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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.
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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: *R. felis*	Sample titrations: *R. typhi*	Days between sampling
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.

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