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# *Coxiella burnetii* in non-Hodgkin lymphoma tissue samples: Innocent until proven otherwise?

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ABSTRACT

*Purpose:* *Coxiella burnetii* has been suggested as a potential cause of B-cell non-Hodgkin lymphoma (B-NHL), as *C. burnetii* was detected in B-NHL tissues. To further investigate this potential relationship, we hypothesized that among subjects previously exposed to *C. burnetii,* the bacterium is more frequently detectable in tissues of patients with B-NHL (cases) compared to patients without B-NHL (controls).

*Methods:* We aimed to evaluate this hypothesis by assessing the presence of *C. burnetii* with polymerase chain reaction (PCR), immunofluorescence staining (IF) and fluorescent in-situ hybridization (FISH). Eligible patients were those previously exposed to *C. burnetii*.

*Results:* Samples were available for 13 cases and 16 controls. *C. burnetii* was demonstrated in tissues of 8/29 patients in total (28%), with either PCR, IF or FISH: in 5/13 cases (38%) and 3/16 controls (19%), p = 0.41. Negative and positive control samples were all negative and positive appropriately for all three diagnostic methods.

*Conclusions:* In patients previously exposed to *C. burnetii* the bacterium was detected in tissue samples from subjects with and without B-NHL, without significant differences in the proportion positive samples. Therefore, we conclude that detection of *C. burnetii* in tissues of patients previously exposed to *C. burnetii* is a non-specific finding.

## 1. Introduction

Q fever is a zoonosis that causes clinically relevant disease in humans and animals (Eldin et al., 2017). After primary infection, individuals may develop a flu-like illness, pneumonia or hepatitis. A small proportion develops a chronic or persistent focalized infection, resulting in endocarditis, vascular infections, lymphadenitis or rarer manifestations (Eldin et al., 2017).

Apart from causing Q fever, *Coxiella burnetii* has been implicated as a potential causative agent in the development of non-Hodgkin lymphoma (NHL) (Melenotte et al., 2016a, 2016b). Non-Hodgkin lymphoma (NHL) is one of the most common cancers in the western world (Shankland et al., 2012). The etiology of NHL is believed to be multifactorial. Factors that are associated with NHL are alcohol consumption, smoking, immunocompromised state, and environmental factors such as exposure to chemicals (Shankland et al., 2012; Czarnota et al., 2015). Moreover, haematological malignancies can be associated with infectious diseases. Hodgkin lymphoma in children, for example, are strongly associated with Epstein-Barr virus (EBV) (Di Napoli et al., 2013). Multiple pathogens have been associated with NHL, such as Human Immunodeficiency Virus (HIV), EBV, *Helicobacter pylori*, and *Borrelia burgdorferi* (Hjalgrim et al., 2003; Beral et al., 1991; Parsonnet et al., 1994; Schöllkopf et al., 2008). An increased incidence of NHL was reported in patients with Q fever infection. Moreover, *C. burnetii* was demonstrated in macrophages and plasmacytoid dendritic cells in NHL tissues. The hypothesis of a causal relationship between *C. burnetii* and

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NHL was postulated (Melenotte et al., 2016a, 2016b). One of the potential mechanisms could be that increased interleukin-10 (IL-10) production during infection with *C. burnetii* induces the development of NHL (Capo et al., 1996; Honstettre et al., 2003; Ghigo et al., 2001; Meghari et al., 2008). It has been suggested that IL-10 plays a role in development of lymphoma, B-cell NHL (B-NHL) specifically, through stimulation of proliferation of B-cells and prevention of apoptosis (Akdis et al., 2016). Another potential explanation for the presence of *C. burnetii* in B-NHL tissue samples is that *C. burnetii* also invades antigen-presenting cells in malignant tissues (e.g. tumor-infiltrating monocytes or macrophages) without playing a direct role in lymphoma development. In one previous study, latent presence of *C. burnetii* in various tissues up to five years after primary infection was reported (Harris et al., 2000).

We hypothesized that if *C. burnetii* induces the development of B-NHL, the bacterium would be significantly more often present in B-NHL tissues than in tissues of patients without NHL. If *C. burnetii* is detected equally frequent in B-NHL tissues and tissues without B-NHL, the finding of *C. burnetii* in B-NHL tissues could be considered non-specific. To test this hypothesis, we investigated the presence of *C. burnetii* in tissues of patients previously exposed to *C. burnetii* both with and without B-NHL, with highly sensitive diagnostic techniques.

## 2. Methods

### 2.1. Study design and population

The presence of *C. burnetii* in tissues of patients previously exposed to *C. burnetii* with and without B-NHL was assessed with different diagnostic techniques: polymerase chain reaction (PCR), immunofluorescence (IF) staining and fluorescent in-situ hybridization (FISH).

We collected tissues of patients previously exposed to *C. burnetii* with B-NHL (cases) and without B-NHL (controls). To identify cases, all B-NHL patients alive after 01-01-2007 diagnosed at the Jeroen Bosch Hospital in' s-Hertogenbosch, located in the heart of the Dutch Q fever outbreak of 2007–2010, were evaluated (Kampschreur et al., 2013). All B-NHL patients ≥18 years of age, with *C. burnetii* serology performed in clinical routine care or with deep frozen serum available from prior venepunctures to perform serological testing, were evaluated. Patients were considered eligible if tissue samples were available. Patients with B-NHL deceased prior to 2007 were not evaluated, because Q fever was rare in the Netherlands before the outbreak and exposure to *C. burnetii* in these patients is highly unlikely (National Institute for Public Health and the Environment, 2017). In patients without serology performed in clinical routine with deep frozen serum available from prior venepunctures, serological testing was performed on a thawed serum sample. Tissues of all selected cases were assessed for presence of *C. burnetii*. If multiple tissues were available, the tissue on which the initial diagnosis was based was selected; if two tissue specimens from one patient were obtained simultaneously, both were selected.

Controls were selected by cross-checking clinical, pathology and microbiology records in the same hospital: all patients exposed to *C. burnetii* confirmed by serology with a tissue biopsy obtained between 2007 and 2015, without history or presence of any type of NHL, were considered eligible. To ensure comparability between cases and controls with regard to tissue type and time since exposure, controls were matched on tissue type and year of retrieval. If matching on year was not possible, the most nearby year was taken. If matching on tissue type was not possible, a lymph node biopsy was selected if available. If multiple eligible tissues were available, all were selected.

### 2.2. Data collection and storage

Design and performance of this study was approved and monitored by the Medical Ethical Committee Brabant in 2016. Waiver for informed consent procedure was obtained, since all tissues and data were collected in routine care and data and tissues were completely anonymised. Performing of serological testing and collection of clinical data from electronically stored medical records occurred between June 2016 and September 2016. Laboratory testing of tissue specimens occurred in December 2016 for B-NHL tissues and in June 2017 for control samples. All data were processed anonymously and stored and analysed in SPSS Version 21.0.

### 2.3. Laboratory diagnostics

*Serum* - Serological testing for *C. burnetii* consisted of indirect fluorescent-antibody assay (IFA; Focus Diagnostics, Inc., Cypress, CA, USA) for phase I and II IgM and IgG, performed by a trained microbiological technician from the Jeroen Bosch Hospital. Titration was performed with binary serial dilutions, with a detection cut-off titer of 1:32. Serum samples were retrieved from the central laboratory freezer, where samples are kept at a temperature of -20C°, and thawed for serological assays.

*Tissues* - Positive control samples were prepared from L929 cells and tissues of a SCID mouse infected with the *C. burnetii* nine mile strain (Melenotte et al., 2016a, 2016b). Negative control samples were prepared from non-infected L929 cells, tissues of a non-infected SCID mouse and tissues of patients without *C. burnetii* antibodies (a sample of a B-NHL patient as well as a sample of a patient without NHL). Diagnoses from histopathological reports generated in clinical routine were used.

#### 2.3.1. Polymerase chain reaction

A laboratory-developed real time PCR for *C. burnetii* was performed on paraffin-embedded lymphoma tissue by a trained microbiological technician from the Jeroen Bosch Hospital (Schneeberger et al., 2010). All samples were analyzed in duplo. This real-time PCR targets the multicopy transposase gene (IS1111a element). A specimen of at least 2 square millimeters (mm2) was used. Paraffin-embedded materials were dewaxed and pre-treated with proteinase K. DNA was extracted using easyMAG (NucliSENS, bioMérieux, Marcy l'Etoile, France). The DNA isolate was used as template for three PCRs: two for detection of *C. burnetii* and one for PhHV to ensure adequate DNA isolation and exclude inhibition.

#### 2.3.2. Immunofluorescence

For IF, paraffin-embedded tissues were dewaxed by heating at 65 °C for 5 min, bathed in Clearify for 5 min, rehydrated in ethanol (three baths of 5 min in 100% ethanol, 95% ethanol and 70% ethanol respectively) and permeabilised in Phosphate Buffered Saline (PBS) for 5 min. Bovine Serum Albumin 3%/PBS was applied for blocking non-specific sites and samples were incubated at 37 °C for 30–60 minutes. After three brief washes in PBS-Tween20 (0.1%), the first antibody (anti-*C. burnetii* rabbit IgG conjugated to Alexa Fluor 555 (red)) was applied (dilution 1/800) and samples were incubated at 37 °C for 1 h. Samples were washed in three subsequent baths of PBS-Tween20 (0.1%) for 5 min, and the second antibody (goat anti-rabbit IgG conjugated to Alexa Fluor 555) was applied (dilution 1/800) and samples were incubated at 37 °C for one hour with again three subsequent baths of PBS-Tween20 (0.1%) for 5 min. After second antibody fixation, 4′,6-diamidino-2-phenylindole (DAPI) staining (blue) was performed and slides were conserved at 4 °C. The process was performed under protection from light.

#### 2.3.3. Fluorescence in situ-hybridization

For FISH targeting specific *C. burnetii* 16S rRNA, paraffin-embedded tissues were first dewaxed by heating at 65 °C for 5 min, bathed in Clearify for 5 min, rehydrated in ethanol (three baths of 5 min in 100% ethanol, 70% ethanol and 50% ethanol respectively) and permeabilised in PBS-Tween20 (0.1%) for 5–10 minutes. Subsequently, samples were hybridized with RNA-probes. The probes, CB-440 (5′- CTTGAGAATTT

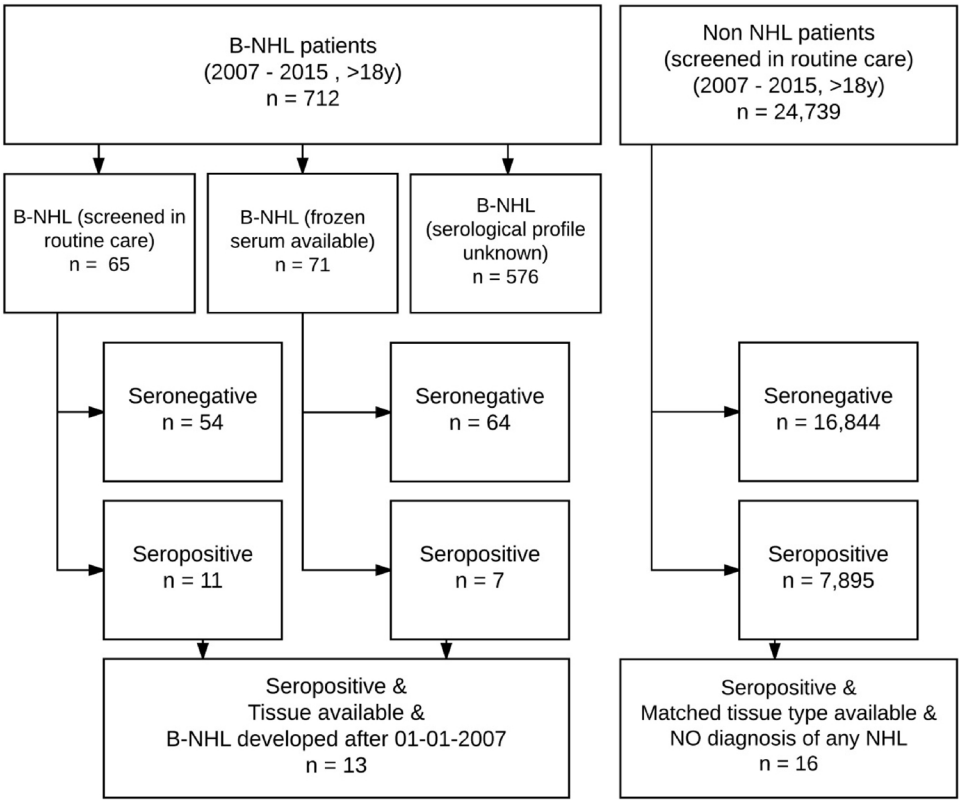


**Fig. 1.** Flowchart of inclusion.
*B-NHL = B-cell non-Hodgkin lymphoma. NHL = non-Hodgkin lymphoma.

CTTCCCC -3′) and CB-189 (5′- CCGAAGATCCCCCGCTTTGC - 3′) specifically target the *C. burnetii* 16S rRNA sequences (green). Furthermore, a probe for detection of bacterial 16S rRNA molecule (EUB-338, specific for most eubacteria, neon-red) and a non-specific probe (non-EUB-338, dark red) were added to exclude nonsense hybridization. A positive signal is yellow, as a result of the co-localization of the universal probe EUB (red) and the specific 16S rRNA *C. burnetii* probe (green). The probes were diluted in hybridization solution (dilution 1/100), that consists of natriumchloride, formamide, 10% sodium dodecyl sulfate (SDS), tris-hydrochloride (pH 8.0) and H2O. Samples were hybridized at 65 °C for 10 min and at 37 °C overnight. After hybridization, samples were washed in Washing Buffer (consisting of natriumchloride, ethylenediamineetetraacetic acid, tris-hydrochloride (pH 8.0), 10% SDS and H2O) for 15 min and briefly rinsed with H2O. After drying, DAPI staining (blue) was performed and slides were conserved at 4 °C. The process was performed under protection from light.

### 2.4. Definitions

#### 2.4.1. Presence of C. burnetii in tissue samples

The primary outcome of this study was the proportion of positive tissue samples, thus samples in which *C. burnetii* was detected, with any of the three highly sensitive and specific diagnostic techniques.

#### 2.4.2. B-cell non-Hodgkin lymphoma

The definition of B-NHL includes all types of mature B-cell lymphoma, including precursor B-cell leukemia and precursor B-cell lymphoma. Mature B-cell lymphoma was defined as diffuse large cell B-cell lymphoma, follicular lymphoma, mantle cell lymphoma, Burkitt lymphoma, extranodal marginal zone B-cell lymphoma or mucosa-associated lymphoid tissue B-cell lymphoma, nodal marginal zone B-cell lymphoma, splenic marginal zone B-cell lymphoma, chronic lymphatic leukemia, hairy cell leukemia, B-cell prolymphocytic leukemia and lymphoplasmacytic lymphoma or Waldenström macroglobulinemia (Swerdlow et al., 2008).

#### 2.4.3. Exposure to C. burnetii

Patients considered exposed to *C. burnetii* are those with positive serology (with or without matching the definition of chronic *or* persistent Q fever). Definition of positive serology *not* matching chronic *or* persisting Q fever is a positive phase II IgG titer (≥1:32), without fulfilling the criteria for chronic *or* persisting Q fever. Not all patients had undergone radiographical imaging, since we used data and samples that were generated in routine clinical care. Therefore, radiographical imaging data were available only if imaging had been performed in clinical practice. Chronic Q fever was defined according to the Dutch consensus guideline (possible, probable or proven chronic Q fever), which implies having at least a phase I IgG titer of ≥ 1:1024 (Wegdam-Blans et al., 2012). The diagnosis of proven or probable chronic Q fever according to these criteria with a defined focus of infection, is comparable to the definition of a definite or possible persistent focalized infection according to the criteria formulated by *Eldin et al* (Eldin et al., 2017). Patients without antibodies against *C. burnetii* (seronegative patients) are considered unexposed to *C. burnetii.* All dates between 01-01-2007 and 31-12-2010 are considered to be during the Dutch Q fever outbreak (National Institute for Public Health and the Environment, 2017).

## 3. Statistics

Differences in proportions of positive tissues for cases and controls were calculated with a Chi-square or Fisher exact test, as appropriate. Calculations were performed in SPSS (Version 21.0). Univariable odds ratio's (OR) with 95% confidence intervals were calculated. P-values below 0.05 were considered to be statistically significant.

## 4. Role of the funding source

The funding source had no role in study design, data collection, analysis or interpretation. The funding source had no role in writing the report or decision were to submit the report.

## 5. Results

In total, 136 B-NHL patients were evaluated for eligibility in this study (Fig. 1). See Table 1 for baseline characteristics of all serologically evaluated B-NHL patients. Tissue samples were available from 13 cases and 16 controls, a description of cases and controls is given in Table 2.

*C. burnetii* was demonstrated in tissues of 8 patients (28%) with PCR, IF or FISH: in 5/13 cases (38%) and 3/16 controls (19%), p = 0.41 (OR for positivity in cases: 2.71, 95% confidence interval (CI) 0.50–14.54). Tissues of 7 patients were positive with IF and FISH: 5 cases (38%) and 2 controls (13%). There was complete agreement between IF and FISH. PCR was negative for all cases and positive in 2 controls (13%). Of 2 PCR positive control tissues, one was IF and FISH

**Table 1**
Baseline characteristics of all B-cell non-Hodgkin lymphoma patients.

| | All | Exposed to *C. burnetii* | Not exposed to *C. burnetii* |
|---|---|---|---|
| **N (%)** | 136 | 18 (13) | 118 (87) |
| **Mean age (standard deviation (sd))** | 61 (15) | 64 (18) | 60 (14) |
| **Male gender (%)** | 77 (57) | 11 (61) | 66 (56) |
| **Residency in high endemic area (%)** | 124 (91) | 17 (94) | 107 (91) |
| **Year of B-NHL diagnosis** | | | |
| Pre-outbreak (%) | 23 (17) | 3 (17) | 20 (17) |
| During outbreak (%) | 20 (15) | 1 (6) | 19 (16) |
| After outbreak (%) | 93 (68) | 14 (78) | 79 (67) |
| **Serological diagnosis** | | | |
| Exposed to *C. burnetii* (%) | 18 (13) | 18 (100) | – |
| Chronic Q fever (%)[a] | 2 (11) | 2 (11) | – |
| Seronegative (%) | 118 (87) | – | 118 (100) |
| **Subtype of lymphoma** | | | |
| Follicular lymphoma (%) | 34 (25) | 7 (39) | 27 (23) |
| Diffuse large cell B-cell lymphoma (%) | 40 (29) | 5 (28) | 35 (30) |
| Mantle cell lymphoma (%) | 16 (12) | 2 (11) | 14 (20) |
| B-acute lymphoblastic leukemia (%) | 7 (5) | 1 (6) | 6 (5) |
| Marginal zone (including extranodal) lymphoma (%) | 12 (9) | 1 (6) | 11 (9) |
| Small cell lymphoma (%) | 5 (4) | – | 5 (4) |
| Mucosa-associated lymphoid tissue lymphoma (%) | 3 (2) | 1 (6) | 2 (2) |
| Burkitt lymphoma (%) | 3 (2) | – | 3 (3) |
| Waldenstrom (%) | 3 (2) | – | 3 (3) |
| Other (%) | 13 (10) | 1 (6) | 12 (10) |
| **Ann Arbor stage** | | | |
| I–II (%) | 52 (38) | 9 (50) | 43 (34) |
| III–IV (%) | 76 (56) | 8 (44) | 68 (58) |
| n/a or unknown (%) | 8 (6) | 1 (6) | 7 (6) |
| **NHL risk factors** | | | |
| Immunocompromised (%)[b] | 11 (8) | 2 (11) | 9 (8) |
| Rheumatoid arthritis (%) | 7 (5) | 2 (11) | 5 (4) |
| Prior chemo-/radiotherapy (for other malignancy) (%) | 5 (4) | – | 5 (4) |
| Use of alcohol (%)[c] | 76 (56) | 11 (61) | 65 (55) |
| History of smoking (%)[d] | 58 (43) | 9 (50) | 49 (42) |
| **Q fever risk factor** | | | |
| Valvulopathy worse than mild / valve prosthesis (%) | 11 (8) | 3 (17) | 8 (7) |
| Aneurysm or vascular prosthesis (%) | 5 (4) | 2 (11) | 3 (3) |
| **Co-infections**[e] | | | |
| Eppstein-Barr virus (EBV) positive (%)[f] | 4 (3) | – | 4 (3) |
| EBV-positive lymphoma tissue sample (%)[g] | 4 (3) | – | 4 (3) |
| **Course of disease** | | | |
| Deceased (%) | 35 (26) | 2 (11) | 33 (28) |
| Treatment with chemo-/radiotherapy (%) | 115 (82) | 14 (78) | 101 (86) |
| Stem cell transplant (%) | 16 (12) | 1 (6) | 15 (13) |

[a] See definitions. Of cases, 85% (n = 11) underwent PET-CT scanning and 31% (n = 4) underwent echocardiography shortly before or after of diagnosis of NHL. Of controls, 25% (n = 4) underwent PET-CT scanning and 38% (n = 6) underwent echocardiography shortly before or after biopsy for various reasons.

[b] 1 post renal transplant, 6 patients with immunosuppressive medication (DMARD, prednisone or biologicals or a combination), 1 hypogamma-globulinemia, 1 patient with both hypogammaglobulinemia and immunosuppressive medication (as previously defined), 1 MDS with severe leukopenia, 1 hemodialysis patient.

[c] Unknown for 18 patients.

[d] Unknown for 8 patients.

[e] No patients with known hepatitis B virus, hepatitis C virus or human immunodeficiency virus infection. Unknown HIV status for 77 patients, unknown HCV status for 70 patients, unknown HBV status for 74 patients. 4 patients with past HBV infection.

[f] Active infection with detectable viral load. Past infection in 61 patients, unknown status for 63 patients.

[g] Epstein-Barr encoding region (EBER) in situ hybridization was standardly performed for all immunocompromised patients, all CD30+ lymphoma, all large cell lymphoma, plasmablastic lymphoma, Burkitt lymphoma, lymphomatoid granulomatosis and NK or T-cell lymphoma.

positive (Table 3). Negative and positive control samples were all negative and positive appropriately, for all three diagnostic methods.

When assessing the number of positive tissues among chronic Q fever patients, 3/6 were positive (50%): 1 case (50%) and 2 controls (50%), p = 0.99, OR 1.00 with 95% CI 0.03 – 29.81. When assessing the number of positive tissues among past Q fever patients, 5/23 (22%) were positive: 4 cases (36%) and 1 control (8%), p = 0.16, OR 6.29 with 95% CI 0.58–68.42.

In supplementary Table 1, specification of pathology diagnoses, tissue types and results of PCR, IF and FISH are shown for all case and control matched tissues. In Fig. 2, microscopic photographs of IF and FISH results are shown for negative controls, positive controls and a selection of positive samples.

## 6. Discussion

Overall, *C. burnetii* was detected in tissues of 38% of cases and 19% of controls. The difference in proportion positive samples between cases and controls was not significant. When assessing past Q fever patients only, 36% of cases and 8% of controls were positive, which difference was also not significant. Therefore, we conclude that the presence of *C. burnetii* in tissue samples is not specific for B-NHL.

When evaluating the Bradford Hill criteria for establishing a causal relationship, evidence supporting some criteria is limited (Hill, 1965; Fedak et al., 2015). The previously reported association between exposure to *C. burnetii* and development of NHL was found to be quite strong with a 25-fold increased risk (Melenotte et al., 2016a, 2016b). However, the consistency of this finding is moderate. In a nationwide Dutch epidemiological study, the risk for was increased in one year only (RR1.16), and not in any other year (Van Roeden et al., 2018). Thus, the height of the excess in the two studies risk ranges between 1 and 25,

**Table 2**
Description of cases (B-NHL patients) and controls (without any NHL).

| | Cases | Controls |
|---|---|---|
| **Number of patients (%)** | 13 (45) | 16 (55) |
| **Number of tissues (%)** | 14 (47) | 16 (53)[a] |
| **Mean age (sd)** | 65 (18) | 59 (19) |
| **Male gender (%)** | 9 (64) | 10 (63) |
| **Malignant tissue (%)** | 14 (100) | 4 (25) |
| **Type of tissue** | | |
| Lymph node (%) | 6 (43) | 8 (50) |
| Bone marrow (%) | 2 (14) | 3 (19) |
| Gastro-intestinal (%) | 2 (14) | 2 (13) |
| Spleen (%) | 1 (7) | 1 (6) |
| Lung (%) | 1 (7) | 1 (6) |
| Other (%) | 2 (14) | 1 (6) |
| **Timing of biopsy** | | |
| Retrieved during epidemic (%) | 1 (7) | 3 (19) |
| Retrieved after epidemic (%) | 13 (93) | 13 (81) |
| **Serological profile** | | |
| Patient with past Q fever infection (%) | 11 (79) | 12 (75) |
| Patient with chronic Q fever (%)[b] | 2 (14) | 4 (25) |
| Vascular focus of infection (%) | 1 (7) | 2 (13) |
| Combined endocarditis and vascular (%)focus | 1 (7) | – |
| No focus of infection (%) | – | 2 (13) |
| Endocarditis (%) | – | – |

[a] Biopsy findings: 2 patients with lung carcinoma, 1 mamma carcinoma, 1 renal cell carcinoma, 2 myelodysplastic syndromes, 2 sarcoidosis, 2 lymphadenopathy observed during vascular surgeries with chronic inflammation, 1 splenectomy performed during vascular surgery because of a complication without pathology, 1 lymphnode after hemistrumectomy (benign pathology), 1 lymphnode found in breast biopsy, 1 stomach biopsy, 1 coecum biopsy, 1 bone marrow biopsy without any pathological diagnosis.

[b] 4 patients with proven chronic Q fever (three with a vascular focus of infection and one with both endocarditis and a vascular focus of infection, 1 patient with probable chronic Q fever (no focus of infection; probable based on immunocompromised state) and 1 patient with possible chronic Q fever (no focus of infection and no risk factors).

**Table 3**
Result of polymerase chain reaction (PCR), immunofluorescence (IF) and fluorescent in-situ hybridization (FISH) on case and control tissue samples.

| | All | Cases | Controls |
|---|---|---|---|
| Number of patients (%) | 29 | 13 (45) | 16 (55) |
| Number of samples (%) | 30 | 14 (47) | 16 (53) |
| Number of patients positive with any test (%)[a] | 8 (28) | 5 (38) | 3 (19) |
| Number of patients with positive PCR (%) | 2 (7) | 0[b] | 2 (13) |
| Number of patients with positive IF (%) | 7 (24) | 5 (38) | 2 (13) |
| Number of patients with positive FISH (%) | 7 (24) | 5 (38) | 2 (13) |

[a] Either PCR, IF or FISH or a combination of those positive.

[b] 1 missing due to repeated inhibition during PCR.

and there is a considerable risk of selection and detection bias in both studies (Melenotte et al., 2016a, 2016b; Van Roeden et al., 2018). Furthermore, the specificity of the potential relation is low, since there are other factors that may explain both diseases (Shankland et al., 2012; Kampschreur et al., 2012; Raoult, 1990; Schoffelen et al., 2014). Temporality between exposure and development of disease is very difficult to confirm definitely, because both diseases can have a considerable diagnostic delay: infection with *C. burnetii* may occur asymptomatically and B-NHL may be difficult to detect at early stages. An experiment would therefore have to be performed to ensure a temporal relationship. The argument of a biological gradient (or dose-response relation) seems to hold: the risk is higher in patients with a chronic or persistent infection compared to those that develop NHL after a primary infection (Melenotte et al., 2016a, 2016b; Van Roeden et al., 2018). Moreover, there is a plausible potential pathophysiological explanation for the association (Eldin et al., 2017; Melenotte et al., 2016a, 2016b). Confirmation of this pathophysiological mechanism, for example by performing an experiment, would be desirable. Finally, there is coherence between epidemiological and laboratory findings (Melenotte et al., 2016a, 2016b; Van Roeden et al., 2018). Altogether, there are arguments supporting causation. However, additional evidence with regard to the temporal relation, specificity of the potential relation and confirmation of the supposed pathophysiological pathway is required. Understanding why *C. burnetii* is latently present more often in NHL tissues and what the effect of the presence of the bacterium is in those tissues, is of vital importance and will provide more definite answers. The lack of a significant difference between the proportion positive samples in cases and controls may be due to a lack of power. Nevertheless, we must conclude that finding *C. burnetii* in tissues is not specific for B-NHL tissues, even if the difference in proportions would have been significant. Among patients (both cases and controls) without chronic *or* persistent Q fever but only previously exposed to *C. burnetii,* the bacterium was demonstrated in over 22% of tissues. Positive tissues were obtained in 2011, 2012, 2014 and 2015 while the Dutch Q fever outbreak occurred between 2007 and 2010, indicating that *C. burnetii* can be detected many years after primary infection in antigen-presenting cells in different types of tissues in the absence of chronic *or* persistent infection. This is in line with a previous study, which reported latent presence of *C. burnetii* in various tissues up to five years after primary infection (Harris et al., 2000). The finding of *C. burnetii* in lymph nodes, bone marrow and gastro-intestinal tissues demonstrates the extensive infiltration of the bacterium in lymphatic tissues. It is obvious that the bacterium can be detected during active infections like acute or chronic Q fever). However, after clearance of a primary infection and in absence of a chronic infection, it is remarkable that *C. burnetii* remains latently present. When comparing the proportion of positive samples of cases and controls with past Q fever, samples of cases were more often positive compared to controls although this difference was also not significant.

Different criteria for diagnosis of chronic Q fever have been developed (Eldin et al., 2017; Wegdam-Blans et al., 2012). For this Dutch cohort, the Dutch chronic Q fever consensus group criteria were used.

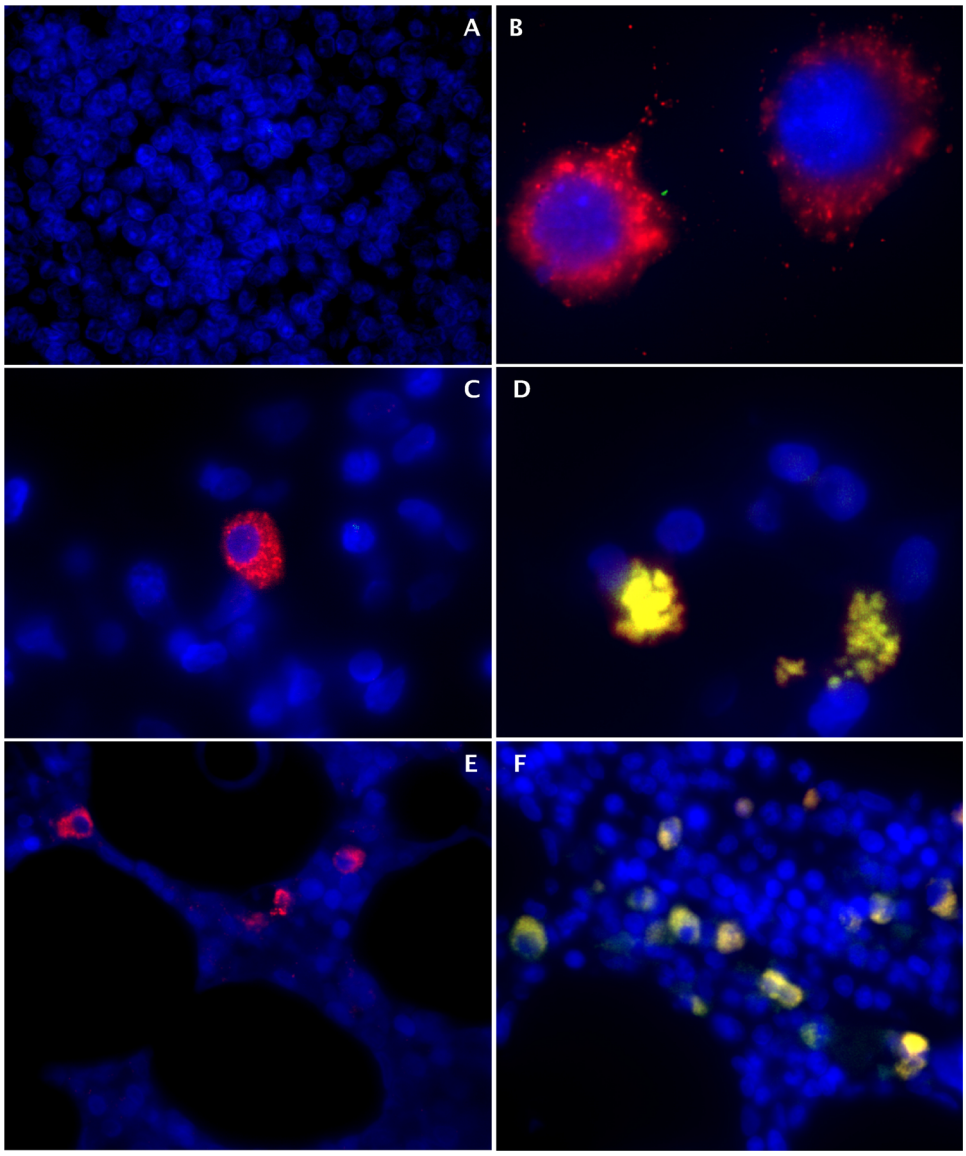


**Fig. 2.** Microscopic photographs of immunofluorescence and fluorescent in-situ hybridization.
**(A)** Microscopic image of immunofluorescence staining (IF) of negative control tissue (patient with sarcoidosis, never exposed to Coxiella burnetii) in which nuclei are stained blue (4′,6-diamidino-2-phenylindole (DAPI)) and no C. burnetii is detected **(B)** Microscopic image of IF of positive control sample (L929 cells infected with C. burnetii) in which nuclei are stained blue (DAPI), while perinuclear C. burnetii is stained red. **(C)** Microscopic image of IF of retroperitoneal lymphoma tissue of a patient with vascular chronic Q fever in which nuclei are stained blue (DAPI), while perinuclear C. burnetii is stained red **(D)** Microscopic image of fluorescence in-situ hybridization (FISH) of the same tissue as (C) in which nuclei are stained blue (DAPI), while C. burnetii, organized in perinuclear vacuoles, is stained yellow. The yellow signal results of the co-localization of the universal probe EUB (red) and the specific 16S rRNA C. burnetii probe (green). **(E)** Microscopic image of IF of a bone marrow biopsy showing follicular non-Hodgkin lymphoma in a patient with past Q fever in which nuclei are stained blue (DAPI), while perinuclear *Coxiella burnetii* is stained red **(F)** Microscopic image of FISH of the same tissue as (E) in which nuclei are stained blue (DAPI), while C. burnetii, organized in perinuclear vacuoles, is stained yellow. The yellow signal results of the co-localization of the universal probe EUB (red) and the specific 16S rRNA C. burnetii probe (green). In all cases, a Leica DMI6000 B microscope was used and original magnification of images was ×100.

When applying the criteria for diagnosing persistent focalized *C. burnetii* infection, formulated by *Eldin et al.,* three (instead of two) cases would potentially classify as having a persistent focalized *C. burnetii* infection. In the control group, three (instead of four) would potentially classify as having a persistent focalized infection. This demonstrates that the definition used for identification of patients influences the description of these study patients. However, since we selected *all* patients that were exposed to *C. burnetii* previously (with either a past or chronic infection), the main outcome of interest would not have changed by using either definition.

This is the second study (with exception of one additional case report) in which the value of IF and FISH to demonstrate *C. burnetii* in human samples was explored (Melenotte et al., 2016a, 2016b; Kumpf et al., 2016). The agreement between IF and FISH was 100% in this study and in the previous study (Melenotte et al., 2016a, 2016b). In the current study, PCR was performed additionally on all tissue samples. IF and FISH were able to detect *C. burnetii* in 6 PCR-negative tissues. Contradictory, PCR was positive in one sample on which IF and FISH were both negative. This may be caused by the fact that this patient was treated with antibiotics at the time of biopsy retrieval for treatment of chronic Q fever. The cycle threshold-values in this case were 39.26 and 41.19, which are marginal positive values. It is possible that due to extensive bacterial and cellular degradation FISH and IF were both negative: both techniques require a specific positive signal in the perinuclear compartment or intracellular vacuoles. Moreover, the difference may rely on sampling error: the bacterial load may vary for different samples. Since microscopic findings with IF and FISH are highly specific, and especially because an additional probe targeting non-specific bacterial DNA was used during FISH to detect false positivity, it is very unlikely that positive IF and FISH are caused by false positivity of these techniques (Kaittanis et al., 2012). The higher detection rate with IF and FISH suggests that these methods may be more sensitive for detection of *C. burnetii* in paraffin-embedded tissues. After the index study, this was the first study to compare presence of *C. burnetii* in both B-NHL tissues and tissues of patients without B-NHL (Melenotte et al., 2016a, 2016b). In the previous study, patients with lymphadenitis were explored as well (Melenotte et al., 2016a, 2016b). In this study, we set out to explore the implication of finding *C. burnetii* in tissue in patients with chronic or past infection, in the absence of primary infection. The large Dutch Q fever outbreak provided the unique opportunity to explore presence of the association between *C. burnetii* and B-NHL, with a relatively large number of samples. We used the best available diagnostic techniques to ensure optimal sensitivity and specificity and to provide insight in the value of these novel diagnostic techniques. Naturally, our study has drawbacks as well. It is likely that our numbers were too small to detect a difference between

patients with and without B-NHL. We observed differences between B-NHL-cases and controls, but none of the differences were significant. Moreover, this is a retrospective study. Therefore, diagnostic work-up for our patients was unstandardized, leading to variable timing of serology and absence of transthoracic echocardiography or PET-CT imaging in part of our patients. Additionally, although Q fever has a worldwide distribution, it remains a relatively rare disease (Eldin et al., 2017). Therefore, the absolute risk for B-NHL after exposure to *C. burnetii* remains small (in a large cohort of 1468 Q fever patients, 7 patients developed B-NHL) (Melenotte et al., 2016a, 2016b). Assuming a causal relationship, the contribution of *C. burnetii* on the total number of diagnosed B-NHL may be limited, since many other factors contribute to the incidence of B-NHL. Nevertheless, from a scientific point of view and for a selected group of patients, it is very relevant to explore the causation of this association. Furthermore, all patients in our study were infected during the Dutch Q fever outbreak, with the Dutch Q fever outbreak strain. It is possible that other *C. burnetii* strains behave differently, leading to different clinical sequelae. Therefore, these results will have to be validated in other populations. Another potential issue is the fact that we used certain diagnostic techniques (FISH and IF) that are very sensitive and specific on theoretical grounds, but clinical data on sensitivity and specificity of these techniques are very scarce. Finally, patients might still develop B-NHL over time, and the presence of any lymphoprolipherative disease has not been excluded systematically in the control groups. These considerations make the interpretation of the implication of detection of *C. burnetii* in tissues challenging.

In conclusion, *C. burnetii* was detected in 38% of B-NHL tissues of patients that were previously exposed to the bacterium. Although *C. burnetii* was detected in more B-NHL tissues than in tissues of control patients (19%), the difference was not significant. Therefore, we conclude that the finding of *C. burnetii* in tissues is a non-specific finding. Experimental research, such as animal experiments with induction of lymphomagenesis or micro-array on oncogenic proteins in antigen-presenting cells, may provide more definite answers.

## Conflicts of interest

Sonja E. van Roeden, Chantal P. Bleeker-Rovers, Jan Jelrik Oosterheert and Peter C. Wever received a research grant from Foundation Q-support. Sonja E. van Roeden and Jan Jelrik Oosterheert received a research grant from Institut Mérieux. Sonja E. van Roeden, Chantal P. Bleeker-Rovers, Jan Jelrik Oosterheert and Peter C. Wever declare no other competing (financial) interests. Mirjam H.A. Hermans, Peet T.G.A. Nooijen, Alexandra Herbers and Andy I.M. Hoepelman declare no other competing (financial) interests.

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## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.imbio.2018.11.012.

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