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Evidence of exposure to *Rickettsia felis* in Australian patients

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ABSTRACT

Rickettsia felis is an emerging zoonosis, causing flea-borne spotted fever (FBSF). Serological diagnosis is typically confounded by cross-reactivity with typhus group rickettsiae and prior to the development of specific serological methods, cases of FBSF in Australia were misdiagnosed.

Patient sera tested between August 2010 and December 2013 and known to be seropositive to *R. typhi* by immunofluorescence antibody testing (IFAT) were subsequently retested against *R. felis* using an *R. felis*-specific IFAT. Sera of 49 patients were of a sufficient quality to be included in re-analysis. A classification of FBSF and murine typhus (MT) was attributed to fourteen and seven patients respectively, based on a minimum four-fold higher antibody titre to *R. felis* than to *R. typhi* and vice versa. Twenty-eight patients were classified as indeterminate for either *R. felis* or *R. typhi* (antibody titres within two-fold of one another).

Historically, it is likely that Australian patients clinically ill with FBSF were misdiagnosed. It is important that medical practitioners consider FBSF as part of their differential diagnoses, and obtain relevant history with regard to patient's exposure to domestic pets and their fleas. Australian microbiology diagnostic laboratories should include serological testing for *R. felis* as part of the diagnostic panel for febrile diseases. Veterinarians are encouraged to increase their awareness of this emerging zoonosis and advocate flea control in pets.

1. Introduction

Rickettsia felis is an obligate intracellular bacterium that is being increasingly recognised as an aetiological agent in human rickettsial disease globally [1]. The agent is transmitted through the bite of an infected vector, typically the cat flea, *Ctenocephalides felis* [2]. Detection of *R. felis* in cat fleas has been reported globally, ranging from 15% in New Zealand [3] to as high as 81% in New Caledonia [4]. In Australia, infection rates in cat fleas range between 20% in metropolitan centres along the coast of eastern Australia to up to 36% in regional centres in Western Australia [5,6].

Disease in humans is variously referred to as flea-borne spotted fever (FBSF), cat flea typhus or cat flea spotted fever. Symptoms of infection range from non-specific flu-like illness (fever, myalgia and headache) to severe multi-systemic disease accompanied by a maculopapular rash, due to widespread vasculitis [7–9]. In 2009, the first described Australian cases of FBSF occurred in a family from Melbourne who had recently acquired *R. felis* positive *C. felis* flea-ridden kittens from a farm in Lara, Victoria [10].

Blood and serum of suspected Australian cases of rickettsiosis were sent to the Australian Rickettsial Reference Laboratory (ARRL) where they underwent immunofluorescence antibody testing (IFAT), the gold standard for rickettsial serological diagnosis, to establish probable exposure. Prior to the successful culture of *R. felis* in Australia in 2011 [11], serological protocols used at the ARRL did not screen specifically for exposure to *R. felis*. As both *R. typhi* and *R. felis* cross-react using IFAT, it is likely that a proportion of *R. felis* cases were misdiagnosed as *R typhi*, a typhus group (TG) organism and agent of murine typhus, which is also endemic to Australia [12].

The aim of this study was to retrospectively determine the exposure and common clinical presentations associated with FBSF attributable to *R. felis* in Australian patients referred to the ARRL in Geelong Australia between August 2010 and December 2013. All *R. typhi* positive samples reported in this period were re-examined in this study.

2. Methods

2.1. Culture

R. felis was cultured in a XTC-2 cell line at 28 °C in Leibovitz-15 media (GIBCO, Rockville, MD), supplemented with 10% foetal calf serum, 2 mM L-glutamine and 5% tryptose phosphate broth [13]. *R. typhi* was cultured

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in a L929 cell line in RPMI media (GIBCO, Rockville, MD), supplemented with 10% foetal calf serum and 2 mM L-glutamine. Once the cell lines reached confluency, they were infected with the appropriate rickettsia and levels in the cell monolayers were monitored using a semi-quantitative qPCR based on the citrate synthase (*glt*A) gene [14]. Species confirmation was achieved through PCR and DNA sequencing of the citrate synthase (*glt*A) gene (Australian Genomic Research Facility Ltd., Australia). Monolayer cells infected with rickettsiae were harvested by physical detachment using cell scrapers, and heat inactivated at 56 °C for 30 min. The harvested material was then pelleted by centrifugation at 3000 *g* for 10 min at room temperature and the pellet resuspended in PBS and evaluated using the IFAT. An optimal working dilution of rickettsial antigen was established through serial doubling dilution of the cell antigen preparations and gauging its fluorescence in the IFAT.

2.2. Ethics approval

Ethics approval for this study was granted through the University of Melbourne Research Ethics Committee (ID: 1443252).

2.3. Sample selection

Following the rickettsial serological testing of patient sera referred to the ARRL, the samples were placed at −20 °C for long term storage. A total of 136 serum samples that previously tested positive for antibodies to *R. typhi* by IFAT between August 2010 and December 2013 were screened, and of these, 69 serum samples corresponding to 49 patients were of sufficient quality (re-tested reactive to *R. felis* or *R. typhi* antigen) to be included for the retrospective assessment of *R. felis* exposure. FBSF infection was confirmed by demonstrating seroconversion in cases where paired sera were available, or a four-fold difference in antibody titre to *R. felis* compared to *R. typhi* when only single serum samples were available. Additionally, data in relation to the patients' age, locality, and clinical presentations were collected.

2.4. Immunofluorescence antibody testing

A previously developed IFAT protocol [15] was modified and carried out as follows; 40 well slides (Scientific Device Laboratory, Des Plaines, IL) were washed in 100% acetic acid and antigen at its optimal concentration was spotted onto each well. Once the antigen had air dried the slides were fixed in 100% acetone for 2 min. Serum samples were serially diluted using 2% casein in PBS with a starting dilution of 1:128 and incubated at 34 °C for 40 min in a humid environment. Positive and negative controls were diluted to 1:128 and included in each assay run. Slides were then washed with 1/10 PBS, air-dried and spotted with a 1:100 dilution of a fluorescein isothiocyanate (FITC)-labelled goat anti-human immunoglobulin IgG (H + L) (Kirkegaard & Perry Laboratories, USA) and incubated at 34 °C for a further 40 min. Following the final washing, the slides were air-dried, covered and stored in a dark environment at 4 °C until read.

Each well was visualised by fluorescence microscopy and the end-point dilution titres determined. Reading was repeated by a second independent observer to control bias, with a third independent observer recruited to resolve any discrepancies.

3. Results

Of the 69 viable sera corresponding to 49 patients, 40 patients were identified to be reactive to *R. felis*, and 14 patients had antibody titres to *R. felis* four-fold or greater to that of *R. typhi* in either the acute or convalescent sera (Table 1). These were classified as cases of FBSF. Of these, nine patients had paired serum samples submitted and within this group, five showed seroconversion, or a significant rise in antibody titre, indicating recent infection.

Forty-five patients remained reactive to *R. typhi*. Single serum samples submitted by seven patients were classified as MT infections, attributed to four-fold greater *R. typhi* antibody titres compared to that of *R. felis*. The remaining serum samples representing 28 patients were classified as indeterminate for either *R. felis* or *R. typhi*, possessing antibody titres within two-fold of one another.

Table 1
Overview of the FBSF-infected patient IFAT testing results.

Patient	Age	Locality	Sample	Sample titrations		Days between sampling
				R. felis	*R. typhi*	
Single sera						
1	83	Sapphire Beach NSW, 2450	1	1:1024	<1:128	N/A
2	72	Fernmount NSW, 2454	1	1:512	<1:128	N/A
3	71	Maleny QLD, 4552	1	1:512	1:128	N/A
4	58	Tamborine North QLD, 4272	1	1:1024	<1:128	N/A
5	36	Lalor VIC, 3075	1	1:1024	1:128	N/A
Paired sera						
6	30	Exmouth WA, 6707	1	1:512	<1:128	14
			2	1:1024	1:256	
7[a]	47	Fern Bay NSW, 2295	1	1:128	1:256	7
			2	1:8192	1:1024	
8[a]	56	Lauderdale TAS, 7021	1	1:256	<1:128	12
			2	1:8192	1:512	
9[a]	52	Charlestown NSW, 2290	1	<1:128	<1:128	10
			2	1:2048	1:256	
10	23	Carey Bay NSW, 2283	1	1:2048	1:256	66
			2	1:1024	1:256	
11	69	Manyana NSW, 2539	1	1:8192	1:2048	30
			2	1:4096	1:256	
12[a]	39	Cawongla NSW, 2474	1	<1:128	1:128	32
			2	1:2048	1:256	
13[a]	67	Eltham VIC, 3095	1	1:128	<1:128	31
			2	1:1024	1:128	
14	55	Mallanganee NSW, 2469	1	1:1024	1:256	17
			2	1:256	1:256	

N/A = not applicable.
[a] Seroconverted paired sera.

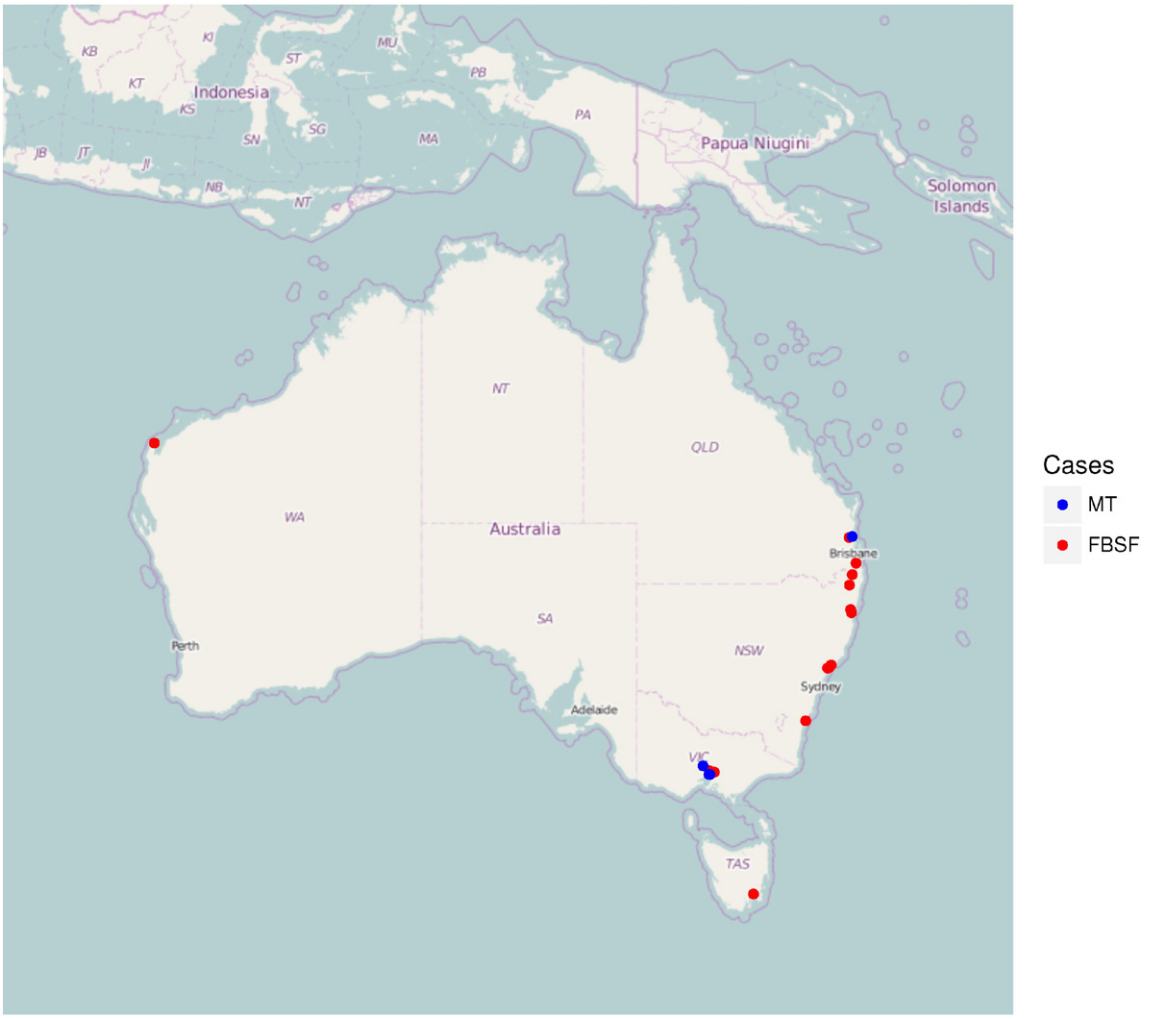

Fig. 1. Location of retrospectively classified cases of flea-borne spotted fever and murine typhus in Australia referred to the Australian Rickettsial Reference Laboratory 2010–2013.

Patients with FBSF were located across the eastern Australian states, Tasmania and Western Australia (Fig. 1). Information on the clinical presentation of FBSF was sparse and poorly recorded. Of the patients with this information, febrile illness ($n = 2$), fatigue ($n = 1$), myalgia ($n = 1$) and response to doxycycline ($n = 1$) were noted at presentation. There was no information recorded on the clinical presentation of the confirmed *R. typhi* patients.

4. Discussion

This is the first study to retrospectively identify fourteen probable cases of *R. felis* infection in patients in Australia between August 2010 and December 2013 using an *R. felis* specific IFAT. We demonstrate that FBSF is a cause of rickettsial disease in Australia and was likely misdiagnosed due to the unavailability of *R. felis* antigen for specific testing at that time. Our results show that FBSF cases in Australia during the period spanning August 2010 to December 2013 were twice as common as those attributed to MT. This suggests the situation in Australia resembles other parts of the world where *R. felis* has been shown to be an important aetiological agent contributing to febrile illness [7,16]. This result is not surprising given the ubiquitous nature and high rates of *R. felis* infection in *C. felis* populations in Australia [5,6].

Clinical data and history can be helpful in establishing a diagnosis of rickettsial disease. In this case much of the data provided as part of the sample submission process was missing or lacking in detail with regard to the clinical syndrome and the patient's history of exposure to fleas. The clinical syndrome of FBSF tends to include a series of non-specific symptoms of varying occurrence: pyrexia, maculopapular rash, myalgia, athralgia, headache, and fatigue [7,17–20]. These clinical signs cannot distinguish from diseases caused by the other rickettsial organisms endemic to Australia including *Rickettsia australis* (Queensland Tick Typhus), *Rickettsia honei* (Flinders Island Spotted Fever), *Rickettsia honei* subspecies *marmionii* (Australian Spotted Fever), *R. typhi* (murine typhus), *Orientia tsutsugamushi* (Scrub Typhus) and *Coxiella burnetii* (Q fever) [12]. While these patients may have been potentially misclassified, the treatment of these rickettsial diseases is identical [21]. It is critical that the distinction be made in order to better define the epidemiology of these diseases which will allow for better medical and laboratory diagnoses.

In contrast to the other tick- and "rodent flea"-borne spotted fevers endemic to Australia, FBSF is associated with exposure to the common domestic dog and cat flea as opposed to arthropods commonly associated with outdoor recreational activities in the Australian 'bush'. The source of these patients' exposure was not clear, mainly due to the lack of relevant clinical history.

Rickettsia felis is transmitted via the bite of an infected flea [2]. The recognised vector, *Ctenocephalides felis* [22], is a widely distributed, ubiquitous parasite of Australian companion animals [23] but will readily feed on humans. In Australia, dogs have been implicated as natural mammalian reservoirs for *R. felis*, based on their ability to harbour naturally occurring, asymptomatic rickettsaemias [11,24,25]; Cats have not been shown to harbour natural *R. felis* rickettsaemias in

Australia [6], but have more recently been implicated as reservoirs in Bangladesh [16].

In this study, we could definitively attribute FBSF infection in nine of fourteen patient's sera found preferentially reactive to *R. felis*. Standard diagnostic laboratory protocol for rickettsial IFAT measures an IgG reaction, which isn't able to provide an indication on the acute nature of an infection. An inherent limitation of the IFAT test is substantial cross reactivity between antibodies to *R. felis* and *R. typhi*, owing to shared genotypic and phenotypic features [26]. The requirement for four-fold differences in titre between *R. felis* and *R. typhi* antibodies was necessary to confidently identify the aetiological agent. This accounted for a large number of patients classified as indeterminate for either *R. felis* and *R. typhi* exposure. Furthermore, it is impossible to attribute the patient's symptoms and signs to an active FBSF infection in the absence of a second convalescent serum sample demonstrating changing titres.

Given the reported contribution of *R. felis* to non-specific febrile illnesses in Australia [10], it is important that medical practitioners consider FBSF as part of their differential diagnoses, and obtain relevant history with regard to patient's exposure to domestic pets and their fleas. Multiple serum samples, collected between two to eight weeks post infection, are needed for the confirmation of FBSF. Australian microbiology diagnostic laboratories should include serological testing for *R. felis* as part of their diagnostic panels for febrile illness. Veterinarians have an important role in advocating flea control in domestic pets and educating clients on the risks of flea exposure amongst themselves and their families.

Competing interests

The authors declare that they have no conflicts of interests.

Authors' contributions

YTT participated in the acquisition of data, analysis and interpretation of data, and drafting the manuscript. SFH and SG assisted with analysis and interpretation of data and revising the article critically for important intellectual content. RR assisted with study design and revising the article critically for important intellectual content. JS and RT assisted with study design, analysis and interpretation of data and revising the article critically for important intellectual content. All authors have read and approved the final version of the manuscript.

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