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An analysis of Q fever patients 6 years after an outbreak in Newport, Wales, UK

H. HUSSAIN-YUSUF[1], A. ISLAM[1], B. HEALY[2], M. LOCKHART[1], C. NGUYEN[1], O. SUKOCHEVA[1], J. STENOS[1] and S. GRAVES[1]

From the [1]Australian Rickettsial Reference Laboratory, Newcastle & Geelong, Australia and [2]Public Health Wales Microbiology, Cardiff, Wales, UK

Address correspondence to Dr S. Graves, Australian Rickettsial Reference Laboratory, Division of Microbiology, Hunter Area Pathology Service, John Hunter Hospital, Newcastle, NSW, Australia.
email: stephen.graves@hnehealth.nsw.gov.au.

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Summary

Background: A cohort of 211 factory workers was exposed to a point source of Q fever in 2002. A total of 38 cases and 14 controls took part in a follow-up study 6 years after the outbreak.
Aim: To compare Q fever serology, the presence of viable *Coxiella burnetii,* its DNA and fatigue between patients and controls.
Design: Laboratory case study.
Methods: Q fever serology was by microimmunofluroescence. Viable *C. burnetii* was detected by VERO cell culture and SCID mice inoculation with patient blood samples. *Coxiella burnetii* DNA was detected by qPCR (com1 gene) on patients' PBMC and on VERO cultures after 6 weeks incubation. Fatigue was measured by the Chalder Fatigue Scale.
Result: At 6 years after the outbreak, 7 of the 38 patients had become seronegative and 4 of the 14 of the controls had become seropositive for Q fever. None of the patient/control peripheral blood mononuclear cells (PBMC) contained viable *C. burnetii* by VERO cell culture or by SCID mouse inoculation (death or splenomegaly) and none contained *C. burnetii* DNA by qPCR.
Conclusion: Six years after acute Q fever, some patients had become seronegative but none contained viable *C. burnetii* or its DNA in their PBMC.

Introduction

Coxiella burnetii is a Gram-negative bacterium that grows intracellularly and causes the zoonotic disease Q fever. It is endemic in animals and arthropods in most parts of the world.[1] The main reservoir for human infection is farm animals. There have been many outbreaks of Q fever reported, often associated with farm animals directly or indirectly. Urban outbreaks of Q fever have also been reported[2,3] whereby the association with an animal reservoir is either tenuous or unclear. The focus of this report is an urban outbreak that occurred in a cardboard box manufacturing plant in South Wales, UK, between July and September 2002.[4] A total of 106 cases of acute Q fever were confirmed at the time. The highest number of cases occurred in the central office of the plant, with fewer cases in adjacent offices and none amongst employees working in and around the periphery or the outside of the

plant where there may have been contact with animals. Although there was no direct evidence, the investigating team hypothesized that contaminated straw board may have been the source of the outbreak, with organism released during drilling into the straw board at the time of office renovations.

Coxiella burnetii can exist in two morphological forms, the large cell variant which is the active form growing in acidified parasitophorous vacuoles of monocytes and macrophages and the spore-like small cell variant which is the metabolically inactive but infectious form that is resistant to extreme environmental conditions and persists in the environment for long periods.[5] *Coxiella burnetii* is highly infectious and the infective dose can be less than 10 organisms for guinea pigs and humans.[6]

Infection with Q fever can take four forms; acute infection, asymptomatic exposure with seroconversion, chronic infection (following previous acute infection) and post Q fever fatigue syndrome (QFS). The later has been only recently recognized.[7,8] Around 10–15% of patients with acute Q fever subsequently develop QFS lasting from 1 to 10 years. Chronic Q fever infection occurs as a complication of acute Q fever infections in some patients, often resulting in endocarditis.[9] Despite much research Q fever remains a poorly understood disease. Most cases of acute Q fever infection probably resolve spontaneously without antimicrobial intervention. Cases of symptomatic Q fever infection often go undiagnosed. Serological follow up of acute cases is deemed important.[10] However there is controversy over diagnostic cut off titres for chronic Q fever as serological assay results vary from laboratory to laboratory.[11] Our recent investigation into the post-QFS[12,13] has provided evidence for the persistence of a non-infective complex of *C. burnetii* antigens, including DNA, in some patients. These studies were carried out on samples taken from patients 12 years after a Q fever outbreak in Birmingham 1989 that was originally described in 1998.[3] To further our understanding of Q fever infection and persistence of *C. burnetii* (viable cells or DNA only), we tested samples taken 6 years after an outbreak of Q fever in Newport 2002. The study was designed to test the current hypothesis on the aetiology of post-Q fever fatigue.[12] This hypothesis states that certain antigens (including DNA) of *C. burnetii* (and probably including the phase 1 lipopolysaccharide (LPS)) remain undegraded in some patients following an episode of acute Q fever. This material, named an 'immunomodulatory complex', perturbs the patient's immune system, by causing persistent and excessive synthesis of pro-inflammatory cytokines.[13] It was proposed that an abnormal cytokine profile produce the symptoms of ongoing fatigue in the patient. The adaptive 'acute fatigue' of acute infection becomes the unwanted 'chronic fatigue' associated with the post-infection period. The 'post-viral fatigue syndrome', seen in many other infections, may have a similar aetiology. By comparing individuals from the cohort with different levels of fatigue with serologically negative individuals who were working at the factory at the same time we hoped to clarify the aetiology of the condition.

Materials and Methods

Patients' samples

All patients came from the cohort of confirmed acute Q-fever cases acquired during the August 2002 Q fever outbreak in Newport, UK. A cohort of 211 factory workers was exposed to a point source of Q fever in 2002. A total of 38 cases, defined as those who had serological evidence of acute Q fever in 2002, and 14 controls, defined as those who worked in the same factory but were serologically negative for Q fever antibodies at the time of the outbreak, consented to take part in a follow-up study 6 years after the outbreak. Patients were categorized according to serological profiles at the time of the original outbreak. A total of 35 of the 106 acute cases, 3 of the 23 patients with uncertain serology and 14 of the 96 serologically negative factory employees without symptoms agreed to participate in the follow-up study. Blood (serum and peripheral blood mononuclear cells (PBMCs)) was sent to the Australian Rickettsial Reference Laboratory (ARRL), Australia, for analysis. Further analysis (by SCID mice inoculation) was conducted on a subset of 18 patient samples (1,3,5,6,7,14, 15,17,31,33,35,45,46,50,52,54,57 and 58) selected at random and one control (#4 (Figure 1)).

Treatment of samples

Q fever serology[14] on sera and qPCR[15] on PBMCs were carried out on all 52 samples.

Nineteen samples were subjected to further analysis by testing the PBMC samples for the presence of viable *C. burnetii.* Each sample was divided into three aliquots; the first aliquot was directly subjected to com1 qPCR. The second aliquot was inoculated into VERO cell culture (2×) followed by com1 qPCR after 6 weeks incubation at 35°C in 5% CO_2 to detect growth. The third aliquot was inoculated into SCID mice (3×), which were euthanased 6 weeks later (as all were still alive) and examined for splenomegaly.

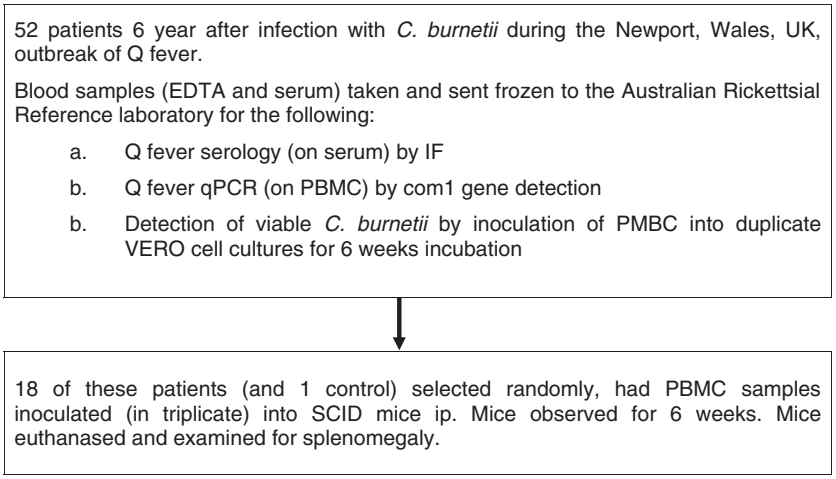

Figure 1. Protocol of study.

The remaining 32 samples were screened only for viable *C. burnetii* by inoculation of the patients PBMCs into VERO cell culture (2×) followed by com1 qPCR after 6 weeks culture to detect growth.

Serology

Micro-immunofluorescence (IF) was used to determine the presence of antibodies to phase I and II of *C. burnetii* in the IgG, IgM, IgA fractions and total antibody. These tests were done in the routine diagnostic laboratory of the ARRL. Serum was diluted in phosphate buffered saline (PBS) with 2% casein, at 1:25 and 1:400 (to detect a prozone) and if positive serially diluted until 1:3200. Sera were applied to slides fixed with *C. burnetii* (phase I and phase II). Rheumatoid factor removal agent was added to serum for the detection of IgM and IgA. After patient serum was applied, the slides were incubated for 30 min in humid conditions at 35°C, washed twice in 1/10 PBS, air dried and conjugate applied (FITC anti-human (diluted 1 in 50 with 2% casein) for IgA or IgM and (diluted 1 in 100 with 2% casein) for IgG or total antibody (IgA + IgG + IgM). The incubation, washing and drying steps were repeated. Slides were covered with mounting fluid, a cover slip added and viewed at 400× magnification with a UV lamp microscope.

Positive results were defined as fluorescence in sera that had been diluted to 1:25 or greater. Negative results were sera that didn't fluoresce at the screening dilutions of 1:25 or 1:400.

qPCR com1

Total genomic DNA was extracted from patient PBMCs and VERO cell samples using the RBC Hi Yield Genomic DNA Mini PCR kit as described by the manufacturer (Real Biotech Corporation, Real Genomics Cat. No. YGB300). One hundred microlitres of serum or PBMCs or VERO cell suspension was used as the starting materials. Fifty microlitres of elution buffer were used to re-suspend the DNA. Real-time PCR was conducted on the extracted DNA targeting the com1 outer membrane protein gene of *C. burnetii*. Each 25 µl reaction mixture was made of 5 µl of extracted DNA, 2.5 µl of forward (AAA ACC TCC GCG TTG TCT TCA) and reverse (GCT AAT GAT ACT TTG GCA GCG TAT TG) primers at 4 µM, dually labelled hydrolysis probe generated using the reported fluorophore 6-carboxyfluorescein (FAM) and Black Hole Quencher 1 (FAM AGA ACT GCC CAT TTT TGG CGG CCA BHQ1) at 2 µM and 12.5 µl of Platinum Quantitative PCR SuperMix-UDG (invitrogen). Primers and probes were synthesized by BioSearch Technologies (Novato, CA). Amplification was performed according to the following parameters: one step of 50°C for 2 min, followed by one step of 95°C for 2 min and 50 cycles of 95°C for 10 s and 60°C for 20 s. The amplicon produced by this PCR was 76-bp long.

VERO cell cultures

Duplicate cultures of VERO (monkey kidney epithelial) cells, in 25 cm^2 flasks, 100% confluent, were each inoculated with 0.1 ml of PBMCs from each patient. The flasks were incubated at 35°C in an atmosphere of 5% CO_2, in RPMI-1640 medium, with 1% glutamine and 10% foetal calf serum, for 6 weeks. Each flask contained 10 ml media, which was changed every 2 weeks. At the end of 6 weeks the complete monolayer was scrapped into a residual volume of 1 ml medium, homogenized and subject to com1 qPCR.

SCID mice inoculation

Triplicate, adult, female SCID mice (NOD.CB17-prkdc scid/JAsmu) were each inoculated intraperitoneally with 0.1 ml of patient's PBMCs. This is an extremely sensitive assay for detecting viable *C. burnetii.*[16] Mice were housed in a Techniplast cage unit (2 ISO12) and kept at 22°C with food and water provided ad libitum for 6 weeks. The cages were fully enclosed, with HEPA filters on the exit air and under negative pressure (Techniplast, Italy). The cages were kept in a BSL-3 laboratory at the Division of Microbiology, Pathology North-Hunter, John Hunter Hospital, Newcastle, NSW, Australia. Animal use was approved by the 'Animal Care and Ethics Committee' of the ARRL. At the end of 6 weeks, each mouse was euthanased, its spleen removed and weighed to detect splenomegaly.

Results

Patients were examined 6 years after acute Q fever. Blood specimens (serum and PBMCs) were processed in a manner that could detect viable *C. burnetii* and DNA of *C. burnetii* (Figure 1).

Serology

Results of microimmunofluorescence serology for *C. burnetii,* on all patients (Table 1) and controls (Table 2) sera showed significant variability. A total of 18% (7/38) had lost their seropositivity after 6 years. Of the remainder, 10 patients had phase I antibodies, and 21 had both phase I and phase II antibodies. Of the 14 controls (who were serologically negative at the time of the outbreak UK serology), 4 now tested positive on the blood taken for this study, 2 with phase II antibodies, 1 with phase II and phase I antibodies and one patient (#43) with phase I antibodies alone, an unusual result. Of the longer lasting phase II antibodies, IgG was the most persistent (34/34), with IgM (8/34), and IgA (5/34) much less so. Clearly, for serological testing of past exposure to *C. burnetii,* phase II IgG is the most useful antibody. These serological results have already been part of a comparative study.[11] Antibody titres were generally modest ($\leqslant$400) but a few patients had high titres ($\geqslant$800) (Patients # 3, 32, 33, 35, 49, 52 and 58), but only against phase 2 *C. burnetii.* The highest titre against phase 1 *C. burnetii* was 400 (Patients # 32, 35, 49 and 58). One might think that these four patients (with high phase 2 and modest phase 1 antibody titres) may have been subject to ongoing antigenic stimulation from residual *C. burnetii* antigen, yet none of these four patients had *C. burnetii* DNA detected in their PBMCs.

Cell culture

Viable *C. burnetii* normally grows well in VERO cells, especially after 6 weeks inoculation. The detection of only 1/51 *C. burnetii* positive cell monolayers (and at a high C_T of 36.7) suggests that no patient PBMC cells contained viable *C. burnetii* detectable by VERO cell culture.

SCID mice inoculation

After inoculation with patients' PBMCs, no mouse died of Q fever/coxiellosis nor had splenomegaly. This supports the view that no viable *C. burnetii* were present in these samples.

Patient fatigue at 6 years post-infection

A Chalder Fatigue Scale score (0 = no fatigue: 11 = extrême fatigue)[17] was allocated to 11 patients; four had significant fatigue (#33, 45, 52 and 54), four had some fatigue (#5, 17, 46 and 50) and three were not fatigued (#15, 31 and 35). There was no detectable relationship between patient fatigue levels and serology at 6 years (Table 1) nor with the persistence of viable *C. burnetii* or DNA from *C. burnetii*, as neither were detected in any sample.

Discussion

This study was undertaken as an opportunistic response to the outbreak of Q fever in Newport, Wales, in 2002. Six years later a subset (n = 52) of the employees working in the factory had blood taken for investigation. Chalder fatigue scores were significantly raised in patients vs. controls.[17] One patient had completed treatment for chronic Q fever endocarditis (#35).[10]

The ARRL was sent aliquots of the blood samples, for serology, PCR and tests for detecting the presence of viable *C. burnetii* by cell culture and SCID mice inoculation.

The serology results were also part of a comparative study of Q fever serology results in three diagnostic laboratories in the UK, France and Australia.[11]

The results obtained were similar to those from investigations of the Birmingham Q fever outbreak in 1989[3,12,13,18] and confirmed the following:

(a) Patient antibody levels drop, often to undetectable levels, some years after infection. In the current study, 18% of patients were seronegative by 6 years

Table 1 Micro-immunofluorescence antibody titres for phase I and phase II antigens of *C. burnetii* and Chalder Fatigue Score (0 no fatigue; 11 extreme fatigue) in patients 6 years after acute Q fever

Patient number	Phase II				Phase I				
	IgM	IgG	IgA	TOTAL	IgM	IgG	IgA	TOTAL	Chalder Fatigue Score
3	–	800	200	800	–	25	–	25	ND
5	–	200	–	200	–	–	–	–	3
6	–	50	–	50	–	–	–	–	4
9	–	–	–	–	–	–	–	–	11
10	–	100	–	100	–	50	–	50	18
11	–	–	–	–	–	–	–	–	2
12	–	50	–	50	–	50	–	50	5
13	–	100	–	100	–	–	–	–	2
14	–	100	–	100	–	–	–	–	11
15	–	400	–	400	–	–	–	–	0
17	25	200	–	200	–	–	–	–	1
19	–	100	–	100	–	–	–	–	0
25	–	50	–	50	–	–	–	–	7
26	–	–	–	–	–	–	–	–	1
29	–	50	–	50	–	–	–	–	4
31	25	200	–	200	–	–	–	–	0
32	50	800	200	800	–	400	100	400	3
33	25	800	–	800	–	–	–	–	6
34	–	25	–	25	–	–	–	–	6
35*	–	800	100	800	–	400	–	400	0
37	–	50	–	50	–	–	–	–	5
41	–	–	–	–	–	–	–	–	0
42	–	50	–	50	–	–	–	–	5
44	–	–	–	–	–	–	–	–	8
45	–	–	–	–	–	–	–	–	6
46	–	400	–	400	–	–	–	–	3
48	–	50	–	50	–	–	–	–	9
49	–	800	–	800	–	400	–	400	5
50	25	25	–	25	–	–	–	–	2
51	–	400	–	400	–	–	–	–	6
52	25	800	25	800	–	–	–	–	11
54	50	50	–	50	–	200	–	200	8
55	–	50	–	50	–		–	–	5
56	–	100	–	100	–	50	–	50	1
57	–	100	–	100	–	50	–	50	ND
58	–	1600	–	1600	–	400	–	400	ND
59	–	–	–	–	–	–	–	–	9
60	–	100	–	100	–	–	–	–	8

N = 38

*This patient originally had Q fever endocarditis. ND: not done.

post infection. This shows the unreliability of serology in detecting post infection/exposure and it cannot be relied upon in situations where such information is vital (e.g. prior to vaccination). Pre-vaccination screening with intradermal skin testing with *C. burnetii* antigen is, therefore, mandatory[19] even if the serology is negative.

(b) When antibody does persist in a patient, the phase II IgG is the most long-lasting immunoglobulin isotype. Virtually always the phase I antibodies decay to undetectable levels before the phase II antibodies do so.

(c) None of the 52 patients met the Australian serological criteria for chronic Q fever at 6 years post-infection.

(d) None of the PBMC samples contained viable *C. burnetii*. This was confirmed by two methods:

(i) Inoculation into VERO cell culture and incubation for 6 weeks. At the end of this period only 1/51 patient samples were PCR positive for the com1 gene of *C. burnetii* and the high C_T value (36.7) would not normally be accepted as positive in our routine diagnostic laboratory. This was likely to be a false positive result.

Table 2 Micro-immunofluorescence antibody titres for phase I and phase II antigens of *C. burnetii* in controls (originally seronegative) 6 years after the outbreak of Q fever

Control number	Phase II				Phase I			
	IgM	IgG	IgA	TOTAL	IgM	IgG	IgA	Total
1*	50	25	–	50	–	–	–	–
4	–	–	–	–	–	–	–	–
7*	–	100	50	100	–	50	–	50
8	–	–	–	–	–	–	–	–
20*	–	25	–	25	–	–	–	–
21	–	–	–	–	–	–	–	–
23	–	–	–	–	–	–	–	–
27	–	–	–	–	–	–	–	–
28	–	–	–	–	–	–	–	–
38	–	–	–	–	–	–	–	–
40	–	–	–	–	–	–	–	–
43*	–	–	–	–	–	50	–	50
47	–	–	–	–	–	–	–	–
53	–	–	–	–	–	–	–	–

$N = 14$

*These patients were either exposed to *C. burnetii* after the outbreak and during the 6 years leading to the current time of testing or the serological reactions are false-positives.

(ii) Inoculation into SCID mice. These mice are extremely sensitive to viable *C. burnetii* [15,20] and will respond to a single (1) viable *C. burnetii* cell with death (between Day 30 and 50 post-inoculation), or splenomegaly (detected on euthanasia).

No viable *C. burnetii* were detected in any of the 38 patient samples, despite the very sensitive detection methods used. This supports earlier findings where PBMCs and bone marrow aspirates[13,18] were negative for viable *C. burnetii*. However, it is possible that these patients were harbouring viable *C. burnetii* organism in other organs that were not sampled, (e.g. heart valves). Cases of Q fever endocarditis in patients with negative PCR are well recognized. There is also a case report of a patient with a strongly positive valve by PCR in whom culture of the organism was not possible.[12] *Coxiella burnetii* is thought to reactivate after a period of dormancy if conditions are favourable (e.g. immunosuppression, pregnancy) giving rise to chronic Q fever. Clearly, in these patients *C. burnetii* did remain, after termination of the acute infection, in a quiescent, but still viable, state and were able to start growing again when conditions became favourable for bacterial reactivation.

One objective of this study was not achieved. As no DNA from *C. burnetii* was detected in any patients' PBMC, it was not possible to examine any correlation between such persistent DNA and the level of post-Q Fever fatigue in the patient. Either more sensitive DNA assays, or more invasive sampling (e.g. bone-marrow aspirates) will be needed to test this hypothesis. Unfortunately, no conclusions pertaining to the hypothesis could be drawn from the current study.

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Conflict of interest: None declared.

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