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Serological Evidence of *Rickettsia* spp. in Western Australian Dogs

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Abstract. It has been claimed that dogs can be useful sentinels for public health monitoring of vector-borne infectious diseases, including *Rickettsia* spp. We used 153 canine blood samples opportunistically collected at Murdoch University Veterinary Hospital and 156 canine sera collected from Aboriginal communities in northwest Western Australia to test for evidence of *Rickettsia* spp. exposure, using microimmunofluorescence (MIF) in the latter case, and both MIF and polymerase chain reaction (PCR) in the former. Conventional and real-time PCR failed to amplify any *Rickettsia* spp. DNA. The seroprevalence for spotted fever group/transitional group *Rickettsia* spp. in Western Australian dogs was 17.3% (54/312), and for typhus group (TG) *Rickettsia* spp., 18.4% (57/310), with a cut-off titer of 1:128. Young dogs (≤ 2 years) from Aboriginal communities had significantly lower seropositivity to TG *Rickettsia* spp. compared with all other groups, and young Perth dogs had a significantly higher seropositivity to TG *Rickettsia* spp. than all Aboriginal community dogs.

INTRODUCTION

Pet dogs can act as sentinels for infectious disease surveillance, including vector-borne zoonotic diseases such as Lyme disease and rickettsioses.[1–3] *Rickettsia* spp. are obligate intracellular, Gram-negative bacteria classified within the α-subdivision of Proteobacteria.[4] Members of the genus *Rickettsia* can be divided into four serogroups: the spotted fever group (SFG), typhus group (TG), transitional group (TRG), and ancestral group (AG).[5] Fully characterized *Rickettsia* spp. that are reportedly endemic in Australia include one TG member, *Rickettsia typhi*[6–9]; two SFG members, *Rickettsia honei*,[10] and *Rickettsia gravesii*[11,12]; and two TRG members, *Rickettsia australis*,[13] and *Rickettsia felis*.[14]

Many of the epidemiological features of vertebrate rickettsial infections depend on the ecology of the invertebrate host associated with the lifecycle of each *Rickettsia* spp.[15] Preferred environment, host specificity and feeding behavior of the ectoparasite vectors are important factors that influence geographical, seasonal, and host species distribution of particular rickettsioses.[15]

Members of the Ixodidae (hard ticks) are the main arthropod vectors for some of the Australian rickettsiae. *Ixodes tasmani*, *Ixodes holocyclus*, and *Ixodes cornuatus* are the major vectors of *R. australis*[16,17]; *Bothriocroton hydrosauri* is a major vector of *R. honei*[18,19]; and *Amblyomma triguttatum* is the main reservoir of *R. gravesii*.[20]

R. felis and *R. typhi* are generally transmitted by fleas. The cat flea, *Ctenocephalides felis* is thought to be the main vector involved in *R. felis* transmission,[14,21,22] whereas the rat flea, *Xenopsylla cheopis*, is the natural vector of *R. typhi*.[23]

The Aboriginal communities in northern Western Australia studied in this article are located in tropical or grassland climatic zones, whereas metropolitan Perth and its surroundings are found in subtropical or temperate climate zones. Of the major arthropod vectors of rickettsiae listed above, only *A. triguttatum* and *C. felis* have been reported in both the southwest and northwest of Western Australia.[14,20,24] The flea species *X. cheopis* and the tick species *Ixodes tasmani* have been reported in the southwest but not in the northwest of Western Australia.[24,25] The tick species *Bothriocroton hydrosauri* has been reported from the southwest of Western Australia, but *I. holocyclus*, and *I. cornuatus* have not been reported in Western Australia to date.[24] Schloderer and others (2006) reported finding *C. felis*, *Echidnophaga gallinacea*, and *C. canis* on dogs from southwest Western Australia in their survey of fleas from companion animals, whereas four dogs from Broome in northwest Western Australia all had only *E. gallinacea*.[14]

The preferred serological test for the diagnosis of previous exposure to rickettsial antigens is the microimmunofluorescence (MIF) test.[26] Although MIF can be useful in detecting prior rickettsial infections, interpretation of test results requires great care and expertise, due to operator variability in reading the tests and the phenomenon of serological cross reactivity between different *Rickettsia* spp.[27,28] This latter phenomenon often prevents diagnosticians from definitively determining a specific etiology for a particular rickettsiosis.[29]

Rickettsaemias can be detected using conventional polymerase chain reaction (PCR), and several such tests have been described.[21,30–33] These tests, when combined with amplicon sequencing, are very specific.[34] Unfortunately, conventional PCR has low sensitivity, because rickettsaemias are transient and usually have low concentrations of circulating organisms.[34,35] A sensitive real-time PCR assay to detect rickettsiae is currently available.[36]

Several recent Australian studies have pointed out the risk to human health posed by rickettsial infections in domestic pets and their ectoparasites. In Victoria, a family was infected with rickettsiae shortly after adopting a kitten with a flea infestation. The owners and cat showed strongly positive results for the presence of TG antibodies in serological tests.[28] PCR evidence of *R. felis* in 9% of tested southern Queensland pound dogs was demonstrated,[37] and in a subsequent paper, the same research group reported on PCR-based detection of *R. felis* in canine blood collected from Aboriginal community dogs in the Northern Territory.[38]

This manuscript reports on serological and genetic evidence of SFG/TRG and TG *Rickettsia* spp. in dogs living in Western Australia and is the first report of rickettsial serology and rickettsaemias in Western Australian companion animals.

MATERIALS AND METHODS

Whole-blood and serum samples from Perth dogs. Ethylenediaminetetraacetic acid (EDTA)-anticoagulated whole-blood paired with plasma or serum samples were opportunistically

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collected from 153 individual dogs that presented to Murdoch University Veterinary Hospital between August 2011 and June 2012. Information was collected on each animal including age, sex, location, and clinical condition. Samples collected from the same dog but at different times of the year were kept aside (excluded from the group of 153 samples). They were then tested and compared with the samples that were collected earlier from the same dog. All samples were stored frozen at –20°C before processing.

Serum samples from Aboriginal community dogs. Serum samples were collected from 156 individual dogs sourced from Ngalinkadji (NG) (18°12′S, 125°34′E), Joy Springs (JS) (18°20′S, 125°42′E), Djarindjin (DJ) (16°31′S, 122°54′E), One Arm Point (OAP) (16°27′S, 123°4′E), and Warmun (W) (17°2′S, 128°13′E) in the northwest of Western Australia, between March 1992 and September 1994. Information collected for each dog included age, sex, location, and the presence or absence of a tick burden at the time of examination. As with those from Perth, samples collected from the same dog but at different times were kept aside, to be tested and compared with the earlier samples. All samples were stored frozen at –20°C before processing.

DNA extraction. DNA was extracted from whole-blood samples collected from dogs into EDTA tubes using the HiYield™ Genomic DNA Mini Kit (YGB300, Real Biotech Corporation, Taiwan) according to the manufacturer's instructions. DNA was eluted in 50 µL of elution buffer (preheated to 70°C) and stored at –20°C until PCR analysis.

Conventional PCR and real-time PCR for detection of *Rickettsia* spp. PCR amplification was performed on extracted DNA to determine presence of rickettsial DNA in the whole-blood samples collected, as previously described.[21] The gene target used in the PCR assay was the *gltA* gene using the primers gltA-F: GCAAGTATTGGTGAGGATGTATC, and gltA-R: CTGCGGCACGTGGGTCATAG. Each 25 µL reaction contained 2 µL of template, 1 µmol/L of each primer, 1× reaction buffer (Fisher Biotec, Australia), 200 µmol/L of each dNTP, and 2 U Taq polymerase. The amplifications were performed in a PCR thermal cycler (Mastercycler personal, Quantum Scientific, Australia) with an initial denaturation step of 3 minutes at 95°C, followed by 50 cycles of denaturation at 95°C for 30 seconds, annealing at 51°C for 30 seconds and extension at 72°C for 1 minute, followed by a final extension of 72°C for 10 minutes. PCR products were electrophoresed through 1% agarose (w/v) gels stained with SYBR-safe (Invitrogen, Australia) for 30 minutes at 100 V and visualized using an ultraviolet transilluminator (CN-WL, Fisher Biotec) and compared against a known positive control (*R. australis*), expected to yield a 381 base pair (bp) band. A no template negative control (2-µL PCR grade water) was included in each reaction run.

To confirm successful DNA extraction, and to ensure that nonspecific PCR inhibitors were not compromising the reaction, previously published dog-specific primers were used: 12SpDOGDIR: AATTGAATCGGGCCATGAA, and 12SpDOGINV: CTCCTCTTGTGTTTTAGTTAAGTTAATCTG.[39] Each 25 µL reaction contained 2 µL of template, 1 µmol/L of each primer, 1× reaction buffer (Fisher Biotec), 2 mmol/L $MgCl_2$, 200 µmol/L of each dNTP, and 2 U Taq polymerase. A PCR thermal cycler was used to run the reactions. The sample was initially heat denatured at 93°C for 2 minutes, followed by 35 cycles of denaturation at 93°C for 30 seconds, 30 seconds at 60°C for primer annealing, and 45 seconds at 72°C for DNA extension. The final extension step was maintained at 72°C for 3 minutes. PCR products were loaded into a 1% agarose gel (w/v) stained with SYBR-safe, and electrophoresed at 85 V for 20 minutes. The gel was then viewed on an ultraviolet transilluminator, to visualize the expected 101 bp bands. Again, a no template negative control (2-µL PCR grade water) was included in each reaction run.

DNA extracts were also tested by real-time PCR to detect the *glt*A gene, following the method of Stenos and others (2005).[36]

Rickettsial antigen and MIF slide preparation. Rickettsial antigens (*R. australis*, *R. honei* strain Brown, *R. gravesii*, *Rickettsia rickettsii*, and *R. typhi*) were kindly provided by the Australian Rickettsial Reference Laboratory. Cell suspensions from rickettsial cultures were heat inactivated at 56°C for 30 minutes and stored at –20°C before use as antigens for MIF. SFG/TRG screening slides were produced from a mixture of *R. australis* and *R. honei* strain Brown antigens. SFG/TRG titration slides were produced from *R. australis*, *R. honei*, *R. gravesii*, and *R. rickettsii* antigens, separately. TG screening and titration slides were produced using *R. typhi* antigens only. Antigen concentrations were adjusted to produce optimal reactivity with control antibodies. In all cases, a 1–2 µL drop of antigen material was applied to each well of a 40 well slide (Scientific Device Laboratory, Chicago, IL), left to air dry, fixed in pure acetone for 2 minutes, and air dried again before storage at 4°C. Slides were set up such that duplicate reactions per test were performed in all cases.

MIF assay. Rickettsial seroprevalence in dogs was investigated using indirect MIF as previously described.[40] For screening tests, canine sera or plasma samples were diluted 1:128 with 2% (w/v) skim milk powder in phosphate buffered saline (PBS) solution, applied to their respective wells and incubated in a humid environment at 37°C for 35 minutes. Slides were then washed with 0.1 × PBS and air dried. A 1:100 dilution of fluorescein isothiocyanate labeled goat antidog immunoglobulin G (02-19-06, KPL, Gaithersburg, MD) with 0.01% (w/v) Evans blue (Sigma, Castle Hill, Australia) was then added to the wells and incubated at 37°C for 35 minutes before being washed twice in 0.1 × PBS and air dried. Slides were then mounted in Faramount Aqueous Mounting Medium (Dako, Carpinteria, CA) and examined using an ultraviolet fluorescent microscope (Olympus BH2-RFCA, Olympus Australia, Notting Hill, Australia). Samples considered positive at the 1:128 screening dilution were subsequently titrated using 2-fold serial dilutions of canine serum or plasma from 1:128 to 1:1024. The same individual prepared and read all MIF slides for this study.

Statistical analysis. The two-tailed Freeman-Halton extension of Fisher's exact test using 2 × 3 contingency tables was used to compare end-point titration results between Perth and Aboriginal community dogs. Fisher's exact test using 2 × 2 contingency tables was used to detect statistically significant differences in the proportions of seropositive dogs (for both SFG/TRG and TG) in the following epidemiological groups: young dogs (≤ 2 years) versus adult dogs (> 2 years) in Perth or Aboriginal communities; males versus females in Perth or Aboriginal communities; presence versus absence of ticks during clinical examination (Aboriginal community dogs only); and each of the study localities (Perth, JS, NG, W, OAP, and DJ). Statistics were calculated using online software available at http://epitools.ausvet.com.au, http://www.danielsoper.com/statcalc3/default.aspx, and

TABLE 1
Summary signalment data of the cohort included in the study

	Sex			Age		
	M	F	UNK	≤ 2 years	> 2 years	Unknown
Perth (*N* = 153)	91	60	2	11	140	2
Joy Springs (*N* = 19)	9	10	0	8	3	8
Ngalinkadji (*N* = 29)	16	13	0	9	13	7
Warmun (*N* = 31)	19	12	0	14	3	14
Djarindjin (*N* = 49)	28	21	0	23	11	15
One Arm Point (*N* = 19)	14	5	0	11	0	8
Aboriginal communities (*N* = 159)	64	95	0	73	31	55

http://www.vassarstats.net/tab2x2.html. Statistical significance was set at $P < 0.05$.

RESULTS

Epidemiological information was collated from all 312 dogs included in the study, and the relevant summary signalment data are presented in Table 1. A wide clinical spectrum of dogs was sampled at Murdoch University Veterinary Hospital, whose presenting health conditions included gastroenterological, neurological, oncological, orthopedic, urological, dermatological, traumatic, and cardiological cases, several uncategorized cases, and some cases of individuals in good health. Clinical data were not collected for Aboriginal community dogs, other than noting in 38/159 (24%) cases, the presence of ticks on the dog at the time of blood sample collection.

Rickettsaemia testing of Perth dogs using conventional PCR and real-time PCR. PCR conducted on DNA extracts from 153 whole-blood samples collected from Perth dogs revealed no (0%) positives for rickettsial DNA (*glt*A), while all (100%) samples tested positive using the dog-specific primers, demonstrating that the DNA extraction technique worked for all samples and there was no significant nonspecific inhibition of the PCR reaction. Real-time PCR confirmed the results of the conventional PCR.

Seroprevalence, seroconversion, and seroreversion. Of the 153 Perth dog samples tested, 28 (18.3%) were positive at 1:128 against the SFG/TRG antigens, and 33 (21.6%) were positive against the TG antigens. Of the 159 Aboriginal community dog samples tested, 26 (16.4%) were positive at 1:128 against the SFG/TRG antigens and 24 (15.1%) were positive against TG antigens (Tables 2 and 3).

There were eight Perth dog cases for which two or more samples were available, that had been collected approximately 3–4 months apart, and 14 Aboriginal community dog cases for which two or more samples had been collected approximately 6–18 months apart. These cases enabled the estimation of seroconversion and seroreversion rates.

No instances of seroreversion were observed. No instances of seroconversion were observed in Perth dogs. There were five instances of seroconversion to SFG/TRG *Rickettsia* spp. antigens identified in Aboriginal community dogs (5/14; 36%), but this was not a statistically significant difference between Perth dogs and Aboriginal community dogs ($P = 0.076$). The longer the interval between the first and subsequent samples, the higher the rate of seroconversion, up to 18 months.

Seroprevalence by age group, sex, and geographical region. There were no statistically significant differences detected between male and female dogs living in Perth or Aboriginal communities with respect to seropositivity to SFG/TRG or TG *Rickettsia* spp. antigens. The overall apparent seroprevalence estimate for SFG/TRG *Rickettsia* spp. in Western Australian dogs was 17.3% (54/312) (95% confidence interval [CI] 13.5–21.9%), whereas the seroprevalence estimate for TG *Rickettsia* spp. was 18.4% (57/310) (95% CI 14.5–23.1%).

There were no statistically significantly differences detected by comparing age groups of dogs living in Perth or Aboriginal communities for seropositivity to SFG/TRG *Rickettsia* spp. antigens, nor was there a significant difference in the rate of seropositivity to SFG/TRG *Rickettsia* spp. antigens in Aboriginal community dogs that had a visible tick burden at the time of blood sample collection (with ticks: 6/38 (16%), without ticks: 20/121 (16.5%); $P = 0.569$).

Young dogs living in Aboriginal communities had significantly lower seropositivity to TG *Rickettsia* spp. antigens compared with all Perth dogs regardless of age ($P = 0.017$), young Perth dogs ($P = 0.007$), adult Perth dogs ($P = 0.035$), and adult dogs living in Aboriginal communities ($P = 0.016$). In stark contrast, young Perth dogs had significantly higher seropositivity to TG *Rickettsia* spp. antigens compared with Aboriginal community dogs regardless of age ($P = 0.023$).

Seroprevalence by location. With respect to specific location, there was a statistically significant higher rate of seropositivity to SFG/TRG *Rickettsia* spp. antigens in dogs from NG compared with JS ($P = 0.047$) and DJ ($P = 0.033$). There was also a statistically significant higher rate of seropositivity to TG

TABLE 2
SFG/TRG serology results analyzed by age category, sex, and geographical region

	Perth				Aboriginal communities			
	Positive	Negative	Total	Rate (%)	Positive	Negative	Total	Rate (%)
≤ 2 years	3	8	11	27	8	65	73	11
> 2 years	25	115	140	17.9	8	23	31	26
All ages	28	125	153	18.3	26	133	159	16.4
Female	11	49	60	18.3	13	51	64	20.3
Male	17	74	91	18.7	13	82	95	13.7
Both sexes	28	125	153	18.3	26	133	159	16.4

TABLE 3
TG serology results analyzed by age category, sex, and geographical region

	Perth				Aboriginal communities			
	Positive	Negative	Total	Rate (%)	Positive	Negative	Total	Rate (%)
≤ 2 years	5	6	11	45[↑]	7	66	73	9.6%[↓+]
> 2 years	28	111	139	20.1[-]	9	22	31	29.0%[-+]
All ages	33	118	151	21.9[-]	24	135	159	15.1[+]
Female	12	48	60	20.0	8	56	64	12.5
Male	21	70	91	23.1	16	79	95	16.8
Both sexes	33	118	151	21.9	24	135	159	15.1

↓ Statistically significantly lower seropositivity compared with categories marked with (-).
↑ Statistically significantly higher seropositivity compared with categories marked with (+).

Rickettsia spp. antigens in dogs from NG compared with DJ ($P < 0.0001$) and W ($P = 0.010$). There was a statistically significant lower seropositivity rate to TG *Rickettsia* spp. antigens in dogs from DJ compared with Perth ($P = 0.0006$) and OAP ($P = 0.0087$) (Table 4).

Endpoint titrations. Perth and Aboriginal community dog sera that were seropositive for SFG/TRG and TG at a 1:128 dilution were titrated using 2-fold serial dilutions from 1:128 to 1:1024. The four SFG/TRG rickettsial species chosen for titrations were *R. rickettsii, R. honei*, *R. australis*, and *R. gravesii*. Titrations for TG *Rickettsia* using *R. typhi* were also performed (Table 5). The maximum titer detected was 1:512. There were no statistically significant differences in the patterns of endpoint titration results.

DISCUSSION

Rickettsaemias in Australian dogs, detected by PCR, have recently been reported in southeast Queensland pound dogs (9%), and Aboriginal community dogs in the Northern Territory of Australia (2.3%), suggesting that dogs have the potential to act as an important reservoir of human zoonotic rickettsial infections.[38,41] In the current study, active rickettsaemias were not detected; however, serological evidence of previous exposure to rickettsial antigens was found.

Using a 1:128 dilution of canine serum, this study estimated an overall seroprevalence of canine anti-SFG/TRG antibodies of 17.3% (95% CI 13.5–21.9%) and an overall seroprevalence of canine anti-TG antibodies of 18.4% (95% CI 14.5–23.1%). Given that the MIF test is imperfect, these should be regarded as likely overestimates.[40] In comparison, a study done in Northern Territory and southern Queensland dogs found a closely comparable seroprevalence of anti-TG antibodies of 18.5% using a serum dilution of 1:128.[41] The seroprevalence of anti-SFG/TRG antibodies in dogs from southeastern Australia was estimated at 11.2%, using a serum dilution of 1:64,[42] while a study in Launceston, Tasmania found 57%, 32%, and 13% seropositivity to SFG rickettsiae in dogs using serum diluted 1:50, 1:100, and 1:200, respectively.[43] The variation in these reported seroprevalences underlines several technical challenges with the use of MIF tests in rickettsial serodiagnostics, namely, inter- and intraoperator variability in the interpretation of slides; the selection of an appropriate dilution of dog serum and/or cut-off titer; and the lack of standardization in methods and materials.[27]

A human serosurvey conducted in the Kimberley region of Western Australia in the late 1990s revealed 13/920 (1.4%) of sera were positive only for SFG rickettsiae; 11/920 sera (1.2%) were positive for both scrub typhus (*Orientia tsutsugamushi*) and SFG rickettsiae, while 4/920 (0.4%) were positive for scrub typhus, SFG, and TG rickettsiae.[7] All seropositivity rates in this human survey were far lower than those found in Aboriginal community dogs in the current study. This finding may be partially accounted for by the difference in cut-off titer for seropositivity (≥ 1:256, versus our ≥ 1:128), but probably also reflects differences in exposure to the relevant vectors between human and canine inhabitants of northwest Western Australia. Pet dogs may therefore act as sentinels of rickettsial infections in our communities, and this is a potential resource that could be explored by public health officials.[1–3,44]

There was a significantly lower seroprevalence of anti-TG antibodies in young Aboriginal community dogs compared with all other groups, and there was a significantly higher seroprevalence of anti-TG antibodies in young Perth dogs, although the number of young Perth dogs in this study was small ($N = 11$). Presumably, this indicates greater exposure of dogs in the Perth region to *R. felis* or *R. typhi*, compared with dogs in Aboriginal communities. However, it should also be remembered that serum samples from young puppies may be seropositive due to the presence of maternally derived antibodies, and this complicates the interpretation of these results.

TABLE 4
SFG/TRG and TG serology results analyzed with respect to location

	Spotted fever group and transitional group				Typhus group			
	Positive	Negative	Total	Rate (%)	Positive	Negative	Total	Rate (%)
Perth	28	125	153	18.3	33	120	153	21.6
Joy Springs	1	18	19	5.3[+]	3	16	19	16
Ngalinkadji	9	23	32	28.1[↑]	12	20	32	37.5[#]
Warmun	8	23	31	25.8	3	28	31	9.7[*]
One Arm Point	2	17	19	10.5	5	14	19	26.3
Djarindjin	6	52	58	11.5[+]	2	56	58	3.4[*↓]

↑ Statistically significantly higher seropositivity compared with categories marked with (+).
Statistically significantly higher seropositivity compared with categories marked with (*).
↓ Statistically significantly lower seropositivity compared with categories marked with (-).

TABLE 5

Endpoint titration results for Perth and Aboriginal community dog serum and plasma samples (cumulative results are presented in parentheses)

	Perth dogs			Aboriginal community dogs		
Antigen	1:128	1:256	1:512	1:128	1:256	1:512
Rickettsia rickettsii	7 (12)	5 (5)	0	7 (8)	1 (1)	0
Rickettsia honei	17 (22)	4 (5)	1	8 (17)	9 (9)	0
Rickettsia gravesii	17 (20)	2 (3)	1	10 (17)	6 (7)	1
Rickettsia australis	12 (15)	2 (3)	1	4 (11)	5 (7)	2
Rickettsia typhi	19 (33)	8 (14)	6	13 (24)	10 (11)	1

It is possible that sample degradation during long-term frozen storage may have occurred, although human serum IgE stored at –20°C for up to 37 years proved to be stable,[45] and only small differences in human IgG stored at –25°C for 25 years were found.[46] Sample degradation would presumably tend to decrease the estimated seroprevalence of anti-*Rickettsia* antibodies, so this should be considered when comparing the estimated seroprevalences between Aboriginal community dogs sampled in 1992–1994, and Perth dogs sampled in 2011–2012. It is important to emphasize that these data provide seroprevalence estimates in the companion dog population up to 20 years apart in two climatically and ecologically distinct regions of Western Australia, so it is remarkable that the estimates are as comparable as they are to each other and to a similar study performed in dogs from Queensland and the Northern Territory from 2009 to 2012.[41] The dynamics of *Rickettsia* spp. and their arthropod vectors' ecology in Western Australia is uncertain, and this is worthy of further scientific investigation.

This study provides serological evidence of relatively common SFG/TRG and TG *Rickettsia* spp. exposure in dogs in both metropolitan settings and remote Aboriginal communities in Western Australia. Because these *Rickettsia* spp. are potentially zoonotic, and dogs are often such close companions of humans, greater awareness of the possible role of dogs and their ectoparasites in the transmission of rickettsioses to humans should be encouraged; however, dogs might also serve as valuable sentinels of rickettsial agents in our communities, and this fact could be exploited in the service of public health monitoring.

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