RESEARCH LETTER

Comparative sensitivity of four different cell lines for the isolation of *Coxiella burnetii*

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Abstract

Coxiella burnetii is an obligate intracellular bacterium that causes the disease Q-fever. This is usually diagnosed by serology (immunofluorescence assay) and/or PCR detection of *C. burnetii* DNA. However, neither of these methods can determine the viability of the bacterium. Four different cell lines were compared for their ability to amplify very low numbers of viable *C. burnetii*. Two different isolates of *C. burnetii* were used. For the Henzerling isolate, DH82 (dog macrophage) cells were the most sensitive with an ID_{50} (dose required to infect 50% of cell cultures) of 14.6 bacterial copies. For the Arandale isolate, Vero (monkey epithelial) cells were the most sensitive with an ID_{50} of less than one bacterium in a 100-μL inoculum. The Vero cell line appeared highly useful as vacuoles could be seen microscopically in unstained infected cells. The findings of this study favour the use of Vero and DH82 tissue culture cell lines for isolation and growth of *C. burnetii in vitro*. The other cell lines, XTC-2 and L929, were less suitable.

Introduction

Q-fever is a worldwide zoonosis caused by the intracellular bacterium *Coxiella burnetii*. Diagnosis of Q-fever is generally made by serological testing by immunofluorescence assay (IFA). It has been shown that polymerase chain reaction (PCR) detection of bacterial DNA may be more sensitive and can be used earlier in the disease before an antibody response can be detected (Fournier & Raoult, 2003). As PCR cannot differentiate between viable and non-viable bacteria, isolation of the infective agent enables further studies to be undertaken and allows viable *C. burnetii* to be detected. Hence, there is value in obtaining viable strains of *C. burnetii* by inoculation of patient samples into cell cultures.

Traditionally embryonated chicken eggs have been used for the isolation and growth of large numbers of *C. burnetii* and other rickettsiae. Advances in cell culture have allowed the growth of intracellular bacteria in flasks or multi-welled trays containing a monolayer of eukaryotic host cells. Cell culture may be more cost-effective and time-efficient than the use of embryonated eggs or animal inoculation. Continuous cell lines such as Vero and L929 cells are useful for growing *C. burnetii* (Burton *et al.*, 1978). Infection does not generally destroy the host cell line, and infected cells have the same cell cycle progression as uninfected cells. This is a result of asymmetric division of infected cells producing one infected and one uninfected daughter cell. This ability of *C. burnetii* has allowed it to persistently infect cell cultures for over 2 years without the addition of uninfected cells (Roman *et al.*, 1986). Amoeba (*Acanthamoeba castellanii*) have also been shown to maintain *C. burnetii* infection (La Scola & Raoult, 2001).

Four cell lines (Vero, L929, DH82, and XTC-2) were used in this study and compared for their ability to amplify very low numbers of *C. burnetii*. Previous studies have shown that different cell lines have different levels of sensitivity to *C. burnetii* infection (Rumin *et al.*, 1990).

Two different isolates of *C. burnetii* were used in this comparative study of four different cell lines as it

has been shown that different strains have different pathogenicities (Stoenner & Lackman, 1960). These were the Henzerling strain (as used in the Australian vaccine Qvax, originally isolated in Italy) and the recent Australian isolate 'Arandale' (isolated from a human case of acute Q-fever). It has been shown that both phase I and phase II cells can persistently infect cell cultures (Baca *et al.*, 1985), but phase I cells revert to phase II during cell passages. It may be possible that cell lines have different sensitivities to *C. burnetii* isolates from different genomic groups. It has been found that 'acute' isolates (with plasmid QpH1) and 'chronic' isolates (with no plasmid) infected cells more readily and caused an increased amount of *C. burnetii* antigen to be displayed on the host cell membrane compared to other isolates also implicated in chronic Q-fever (such as Priscilla Q177 and F Q228, both with the plasmid QpRS) (Roman *et al.*, 1991).

Materials and methods

Dilutions of *C. burnetii* for inoculation

Tenfold dilutions were made from a suspension of both *C. burnetii* isolates. The starting material for the Henzerling isolate was a homogenate of infected egg yolk sack (courtesy of Commonwealth Serum Laboratories, Australia). The starting material for the 'Arandale' isolate was a homogenate of spleen from infected severe combined immunodeficient (SCID) mice. Tenfold dilutions of each starting material were made in Hanks' balanced salt solution (HBSS; Gibco, Australia). The actual dilutions of the *C. burnetii* suspensions selected to inoculate into cell culture were based on preliminary testing (data not shown). All experiments with *C. burnetii* were carried out in a biocontainment level 3 laboratory at the Department of Microbiology, John Hunter Hospital, Newcastle.

Cell lines

African green monkey epithelial cells (Vero), mouse fibroblast cells (L929) and canine macrophage cells (DH82) were grown in RPMI-1640 medium (Gibco) supplemented with 10% new born calf serum (NBC; Gibco) and 1% L-glutamine (Gibco) and incubated at 35 °C in 5% CO_2. The South African clawed frog epithelial cells (XTC-2) were grown in Leibovitz L-15 (Gibco) medium supplemented with 10% NBC (Gibco), 0.4% tryptose phosphate broth (Oxoid, UK) and 1% L-glutamine (Gibco) and incubated at 28 °C in 5% CO_2.

Six 24-well trays (IWAKI, Japan) each containing the four cell culture types were grown to confluency. Each well contained 2 mL of medium. Dilutions 10^{-6}–10^{-11} (Arandale isolate) or 10^{-5}–10^{-10} (Henzerling strain) were used to infect the cell cultures. Six wells of each cell culture type were inoculated with 100 μL of each dilution of *C. burnetii*. Cultures were incubated for 6 weeks before the monolayer from each well was harvested by scraping. Cells were pelleted by centrifugation for 5 min at 4500 ***g*** and resuspended in 300 μL of phosphate-buffered saline (PBS; Oxoid) and analysed by DNA extraction and Com1 PCR.

Analysis of *C. burnetii* growth

The DNA was extracted from 200 μL by Qiagen Extraction Kit (Qiagen, Germany), following the manufacturers' instructions, eluted into 50 μL and analysed by specific PCR targeting a 76-bp sequence of the *com1* gene (Lockhart *et al.*, 2011). Extracted DNA (5 μL) was analysed for each reaction. The cycling threshold resulting form the PCR was used to calculate the approximate *C. burnetii* DNA concentration (μg μL^{-1}) in each reaction. The *C. burnetii* dose that would infect 50% of cultures (ID_{50}) was calculated using the Spearman–Kärber method (Anellis & Werkowski, 1968). The dilutions of the inoculum were analysed by PCR, and a standard curve was made (data not shown) and used to convert the ID_{50} calculation from a dilution into a number of bacterial copies required for 50% infection.

Results

By determining which wells contained *C. burnetii* DNA in amounts to suggest growth of the bacteria, the ID_{50} could be determined for each cell line and *C. burnetii* isolate. The cell line most susceptible (sensitive) to infection was different for the two *C. burnetii* isolates (Table 1). For the Arandale isolate the Vero cell line was the most sensitive with an ID_{50} of 0.1 copies of *C. burnetii*, followed by the L929 cell line with an ID_{50} of 3.2 copies. For the Henzerling strain, the DH82 cell line was the most sensitive with an ID_{50} of 14.6 copies of *C. burnetii* followed by the L929 cell line with an ID_{50} of 22.0 copies.

During the growth of *C. burnetii* the monolayers were routinely observed under light microscopy. Only in Veros could infection with *C. burnetii* be seen as large vacuoles in the cell cytoplasm. Although they were subsequently shown by PCR to be infected, the other cell lines showed little or no morphological indication of infection.

Discussion

The gold standard and most widely used technique for the diagnosis of Q-fever is serology by IFA. Diagnosis by PCR is useful in the first 2 weeks of infection (Fournier & Raoult, 2003). While PCR is most useful in establishing

Table 1. Infective dose$_{50}$ (ID_{50}) of two isolates of *Coxiella burnetii* in four different cell lines

	Cell line			
C. burnetii isolate and dilution	DH82	L929	Vero	XTC-2
Henzerling isolate				
10^{-5}	3/3	6/6	3/3	6/6
10^{-6}	2/3	2/6	0/3	2/6
10^{-7}	1/3	2/6	0/3	1/6
10^{-8}	0/3	1/6	0/3	0/6
10^{-9}	0/3	0/6	0/3	0/6
Dilution for 50% infection	**3.2×10^6**	2.2×10^6	3.2×10^5	1.0×10^6
Number of *C. burnetii* (copy numbers per 100 μL) required for 50% infection of cell line	**14.6**	22.0	170.0	49.8
Arandale isolate				
10^{-7}	3/3	6/6	6/6	4/6
10^{-8}	2/3	6/6	6/6	0/6
10^{-9}	0/3	1/6	6/6	0/6
10^{-10}	0/3	0/6	4/6	0/6
10^{-11}	0/3	0/6	0/6	0/6
Dilution for 50% infection	1.5×10^8	4.6×10^8	**1.5×10^{10}**	1.5×10^7
Number of *C. burnetii* (copy numbers per 100 μL) required for 50% infection of cell line	11.7	3.2	**0.1**	157.7

The ID_{50}s were calculated using the Spearman–Kärber method described (Anellis & Werkowski, 1968). These numbers are also converted to copy numbers of *C. burnetii* in 100 μL. The most sensitive cell line is indicated in bold.

a microbial diagnosis for samples that may include other bacteria, PCR cannot distinguish between living and dead bacteria. The isolation of *C. burnetii* definitively demonstrates a current infection with viable bacteria. In this study the use of cell culture for the isolation of *C. burnetii* was investigated. Four different cell lines were compared for their sensitivity for the detection of very low numbers of *C. burnetii*, as might occur in a genuine clinical sample.

Six 10-fold serial dilutions of both *C. burnetii* suspensions were used to infect confluent monolayers of four different cell lines. Two *C. burnetii* isolates were used as it has been shown that different strains have different pathogenicity (Stoenner & Lackman, 1960) that may affect their interactions with the cell lines. The results of this study demonstrate that the Vero cell line was the most sensitive for detection and growth of the Arandale isolate, while the DH82 cell line was the most sensitive for detection and growth of the Henzerling strain.

Continuous cell lines including Vero and L929 cells are very useful in the growth of *C. burnetii* as they are capable of persistent infection (Burton *et al.*, 1978). The difference demonstrated between the two isolates used agreed with previous studies showing a difference in pathogenicity amongst isolates of *C. burnetii* (Stoenner & Lackman, 1960). The Henzerling isolate had been shown to have a higher infectivity for Vero cells compared to the Zamosc isolate (Rumin *et al.*, 1990). Vero cells are widely used, are easy to grow, and when infected with *C. burnetii* vacuole inclusions could be seen in unstained cells under 100 × magnification with a light microscope. Such vacuoles were not visible in the DH82, L929 or XTC-2 cells.

Concluding remarks

Although not commonly used for diagnosis, obtaining *C. burnetii* isolates is crucial for studies on the viable whole bacterium. The results of this study show the advantage of using Vero and DH82 cell lines for the isolation of *C. burnetii* strains from clinical samples. Recently *C. burnetii* has been grown without the use of host cells (Omsland *et al.*, 2009) but not yet from clinical samples (G. Vincent, pers. commun.). The results of the current study could be used in comparison with cell-free media to determine which is more sensitive for the detection of low numbers of viable *C. burnetii* in clinical samples from infected patients.

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