

The Presence of Intracellular *Coxiellae burnetii* as Polymorphic Cell Wall Deficient Bacteria in the Blood of Patients with Q-Fever Fatigue Syndrome Determined Using FISH Technology

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Abstract

Since 2007 the Netherlands has been faced with serious public health issues resulting from an epidemic outbreak of Q-fever. Although acute Q-fever may etiologically be linked to *Coxiella burnetii*, there is currently no expert consensus on the primary cause of the pathogenic clinical manifestation in patients suffering from Q-fever fatigue syndrome (QFS). Scientists have been searching for the cause of QFS for many years. In the Netherlands, negative qPCR results and serology tests have led to the conclusion that there were no viable *C. burnetii* present in patients suffering from the Q-fever fatigue syndrome. In another study, infecting test animals with bacterial remnants taken from QFS patients did not result in a transfer of infection. The conclusion is that no viable bacteria are present and that the clinical condition should be attributed to an immuno-modulatory complex (IMC).

The QFS subjects in this study had been previously serologically diagnosed as having Q-fever. Using Fluorescence *in situ* Hybridization technology, the authors have found that QFS significantly correlated with the presence of viable large, cell variants (LCV) of *C. burnetii*. Other studies have shown that the host cells for these LCVs are macrophages, the part of the immune system designed to ingest and destroy pathogens. The cell wall of an LCV has a very limited amount of peptidoglycan and is, in fact, a cell wall deficient bacteria (CWDB). These CWDB hosted in the macrophages can multiply within the lysosome, and eventually revert back to the classical bacterial form given the right conditions.

This article demonstrates that automated Fluorescence in situ Hybridization technology can be used as a method to determine the presence of bacterial DNA of viable L-form bacteria in white blood cells derived from patients suffering from Q-fever Fatigue Syndrome.

Keywords: Q-fever; Q-fever fatigue syndrome (QFS); *Coxiella burnetii*; Pleiomorphism fluorescence; In situ hybridization; Cell wall deficient bacteria

Introduction

Coxiella burnetii was described in 1981 as a “gram-variable obligate intracellular bacterium which carries out its development cycle in the phagolysosome of eukaryotic cells” [1]. Howe et al. referred to the host cell as a macrophage [2]. The bacteria penetrate the host cells in their classic small cellular variant (SCV) and undergo an intracellular developmental cycle involving diverse variants of itself, the main form being the large cell variant (LCV) which functions as breeding hatchery for a new generation of these bacteria [3]. The illustration in Figure 1 was based on a chart made by Domingue to illustrate his hypothesis about the developmental cycle of CWDB found in the intracellular environment [4]. With the permission of the author, this chart was adjusted by the authors to include the latest insights into CWDB behavior in cells.

In past years, researchers using Fluorescence in situ Hybridization (FISH) have published work showing the successful use of probes directed at ribosomal RNA to demonstrate the presence of viable *C. burnetii* in chronic inflammations in infected tissues [5-7]. In comparative studies, it appeared that this analytical principle has a superior tracking sensitivity to the presence of bacterial RNA to that of the quantitative Polymerase Chain Reaction (qPCR). The testing in question was done on tissue from excised heart valves, from aneurisms and from thromboses in patients with vascular infections [8,9]. Research into the application of FISH as analytical tool in order to identify and quantify Cell Wall Deficient Bacteria (CWDB) in venous

blood has not been published to date. The study undertaken by the authors of the present article was designed to prove, beyond doubt, the presence of viable CWDB of *C. burnetii* in the blood of patients suffering from QFS. This procedure is less invasive than testing of tissues and potentially more practical for use in diagnostics in general medical practice.

Material and Methods

Subjects

The control group consisted of 10 healthy volunteers. Four men and 6 women between the ages of 23 and 52 were selected on the basis of an extensive anamnesis. They had never had Q-fever or anything like Q-fever symptoms, and had never visited regions of the Netherlands where Q-fever is/was endemic.

The patient group consisted of 10 patients; 6 women and 4 men between the ages of 19-69, who demonstrated in anamneses and

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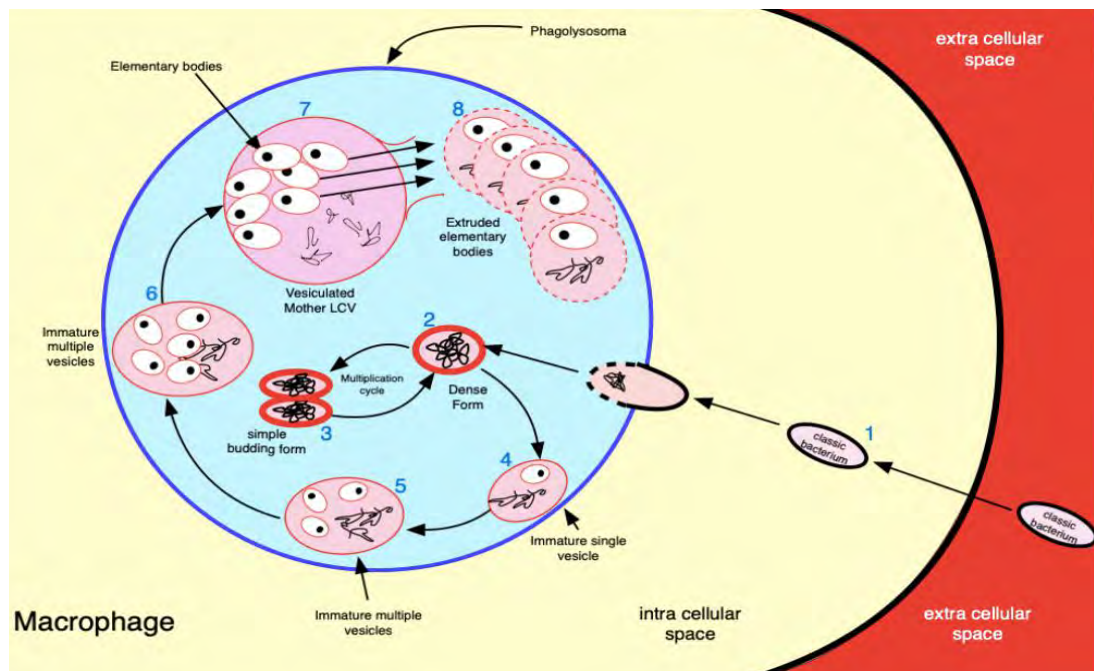


Figure 1: Hypothesized reproductive life-cycle of L-forms within the phagolysosome of a white blood cell, developed by Domingue. Follow the arrows. The classical bacterium enters a macrophage and arrives in the dense L-form [1,2]. From there two options are possible: a multiplication cycle [3] or a maturation cycle [4-7]. Elementary bodies [8] extruded from mother L-forms have several options: they are capable of maturing into vesiculated mother-forms and reproduction [3].

serology tests to be confirmed to have had Q-fever and to be suffering from Q-fever fatigue syndrome.

The study was observational in that the administration of antimicrobial agents, and other therapeutic management was controlled by the patient's physician and not the researchers. The study took place in the period 04-23-2018 to 07-11-2018. Informed consent was obtained from both the patients and the volunteers.

Sampling details

A minimum of 4 ml venous blood was taken using a heparinized plastic test-tube container using the vacutainer system. The blood was directly stored at 4° Celsius and was analyzed within 8 hours of being taken. The blood was taken by the patient's own doctor. The blood samples were anonymized and sent via the TranspoSafe delivery system by courier to the laboratory for testing using Fluorescence in situ Hybridization as the underlying analytical principle. The actual analyses were performed by means of a specialized analyzer system: Biotrack-MED.

Fluorescence in situ Hybridization using Biotrack-MED™

Sample preparation: The analyses were based on patented and proprietary methods and technologies of Biotrack as incorporated in an autonomous analyzer with the brand name Biotrack-MED Mark-2 using standard settings. Ten µl of full blood was thoroughly mixed with 90 µl of physiological salt solution. Subsequently, the diluted blood was pipetted onto an enclosed analytical substrate after which fixation by means of a fixation fluid 1 was performed autonomously. Finally, a drying step was executed at 50° Celsius to discard the fixation fluid by evaporation.

Hybridization: Following the controlled drying procedure, the

enclosed analytical substrate was fed with a solution of a proprietary hybridization buffer 2 containing a DNA-based *C. burnetii* 16S ribosomal RNA targeted probe. The sequence of this buffer is based on the specifications of Melenotte et al. (www.bloodjournal.org), October 2018 (5'-CACCGCGACATGCTGATTGCGG-3') and fluorescently labeled at 5'. The enclosed analytical substrate was then incubated using standard settings of the thermostated processing module. A module is used for the enclosed analytical substrate combined with the internal atmosphere in the module preventing evaporation of the hybridization buffer. After completion of the hybridization procedure, 10 ml of a washing solution 3 was used on discard the washing solution by evaporation.

Image acquisition: The analyzer is equipped with a multilevel fluorescence detector module. Under ultra-violet illumination this module produces a stack of up to 300 digital microphotographs resulting in a 3D-image. This image-cube is subsequently stored on the systems' SSD for archiving purposes or further morphometry.

A microphotograph taken during the *in situ*-labelling of the full blood of one of the QFS patients tested, using a *C. burnetii* probe is shown in Figure 2.

Morphometric analysis: This analysis was done on each image cube, using the following procedure:

1. Fluorescent objects were identified and selected using the recent image segmentation algorithm formulated by Tamminga et al. [10]. This resulted in a binary image comprising of only 0 or 1 values.
2. A series of morphometric parameters was calculated for each digitalized object. These parameters comprised of, at least, but not limited to: area (µm²), perimeter (µm²), roundness and convex hull.

3. The parameter scores per object were subsequently fed into a trained neural network. This network calculates the likeliness of an object to correlate with the morphometric characteristics of a CWDB based on these parameter scores. The fact that these objects also fluoresce (and have thus positively hybridized with the specific DNA-probe) determines the identity of the CWDB.
4. A total of minimum 50 white blood cells (WBCs) per sample were analyzed according to this procedure, by definition, a sample containing 3 white blood cells positive for CWDB was scored as positive for *C. burnetii* CWDB.

The method presented here was applied to 20 blood samples. These samples (10 patients diagnosed with Q-fever fatigue syndrome according to current clinical standards and 10 healthy volunteers) were taken from the author’s medical practice in Gelderland, in the Netherlands. The samples were anonymized and consisted of a minimum of 4 ml heparinized blood in a plastic vacutainer. The results are presented in Table 1.

In compliance with the Wilcoxon-test ($p=0.05$), it may be concluded from these results that the number of CWDB-positive white blood cells in Q-fever patients (QFS) is significantly higher than those found in the healthy control group. This suggests that the combination of in situ rRNA-labeling and morphometrics is a potential tool for explicating and diagnosing QFS. It has also shown specifically that the white blood cells of patients suffering from QFS harbor CWDB objects that hybridize with the *C. burnetii* probe.

The authors conclude that the result of this study justifies the instigation of a larger multicenter study to confirm these results, and in that way, underscore the value of using this analytical procedure in full blood samples to establish an exact diagnosis for Q-fever in patients – and in particular for diagnosing QFS.

Discussion

Generally speaking, classical bacterial forms have a peptidoglycan exterior membrane that triggers an immunological response and makes an interaction with antibiotics possible. CWDB have a much thinner peptidoglycan exterior and do not evoke the same reactions. When subject to stress, classical bacteria can modify their exterior membrane under the influence of phagolysosome present within the macrophage, but still retain their unique DNA. This allows the bacteria to develop intracellularly, under the radar of the immune system or other defense mechanisms.

Intracellular CWDB variants devolving from bacteria such as *C.*

burnetii cannot be cultured using the standard methods in clinical bacteriology. Serology can only determine those anti-*C. burnetii* immunoglobulins (isotypes IgG and IgM) that are circulating in the extra-cellular space. Intracellular viable forms of *C. burnetii* are not recognized as a pathogen by the immune system and thus generate no humoral immune response which would be revealed in serology tests.

CWDB have been linked to various chronic inflammatory diseases in a multitude of studies done over the last 80 years, this connection has not yet been explicated and remains the subject of scientific dispute [11]. It is interesting to note that the Australian Q-fever researchers suggest that finding the causal factor for QFS may well explain the source of various other chronic conditions [12]. The application of the diagnostic FISH test is not restricted to the detection of *C. burnetii*. In various other cases of chronic inflammatory disease, similar images to that of Figure 2 have been seen during the in situ-labeling of full blood utilizing ‘fluorescent microphotography of macrophages’ by these authors.

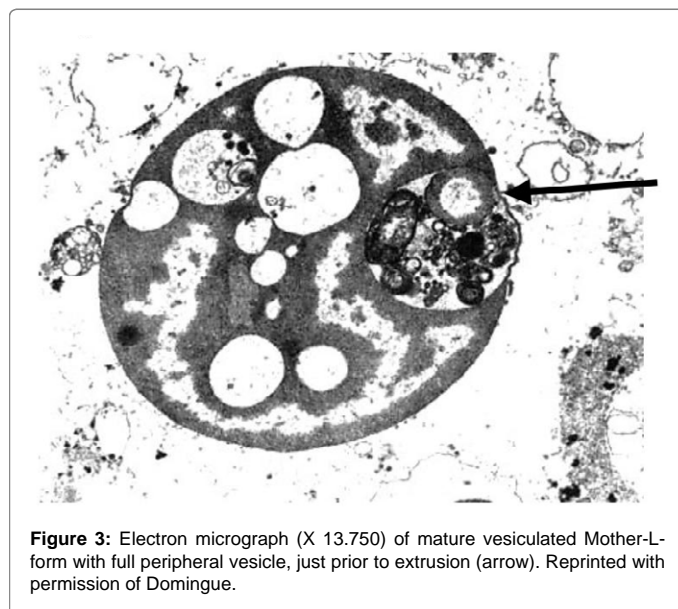
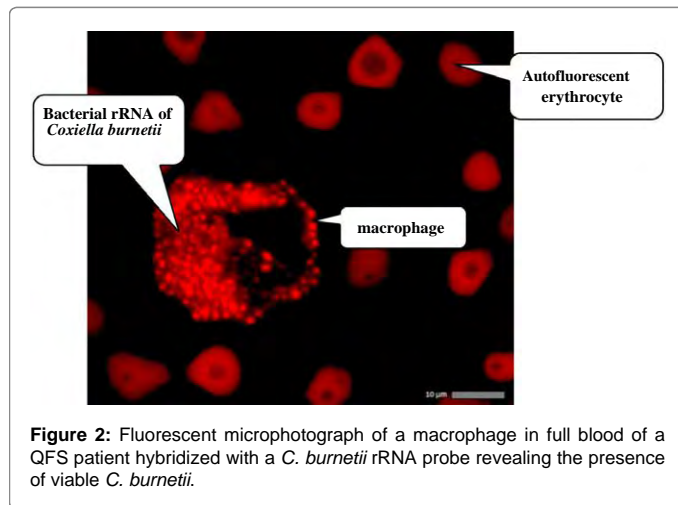
The existence of Cell Wall Deficient Bacterial forms has been demonstrated convincingly by Domingue et al., who, in the course of decades of research in the last century, have determined that various bacteria are found to possess an intracellular development cycle – similar to that of *C. burnetii* – particularly in cases involving chronic inflammatory disease, which indicated that they likely played a causal role in that condition [13]. See Figure 3 for an electron micrograph of one part of that developmental cycle showing the vesiculated mother L-form.

A recent study published in Nature Communications, reiterates that these cell wall deficient L-forms present an adaptive morphology allowing them to overcome the specific environmental challenges posed by antibiotic treatments, hyperosmotic stress conditions, or stress caused by the immune system. L-forms of many different bacteria have been successfully propagated for many years which demonstrates that they are indeed viable microorganisms and not just isolated genetic remnants [14,15]. This knowledge has not yet made its transition into medical practice, largely because the clinical significance of these findings has until now been frustrated by a lack of molecular evidence for the role CWDB might play in pathology.

During the recent Q-fever epidemic in the Netherlands, many infected individuals underwent an acute phase of the illness in the form of a serious infection of the airways which in the majority of cases healed completely. However, 20% of patients developed chronic fatigue in combination with complaints relating to a variety of organs which manifested after the acute phase had passed. This Q-fever

#Q-fever patient code	Number of positive WBCs	#Healthy control subject code	Number of positive WBCs
1	3	1	0
2	5	2	1
3	8	3	1
4	6	4	0
5	7	5	0
6	7	6	1
7	4	7	0
8	9	8	0
9	3	9	1
10	4	10	0
Total	56	Total	4
Mean Total	5.6	Mean Total	0.4

Table 1: WBC scores (i.e., the number of *C. burnetii* CWDB positive tests in 50 observed white blood cells (WBCs) in the cohort of 10 Q-fever patients) and 10 healthy control subjects.



fatigue syndrome (QFS) often led to permanent invalidity. The lack of microbiological evidence for a pathogenic microorganism as causal factor has led to the conclusion that, 'no viable *C. burnetii* are present in patients suffering from the Q-fever fatigue syndrome'. A conclusion based on negative results of PCR testing in the patients' serum [16].

The search for that causal factor led the Q-fever Research Group from Australia to conclude that QFS was a manifestation of an immunomodulatory complex (IMC) of antigenic residue derived from *Coxiella* cells that can persist in the mononuclear phagocytes and in dendritic cells. As a logical consequence, it was said that QFS reflects a mechanism for the maintenance of antibody and cell-mediated immunity issues long after an acute attack and in the absence of demonstrably viable organisms. They had described the detection of *C. burnetii* cells in bone marrow aspirates, buffy coats or in peripheral blood mononuclear cells (PMBC) long after initial infection of Q-fever in patients [17].

Merely detecting the presence of bacterial DNA in the blood of subjects does not necessarily have a clinical significance. In the past, hundreds of new viral and bacterial RNA or DNA have been identified using advanced testing methods for the presence of DNA, representing

previously unknown components of the human microbiome. It has been demonstrated that all the various types of microbiological life forms have deposited their genetic footprint in the blood at some point in the past and this DNA has become an integral part of the human microbiome [18].

The test method applied in this study has been used to find *specific* pathogenic bacteriological DNA in both intracellular and extracellular space in the blood of patients demonstrating clinical symptoms, whereby the causal bacterial cell forms cannot be detected using standard methods. The focus for the search for the presence of any viable BDNA in their L-form (CWDB) is achieved by basing it on a thorough anamnesis and specific serological tests of the patient's blood. As it was done for this study.

Conclusion

The results of this small-scale study indicate that using Fluorescence in situ Hybridization with *C. burnetii*-specific DNA probes on 16S-ribosomal RNA combined with morphometrics gives a reliable specific diagnostic test result for the presence of L-forms of *C. burnetii* during and after an epidemic phase of an illness such as Q-fever. It is essential to realize that because it is rRNA that is revealed by the FISH test, this is irrefutable evidence that the bacterial forms found are viable micro-organisms.

Finding rRNA of *C. burnetii* in a patient suffering from QFS provides molecular evidence for an exact diagnosis of the pathogen involved, however, the possibility that this pathogen may also be infectious has not been studied. It does raise the issue of whether the potential for cross-contamination from patients that are still infected with viable bacterial forms should lead to the implementation of preventative measures against the spread of these 'hidden' infections via blood transfusions, organ donations, and even pregnancy and birth. It would certainly be a relevant subject for further study.

Our recommendation is that FISH-testing should become a standard complement to the current instruments for the diagnosis of Q-fever by medical professionals. New perspectives for the development of therapeutic applications for QFS and other chronic conditions can be formed on the basis of these findings.

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