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# Markers of exposure to spotted fever rickettsiae in patients with chronic illness, including fatigue, in two Australian populations

N. UNSWORTH[1], S. GRAVES[1], C. NGUYEN[1], G. KEMP[2], J. GRAHAM[3] and J. STENOS[1]

*From the [1]Australian Rickettsial Reference Laboratory, Barwon Biomedical Research, Geelong, [2]The Burke Road Medical Centre, Camberwell, and [3]The School of Medicine, Flinders University, Adelaide, Australia*

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## Summary

**Background:** Some investigators believe that a proportion of chronically unwell patients, many with fatigue, have an underlying rickettsial disease.
**Aim:** To investigate the prevalence of markers of rickettsial infection in patients with chronic illnesses.
**Design:** Observational study.
**Methods:** A 526 patient cohort with chronic illnesses from Melbourne, Australia and 400 control patients from Newcastle, Australia were assessed using serology, culture and PCR for the detection of rickettsiae. Rickettsial serology was performed on another cohort of 581 chronically unwell patients (and 34 non-fatigued patients from the same practice) from Adelaide, Australia.
**Results:** Of the Melbourne patient cohort, 14/526 (3%) were real-time PCR positive for rickettsial DNA compared to none of the 400 control patients ($P<0.001$). Of these 14 patients, *Rickettsia honei* strain 'marmionii' was detected in 5 and isolated from 2. Rickettsaemia was seasonal, with more in winter (8/145; $P<0.03$) and less in spring (0/143; $P<0.03$). Positive rickettsial serology titres of $\geqslant$1:256 were seen in 206 (39%) patients. Of the Adelaide patient cohort, 238/581 (41%) had positive rickettsial antibodies titres. Of the 34 control sera, 5 (15%) were serologically positive ($P<0.002$). Both Melbourne and Adelaide patient cohorts had significantly higher seropositivity than the Newcastle control cohort (3/399; $P<0.0001$).
**Conclusions:** In patients with chronic illness, rickettsial DNA in peripheral blood and/or rickettsial seropositivity may represent exposure to rickettsiae or underlying rickettsial diseases. It is not known whether the presence of rickettsiae is causally related to the patients' chronic illnesses, or reactivation of a latent rickettsial infection.

## Introduction

Chronic illness can be frustrating for patient and doctor alike. This is only accentuated when no diagnosis can be made.[1–3] Often the patient feels as if no one can give them a credible diagnosis and they often seek assistance from 'alternative medicine'.

It has been claimed that some patients with chronic illnesses, including those with post-infection fatigue syndrome, have underlying rickettsial disease, although control group investigations are lacking.[4] Although this view requires further evidence, it is well known that an infectious disease may lead to chronic infection and chronic illness. Such infections include Epstein-Barr Virus (EBV),[5] Cytomegalovirus (CMV),[6] Parvovirus B-19,[7] Barmah

*Address correspondence to Dr S. Graves, Australian Rickettsial Reference Laboratory, Barwon Biomedical Research, the Geelong Hospital, Geelong, Victoria, Australia 3220.*
*email: Stephen.Graves@hnehealth.nsw.gov.au*

Forest Virus,[8] Human Herpes type 6 Virus (HHV-6),[9] Brucellosis (including *Brucella melitenis*),[10] Lyme disease (*Borrelia burgdorferi*),[11–12] *Mycoplasma* species (especially *M. pneumoniae* and *M. fermentans*)[13] and *Chlamydia pneumoniae*.[14] Post-infection fatigue syndrome after Q-fever (*Coxiella burnetii*) has also been well documented in both adults[15–18] and children.[19] It has associations with cytokine aberrations,[20] specific immune system genes alleles[21] and the persistence of *C. burnetii* DNA.[22–24]

The microbial agents listed above grow mainly within an intracellular environment or in the case of mycoplasmas, on the cell surface. Rickettsiae are small obligate intracellular bacteria usually transmitted to a human via the bite of an arthropod, often a tick. Rickettsial disease usually has an acute onset and has major symptoms of headache, rash, fever, myalgia, arthralgia and fatigue.[25]

Most cases of acute rickettsial disease resolve without complication after antibiotic therapy, however, cases of Brill–Zinsser disease, a recurrent form of epidemic typhus (*Rickettsia prowazekii*), are well documented decades after the initial infection.[26] Cases of persistent scrub typhus (*Orientia tsutsugamushi*) infections in humans[27] and rickettsial spotted fever infections in dogs[28] have also been documented. Thus it is reasonable to speculate that spotted fever group (SFG) rickettsia may also be able to cause a chronic infection or be associated with a chronic illness.

To test the hypothesis that some chronically unwell patients have underlying rickettsial disease, two groups of chronically ill patients were studied. The first cohort was from Victoria, Australia, under the care of Dr Geoff Kemp was tested for the presence of rickettsia, by *in vitro* culture, rickettsial DNA via real-time PCR and serology. The second cohort of patients from South Australia under the care of Dr John Graham was tested serologically for rickettsial antibodies but not by rickettsial PCR or culture.

## Methods

A patient cohort of 526 was recruited from the practice of Dr Geoff Kemp in Camberwell, Victoria from February 2003 to February 2005 ('Melbourne cohort'). Patients came from both urban and rural areas. Patients were added to the study *ad seriatim* as they presented. Dr Kemp's usual diagnostic tests included investigations on peripheral blood for rickettsial DNA (by real-time PCR), viable rickettsia (by culture) and rickettsial serological tests. The patients of Dr Kemp had actively sought his advice due to his expertise in treating chronic conditions and had thus come from all over Victoria. As such they did not represent a typical patient cohort.

A control group of 400 peripheral blood samples were randomly selected from another medical practice in Newcastle, New South Wales and subjected to the same rickettsial real-time PCR and serological tests. The investigators had no details on any of these control patients but it can be assumed that they were not completely well and had their blood taken for purposes that may have included full blood counts, blood grouping/cross matching, HbAlc or red cell folate testing, as they were discarded bloods from a diagnostic haematology laboratory.

SFG rickettsial serological assays were performed using the indirect microimmunofluorescence assay with the antigens *R. akari, R. australis, R. conorii, R. honei, R. rickettsii* and *R. sibirica* using previously described methods.[29] SFG rickettsial titres of $=256$ were considered positive.

Rickettsial culture was performed only on the patient group (not controls) using previously described methods.[30]

DNA was extracted from buffy coat specimens from the patient and control groups as well as positive rickettsial cultures with a DNA extraction kit (Gentra, USA) using the manufacturer's protocols. Rickettsial specific citrate synthase real-time PCR was performed on each DNA extract and rickettsial cell culture as previously described.[31] To rule out any false positive results, positive samples were repeated on two other occasions for confirmation. Additionally, UDG was incorporated in all master mixes in order to prevent carryover contamination from amplified products. Conventional gel-based PCR for the rickettsial 17 kDa antigen gene using the primers MTO-1 and MTO-2 was performed on real-time PCR positive cultures and buffy coat extracts as previously described, except with an annealing temperature of 51°C.[32] Subsequent sequencing of the 17 kDa antigen gene amplicons was used to identify the aetiological rickettsia in PCR positive cultures and buffy coat specimens (Newcastle DNA, Newcastle, Australia).

The second cohort of 581 chronically unwell patients was recruited from the practice of Dr John Graham, a physician specializing in chronic illnesses, in Adelaide, South Australia ('Adelaide cohort'). These patients, like Dr Kemp's, had rickettsial serological testing performed as apart of their routine diagnostic testing. Testing for rickettsial DNA and culture was not done. A group of 34 patients without a chronic condition, from Dr Graham's practice, was used as controls for serological comparisons. All patients had rickettsial serology performed using the methods described before.

**Table 1** Rickettsial DNA positive patients from the 'Melbourne cohort'; their clinical and laboratory findings

| Main clinical features and laboratory findings | Case | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | % + |
| Clinical symptoms | | | | | | | | | | | | | | | |
| Chronic pain | | | | | | | | | | | | | | | |
| Chronic pain/spondylitis | + | + | + | + | + | + | + | + | + | + | + | + | + | + | 100 |
| Rheumatoid arthritis/arthralgia | + | − | + | − | − | + | + | + | + | + | + | + | + | − | 71 |
| Myalgia/fibromyalgia | + | − | − | + | + | + | − | + | − | − | − | + | + | − | 50 |
| Headaches | + | − | − | + | + | − | − | − | − | − | + | − | − | + | 36 |
| Fatigue | | | | | | | | | | | | | | | |
| Fatigue/tiredness (<6 months) | + | − | − | + | + | + | − | + | + | + | + | + | − | + | 71 |
| Chronic fatigue (>6 months) | + | − | − | + | + | + | − | − | − | + | + | − | − | + | 50 |
| Depression | | | | | | | | | | | | | | | |
| Abnormal laboratory findings | + | + | − | + | + | + | − | + | + | + | + | − | − | − | 64 |
| ESR/CRP | + | − | − | + | + | − | + | + | + | + | + | + | − | − | 64 |
| Liver function tests | + | − | − | + | + | + | − | − | + | − | + | − | − | − | 43 |
| Blood film | − | + | − | + | − | − | + | + | − | + | + | − | − | − | 43 |
| Rickettsial tests | | | | | | | | | | | | | | | |
| PCR | | | | | | | | | | | | | | | |
| Real-time (citrate synthase) | + | + | + | + | + | + | + | + | + | + | + | + | + | + | 100 |
| Traditional (17kDa gene) | + | + | + | + | + | − | − | − | − | − | − | − | − | − | 36 |
| Rickettsial culture | + | + | − | − | − | − | − | − | − | − | − | − | − | − | 14 |
| SFG serology titre | − | − | − | 256 | − | 256 | − | 256 | − | 256 | − | − | 1024 | − | 36 |

ESR: Erythrocyte Sedimentation Rate, CRP: C-Reactive Protein. + Symptom/finding present. − Symptom/finding absent/ seronegative.

## Results

Examination of the 'Melbourne cohort' by citrate synthase real-time PCR revealed that 14/526 (3%) had detectable rickettsial DNA in their peripheral blood, compared to none of the Newcastle control cohort ($P < 0.001$; chi-squared test; Table 1). Of the 14 patients positive by rickettsial real-time PCR, only 5 had a 420bp 17 kDa antigen gene product amplified by traditional, gel-based PCR that was subsequently sequenced (Table 1). A rickettsial isolate was also obtained from 2 of the 14 patients (Table 1), but neither isolate could be maintained in continuous culture. Each of the 17 kDa PCR products from the five positive buffy coat specimens and the two rickettsial isolates yielded a 400 bp DNA sequence that was 100% homologous with the *Rickettsia honei* strain 'marmionii' 17 kDa antigen gene (GenBank accession number AY37683).[33]

Of the 14 patients positive by real-time PCR, significantly fewer were detected in spring than all other seasons ($P < 0.03$; Fisher's exact test) and significantly more were detected in winter than all other seasons ($P < 0.03$; Fisher's exact test) (Figure 1).

The summary of the 14 rickettsial DNA positive patients in the 'Melbourne cohort' is shown in Table 2. Common clinical features from these 14 patients included arthralgia, fatigue or tiredness and clinical depression. Other symptoms included myalgia or fibromyalgia, and chronic headache (Table 1). The most common laboratory abnormality detected was raised inflammatory markers (erythrocyte sedimentation rate and C-reactive protein; Table 1). Females were more commonly afflicted than males (11:3) with most patients being middle-aged (mean 43 years). The average length of

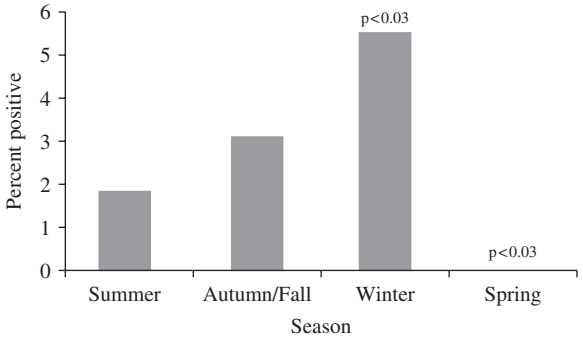


**Figure 1.** A graph showing the seasonal incidence of rickettsial DNA positive patients from the 'Melbourne cohort'. *P*-values are shown above statistically significant seasons.

**Table 2** Summary of the 14 patients of the 'Melbourne cohort' with rickettsial DNA in their peripheral blood

| Case | Age (years) | Sex | Approximate length of illness (years) | Patient residence |
|---|---|---|---|---|
| 1 | 73 | F | 10 | Melbourne |
| 2 | 37 | F | 2 | Melbourne |
| 3 | 32 | M | 1 | Rural Victoria |
| 4 | 19 | F | 1 | Rural Victoria |
| 5 | 22 | F | 2 | Rural Victoria |
| 6 | 39 | M | 14 | Melbourne (originally from Queensland) |
| 7 | 25 | F | 4 | Melbourne |
| 8 | 70 | F | 20 | Rural Victoria |
| 9 | 50 | F | 5 | Rural Victoria |
| 10 | 58 | F | 8 | Melbourne (grew up in Rhodesia) |
| 11 | 50 | F | 20 | Melbourne |
| 12 | 45 | F | <1 | Melbourne |
| 13 | 22 | M | 8 | Melbourne (originally from Poland) |
| 14 | 55 | F | 17 | Melbourne |
| Mean | 43 | M:F = 1:4 | 8 | Rural:Urban = 5:9 |

illnesses was 8 years. Of the 14 patients, 9 were from urban Victoria (Melbourne) and the remaining 5 from rural areas (Table 2).

Positive rickettsial serology titres (=256) were seen in only 5/14 (36%) of the patients who were positive in the rickettsial real-time PCR assay (Table 1). Serologically the 'Melbourne cohort' had 206/521 (39%) patients with positive rickettsial titres. When compared to serological results of the Newcastle control cohort, the latter contained significantly less patients (3/399; 1%) with positive rickettsial titres ($P < 0.0001$; two sample test for binomial proportions) (Figure 2).

A significantly higher proportion of patients in the 'Adelaide cohort' (238/581; 41%) had positive rickettsial serology, when compared to the Newcastle control cohort (3/399; 1%) ($P < 0.0001$; two sample test for binomial proportions) (Figure 2). The control cohort from the same Adelaide clinic also had significantly fewer patients with positive rickettsial serology (5/34; 15%) than the 'Adelaide cohort' of patients ($P < 0.002$; two sample test for binomial proportions).

## Discussion

We now report that 14 of 526 (3%) chronically unwell patients from a medical practice in Melbourne, Victoria had rickettsial DNA in their blood. Of the 14 patients, 5 had a *R. honei* strain 'marmionii' DNA sequence amplified, an aetiological agent of Flinders Island Spotted Fever (FISF).[33] *Rickettsia honei* strain 'marmionii' was able to be cultivated from a further two patients. As a whole, both the Melbourne and Adelaide cohorts had a significantly higher proportion of rickettsial seropositivity than the control cohorts.

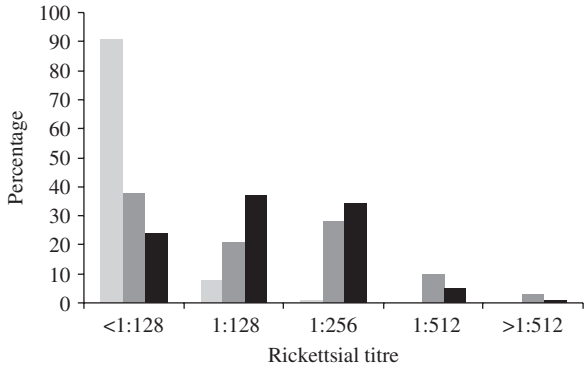


**Figure 2.** A graph showing the distribution of rickettsial serology titres from the control cohort of Newcastle (light) and chronically ill patient cohorts of Adelaide (medium) and Melbourne (dark). Only titres of $\geqslant 256$ were considered genuine positives.

The 14 cases of rickettsaemia in the current study had similar symptoms to cases of acute rickettsial infection (e.g. myalgia, arthralgia, headache and lethargy).[25] Abnormal laboratory findings (e.g. thrombocytopenia, aberrations in leukocyte and neutrophil counts, raised C-reactive protein and liver transaminase levels) were also found in both rickettsaemic patients within our study and acute rickettsioses.[34] The pattern of rickettsial serology in the 14 patients who were rickettsial DNA positive (only 5/14 seropositive) was not dissimilar to the seven acute rickettsial patients with FISF.[33] In the current 14 cases the failure of some patients to seroconvert is not understood and may have been due to the extremely low quantity of rickettsial DNA/organisms circulating in the patient's blood. The Melbourne and Adelaide cohorts' serology findings were not dissimilar, with 39 and 41%, respectively having positive rickettsial serology. Such a high sero-prevalence could be explained by Adelaide and surrounding areas, being endemic for spotted fever illnesses.[30,34] However, endemic rickettsial areas in Victoria are unknown with the exception of Gippsland.[35] The significantly higher proportion of chronically unwell patients that were seropositive for rickettsia suggests that exposure to rickettsiae may be causally related to their chronic conditions. It is unclear if the underlying rickettsaemia is (partially) responsible for the patients' chronic ailments or simply a reactivation of a latent infection

caused by immunosuppression generated from the chronic condition itself or the treatment thereof. Regarding the later, bacterial diseases such as tuberculosis,[36] syphilis,[37] brucellosis,[38] Q-fever[22–24] and, with most relevance, typhus (Brill–Zinsser disease)[26] are known to cause latent infections using yet to be defined mechanisms. A rickettsial infection would normally be eradicated from the host, unless there is an aberration in the host's immune response. Gene expression studies in patients with chronic fatigue syndrome revealed an increase in T-cell activation.[39] Although a cytotoxic T-cell response is an important factor for eradicating a rickettsial infections,[40] chronic antigenic stimulation of T-cells may be responsible for fatigue induced as a result of an aberration in the patient's immune response. It has been postulated that post Q-fever fatigue syndrome, along with its cytokine dysregulation, may be caused by a similar chronic antigenic stimulation.[20,22–24]

The number of rickettsia found in the patients' blood was very low, being detectable in most cases only by a highly sensitive and specific rickettsial real-time PCR.[31] The amplicons of the citrate synthase real-time PCR assay are very small (74 base pairs) and cannot be sequenced. The low sensitivity of the traditional gel-based 17 kDa PCR may explain why only 5/14 real-time PCR positive patients were positive with the traditional assay. Hence it was not possible to determine the rickettsial species present in the other nine patients. The two isolates of *R. honei* strain 'marmionii' were initially isolated in Vero cell culture and then grown for another two passages in XTC-2 cells before they could no longer be passaged in cell culture. This phenomenon has been noted with previous isolates of *R. honei* strain 'marmionii' from acute cases.[33] Unfortunately it was not possible to obtain control patient specimens from Melbourne (rather than Newcastle) for the 'Melbourne cohort', although this would have been ideal.

To complement the current study additional cohort and prospective studies are needed. As apart of these additional studies a more comprehensive effort needs to be taken in examining patient blood for rickettsaemia via real-time PCR. Further study of patient immune gene alleles and cytokine concentration should determine whether immune system dysfunction may be contributing to the patients' chronic illnesses and whether the persistence of rickettsaemia is causal. The presence of rickettsaemia and a high proportion of rickettsial seropositivity within two cohorts of chronically ill patients suggest that rickettsiae may be an underlying factor to some of the patient's illnesses.

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*Conflict of interest*: None declared.

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