Invasion of Phagocytic and Non-Phagocytic Cells by '*Candidatus* Berkiella cookevillensis'

By

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Abstract

Bacterium '*Candidatus* Berkiella cookevillensis' (strain CC99) was isolated from an amoeba in cooling tower biofilm. CC99 is an obligate intracellular pathogen growing within vacuoles closely associated with the host cell nucleus and typically lysing its amoeba host in 3-4 days. CC99 that lyse from amoebae can infect both THP-1 and U937 human macrophage-like cell lines, but whether bacteria from human cell lines are transmissible to other human cells had not been investigated. In this study, CC99 bacteria lysed from amoebae productively infected both THP-1 and HeLa human epithelial cells, as evidenced by nuclear-associated vacuoles filled with bacteria. At least 50% of the cell populations were positive for CC99 at 72 hours as determined by histochemical staining. Furthermore, CC99 DNA was detected in these treated cells using quantitative PCR (qPCR). However, when CC99 was grown in either THP-1 or HeLa cells, no bacteria were detectable in these cells either by histochemical staining or by qPCR at 72 hours. These results suggest that although bacterium CC99 may be transmissible to human cells when lysed from amoebae, CC99 propagated in human cells may not be directly transmissible to other human cells. Therefore, this study has important implications for the safety procedures surrounding amoeba-derived infections, such as *Legionella spp.*

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Introduction

Free-living amoebae (FLA) are single-celled, eukaryotic microorganisms that inhabit air, soil, and water environments without the need of a host for survival. Amoebae can also act as opportunistic pathogens that are the causative agents of granulomatous amebic encephalitis and amebic keratitis associated with cutaneous lesions and sinusitis (Marciano-Cabral and Cabral 2003). FLA ingest bacteria but some microorganisms have become resistant to amoeba digestion and use the amoeba host for intracellular replication, potentially becoming lytic for the host, while also acquiring a uniquely hidden environment for replication (Greub and Raoult 2004; Ogata et al. 2006; Schulz et al. 2014). These organisms are referred to as amoeba-resistant bacteria (ARB) and the FLA which enclose them protect the internalized bacteria from chlorine and other biocides in human-constructed environments (Greub and Raoult 2004; Schulz and Horn 2015). FLA may even play a role in the selection of virulence traits and in adaption to survival in eukaryotic macrophages (Greub and Raoult 2004). Some of these bacterial pathogens use similar mechanisms to invade both protozoan and mammalian cells (Molmeret et al. 2005). Two historically important bacterial intracellular pathogens are *Legionella pneumophila* and *Coxiella burnetti* as they both cause infection in various host cells.

Legionella pneumophila has been observed as an intracellular parasite of amoebae but also resides in specialized phagosomes when internalized by alveolar macrophages (Khweek and Amer 2010). It is the causative agent of a severe pneumonia in humans

called Legionnaires' Disease or a milder, flu-like illness known as Pontiac Fever (Bandyopadhyay et al. 2004; Chauhan and Shames 2021). Prior to mammalian infection, the amoeba host provides *L. pneumophila* protection from the environment thus enhancing bacterial growth through resistance to chemical disinfectants, biocides and antibiotics (Khweek and Amer 2010). Amoeba-grown *L. pneumophila* enter monocytes via an altered mechanism (coiling phagocytosis) and display enhanced growth in the lungs of mice which suggested that protozoan-grown bacteria are responsible for the production of Legionnaires' disease in humans (Cirillo et al. 1999). Human infection of *L. pneumophila* likely occurs through the aspiration of contaminated water but person-to-person transmission is rare (Newton et al. 2010).

Successful intracellular replication occurs by the generation of a replication-permissive compartment called the *Legionella*-containing vacuole (LCV) through the action of the Dot/Icm-translocated effector proteins (Chauhan and Shames 2021). The Dot/Icm system is a highly conserved type IV secretion system (T4SS) that is co-opted by the bacterium to effectively avoid lysosomal degradation and allow the bacteria to acquire nutrients from the host cell (Chauhan and Shames 2021; Swanson and Hammer 2000). *L. pneumophila* replicates in protozoan hosts as well in various human-derived cell lines such as macrophage-like HL-60 cells, monocytic U937 cells, macrophage-like THP-1 cells, and human epithelial HeLa cells (Khweek and Amer 2010; Swanson and Hammer 2000). Although *L. pneumophila* has unique interactions within amoebae and mammalian cell lines, infection and the formation of a Legionella-containing vacuole occurs across all hosts (Khweek and Amer 2010).

Another bacterium that can infect various host cell lines is *Coxiella burnetii*, the causative agent of the zoonotic disease Q (query) fever. Although *C. burnetii* can infect amoebae, this bacterium is primarily transmitted between mammals by tick vectors (Wang et al. 2023). Fleas also serve as a vector to transmit the bacterium, especially during human epidemics, but there are also cases of Q fever transmission by release of aerosolized bacteria from phagocytic amoebae, as is seen with *L. pneumophila* (Burette and Bonazzi 2020; Raoult 2010; Wang et al. 2023). Another similarity with *Legionella* is that *C. burnetii* also relies on a Dot/Icm T4SS to translocate effector proteins into host cells, but the actions of the effectors differ (Qiu and Luo 2017; Thomas et al. 2020). During *Legionella* infection, the bacterium uses its effectors to inhibit host immune recognition by disrupting autophagy to avoid lysosome fusion, alternatively producing an endoplasmic reticulum-derived vacuole. However, *C. burnetii* controls autophagy to have a continual supply of nutrients and membranes to support the *Coxiella*-containing vacuole (Segal et al. 2005; Thomas et al. 2020; Zusman et al. 2003). Although both *L. pneumophila* and *C. burnetii* utilize similar strategies for infection, one difference is the host range, with *Coxiella* infecting a greater range of hosts compared to *L. pneumophila* (Segal et al. 2005; Thomas et al. 2020; Zusman et al. 2003). Thus, even though these two bacteria are genetically similar and use comparable T4SS structures to cause infection, it is important to thoroughly investigate mechanisms of infection and intracellular survival to understand their importance in relation to human and zoonotic disease.

In comparison to these more historical amoeba-derived bacteria, a more recently identified bacterium is ‘*Candidatus* Berkella cookevillensis’ (type strain CC99), a novel gram-negative coccus to coccobacillus-shaped bacterium that was isolated from an

amoeba found in the biofilm of a cooling tower (Mehari et al. 2016). This bacterium is unusual in that it is intranuclear when maintained in the amoeba host *Acanthamoeba polyphaga,* but unculturable on conventional laboratory media. Whole genome sequencing was used to identify it as a member of the phylum Gamma-proteobacterium and order Legionellales (Mehari et al. 2016). Similar bacteria resembling *Legionella* spp. have been recovered from free-living amoeba, but unlike these *Legionella* spp., '*Ca.* B. cookevillensis' invades and replicates in a bacteria-containing vacuole that either associates with or enters the host nucleus, resulting in complete lysis of the amoeba host cells within 2-4 days (Mehari et al. 2016).

'*Ca.* B. cookevillensis' was further characterized by demonstrating association with nuclei of human phagocytic and non-phagocytic cell lines (Chamberlain et al. 2019). Evaluation of intracellular trafficking through these human cell lines showed that '*Ca.* B. cookevillensis' was first found within cytoplasmic vacuoles where numbers of bacteria increase. These bacteria-containing vacuoles (BCVs) then associate with the host cell nucleus and either form a deep nucleus-associated pocket or enter as an intact vacuole into the nucleoplasm, ultimately resulting in host cell lysis (Chamberlain et al. 2019). This study was also significant in that it was the first description of a bacterium infecting the nuclei of both protozoan and mammalian cells.

'*Ca* B. cookevillensis' infects the nuclei of amoebal, murine, and human cells with varying efficiencies (Mehari et al. 2016, Chamberlain et al. 2019). In amoebae, 97% of cells are generally infected by 72 hours with 95% of amoeba having infected nuclei. In contrast, U937 human macrophage-like cells generally have 55% infection of cells with 42% having infected nuclei, while the non-phagocytic HeLa epithelial cells have only

47% of cells infected with 38% having infected nuclei (Chamberlain et al. 2019). These data suggest the bacterium is less successful in establishing infections in mammalian cells compared to the amoeba host. Like '*Ca* B. cookevillensis,' *L. pneumophila* also infects both amoebae and mammalian cells (Cirillo et al. 1999), but while *L. pneumophila* is highly transmissible after lysing out of amoebae, bacteria lysing out of mammalian cells showed a lack of transmissibility to mammalian cells (Cirillo et al. 1999). It is currently unknown whether '*Ca.* B. cookevillensis' also shares this characteristic of losing its infectious capabilities when propagated in mammalian cells rather than amoebae.

The overarching goal of this proposal is to assess how bacterium CC99 may be transmitted between human phagocytic and non-phagocytic cell lines (Chanput et al. 2014; Fountoulakis et al. 2004). For this study, infection is defined as successful entry and evidence of replication of bacterium CC99 within host cells. The success with which the bacterium is transferred from amoebae to human cells and between human cell types can provide clues as to the pathogenesis of this bacterium, which is currently not associated with any known disease. To achieve this goal, the following aims were undertaken:

Aim 1: Determine the transmissibility of bacterium CC99 between human phagocytic cells using the macrophage-like cell line, THP-1.

Aim 2: Determine the transmissibility of bacterium CC99 between phagocytic and nonphagocytic human cells using THP-1 macrophage-like cells and HeLa epithelial-like cells.

Aim 3: Determine the transmissibility of bacterium CC99 from a nonphagocytic HeLa epithelial-like cell to phagocytic (THP-1) and nonphagocytic cells (HeLa).

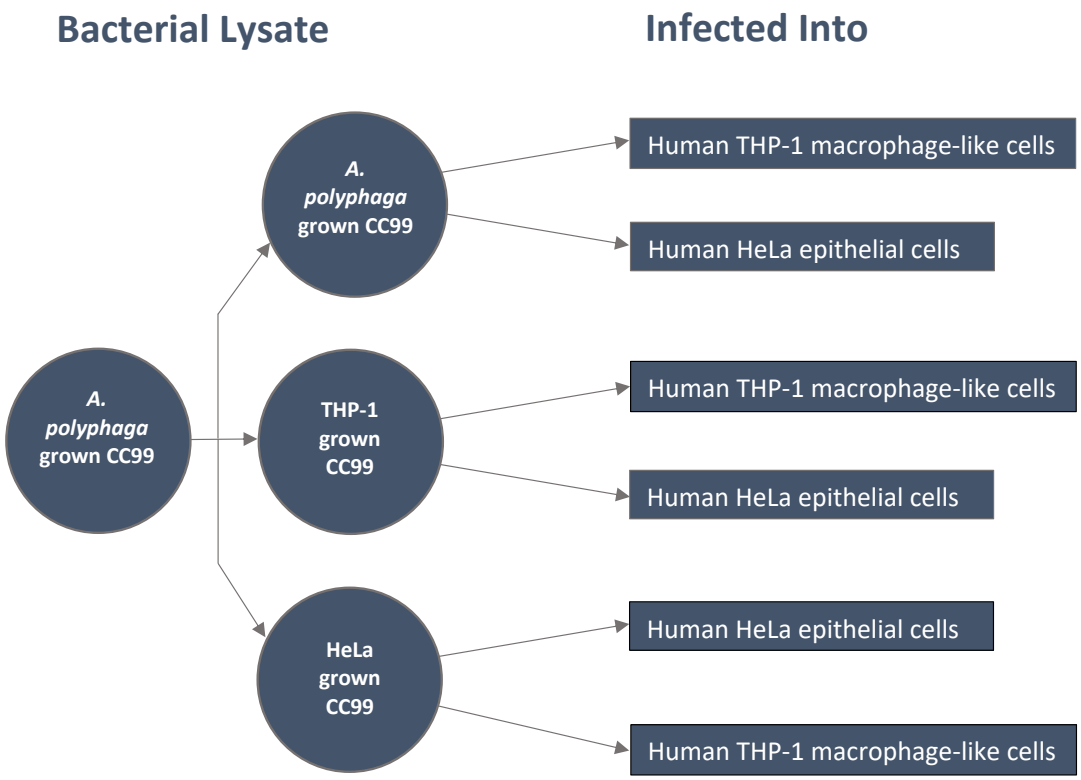


Figure 1. Overview of Host Cell and Bacterial Sources for Transmissibility Studies.

Methods

***Acanthamoeba polyphaga* maintenance.** Amoebal cultures of *A. polyphaga* were grown at room temperature in 5 mL of sterile trypticase soy broth (TSB) in a 25 cm^2 flat-bottomed cell culture flask (T25) for adherent cells. Once the cells were visually 80% confluent by microscopic observation, the cells were passaged into additional T25 flasks. In order to do this, flasks were gently tapped against a hard surface to force adherent cells into suspension, and a 0.5 mL volume of the suspension was added into a new T25 flask along with fresh 4.5mL of sterile TSB. Cells were incubated at 25°C.

Infection of *A. polyphaga* with bacterium CC99. *A. polyphaga* in a T25 flask were allowed to reach 80% confluency and then moved to the biosafety cabinet (BSC) as to be careful not to disturb the adherent monolayer. The TSB was removed to a waste tube and the monolayer was gently washed using 1-2 mL of sterile spring water (SSW; Carolina Biological Supply Company) which, after a gentle rocking across the monolayer, was removed. After an additional wash step, 4.9 mL of SSW was added to the flask along with CC99 lysate from a previous amoeba1 infection for an MOI of 100. SSW was added to induce starvation of the amoebae and ingestion of the CC99 bacteria. Complete lysis of the amoeba layer by the bacteria was typically observed in 3 days.

Enumeration of bacterium CC99. Amoeba or mammalian cell lysates containing CC99 were passed through a 5 μm nylon syringe filter (Fisher Scientific) to ensure the removal of remaining amoebal or mammalian cells. Bacteria were diluted in SSW resulting in a 1:1000 dilution of the filtered supernatant in a volume of 1 mL. Next, 3 μL of BacLight

(BacLight Bacterial Viability kit, Thermo Scientific) was added to the supernatant and was allowed to incubate in the dark for 20 minutes. During this time the filter apparatus was prepared by assembling a filter funnel system with a 0.1 μ black nucleopore filter (Millipore) which was inserted into a vacuum flask. A volume of 2 mL of SSW was added to rinse the filter before the 1 mL of stained bacterial solution was added and pulled through the filter by vacuum. Then, the filter was rinsed with a final 1 mL of SSW. The filter was carefully removed from the filter system and added to the pre-prepared microscope slide with BacLight mounting oil. The slide was kept in the dark. Finally, the bacteria on the filter slide were counted using immunofluorescence microscopy (Olympus BX60 epifluorescent microscope fitted with an ocular reticle) and counting ten reticle fields at 1000X magnification. The concentration of bacteria in the centrifuge tube was determined by multiplying the average number of bacteria on ten fields by the dilution and conversion factors for the ocular reticle and field size of the 1000X oil immersion lens.

THP-1 cell maintenance. THP-1 cells (ATCC TIB-202) were grown in a 5% CO_2 (95% room air), 37°C humidified incubator in a T25 flat-bottomed cell culture flask with the cap of the flask loosened very slightly to allow proper CO_2 exchange. THP-1 cells were cultured in 5 mL of RPMI 1640 complete medium with additions of 10% fetal bovine serum (FBS; Fisher Scientific) and 100 units/mL of penicillin and 100 μg/mL of streptomycin (P/S; Sigma-Aldrich). The cells were allowed to reach 80% confluency before splitting into a new T25 flask with 4 mL of warmed, fresh RPMI 1640 complete medium and 1 mL of the previous THP-1 culture. Flasks were incubated as above.

Differentiation of THP-1 cells. The cells were first grown to 80% confluency in RPMI complete media and then pipetted into a 15 mL conical tube and centrifuged at 300 *x g* in a Thermo Sorvall Legend XTR refrigerated centrifuge with a BIOliner swinging bucket rotor for 5 minutes. The supernatant was removed, being careful not to disturb the pellet at the bottom of the tube and replaced with 5 mL of RPMI with 10% FBS but no antibiotics (RPMI+FBS), using this volume to triturate and resuspend the pellet. Next, 0.2 mL of the cells and 0.2 mL of trypan blue were combined in a microcentrifuge tube and a volume of 10 µL of this mixture was added to a hematocytometer to count and calculate the cell concentration. Cells were adjusted to the appropriate concentration such that 5 x 10^5 cells/mL would be added per well of a 6-well plate (Fisher Scientific). Following cell adjustment, 2 µL per mL of cells of a 50 µM phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich) stock solution was added to the tube of cells (Park et al. 2007). The cells were then moved to a 6-well plate promptly as differentiation into adherent macrophages would cause cells to be lost in the conical tube. The cells were observed for at least 80% confluency and allowed to differentiate for an additional 48 hours in the CO_2 incubator at 37°C.

Cell counting prior to infection with bacterium CC99. To obtain the cell concentration prior to infection, *A. polyphaga* flasks were gently tapped to dislodge the cells. THP-1 monocytic cells were already free-floating so they could easily be removed for counting. HeLa cells were detached from the flask using trypsinization as described below. Following detachment or removal of cells from the flask, 0.2 mL of the cell sample was added into a microcentrifuge tube which contained 0.2mL of 0.08% trypan blue stain

(Sigma-Aldrich). A volume of 10 μL was then loaded into each side of a hemacytometer slide with a coverslip. Next, a total of ten grids were counted using the center and four corner grids of each side of the hemocytometer. Cells that were alive appeared clear/yellowish and dead cells were stained blue. Cell concentration was determined by taking the live cell count and multiplying by chamber conversion factor and the dilution factor. Percent viability was determined by calculating the number of live cells divided by the total number of cells and multiplying by 100.

Infection of THP-1 cells with bacterium CC99. After observing that there was a confluent monolayer of adherent differentiated macrophages, the old media was removed from each well, careful not to disturb the cells attached. Media from each well was replaced with 1 mL of phosphate buffered saline (PBS; Millipore Sigma) to wash and remove any non-adherent cells. After two washes with PBS, 0.9 mL of RPMI with no additions but containing bacterium CC99 at a multiplicity of infection (MOI) of 100 was added to appropriate wells. Mock-infected control wells were treated with RPMI medium with no additions only. The CC99 mixture or media was left on the cells for 2 hours with incubation in the CO_2 incubator at 37°C. After incubation, the medium from all wells was removed and replaced with 2 mL of RPMI containing 10% FBS. Cells were then returned to the CO_2 incubator at 37°C for 24, 48, and 72 hours.

HeLa cell maintenance. Human HeLa S3 epithelial cells (ATCC CCL-2.2) were grown at 37°C, and 5% CO_2 in a T25 tissue culture flask with RPMI 1640 complete medium. To passage cells, the medium was removed from the flask and replaced with 1 mL of 1X

trypsin (Sigma-Aldrich), and the flask was incubated at 37ºC and 5% CO_2 for 15 min. The flask was then gently tapped on a hard surface to detach cells from the flask surface, and the dislodged cells were then resuspended in 5 mL of fresh RPMI 1640 complete medium. In a new flask, 0.5mL of the resuspended cells were added along with 4.5mL of fresh warm RPMI 1640 complete medium, and this flask was incubated at 37ºC in 5% CO_2.

Infection of HeLa cells with bacterium CC99. After observing that there was a confluent monolayer of adherent Hela cells in the flask, the Hela cells were trypsinized as described above and added to a 15 mL conical tube. The cells were centrifuged at 500 *x g* for 3 min in a Thermo Sorvall Legend XTR refrigerated centrifuge with a BIOliner swinging bucket rotor, and the old medium was removed and replaced with RPMI with no additions. A total of 2 mL of cells were then added to 6-well plates at a concentration of 5 x 10^5 cells/mL and were grown in the incubator for no more than 12 hours to promote attachment. The cells were then infected with bacterium CC99 at an MOI of 100 for 2 hours in the CO_2 incubator at 37°C. Mock-infected control wells were treated with RPMI with no additions for 2 hours in the CO_2 incubator. Following incubation, the RPMI or CC99-containing media were removed from culture wells and replaced with 2 mL RPMI with 10% FBS as described above for the THP-1 cells. Cells were then returned to the CO_2 incubator at 37°C.

Harvesting bacteria and host cells following infection with bacterium CC99. Following initiation of bacterial infection, the plates were placed in the incubator and

allowed to incubate for 24, 48, and 72 hours. To remove the host cells and bacteria, *A. polyphaga*, THP-1, or HeLa cell control and CC99-infected wells were scraped to dislodge anything that remained following CC99 infection. Host cell-CC99 and control treatments occurred in volumes of 2 mL, and that volume along with cells that were gently scraped from the well surface was moved into a microcentrifuge tube for each well. A volume of 0.2 mL of this mixture was combined with 0.08% trypan blue stain to assess host cell number and viability. A 0.1 mL volume was removed and added into a cytofunnel, and the cells pelleted onto a glass slide using a Thermo Shandon Cytospin 3 for Giemsa staining to determine the levels of infection as indicated by the presence of intracellular bacteria in the cytoplasm and nuclei of infected cells. The remaining volume was centrifuged at 8000 rpm for 10 minutes in a Fisher Scientific AccuSpin Micro17 centrifuge. The supernatant was replaced with 100 μL of sterile nuclease-free water and frozen at -20°C.

Viability counts of host cells following infection with bacterium CC99. CC99-infected and mock-infected control cells were harvested in triplicate at 24-, 48-, and 72-hours post-infection. During harvesting of CC99-infected cells and controls, 0.2 mL aliquots of trypan blue were prepared in microcentrifuge tubes such that there would be an individual tube for cells from each CC99-infected and each control well. Following resuspension of harvested control and CC99-infected cells, 0.2 mL from each well was pipetted into a microcentrifuge tube with trypan blue. Total cell and viability counts were determined as described above.

Giemsa staining. For each timepoint at which control and infected cells were harvested, a 100 μL aliquot from each tube containing the contents of a harvested control or infection well was added into a cytofunnel attached to a glass microscope slide by a cytoclip. Slides and cytofunnels were centrifuged in a Thermo Shandon Cytospin 3 at 800 rpm for 3 min. Giemsa stain working solution was prepared by adding 19 mL of distilled water and 1 mL of Fluka Giemsa stain (Fisher Scientific) into a Coplin jar. After centrifugation, slides were removed from the cytoclips and placed onto a paper towel to air dry. Next, each concentrated cell spot on the slide was covered with 0.1 mL of methanol for 1 minute for fixation and then promptly rinsed in a Coplin jar with distilled water. Following a brief air dry, the slides were stained for 1 hour in the Giemsa stain solution. Once removed, the slides were rinsed using distilled water and allowed to air dry overnight on the benchtop for later analysis by light microscopy.

Quantification of bacterial numbers by real time quantitative PCR. Following viability counts and Giemsa staining, the remainder of the volume (1.7 mL) from each well which had contained host cells infected with CC99 or mock-infected control cells was pipetted into 2 mL microcentrifuge tubes and centrifuged at 8,000 rpm for 10 minutes. After ensuring that there was a visible pellet, 1 mL of media was removed being careful not to disturb the pellet. Then 100 μL of nuclease-free water was added to cover the pellet, and these pellets were frozen at -20ºC for real time quantitative PCR (qPCR).

Because bacterium CC99 is unculturable, genomic equivalents (GE) were used to quantitate numbers of CC99 bacteria. This method has been used to enumerate the closely related bacterium *Coxiella burnetti* (Brennan and Samuel 2003, Coleman et al.

2004). Because genomic sequencing has shown that bacterium CC99 has only a single copy of the gene for 16S rRNA, a unique sequence of this gene was used for quantification of genomic equivalents by qPCR (Chamberlain et al. 2019). To quantify the amount of CC99, primers CC722F (5'-GGCTTCCTGGACCATAACTGA-3') and CC832R (5'-CGATACCAGATGGTCTAA-3') were used. The primers were synthesized by Eurofins and amplify a 110-bp fragment of the gene. Reactions for qPCR consisted of 5 µL of 2X Maxima master mix (Thermo-Fisher), 1 µL of each primer (final concentration 500 nM), 1 µL of nuclease-free water, and 2 µL of either a dilution of enumerated CC99 (heated for 10 min at 95-99ºC) or a frozen aliquot harvested from CC99 or mock-infected amoebae or mammalian cells. No template control wells (negative control) contained 2 µL of nuclease-free water instead of a cellular sample.

For quantification, ten-fold dilutions of a known amount of CC99 bacteria were amplified by qPCR to generate a standard curve and used to assess that amount of bacterium CC99. Standard curves had at least four enumerated bacterial points with each point representing the average of duplicate samples that covered the range of bacteria detected in samples and with an R^2 value of > 0.97. The lowest detectable concentration of CC99 DNA using the qPCR assay was equivalent to 1000 GE/mL (Appendix A1). Samples used to generate the standard curve were included on every 96-well plate used for qPCR and the standard curve had to meet the above requirements to validate sample qPCR values. The standard curve samples also served as the positive control for amplification of CC99 DNA. The equation of the line for the standard curve was used to estimate the amount of nucleic acid in the original sample. This worked by evaluation of the Ct values which correlated to the cycle at which the fluorescence signal crossed a

predefined threshold and were inversely proportional to the initial amount of target nucleic acid in the original sample. Using the values from the line of the standard curve, the linear regression equation was used to estimate the concentration of genomic equivalents of bacteria in each sample.

Statistical Analyses. For each experiment, all infections, including controls, were performed in triplicate for each timepoint. Since all infections were performed using cultured cell lines that exhibit low variability, triplicate biological replicates were acceptable (Hartman et al. 2023). All experiments were repeated at least two times. All values were reported as a mean ± standard deviation of triplicate samples, and statistical analyses were completed on all infection groups. Trypan blue staining was used to evaluate the cell viability of uninfected control and CC99-infected cells. A two-tailed unpaired student's t test (Analysis Tool-Pak in Microsoft Excel 2016) was used that determined significant differences between the control and infection wells.

Results

Infection of THP-1 Cells with Lysates of CC99 Bacteria from *A. polyphaga*

To establish that the infection system was intact, a positive control experiment was conducted in which infection of THP-1 mammalain macrophage-like cells were infected by bacteria derived from amoebae. Target cell viability, levels of infection, and yields of bacteria were then measured at 48 and 72 hours post infection.

In two independent trials using lysates of CC99 from *A. polyphaga,* mock-infected cells remained >80% viable over the course of the 72 hours, but the CC99-infected cells had statistically significant decreases in viability over the same time ($\eta=2$) (Fig. 2). CC99-infected cells were observed to be 40 to 50% viable at 48 hours which then decreased to below 40% by 72 hours (Fig.2).

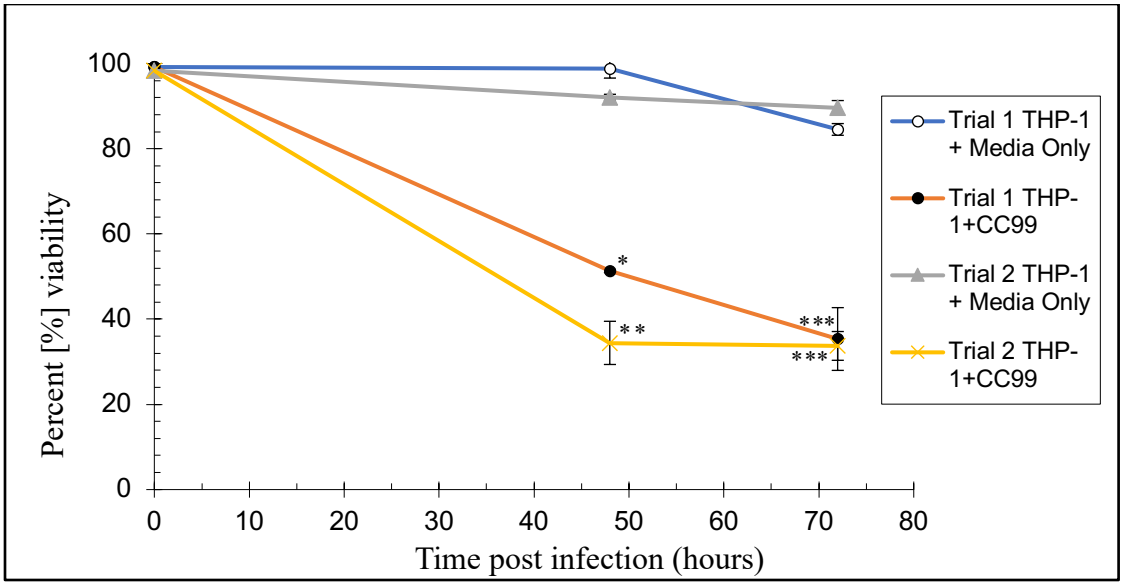


Figure 2. Viability of THP-1 cells infected with A. *polyphaga*-grown CC99 bacteria: Trials 1-2. Trypan blue staining was used to assess viability of THP-1 cells infected with an MOI of 100 of a CC99 lysate from *A. polyphaga.* Cells were infected for 2 hours with CC99 lysate or media alone, after which time supernatants were removed, cells were gently washed, and fresh media added to the cells. This timepoint was considered time 0. At time 0 and subsequent timepoints, cells were removed from wells by gentle scraping, stained with trypan blue, and counted on a hemocytometer. Triplicate wells were used for each timepoint for both infected and control wells. Statistical significance between CC99-infected and mock-infected control cells at each timepoint is indicated by *$p < 0.05$, **p < 0.01, *** $p < 0.001$.

Next, to localize and confirm intracellular bacterial infection, Giemsa stains of THP-1 cells infected with *A. polyphaga*-lysed CC99 were examined. Giemsa stains of mock-infected control cells had no visible bacteria whereas CC99 infected THP-1 cells showed dark purple coccoid bacteria were visible within light bluish cytoplasm, or more commonly within the pinkish-purple nucleus (Fig. 3). In the later stages of infection, nuclear-associated CC99 overloaded the THP-1 nucleus such that the entire nucleus appeared dark purple (Fig. 3).

Microscopic counts of Giemsa stains of CC99-infected cells at 48 hours show that 36-49% of THP-1 nuclei have intranuclear or nuclear-associated bacteria with 48-65% of total cells infected (Fig. 4). At 72 hours post-infection, the bacteria appeared in the nuclei of 45-51% of host cells with 59-76% of total cells showing infection (Fig. 4). Both the cell viability of THP-1 cells infected with CC99 harvested from *A. polyphaga* and the percentages of infection were similar to previous reports (Chamberlain et al. 2019). With these controls showing expected results, the next step was to quantify the level of infection using qPCR.

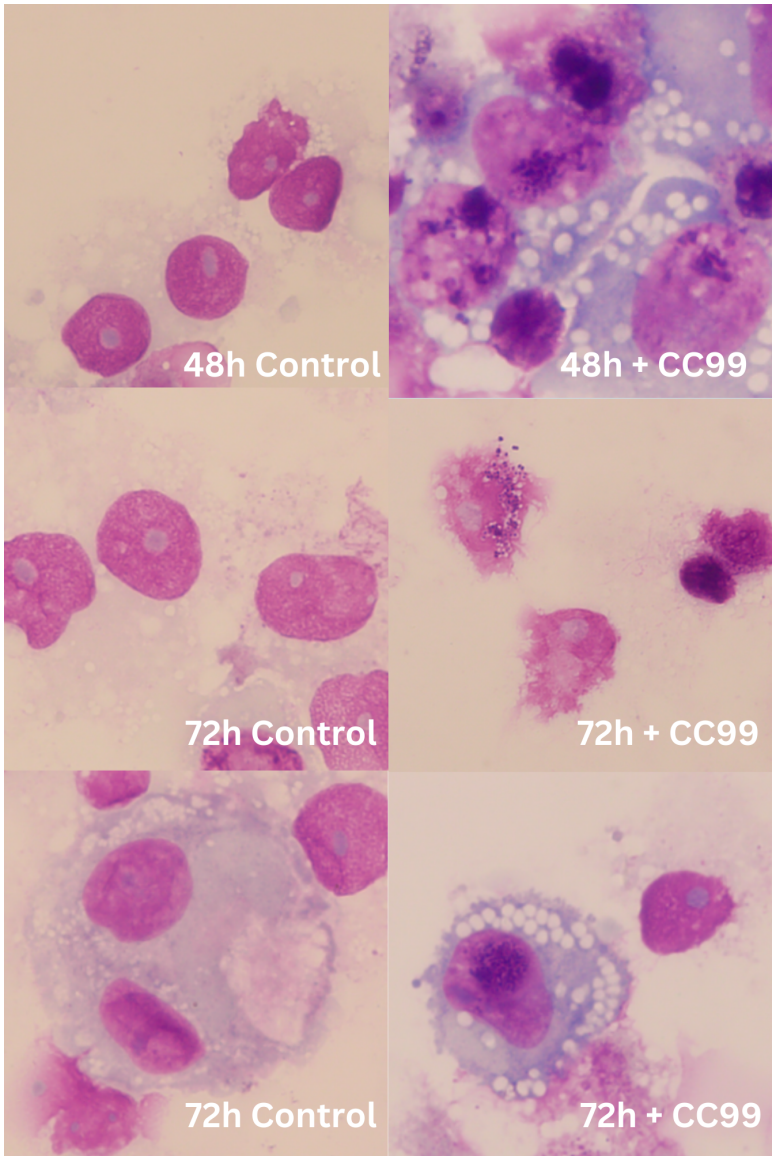


Figure 3. Giemsa stains to determine CC99 infectivity of THP-1 cells infected with *A. polyphaga*-grown CC99 bacteria. Representative Giemsa stains of THP-1 cells infected with *A. polyphaga*-grown CC99. Control images show examples of mock infected THP-1 cells. The 48- and 72-hour CC99 images depict THP-1 cells infected with CC99 from amoebae (MOI 100). Cytoplasmic and nuclear-associated bacteria are visible as dark purple cocci.

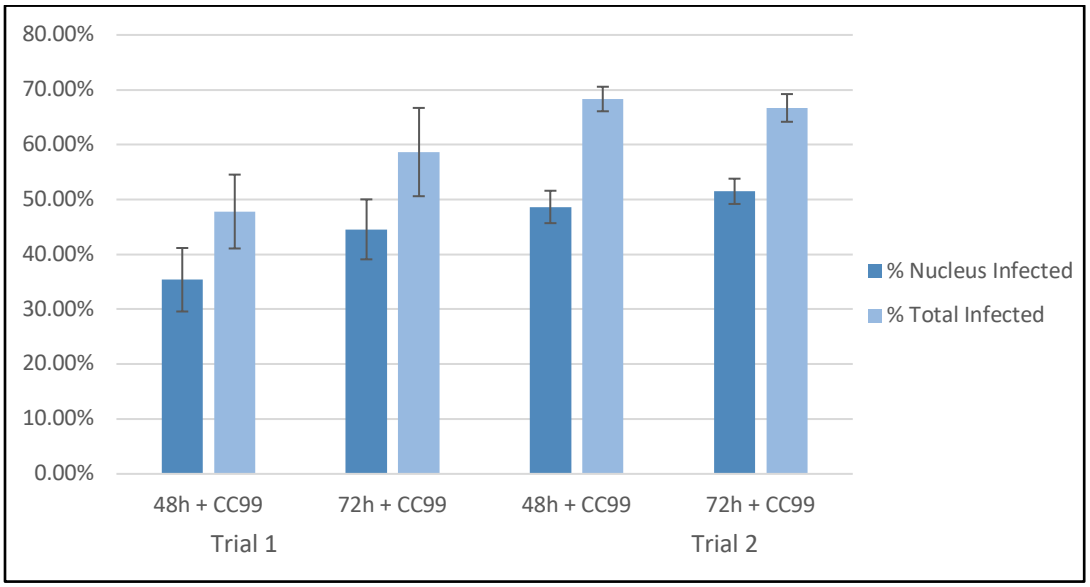


Figure 4. Percentages of infected THP-1 cells and THP-1 cells with bacteria in the nuclei following infection with *A. polyphaga*-grown CC99 bacteria: Trials 1-2. Giemsa staining and microscopy were used to assess percentages of cells infected by CC99 when cells were harvested at 48- and 72-hours post-infection with CC99 harvested from *A. polyphaga* at an MOI of 100. Dark blue bars represent cells with nuclear bacteria; Light blue bars represent cells with bacteria in the cytoplasm with or without infection of the nucleus, or the total number of cells infected. Values are the average of three replicate wells. No statistically significant differences in percentages of infection were observed between 48- and 72-hours post-infection.

To establish a standard curve for quantification of CC99 by qPCR, a stock of enumerated CC99 was diluted tenfold to concentrations of $1x10^8$ to $1x10^3$ cells/mL in nuclease-free water, such that 1 µL of each dilution corresponded to a defined number of bacteria. Thus, for each dilution of bacteria, the Cq values from qPCR would correspond to a defined number of bacteria. The concentration of bacteria in experimental samples could then be interpolated from the standard curve by using the slope intercept equation of the standard curve and linear regression to quantify the concentration of bacterial genomic equivalents (GE/mL) in samples from the CC99-infected wells. Duplicate samples of the standard curve were included on each PCR plate.

Quantification of CC99 in THP-1 cells that were infected with *A. polyphaga*-lysed CC99 showed that cells in CC99-infected wells had significant amounts of detectable CC99 DNA. To interpret the data, it was important to refer to the melt curve when values for the experimental wells were observed because bacterial melt curves should peak at a specific melt temperature (79.5 ± 0.5°C). Samples that crossed the cycle threshold and had peaks of the appropriate melt temperature were considered positive for CC99 if they crossed the cycle threshold before cycle 35. In contrast, control wells were observed to have little to no amplification or if amplification occurred, the fluorescence corresponded to melt curves with lower melt temperatures inconsistent with the expected melt temperatures for the CC99-specific primers when compared to the amplification peaks for the CC99 standard curve and positive control wells (Appendices A1 & B1-B2). These lower temperature melt peaks most likely represent primer dimers or non-specific amplification due to absence of target CC99 DNA and consequential primer excess (Appendices B1-B2). CC99-infected samples were all observed to have peaks with the

same melt temperature as the standard curve and positive control wells (Appendices B1 & B2).

When DNA in the samples from CC99 and mock-infected THP-1 cells was amplified by qPCR using CC99-specific primers, the logarithmic base 10 (log10) concentrations of CC99 at 48 hours post-infection were calculated using the standard curve as 3.5 and 4.1 GE/mL for Trials 1 and 2 respectively (Fig. 5), and after 72 hours the values GE/mL of CC99 were 4.1 and 6.2 respectively (Fig. 5). Melt curves for these samples showed the expected melt temperature of 79.5°C confirming that these quantifications were of CC99 DNA. The presence of CC99 DNA supports the observations of bacteria within Giemsa-stained cells and supports previous observations that amoeba-lysed CC99 can infect THP-1 cells (Chamberlain et al. 2019). Having established that amoeba-derived CC99 can infect THP-1 cells, a phagocytic call line, we next attempted to confirm that amoeba-derived CC99 were able to infect the non-phagocytic HeLa cell line.

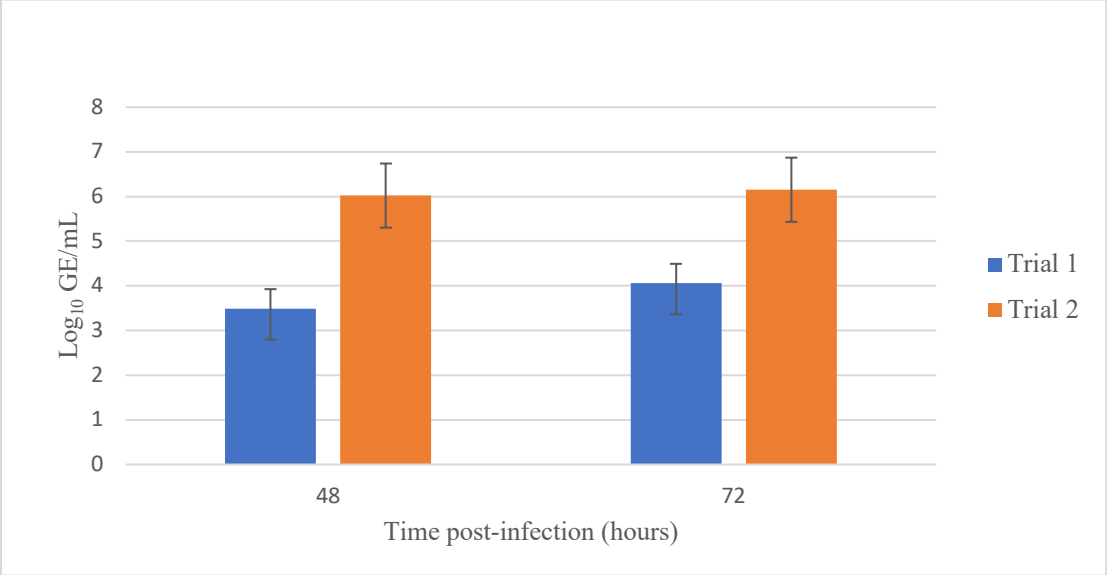


Figure 5. Quantification of CC99 bacteria harvested from THP-1 cells infected with A. *polyphaga*-grown CC99 bacteria: Trials 1-2. To determine the yields of bacteria from CC99-infected THP-1 cells, aliquots from triplicate sample wells were assessed for bacterial load using qPCR. Primers for unique regions of the gene for CC99 16S rRNA were used to determine the amount of DNA present in the sample. Ten-fold dilutions of a known concentration of bacterium CC99 were also amplified as part of the same set of qPCR reactions using the same primers. Linear regression analyses using a standard curve of known concentrations of bacteria were used to assess concentrations of CC99 in the samples in GE/mL.

Infection of HeLa Cells with Lysates of CC99 Bacteria from *A. polyphaga*

Human HeLa epithelial cells were infected with CC99 bacteria harvested from *A. polyphaga*. As for the THP-1 macrophage-like cells, infected HeLa cells were assessed for host cell viability, percentages of infected cells, and yields of bacteria from the cells using qPCR.

HeLa cells from control wells exhibited viabilities of 89-92% over the 72-hour period for Trials 1 and 2 (Fig. 6). HeLa cells that were infected with CC99 bacteria from *A. polyphaga* were 73% and 68% viable after 24 hours, 58% and 56% at 48 hours, and 50% and 49% at the final 72-hour time point in Trials 1 and 2, respectively (Fig. 6). There was a statistically significant decrease in viability in the CC99 infection wells compared to mock-infected control cells at all three timepoints.

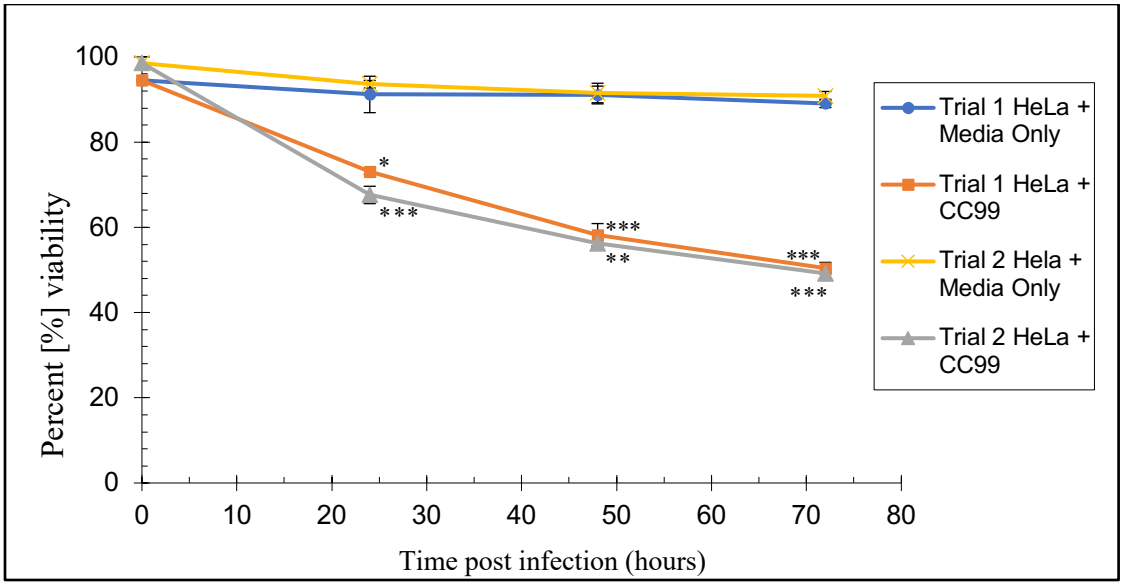


Figure 6. Viability of HeLa cells infected with *A. polyphaga*-grown CC99 bacteria: Trials 1-2. Trypan blue staining was used to assess viability of cells HeLa cells infected with an MOI of 100 of a CC99 lysate from *A. polyphaga.* Cells were allowed to infect for 2 h with CC99 or media alone, after which time supernatants were removed, cells gently washed, and fresh media added to the cells. This timepoint was considered time 0. At time 0 and subsequent timepoints, cells were removed from wells by gentle scraping, stained with trypan blue, and counted on a hemocytometer. Triplicate wells were used for each timepoint. Statistical significance between treated and mock treated control cells at each timepoint is indicated by *$p < 0.05$, **$p < 0.01$, *** $p < 0.001$.

Next, to localize and confirm intracellular infection, Giemsa staining of HeLa cells infected with *A. polyphaga*-lysed CC99 or mock infected were examined. Over the course of the 72 hours the bacteria were able to infect HeLa host cells similarly to the THP-1 cells (Fig. 7). Images of mock-infected control cells have no visible bacteria whereas in CC99-infected cells, dark purple coccoid bacteria are visible in the light cytoplasm but more often within the pinkish-purple nuclei (Fig. 7).

When light microscopy was used to count the numbers of infected cells in Giemsa-stained cells, at 24 hours CC99 bacteria appeared to be in the nuclei of the host cell approximately 35% of the time (Fig. 8; Trials 1 & 2). At the same time point it was observed that 41-42% of total cells were infected with bacteria either in the cytoplasm, the nucleus, or in both cellular compartments (Fig. 8; Trials 2 & 1, respectively). At 48 hours, CC99 bacteria were intranuclear at percentages of 37% and 41% with 48-57% of total cells infected in Trials 1 and 2, respectively (Fig. 8). This represented a statistically significant increase of total infected cells by 48 h in Trial 1. By 72 hours bacteria appeared to be in the nuclei of 42-46% of cells (Fig. 8; Trials 1 & 2), which was a significant increase in nuclear infection from 24 h in Trial 1. At 72 hours, 52-58% of total cells were infected (Fig. 8; Trials 1 & 2).

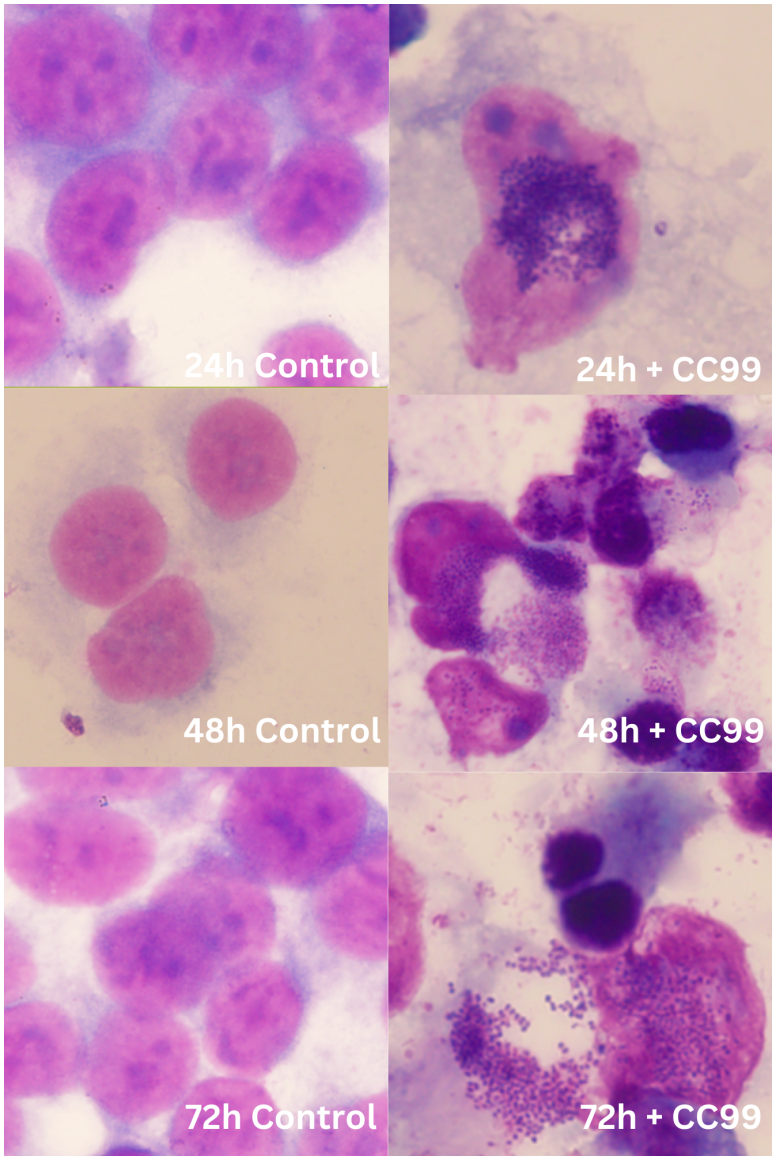


Figure 7. Giemsa stains to determine CC99 infectivity of HeLa cells infected with *A. polyphaga*-grown CC99 bacteria. Representative Giemsa stains of HeLa cells infected with *A. polyphaga*-grown CC99. Control images show examples of mock infected HeLa cells. The 24-, 48-, and 72-hour CC99 images depict HeLa cells infected with CC99 from amoebae (MOI 100). Cytoplasmic and nuclear-associated bacteria are visible as dark purple cocci.

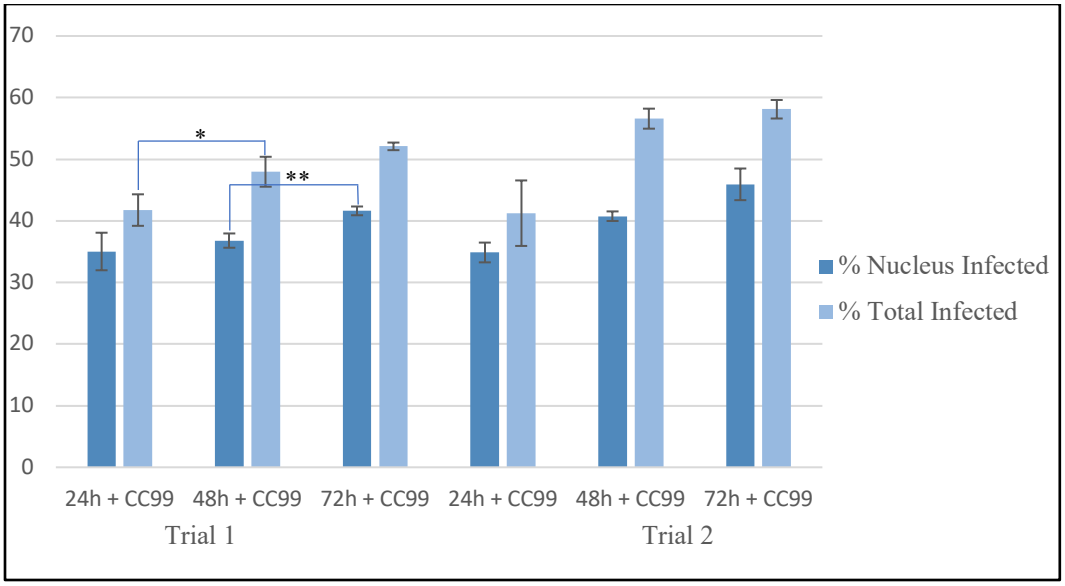


Figure 8. Percentages of infected HeLa cells and HeLa cells with bacteria in the nuclei following infection with *A. polyphaga*-grown CC99 bacteria: Trials 1-2. Levels of infection by bacterium CC99 were assessed by Giemsa staining at 48 and 72 post-infection with CC99 harvested from *A. polyphaga* at an MOI of 100. The dark blue bars represent cells with nuclear bacteria; The light blue bars represent cells with bacteria in the cytoplasm with or without infection of the nucleus. Values are the average of three replicate wells. Statistical significance is indicated by *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Finally, the levels of CC99 bacteria were quantified using qPCR. CC99 DNA was detected in samples from infected but not mock-infected control cells. As with the THP-1 infected cells, it was important to refer to the standard curve when the experimental wells were observed to ensure that the detected fluorescence corresponded to the expected melt curves for amplified CC99 DNA (Appendices B3 & B4). CC99-infected wells were observed to have peaks with the same melt temperature as the standard curve and positive control samples and values within the range of standard curve samples (Appendices B3 & B4). Linear regression analyses using the standard curve for CC99 DNA estimated between log10 4.1-5.5 GE/mL at 24 hours (Fig. 9; Trials 2 & 1, respectively). At 48 hours, the concentrations of CC99 were calculated as log10 4.6-5.5 GE/mL (Fig. 9; Trials 2 & 1, respectively) and by 72 hours concentrations of CC99 were estimated to be between log10 4.1-5.2 GE/mL (Fig. 9; Trials 2 & 1, respectively). Although there were no significant differences in the amounts of CC99 DNA between time points or between replicate trials, the genomic equivalents were comparable to those from THP-1 cells.

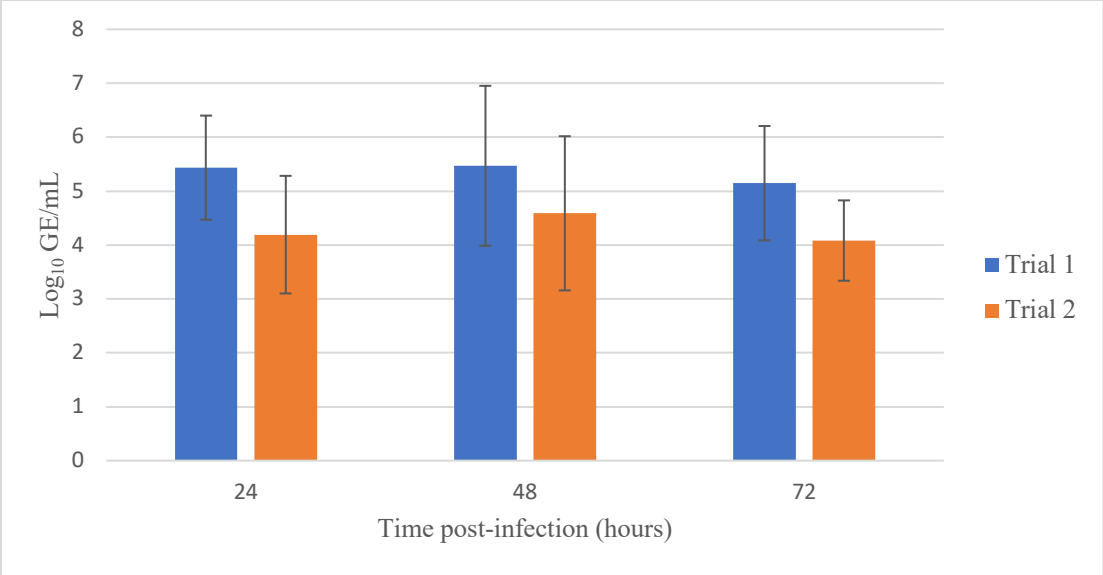


Figure 9. Quantification of CC99 bacteria harvested from HeLa cells infected with A. *polyphaga*-grown CC99 bacteria: Trials 1-2. To determine the yields of bacteria from CC99-infected HeLa cells, aliquots from triplicate sample wells were assessed for bacterial load using qPCR. Primers for unique regions of the gene for CC99 16S rRNA were used to determine the amount of DNA present in the sample. Ten-fold dilutions of a known concentration of bacterium CC99 were also amplified as part of the same set of qPCR reactions using the same primers. Linear regression analyses using a standard curve of known concentrations of bacteria were used to assess concentrations of CC99 in the samples in GE/mL.

Infection of THP-1 Cells with Lysates of CC99 Bacteria from THP-1 Cells

Having established that both phagocytic (THP-1) and non-phagocytic (HeLa) cells can be infected by CC99 derived from amoeba, we began a series of experiments to determine whether the source of the CC99 changed its infection capabilities. CC99 derived from prior THP-1 infections and CC99 derived from prior HeLa infections were used as the infectious particles.

Mock-infected THP-1 control cells remained > 89% viable throughout the 72-hour treatment period for Trials 1 and 2 (Fig. 10) as assessed by trypan blue viability staining. THP-1 cells infected with the THP-1-derived CC99 significantly decreased in viability to between 84-85% by the 72-hour timepoint for both Trials 1 and 2 (Fig. 10). Statistically significant decreases in cell viability were also observed at the 24- and 48-hour timepoints when compared to untreated cells in both trials (Fig. 10).

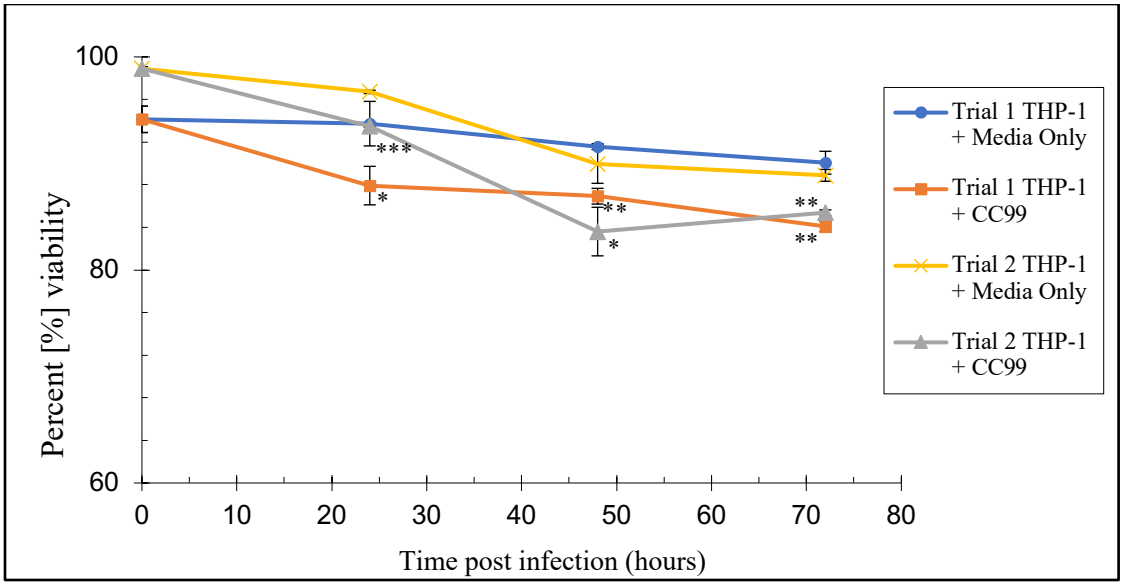


Figure 10. Viability of THP-1 cells infected with THP-1-grown CC99 bacteria: Trials 1-2. Trypan blue staining was used to assess viability of THP-1 cells infected at an MOI of 100 with a CC99 lysate from THP-1 cells. Cells were infected with CC99 bacteria or media alone, after which time, supernatants were removed, cells gently washed, and fresh media added to the cells. This timepoint was considered time 0. At time 0 and subsequent timepoints, cells were removed from wells by gentle scraping, stained with trypan blue, and counted with a hemocytometer. Triplicate wells were counted for each timepoint. Statistical significance is indicated by *$p < 0.05$, **p < 0.01, ***$p < 0.001$.

Giemsa stains for THP-1 cells, which were infected with CC99 collected from lysed THP-1 cells, showed that cells in mock-infected control wells looked as expected with light bluish-purple cytoplasm and darker pinkish-purple nuclei (Fig. 11 & 12). Giemsa stains of THP-1 cells infected with the CC99 from THP-1 cells appeared similarly to the uninfected cells with no evidence of intracellular dark purple coccoid bacteria (Fig. 11 & 12). The entire area of cells on the cytospin slide was scanned for infected cells, but no infected cells were observed on any of the triplicate slides.

Although there were slight, but statistically significant, decreases in viability of THP-1 cells infected with the CC99 lysate from THP-1 cells, the failure to detect infected cells in Giemsa stains from two trials was the first compelling indicator that for infections with CC99 lysed from THP-1 cells there was no subsequent infection of THP-1 cells. Although no infected cells were found on the Giemsa-stained slides, there is still a possibility that there were cells that may have been infected but which were not in aliquots used to prepare slides for Giemsa staining. The decrease in cell viability of the THP-1 cells infected with the CC99 lysate from THP-1 cells could suggest that by 72 hours, there were low levels of infection. However, the decrease in host cell viability could have also been due to the presence of CC99 bacteria in the cell culture. THP-1 cells that engulf avirulent strains of the intracellular bacterium *Mycobacterium bovis* become apoptotic, and as CC99 are Gram-negative bacteria, the presence of bacterial LPS in the cell culture could also induce apoptotic cell death (Monguio-Tortajada et al. 2018, Riendeau and Kornfield 2003). It is also possible, that although they may be engulfed and digested by the THP-1 cells, molecules that the bacteria secrete or utilize either prior to or during engulfment, such as pore-forming molecules, could also damage the macrophage

cells. For example, the related pathogens *C. burnetii* and *L. pneumophila* both have molecules with pore-forming activity against phagocytic cells (Banerjee-Bhatnagar et al. 1996, Kirby et al. 1998. Silveira and Zamboni 2010).

The qPCR analysis along with host cell viability counts and Giemsa stains helped to confirm that infection by CC99 did not occur when THP-1-lysed bacteria were used to treat THP-1 cells. Additionally, the experimental wells with THP-1 cells that had been infected with CC99 did not have melt curve peaks at the expected melt temperature of 79.5 °C, although the control and standard curve samples had peaks at the expected melt temperatures, which supported that there was not any target CC99 DNA for the CC99 primers in to bind in the qPCR reactions of the experimental wells (Appendices B5 and B6). These studies indicate that CC99 bacteria harvested from THP-1 cells do not produce a detectable, productive infection in THP-1 cells within a 72-hour period.

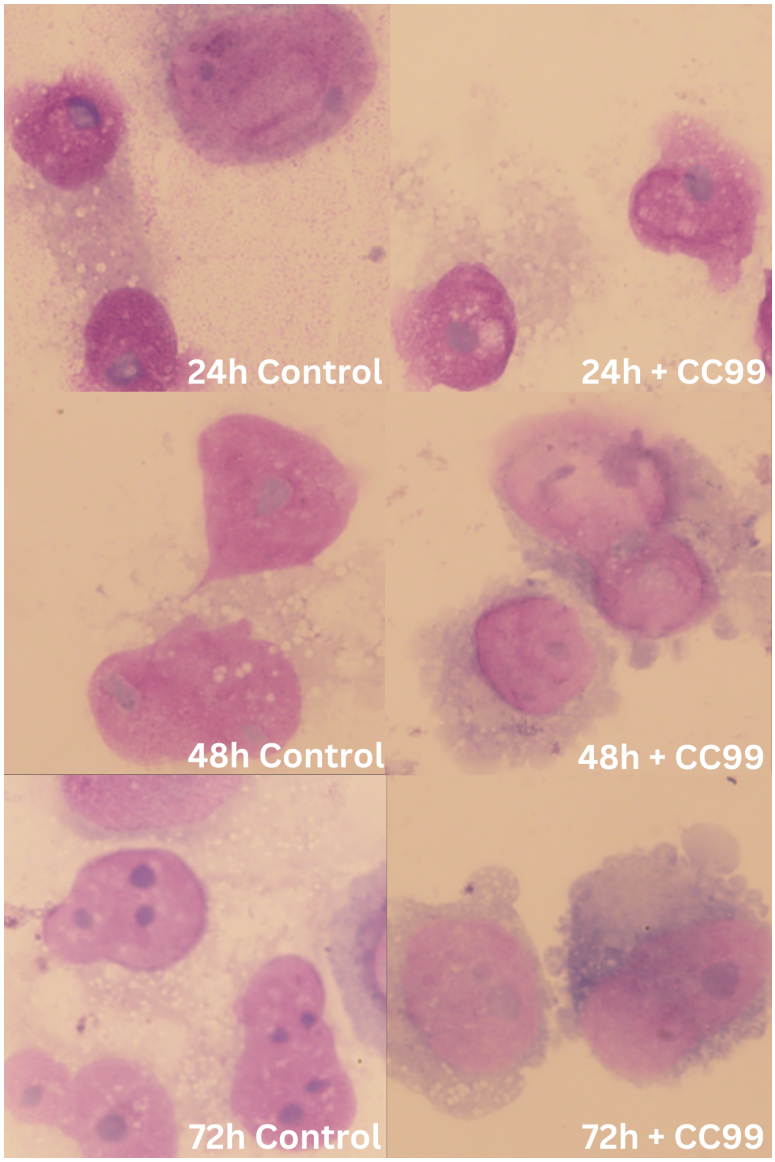


Figure 11. Giemsa stains to determine CC99 infectivity of THP-1 cells infected with THP-1-grown CC99 bacteria: Trial 1. Giemsa stains of THP-1 cells infected with bacterium CC99 grown in THP-1 cells. Representative Giemsa stains of control and infected cells from the 24-, 48-, and 72-hour timepoints do not show evidence of infection by CC99 bacteria.

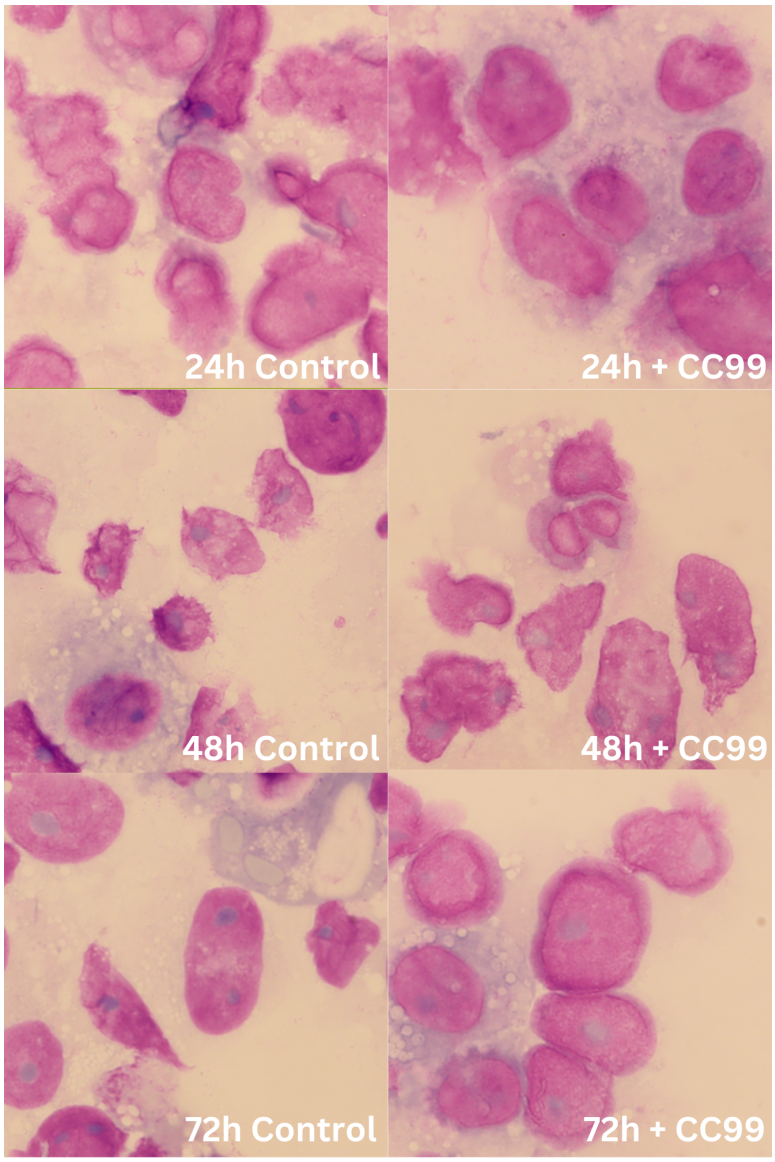


Figure 12. Giemsa stains to determine CC99 infectivity of THP-1 cells infected with THP-1-grown CC99 bacteria: Trial 2. Giemsa stains of THP-1 cells infected with bacterium CC99 grown in THP-1 cells. Representative Giemsa stains of control and infected cells from the 24-, 48-, and 72-hour timepoints do not show evidence of infection by CC99 bacteria.

Infection of HeLa Cells with Lysates of CC99 Bacteria from THP-1 cells

Although the bacteria grown in THP-1 cells did not infect the THP-1 macrophages, it is possible these bacteria might be able to infect HeLa cells, which are not naturally phagocytic, but can be infected by amoeba-grown CC99 bacteria.

Trypan blue viability counts for the HeLa cells that were infected with CC99 that lysed from THP-1 showed that for both trials cells in the control wells remained around 87-90% viable throughout the 72-hour treatment period, whereas the viability of cells in CC99-infected wells statistically significantly decreased to 71% viability by 72 hours (Fig. 13). The decrease in viability was slightly higher than for the THP-1 cells (Fig. 10). This decrease in viability in the infected cells again could suggest that infection occurred. However, as for the THP-1 cells, HeLa cells have also been reported to become apoptotic in response to LPS (Santos et al. 2020) or may also be affected by pore-forming proteins as has been described for *C. burnetti* and *L. pneumophila* (Banerjee-Bhatnagar et al. 1996, Kirby et al. 1998, Silveira and Zamboni 2010). Giemsa staining and real-time qPCR were next used to determine whether CC99 bacteria could be detected in the infected cells.

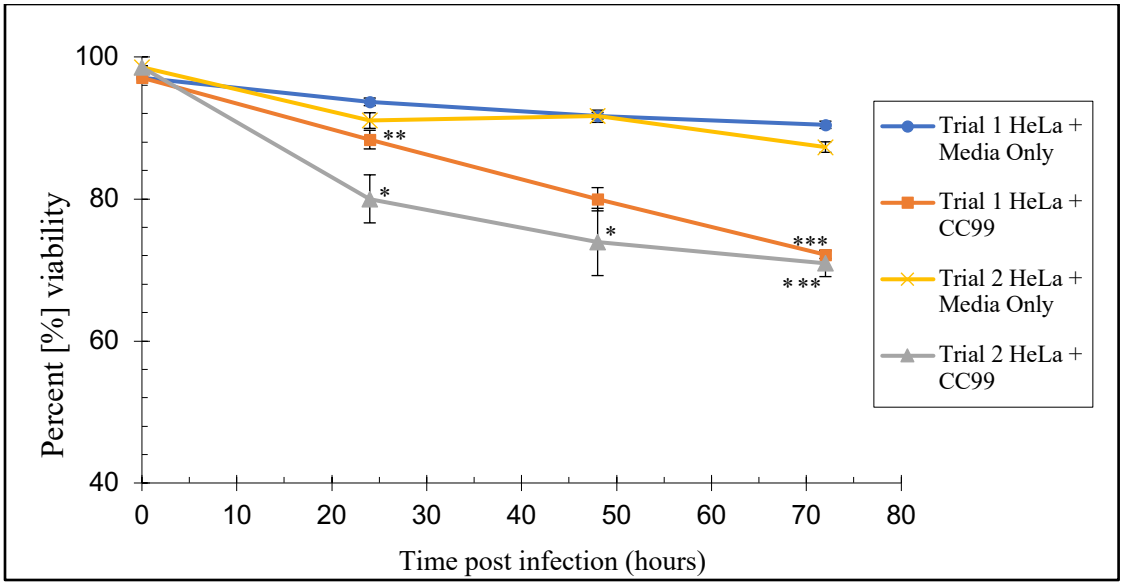


Figure 13. Viability of HeLa cells infected with THP-1-grown CC99 bacteria: Trials 1-2. Trypan blue staining was used to assess viability of HeLa cells infected at an MOI of 100 with a CC99 lysate from THP-1 cells. Cells were infected with CC99 bacteria or media alone, after which time, supernatants were removed, cells gently washed, and fresh media added to the cells. This timepoint was considered time 0. At time 0 and subsequent timepoints, cells were removed from wells by gentle scraping, stained with trypan blue, and counted with a hemocytometer. Triplicate wells were counted for each timepoint. Statistical significance is indicated by *$p < 0.05$, **$p < 0.01$, *** $p < 0.001$.

Giemsa stains for HeLa cells infected with CC99 which had lysed from THP-1 cells showed no differences in the appearances of cells between the control and CC99-infected wells. Both infected and uninfected HeLa cells looked as expected with pinkish-purple nuclei and light blue cytoplasm and no evidence of infection (Fig. 14 & 15). No infected cells were detected on any of the triplicate slides for each timepoint for either trial. It should be noted that HeLa cells have often have multiple densely staining nucleoli within the nucleus (Lafontaine et al. 2021), which should not be mistaken for intranuclear CC99-containing vacuoles.

When HeLa cells infected with the CC99 lysate from THP-1 cells were quantified by qPCR for CC99 DNA, no measurable CC99 DNA was detected in the samples (Appendices B7 & B8), leading to the conclusion that productive infection of HeLa cells by CC99 did not occur when THP-1-lysed bacteria were used to infect the HeLa cells.

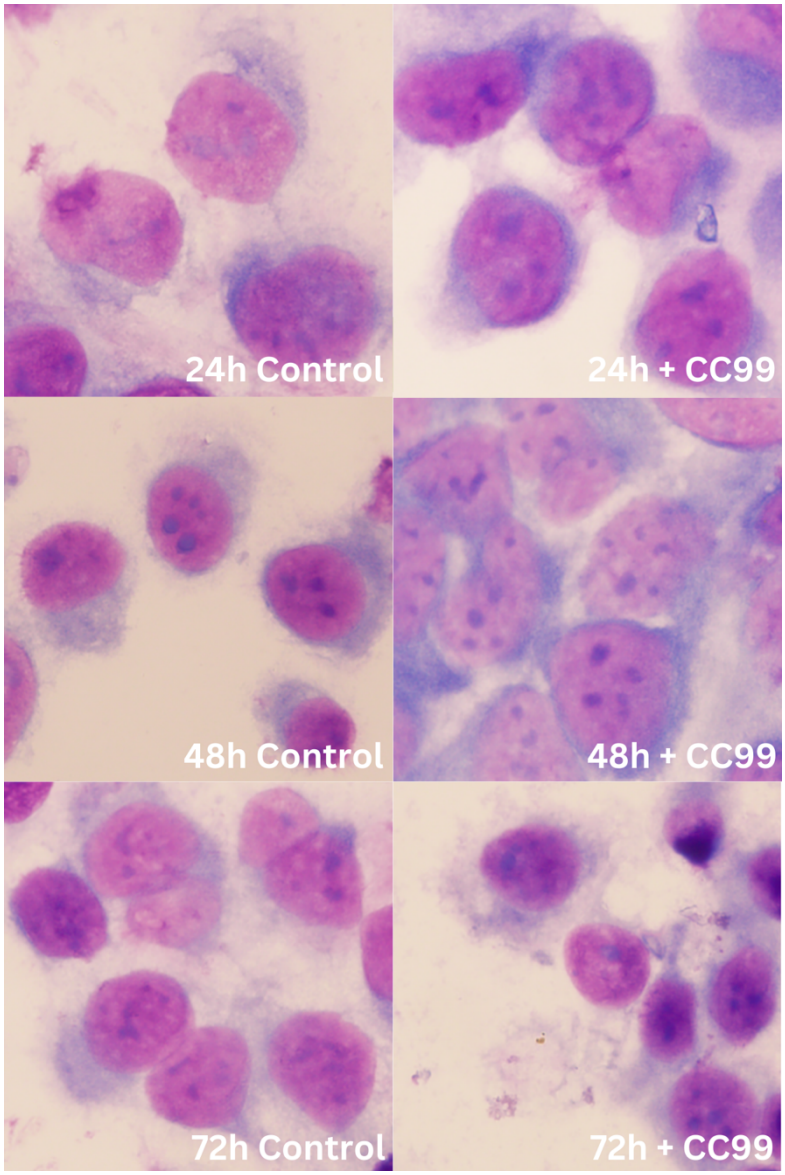


Figure 14. Giemsa stains to determine CC99 infectivity of HeLa cells infected with THP-1-grown CC99 bacteria: Trial 1. Giemsa stains of HeLa cells infected with bacterium CC99 grown in THP-1 cells. Representative Giemsa stains of control and infected cells from the 24-, 48-, and 72-hour timepoints do not show evidence of infection by CC99 bacteria.

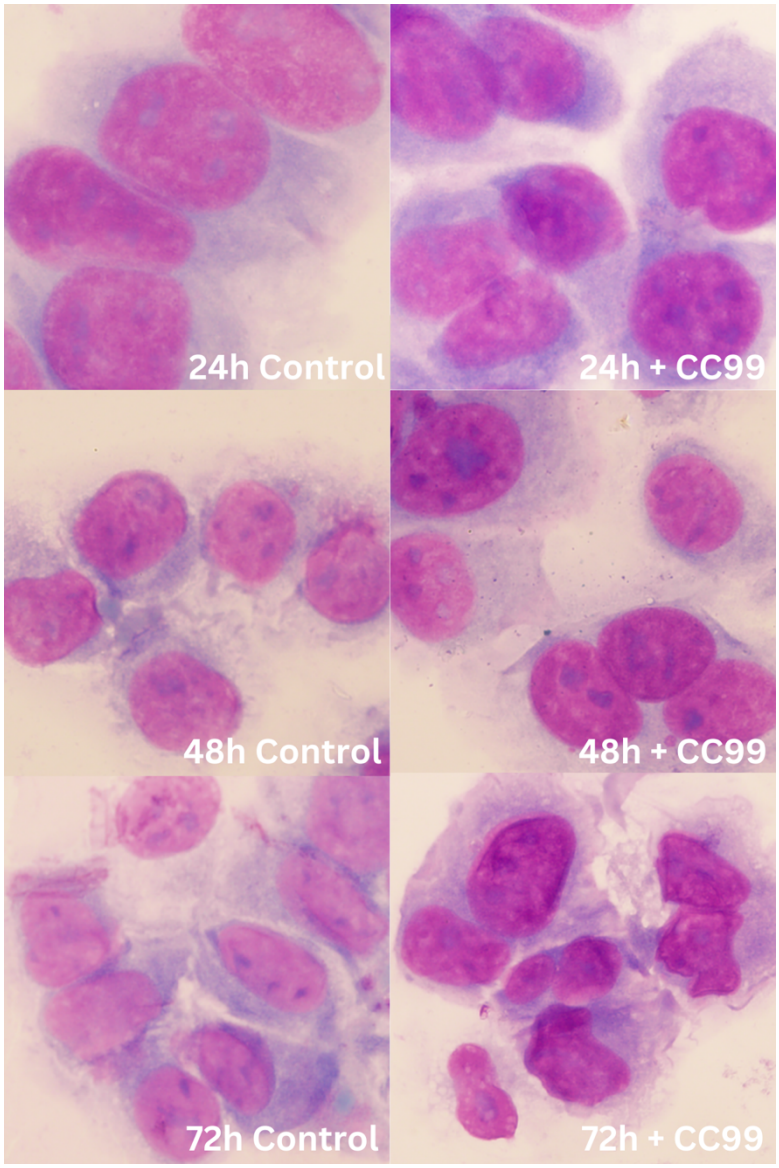


Figure 15. Giemsa stains to determine CC99 infectivity of HeLa cells infected with THP-1-grown CC99 bacteria: Trial 2. Giemsa stains of HeLa cells infected with bacterium CC99 grown in THP-1 cells. Representative Giemsa stains of control and infected cells from the 24-, 48-, and 72-hour timepoints do not show evidence of infection by CC99 bacteria.

Infection of THP-1 Cells with Lysates of CC99 Bacteria from HeLa Cells

To determine whether bacteria from non-phagocytic mammalian cells can be transmitted to other mammalian cells, CC99 from amoebae was grown in HeLa cells. CC99 bacteria were collected from the lysed HeLa cells for infection of both THP-1 and HeLa cells. Although intracellular killing of microbes is a function of phagocytic cells, and non-phagocytic HeLa cells do not have the membrane pathways to degrade bacteria, CC99 and other bacteria, including *C. burnetii* and *L. pneumophila*, can form membrane-bound bacteria-containing vacuoles within HeLa cells (Chamberlain et al. 2019, Garduño et al. 1998, Miller et al. 2019). For the current studies, infections using HeLa cells as the host for growth of CC99 (which had first lysed from amoebae) were performed to assess whether growth in constructed compartments in non-phagocytic cells would permit transmission to mammalian cells. HeLa cells which had been infected with CC99 from amoebae produced sufficient concentrations of CC99 for these treatments.

When infected with HeLa cell-derived CC99 or media alone, the mock-infected THP-1 cells remained at least 90% viable through 72 hours, whereas CC99-infected cells were only 79% and 88% viable in Trials 2 and 1, respectively, which was statistically significantly different when compared to mock-infected control cells (Fig. 16). Statistically significant decreases in viability of infected cells also occurred at 48 hours for cells in Trials 1 and 2. These decreases were similar to those observed for the infections using CC99 lysates from THP-1 cells, and as for those infections, the decrease in host viability could indicate that low levels of infection are occurring, that the bacterium, although not endocytosed by the HeLa cells, is producing molecules that may

be harmful to the HeLa cells, or that the presence of bacterial LPS in the HeLa cell environment might induce apoptotic cell death.

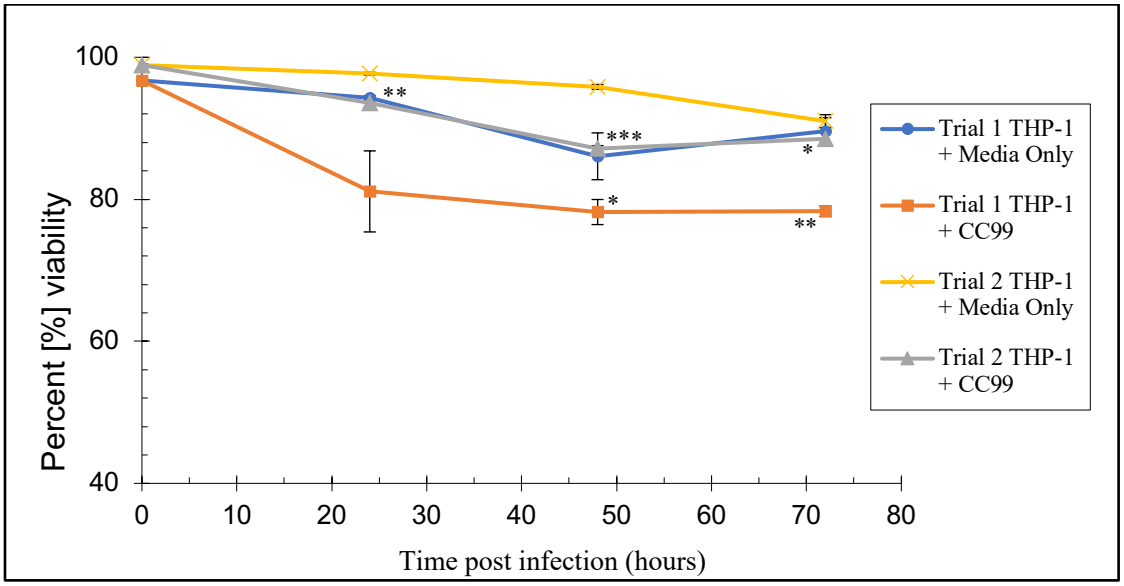


Figure 16. Viability of THP-1 cells infected with HeLa-grown CC99 bacteria: Trials 1-2. Trypan blue staining was used to assess viability of THP-1 cells infected with an MOI of 100 with a CC99 lysate from HeLa cells. Cells were infected with CC99 bacteria or media alone, after which time, supernatants were removed, cells gently washed, and fresh media added to the cells. This timepoint was considered time 0. At time 0 and subsequent timepoints, cells were removed from wells by gentle scraping, stained with trypan blue, and counted with a hemocytometer. Triplicate wells were counted for each timepoint. Statistical significance is indicated by *$p < 0.05$, **$p < 0.01$, *** $p < 0.001$.

Giemsa stains of HeLa cells infected with CC99 derived from HeLa cells showed no differences in the appearances of mock- or CC99-infected cells. Both infected and uninfected THP-1 cells looked as expected with pinkish-purple nuclei and light blue cytoplasm with no evidence of infection (Fig. 17 & 18). No infected cells were detected on any of the triplicate slides for each timepoint for either trial. When THP-1 cells treated with the CC99 lysate from HeLa cells were quantified by qPCR for CC99 DNA, no quantifiable CC99 DNA was detected in the samples (Appendices B9 & B10), leading to the conclusion that productive infection of THP-1 cells by CC99 did not occur when HeLa-lysed bacteria were used to infect THP-1 cells.

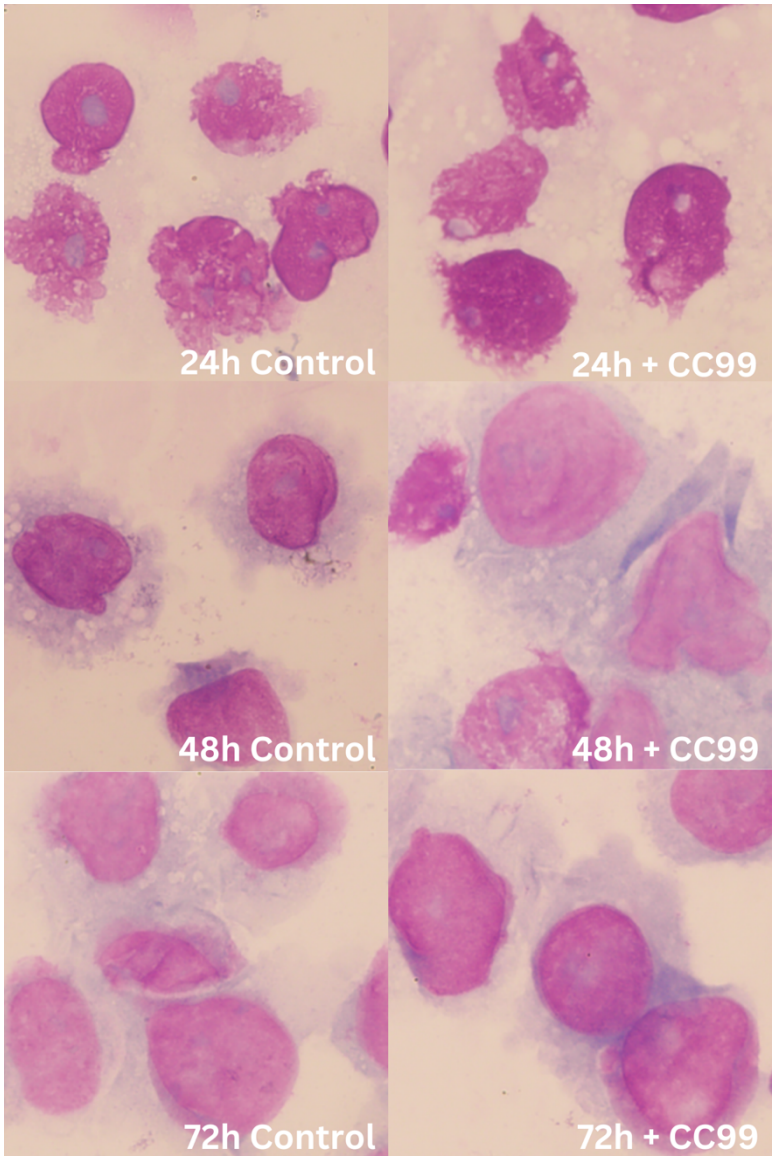


Figure 17. Giemsa stains to determine CC99 infectivity of THP-1 cells infected with HeLa-grown CC99 bacteria: Trial 1. Giemsa stains of THP-1 cells infected with bacterium CC99 grown in HeLa cells. Representative Giemsa stains of control and infected cells from the 24-, 48-, and 72-hour timepoints do not show evidence of infection by CC99 bacteria.

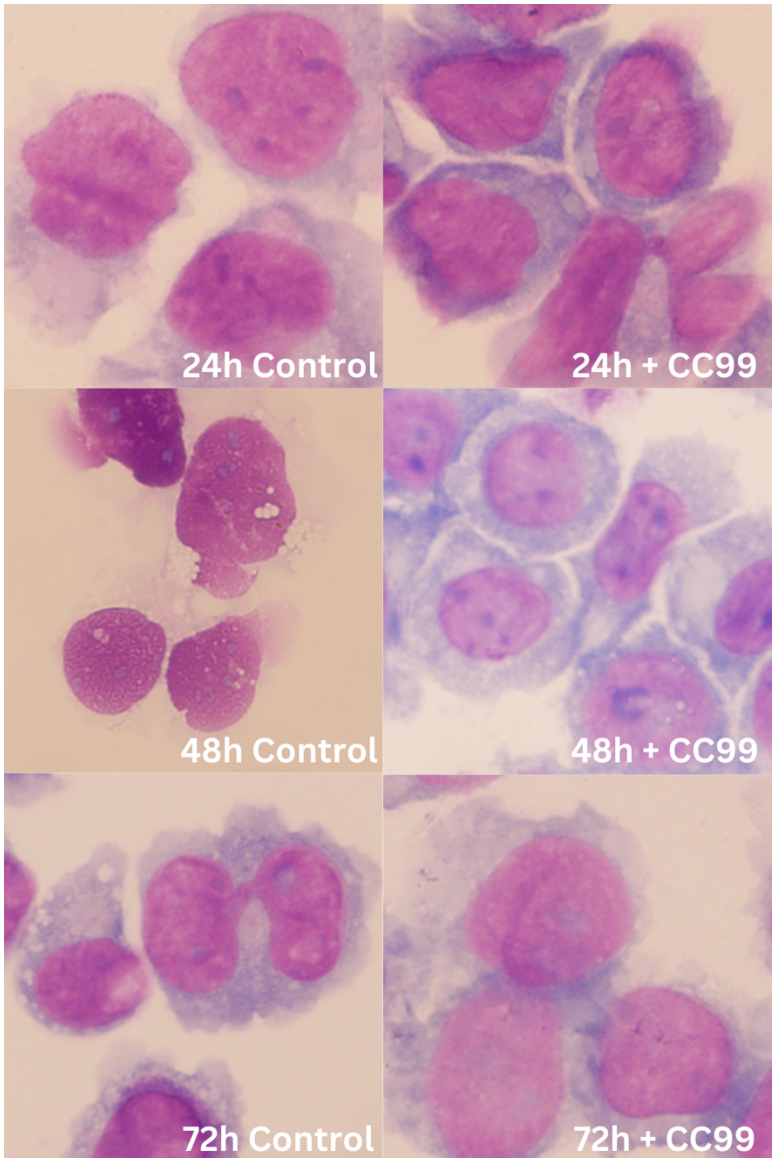


Figure 18. Giemsa stains to determine CC99 infectivity of THP-1 cells infected with HeLa-grown CC99 bacteria: Trial 2. Giemsa stains of THP-1 cells infected with bacterium CC99 grown in HeLa cells. Representative Giemsa stains of control and infected cells from the 24-, 48-, and 72-hour timepoints do not show evidence of infection by CC99 bacteria.

Infection of HeLa Cells with Lysates of CC99 Bacteria from HeLa Cells

To determine whether CC99 bacteria that had lysed from HeLa cells could enter and infect other HeLa cells, HeLa cells were infected with the HeLa-derived CC99 lysate. Infected HeLa cells were assessed for cell viability, the presence of intracellular CC99, and detectable CC99 DNA in the infected cells.

When infected with HeLa cell-derived CC99 or media alone, mock-infected HeLa cells remained 86-88% viable through 72 hours, whereas CC99-infected cells statistically significantly decreased to 73% and 77% viability (Fig. 19; Trials 1 & 2). Statistically significant decreases in cell viability also occurred for both Trials 1 and 2 at 24- and 48-hours post-treatment (Fig. 19). The decrease in viability indicated that infection may have occurred or that the LPS from the bacterial cell wall or other bacterial molecules were affecting cell viability. Giemsa staining and detection of CC99 by qPCR were next used to assess whether infection of the HeLa cells occurred.

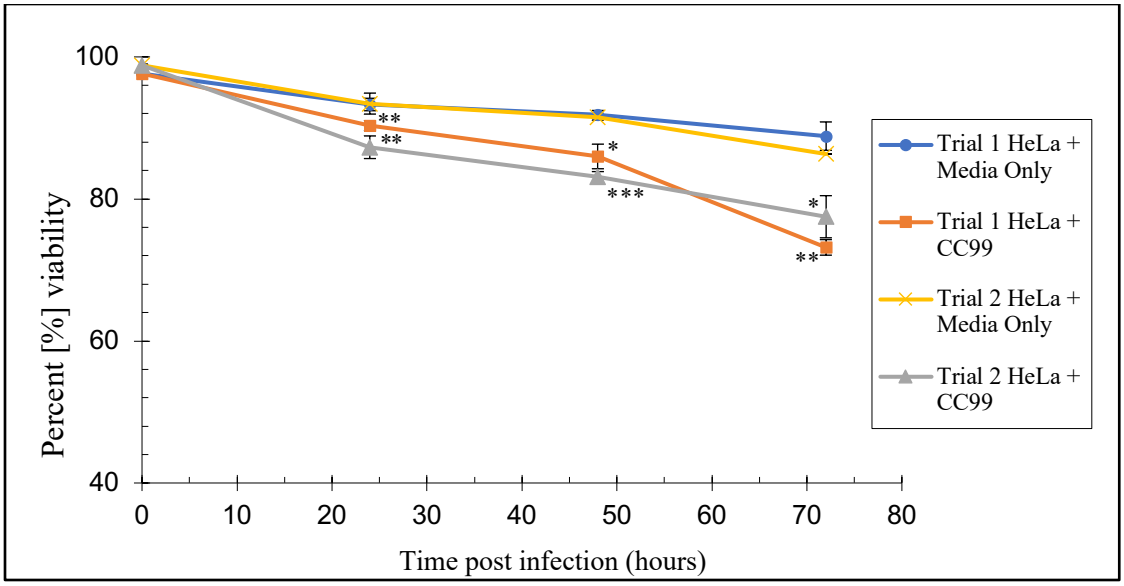


Figure 19. Viability of HeLa cells infection with HeLa-grown CC99 bacteria: Trials 1-2. Trypan blue staining was used to assess viability of THP-1 cells infected with an MOI of 100 with a CC99 lysate from HeLa cells. Cells were infected with CC99 bacteria or media alone, after which time, supernatants were removed, cells gently washed, and fresh media added to the cells. This timepoint was considered time 0. At time 0 and subsequent timepoints, cells were removed from wells by gentle scraping, stained with trypan blue, and counted with a hemocytometer. Triplicate wells were counted for each timepoint. Statistical significance is indicated by *$p < 0.05$, **p < 0.01, *** $p < 0.001$.

No differences between HeLa cells that were either mock-treated or infected with HeLa cell-derived CC99 were visible on Giemsa stains from replicate wells (Fig. 20 & 21). As with the cell infections uisng CC99 from THP-1 cell lysates (Fig. 13-19), this was a strong indication that CC99 may not be transmissible between mammalian cells. Furthermore, qPCR amplification of DNA from control and CC99 treatments did not result in amplification of CC99 DNA from any of the mock- or CC99-infected experimental samples. Samples from experimental wells with CC99-infected HeLa cells did not exhibit peaks of the expected melt temperature of 79.5 °C, although standard curve and positive control samples had appropriate melt peaks (Appendices B11 & B12).

Only lysates from *A. polyphaga* were able to replicate within THP-1 macrophage-like cells or HeLa epithelial cells. When lysates of CC99 bacteria derived from THP-1 or HeLa cells were used to infect either mammalian cell line, no productive CC99 infections in the mammalian cells were observed (Figure 22).

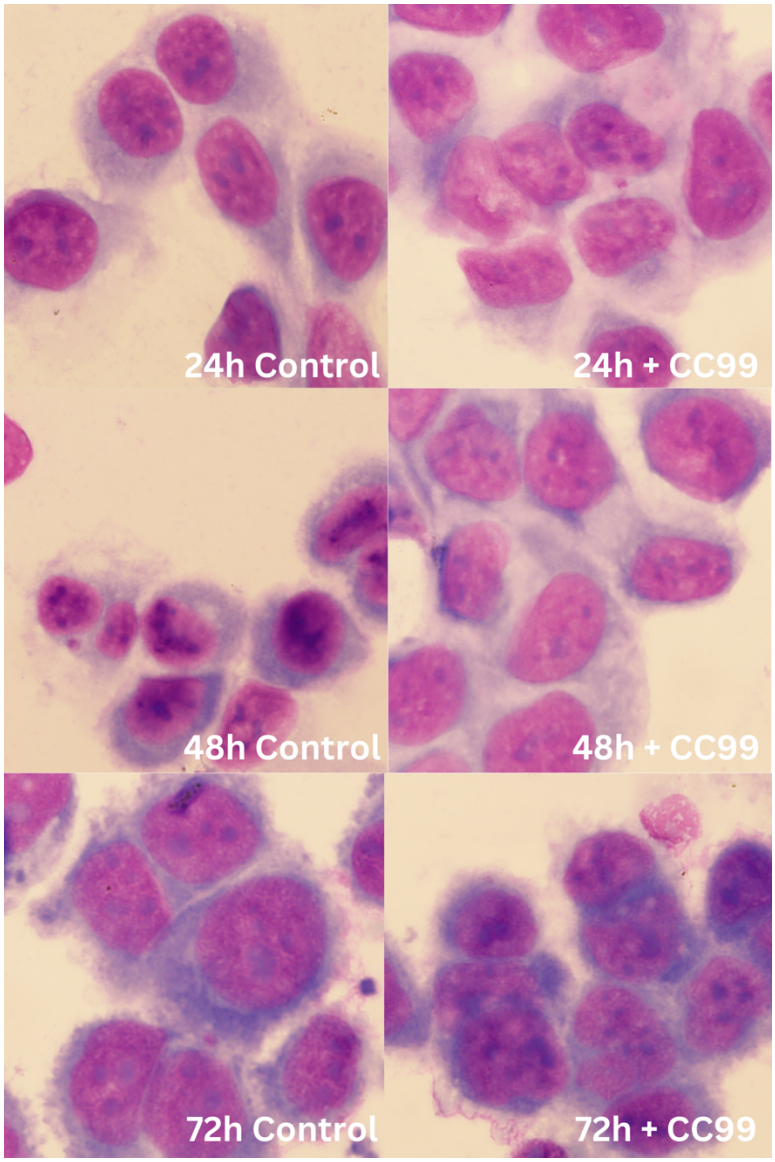


Figure 20. Giemsa stains to determine CC99 infectivity of HeLa cells infected with HeLa-grown CC99 bacteria: Trial 1. Giemsa stains of HeLa cells infected with bacterium CC99 grown in HeLa cells. Representative Giemsa stains of control and infected cells from the 24-, 48-, and 72-hour timepoints do not show evidence of infection by CC99 bacteria.

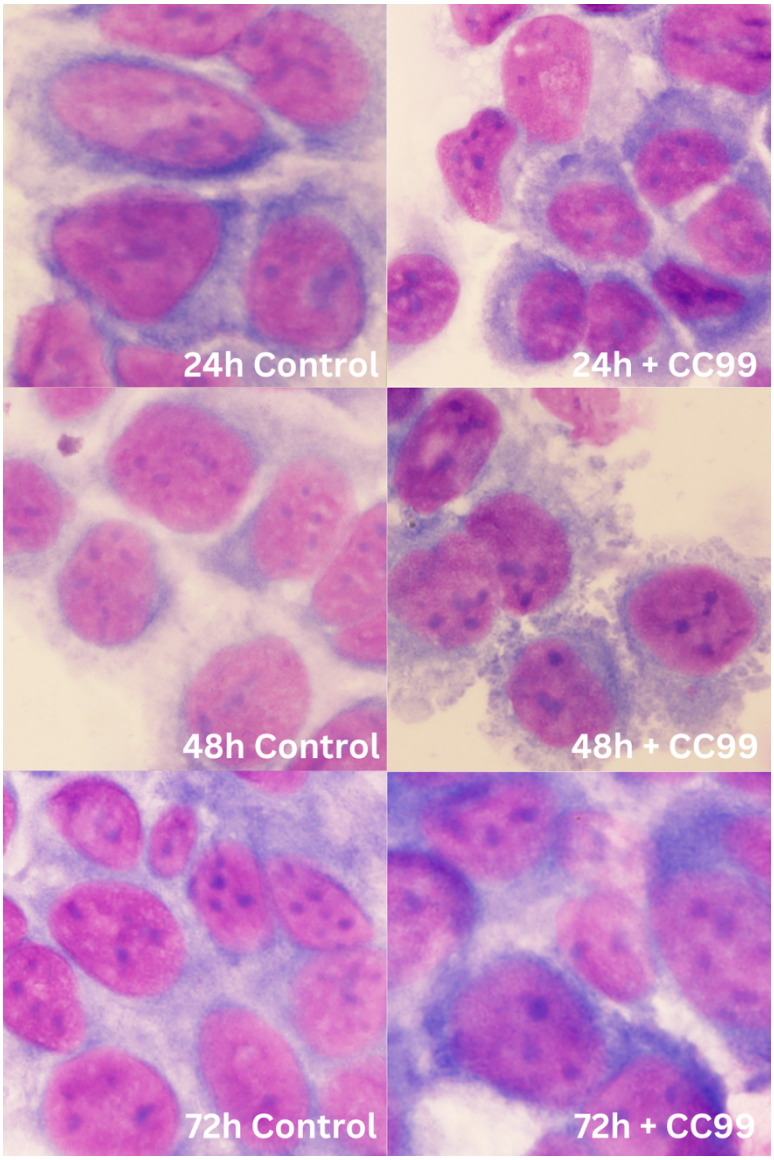


Figure 21. Giemsa stains to determine CC99 infectivity of HeLa cells infected with HeLa-grown CC99 bacteria: Trial 2. Giemsa stains of HeLa cells infected with bacterium CC99 grown in HeLa cells. Representative Giemsa stains of control and infected cells from the 24-, 48-, and 72-hour timepoints do not show evidence of infection by CC99 bacteria.

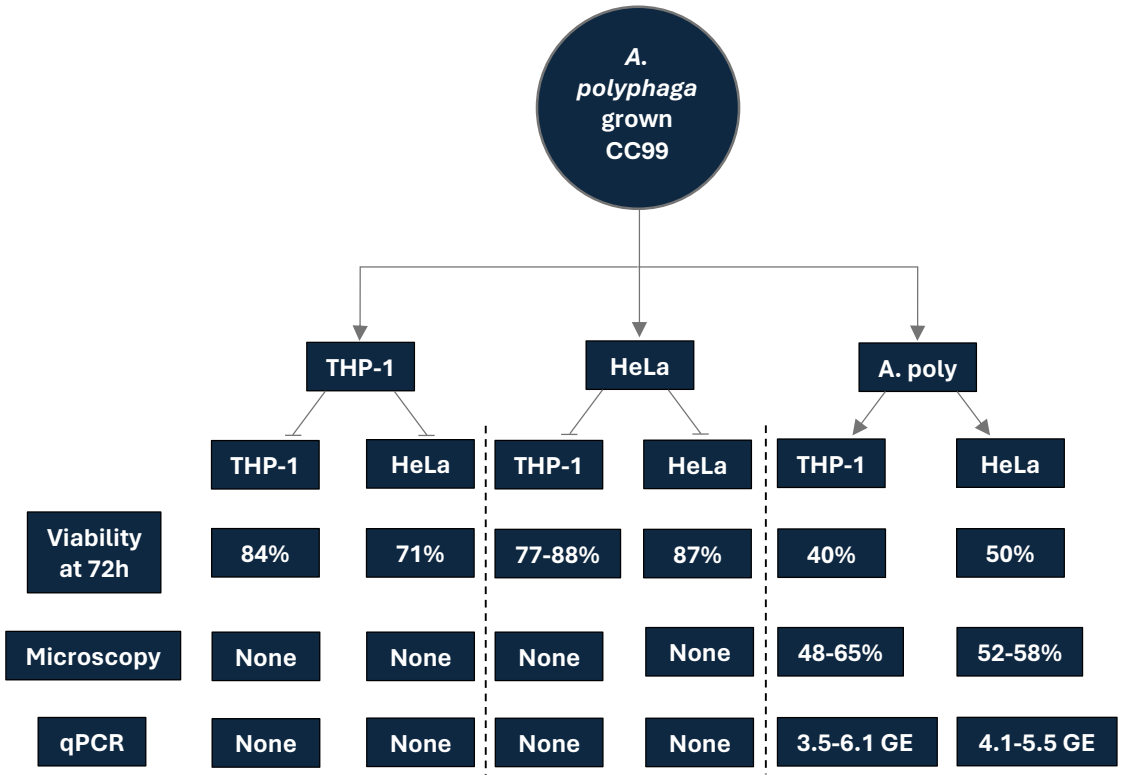


Figure 22. Overview of Host Cell and Bacterial Sources for Transmissibility Studies and Summary of Results.

Discussion

'*Ca.* Berkiella cookevillensis' is found in in the nuclei of both phagocytic and nonphagocytic mammalian cells (Chamberlain et al. 2019). Giemsa histochemical staining, trypan blue viability counts, and real-time qPCR showed *A. polyphaga*-lysed bacteria could infect differentiated THP-1 macrophages and HeLa epithelial cells.

This study expands on this foundational work by examining the transmission of infection when the infection particles are derived not from amoeba, but from the infected phagocytic or non-phagocytic mammalain cells. The initial studies for this project involved repeating treatments in both differentiated THP-1 macrophage-like cells and HeLa epithelial-like cells using a CC99 lysate from amoebae. CC99 lysates from *A. polyphaga* showed decreases in host cell viability and higher percentages of infection as assessed by Giemsa staining and qPCR for genomic bacterial DNA in both infected THP-1 and infected HeLa compared to mock-infected cells (Figures 3 and 7).

When CC99 from THP-1 cells were used to infect either THP-1 cells or HeLa cells, both cell types did exhibit statistically significant decreases in cell viability compared to the mock-treated cells by 72 hours post-treatment. This decrease in viability could be a result of infection of these cells by CC99; however, Giemsa stains and qPCR to detect CC99 bacteria and DNA, did not provide evidence of infection of either cell type by 72 hours post initial treatment. The decrease in cell viability could be due to '*Ca.* B. cookevillensis' being a Gram-negative bacterium and consequently has the toxin LPS (lipopolysaccharide) as part of its outer membrane. If the THP-1 macrophages can digest the bacteria, LPS is released as part of exocytosis. Exposure of THP-1 cells to LPS has been shown to induce apoptotic cell death (Monguio-Tortajada et al. 2018, Riendeau and

Kornfield 2003). It is also possible, that as the bacteria either attach to THP-1 macrophages or are engulfed and digested by the THP-1 cells, molecules that the bacteria secrete or utilize either prior to or during engulfment, such as pore-forming molecules, might damage the cells. Indeed, *C. burnetii* and *L. pneumophila* both have molecules with pore-forming activity against phagocytic cells (Banerjee-Bhatnagar et al. 1996, Kirby et al. 1998, Silveira and Zamboni 2010) suggesting that CC99 might have similar types of molecules. Analysis of recent whole transcriptome sequence data from THP-1 cells infected with CC99 bacteria might help to identify potential molecules.

HeLa cells, while not naturally phagocytic, might also be sensitive to the presence of LPS in the cellular environment (Santos et al. 2020) or their viability may be affected by molecules secreted by the extracellular CC99 bacteria. Although CC99 may not be able to grow and divide extracellularly, the bacteria remain viable for up to three months in only a sterile spring water environment (Mehari et al. 2016). Thus, although not dividing, it is possible that CC99 bacteria secrete molecules that may be toxic or inhibitory to mammalian cells, such as for the related bacterium, *L. pneumophila,* which uses a type II secretion system to secrete degradative enzymes when in an extracellular or adverse environment (Soderberg et al. 2008).

The final set of infections utilized CC99 derived from HeLa cells to infect THP-1 and HeLa cells. Just as with the THP-1-derived CC99 lysates, Giemsa stains, cell viability counts, and qPCR quantification showed that no productive infection with CC99 occurred over 72 hours for either cell type. As before for the THP-1-derived CC99, there was a decrease in cell viability, but as described above, this may have been the result of LPS toxicity or other molecules associated with or secreted by CC99 bacteria.

The inability of bacterium CC99 derived from mammalian cells (THP-1 and HeLa cells) to produce infection in other mammalian host cells is intriguing. One possibility is that differences in intracellular growth compartments between amoebae and mammalian cells alter gene expression for virulence-related proteins. Transcriptome analysis of RNA from bacteria grown in *A. polyphaga* and mammalian hosts would provide a list of differentially expressed genes and the pathways to investigate.

Genetic variation in the CC99 bacterium should also be investigated. As the CC99 bacteria are passed between amoeba and mammalian cell lines, variants that can either restrict or permit replication in the mammalian cells can arise. For many bacteria, serial passage can result in decreased virulence (Duanguari et al. 2020). For example, in a study using forced evolution of *L. pneumophila* for growth in murine macrophages, spontaneous mutations arose in *L. pneumophila* that resulted in improved fitness within the macrophages (Ensminger et al. 2012). This evolution of genetic variants was within a relatively short time of 180 days. It is possible that the frequent passage of CC99 bacteria within amoebae may have selected for variants unable to be transmitted between mammalian cells. Genomic sequence comparisons of CC99 that have been frequently passed through amoebae in the laboratory setting should be compared to the sequence of the initially isolated bacterium. Likewise, it may be possible to select for bacteria that can grow continuously by passage through macrophage cell lines.

Bacterium CC99 shares more genomic similarity with the bacterium *C. burnetii* (~90%) compared to *L. pneumophila* (~88%), and whole genome sequencing originally placed CC99 within the family Legionellaceae and order Legionalles along with *Legionella* spp. and *C. burnetii*. However, more recent genomic analyses by the Farone

lab and others have placed CC99 either in a deeper branching family or order within the Gammaproteobacteria (Hugoson et al. 2022, Saini and Gupta 2021), suggesting that the Berkiella species are ancestral to Legionella and Coxiella, whereas another Gammaproteobacterial intracellular pathogen, Francisella, diverged prior to Berkiella.

The ancestral lineage from Francisella to '*Ca.* Berkiella' to Legionella and Coxiella alludes to insect vectors as a possible vector for the spread of bacterium CC99 and is currently being investigated. The bacterium *Coxiella burnetii* is an intracellular bacterium responsible for the zoonotic disease, Q (query) fever. It is acquired via ticks or inhalation of aerosols from infected animals, but human-to-human transmission is rare and although it can survive within amoebae, it has limited replication in those cells in vitro (Eldin et al. 2017). Other obligate or facultative intracellular parasites are also transmitted by insect vectors, including the Gammaproteobacterium, *Francisella tularensis*. *Francisella* transmission to humans can occur through arthropod or insect vectors, by ingestion of contaminated food, or by inhalation of aerosolized bacteria. *Francisella* spp. can also resist digestion by amoebae (Hennebique et al. 2021). However, as for both Legionella and Coxiella, human-to-human transmission of *Franscisella* spp. has not been documented (Celli and Zahrt 2013).

Although these present studies have shown that CC99 is not likely transmitted directly between human cell lines and thus may not transmitted person-to-person, there is the possibility, that like Legionella, Coxiella, and Francisella, CC99 could be transmitted via aerosols or may be able to infect cells of other mammals or vertebrates. Because bacterium CC99 is not yet culturable, the development of an axenic growth medium will be critical to studying this bacterium at the genetic level and determining whether this

bacterium is associated with disease. Also, as global warming and climate change progress, concentrations of amoebae and their associated pathogens may increase as water evaporates. As temperatures increase globally, the need for human-constructed cooling systems, which harbor both amoebae and their intracellular pathogens, will also increase. A better understanding of the intracellular pathogens that thrive in amoebae will be important for protecting human health. Conversely, if '*Ca.* Berkiella spp.' are not pathogenic for humans or other animal species, their anti-amoebal activity may be exploited as a mechanism to control amoebae growth.

References

Bandyopadhyay P, Xiao H, Coleman HA, Price-Whelan A, Steinman HM. 2004. Icm/Dot-independent entry of *Legionella pneumophila* into amoeba and macrophage hosts. Infection and Immunity **72:** 4541–4551.

Banerjee-Bhatnagar N, Bolt CR, Williams JC. 1996. Pore-forming activity of *Coxiella burnetii* outer membrane protein oligomer comprised of 29.5- and 31-kDa polypeptides. Inhibition of porin activity by monoclonal antibodies 4E8 and 4D6. Annals of the New York Academy of Science **791**:378-401.

Brennan RE, Samuel JE. 2003. Evaluation of *Coxiella burnetii* antibiotic susceptibilities by real-time PCR assay. Journal of Clinical Microbiology: **41**:1869-74.

Burette M, Bonazzi M. 2020. From neglected to dissected: how technological advances are leading the way to the study of *Coxiella burnetii* pathogenesis. Cellular Microbiology **22**.

Celli J, Zahrt TC. 2013. Mechanisms of *Francisella tularensis* intracellular pathogenesis. Cold Spring Harbor Perspectives in Medicine **3**: a010314.

Chamberlain NB, Mehari YT, Hayes BJ, Roden CM, Kidane DT, Swehla AJ, Lorenzana-DeWitt MA, Farone AL, Gunderson JH, Berk SG, Farone MB. 2019. Infection and nuclear interaction in mammalian cells by '*Candidatus* Berkiella cookevillensis', a novel bacterium isolated from amoebae. BMC Microbiology **19**:91.

Chanput W, Mes JJ, Wichers HJ. 2014. THP-1 cell line: An in vitro cell model for immune modulation approach. International Immunopharmacology **23**:37–45.

Chauhan D, Shames S. 2021 Pathogenicity and virulence of Legionella: intracellular replication and host response. Virulence, **12**: 1122-1144.

Cirillo JD, Cirillo SL, Yan L, Bermudez LE, Falkow S, Tompkins LS. 1999. Intracellular growth in *Acanthamoeba castellanii* affects monocyte entry mechanisms and enhances virulence of *Legionella pneumophila*. Infection and Immunity **67**:4427–4434.

Coleman SA, Fischer ER, Howe D, Mead DJ, Heinzen RA. 2004. Temporal analysis of *Coxiella burnetii* morphological differentiation. Journal of Bacteriology **186**:7344–7352

Duangurai T, Reamtong O, Rungruengkitkun A, Srinon V, Boonyuen U, Limmathurotsakul D, Chantratita N, Pumirat P. 2020. In vitro passage alters virulence, immune activation and proteomic profiles of *Burkholderia pseudomallei*. Scientific Reports **10:**8320.

Eldin C, Mélenotte C, Mediannikov O, Ghigo E, Million M, Edouard S, Mege J, Maurin M, Raoult D. 2017. From Q Fever to *Coxiella burnetii* Infection: a Paradigm Change. Clinical Microbiology Reviews **30**.

Ensminger AW, Yassin Y, Miron A, Isberg RR. 2012. Experimental evolution of *Legionella pneumophila* in mouse macrophages leads to strains with altered determinants of environmental survival. PLoS Pathogens **8**: e1002731.

Fountoulakis M, Tsangaris G, Oh J-eun, Maris A, Lubec G. 2004. Protein profile of the Hela cell line. Journal of Chromatography A **1038**:247–265.

Garduño RA, Quinn FD, Hoffman PS. 1998. HeLa cells as a model to study the invasiveness and biology of *Legionella pneumophila*. Canadian Journal of Microbiology, **44**:430-440.

Greub G, Raoult D. 2004. Microorganisms Resistant to Free-Living Amoebae. Clinical Microbiology Reviews **17**: 413–433.

Hartmann C, Herling L, Hartmann A, Köckritz V, Fuellen G, Walter M, Hermann A. 2023. Systematic estimation of biological age of in vitro cell culture systems by an age-associated marker panel. Frontiers in Aging, **4.**

Hennebique A, Peyroux J, Brunet C, Martin A, Henry T, Knezevic M, Santic M, Boisset S, Maurin M. 2021. Amoebae can promote the survival of *Francisella* species in the aquatic environment. Emerging Microbes and Infections, **10**:277-290.

Hugoson E, Guliaev A, Ammunét T, Guy L. 2022. Host adaptation in Legionellales is 1.9 Ga coincident with eukaryogenesis. Molecular Biology and Evolution **39**: msac037.

Khweek AA, Amer A. 2010. Replication of *Legionella pneumophila* in human cells: why are we susceptible? Frontiers in Microbiology **1.**

Kidane DT, Mehari YT, Rice FC, Arivett BA, Gunderson JH, Farone AL, Farone MB. 2022. The inside scoop: Comparative genomics of two intranuclear bacteria, "*Candidatus* Berkiella cookevillensis" and "*Candidatus* Berkiella aquae. PLoS One **17:** e0278206-e0278206.

Kirby JE, Vogel JP, Andrews HL, Isberg RR. 1998. Evidence for pore-forming ability by *Legionella pneumophila*. Molecular Microbiology **27:** 323-336.

Lafontaine DLJ, Riback JA, Bascetin R, Brangwynne CP. 2021. The nucleolus as a multiphase liquid condensate. Nature Reviews Molecular Cell Biology **22**: 165-182.

Marciano-Cabral F, Cabral G. 2003. *Acanthamoeba* spp. as agents of disease in humans. Clinical Microbiology Reviews **16**: 273–307.

Mehari YT, Arivett BA, Farone AL, Gunderson JH, Farone MB. 2016. Draft genome sequences of two novel amoeba-resistant intranuclear bacteria, "*Candidatus* Berkiella cookevillensis" and "*Candidatus* Berkiella aquae." Genome Announcements **4**: e01732-15.

Mehari YT, Jason Hayes B, Redding KS, Mariappan PV, Gunderson JH, Farone AL, Farone MB. 2016. Description of '*Candidatus* Berkiella aquae' and '*Candidatus* Berkiella cookevillensis', two intranuclear bacteria of freshwater amoebae. International Journal of Systematic and Evolutionary Microbiology **66**: 536–541.

Miller HE, Hoyt FH, Heinzen RA. 2019. Replication of *Coxiella burnetii* in a lysosome-like vacuole does not require lysosomal hydrolases. Infection and Immunity, **87**:10.1128/iai.00493-19.

Molmeret, M, Horn M, Wagner M, Santic M, Abu Kwaik Y. (005. Amoebae as training grounds for intracellular bacterial pathogens. Applied and Environmental Microbiology **71**: 20–28.

Monguió-Tortajada M, Franquesa M, Sarrias MR, Borras FE. 2018. Low doses of LPS exacerbate the inflammatory response and trigger death on TLR3-primed human monocytes. Cell Death and Disease **9**: 499.

Newton HJ, Ang DK, van Driel IR, Hartland EL. 2010. Molecular pathogenesis of infections caused by *Legionella pneumophila*. Clinical Microbiology Reviews **23**:274–298.

Ogata H, La Scola B, Audic S, Renesto P, Blanc G, Robert C, Fournier P-E, Claverie J-M, Raoult D. 2006. Genome sequence of *Rickettsia bellii* illuminates the role of amoebae in gene exchanges between intracellular pathogens. PLoS Genetics **2**: e76.

Park EK, Jung HS, Yang HI, Yoo MC, Kim C, Kim KS. 2007. Optimized THP-1 differentiation is required for the detection of responses to weak stimuli. Inflammation Research **56**:45–50.

Qiu J, Luo Z-Q. 2017. *Legionella* and *Coxiella* effectors: strength in diversity and activity. Nature Reviews Microbiology **15**:591–605.

Raoult, D. (2010). Q fever, free amoeba, and air conditioning. Clinical Infectious Diseases, **51**: 869–870.

Riendeau CJ, Kornfeld H. 2003. THP-1 cell apoptosis in response to Mycobacterial infection. Infection and Immunity. **71:** 254-259.

Saini N, Gupta RS. 2021. A robust phylogenetic framework for members of the order Legionellales and its main genera (Legionella, Aquicella, Coxiella and Rickettsiella) based on phylogenomic analyses and identification of molecular markers demarcating different clades. Antonie Van Leeuwenhoek **4**: 957-982.

Santos JC, Boucher D, Schneider LK, Demarco B, Dilucca M, Shkarina K, Heilig R, Chen KW, Lim RYH, Broz P. 2020. Human GBP1 binds LPS to initiate assembly of a caspase-4 activating platform on cytosolic bacteria. Nature Communications **11**: 3276.

Schulz F, Horn M. 2015. Intranuclear bacteria: Inside the cellular control center of Eukaryotes. Trends in Cell Biology **25**: 339–346.

Schulz F, Lagkouvardos I, Wascher F, Aistleitner K, Kostanjšek R, Horn M. 2014. Life in an unusual intracellular niche: A bacterial symbiont infecting the nucleus of amoebae. The ISME Journal **8**: 1634–1644.

Segal G, Feldman M, Zusman T. 2005. The ICM/dot type-IV secretion systems of *Legionella pneumophila* and *Coxiella burnetii*. FEMS Microbiology Reviews **29**: 65–81.

Silveira TN, Zamboni DS. 2010. Pore formation triggered by *Legionella* spp. is an Nlrc4 inflammasome-dependent host cell response that precedes pyroptosis. Infection and Immunity **78**: 1403-13.

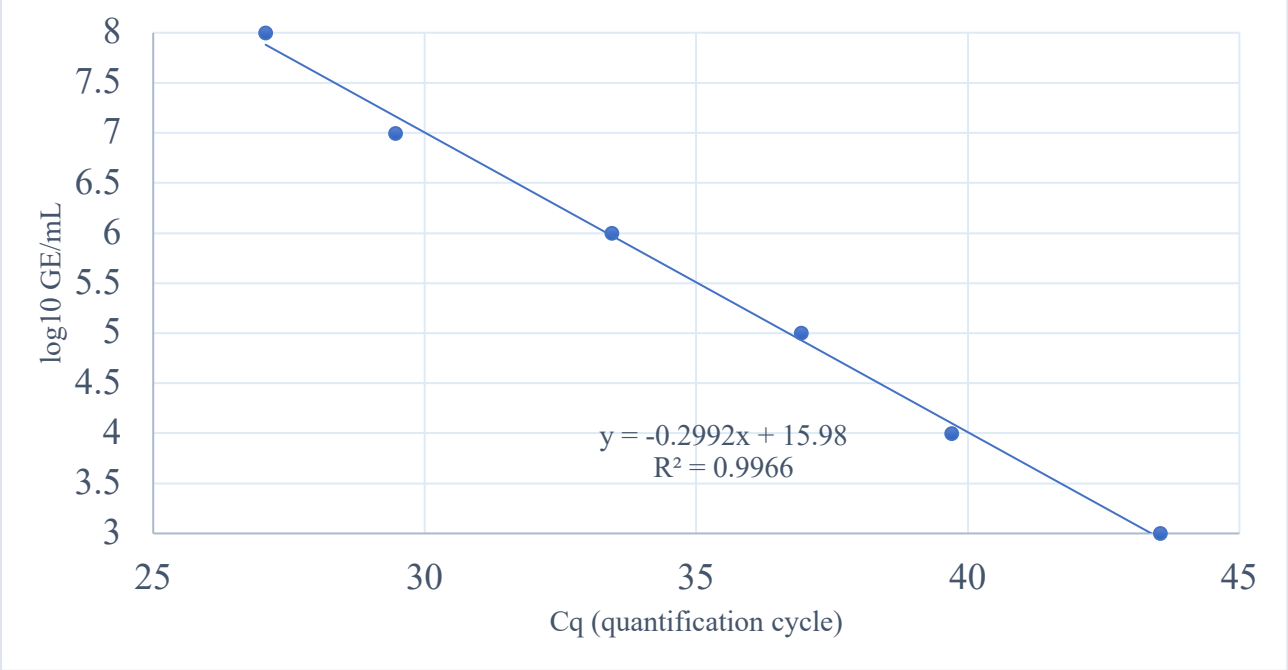
Soderberg MA, Dao J, Starkenburg SR, Cianciotto NP. 2008. Importance of type II secretion for survival of *Legionella pneumophila* in tap water and in amoebae at low temperatures. Applied and Environmental Microbiology **74**: 5583–5588.

Swanson MS, Hammer BK. 2000. *Legionella pneumophila* pathogenesis: A fateful journey from amoebae to macrophages. Annual Review of Microbiology **54**: 567–613.

Thomas DR, Newton P, Lau N, Newton HJ. 2020. Interfering with autophagy: the opposing strategies deployed by *Legionella pneumophila* and *Coxiella burnetii* effector proteins. Frontiers in Cellular and Infection Microbiology, **10.**

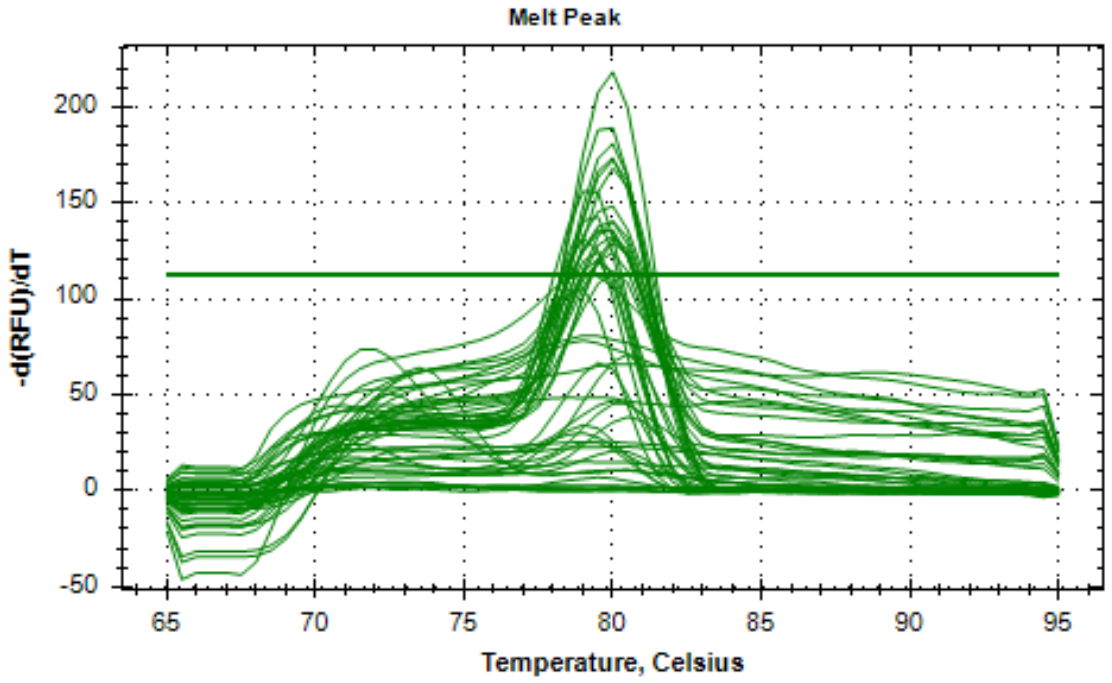
Zusman T, Yerushalmi G, Segal G. 2003. Functional similarities between the *ICM/dot* pathogenesis systems of *Coxiella burnetii* and *Legionella pneumophila*. Infection and Immunity **71**: 3714–3723.

Appendix A: Representative Standand Curve for Real-time qPCR Assays to Quantify Concentrations of '*Candidatus* Berkiella cookevillensis' (Strain CC99) Bacteria in Mammalian Cells

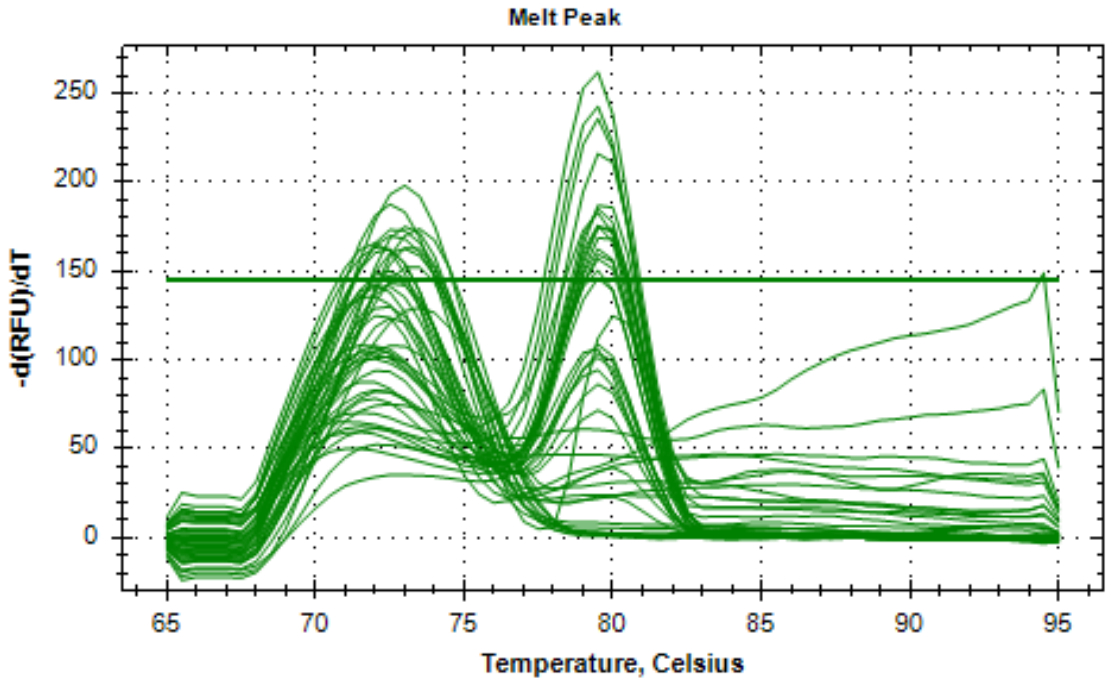


Appendix A1. Representative standard curve for quantification of genomic equivalents/mL of bacterium CC99 by real-time qPCR.

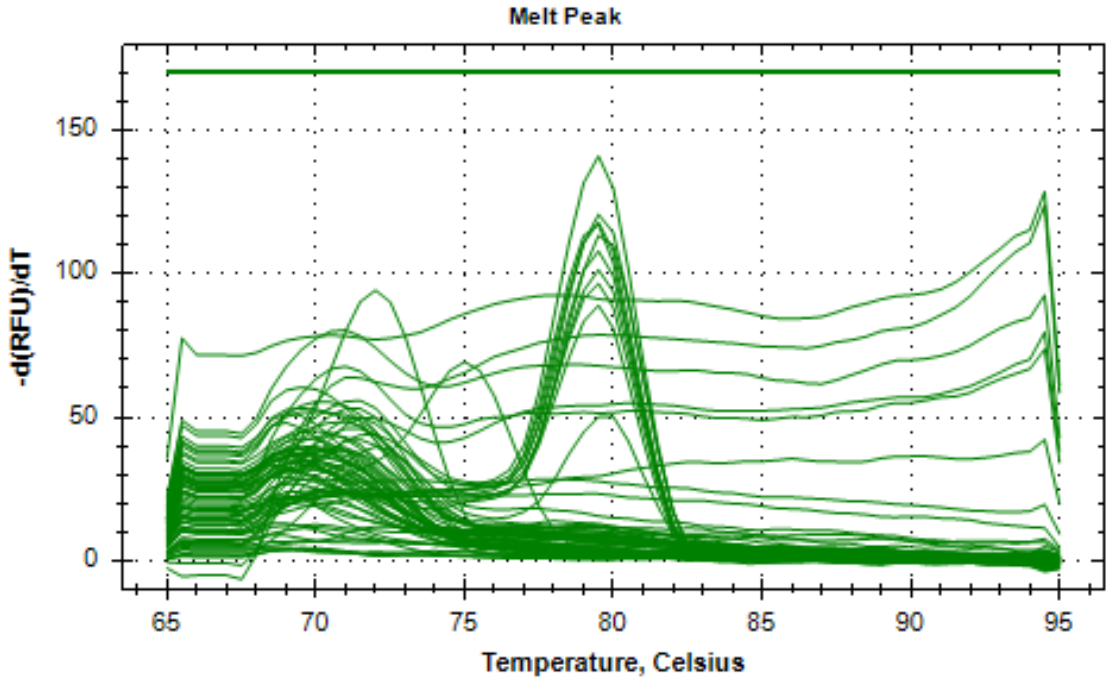
Appendix B: Melt Curves for Real-time qPCR Assays to Quantify Concentrations of '*Candidatus* Berkiella cookevillensis' (Strain CC99) Bacteria in Mammalian Cells



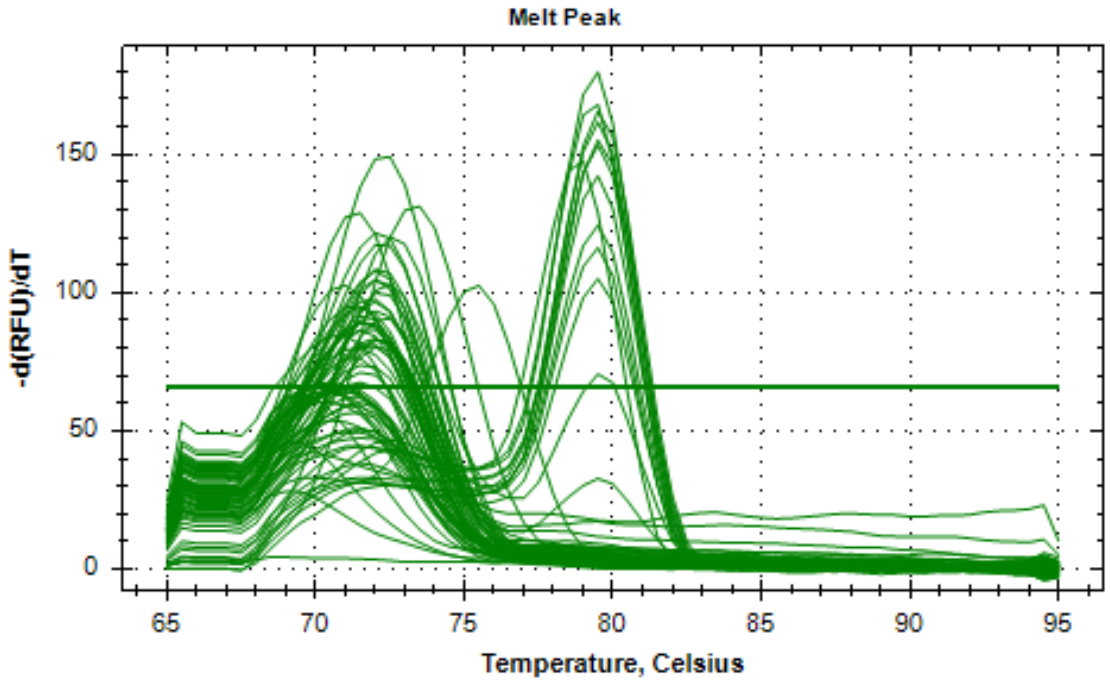
Appendix B1. *A. polyphaga* to THP-1 Trial 1 qPCR melt temperature graph.



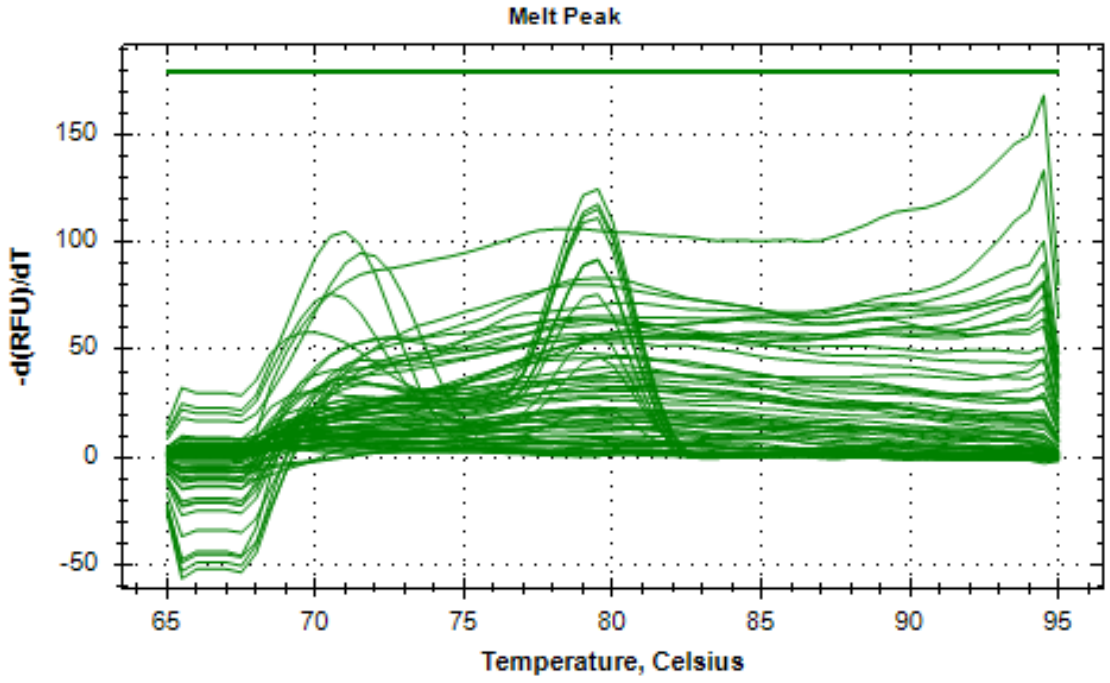
Appendix B2. *A. polyphaga* to THP-1 Trial 2 qPCR melt temperature graph.



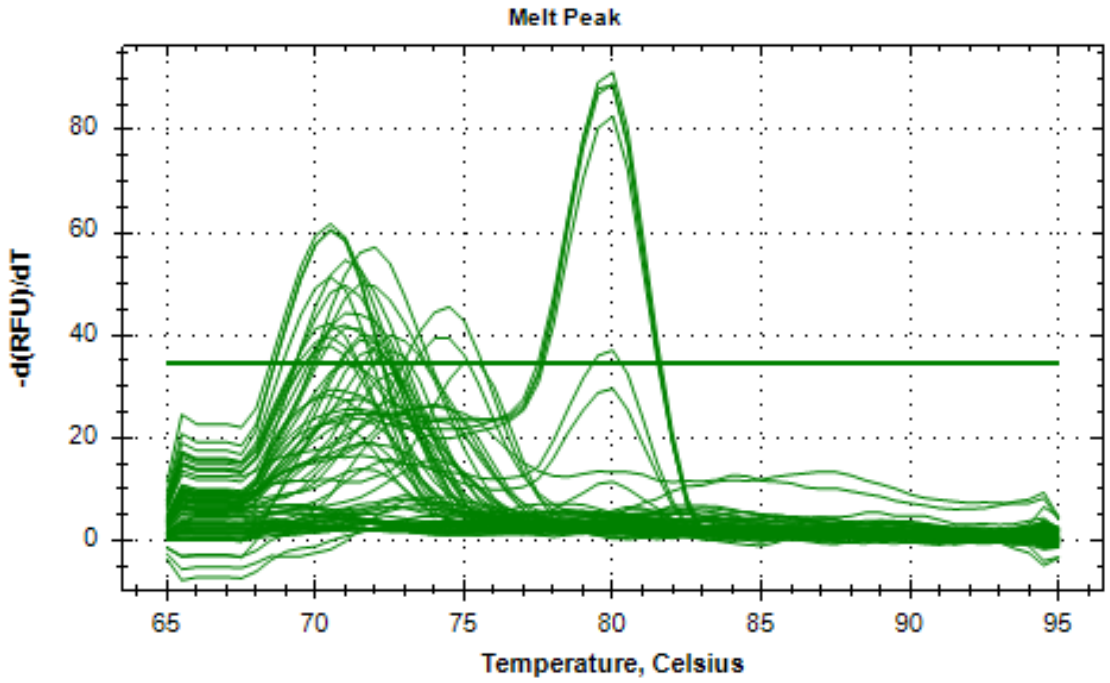
Appendix B3. *A. polyphaga* to HeLa Trial 1 qPCR melt temperature graph.



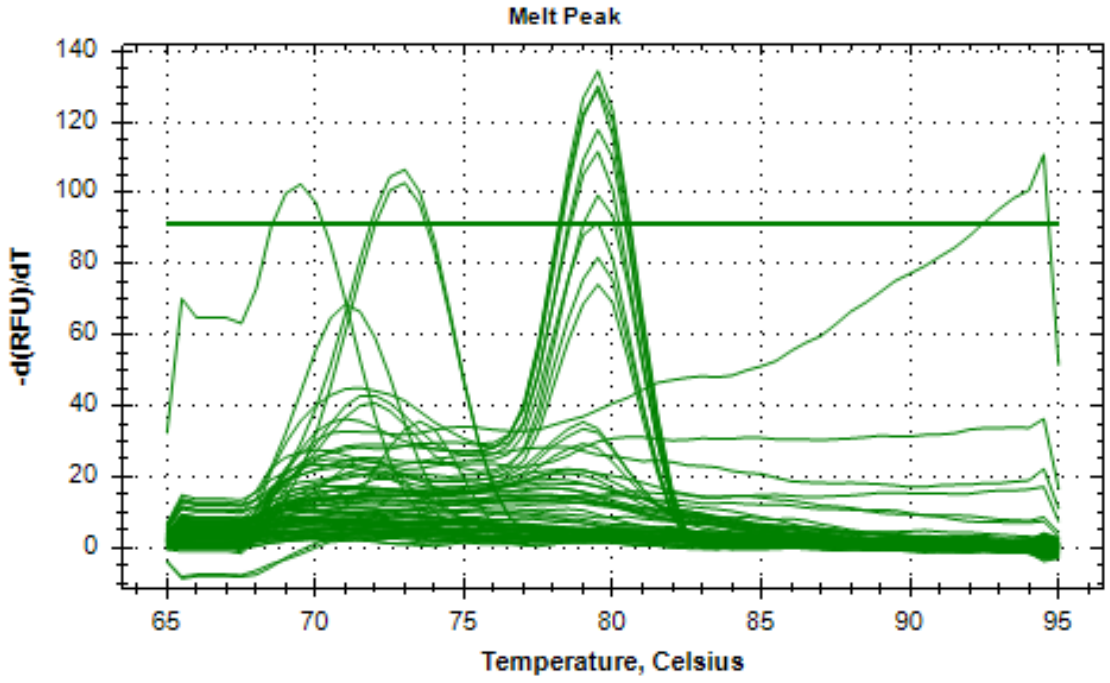
Appendix B4. *A. polyphaga* to HeLa Trial 2 qPCR melt temperature graph.



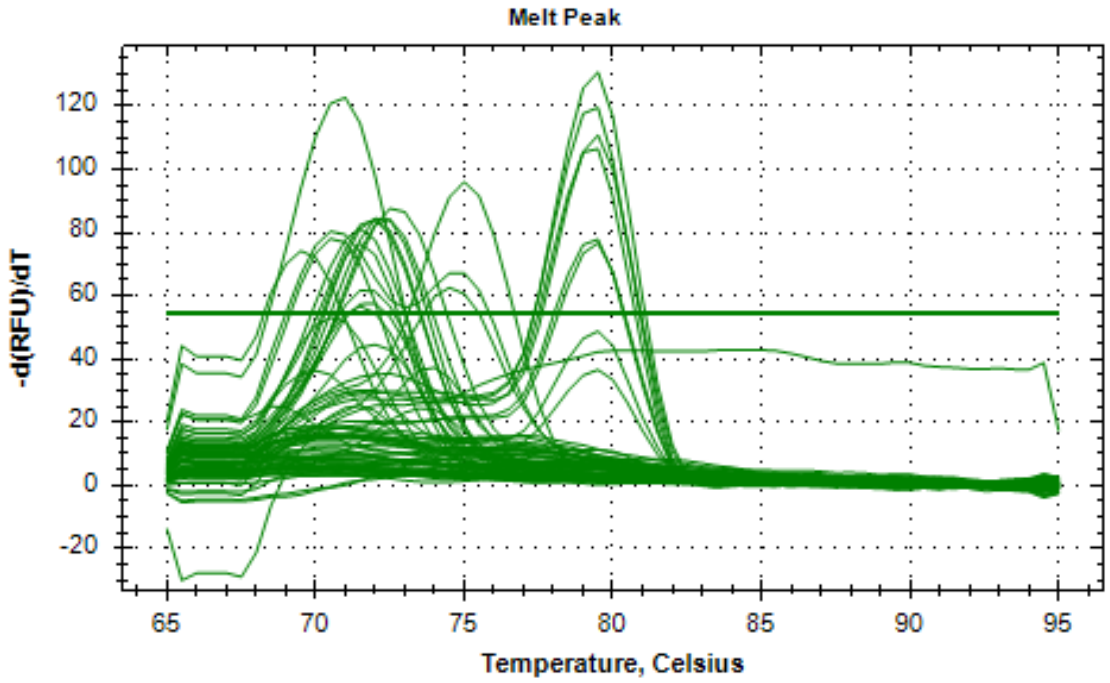
Appendix B5. THP-1 to THP-1 Trial 1 qPCR melt temperature graph.



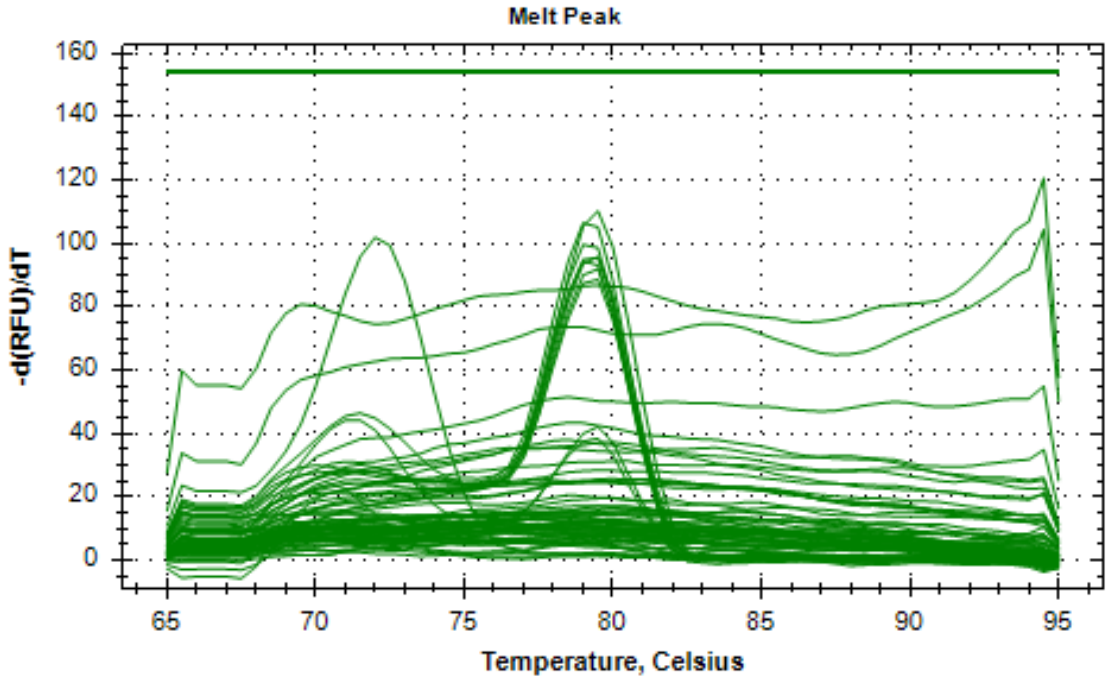
Appendix B6. THP-1 to THP-1 Trial 2 qPCR melt temperature graph.



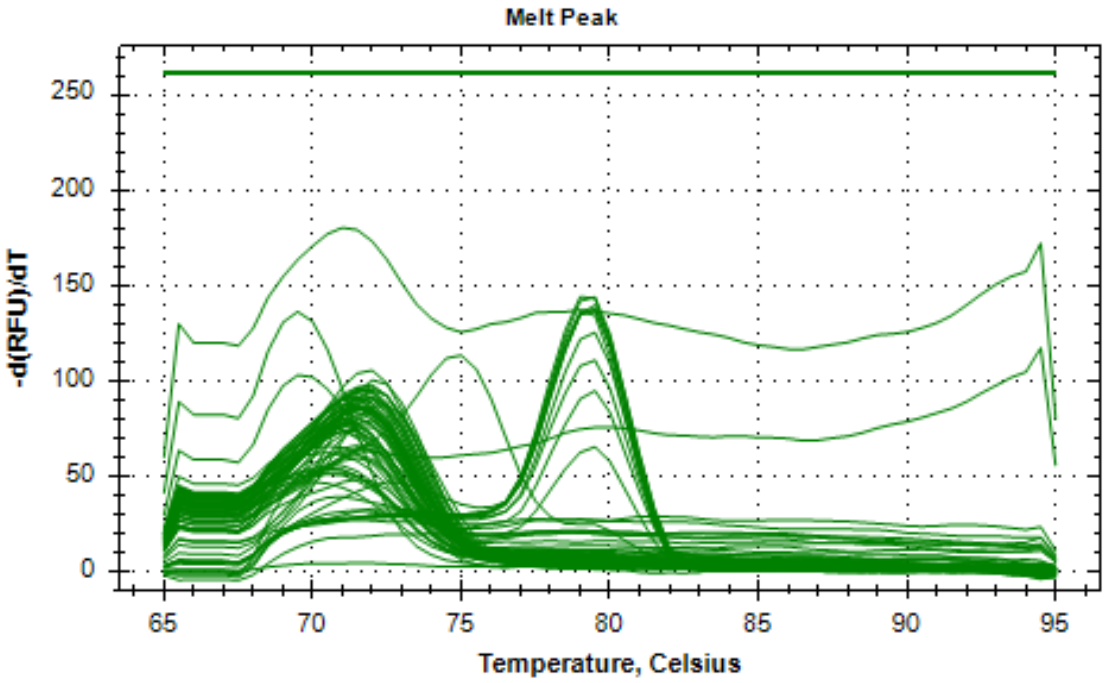
Appendix B7. THP-1 to HeLa Trial 1 qPCR melt temperature graph.



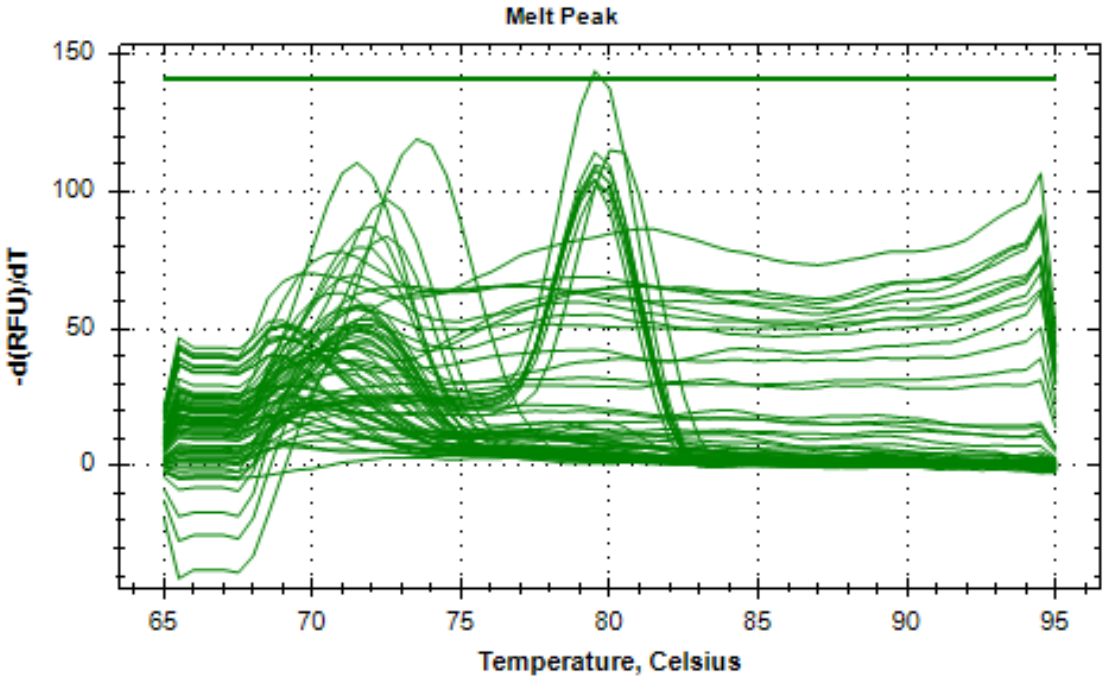
Appendix B8. THP-1 to HeLa Trial 2 qPCR melt temperature graph.



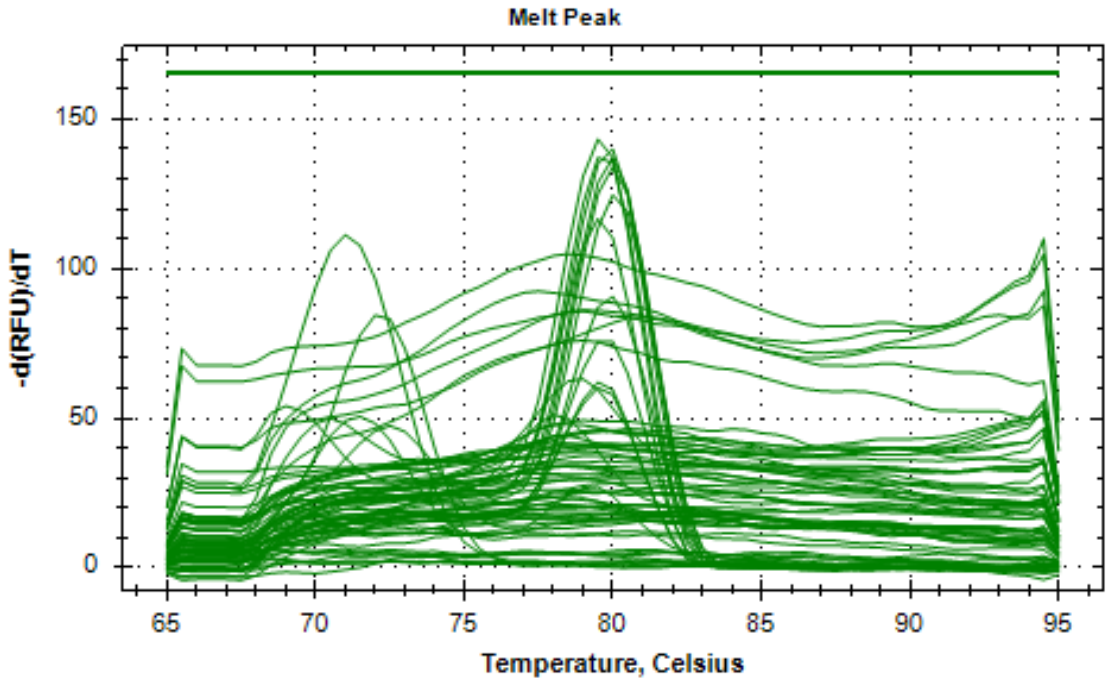
Appendix B9. HeLa to THP-1 Trial 1 qPCR melt temperature graph.



Appendix B10. HeLa to THP-1 Trial 2 qPCR melt temperature graph.



Appendix B11. HeLa to HeLa Trial 1 qPCR melt temperature graph.



Appendix B12. HeLa to HeLa Trial 2 qPCR melt temperature graph.