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RESEARCH ARTICLE

# Developing a new clinical tool for diagnosing chronic Q fever: the *Coxiella* ELISPOT

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Q fever; *Coxiella burnetii*; ELISPOT.

## Abstract

Definitively establishing a clinical diagnosis of chronic Q fever remains challenging, as the diagnostic performance of both conventional serological tests and PCR is limited. Given the importance of an early diagnosis of chronic Q fever, there is a need for a reliable diagnostic test. We developed an enzyme-linked immunospot assay to measure *Coxiella burnetii* (*C. burnetii*)-specific T-cell responses (*Coxiella* ELISPOT) to both phase I and phase II antigens and tested convalescent Q fever patients (without chronic disease, $n = 9$) and patients with an established diagnosis of chronic Q fever ($n = 3$). The *Coxiella* ELISPOT adequately identified convalescent Q fever patients from healthy controls by demonstrating *C. burnetii*-specific T-cell interferon-γ production to both phase I and phase II antigens. Compared to convalescent Q fever patients, chronic Q fever patients showed a distinct *Coxiella* ELISPOT profile characterized by a much higher spot count for both phase I and phase II (18-fold for phase II, 8-fold higher for phase I) and a consistent shift towards more phase I reactivity. The diagnostic potential of the *Coxiella* ELISPOT is promising and warrants further investigation.

## Introduction

In the Netherlands, annual Q fever outbreaks associated with goat farming have amounted to more than 4000 new human Q fever cases since 2007 (Delsing *et al.*, 2010). With an expected 2–5% of patients developing chronic Q fever, clinicians now increasingly face the challenges in accurately diagnosing this rare but potentially fatal complication of *Coxiella burnetii* (*C. burnetii*) infection (Delsing *et al.*, 2010; Frankel *et al.*, 2011). The main clinical manifestations of chronic disease following the Dutch outbreaks are infection of vascular aneurysms and prostheses, closely followed by endocarditis (Delsing *et al.*, 2010). In routine clinical practice, definitively establishing a diagnosis of chronic Q fever diagnosis is challenging and relies heavily on the demonstration of a vigorous humoral immune response to the antigenic phase I *C. burnetii*, as evidenced by high levels of phase I IgG antibodies (Frankel *et al.*, 2011). In conjunction with suggestive serology, a positive serum PCR for *Coxiella* DNA is considered diagnostic for chronic disease (Fenollar *et al.*, 2004; Wegdam-Blans *et al.*, 2011). This poses an important clinical problem, as the performance of serological tests and PCR is limited in both establishing and excluding a diagnosis of chronic disease. A possible explanation for the underperformance of current serological assays may be the fact that these tests only reflect the host's humoral immunity, whereas the most relevant immunological response to chronic infection with the obligate intracellular *C. burnetii* is first and foremost cell-mediated (Andoh *et al.*, 2007; Marmion *et al.*, 2009).

Given the importance of an early diagnosis of chronic Q fever timely prompting long-term antibiotics and/or surgical intervention, testing a patient's cellular rather than humoral immunological response to *C. burnetii* might be a more reliable tool for diagnosing chronic Q fever in a clinical setting.

A recently developed paradigm indeed proposes clinical outcome after *C. burnetii* infection as a function of host-immunity/pathogen interaction with a strong emphasis on dysfunctional cellular immunity (Marmion *et al.*, 2009).



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T-cells and their associated cytokines, interferon-γ (IFN-γ) being the most potent, have a pivotal role in dealing with primary infection and subsequent clearance or control of intracellular pathogens such as *Mycobacterium tuberculosis*, *Legionella pneumophila*, *Chlamydophila* and *C. burnetii* (Andoh *et al.*, 2007). The long-term clinical outcome of Q fever might, therefore, be better characterized by measuring the host's *C. burnetii*-specific T-cell response. IFN-γ release assays (IGRA) such as ELISPOT can detect the IFN-γ production by antigen-specific T-cells *ex vivo* at an individual cell level. In tuberculosis, IFN-γ-based tests are superior to tuberculin skin testing in detecting patients with latent infection and are better predictors of subsequent development of active disease (Santín Cerezales & Benítez, 2011). We hypothesize that different Q fever clinical outcomes (nonchronic past infection and chronic disease) are similarly associated with marked differences in a patient's *C. burnetii* antigen-specific T-cell IFN-γ production.

## Aim

The first aim of this study was to develop an enzyme-linked immunospot assay to measure *C. burnetii*-specific T-cell responses (*Coxiella* ELISPOT) in convalescent Q fever patients (without any evidence of chronic Q fever). The second aim was to test the *Coxiella* ELISPOT in patients with an established diagnosis of chronic Q fever.

## Materials and methods

### *Coxiella* ELISPOT

Mononuclear cells were isolated from whole blood specimens (lithium heparin) drawn at regular phlebotomy, using Leucosep tubes (Greiner Bio-One Ltd # 163288) in combination with T-cell *Xtend* (TTK.610; Oxford Imunnotec Ltd, Abingdon, UK). As stimulating antigens, we used the commercially available lyophilized phase I and phase II antigens (#1227 and #1123 respectively; Virion-Serion Immunodiagnostica GmbH, Wurzburg, Germany). Phytohaemagglutinin (PHA; Sigma L4144), anti-CD3 Mab (Mabtech, Sweden) and CEF-pool (CT370; U-Cy-Tech Biosciences, Utrecht, the Netherlands; a combination of CMV, EBV and influenza peptides) were used as stimulation controls. Negative control wells only contained mononuclear cells without stimulating antigens. Subsequently, 100 μL of mononuclear cells were seeded at a density of 250 000 cells per well in precoated wells of PVDF strip plates (ELISpot$^{PRO}$; Mabtech) and incubated with 50 μL of antigens or controls during 16–20 h at 37 °C and 5% $CO_2$. The resulting number of spots corresponds with the number of individual T-cells producing IFN-γ after stimulation with *C. burnetii* antigens. After detection spots were enumerated using an ELISpot reader (Auto Immun Diagnostika GmbH, Strassberg, Germany). This work was a proof of principle study for the TRIQ (T-cell Response In Q fever) study approved by the local Ethical Committee (VCMO NL35867.100.11). All study subjects gave informed consent.

### Study population

Study subjects were classified into three groups. Healthy control subjects ($n$ = 9): negative serology (immunofluorescence assay: IFA, Focus Diagnostics or ELISA, Serion Immundiagnostica) for *C. burnetii* and no pre-existing comorbidities. Convalescent Q fever patients ($n$ = 9): a documented history of Q fever with complete clinical recovery; no residual complaints or clinical signs of chronic Q fever, serological evidence of a past *C. burnetii* infection and no evidence of chronic disease (PCR negative and IFA phase I IgG < 1024 at ≥6 months follow-up). Chronic Q fever patients ($n$ = 3) fulfilled the diagnostic criteria of the Dutch consensus statement on chronic Q fever (Wegdam-Blans *et al.*, 2011): a positive *C. burnetii* PCR in tissue and/or serum in the absence of acute Q fever infection ($n$ = 2), or an IFA phase I IgG titre of ≥ 1024 and an endocarditis diagnosis using the Duke criteria or evidence of a vascular infection on PET/CT or ultrasound examination ($n$ = 1).

### Main study endpoint

The spot count in the *Coxiella* ELISPOT for the three groups were assessed: healthy controls, convalescent patients and chronic Q fever patients. Assuming a Gaussian distribution, differences in mean spot counts between groups were assessed by one-way ANOVA.

## Results

*Coxiella burnetii*-specific T-cell responses (mean ± SE) in convalescent Q fever patients were 10 ± 5 spots to phase I antigen and 28 ± 13 spots to phase II antigen (Table 1). Using a cut-off value of five spots for phase II reactivity, ELISPOT sensitivity for a past Q fever infection was 89%.

*Coxiella burnetii*-specific T-cell responses in chronic Q fever patients were more pronounced: 194 ± 46 spots to phase I antigen and 219 ± 36 spots to phase II antigen. T-cell responses in chronic Q fever patients were significantly higher than those in convalescent patients: 18-fold for phase I antigen ($P < 0.0001$) and 8-fold for phase II antigen ($P$ 0.0002). The proportion of phase II to phase I response was expressed as a ratio or stimulation index (SI = phase II spots/phase I spots). The SI value in

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**Table 1.** Clinical characteristics and *Coxiella* ELISPOT results

| | Healthy controls<br>*n* = 9 | Convalescent Q fever<br>*n* = 9 | Chronic Q fever<br>*n* = 3 | *P*-value* |
|---|---|---|---|---|
| Mean (± SD) age, range (years) | 44.2 (10.8), 31–64 | 57.0 (15.5), 28–82 | 52.6 (15.3), 31–64 | |
| Female/male | 3/6 | 3/6 | 0/3 | |
| Median months since diagnosis (range) | n/a | 8 (6–22) | 1 (0–1) | |
| Phase I spots mean (± SD), range | 0.8 (± 1.0), 0–3 | 10 (± 5), 1–42 | 194 (± 46), 104–217 | < 0.01 |
| Phase II spots mean (± SD), range | 0.2 (± 0.4), 0–1 | 28 (± 13), 1–120 | 219 (± 36), 164–205 | < 0.01 |
| PHA positive control spots mean (± SD), range | 240 (± 56), 58–350 | 272 (± 25), 102–350 | 240 (± 56), 172–350 | 0.87 |
| CEF positive control spots mean (± SD), range | 126 (± 54), 16–211 | 101 (± 62), 0–233 | 104 (± 67), 18–236 | 0.95 |
| Negative control spots mean (± SD), range | 0 | 0 | 0 | |

*Differences between means of all three groups, significance set at $P < 0.05$.

convalescent patients was 2.8 compared to 1.1 for chronic patients, indicating a tendency to shift to a phase I predominance in chronic disease.

Stimulation controls in all patients and controls indicated adequate T-cell responses to the stimuli. Mean (± SE) T-cell responses in PHA-stimulated PBMCs (peripheral blood mononuclear cells) were adequate and not significantly different between all groups (*P* 0.87). Mean (± SE) T-cell responses in CEF-stimulated PBMCs did not significantly differ between all three groups (*P* 0.95). Responses in the healthy control group were negligible. We did not detect more than one spot after stimulation with either phase I or phase II antigens.

## Discussion

In the Netherlands, a definitive chronic Q fever diagnosis currently is made on the basis of serology in combination with a positive serum PCR and in most cases, the presence of symptoms and a compatible clinical picture of endocarditis or vascular infection (Wegdam-Blans *et al.*, 2011). In many Q fever patients, however, high levels of IgG phase I antibodies are found without a positive PCR and/or clinical symptoms (Delsing *et al.*, 2010). The positive predictive value (PPV) of the previously widely advocated IFA phase I IgG titre of $\geq 800$ for diagnosing chronic Q fever is 37%. Recent adjustment of this cut-off titre to $\geq 1600$ only increased the PPV to 59% (Frankel *et al.*, 2011). The sensitivity of PCR on a fresh serum sample in chronic Q fever is 64% (specificity 100%) (Fenollar *et al.*, 2004). Therefore, a negative PCR in the presence of highly elevated antibody levels does not exclude chronic disease. For clinicians, there clearly is a need for a more reliable diagnostic test for chronic Q fever.

In this exploratory study, the *Coxiella* ELISPOT adequately identified convalescent Q fever patients from healthy controls by demonstrating *C. burnetii*-specific T-cell IFN-γ production to both phase I and phase II antigens. Analogous to conventional serology, there was a clear phase II predominance. Specificity of the *Coxiella* ELISPOT for a past Q fever infection appears to be high, as T-cells from healthy controls did not react to both phase I and phase II *C. burnetii* antigens. False positive results because of cross-reactivity of the *Coxiella* ELISPOTs with other pathogens, however, remain a theoretical possibility.

Chronic Q fever patients showed a distinct *Coxiella* ELISPOT profile. First, spot count for both phase I and phase II was much higher (18-fold for phase II, 8-fold higher for phase I) indicating a more vigorous immune response *in vitro* to both antigenic stimuli. This is analogous to conventional serology, classically showing both highly elevated IFA phase I and phase II IgG antibody levels (Delsing *et al.*, 2010; Frankel *et al.*, 2011). Second, *Coxiella* ELISPOT results from chronic Q fever patients were consistently shifted towards more phase I reactivity as evidenced by a significantly lower SI (phase II/phase I spots). This balanced phase I and phase II reactivity is not a consistent feature in the conventionally assayed IFA antibody titres (Delsing *et al.*, 2010).

There are some limitations to this pilot study. First, the limited number of patients does not allow for determination of a cut-off value for *Coxiella* ELISPOT results for diagnosing chronic Q fever. The consistent findings in phase I and phase II reactivity in chronic disease, however, are already statistically significant, but need confirmation in a larger cohort. Second, we used commercially available lyophilized *C. burnetii* antigens. These antigens are isolated from infected cells and can still contain tissue cell substrates, which could lead to false positive results. As we did not observe any reactivity for both antigens in healthy controls, such an aspecific reaction to these 'impure' antigens in the *Coxiella* ELISPOT is highly unlikely. Third, in contrast to the well-characterized conventional serological response to *C. burnetii* infection in time, the kinetic properties of the *Coxiella* ELISPOT assay are not yet known, precluding determination of cut-off values for diagnosing acute or chronic Q fever.

Anticipating further research, we hypothesize that measuring a patient's *C. burnetii*-specific T-cell responsiveness

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may better aid the clinician in accurately diagnosing a chronic from a nonchronic Q fever infection. In addition, such a test might prove a better tool for monitoring and guiding therapy than conventional serology. In the Netherlands, following the annual major Q fever outbreaks from 2007 to 2010, already more than 200 patients with a definite or probable diagnosis of chronic Q fever have been identified (L.M. Kampschreur, pers. commun.). To validate the *Coxiella* ELISPOT for clinical use, a study comparing this assay to conventional serodiagnostics (serology and PCR) in a large cohort of Q fever patients is currently underway (the TRIQ study).

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