Journal of Microbiological Methods 151 (2018) 39–43

Contents lists available at ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

Note

Flow cytometry as a new complementary tool to study *Coxiella burnetii* in cell cultures

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ARTICLE INFO

Keywords:
Coxiella bunetii
Cell culture
Flow cytometry
qPCR

ABSTRACT

Flow cytometry enables the analysis of cells by labeling them with fluorescent probes. We describe a novel flow cytometric approach permitting reliable analysis of *Coxiella (C.) burnetii*-infected cells. The method quantifies infection-forming units (IFUs) in a dose-dependent manner and allows for the specific detection of infection/replication-competent coxiella in cell cultures.

Coxiella (*C.*) *burnetii* is a Gram-negative, obligate intracellular pathogen that causes Q fever (Maurin and Raoult, 1999), a zoonotic disease with worldwide prevalence (Eldin et al., 2017) posing serious and often underestimated health problems in both humans and livestock. Q fever is often a mild and self-limited flu-like illness, but can also cause interstitial pneumonia or hepatitis (Eldin et al., 2017). Even though most transmissions to humans cause asymptomatic infections, they can also result in severe sequelae, such as endocarditis (Hurley et al., 1970). The bacterial pathogen is highly infectious via inhalation of contaminated aerosols (Maurin and Raoult, 1999) and can survive for long periods in its natural environment. Goats, cattle, and sheep are the primary reservoirs for human infection (Guatteo et al., 2011; Maurin and Raoult, 1999). *C. burnetii* infects alveolar macrophages and replicate within a host cell-derived *C. burnetii*-containing vacuole (CCV) that retains several characteristic properties of phagolysosomes. *C. burnetii* seems to have an arsenal of virulence factors, including the dot/icm system and LPS (van Schaik et al., 2013). The latter is postulated to be a key factor for immune subversion of LPS-bearing phase I bacteria with complete O-Antigen (van Schaik et al., 2013).

Chronic Q fever can develop years after primary infection and results from the ability of *C. burnetii* to persist within the host (Eldin et al., 2017). In addition to macrophages, *C. burnetii* is able to infect many different cell types, including epithelial cells, monocytes and dendritic cells (Eldin et al., 2017). In the phagolysosome of eukaryotic cells, the organism undergoes a biphasic developmental program (Coleman et al., 2004) characterized by two morphological types: a large cell variant (LCV) and a small cell variant (SCV) (McCaul and Williams, 1981). *C. burnetii* is environmentally stable and highly resistant to physical as well as biochemical stresses (Eldin et al., 2017), which is attributed to the SCV. The infectious cycle of *C. burnetii* lasts about one week (Coleman et al., 2004) and is initiated by the invasion of the host cell via the environmentally stable and metabolic inactive SCVs. This leads to the formation of an acidified CCV, within which SCV differentiation into large metabolic active LCVs occurs. The developmental early mid phase (48–72 hpi) of the *C. burnetii* infection cycle is characterized by detectable CCV enlargement (Fig. 1A/C) as well as bacterial growth. Finally, the cycle terminates with asynchronous LCV to SCV differentiation and bacterial release from the host cell. *C. burnetii* grown in eggs for serial passages (Fiset, 1957) is characterized by defective lipoglycan synthesis and incompletely synthesized lipopolysaccharides (LPS) (Schramek and Brezina, 1979). These so called attenuated phase II bacteria (Brezina, 1958) display only low virulence (Kazar et al., 1974) in immunocompetent animal models and/or humans and are frequently used for cell biological studies (Amano and Williams, 1984).

Due to the obligate intracellular lifestyle of *C. burnetii*, the quantitation of infectious units (IFUs) or of genome equivalents (GEs) is routinely used for bacterial titer determination (Moos and Hackstadt, 1987; Schulze-Luehrmann et al., 2016). A standard method for measuring GEs is quantitative real-time PCR (qPCR), while detection of IFUs can be performed by immunofluorescence microscopy using *C. burnetii* specific antibodies (Hahon and Cooke, 1966; Raoult et al., 1990). Although both methods are widely used (Fournier et al., 1998), they have critical limitations. For instance, the qPCR method does not differentiate between infection/replication-incompetent and -competent *C. burnetii*. In contrast, immunofluorescence enables single-cell analysis as well as to some extent also the quantitation of infection/replication-

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https://doi.org/10.1016/j.mimet.2018.05.022
Received 2 April 2018; Received in revised form 27 May 2018; Accepted 28 May 2018
Available online 29 May 2018

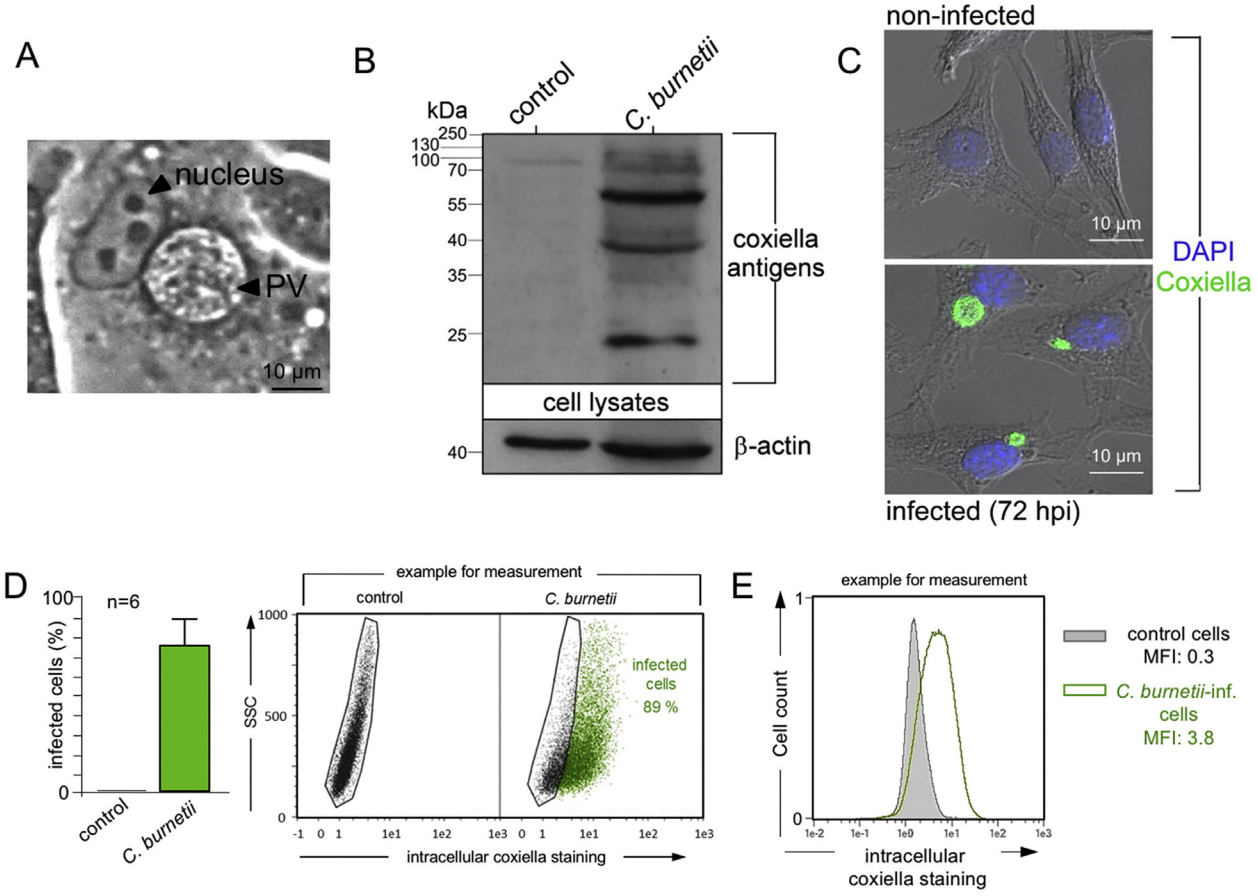

Fig. 1. Distinct applications for the used anti-cox II antiserum. (A) Light microscopic view of a *C. burnetii*-infected L-929 cell (72 hpi) containing a parasitophorous vacuole (PV), also termed CCV. The rabbit anti-cox II antiserum was tested for its ability to recognize *C. burnetii* antigens in immunoblots (B), immunofluorescence (C) and flow cytometry (side-scatter (SSC) vs. fluorescence dot plots and fluorescence histograms) (D/E). Six independent flow cytometry experiments were performed on different days with distinct coxiella stocks (D, left panel). To detect and quantify *C. burnetii*-positive L-929 cells, negative cells were identified/gated via corresponding non-infected controls and then subtracted from the total cell population (D, representative example in right panel). In the case of the depicted immunoblot (B), lysates corresponding to 1.5×10^5 L-929 cells (non-infected and infected (72 hpi)) were stained with a 1:10000 dilution of anti-cox II antiserum. Anti-β-actin staining served as a loading control. A dilution of 1:5000 of the antiserum was used for immunofluorescence studies (C) (comparison of non-infected and infected L-929 cells (72 hpi)).

competent *C. burnetii* (Hahon and Cooke, 1966; Moos and Hackstadt, 1987). However, immunofluorescence is a rather subjective visual method requiring empirical experience and typically only allows the analysis of a small number of cells. Moreover, it is based on time-consuming microscopic inspection, which is rather unsuitable for screening large numbers of samples (Fournier et al., 1998).

> 30 years ago, Waldmann and colleagues could demonstrate that flow cytometry is a useful method to analyze chlamydial infections in cultured cells (Waldman et al., 1987). In our present study, we established a flow cytometric approach that permits fast, efficient and straightforward analysis of *C. burnetii* infections in cell cultures.

For preparation of bacterial stocks, a plaque-purified isolate of the avirulent *C. burnetii* (Nine Mile phase II RSA 439 strain) (Hackstadt et al., 1985) was propagated in murine fibroblastic L-929 cells (Sanford et al., 1948) (obtained from ATCC, #CCL-1), a well-known infection model system (Baca et al., 1985), to validate the detection of infected cells. To this end, 1×10^5 cells were infected with a very high dose of *C. burnetii* (500 GEs/cell) at 37 °C/7.5% CO_2 in IMDM medium containing 10% fetal bovine serum (Bio & Sell) and cultured for two weeks. During this cultivation phase no detectable cytotoxicity or effects on cell viability/growth were observed in these highly infected L-929 cells. After the first passage, a large number of cells contained vacuoles with replicating *C. burnetii*, as determined by light microscopy (Fig. 1A). This level of infection with large singular CCVs was maintained upon further passaging. After 14 days of culture, infectious *C. burnetii* were harvested by hypoosmotic cell lysis (45 min, 4 °C), purified, and resuspended in PBS before immediate storage at −80 °C.

For analysis, we used a rabbit polyclonal anti-*C. burnetii* antiserum (anti-cox II antiserum) (Schulze-Luehrmann et al., 2016), which is able to detect cell infection via immunoblotting (Fig. 1B) and immunofluorescence (Fig. 1C). In particular, the anti-cox II antiserum (1:10000 diluted) specifically recognizes three distinct *C. burnetii*-associated protein bands (24, 39 and 60 KDa) in Western blot (Fig. 1B) and labels CCVs 72 hpi with no detectable background in immunofluorescence (Fig. 1C). Our microscopic observations (Figs. 1A/C) showed that the majority of *C. burnetii*-infected cells display a large single perinuclear CCV structure at 72 hpi, which is most likely generated by homotypic fusion of distinct *C. burnetii*-containing vacuoles (Martinez et al., 2016). Based on this, the anti-cox II antiserum was further tested for flow cytometric detection of *C. burnetii*-infected cells. Hence, L-929 cells were infected with freshly prepared *C. burnetii* stocks (100 GEs/cell) and cultured for 72 h. Before antibody staining and flow cytometry, the cell culture medium of infected L-929 cells was aspirated and the adherent cells were washed several times with PBS to remove cell debris and remaining extracellular bacterial particles. Subsequently, the cell monolayer was trypsinized and L-929 cells were mildly fixed with 2% formaldehyde (Sigma Aldrich) for 20 min at room temperature (RT). Formaldehyde was removed by washing with PBS and cells were