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Long-term persistence of *Coxiella burnetii* in the host after primary Q fever

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SUMMARY

After a primary infection *Coxiella burnetii* may persist covertly in animals and recrudesce at parturition to be shed in the products of conception and the milk. Similar latent persistence and recrudescence occurs in man: namely, infection of placenta, heart valve or mural endocardium, bone or liver. The numbers of organisms, their viability and cellular form, and the underlying organ sites of latent infection for the coxiella are obscure. During investigations of 29 patients with a chronic sequel to acute Q fever, the post-Q fever fatigue syndrome (QFS) [1–3], sensitive conventional and TaqMan-based PCR revealed low levels of *C. burnetii* DNA in blood mononuclear cells (5/29; 17%), thin needle liver biopsies (2/14; 14%) and, notably, in bone marrow aspirates (13/20; 65%). Irrespective of the ultimate significance of coxiella persistence for QFS, the detection of *C. burnetii* genomic DNA in bone marrow several years after a primary infection unveils a new pathological dimension for Q fever.

INTRODUCTION

It has been evident since the 1940s that *Coxiella burnetii* can persist in animals after an initial clinical or subclinical infection. For example, guinea-pigs inoculated with *C. burnetii* developed persistent infection in liver, spleen, kidney (up to 120 days after inoculation), in testes and seminal vesicles (100 days) [4], or in brain (500 days) [5]. Cryptic infection could be reactivated in guinea pigs by pregnancy, X-irradiation or cortisone treatment [6–9]. Again, after experimental inoculation of sheep in early pregnancy, *C. burnetii* was recovered from liver, spleen, kidney, lymph nodes, bone marrow, and intestine up to week 13 of pregnancy but not thereafter. Placental and foetal samplings were negative from 1–15 weeks but became positive just before parturition at 19–20 weeks [6, 10]. Despite the evidence that *C. burnetii* can produce latent or recrudescent infection in animals, human Q fever is not infrequently viewed as an unpleasant but essentially self-limiting infection with an occasional case of Q fever endocarditis [11] or of placental infection [12] as an aberrant outcome. Other infrequent manifestations of chronic Q fever infection in man are osteoarticular infection [13, 14], colonization of vascular prostheses [15], chronic granulomatous hepatitis in the absence of endocarditis [16, 17], and, probably, infection of the genital tract [18].

We do not know whether persistent symptomless infection is the outcome in most or all human cases of Q fever or if only a minority of patients fail to clear the infection and remain symptomatic. Again, in both the animal models and in man, the organ sites for latent infection are essentially unknown. The biological form of the persistent organisms is also unresolved. For example, they may be the highly

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infective, resistant, small cell variant (SCV) of *C. burnetii*, readily cultured and visualized by IFA staining or electron microscopy [19]. Hypothetically, they might be minute forms [20] of the coxiella claimed to mature in culture to SCV, or conceivably even the morphological spores seen in coxiella cells in culture or in human tissue [19, 21], of unknown infectivity, but containing DNA, presumably genomic and, if so, detectable by a suitably targeted PCR assay.

These issues have been explored as a first phase of an ongoing study of the possible role of persistent infection with *C. burnetii* in a newly recognized, common (10–15% of acute cases), sequel to acute Q fever – a prolonged (5–10 years or more) post-infection fatigue syndrome (QFS) [1–3]. Preliminary tests using PCR amplification of a sequence in the single copy superoxide dismutase gene of *C. burnetii* gave positive results with peripheral blood mononuclear cells (PBMC) and thin needle liver biopsies from QFS patients [22]. We now report results with a wider range of samples and a more sensitive PCR assay targeted to an insertion sequence IS*1111a* [23] present in multiple copies in the *C. burnetii* genome.

GROUPS STUDIED AND METHODS

Patients and control subjects

Details of the recruitment, after informed consent, of the core group of QFS patients are given by Penttila and colleagues [3]. The 18 patients in this group were later supplemented with a further 11 with QFS after acute Q fever. All patients except two had an initial illness clinically compatible with acute primary Q fever and confirmed by serological testing [3]. Detection of *C. burnetii* DNA and epidemiological evidence suggested that the two exceptions were infected subclinically at first and later developed QFS on re-exposure to *C. burnetii* [3]. Venous blood samples were collected from all 29 patients and peripheral blood mononuclear cells (PBMC) separated and stored in liquid nitrogen [3]. In addition thin needle liver biopsies and bone marrow (BM) aspirates were collected from 14 and 20 patients, respectively. Two patients were sampled 9 months after the acute Q fever and the remainder 12 months or more after onset; the mean period after onset was 37 months.

As controls, we tested PBMC packs from our previous study [3] and bone marrow aspirates from patients with unrelated diseases. They were included primarily to check PCR assay specificity and to detect cross contamination with amplicon or positive specimens in PCR assays, not to assess the aetiological significance of results for QFS. There were PBMC packs from: (1) 5 patients who had had acute Q fever but subsequently had not developed QFS, (2) 7 subjects who had been vaccinated against Q fever but were not infected with the coxiella and (3) 6 healthy Q fever seronegative subjects without chronic fatigue syndrome. Bone marrow aspirates were collected from 6 patients with diseases other than Q fever. As positive controls for the PCR assays, specimens were tested from 10 patients with either Q fever endocarditis or recrudescent infection in pregnancy. Most of these samples were positive for *C. burnetii* by cell culture [24] or guinea-pig inoculation or the donors had appropriate serological evidence of Q fever [25].

Ethical approval for the study was given by the Human Ethics Committee of the Royal Adelaide Hospital, Adelaide, South Australia 5000.

Detection of *C. burnetii* genomic DNA by PCR

PCR detection of a target in the superoxide dismutase gene of the coxiella used the sequence data of Heinzen and colleagues [26] to design, in Adelaide, two primer sets giving products of 128 and 186 bp respectively. As the resulting PCR assays proved to be of lower sensitivity than one based on the insertion element IS*1111a*, further details are not given.

Several primer sets for IS*1111a* were prepared for use in a conventional PCR, i.e. amplification in a 9600 thermal cycler (Perkin–Elmer, PE) and sizing of products by agarose gel electrophoresis, and also for use in the PE AB Prism 7700 Sequence Detection System ('TaqMan' PCR system) which provided probe based detection of amplicons and quantification of initial target numbers.

Primers for, and target sequences in *C. burnetii* DNA

Conventional insertion sequence (IS) PCR

Target: *C. burnetii* IS*1111a*: 73 bp;
P1 717 f = TCATCGTTCCCGGCAGTT;
P2 789 r = CACCTCCTTATTCCCACTCGAA.

Reaction conditions. C. burnetii IS PCR: P1, P2 150 nM, $MgCl_2$ 5·0 mM, KCl 50 mM, Tris–HCl 10 mM, pH 8·3, and 0·001% w/v gelatin, dNTP 200 μM each and, when substituted for TTP, dUTP 400 μM, 5 units AmpliTaq Gold (PE) with thermal programme of 95 °C, 2 min (AmpliTaq activation), 95 °C, 30 s;

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58 °C, 30 s; 72 °C, 45 s, with 3 s/cycle increase, 60 cycles and finally 72 °C, 7·5 min.

TaqMan insertion sequence (IS) PCR

Target: *C. burnetii* IS*1111a*: 61 bp;
P1671f = TAACGGCGCTCTCGGTTT;
P2731r = TGCCGGGAACGATGAAA;
Probe 713–690: 5′-FAM-TGATGAATGTCACCCA-CGCTCGCA-TAMRA-3′ (PE).

Reaction conditions. TaqMan *C. burnetii* IS PCR: P1 and P2 150 nM, TaqMan *C. burnetii* IS 24 bp probe, 100 nM, $MgCl_2$ 5·0 mM, KCl 50 mM, Tris–HCl 10 mM pH 8·3, EDTA 0·01 mM, dNTP 200 μM each and when substituted for TTP, dUTP 400 μM, ROX (passive reference dye) 60 nM, AmpErase™ uracil *N*-glycosylase (UNG), 1 U/reaction (basically PE TaqMan Core Reagents kit), 2·5 U AmpliTaq Gold (PE) with a thermal programme of 50 °C, 2 min for AmpErase™ inactivation of any contaminating amplicons, then 90 °C, 7·5 min (AmpliTaq activation); 90 °C, 30 s; 58 °C, 1 min; 60 cycles and finally an extension step of 65 °C, 5 min.

Artificial dU-IS 671–731 target (61 bp)

This consists of an artificial construct of the same length and sequence as the natural target except for dU substituted for T and an introduced restriction site (see Discussion).

Preparation of DNA from QFS patient and control samples

Bone marrow (BM), liver, PBMC and endocardial/placental (E/P) samples were initially heated to 95 °C, 15 min, to inactivate DNase and then Proteinase K (0·5 mgm/ml) and SDS (0·025 %) treated at 50 °C for up to 4 days. The digest buffer contained 5 mM Ca^{2+} for optimal protease activity. The Ca^{2+} was chelated post-digestion with 10 mM EGTA. To permit PCR on 'raw' digests, Proteinase K was thermally inactivated, 95 °C, 15 min. DNA from BM, PBMC and E/P were prepared by standard phenol–chloroform extraction. The BM DNA was also purified by column chromatography. Anionic columns (Genomic tip 100G; Qiagen Ltd, Sydney, Australia) removed most of the residual haem remaining after phenol-chloroform treatment and Chroma Spin™ 200 column (Integrated Sciences Pty Ltd, Willoughby, NSW, Australia) substantially reduced the remainder. Because of the small amount of tissue present, thin needle liver biopsies were processed only with anionic columns. All ethanol-precipitated DNA pellets were ethanol (70–80 % v/v) washed, air dried, dissolved in TE8 and stored at −60 °C.

Amplicon containment – minimization of false positives

Stringent measures were used to avoid false positives from amplicon contamination. These included the multiple room approach and increasingly, use of the uracil *N*-glycosylase method together with the introduction of dU-artificial targets (see Discussion).

RESULTS

Both the conventional and the TaqMan IS PCR assays were optimized for sensitivity. This was quantified by use of falling dilutions of either Proteinase K treated *C. burnetii* Henzerling strain (Q-Vax CSL Ltd, Parkville, Victoria, Australia) DNA or the artificial IS dU-target. The artificial dU-target and the natural 'wild-type' targets were shown to be equivalent: the optimized PCRs detected as little as one target. Comparative titrations confirmed that the IS PCR, given the multiple copy number of IS*1111a* in the *C. burnetii* genome i.e. ~20, was more sensitive than the PCR targeted to the superoxide dismutase gene.

Examination of specimens

Table 1 shows that the conventional IS PCR detected 10/10 cases in the endocarditis/placentitis category. The superoxide dismutase PCR was positive in 8/10 and HEL cell culture only in 5/8 of the endocarditis samples.

With the QFS samples (see [3] and Patients and Controls above) positives were detected in PBMC (5/29), liver biopsies (2/14) and notably, in BM (13/20, 65 %). Of the 13 positives in the BM group, 2 were positive only on testing numerous replicates over a range of dilutions in the TaqMan system so as to overcome the PCR inhibition found with the neat extract. Three were confirmed by sequencing amplicons. Ten were chosen at random from the 13 BM positives and confirmed by sequencing or specific probe hybridization in the TaqMan system.

Inhibition of the PCR reaction by human DNA and quantification of *C. burnetii* cells

With samples from the QFS patients, the high number of thermal cycles required to give a positive result

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Table 1. *Detection of* Coxiella burnetii *genome sequences in bone marrow and other samples from QFS patients and control subjects*

Clinical category	Specimens	Detection of *C. burnetii* sequences by insertion element PCR*
QFS	PBMC	5/29†
	Liver biopsies	2/14†
	Bone marrow aspirates	13/20‡
Q fever without QFS	PBMC	0/5
Q fever vaccinees	PBMC	0/7
Seronegative subjects	PBMC	0/6
Disease other than Q fever	Bone marrow aspirates	0/6
Q fever endocarditis	Valve vegetations	
Q fever placentitis	Placental cotyledons amniotic fluid	10/10

* No. positive/total.
† Conventional PCR, 73 bp target one PBMC and liver biopsy positive by superoxide dismutase PCR only.
‡ Two positive by Taqman PCR after dilution.

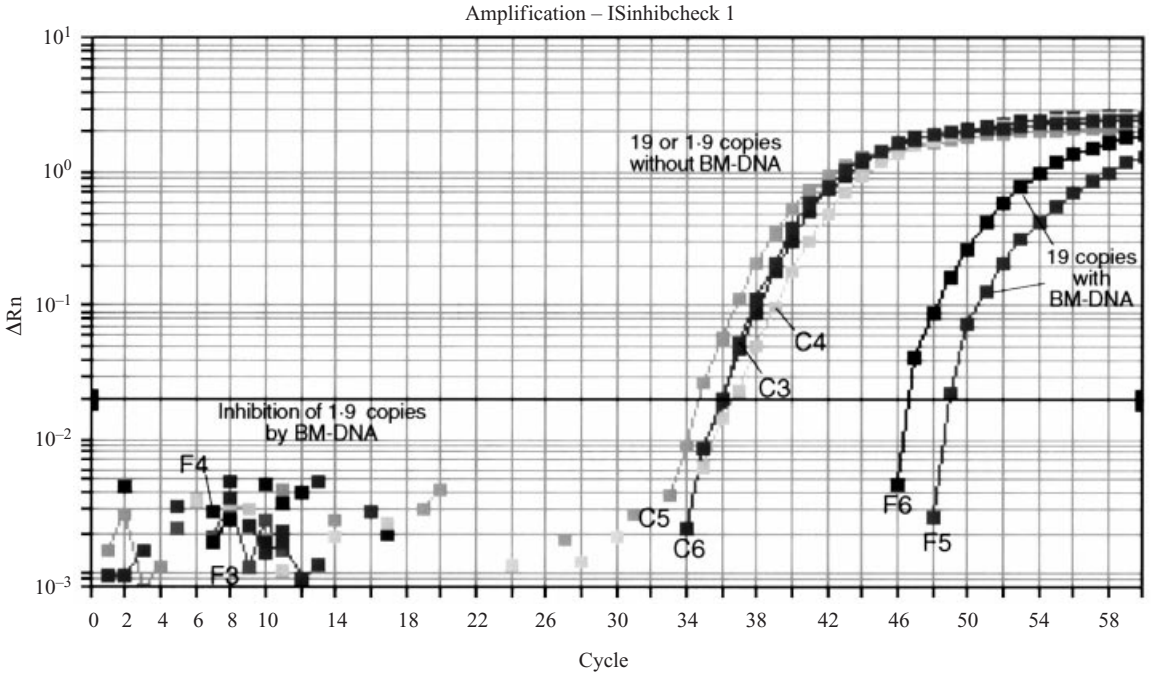

Fig. 1. Inhibition of TaqMan PCR amplification of *C. burnetii* (IS*1111a* target) by bone marrow DNA. Assays were made in duplicate and curves may overlap. [F3/F4 = 1·9 copies plus BM-DNA; C3/C4 = 1·9 copies no BM-DNA; C5/C6 = 19 copies plus BM-DNA; F5/F6 = 19 copies no BM-DNA (see text). [$\triangle Rn$ = (fluorescence − background)/background].

suggested not only low target numbers but also a probable inhibition of the PCR by the large amounts of human cell components, particularly DNA, which were present in the samples. As it was intended to use the TaqMan AB Sequence Detector to estimate the number of targets present in samples the effect of the inhibition by BM extracts on the amplification patterns of a known number of *C. burnetii* cells was assessed. Duplicate dilutions of inactivated, highly purified Henzerling strain, of known cell content, in the form of Q fever vaccine (Q-Vax $\sim 1 \times 10^9$ cells per 25 μg), were prepared to contain 1·9, 19, 190 and 1900 IS DNA copies and portions mixed either with an equal volume of DNA purified from the BM aspirate

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of a Q fever negative subject, or with buffer without BM DNA. The final concentration of BM DNA in the PCR reaction was 1·2 μg/50 μl. The DNA was prepared by exactly the same technique as those from the QFS patients. The final purification step on the Chroma Spin 200 column would remove small molecules, e.g. phenol, ethanol, as well as small and large proteins. Figure 1 shows that in the AB7700 TaqMan instrument, the 1·9 copies were not amplified in the presence of BM DNA and also that the curve for 19 copies mixed with BM DNA crossed the C_T line $\sim$12 cycles later compared to the control curves with buffer alone. Similar shifts to higher cycle numbers were observed with Q-Vax DNA dilutions containing BM DNA and 190 or 1900 copies (data not shown). The differences in cycle numbers required to reach the C_T value in the presence and absence of BM-DNA indicated a 36% per cycle reduction in PCR efficiency by BM-DNA. Using the same experimental design it was also found that treatment of the bone marrow suspension with purified DNase (Pulmozyme, Roche: 30 units, 37 °C, 1 h), in the presence of the small numbers of *C. burnetii* cells, removed the PCR inhibitory effect.

DISCUSSION

Detection of *C. burnetii* DNA in samples taken from subjects at 0·75 to 5 years post acute Q fever is, we feel, an important finding irrespective of any final clinical implications. The work does not define the state of the coxiellas present – they may be live or dead (with entombed DNA perhaps protected by the cell wall) or other bioentities (see Introduction).

The finding of small numbers of *C. burnetii* in clinical specimens when using an ultra-sensitive PCR inevitably leads to questions as to whether the results are due to contamination, either from PCR product (amplicons) from previous tests, or cross-contamination from other specimens or control suspensions (e.g. killed suspension of *C. burnetii* used for extraction of known positive DNA as a standard for comparisons of amplicon size on gels or for quantification of test sensitivity). To guard against such hazards: reagent preparation, test set up, sample addition, harvesting of product and gel analysis were each undertaken in separate locations with equipment, gowns and gloves, etc., dedicated to each area. Numerous negative controls (tubes with reagents but no target) were included in each run and only closed after the addition of specimen extracts. In addition, the development and application of the dU-artificial target for use in positive controls and quantification standards is a novel and useful addition to PCR contamination control (Harris and Storm, unpublished). Optimally, the dU-artificial target is constructed in a manner such that only full length dUMP containing amplicons are generated in the laboratory and are thus susceptible to subsequent inactivation by uracil *N*-glycosylase. Additionally a unique restriction site is introduced (outside the primer binding regions) so that any suspected false positive arising from contamination from positive control reagents or derived amplicons can be easily identified by restriction enzyme treatment of the PCR product and demonstration of fragments of characteristic size on gel electrophoresis.

A preliminary estimate of the numbers of *C. burnetii* cells in the BM aspirates, correcting for the inhibitory effect of the BM, was attempted by comparing the TaqMan C_T values given by four of the QFS BM samples, tested neat, with a linear response curve constructed from the C_T values from the titration of Q-Vax cells in the presence of normal BM. This indicated numbers of *C. burnetii* cells in the range of 40–700 coxiella genomes/ml of BM aspirate with an average number of 230. The estimate is probably conservative as column purification of the DNA restricts the amount bound to 100 μg thereby placing an upper limit on the targets available. Nevertheless the number is quite low as compared, for example, with that (10^6 GP ID50/ml: one ID50 $\cong$ 1–5 organisms) of a valve suspension from a patient with Q fever endocarditis although in the same range as the infectivity titres in the patient's other organs [11]. Broadly speaking, the pattern suggests a paucibaccillary infection presumably under immune control, but not finally eliminated.

Two recent studies [27, 28] on the PCR detection of small numbers of mycobacteria in Crohn's disease (*M. paratuberculosis*) and of a paucibaccillary form of human tuberculosis, pleural effusion, offer some striking parallels to our findings with *C. burnetii* in BM. The proportion of positives and the low numbers of organisms per g or ml of tissue or exudate are similar; there were difficulties from inhibition of PCR by tissue DNA. There was also a dichotomy between the intensity of disease and the number of organisms present, suggesting perhaps indirect amplification of disease by immune mechanisms.

A recent report [29] from Japan describes detection by nested PCR of *C. burnetii* DNA in whole blood

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samples from 17 of 52 patients with an illness like QFS, a rate not too dissimilar ($P = 0{\cdot}19$) to that in PBMC from our QFS patients.

Precise quantification of the number of coxiellas in BM is being sought by processes to remove inhibitors and by parallel assay of residual PCR inhibitors in each sample with a standard quantity of artificial target.

In general terms, the identification of BM as a potential focus of cryptic infection from which endocardium, placenta or other sites may be seeded for recrudescent infection may close one gap in our knowledge of the pathogenesis of chronic Q fever. The lower frequency ($P = 0{\cdot}0028$) of *C. burnetii* DNA positives in PBMC and liver suggests seeding from BM rather than a uniform seeding of all three sites via the blood stream from some other source. BM is sometimes overtly involved in acute Q fever [30–32]. It contains immature cells in the monocyte-macrophage lineage, the presumptive cell for coxiella replication, which may not be as effectively activated to clear coxiella infection as fully differentiated macrophages. The traffic pathways of macrophages from BM to the periphery may explain the seeding of other tissues, e.g. placenta, endocardium, bone, synovium and liver.

In terms of the clinical problem, QFS, under study, the demonstration of the persistence of *C. burnetii* in man is in line with the hypotheses advanced in previous reports [1, 3, 22] of a relationship between QFS, cytokine dysregulation and immunomodulation from persistence of *C. burnetii*. However before final conclusions are drawn it is necessary (*a*) to examine BM aspirates from patients who had Q fever but who did not subsequently develop QFS to see whether coxiella persistence is a feature of all Q fever infections or if is limited to those with QFS or other chronic sequelae, and (*b*) to determine whether infective coxiellas are present. These studies are in progress as part of collaborative prospective studies of acute Q fever and its long-term outcomes in Australia and the UK.

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