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Isolation of *Coxiella burnetii* from serum of patients with acute Q fever

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Running head: *Coxiella burnetii* isolation from acute serum

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ABSTRACT

Worldwide there are few isolate collections of the intracellular bacterium *Coxiella burnetii*, due to the difficulties associated with working with the organism and the scarcity of suitable samples from which to attempt isolation. Particularly lacking are isolates from acute Q fever patients. The aim of this study was to evaluate whether the serum samples taken from patients with confirmed acute Q fever during the early stage of their disease represented a potential source of viable *C. burnetii*. Isolation

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was attempted from 65 of these samples by inoculation of the serum into Vero cell culture and was successful in 36 cases (55%). This high success rate was likely due to extended incubation of up to twelve weeks of the inoculated cultures, allowing the growth of the organism to levels detectable by PCR. Retrospective analysis of the time the sera was stored prior to inoculation into culture demonstrated that *C. burnetii* remained viable for 224 days in samples stored refrigerated and 371 days in samples stored frozen at -20°C. These results demonstrate that standard serum samples taken from acute Q fever patients are a valuable source of new isolates of *C. burnetii*, with no special handling of the specimens required to maintain the organism's viability.

KEYWORDS

Coxiella burnetii; Q fever; isolation; cell culture

1. BACKGROUND

Coxiella burnetii is the intracellular bacterium responsible for the zoonotic disease Q fever. Human infection is usually associated with the inhalation of aerosols containing bacteria originating from infected animals. Domestic ruminants such as sheep, cattle and goats are considered to be the main reservoirs of human infection in most parts of the world (Guatteo *et al.*, 2011). However, evidence of *C. burnetii* has been found in a wide and diverse range of animal and bird species (Lang, 1990). It is estimated that around 60% of human *C. burnetii* infections are asymptomatic or result in a brief, unremarkable illness not requiring medical attention (Maurin and Raoult, 1999). Symptomatic infections can be classified as acute or chronic. Acute Q fever is characterized by an influenza-like illness, often with pneumonia or hepatitis, whereas the chronic form of the disease primarily manifests as endocarditis or vascular

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infection. Although acute Q fever is around twenty times more common than the chronic disease (Leroy *et al.*, 2011), the few existing *C. burnetii* isolate collections are dominated by isolates from chronic patients due to the scarcity of suitable samples from acute patients from which to attempt isolation.

The Australian Rickettsial Reference Laboratory (ARRL) performs diagnostic testing for Q fever, most commonly receiving serum rather than whole blood specimens. PCR testing of serum has proven to be extremely useful for the early diagnosis of acute Q fever, with results demonstrating that DNA from *C. burnetii* is present in the serum during the early stages of the disease (Turra *et al.*, 2006, Schneeberger *et al.*, 2010). It had been postulated that as most *C. burnetii* would be within circulating monocytes, the DNA detected by PCR represented fragmented genomes rather than intact organisms (Schneeberger *et al.*, 2010). However, successful isolation of *C. burnetii* has been achieved by inoculation of serum from Q fever patients into immunosuppressed A/J mice (Nagaoka *et al.*, 1996), demonstrating that live organisms were present in the extracellular fraction of the blood during infection. The aim of the current study was to determine whether serum specimens submitted for routine diagnostic testing represented a potential source of viable *C. burnetii* that could be isolated using a cell culture method and used to establish new isolates of the organism.

2. METHODS

2.1 Sample selection

Isolation was attempted from 64 acute serum samples from Australian patients with confirmed acute Q fever. Samples were collected between 2010-2012. Diagnosis was

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made by either PCR or an indirect immunofluorescence assay (IFA). PCRs were performed on DNA extracted from the serum sample or from the buffy coat fraction of an accompanying blood sample. The result was reported as positive when a Ct value of ≤ 40 was obtained in both the diagnostic qPCR assays targeting the *com1* gene (Lockhart *et al*., 2011) and the heat-shock operon *htpAB* gene (Sullivan Nicolaides Pathology in-house assay), or a Ct value of ≤ 35 was obtained in the *com1* assay only. For diagnosis by serology, a National Association of Testing Authorities (NATA) accredited in-house IFA was used to test paired acute and convalescent serum samples. The acute specimen was either negative or had low titres (≤ 400) of *C. burnetii* Phase II antibodies and a seroconversion or four-fold rise in antibody titre was observed in the paired convalescent sample. Isolation was attempted from the acute sample only.

Isolation was also attempted from one acute unpaired sample that was seronegative and PCR-negative. It was included in the study after retrospective analysis of the case by a pathologist who suspected that the Australian patient from whom the sample originated may have had Q fever.

Samples were transported to the laboratory at 4°C or at ambient temperature then stored at 4°C after routine diagnostic testing. Five additional specimens obtained from another laboratory (Sullivan Nicolaides Pathology) were stored frozen at -20°C after diagnostic testing. They were transported to the ARRL on dry ice then stored at -20°C until isolation was attempted.

2.2 Isolation of *Coxiella burnetii*

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Fresh, confluent Vero cell monolayers were prepared in 12.5cm^2 non-vented tissue culture flasks. The culture medium (RPMI supplemented with 3% foetal calf serum, 4mM L-Glutamine and 25mM HEPES) was removed and 100µL serum was added. Flasks were centrifuged at room temperature at 500 x *g* for 30 minutes to promote attachment of any *C. burnetii* organisms to the cells. Following centrifugation, 9mL medium was added and the flasks were incubated at 35°C for up to 12 weeks, with a change of the medium performed every 14 days. Up to 11 flasks were inoculated and incubated simultaneously. In each batch of isolation attempts a negative control flask inoculated with 100µL PBS was included to monitor for contamination.

2.3 Monitoring of cultures for *C. burnetii* growth

The spent medium removed from the cultures after two weeks of incubation was discarded without testing as it may have contained *C. burnetii* organisms or DNA from the original inoculum. The spent medium removed in subsequent weeks was centrifuged at 5445 x *g* for 20 minutes and the supernatant removed leaving 1mL in which to resuspend the pellet. DNA was extracted from 100µL of this material using a HiYield Genomic DNA Mini Extraction Kit (RBC Bioscience, New Taipai City, Taiwan) according to the manufacturer's tissue protocol, with the incubation period for the 60°C cell lysis step extended from 30 minutes to overnight. Extracted DNA was tested for the presence of *C. burnetii* DNA with the *com1*qPCR assay and isolation was recorded as positive when a positive result (Ct < 40) was obtained on two consecutive occasions. A standard curve was created as described previously (Brennan and Samuel, 2003) from a plasmid preparation of the *com1* assay target cloned into the vector pCR 2.1 TOPO, with concentrations in the range of 2 to 2 x 10^7

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genome copies/µL. This curve was used to determine the number of genome copies present in positive samples.

Cultures were also monitored by light microscopy at 100X magnification. Previous experience in our laboratory showed that bacteria-filled vacuoles were visible in the cytoplasm of *C. burnetii* infected Vero cells, with no staining required for this observation (Lockhart *et al.*, 2012).

2.4 Statistical analysis

A two-tailed Fisher's exact test was performed using the GraphPad software online calculator (http://graphpad.com/quickcalcs/contingency1/). Non-parametric Mann-Whitney U testing was performed using StatPlus v2009 (AnalystSoft Inc., Walnut, CA, USA), with a p value < 0.05 considered significant. Poisson regression analysis was conducted in R (R Development Core Team, 2013). The Poisson model fit was $log(\mathrm{E}[Y_i|t_i]) = log(N_i) + \lambda + \beta t_i$ where Y_i was the number of samples that yielded an isolate at time t_i out of N_i samples tested, λ was the overall positivity rate and β was the effect on the positivity rate of a change of one unit of time (10 days).

3. RESULTS

3.1 Successful isolation of *C. burnetii* from serum using Vero cell culture

In total, 16 batches of isolation attempts were performed from 65 serum samples. Isolation of *C. burnetii* was successful from 36 of the specimens giving an overall successful isolation rate of 55%. A positive result was never obtained from any of the negative control flasks.

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The positive isolation cultures were maintained in the original flasks with fortnightly changes of the medium and PCR testing of the spent medium continued. At the initial detection of *C. burnetii* in the flasks, the Ct values in the *com1* qPCRs were high (35-40) indicating the presence of 10-450 genome copies/ml. These Ct values decreased in subsequent weeks, indicating an increase in the amount of *C. burnetii* DNA, corresponding to bacterial growth. In many cases, *C. burnetii* filled vacuoles were visible by light microscopy as the infection progressed. When the Ct value had dropped to < 30 (approximately 1.5 x 10^4 copies/ml), the positive culture was passaged to flasks containing fresh Vero monolayers and *C. burnetii* growth in the new flasks was monitored by *com1* qPCR. In heavily infected flasks the Ct values in the *com1* qPCRs performed on DNA extracted from the spent medium were < 25 (more than 5 x 10^5 copies/ml). From the 36 positive isolation flasks, 34 isolates were successfully established in Vero cell culture. The remaining two flasks became contaminated and were discarded.

3.2 Relationship between diagnostic test result and isolation success

All the samples used in this study had been tested with the diagnostic IFA and PCR assays. Analysis of the isolation results in conjunction with these diagnostic testing results showed that isolation of *C. burnetii* was highly likely (>80%) from serum that had tested positive in the diagnostic PCR. A significantly higher proportion of successful isolations was observed in those cultures inoculated with PCR-positive serum compared with those inoculated with PCR-negative serum (Table 1). There was no significant difference in the proportion of positive isolations from seropositive and seronegative serum (Table 1). It should be noted however that the seropositive samples had low antibody titres (≤ 400 of Phase II antibodies only).

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Table 1 Relationship between IFA and PCR diagnostic assay outcome and the successful isolation of* C. burnetii *from serum samples

*Serum samples were grouped according to the results of the diagnostic IFA and PCR assays performed on them prior to their inclusion in the study. The difference in proportion of positive isolations from the groups of specimens was compared and the 95% confidence interval (CI) calculated. A 95% CI that does not contain the value 0 indicates that the difference in proportions was statistically significant at this confidence level and is denoted by *. P values were calculated using Fisher's exact test.*

Diagnostic testing result		Number of samples	Number of positive isolations (%)	Difference in proportion of positive isolations	95% CI	P value
IFA	Positive	20	10 (50)	8	-17.0, 31.8	0.5981
	Negative	45	26 (58)			
PCR	Positive	27	22 (81)	44	20.5, 61.6*	0.0004
	Negative	38	14 (37)			

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3.3 Time to culture positivity

It was observed that amongst the positive isolation flasks there was some variation in the length of incubation required before *C. burnetii* DNA was first detected in the culture medium (Table 2). A Mann-Whitney U test performed on the ranked distributions of the time to positivity showed that it was significantly longer ($p = 0.004$) before *C. burnetii* DNA was detected in the cultures inoculated with PCR-negative serum compared to those inoculated with PCR-positive specimens. Overall only 50% of the cultures that yielded an isolate were positive at the first test point

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after four weeks of incubation. However, this value rose to 94% and 100% after eight and twelve weeks of incubation respectively.

Table 2. Length of incubation before the first detection of* C. burnetii *DNA in the flasks yielding isolates (n=36)

Value in brackets indicates the cumulative percentage of positive flasks at each time point.

Weeks of incubation	Inoculum of PCR-positive serum (n=22)		Inoculum of PCR-negative serum (n=14)		Overall (n=36)	
	First detection of *C. burnetii* DNA by qPCR	Cumulative percentage of positive flasks	First detection of *C. burnetii* DNA by qPCR	Cumulative percentage of positive flasks	First detection of *C. burnetii* DNA by qPCR	Cumulative percentage of positive flasks
4	15	68%	3	21%	18	50%
6	7	100%	6	64%	13	86%
8	n/a	n/a	3	86%	3	94%
10	n/a	n/a	0	86%	0	94%
12	n/a	n/a	2	100%	2	100%
Median time to positivity	4 weeks		6 weeks		5 weeks	

n/a- not applicable as all the cultures had become positive prior to this time point

3.4 Stability of viable *C. burnetii* in serum

As the isolation attempts were batched many of the samples were stored for weeks or even months before their inoculation into Vero cell culture. Storage time was defined as the number of days between the date of sample collection and the inoculation of the serum into Vero cell culture. Analysis of the storage times was performed

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retrospectively as the study was not designed to investigate a particular range of storage times. Storage of the frozen samples (n=5) was for an average of 244 days (range 52-371 days), with all of them yielding an isolate of *C. burnetii*. Of the refrigerated samples (n=60), those yielding isolates (n=31) were stored for an average of 74 days (range 17-224 days) and isolation of *C. burnetii* was successful from samples across the whole range of storage times (Table 3). Poisson regression analysis performed on these results demonstrated that the serum storage time had no effect on the isolation success rate (β = 1.00; 95% CI 0.99-1.01), thus successful isolation of *C. burnetii* was not more likely from a serum sample that had been stored refrigerated for a shorter period of time (Figure 1).

Table 3. Distribution of the storage time of refrigerated serum samples (n=60) and* C. burnetii *isolation outcome

Storage time was grouped into periods of ten days. Not all consecutive time periods were represented by samples in this study.

Storage time (days)	Number of samples (n=60)	Isolation outcome		Isolation success rate
		Positive (n=31)	Negative (n=29)	
11-20	6	4	2	67%
21-30	7	5	2	71%
31-40	4	3	1	75%
41-50	6	3	3	50%
51-60	5	3	2	60%
61-70	5	3	2	60%
71-80	4	0	4	0%
81-90	4	0	4	0%
91-100	5	3	2	60%
101-110	4	2	2	50%
111-120	2	0	2	0%
121-130	2	0	2	0%
161-170	1	1	0	100%
181-190	1	1	0	100%
191-200	1	1	0	100%
>200	3	2	1	67%

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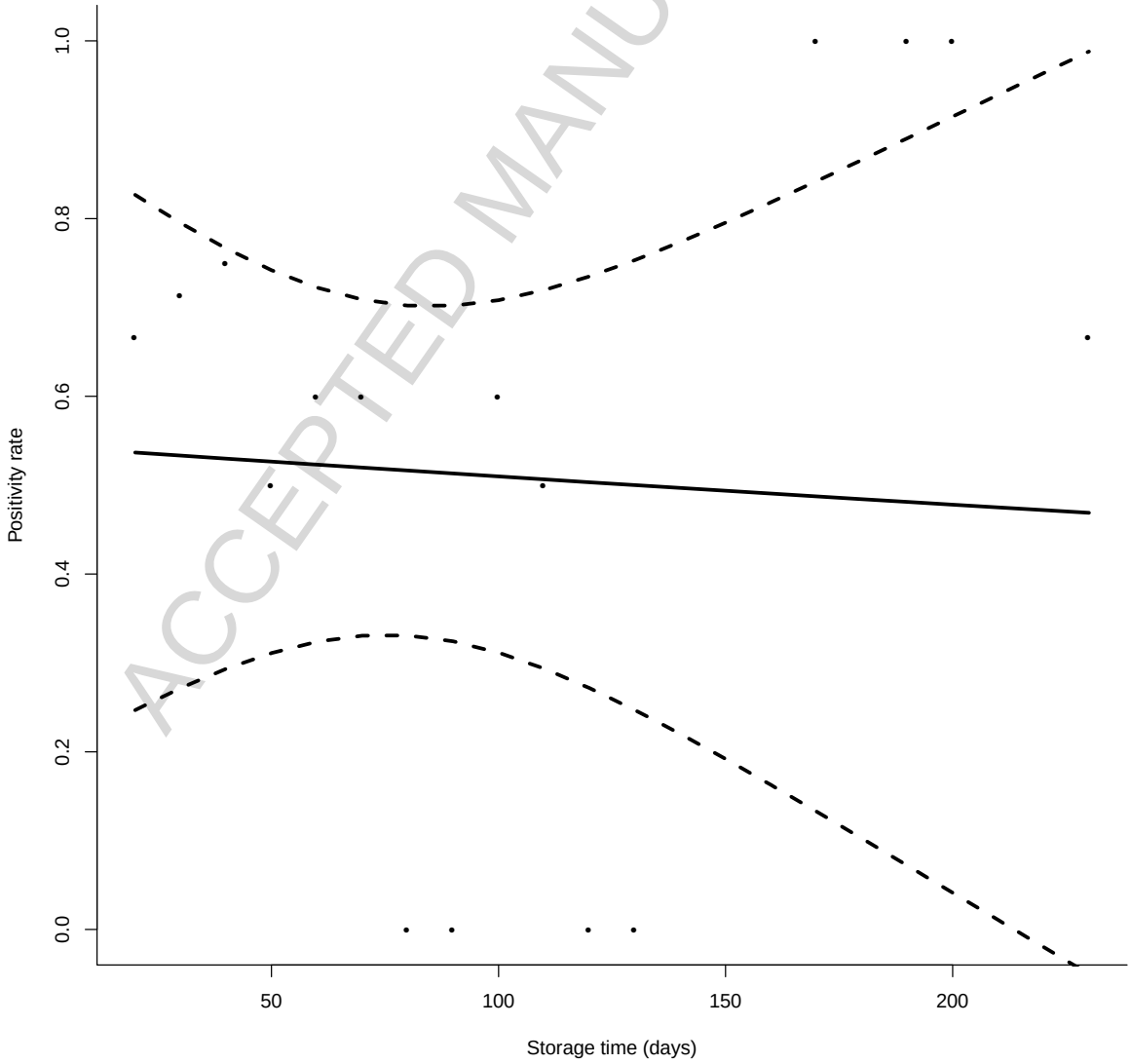
Figure 1 Effect of serum storage time on* C. burnetii *isolation success

Data points show the isolation success rate for each of the sixteen ten-day storage time periods represented by the n=60 refrigerated samples. Solid line indicates the mean isolation success rate as a function of storage time as calculated by Poisson regression analysis, with the dashed line indicating the 95% confidence limits of this rate.



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4. DISCUSSION

A single detection of *C. burnetii* DNA by PCR is not convincing evidence of the presence of viable bacteria, particularly if the Ct values are high (35-40). However, repeated detection accompanied by a decrease in the Ct value is indicative of an increase in the number of *C. burnetii* genome copies and therefore a good indication of bacterial growth. Previous studies have demonstrated the utility of qPCR for monitoring *C. burnetii* growth in a cell culture model, showing that it was comparable to other methods such as the direct IFA and the replating focus forming unit assay (Brennan and Samuel, 2003, Coleman *et al.*, 2004). The decreasing Ct values observed in our study in both the original positive isolation flasks and subsequent passaged flasks confirmed that viable *C. burnetii* were isolated from the serum samples of acute Q fever patients and successfully amplified. The number of *C. burnetii* genome copies was more than one thousand fold greater in the medium of heavily infected flasks compared to the number observed when *C. burnetii* DNA was initially detected in the flask. These successful isolations provide further evidence that viable organisms are present in the extracellular portion of human blood during the acute phase of infection. Since the infectious dose of *C. burnetii* can be as low as one organism (Jones *et al.*, 2006), these specimens present a potential source of infection for laboratory personnel. Vaccination of staff handling these specimens would be advisable, although the current human vaccine Q-Vax is only licensed for use in Australia.

The serum samples in which *C. burnetii* DNA was detected by PCR yielded a significantly higher proportion of isolates than the PCR-negative samples. This was likely due to the PCR-positive specimens having a higher bacterial load, thus the

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cultures inoculated with these samples had a higher multiplicity of infection. The successful isolation from PCR-negative sera demonstrates that in many cases the Vero cell culture was more sensitive than the diagnostic qPCR assays for the detection of *C. burnetii.* This supports previous results from our laboratory, which determined the ID_{50} for Vero cells to be one bacterium in a 100µL inoculum (0.01 GE/µL) (Lockhart *et al.*, 2012), whereas the *com1* qPCR limit of detection is known to be 6 GEs/reaction (1.25 GE/µL) (data not shown).

The long incubation time of up to 12 weeks may raise concerns about possible laboratory contamination of the cultures. However, the negative control flask included in each batch of isolation attempts was always handled last when any manipulations were performed and *C. burnetii* DNA was not detected in any of the samples from these flasks. In addition, the only other strain of *C. burnetii* being grown in the laboratory at the same time as the isolation cultures was Nine Mile Phase II Clone 4 (RSA439). Molecular characterisation of the 34 new isolates using published methods for plasmid typing (Zhang *et al.*, 1998), SNP analysis (Hornstra *et al.*, 2011) and MLVA (Arricau-Bouvery *et al.*, 2006) showed that none was the same genotype as the Nine Mile strain, with several different genotypes observed amongst the collection (manuscript in preparation). The extended incubation period of up to 12 weeks in our study may have been the reason for the high isolation success rate of 55%. In a similar study where isolation was attempted from the heparinized blood supernatant of acute Q fever patients, isolation was only successful in 17% of attempts but incubation of the cultures was for only 14 days (Musso and Raoult, 1995). Our results showed that only 50% of the flasks that yielded an isolate were positive after four weeks of incubation; therefore 18 of our isolates would not have been obtained

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without the extended incubation. Strain specific differences in the growth of *C. burnetii* in cell culture have been demonstrated previously. Established strains containing the QpRS plasmid took 10 days post-inoculation to establish a detectable infection in L929 cells, whereas strains containing QpH1 or an integrated plasmid-like sequence required only two days (Roman *et al.*, 1991). It seems likely therefore that primary isolates not adapted to cell culture may be expected to grow slowly, particularly if they carry QpRS. Infection of the cell cultures may have been detected sooner if the monolayer had been sampled and tested, using either PCR or direct IFA. However, since *C. burnetii* has been shown to initiate foci of infection (Roberts and Downs, 1959), destructive sampling of the monolayer may have resulted in the removal of infected cells and the isolates may not have become established. In most cases there was an insufficient volume of serum to inoculate multiple flasks with the same sample, so in our study the non-destructive sampling method used was considered to be the best way to ensure infection of the cells would be established if viable organisms were present.

The stability of *C. burnetii* in whole human blood and its various fractions has been investigated previously, with results demonstrating that viability was maintained for up to 6 weeks at 1-6°C (Kersh *et al.*, 2013). However, the infected samples were created in the laboratory and may not have been representative of the *in vivo* situation. Our results using naturally infected serum show that the viability of *C. burnetii* in serum was maintained for 224 days under normal transport and storage conditions, and for over a year when the serum was frozen. As our analysis was retrospective, further work was not carried out to determine a cut-off storage time for maintaining viability of the organism, but it is possible it could be longer than the figures

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observed. The long-term stability can be attributed to the small cell variant of the bacterium, which has spore-like properties enabling its survival in the absence of host cells. Our observations may be valuable for other researchers with stored serum from acute Q fever patients as retrospective isolation of *C. burnetii* from these samples may be possible.

Since the isolation of *C. burnetii* is time consuming and can only be performed by specialized laboratories with BSL-3 and cell culture facilities, it is not used routinely for the diagnosis of Q fever. However, it can be useful in some cases. For example, isolation of *C. burnetii* from the one unpaired seronegative and PCR-negative sample in this study provided the diagnosis of Q fever for the patient, who was still experiencing symptoms at the time the isolation result was obtained. The isolation of the organism was the first indication that this patient had Q fever and assisted in the provision of correct treatment. Subsequent to this study, the described technique was used to isolate *C. burnetii* from the serum of a patient suffering from a supposed reaction to the Q fever vaccine, Q-Vax. This confirmed the clinician's suspicions that the patient's illness was in fact acute Q fever acquired before he received the vaccination.

Although the method of isolation of *C. burnetii* described here has limited use as a diagnostic tool, the establishment of isolate collections is important to enable further studies to be performed. The ability to grow the organism allows the investigation of properties such as antibiotic resistance and provides an unlimited source of DNA for phylogenetic studies.

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5. CONCLUSIONS

The results of this study demonstrate that early serum samples from acute Q fever patients are infectious and a valuable source of viable *C. burnetii*. Whilst the slow growth of the organism from samples containing low numbers of bacteria limits the described technique as a first line diagnostic tool, it may be useful for confirmatory purposes and for establishing new and larger collections of *C. burnetii* isolates from acute Q fever patients.

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Highlights

- Viable *C. burnetii* was successfully isolated from diagnostic serum samples.
- Isolation success rate was 81% from PCR-positive specimens.
- Isolation success was increased by extended incubation of isolation cultures.
- Long term viability of *C. burnetii* in serum (>200 days) was demonstrated.

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