Growth of *Coxiella burnetii* in the *Ixodes scapularis*–Derived IDE8 Tick Cell Line

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Abstract

Q fever, a zoonotic disease, is caused by a gram-negative intracellular bacterium, *Coxiella burnetii*. Although normally transmitted during exposure to infectious aerosols, *C. burnetii* is also found in arthropod vectors. In the environment, ticks are thought to play a crucial role in bacterial maintenance and transmission by infecting various mammalian species. However, the nature of the pathogen–tick relationship is not well defined. To determine *C. burnetii*'s interactions with a cultured tick cell line, we introduced purified *C. burnetii* NMII into *Ixodes scapularis*–derived IDE8 cells and assayed for bacterial presence, replication, gene expression, and subsequent infectivity for mammalian cells. Tick cells were harvested at 24 h, 72 h, 7 days, and 11 days postinfection (PI). *C. burnetii* uptake and subsequent replication was demonstrated by indirect immunofluorescence assay, electron microscopy, and real-time polymerase chain reaction (PCR). When a genome equivalent multiplicity of infection of 30 was used, 30%–40% of exposed cells were seen to have small, rounded, vacuoles at 72 h PI, whereas at 7 and 11 days PI, 60%–70% of cells contained enlarged vacuoles harboring large numbers of bacteria. Quantitative PCR analysis of total genomic DNA confirmed that *C. burnetii* genome numbers increased significantly from 24 h to 11 days PI. Expression of *C. burnetii* type four secretion system homologs at 7 days PI was demonstrated by reverse transcriptase PCR. Finally, indirect immunofluorescence assay demonstrated that *C. burnetii* propagated within IDE8 cells were infectious for mammalian cells. These studies demonstrate the utility of cultured tick cell lines as a model to investigate *C. burnetii*'s molecular interactions with its arthropod vectors.

Key Words: *Coxiella*—IDE8 cells—Intracellular replication.

Introduction

Q FEVER IS A zoonotic disease found throughout the world, with the exception of New Zealand (Kaplan and Bertagna 1955, Hilbink et al. 1993). The disease is caused by the Gram-negative intracellular bacterial pathogen *Coxiella burnetii*. Human infection occurs mainly through inhalation of contaminated particulates shed from infected goats, sheep, and cattle (Babudieri 1959, Woldehiwet 2004). Transmission to animals and humans is facilitated by the ability of *C. burnetii* to survive for extended periods in a spore-like state on objects contaminated with infected tick feces, in water, and in soil (McCaul and Williams 1981). Additionally, wild and domestic mammals, birds, and ticks act as reservoirs for the bacterium (Babudieri 1959, Maurin and Raoult 1999). *C. burnetii* infections are usually not clinically apparent in animals; however, acute and chronic infection can lead to abortion in sheep and goats, and low birth weights and infertility in cattle (Aitken 1989). Since ticks are a reservoir, it is thought that they

act as vectors in the transmission of *C. burnetii* among animals (Eklund et al. 1947, Babudieri 1959, Beaman and Hung 1989) as well as maintaining the pathogen in the environment. Early investigations indicate that *C. burnetii* may replicate in the middle gut or stomach of ticks and subsequently be excreted in the feces (Kordova and Rehacek 1959). Moreover, studies indicate that transovarial and transstadial transmission of *C. burnetii* may occur in *Hyalomma asiaticum*, *Hyalomma lusitanicum*, and *Dermacentor marginatus* (Daiter 1977, Walker and Fishbein 1991, Toledo et al. 2009). Although there is evidence that *C. burnetii* is able to replicate in crude primary tick cell cultures (Rehacek and Brezina 1964), recently established continuous tick cell lines have not been employed to study the host cell–pathogen interactions of *C. burnetii* and these vectors.

Blood feeding Ixodid ticks (subphylum Chelicerata; class Arachnida; subclass Acari; family Ixodidae) are known to transmit a variety of bacterial, rickettsial, viral, and protozoan diseases (Estrada-Pena and Jongejan 1999). Ixodid ticks have

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recently been shown to harbor *Coxiella* spp. and *Coxiella*-like pathogens in the wild (Vilcins et al. 2009). Due to the efficiency of *Ixodes* spp. ticks as vectors of pathogens and their worldwide distribution, we have chosen an *Ixodes scapularis*–derived cell line (IDE8) to investigate as an *in vitro* model for studying the tick-pathogen cellular interactions of *C. burnetii*. This cell line has been used to successfully propagate multiple tick-borne pathogens, including *Anaplasma phagocytophilum*, *A. marginale*, *Ehrlichia canis*, *E. ruminantium*, *Borrelia* spp., and *Rickettsia* spp. (Bell-Sakyi et al. 2007). In the current study, we sought to determine *C. burnetii*'s infectivity, growth rate, gene expression, as well as its ability to reinfect mammalian cells after growth in cultured tick cells. Using the indirect fluorescent antibody (IFA) microscopy assay, electron microscopy (EM), quantitative polymerase chain reaction (qPCR), and reverse transcriptase-PCR (RT-PCR), we determined the ability of *C. burnetii* to invade and replicate within the IDE8 tick cell line, expression levels of genes of the type four secretion system (T4SS) within tick cells, and the ability of tick cell-derived *C. burnetii* to invade mammalian cells.

Materials and Methods

Bacterial cultivation and purification

Coxiella burnetii Nine Mile Phase II Clone 4 (NMII) was propagated in African green monkey kidney (Vero) cells in RPMI 1640 medium and 5% fetal bovine serum (FBS) at 37°C in an atmosphere of 5% CO_2, and the small cell variant (SCV) form of the organism was isolated as previously described (Coleman et al. 2004). The SCVs were resuspended in SPG buffer (0.7 M sucrose, 3.7 mM KH_2PO_4, 6.0 mM K_2HPO_4, 0.15 M KCl, and 5.0 mM glutamic acid, pH 7.4) and stored at −80°C. *C. burnetii* genome equivalents were calculated using qPCR (Brennan and Samuel 2003).

Tissue culture cells

Uninfected Vero cells were propagated as described in the medium containing 20 μg/mL gentamicin. The medium was exchanged with fresh RPMI 1640 and 5% FBS without antibiotics 2 h before bacterial infection. The tick cell line IDE8 (ATCC CRL 11973), derived from embryos of *I. scapularis* and maintained in continuous passage for several years, was maintained in a modified Liebovitz's L15 medium at 34°C following the procedures of Munderloh et al. (1994). Cultures were washed with the antibiotic-free medium before *C. burnetii* infections.

Infection of IDE8 tick cells

The optimal *C. burnetii* multiplicity of infection (MOI, based on genome equivalents) for IDE8 cells was empirically determine (data not shown). Thereafter, 25 cm^2 flasks containing 1×10^7 IDE8 cells were infected with *C. burnetii* NMII at a genome equivalent MOI of 30 in 2 mL of L15 medium at 34°C for 4 h. The flask volume was then brought up to a total of 5 mL with L15 medium. Infected cells were incubated at 34°C with culture flask caps closed. The medium was replaced every 24–48 h as needed.

IDE8 cell sample harvest

The 25 cm^2 flasks containing 1×10^7 IDE8 cells were divided into five sections. One section of the flask was harvested by scraping just before infection (uninfected), and 24, 72, 168 (7 days), and 264 h (11 days) postinfection (PI). The medium was removed before each sampling and replaced immediately afterward, and flasks returned to incubation. Parallel aliquots of infected cells from each time point were (1) seeded in 24-well plastic tissue culture plates for 3–4 h at 34°C to allow for re-attachment, and then fixed with a 4% paraformaldehyde, Tween 20 (0.05%), and phosphate-buffered saline; (2) fixed to glass slides using a cytospin centrifuge followed by fixation for 10 min using ice-cold methanol; and (3) centrifuged and the total genomic DNA isolated using the Genomic Isolation Kit (Promega). A minimum of three biological samples were isolated for each condition and time point.

Indirect immunofluorescence antibody assay

The 24-well plate was seeded and cytospin samples were analyzed by IFA microscopy using rabbit polyclonal antibody against whole-killed *C. burnetii* NMII followed by an Alexa-fluor 488–tagged goat anti-rabbit IgG (Molecular Probes). Fluorescent images were captured at 400× magnification using a Nikon eclipse TE-2000 S inverted microscope equipped with a Nikon DS-Fi1 digital camera.

Vero cells seeded on 24-well tissue culture plates were inoculated with *C. burnetii* isolated from 7-day PI IDE8 cell lysates. Lysates were created by scraping *C. burnetii*–infected IDE8 cells into phosphate-buffered saline, and freeze–thawing the cells twice at −80°C followed by repeatedly passing the thawed cells through a 26.5-gauge needle. *C. burnetii* were separated from cell debris by differential centrifugation, and resuspended in RPMI 1640 medium and 5% FBS, which was used to inoculate the Vero cells within 24-well culture plates. Cultures were grown at 37°C in an atmosphere of 5% CO_2, for 72 h, and then fixed to the culture plates using methanol. IFA microscopy analysis was performed directly in the culture plates as described above.

qPCR analysis

Ten-fold serial dilutions of purified *C. burnetii* genomic DNA (10^6, 10^5, 10^4, and 10^3 genomes/sample well) were used to generate a standard quantitative curve in each experiment. *C. burnetii* genome equivalents in infected IDE8 cell samples were estimated using qPCR and the SYBR Green Master Mix kit (Applied Biosystems) in an Applied Biosystems 7500 real-time cycler, with forward [f] and reverse [r] primers CB594—5′-CGCTTCATGAATTAGCAGCA-3′[f] and CB595—5′-TGCAG TCAAACGGTTCTTCA-3′[r]. These primers target the *C. burnetii icm*W gene (GenBank accession no. AF318146). Briefly, the reaction mixture contained 0.3 μM of each primer, and 10 ng of sample template DNA in a total volume of 15 μL. The resulting fluorescent plots were analyzed, and estimated numbers of *C. burnetii* genomes in the experimental samples were determined based on the standard curve. An increase in genome equivalents was observed relative to infected IDE8 cells collected 24 h PI. A minimum of three biological and three technical samples were used in the analysis of each time point.

RNA isolation and quality control

One half of a 25 cm^2 flask containing infected IDE8 cells was scraped at 7 days PI and cells were pelleted by centrifugation. Total RNA was then harvested using Tri Reagent (Ambion)

following the manufacturer's recommendations. All RNA samples were DNase treated to remove contaminating DNA with RQ1 DNase (Promega) and confirmed DNA-free by PCR before RNA analysis assays.

RT-PCR analysis

RT-PCR analysis was carried out using the Access Quick RT-PCR Kit (Promega) and total RNA isolated from *C. burnetii*–infected IDE8 cells following the manufacturer's directions. Primers CB40, 5′-ATGCCAGATCTGTCGC-3′[f] and CB41, 5′-TAAACCACCTTCCTCAAGAG-3′[r] (*icmW*), CB70, 5′-ATGATTCTTTTGGAGTCTTCC-3′[f] and CB71, 5′-TTG

TTTGGACCCCTTAAAGGTG-3′[r] (*icmV*), and CB703, 5′-A TTGGGGCCAGTATCATTCC-3′[f] and CB696, 5′-ATGGA GTGTGCGGATTTGAT-3′[r] (*dotH*) were used in RT-PCR analysis.

Electron microscope analysis

One half of a 25 cm² flask containing infected IDE8 cells was scraped at 7 days PI and cells were pelleted by centrifugation. The *C. burnetii*–infected IDE8 cells were fixed with 2.5% paraformaldehyde (v/v)/2.5% glutaraldehyde (v/v) for transmission EM analysis as previously described (Morgan et al. 2010). The Imaging Facility in the Department of
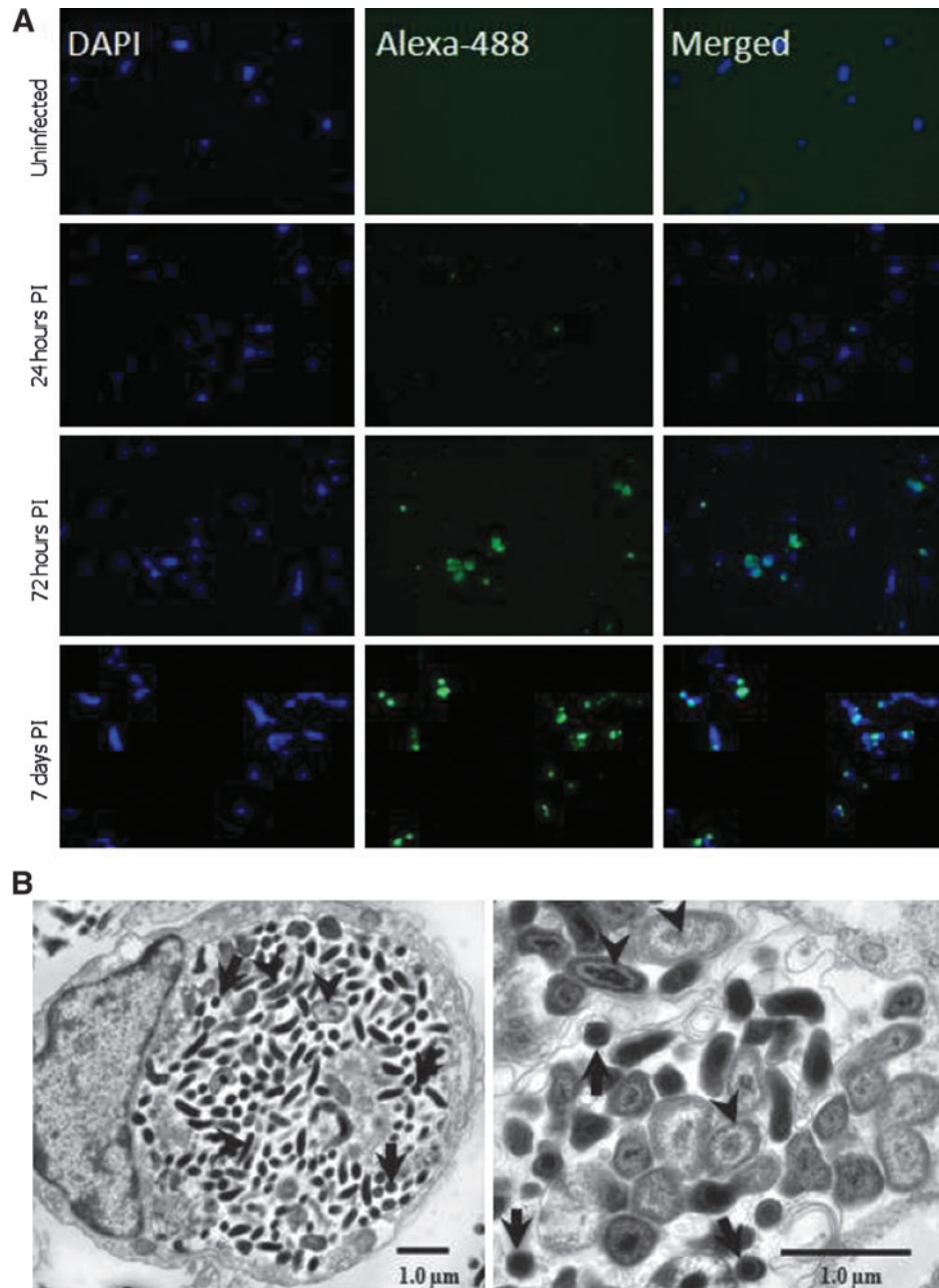
FIG. 1. IFA of *Coxiella burnetii* NMII infection of IDE8 cells. **(A)** Left panel, DAPI-stained cells. Middle panel, Alexa-488 labeling of *C. burnetii*. Right panel, merge of left and middle panels. Time of fixation PI is indicated at the left of each corresponding row. **(B)** Electron micrograph of *C. burnetii*–infected IDE8 cells fixed at 7 days PI. Arrows indicate *C. burnetii* large cell variants. Arrowheads indicate *C. burnetii* small cell variants. Scale bar is 1.0 μm. PI, postinfection.

Molecular Microbiology Center for Infectious Disease Research, Washington University, St. Louis, MO, performed the subsequent sample processing and transmission EM analyses following published techniques (Belland et al. 2003).

Results

C. burnetii *infection of IDE8 tick cells*

To determine whether *C. burnetii* infects IDE8 cells, we used an approximate genome equivalent MOI of 30. The infected cells were cytospun to a microscope slide followed by methanol fixation and IFA. These analyses indicate that *C. burnetii* containing vacuoles are present by 72 h PI and large, spacious, immunostained *C. burnetii* vacuoles were prominent by 7 days PI (data not shown). Although this indicated that *C. burnetii* were infecting IDE8 cells and replicating within them, the cytospin centrifugation method causes distortion and/or disruption of infected cells, resulting in dispersion of many of the bacteria.

To observe infected IDE8 cells that are physiologically intact, an alternative method was employed where the tick cells were re-seeded to 24-well tissue culture plates and allowed to adhere before fixation and IFA analysis. Figure 1A shows that after 72 h PI ~30%–40% of infected cells had small, rounded vacuoles, and at 168 h (7 days) PI, swollen, enlarged vacuoles containing large numbers of bacteria were present. By 264 h (11 days) PI, the infected IDE8 cells had large, fragile vacuoles such that intact infected cells could not be transferred from larger flasks to 24-well culture plates for microscopy analysis without rupturing the cells.

To determine whether *C. burnetii* were growing within membrane-bound parasitophorous vacuoles, EM was performed on infected IDE8 cells fixed at 7 days PI (Fig. 1B). EM micrographs indicate that *C. burnetii* is replicating in a membrane-bound compartment (Fig. 1B, left panel) and that both replicative large cell variants and environmentally stable SCV forms of the bacteria appear to be present within the vacuole (Fig. 1B arrows and arrowheads, respectively) at 7 days PI. Combined, these experiments demonstrate that
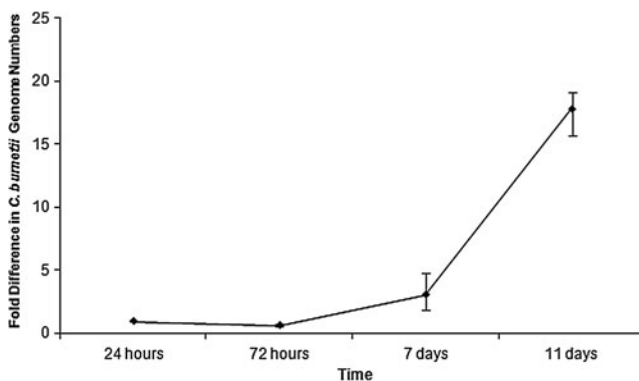
FIG. 2. *C. burnetii* genome levels during infection of IDE8 cells. Fold changes in genome numbers relative to 24 h PI. An equal amount of total genomic DNA from each sample was analyzed by quantitative qPCR. The time (in hours and days) PI when DNA was harvested is indicated below the X-axis. Results represent the mean of three biological samples with no fewer than three technical replicates of each sample. Standard error bars represent the combined standard error of the mean per time point. PCR, polymerase chain reaction.

C. burnetii can be internalized, survive, and grow within IDE8 tick cells *in vitro*. The appearance of spacious vacuoles at the beginning of what might be thought of as the exponential growth phase (72 h PI) is similar to that seen in *C. burnetii* infection of cultured mammalian cells (Coleman et al. 2004).

### C. burnetii *genome numbers increase after an extended lag phase*

In an effort to quantitate the growth characteristics of *C. burnetii* NMII in IDE8 tick cells, we estimated the number of *C. burnetii* genomes during the course of infection using qPCR. Using primers designed to the *C. burnetii* icmW homolog, and 24 h PI as a base line, *C. burnetii* genome equivalents were observed to decrease slightly between 24 and 72 h PI, although the decrease was not statistically significant ($p < 0.05$). This was followed by a 3.10 and 17.83-fold increase at 7 and 11 days PI (Fig. 2), respectively. After a lag, *C. burnetii* double every 10 h in mammalian cell models (Zamboni et al. 2001, Coleman et al. 2004). Using our data to calculate the replication rate of *C. burnetii* in the IDE8 cells, a doubling time of nearly 40 h can be derived over the entire time period. However, if the calculation is made following the ~72 h lag phase, *C. burnetii* genomes double every 10.87 h (Fig. 2) in the IDE8 cells. This rate is very similar to the 10.2 h (qPCR assay) rate found during the exponential phase of *C. burnetii* growth in mammalian cells following a 48 h lag phase (Coleman et al. 2004).

Expression of C. burnetii *T4SS during infection of IDE8 cells*

Secretion systems have been shown to be crucial for the delivery of effector proteins in a number of bacterial pathogens. In particular, the type-three secretion system is required for the virulence of bacteria, including *Escherichia coli*, *Shigella*, and *Salmonella* spp. (Hueck 1998). *C. burnetii* possesses T4SS homologs, a system that has been shown to be required for virulence in its closely related neighbor, *Legionella pneumophila* (Marra et al. 1992, Berger and Isberg 1993, Ensminger and Isberg 2009). *C. burnetii* T4SS homologs are expressed at the RNA and protein level during infection of mammalian cells in culture (Zamboni et al. 2003, Zusman et al. 2003, Coleman et al. 2004, Morgan et al. 2010). To determine whether this virulence determinant is expressed by *C. burnetii* during infection of IDE8 cells, RT-PCR was used to analyze total RNA isolated from infected cells 7 days PI (Fig. 3). Amplification products following RT-PCR clearly demonstrate that *icmW*, *icmV*, and *dotH* are expressed by the bacterium during infection of the IDE8 cell line.

IDE8-derived C. burnetii *infectivity for mammalian cells*

To determine whether the *C. burnetii* surviving within IDE8 cells were infectious for mammalian cells, IFA microscopy analysis was performed on Vero cells that were inoculated with lysates harvested from *C. burnetii*–infected IDE8 cells 7 days PI. Figure 4 shows an IFA of Vero cells infected with IDE8-derived *C. burnetii*. In this qualitative analysis, it is evident that the *C. burnetii* growing within IDE8 cells are infectious for mammalian cells. Large parasitophorous vacuoles containing multiple bacteria are indicated by the large, green fluorescing vacuoles present within the Vero cells
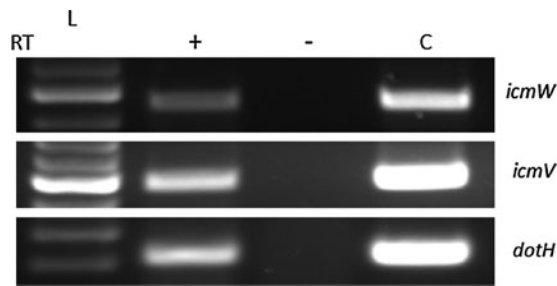
FIG. 3. RT-PCR detection of *C. burnetii* T4BSS transcripts *icmW*, *icmV*, and *dotH* during infection of IDE8 cells. Total RNA template was isolated at 7 days PI from *C. burnetii*–infected IDE8 cells. L, 100 bp DNA ladder. RT, reverse transcriptase; +RT, with reverse transcriptase; –RT, without reverse transcriptase; C, genomic DNA-positive PCR control.

(Fig. 4). This finding indicates that the *C. burnetii* growing within tick cells are readily infectious for mammalian cells, increasing the likelihood that tick-borne exposure to the pathogen could lead to disease.

Discussion

Since the earliest studies of *C. burnetii*, it has been known that this pathogen has an association with arthropod vectors (Davis and Cox 1938, Davis 1939). However, an understanding of whether *C. burnetii* is passively carried in ticks or it is amplified by replication within the tick is not clear. Additionally, it has been demonstrated that *C. burnetii* can grow in a myriad of mammalian cell lines (Brezina et al. 1969, Burton et al. 1978, Baca et al. 1981, Voth et al. 2007), yet its ability to invade, replicate, and produce infectious progeny in tick cell lines had not been reported.

Our findings indicate that *C. burnetii* readily infects cultured IDE8 cells. qPCR of the *icmW* gene indicates that *C. burnetii* grown in IDE8 cells undergoes a prolonged lag phase before replication begins relative to growth in mammalian cells (Coleman et al. 2004). When replication does begin, *C. burnetii*'s doubling time in IDE8 cells appears to approach the ~10 h observed in mammalian cells. These findings suggest that a period of adjustment may be required for successful growth. It may be that the organism has to adjust to the lower temperature (34°C) of IDE8 cell culture. It could also be hypothesized that the bacterium has to adjust to a substantially different host cell environment relative to *in vitro* growth in a mammalian cell line. Both factors may

influence *C. burnetii*'s replication during early infection of these tick cells. Interestingly, while *C. burnetii* genome numbers remained relatively constant between 24 and 72 h of infection (Fig. 2), vacuoles with appreciable numbers of *C. burnetii* are evident in 72 h PI IFA analyses (Fig. 1). Although difficult to appreciate in the low light fluorescent images (Fig. 1), a large number of free, or single, bacteria were observed 24 after *C. burnetii* infection of IDE8 cells, suggesting that many of the bacteria used to initiate an infection did not cause a productive infection within a given cell. We speculate that this is the result of overestimation of the MOI due to the inability of the qPCR assay to discriminate between DNA from viable and nonviable bacteria, or that the IDE8 cells were capable of ingesting and killing a portion of the bacteria. In either case, the bacteria that invaded and survived within the IDE8 cells had formed visible vacuoles by 72 h PI (Fig. 1).

The ability of facultative and obligate intracellular pathogens to subvert host cell processes is crucial to their survival. Secretion systems are one of the primary means by which pathogens interact with the host. Our evidence that *C. burnetii* is expressing RNA for the production of a T4SS during infection of IDE8 cells (Fig. 3) leads us to hypothesize that the pathogen is interacting with the tick cells and are likely manipulating the host cell response, as in mammalian cells (Voth and Heinzen 2009). In mammalian cells, expression of T4SS homologs has been shown at the RNA level throughout infection and that the T4SS machinery is localized to the poles of the bacterial cell (Zamboni et al. 2003, Zusman et al. 2003, Coleman et al. 2004, Morgan et al. 2010). Moreover, it is likely that the *C. burnetii* T4SS is crucial to the pathogens ability to subvert cellular pathways for its own benefit.

Ultimately, *C. burnetii*'s ability to infect a mammalian host after replication within a tick vector makes ticks a viable and crucial environmental reservoir of this pathogen. Our demonstration that *C. burnetii* isolated from IDE8 cells could readily infect cultured Vero cells (Fig. 4) would indicate that the *C. burnetii* propagated within a tick would be capable of causing disease in humans and other mammals. In addition, the production of environmentally stable SCV forms of the pathogen would enable a tick vector to shed infectious particles with the ability to cause disease long after they enter the environment.

The results of these experiments show that *C. burnetii* capable of infecting mammalian cells are produced IDE8 tick cells after an extended lag phase. Further, we demonstrate that *C. burnetii* expresses homologs of a suggested virulence
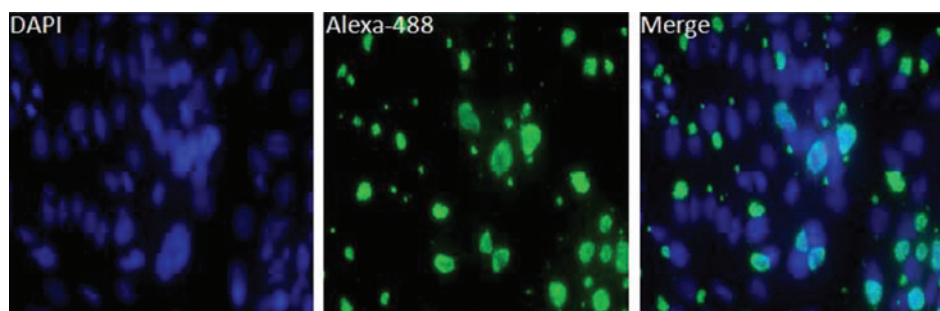
FIG. 4. IFA of IDE8-derived *C. burnetii* NMII infecting Vero cells. Left panel, DAPI (4′,6 diamidino-2-phenylindole)-stained cells. Middle panel, Alexa-488 labeling of *C. burnetii*. Right panel, merge of left and middle panels. Infected cells were fixed at 72 h PI.

determinant during infection of the IDE8 cultured cells. These findings demonstrate that cultured tick cells represent a viable *in vitro* model to study the pathogens cellular interactions with tick cells in comparison to those found in mammalian cells while expanding our understanding of *C. burnetii* growth within the tick vector.

Acknowledgments

Disclosure Statement

No competing financial interests exist.

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