average annual temperature of almost 2ºC, with the most evident warming occurring during winter. Additionally, there has been a significant increase in the number of days with maximum temperatures exceeding 25ºC, indicating a trend towards heatwaves. In the projected scenarios for this 21st century, an increase of 0.4ºC per decade is expected during winter and 0.6–0.7ºC in summer [1,5]. On the other hand, there are several examples in Spain that illustrate an expansion of the

distribution area of certain vectors and their pathogens associated with this temperature increase. For example, the ceratopogonid *Culicoides imicola*, the most important vector of viral diseases in livestock, such as bluetongue and African horse sickness viruses, several species of the genus *Phlebotomus* that transmit *Leishmania infantum*, and especially exotic invasive Aedine mosquitoes that are reaching the Old Continent, such as *Aedes atropalpus* or *Aedes triseratus* [6–10]. We also should highlight the case of the Crimean-Congo hemorrhagic fever (CCHF), a tickborne viral disease caused by the CCHF virus. This is considered an emerging infectious disease in Spain because of the expanding distribution of its main vector, ticks of the genus *Hyalomma*, therefore, recently, the epidemiologic pattern of CCHF in Spain has been assessed based on occasional cases with an elevated mortality rate in the last ten years [11].

Within this context, it is worth highlighting the significant role that fleas can play as vectors of important pathogenic bacteria, some of which caused significant global outbreaks, such as plague caused by the gram-negative bacterium *Yersinia pestis* or murine typhus caused by infection with *Rickettsia typhi* [12]. The ability of fleas to serve as vectors for emerging diseases has also been demonstrated, such as spotted fever caused by *Rickettsia felis* [13] or various bartonelloses [14]. Particularly, *Bartonella henselae* and *Bartonella clarridgeiae* are considered the main causative agents of cat scratch disease, while *Bartonella quintana*, *Bartonella elizabethae*, or *Bartonella koehlerae* have been related to human and animal endocarditis in several countries [15,16]. In spite of that, although the list of flea species found naturally infected with various species of *Bartonella* continues to grow, much remains to be learned about the role of fleas as truly vectors of these pathogens [12,17]. On the other hand, although Rickettsioses and Bartonelloses are the most common genera of bacteria found or transmitted by fleas, other pathogens have been detected in fleas such as *Coxiella burnetii* [18], *L. infantum* [19], *Francisella tularensis* [20], *Mycobacterium* sp. [17], or *Borrelia burgdorferi* [21].

Therefore, numerous species of fleas are known to transmit various infectious diseases, and every day, more studies are being published detecting numerous pathogenic bacteria in different flea species where they had never been detected before, raising concerns about the possible implication of these species in the transmission of certain pathogenic bacteria [12,22,23]. In this regard, special attention should be given to those flea species that parasitize domestic and peridomestic animals and have a close relationship with humans, whether it is within the household, workplace, or even during leisure activities [24–26]. Moreover, epidemiological data is still missing for several flea species which may play an important role in the transmission of zoonotic vector borne pathogens [17]. Therefore, understanding and mitigating the potential health risks to humans associated with these fleas become of vital importance.

Taking these precedents as a starting point, the main objective of our study was to detect the presence and assess the prevalence of numerous pathogenic bacteria, the transmission of which could be linked to species within the Order Siphonaptera. For this purpose, our efforts focused on detecting and identifying a total of 9 bacterial genera (*Rickettsia* sp.; *Bartonella* sp.; *Yersinia* sp.; *Wolbachia* sp., *Mycobacterium* sp., *Leishmania* sp., *Borrelia* sp., *Francisella* sp. and *Coxiella* sp.) within fleas isolated from domestic and peridomestic animals in the southwestern region of Spain (Andalusia).

2 Material and methods

2.1 Ethical statement, sample collection and morphological identification

Over a 19-months period, we obtained flea samples from 182 dogs (*Canis lupus familiaris*), 78 cats (*Felis silvestris catus*) and one hedgehog (*Erinaceus europaeus*) that coexisted with other dogs. To collect fleas from all these hosts, we contacted various veterinary clinics, veterinary hospitals, pet shelters and some pet owners. To conduct our study, we contacted a total of 145 veterinary clinics and 30 pet shelters and kennels. Out of these, 18 centers agreed to collaborate in the collection of samples (see Acknowledgements). They all participated in this sampling voluntarily. Only animals parasitized by fleas were sampled. Veterinary practitioners performed an initial inspection of dogs and cats brought to their practices. Each pet was inspected for fleas and examined by a veterinarian who recorded clinical signs related to flea infestation. Adult flea counts on dogs and cats were conducted as described in the World Association for the Advancement of Veterinary Parasitology (WAAVP) guidelines [27]. Briefly, dogs and cats were combed over the entire body with a fine-toothed comb for 5–10 min. The fleas obtained from hedgehogs were collected from an individual that was found killed on the road during the sampling period. Then it was moved to our lab where fleas were removed from the hedgehogs using topical antiparasitic sprays and gently shaking the animal over the white sheet of paper. No specific approval of any Institutional Animal Care and Use Committee were needed in this study since no anesthesia substances were applied for animal handling procedures.

All veterinary centers and individuals that participated in our study were located in the Western region of Andalusia, Spain, specifically in different towns in the provinces of Seville, Cádiz, Córdoba and Huelva (Table 1) (Fig. 1). Within the province of Seville, fleas were collected from a total of 30 different localities; whereas, in Córdoba, Huelva, and Cádiz, the number of localities included in this study was 5, 14, and 2, respectively (Table 1).

The sample collection period took from late June 2021 to January 2023. Veterinarian practitioners used to send fleas collected every three months from infested dogs and cats visiting the veterinary clinic and hospitals. Furthermore, the collaborating pet shelters and kennels sent their samples every 2–3 months, and sometimes even more frequently when they encountered a host with a high infestation rate. For each parasitized host, an epidemiological survey was completed, including the following information: Geographical origin, breed, age, sex, rural or urban habitat, type of animal's dwelling (domestic, stray hosts, wild/feral, farm, house with garden, apartment or others), health status, cohabiting or not with other animals, host activities, and the total number of collected fleas. All captured fleas from each infested host were transferred to a small plastic 1.5 ml tube containing 96% ethanol until processing. Then in our lab, fleas were sexed and identified to species using a CX21 microscope (Olympus, Tokyo, Japan). Diagnostic morphological characters of all the samples were studied by comparison with figures, keys, and descriptions reported by Hopkins and Rothschild [28] and Beaucournu and Launay [29].

2.2 Molecular detection of pathogens

After morphological identification, total DNA of each flea sample was extracted from fleas using the DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer's protocol. Then, genomic DNA was checked using an electrophoresis in 0.8% agarose gel electrophoresis infused with SYBR Safe and was either immediately used or stored at −20°C until use.

Quantitative PCR was individually performed according to the manufacturer's protocol using a PCR detection system: a CFX Connect™ Real-Time (Bio-Rad) using iTaq Universal probes supermix (Bio-Rad) and IDT Prime time® standard qPCR assay (Integrated DNA Technologies). The qPCR reaction contained 1 µl of IDT Prime time® standard qPCR assay containing 500 nM of each primer and 250 nM of probe, 10 µl of iTaq Universal probes supermix (Bio-Rad), 3.5 µl of sterile distilled water, 0.5 µl of Uracil-DNA-glycosylase (UDG) and 5 µl of the DNA extract. The cycling protocol used for qPCR analysis was described by Diarra et al. [30], using it for all the qPCR assays conducted in this study. All samples were screened using primers and probes targeting specific sequences of the following microorganisms: *Rickettsia* spp., *R. felis*, *R. typhi*, *Wolbachia* sp., *Borrelia* spp., *Bartonella* spp., *B. henselae*, *B. quintana*, *C. burnetii*, *L. infantum*, *Y. pestis*, *F. tularensis* and

Table 1

Species, geographical origin, gender and hosts of the total flea samples collected in this study. N= Total number of hosts.

Species	Locality/province	Number of fleas (female/male)	Hosts (N)
Archaeopsylla erinacei	Dos Hermanas, Sevilla	14 (8/6)	Hedgehog (1)
	Dos Hermanas, Sevilla	23 (15/8)	Dogs (3)
	Fuente Palmeras, Córdoba	2 (1/1)	
		Total: 39 (24/15)	
Pulex irritans	Cazalla de la sierra, Sevilla	5 (5/0)	Dogs (21)
	Alcalá del Río, Sevilla	8 (5/3)	
	Cantillana, Sevilla	4 (3/1)	
	Arahal, Sevilla	1 (0/1)	
	Aguadulce, Sevilla	2 (2/0)	
	Herrera, Sevilla	1 (0/1)	
	Jabugo, Huelva	2 (2/0)	
	Galaroza, Huelva	6 (4/2)	
	Ayamonte, Huelva	1 (0/1)	
	Fuente Palmeras, Córdoba	14 (7/7)	
		Total: 44 (28/16)	
Spilopsyllus cuniculi	Fuente Palmeras, Córdoba	10 (6/4)	Cat (1)
		Total: 10 (6/4)	
Ctenocephalides canis	Cazalla de la sierra, Sevilla	3 (2/1)	Dogs (4)
	El Repilado, Huelva	2 (1/1)	
	Galaroza, Huelva	1 (1/0)	
		Total: 6 (4/2)	
Ctenocephalides felis	Sevilla, Dos Hermanas	8 (4/4)	Cats (77)
	Sevilla, Sevilla	85 (65/20)	
	Tomares, Sevilla	25 (20/5)	
	Valencina de la Concepción, Sevilla	7 (6/1)	
	Aznalcázar, Sevilla	1 (1/0)	
	Alcalá de Guadaira, Sevilla	6 (5/1)	
	Brenes, Sevilla	1 (1/0)	
	Espartinas, Sevilla	1 (1/0)	
	Écija, Sevilla	13 (11/2)	
	Lantejuela, Sevilla	7 (6/1)	
	El Viso del Alcor, Sevilla	3 (3/0)	
	Tocina, Sevilla	1 (1/0)	
	Los Rosales, Sevilla	1 (0/1)	
	La Puebla del Río, Sevilla	41 (34/7)	
	Montequinto, Sevilla	2 (2/0)	
	El Puerto de Santa María, Cádiz	10 (7/3)	
	Galaroza, Huelva	2 (1/1)	
	Huelva, Huelva	1 (1/0)	
	Minas de Río Tinto, Huelva	1 (1/0)	
	Nerva, Huelva	2 (1/1)	
	Ayamonte, Huelva	12 (12/0)	
	Fuente Palmeras, Córdoba	11 (8/3)	
	Posadas, Córdoba	30 (26/4)	
		Total: 271 (217/54)	
	Sevilla, Sevilla	5 (3/2)	Dogs (162)
	Tomares, Sevilla	1 (1/0)	
	San Juan de Aznalfarache, Sevilla	1 (1/0)	
	Palomares, Sevilla	29 (19/10)	
	Cazalla de la sierra, Sevilla	30 (21/9)	
	El Pedroso, Sevilla	4 (2/2)	
	Constantina, Sevilla	6 (3/3)	
	Alcalá del Río, Sevilla	1 (1/0)	
	El Viso del Alcor, Sevilla	8 (6/2)	
	Pilas, Sevilla	42 (36/6)	
	Carrión de los Céspedes, Sevilla	1 (1/0)	
	Huévar del Aljarafe, Sevilla	1 (1/0)	
	Villamanrique de la Condesa, Sevilla	1 (1/0)	
	Arahal, Sevilla	4 (0/4)	
	Écija, Sevilla	20 (16/4)	
	Aguadulce, Sevilla	1 (0/1)	
	Marinaleda, Sevilla	1 (1/0)	
	Estepa, Sevilla	12 (10/2)	
	San Fernando, Cádiz	2 (2/0)	
	El Puerto de Santa María, Cádiz	55 (42/13)	
	Galaroza, Huelva	5 (4/1)	
	Jabugo, Huelva	1 (1/0)	
	Fuenteheridos, Huelva	2 (2/0)	
	Alájar, Huelva	18 (9/9)	
	Santa Ana la Real, Huelva	2 (0/2)	
	Higuera de la Sierra, Huelva	6 (5/1)	
	Cortegana, Huelva	8 (4/4)	
	Campofrío, Huelva	1 (1/0)	
	Nerva, Huelva	5 (2/3)	
	Minas de Río Tinto, Huelva	3 (3/0)	
	Ayamonte, Huelva	21 (18/3)	
	Sanlúcar de Guadiana, Huelva	1 (1/0)	
	Fuente Palmeras, Córdoba	85 (68/17)	
	Posadas, Córdoba	44 (31/13)	
	Almodóvar del Río, Córdoba	9 (7/2)	
	Rivero de Posadas, Córdoba	4 (3/1)	
	La Carlota, Córdoba	2 (2/0)	
		Total: 442 (328/114)	

Mycobacterium spp. As qPCR positive controls, we designed a specific gBlock Gene Fragment (Integrated DNA Technologies) for each of the target pathogenic bacteria, except for the detection of *Wolbachia* sp., *Rickettsia* spp. and *Bartonella* spp. For these latter cases, we utilized positive DNA flea samples available in our laboratory from previous studies. On the other hand, for each assay, as negative controls we used 5 µl of sterile distilled water replacing the DNA extract. The primers, probes and the targeted sequences used to detect each pathogen by real-time quantitative PCR are summarized in Table 2. For all samples tested by qPCR, only those with a cycle threshold (Ct) value lower than 33 were considered positive. The *Bartonella* spp.-positive samples that were negative for *B. henselae*- and *B. quintana*-specific qPCRs were subsequently subjected to standard PCR (amplifying ^{16}S–^{23}S ribosomal RNA Intergenic Spacer) prior to sequencing in order to obtain identification to the species level. The same protocol was carried out for *Rickettsia* spp., but two different genes (*citrate synthase* (*gltA*) and ^{23}S–^{5}S ribosomal RNA Intergenic Spacer) were tested in standard PCR in order to avoid false positives, thus only the samples positive for one of these two genes were considered positive. A thermal cycler (Eppendorf AG; Eppendorf, Hamburg, Germany) was used to amplify the target sequences prior to sequencing. Details about the primers and conditions used for standard PCR in this study are also listed in Table 2. The PCR products were checked on SYBR Safe stained 2% Tris–borate– ethylenediaminetetraacetic acid (TBE) agarose gels. PCR products were purified using the QWizard SV Gel and PCR Clean-Up System Kit (Promega, Madison, WI, U.S.A.). Once purified, these products were sent to the commercial company Stab Vida (Lisbon, Portugal) for sequencing process. We separately sent purified PCR products and 20 µl of 100 µM of each pair of primers (see Table 2) for each molecular marker. Sanger
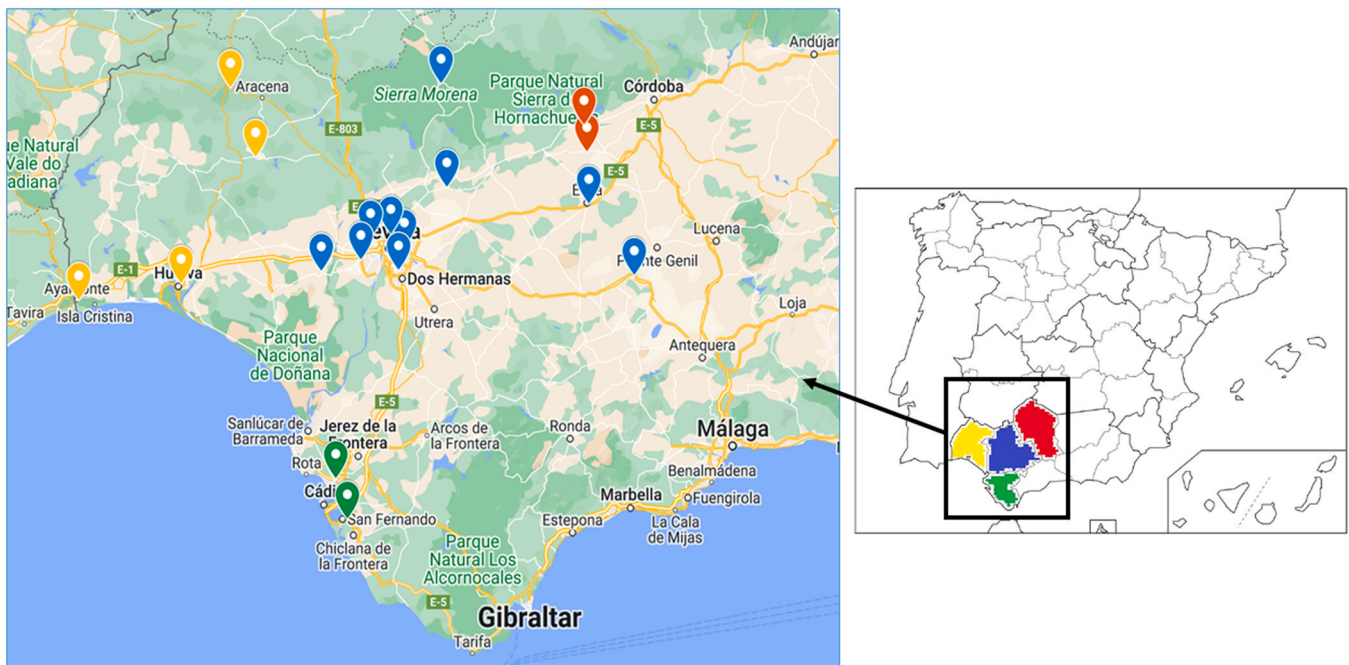
Fig. 1. Overview of the sampling locations in the four Andalusian provinces of Spain: Cádiz (Green spots), Córdoba (Red spots), Huelva (Yellow spots), Sevilla (Blue spots). Map edited using the application ``MyMaps-Google Maps (https://www.google.es/maps)'', ``Map data ©2023 Google''.

sequencing was carried out using an automatic LI-COR ® DNA sequencer. Sequences were aligned with the MUSCLE alignment method (Edgar, 2004) in MEGA, version 5.2 [36]. Alignment settings comprised a gap open = −400.00, a gap extend = 0.0, an UPGMA (Unweighted Pair Group Method with Arithmetic Mean) as a cluster method and a minimum Diagonal length = 24. Finally, the sequences were compared with sequences available in the GenBank database using the BLAST algorithm (http://blast.ncbi.nlm. nih.gov/Blast.cgi).

3 Results

3.1 Morphological identification

A total of 812 fleas was collected for this study. Five different species were morphologically identified, including *Ctenocephalides felis*, *Ctenocephalides canis*, *Spilopsyllus cuniculi*, *Pulex irritans*, and *Archaeopsylla erinacei* (Table 1) (Fig. 2). The majority species was *C. felis* with a total of 713 collected specimens, which accounted for 88% of the total fleas obtained. The next most abundant species was *P. irritans*, with a total of 44 collected fleas (5% of the total). Lastly, the remaining species identified were *A. erinacei* with 39 collected specimens, 10 fleas classified as *S. cuniculi*, and 6 fleas as *C. canis*, which accounted for 5%, 1%, and 1%, respectively of the total fleas collected (Table 1). Of all the fleas collected from cats, 96% of them (n=271) were identified as belonging to the species *C. felis*, while the remaining fleas were attributed to the species *S. cuniculi*. On the other hand, from the fleas collected from dogs, a total of four species were identified (*C. felis*, *C. canis*, *P. irritans*, and *A. erinacei*), with *C. felis* being the predominant species, representing 85% of all fleas isolated from dogs. It is noteworthy that *A. erinacei* was the only flea species isolated from the hedgehog analyzed in our study (Table 1). Regarding the number of parasitized host animals, samples were obtained from a total of 261 different hosts. Of these, 69.7% (n=182) were dogs, while 29.9% (n=78) were cats, and the remaining samples were obtained from a single hedgehog. Out of the total of 812 fleas obtained, 75% of them (n=608) were females, while 25% (n=204) were identified as males (Table 1).

3.2 Molecular detection of microorganisms

All fleas were individually tested to assess the prevalence of known vector-borne microorganisms using molecular methods (Table 3). All the samples showed qPCR negative results for the presence of *R. typhi*, *B. quintana*, *F. tularensis*, *Mycobacterium* spp., *C. burnetii* and *Borrelia* spp. Using qPCR approaches, the endosymbiont *Wolbachia* sp. was detected in all five species identified in our study. In general, a total of 702 fleas tested positive for the presence of this endosymbiont, which corresponds to a total prevalence rate of 86%, while if we analyzed these results by species, the percentages obtained in most cases were quite high, ranging from 40% observed in the case of the species *S. cuniculi* to 100% observed in *P. irritans* (Table 3). Analyzing the prevalence based on the sex of the assessed fleas, it was observed that the presence of *Wolbachia* sp. was higher in females (94%) than in males (64%).

Regarding to *Rickettsia* spp., a total of 181 samples were positive for this bacterial genus, corresponding with a total prevalence of 22%. Within this genus, two different species, *R. felis* and *R. asembonensis*, were detected. *R. felis* was primarily detected using qPCR techniques; however, in some cases, species-level detection could only be achieved through the amplification and sequencing of the ^{23}S–^{5}S ribosomal RNA Intergenic Spacer using standard PCR techniques. In these cases, different BLAST analysis were carried out showing a percentage of identity of 100% with a reference sequence of *R. felis* (GenBank accession number: KF245441). *R. felis* was detected with a percentage of prevalence of 34% in *C. felis* isolated from cats, 12% in *C. felis* isolated from dogs, and 1% in *A. erinacei* collected from dogs. On the other hand, *R. asembonensis* was exclusively detected by standard PCR in the species *A. erinacei*, isolated from both hedgehogs and dogs with very high percentages of prevalence (100% and 92%, respectively) (Table 3). The molecular detection of this species was carried out amplifying and sequencing at least one of the both molecular markers *citrate synthase* (*gltA*) gene and ^{23}S–^{5}S ribosomal RNA Intergenic Spacer. BLAST analysis were carried out showing a percentage of identity of 100% with a reference sequence of *R. asembonensis* (GenBank accession numbers: CP116496 and MN003394). All the *Rickettsia* spp. sequences obtained in this study are now available in GenBank database (GenBank Accession numbers: OR523789, OR523790, OR523791, OR523792 and

Table 2

Primers, probes and targeted sequences used for real-time quantitative and standard PCR in this study. Fwd: Forward primer; Rvs: Reverse primer; Pb: Probe.

qPCR		
Microorganism	**Targeted sequence**	**Primers and probes**
Rickettsia spp.	*Citrate synthase* (*gltA*)	Fwd: RickgltaF: 5´GTGAATGAAAGATTACACTATTTAT 3´
		Rvs: RickgltaR: 5´GTATCTTAGCAATCATTCTAATAGC 3´
		Pb: RickgltaP: 5´CTATTATGCTTGCGGCTGTCGGTTC 3´
		[31]
R. felis	*Phosphatase gene*	Fwd: 5´GGCGTAGTTCTGAGCGAATAA 3´
		Rvs: 5´ATTCTTGGTCCCACGGATAAC 3´
		Pb: 5´ CTCATCAATTTCACCGATGGTTGCACC 3´
R. typhi	Hypothetical protein	(Designed in this study) Fwd: 5´TGCTTCCTCTACTGTATCATATTG 3´
		Rvs: 5´GCAGAGCATAACATCTCTTCTAA 3´
		Pb: 5´TGCTTCTCGTGCTTCAGATTCGTGT 3´
Bartonella spp.	ITS2	(Designed in this study) Fwd: BartoITS2F: 5´GATGCCGGGGAAGGTTTTC 3´
		Rvs: BartoITS2R: 5´GCCTGGGAGGACTTGAACCT 3´
		Pb: BartoITS2P: 5´GCGCGCGCTTGATAAGCGTG 3´
		[32]
B. henselae	*Hemin binding protein A* (*hbpA*)	Fwd: 5´TGGCGGTGGTGTTGATTT 3´
		Rvs: 5´GCTCAGTTCGCTTCCTTTCT 3´
		Pb: 5´TGCGTGCAGAATACCGTTACTCAGA 3´
B. quintana	Hypothetical protein	(Designed in this study) Fwd: 5´ATAGCGGAGTTCAATCTTCCAG 3´
		Rvs: 5´AGGATGTAGGTTCAAGCAAGAG 3´
		Pb: 5´TCGATCATCAAACCGTTGCCGACA 3´
Borrelia spp.	16 S	(Designed in this study) Fwd: 5´CGAGCGTTGTTCGGGATTAT 3´
		Rvs: 5´CCAACATAGGTCCACAGTTGAG 3´
		Pb: 5´ATCCGCCTACTCACCCTTTACGC 3´
Wolbachia sp.	23 S	(Designed in this study) Fwd: Wol16SF: 5´CCAGCAGCCGCGGTAAT 3´
		Rvs: Wol16SR: 5´CGCCCTTTACGCCCAAT 3´
		Pb: Wol16SP: 5´CGGAGAGGGCTAGCGTTATTCGGAATT 3´
		[33]
L. infantum	Minicircle kinetoplast sequence	Fwd: 5´GTGTGTGGGATTGGTAGTAGTG 3´
		Rvs: 5´GTCCGATACGTCAGCACATT 3´

Table 2 (*continued*)

qPCR		
Microorganism	**Targeted sequence**	**Primers and probes**
		Pb: 5´ATTGGGTTGCCGTGATTTGCCTTC 3´
C. burnetii	IS1111 gene	(Designed in this study) Fwd: 5´GAAGCCGATAGCCCGATAAG 3´
		Rvs: 5´GAAAGCGGTTGCATTCGTATATC 3´
		Pb: 5´ATTCATCAAGGCACCAATGGTGGC 3´
Mycobacterium spp.	ITS	(Designed in this study) Fwd: 5´GGCGTGTTCTTTGTGCAATA 3´
		Rvs: 5´CGTCCTTCATCGGCTCTC 3´
		Pb: 5´TAAGTGTCTAAGGGCGCATGGTGG 3´
Y. pestis	*pla* gene	(Designed in this study) Fwd: 5´ACAGCAGGATATCAGGAAACAC 3´
		Rvs: 5´CCTATTACCCGCACTCCTTTC 3´
		Pb: 5´ACCACCTGTAGCTGTCCAACTGAAAC 3´
F. tularensis	*yqaB* gene	(Designed in this study) Fwd: 5´ GCTGATGATAATCACCCGAGTAAAA 3´
		Rvs: 5´TCCTGGAACACCATCTTCAAAAA 3´
		Pb: 5´CCCCAAGGCGTTTACTTTGATCGCA 3´
		(Designed in this study)
Standard PCR *Bartonella* spp.	16S–23S ribosomal RNA Intergenic Spacer	Fwd: URBarto1: 5´CTTCGTTTCTCTTTCTTCA 3´
		Rvs: URBarto2: 5´CTTCTCTTCACAATTTCAAT 3´
		Primers and PCR conditions defined by Rolain et al. [32]
Rickettsia spp.	23S–5S ribosomal RNA Intergenic Spacer	Fwd: RCK/23–5 F: 5´GATAGGTCRGRTGTGGAAGCAC 3´
		Rvs: RCK/23–5 R: 5´TCGGGAYGGGATCGTGTGTTTC 3´
		Primers and PCR conditions defined by Jado et al. [34]
	citrate synthase (*gltA*)	Fwd: RpCS.877p: 5´GGGGGCCTGCTCACGGCGG 3´
		Rvs: RpCS.1258 n: 5´ATTGCAAAAAGTACAGTGAACA 3´
		Primers and PCR conditions defined by Regnery et al. [35]

OR523793).

Our study revealed a total of 131 fleas testing positive for the presence of *Bartonella* sp., representing a prevalence rate of 16% for this genus within our study. Using qPCR and standard PCR (only for 3 qPCR *Bartonella* spp.-positive samples) methods, we detected the species *B. henselae* exclusively in *C. felis*. The prevalence of this pathogenic species was slightly higher in specimens isolated from cats (7%) than in those isolated from dogs (1%). On the other hand, through the amplification and sequencing of the 16S–23S ribosomal RNA Intergenic Spacer, we detected the species *B. clarridgeiae* just in 75 specimens of *C. felis*, representing a prevalence rate of 11% for this bacterium in this flea species. If we delve deeper, we find that the prevalence of this bacterial
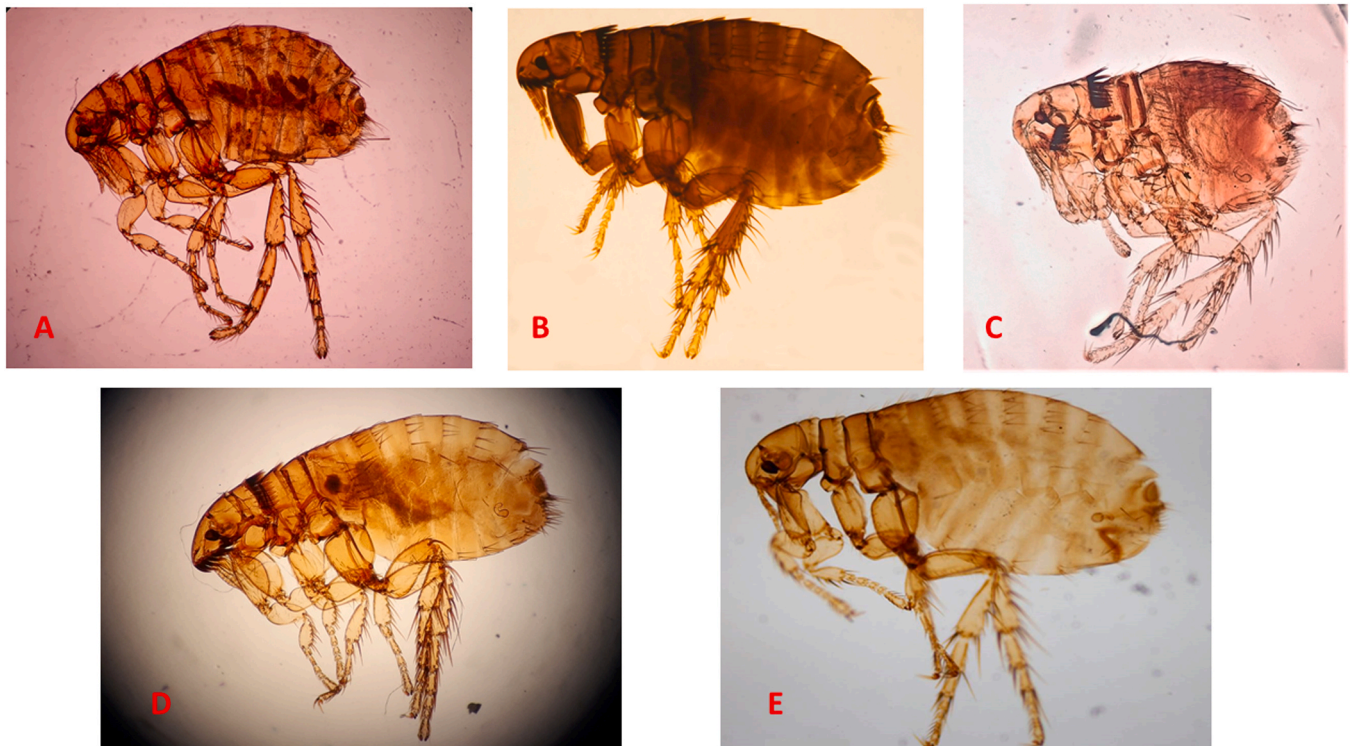
Fig. 2. Photos of flea species identified in this study. A. Female of *A. erinacei*. B. Female of *C. canis*. C. Female of *S. cuniculi*. D. Female of *C. felis*. E. Female of *P. irritans*.

Table 3
Percentage of microorganism-positive fleas detected by qPCR and standard PCR in this study.

	Wolbachia sp.	*R. felis*	*R. asembonensis*	*Bartonella* spp.	*B. henselae*	*B. clarridgeiae*	*Y. pestis*	*L. infantum*
C. felis collected from cats	243/271 (90%)	92/271 (34%)	-	3/271 (1%)	19/271 (7%)	61/271(23%)	-	-
C. felis collected from dogs	381/442 (86%)	51/442 (12%)	-	26/442 (6%)	4/442 (1%)	14/442 (3%)	-	2/442 (0,5%)
P. irritans collected from dogs	44/44 (100%)	-	-	3/44 (7%)	-	-	1/44 (2%)	-
S. cuniculi collected from cats	4/10 (40%)	-	-	-	-	-	-	-
A. erinacei collected from hedgehogs	8/14 (57%)	-	14/14 (100%)	1/14 (7%)	-	-	-	-
A. erinacei collected from dogs	17/25 (68%)	1/25 (4%)	23/25 (92%)	-		-	-	-
C. canis collected from dogs	5/6 (83%)	-	-	-		-	-	-

species was higher in *C. felis* isolated from cats (23%) than in those isolated from dogs (3%) (Table 3). BLAST analysis was carried out using the *Bartonella*-ITS sequences obtained showing a percentage of identity of 100% with a reference sequence of *B. clarridgeiae* (GenBank accession number: AF167989) and a percentage of identity of 100% with a reference sequences of *B. henselae* (GenBank accession numbers: JN646684 and AJ439688). Unfortunately, we were not able to amplify the ITS region of 33 qPCR *Bartonella* spp.-positive samples, thus, in these cases we did not manage to identify these pathogens at species level (Table 3). All the *Bartonella* spp. sequences obtained in this study are now available in GenBank database (GenBank accession numbers: OR505861, OR505862, OR505863 and OR505864).

One of the pathogenic bacteria detected in our study was *Y. pestis*. This species was exclusively found in a specimen of *P. irritans*. This specimen was isolated from a healthy 4-month-old puppy dog in Estepa (Seville, Spain), which was living as a companion animal on a farm with no other animals in proximity. Additionally, through our study, we were also able to detect the presence of *L. infantum* in two fleas identified as *C. felis* isolated from two different dogs. From the epidemiological study

conducted on each of the hosts, it was observed that both dogs originated from Puerto de Santa María (Cádiz) and Sevilla. One of them was a healthy companion dog living in an urban environment (house with a garden), while the second one was also a companion dog in an urban setting but, upon initial veterinary analysis, showed some general weakness and significant signs of dermatitis.

4 Discussion

Of all the zoonotic bacterial agents analyzed in our study, only six of them were not detected in any of the fleas under investigation (*R. typhi*, *B. quintana*, *F. tularensis*, *Mycobacterium* spp., *C. burnetii* and *Borrelia* spp.). In spite of that, as we mentioned in the introduction section, all these bacterial species have previously been detected in various flea species in different geographic regions [17–21]. Therefore, we should not rule out the potential vectorial role that Siphonaptera specimens could play in the transmission of these pathogenic agents among animals and humans. On the other hand, we must always consider some limitations, such as, the possibility of false negatives in this kind of study and

the fact that the prevalence of certain pathogenic bacteria in vector arthropods can vary considerably depending on the species and geographic area under study.

In our study, we have once again confirmed the widespread presence of the endosymbiont *Wolbachia* sp. within numerous species of the Order Siphonaptera. This bacterial genus has been detected with high prevalence values in many flea genera and species such as *C. felis* [37–39], *C. canis* [40], *P. irritans* [40,41], *A. erinacei* [42], *Ctenophthalmus* spp. [17], *Tunga penetrans* [43], *Xenopsylla* spp. [41,44], *Nosopsyllus* spp. [17, 44] or *Echidnophaga gallinacea* [45]. As far as we know, in this study we detected for the first time *Wolbachia* sp. in *S. cuniculi*, supporting the hypothesis of Gorham et al. [45] and Dittmar & Whiting [41] who considered *Wolbachia* sp. as a common symbiont related with Siphonaptera Order. Furthermore, it was observed that the prevalence percentage for this endosymbiont was much higher in females, with values closed to 100%, than in males. These results align with those observed in subsequent studies [37,42,45], highlighting a higher prevalence of infestation by *Wolbachia* sp. in female fleas compared to what is observed in males. Although this bacterium is not considered a pathogenic agent, it has had considerable interest due to the ability of this bacterium to manipulate the reproduction of its hosts, influencing critical processes such as sex determination, cell cycle and speciation [46, 47]. These effects on its host can include parthenogenesis, feminization, male killing and sperm egg cytoplasmic incompatibility (CI). Further, a mutualistic relationship between *Wolbachia* sp. and some host species has also been described [48]. All these remarkable characteristics have placed it in a position of honor in biological research, not only in basic science but also in applied fields, as it possesses several properties that mark it as a potential tool for the control of pest insect populations and disease vectors [49]. However, the role and influence of the presence of *Wolbachia* sp. in flea biology still remain unclear. Therefore, some authors have recently conducted various experimental studies that have either refuted or at least failed to substantiate various traditionally accepted hypotheses such as that the interaction with the endosymbiont is not obligatory for either female or male hosts; there is no evidence for fitness advantages for female hosts carrying the endosymbiont; or there is no indication for reproductive manipulation inducing female-biased sex ratio [50,51]. In this sense, further studies should be carried out in order to clarify the role of this endosymbiont in the biology of fleas and other arthropods.

R. felis is another common pathogenic bacterium found in fleas from domestic and peridomestic animals. This species is responsible for a characteristic symptoms illness in humans causing fever, fatigue, headache, and generalized maculopapular rash and inoculation eschar [52] as well as it is considered an emerging disease [53,54]. Its description and identification as the etiological agent occurred in the early 1990 s, when it was observed, by the first time, by transmission electron microscopy in various tissues of *C. felis* [55] and nowadays it has been considered an important neglected agent of fever in sub-Saharan Africa [13]. Currently, dogs and cats are suspected of serving as reservoir hosts for *R. felis* whereas, the cat flea *C. felis* (cat flea) is still considered the primary vector in the transmission of this bacterial agent [53,56]. Although *R. felis* has been detected in numerous flea species such as *A. erinacei*, *P. irritans*, *Xenopsylla cheopis* or *Leptopsylla segnis* [54] its vectorial role in the transmission of this pathogen has not yet been confirmed through experimental techniques in these species. Our study confirms that *R. felis* is well established as a common pathogen in *C. felis* in southwestern Spain, with detection rates of up to 34% in specimens isolated from cats. These data coincide with those published by other authors in Andalusian region (South of Spain) [17,57] and other regions of the world [54] highlighting the need to control the increase in flea infestation in domestic animals that have close contact with humans. It has been widely proved that exposure to companion animals and their ectoparasites, especially fleas, is a common risk factor associated with *R. felis* rickettsiosis and direct contact with flea infested dogs and cats has been reported in several cases with patients described

flea bites three-four days before fever onset [58–61]. In direct correlation with our study, it is worth mentioning that conducted by Bernabeu-Wittel et al. [60]. These authors carried out a large-scale serological study in patients from the province of Seville (Southwestern of Spain) to detect possible past infections with *R. felis*, *R. typhi*, and *R. conorii*. They observed a prevalence of 6.5% of past infections due to *R. felis* in the study area, especially in people who had close contact with dogs and cats, either due to their lifestyle or occupational activity. Additionally, our study, in accordance with others formerly published [17,62,63], once again confirmed the presence of *R. felis* in the species *A. erinacei* in south of Europe. While this flea species is considered a specific parasite of hedgehogs, it can also infest dogs and cats that share habitats with them, as demonstrated in our study. For this reason, the control of peridomestic animals and their ectoparasites should also be considered when establishing control and prophylaxis measures to prevent the spread of these rickettsioses among the humans and their pet populations that may have some contact with them.

The other *Rickettsia* species detected in the present study was *R. asembonensis*, a Gram negative, obligate intracellular bacteria grouped with other *R. felis*-like organisms (RFLO) or the spotted fever group rickettsiae (SFGR) together with *R. felis* and "*Candidatus Rickettsia senegalensis*". The RFLOs and SFGRs are genetically related to *R. felis* but consist of a unique group of rickettsiae that are associated with various arthropods for which knowledge of their biology and pathogenicity is limited and not always available [64,65]. While the pathogenicity and biology of *R. felis* have been extensively studied, little is still known about the symptoms and lesions caused by *R. asembonensis* or "*Candidatus Rickettsia senegalensis*" can induce in humans and other hosts. These three SFGR agents have worldwide distribution, are often sympatric and most often found parasitizing cat and dog fleas [64]. Thus, *R. asembonensis* has mainly been detected worldwide in recent years in the DNA of fleas of the species *C. felis* in Asia [66], America [67,68] or Africa [69]. Although most studies have been focused in *C. felis*, it has also been detected in several flea genera and species, mainly belonging to Pulicidae and Ceratophyllidae families for example: *Ctenocephalides* (*C. felis*, *C. canis*, and *C. orientis*); *Xenopsylla* (*X. cheopis*, *X. ramesis*, and *X. gerbilli*); *Archaeopsylla* (*A. erinacei*); *Pulex* (*P. irritans*); *Ceratophyllus* (*C. fasciatus*) or *Nosopsyllus* (*N. laeviceps*) [64]. Despite its wide distribution, as far as we know, to date, only one study has reported the presence of this pathogen in fleas in Europe [70]. These authors detected this pathogenic bacterium in fleas of the species *A. erinacei* isolated from wild hedgehogs from Portugal, with a total prevalence of 47%. Our results are perfectly in agreement with those provided by Barrada et al. [70] since we detected the presence of *R. asembonensis* in *A. erinacei* isolated from both hedgehogs and dogs, with very high prevalence rates (100% and 92%, respectively). These results constitute the first evidence of the presence of *R. asembonensis* in fleas in Spain. Furthermore, the high prevalence rates obtained suggest that *R. asembonensis* could be well-established in *A. erinacei* in the southern region of Spain, with hedgehogs being the primary reservoir for this pathogen. It's worth noting that many of these infected fleas were isolated from dogs that coexisted with hedgehogs and other animals in urban or rural areas. Therefore, future studies are necessary to confirm the role of dogs as reservoirs for this rickettsiosis and to investigate whether other common flea species found on domestic and peridomestic animals, such as *C. felis*, *C. canis*, or *P. irritans*, may be involved in the maintenance and spread of this pathogenic bacteria in this geographical area.

The *Bartonella* genus has always been closely linked to fleas from a medical and epidemiological perspective because many diseases transmitted by these hematophagous arthropods are primarily caused by numerous species of this bacterial genus [71]. These infections can result in significant complications such as endocarditis, bacillary angiomatosis, peliosis hepatis, chronic bacteremia, chronic lymphadenopathy, or neurological disorders [72]. Currently, it is known that fleas are the main vector in the transmission of important bartonelloses such as the well-known Cat Scratch Disease (CSD) caused by *B. henselae* or

Trench Fever caused by *B. quintana*. Furthermore, in recent years, the number of *Bartonella* species detected in different flea species continues to increase, with notable ones including *B. clarridgeiae, Bartonella grahamii, B. taylorii,* and *B. elizabethae* [73–75]. The studies conducted in Spain regarding the presence and distribution of *Bartonella* sp. have provided very interesting data so far. These studies have yielded valuable information regarding both, the number of flea species that could act as vectors, and the potential mammals that could serve as reservoirs, thereby promoting the maintenance and distribution of various bartonelloses in this country and the entire geographic region it encompasses [17,76–79]. In Andalusia (Southern Spain), Márquez et al. [76] and Márquez [80] detected the presence of *B. henselae* and *B. clarridgeiae* in the DNA of *C. felis*; *Bartonella alsatica* in *S. cuniculi*; and even amplified and sequenced the ITS of *Bartonella* in the DNA of *P. irritans*, which showed a 99% similarity with *Bartonella rochalimae*. Recently, in this same region, Zurita et al. [17] detected the presence of *Bartonella* sp. in the species *P. irritans* with a prevalence close to 9%. However, these last authors were unable to reach a species-level identification. In the same way, unfortunately in our study, we also encountered the inability to achieve species-level diagnosis. Thus, a total of 33 DNA samples from fleas of the species *C. felis*, *P. irritans*, and *A. erinacei* tested positive for *Bartonella* spp. using qPCR techniques; however, they yielded negative results when attempted to amplify and sequence the ITS using standard PCR techniques. This fact could be explained by the higher sensitivity of qPCR compared to standard PCR, specially using low-concentrated DNA [81]. In our study, we observed significantly different prevalence percentages for the two *Bartonella* species identified. These ranged from 1% for *B. henselae* in DNA from *C. felis* isolated from dogs to 23% prevalence observed for the species *B. clarridgeiae* in *C. felis* isolated from cats. It should be highlighted the higher prevalence observed for this bacteria species in *C. felis* isolated from cats compared to those isolated from dogs. This epidemiological patterns observed for *B. clarridgeiae* has indeed been documented in other studies [73,82], indicating a greater distribution of this pathogenic species in cats compared to dogs. Based on your results, it can be confirmed that various species of the genus *Bartonella* could continue to spread in Southern Spain among different species of fleas that parasitize our pets, with cats possibly serving as the main reservoir for these infections and the cat flea (*C. felis*) as the main vector. The detection of *B. henselae* and *B. clarridgeiae*, both causative agents of significant bartonelloses, should alert us to the need for control and prophylaxis measures to prevent infestation of our pets by various flea species that could be involved in the transmission of these bacterial agents, not only among domestic and peridomestic mammals but also potentially to humans with whom they may have close contact.

Human leishmaniosis caused by *L. infantum* is an important health problem worldwide. In Spain, traditionally, human leishmaniosis was primarily associated with children, but after the implementation of control programs, the number of cases was greatly reduced [83]. However, some outbreaks have been reported since then in this country, for example during the 1980 s, the outbreak of coinfection with HIV led to a large epidemic of cases associated with *L. infantum* infection accounting for the highest number of cases of coinfection in Europe [84–86]. Another famous outbreak took place in the southwestern region of Madrid (Central Spain), from 2009 to 2014, where an unusual increase in human leishmaniosis cases was reported, considering, to this date, as the largest outbreak of human leishmaniosis registered in Europe [85,87]. In that case a significant increase in vector densities was linked with an increase in the populations of hares and rabbits as a consequence of landscape modifications [88]. In spite of that, in Spain, canine leishmaniosis is still endemic with dogs remain as the main domestic reservoir of *L. infantum*, playing a key role in its transmission practically all over the country [89]. In this context, some authors have investigated the possible vectorial role or direct influence that certain ectoparasites of dogs, such as fleas and ticks, could play in the maintenance and transmission of leishmaniasis [90,91]. A few studies have been published so far, where the presence of *L. infantum* has been detected in fleas of the species *C. felis* and *C. canis* in different geographic regions from Brazil and Iran [19,92,93]. On the other hand, Ferreira et al. [93] conducted various experimental studies with hamsters and parasitized dogs, suggesting that even fleas could effectively transmit leishmaniasis from one host to another. However, it was not clear whether the parasite could survive inside the flea and in these hosts. This topic remains controversial today, and Otranto & Danta-Torres [94], ruled out this possibility but mentioned that further research would be required in the future to investigate the potential vectorial role of fleas as transmitters of *L. infantum*. To the best of our knowledge, this study represents the first attempt to detect the presence of *L. infantum* in various species of fleas in Spain parasitizing domestic and peridomestic animals. Our results confirmed the presence of this parasite in the species *C. felis* isolated from dogs, as might be expected based on previous research and given that Spain is an endemic area. In agreement with Otranto & Danta-Torres [94], further experimental studies should be conducted to confirm or discard the vectorial capacity of these ectoparasites as vectors of leishmaniasis, with the aim of adopting possible future containment and control measures, especially in endemic areas like Spain.

The last pathogenic bacterium detected in our epidemiological study was *Y. pestis*, a gram-negative bacillus that may occasionally be transmitted from rodents to humans causing Bubonic plague as the most common clinical presentation among humans [95]. Human plague cases are often preceded by epizootics when large numbers of susceptible rodents die of infection and fleas seek human hosts [12]. Therefore, conducting surveillance for *Y. pestis* circulation in animals and fleas could be useful for monitoring the risk of plague transmission to humans [96]. For that reason, a large group of flea species have been confirmed as plague vectors by experimental methods in endemics area. These flea species included synanthropic fleas as *C. felis*, *P. irritans* and *E. gallinaceaj* [12,95]. Although fleas have been considered the main vectors of host-to-human transmission, this may depend on different contexts. For example, rats and fleas were found to be effective vectors in India in the past, but this pattern could not be applied to effective reservoirs and vectors in most recent plague outbreaks. For example, the plague in the United States seems to be linked mainly to squirrels or chipmunks whereas in North Africa and the Middle East, outbreaks are related to the consumption of poorly cooked meat from camels or goats [97]. Our study represents the first evidence of the presence of *Y. pestis* in fleas in Europe. In this case, we experimentally demonstrated the presence of this bacterium in the species *P. irritans* isolated from dogs. This fact holds significant epidemiological importance, considering that *P. irritans* is a species whose vector capacity in the transmission of plague has been previously demonstrated in North America [98] as well as, another authors suggests that inter-human transmission through ectoparasites (*P. irritans* and *Pediculus humanus*) may have played a predominant role during the historical Black Death Pandemic [95,99]. On the other hand, recently, Yue and Lee [100] concluded that France, Italy, and Spain are expected to face increased risk of plague outbreak or rodent-borne disease outbreak in 2021–2050 because of existing climate change specially focused on the rising trend of drought in Europe in terms of its magnitude, duration, and spatial extent.

The present work demonstrates that the presence of certain infectious agents in fleas, along with the demonstration of their vectorial role in the transmission of numerous diseases, underscores once again the importance of this group of arthropods for both human and animal health. In this regard, the number of pathogenic bacteria detected, the number of potential flea species implicated in their transmission, and the emergence of new potential endemic areas continue to increase year after year. This further emphasizes the need to implement control and prophylaxis measures to prevent infestation of our pets by these ectoparasites, as well as limiting contact with infested areas or environments favorable to fleas. Additionally, these studies reinforce the need to further delve into providing new updated data that allow us to confirm the possibility that fleas may play an active vectorial role in the

transmission of certain diseases such as leishmaniasis.

5 Conclusions

In the present study, a comprehensive epidemiological investigation was conducted to determine the prevalence of certain pathogenic bacteria that could be transmitted by different species of fleas commonly parasitizing domestic and peridomestic animals with a close relationship to humans in Spain. In total, five different species of fleas were identified, and assays were conducted to detect the presence of species belonging to a total of 9 bacterial genera that can potentially cause zoonotic infectious diseases in humans, varying in severity depending on the pathogenic species or the immune status of the affected host. Lastly, the list of bacterial agents found in fleas in Spain has been updated, emphasizing the need to continue conducting future experimental studies to assess and confirm the potential vectorial role that certain synanthropic fleas like *C. felis*, *A. erinacei*, *C. canis*, *S. cuniculi*, or *P. irritans* could have in this geographical area.

Ethical Statement

All procedures performed in studies did not involve human participants, neither animal experimentation and does not require the approval of animal ethics committee.

CRediT authorship contribution statement

Cristina Cutillas: Conceptualization, Formal analysis, Funding acquisition, Project administration, Resources, Supervision, Validation, Visualization, Writing – review & editing. **Ignacio Trujillo:** Data curation, Formal analysis, Investigation, Methodology, Software. **Antonio Zurita:** Conceptualization, Formal analysis, Investigation, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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