Zoonotic Bacteria in Fleas Parasitizing Common Voles, Northwestern Spain

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We detected *Francisella tularensis* and *Bartonella* spp. in fleas parasitizing common voles (*Microtus arvalis*) from northwestern Spain; mean prevalence was 6.1% for *F. tularensis* and 51% for *Bartonella* spp. Contrasted vector–host associations in the prevalence of these bacteria suggest that fleas have distinct roles in the transmission cycle of each pathogen in nature.

A dynamic prevalence of *Francisella tularensis* and *Bartonella* spp. was reported in irruptive common vole (*Microtus arvalis*) populations during 2013–2015 from agricultural landscapes of northwestern Spain (*1*,*2*). In that area, notifiable tularemia has been endemic since 1997, and human cases periodically occur during outbreaks in voles (*3*,*4*). Prevalence of *F. tularensis* and *Bartonella* spp. in voles increases with vole density (*1,2*), highlighting the key role of fluctuating rodents in shaping zoonoses dynamics (*1*–*4*). Rodent ectoparasites often play a major role in transmitting zoonotic pathogens. In the population studied, ticks rarely infest voles (2% prevalence), whereas fleas are much more prevalent (68%) (*2*). Nevertheless, any potential role for vole fleas in the circulation of *F. tularensis* or *Bartonella* spp. in natural environments remains unknown. To elucidate realistic transmission route scenarios in host-dynamic environments (*5*–*8*), we investigated whether zoonotic bacteria occur concomitantly in voles and fleas.

Our main goal was to study the prevalence of *F. tularensis* in fleas collected from voles previously tested for tularemia (*1*). We screened flea DNA in search of 6 main zoonotic bacteria simultaneously (*Anaplasma phagocytophilum*, *Bartonella* spp., *Borrelia* spp., *Coxiella burnetii*, *F. tularensis*, and *Rickettsia* spp.), following the same molecular procedure (multiplex PCR) (*9*) previously used to screen vole pathogens (*1,2*). Voles and fleas were live-trapped in northwestern Spain during March 2013–March 2015 (Appendix, https://wwwnc.cdc.gov/EID/article/25/7/18-1646-App1.pdf). We collected fleas from each individual vole and identified and grouped them in pools (pool = total fleas/vole). Three flea species parasitize common voles in the area: *Ctenophthalmus apertus*, *Nosopsyllus fasciatus*, and *Leptopsylla taschenbergi* (*2*). We screened monospecific pools (all fleas in a pool belonged to the same species and came from the same vole host), for a sample size of 90 vole hosts (pools) and 191 fleas. We screened 78 *C. apertus* fleas (39 pools) and 113 *N. fasciatus* fleas (51 pools). Among the 90 voles providing fleas, 27 were *F. tularensis* PCR–positive; the remaining 63 were negative (*1*). Of these same 90 voles, 45 were *Bartonella* PCR–positive and 45 were negative. Seventeen were positive for both *F. tularensis* and *Bartonella* spp. (*2*).

Flea pools had an average of 2.12 fleas (range 1–9); however, most (>70%) contained 1 (51%) or 2 (22%) fleas (Table). We did not detect DNA from pathogens other than *F. tularensis* and *Bartonella* spp. in fleas. Three (3%) flea pools harbored *F. tularensis* DNA; we estimated the overall prevalence at 6%. *F. tularensis* prevalence in both flea species was low (1 positive pool of 51 in *N. fasciatus* and 2 of 39 in *C. apertus*). All *F. tularensis* PCR–positive flea pools came from *F. tularensis* PCR–positive voles, and prevalence of *F. tularensis* in fleas was significantly associated with its prevalence in voles (analysis of variance [ANOVA], $R^2 = 0.072$, $F_{0.05, 1, 88} = 6.81$; $p = 0.011$). Of note, all fleas containing *F. tularensis* DNA were collected during July 2014, when vole populations reached top densities and tularemia prevalence peaked among them (33%) (*1*). The low prevalence of *F. tularensis* detected in fleas carried by infected hosts (3 of 27 pools) and the detection of infected flea pools only when abundance of the bacterium in the environment was highest (during vole peaks) (*1*,*4*) suggest that the quantitative role of fleas in the circulation of *F. tularensis* might be modest.

Conversely, the role of fleas in the circulation of *Bartonella* spp. seems much more relevant. We detected *Bartonella* spp. in 28 (37%) flea pools and in both flea species (37% of *N. fasciatus* and 23% of *C. apertus*) (Table). We detected *Bartonella* spp. in fleas collected from *Bartonella* PCR–positive and *Bartonella* PCR–negative voles in nearly equal proportions (51% vs. 44%) (Table). The average prevalence of *Bartonella* spp. in fleas was not associated with its prevalence in voles (ANOVA, $R^2 = 0.006$, $F_{0.05, 1, 88} = 0.53$; $p = 0.467$). We found a higher *Bartonella* spp. prevalence in *N. fasciatus* (65%) than in *C. apertus* (33%).

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Table. Detection of *Francisella tularensis* and *Bartonella* spp. in 2 species of fleas from live common voles (*Microtus arvalis*), northwestern Spain, 2013–2015*

		Flea pools			Fleas		
Voles	Flea species	No.	*F. tularensis*–positive, %	*Bartonella* spp.–positive, %	No.	*F. tularensis* prevalence, % (range)	*Bartonella* spp. prevalence, % (range)
All	All	90	3.3	31.1	191	6.1 (3.3–8.8)	51.1([31.1–71.1)
	Nosopsyllus fasciatus	51	2.6	37.3	113	6.9 (3.9–9.8)	64.7 (37.3–92.2)
	Ctenophthalmus apertus	39	3.9	23.1	78	5.1 (2.6–7.7)	33.3 (23.1–43.6)
F. tularensis–negative	All	63			127	0	
	N. fasciatus	32			71	0	
	C. apertus	31			56	0	
F. tularensis–positive	All	27			64	20.4 (11.1–29.6)	
	N. fasciatus	19			42	18.4 (10.5–26.3)	
	C. apertus	8			22	25.0 (12.5–37.5)	
Bartonella spp.–negative	All	45			93		44.4 (26.7–62.2)
	N. fasciatus	21			53		71.4 (38.1–100)
	C. apertus	24			40		20.8 (16.7–25.0)
Bartonella spp.–positive	All	45			98		51.1 (31.1–71.1)
	N. fasciatus	30			60		60 (36.7–83.3)
	C. apertus	15			38		53.3 (33.3–73.3)

*Blank cells indicate that nothing can be calculated for that option.

We identified 3 *Bartonella* species among fleas (*B. taylorii* [17%], *B. grahamii* [14%], and *B. rochalimae* [3%]), as well as mixed infections (Appendix). These findings are in accordance with other research showing fleas as a main vector of *Bartonella* spp. (*5*). Although *F. tularensis* and *Bartonella* spp. have been simultaneously detected in ≈13% of voles during population density peaks (*2*), we identified no co-infection among flea pools (ANOVA, $R^2 = 0.011$, $F_{0.05, 1, 88} = 0.97$; $p = 0.328$).

Our data show that *F. tularensis* and *Bartonella* spp. occur in the fleas infesting wild common voles in northwestern Spain, with notable differences in prevalence (6% and 51%, respectively) and associations with prevalence in vole hosts. Future studies are needed to determine the role of fleas in the circulation of these pathogens in nature and in particular to ascertain any effective vectoring of *F. tularensis*.

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Mycobacterium bovis Infection in African Wild Dogs, Kruger National Park, South Africa

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We screened African wild dogs (*Lycaon pictus*) in Kruger National Park, South Africa, for *Mycobacterium bovis* infection using an interferon-gamma release assay. We detected *M. bovis* sensitization in 20 of 21 packs; overall apparent infection prevalence was 83%. These animals experience high infection pressure, which may affect long-term survival and conservation strategies.

The African wild dog (*Lycaon pictus*) is an endangered carnivore occurring in fragmented, small populations (in South Africa, <500 animals). These factors make them susceptible to adverse factors, such as infectious diseases, that may threaten their long-term survival (*1,2*). Of particular concern are diseases caused by multihost pathogens that are capable of persisting in reservoir host species, such as *Mycobacterium bovis*, the causative agent of bovine tuberculosis (bTB). This pathogen may pose a major threat to the conservation of endangered host populations (*3*).

Since 2012, sporadic cases of wild dogs with macroscopic and histological lesions consistent with tuberculosis (TB) have been recorded in South Africa, specifically in Kruger National Park (KNP; n = 8), uMkuze Game Reserve (n = 1), and Hluhluwe-iMfolozi Park (HiP; n = 2). *M. bovis* infection is endemic in these parks and occurs in multiple species that are preyed upon by wild dogs, such as warthogs, which have an estimated *M. bovis* seroprevalence up to 38% in KNP (*4,5*). In 2 cases from KNP, acid-fast bacilli were associated with granulomatous lymphadenitis, and spoligotype analysis of *M. bovis* isolates from lesions in affected wild dogs from KNP (strain type SB0121) and HiP (strain type SB0130) were the same as those found in local prey (*6*).

M. bovis is a novel pathogen of wild dogs; understanding the impact of bTB disease in wild dogs is imperative to making informed management decisions regarding these animals' conservation. Estimation of prevalence would provide a starting point for this investigation but requires diagnostic tools for accurate detection of *M. bovis* infection. To estimate prevalence in the KNP wild dog population, we assessed sensitization to TB antigens ESAT-6 and CFP-10.

During July 2016–January 2018, we tested blood samples from 77 wild dogs from KNP using an interferon-gamma release assay (IGRA) developed by our group (*7*). We tested animals from 21 wild dog packs; 20 of these included ≥1 IGRA-positive animal, indicating widespread exposure to *M. bovis* throughout KNP (Figure). We observed no significant difference in IGRA results based on sex (p = 0.79 by 2-tailed Mann-Whitney test). Overall, the apparent prevalence of *M. bovis* infection was 82% (63/77; 95% CI 72%–89% by modified Wald test).

Few reports of active bTB disease and related deaths have been documented in wild dogs, so the high apparent

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Appendix

Study Area

The study was conducted in an intensified agricultural landscape in northwestern Spain. Fieldwork was conducted in 2 areas of 40 km^2 each in Palencia province, Castilla-y-León region (42°01′N, 4°42′W). The farmland of the study areas consists of a mosaic of crops dominated by non-irrigated cereals (≈48% of the agricultural surface), scattered with irrigated and non-irrigated alfalfa crops (≈10%) and other herbaceous crops. Natural and semi-natural habitats are reduced to small and dispersed patches of uncultivated land, pastures, or meadows (≈21% of the agricultural area) and a network of field margins (covering <5% of the agrarian surface) (for more details about the study area see [*1*]).

Sample Collection

We held all the necessary licenses and permits for conducting this work: J.J.L.L., F.M., and R.R.P. held official animal experimentation licenses of level B-C for Spain, and capture permission (permit number 4801646) was provided by the Dirección General del Medio Natural, Junta de Castilla-y-León, Spain.

Common voles were live trapped in an agricultural area using LFAHD Sherman traps (8 cm × 9 cm × 23 cm) every 4 months (in March, July, and November) between March 2013 and March 2015 as is described in Rodríguez-Pastor et al. (*1*). Captured voles were taken to the lab alive, where they were euthanatized through medical CO_2 inhalation, following a protocol approved by our institution ethics committee (CEEBA, Universidad de Valladolid; authorization code: 4801646). Immediately after death, the voles' fur was inspected carefully for fleas through

careful visual inspection and by gently blowing the vole's fur while holding the animal over a white plastic tray (520 × 420 × 95 mm) filled with water. We counted, collected and preserved in labeled tubes with 70% ethanol all the fleas collected from 225 individual voles. Each flea was later identified using a binocular microscope based on morphologic criteria following Gómez et al. (*2*). Although in a previous study, 240 common voles were screened for the occurrence of *Francisella tularensis* (*3*), for this current study, we used fleas collected from these same voles, but we only considered those animals that arrived alive to the lab to have a reliable estimation of flea infestation in the voles. Individual fleas often abandon carcasses of hosts that die in traps or during transport. From the 225 voles (141 voles were infested with fleas and 84 were not infested; Appendix Table 1), we selected voles that were infested with only 1 flea species, reducing the sample size to 90 individual voles (Appendix Table 1). Fleas from each vole were grouped in pools, and each pool was analyzed at molecular level (191 fleas in total, regrouped in 90 pools). A given pool consisted of fleas belonging to the same individual vole and flea species, i.e., 78 fleas in 39 pools were identified as *Ctenophthalmus apertus* (40.8%) and 113 in 51 pools as *Nosopsyllus fasciatus* (59.2%). Thus, the number of flea pools was equivalent to the number of sampled voles (n = 90) (Appendix Table 1). We did not analyze pools containing a mix of the 2 flea species, i.e., voles that simultaneously had fleas of both species were not considered in this study.

Flea pools were selected based on an a priori knowledge of *F. tularensis* prevalence in the voles that hosted them (*3*). In particular, from the initial 225 voles, 48 were *F.tularensis* PCR–positive and 177 were *F. tularensis* PCR–negative (Appendix Table 1). The proportion of voles infested with fleas and *F. tularensis* PCR–positive was 70.8% (34/48); while the proportion of voles infested with fleas and *F. tularensis* PCR–positive was ≈60.5% (107/177). We found that *F. tularensis* infection did not affect flea infestation. There were no significant difference between the proportions (χ^2 1.74, g.l. = 1, p>0.05). The selected 90 monospecific flea pools were made up of 27 flea pools from *F. tularensis* PCR–positive voles and 63 pools from *F. tularensis* PCR–negative voles. Since in a previous study we used a multiplex PCR method to analyze the DNA of the common voles that hosted the fleas studied here (see [*4*]; and PCR methods sub-section below), we also knew the prevalence of other zoonotic pathogens in these common voles, specifically *Anaplasma phagocytophilum*, *Bartonella* spp., *Borrelia* spp., *Coxiella burnetii*, *F. tularensis*, and *Rickettsia* spp.

DNA Extraction

DNA from each flea pool was extracted using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the standard procedures of the manufacturer.

PCR Methods

Pathogen detection in the DNA extracted from fleas was carried out using a multiplex PCR that simultaneously detected 6 vectorborne pathogens (*A. phagocytophilum*, *Bartonella* spp., *Borrelia* spp., *C. burnetii*, *F. tularensis*, and *Rickettsia* spp.), combined with a reverse line blotting (RLB), as previously described (*5*,*6*). Sensitivity of the multiplex PCR was between 10 and 100 GE (Genome Equivalents), and specificity with unrelated bacteria, mammals and arthropods was 100% (*5*). The same methodology was used to detect these same pathogens in common voles (*4*), including those hosting the fleas analyzed here. All the samples that tested positive to any given pathogen were further tested separately using specific probes with an individual PCR and subsequent RLB.

For detection of *F. tularensis* DNA in a flea pool, a phylogenetically informative region of *lpnA* (231 bp) was amplified by conventional PCR and further hybridization with specific probes by RLB, as previously described in Escudero et al. (*7*). Positive samples were tested for confirmation of the results using a real-time multitarget TaqMan PCR, targeting *tul4* and *ISFtu2* assays (*8*). A negative PCR control, as well as a negative control for DNA extraction, was included in each group of samples tested.

Identification of *Bartonella* Species

Bartonella-positive samples were further analyzed using a multiplex PCR targeting the 16S rRNA and the intergenic transcribed spacer (ITS) 16S-23S rRNA. Subsequently, amplicons were analyzed with a RLB that included 36 probes for the identification of the different genotypes and species of *Bartonella* (*9*,*10*). Results are shown in Appendix Table 2.

Statistical Analyses

As the number of fleas per pool ranged from 1 to 9, and all the fleas in each pool were screened together, we estimated an average pathogen prevalence per pool as the mean prevalence between the minimum and maximum prevalence. We assumed that either only 1 of the fleas was positive (minimum prevalence estimate) or that all the fleas from the pool were positive (maximum prevalence estimate). Average pathogen prevalence was estimated for all the fleas and for each flea species separately.

We used an analysis of variance (ANOVA) to test whether the pathogen prevalence in voles had an effect on the average pathogen prevalence in fleas. We also tested whether the average prevalence of a pathogen in fleas was related to the average prevalence of other pathogens in fleas. A $p<0.05$ was considered significant. Analyses were done with R v3.5.1 (*11*).

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Appendix Table 1. Distribution of common voles according to flea-infestation and *Francisella tularensis* PCR results in voles, northwestern Spain, 2013–2015

Fleas in voles	*F. tularensis* PCR–positive voles	*F. tularensis* PCR–negative voles	Total voles	Observation
Only 1 flea species	27	63	90	Fleas used in this study
Mixed flea species	7	44	51	
Not infested voles	14	70	84	
Total	48	177	225	

Appendix Table 2. Species-specific occurrence of *Bartonella* species in flea pools (n = 28), *Nosopsyllus fasciatus* pools, and *Ctenophthalmus apertus* pools according to infection type: single *Bartonella* species infection, or mixed-*Bartonella* species infection, northwestern Spain, 2013–2015

Bartonella species	No. (%)	*N. fasciatus* (%)	*C. apertus* (%)
B. grahamii	4 (14.3)	4 (21.1)	0
With *B. rochalimae*	2 (7.1)	2 (10.5)	0
With *B. rochalimae* and *B. taylorii*	7 (25)	7 (36.8)	0
With *B. taylorii*	6 (21.4)	1 (5.3)	5 (55.6)
With *B. elizabethae*	3 (10.7)	3 (15.8)	0
B. taylorii	5 (17.9)	1 (5.3)	4 (44.4)
B. rochalimae	1 (3.6)	1 (5.3)	0
Total	28 (100)	19 (100)	9 (100)