Infectivity of Novel Intracellular Bacteria for Eukaryotic Cells

By

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ABSTRACT

Three different cell lines, *Dictyostelium discoideum*, *Acanthamoeba polyphaga*, and U937 cells, were infected with a novel bacterium, *Candidatus* Berkiella cookevillensis (CC99). The level of infectivity within the cells was observed using Giemsa staining combined with microscopic observation. The first goal of the study was to determine if the bacteria would infect the cells of the model organism *D. discoideum*, so that future studies could be done on pathways of infection. The second goal of the study was to determine the ability of the bacteria to pass between different cell types or hosts. CC99 was passed between *A. polyphaga* cells, from *A. polyphaga* cells to U937 cells, and between U937 cells. The bacteria were observed in the nucleus of *D. discoideum* cells by 24 hours post-infection, and after 48 hours all nuclei were infected. The CC99 bacterium was shown to be capable of passing between *A. polyphaga* cells, from *A. polyphaga* cells to U937 cells, and between U937 cells. The nuclei of *A. polyphaga* cells became fully infected by 48 hours post-infection, while the nuclei of the U937 cells did not show significant levels of infection in the nuclei until 72 hours post-infection.

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INTRODUCTION

The goal of this study was to better understand the ability of a novel strain of bacteria to infect various types of host cells, and to determine the life stages at which the bacteria is infectious. The experiments essentially involve infecting healthy host cells with the bacteria, and analyzing the results of the infections on the cells using cellular staining and microscopy methods. This allows for a deeper understanding of not only the interaction of this bacterium with different host cells but also the infectivity of the bacteria, which may be important for disease prevention and treatment.

Bacterial pathogens are able to survive and reproduce in a variety of different hosts. One such host type which is often infected with these pathogenic bacteria is amoebae. Amoebae use bacteria as a food source, but sometimes the bacteria that they feed upon are pathogenic and cannot be digested by their lysosomes. This results in the bacteria thriving within the amoebal host and eventually lysing (breaking apart) the cell, releasing a large number of bacteria into the surrounding environment (Molmeret *et al.,* 2005). If these bacteria are released into the human body they can potentially enter human cells, causing an infection. Studies have demonstrated that the prior growth of the bacterium *Legionella pneumophila* in amoebae provides those bacteria that survive with greater virulence, resistance, and motility than bacteria that have not been engulfed by the amoebae (Cirillo *et al*., 1994: Gao *et al.,* 1997; Swanson *et al.,* 2000). This results in greater adaptability and aids the bacteria in gaining entry into human cells. Numerous studies have been done on the infectivity of amoebae as well as macrophages with pathogenic bacteria, as these infected hosts are responsible for a number of human diseases (Anacarso *et al.,* 2012; Fajardo *et al*., 2004; Takemura *et al.,* 2000). In these

studies, much importance is placed on the stages and mechanisms of bacterial infectivity within the host, which provides information that can be used to understand infection resistance and can aid in preventing disease.

Recently, a new strain of pathogenic bacteria was discovered within a sample of amoebae from a cooling tower in Cookeville, Tennessee (Mehari *et al.*, 2016). The bacterium, *Candidatus* Berkiella cookevillensis (CC99), belongs to the phylum *Proteobacteria*, order *Legionellales*, and family *Coxiellaceae*. Growth of CC99 on traditional laboratory growth media was unsuccessful and the bacteria were found to only grow within a living host. The host used was *Acanthamoeba polyphaga*, an amoeba which readily engulfs the bacteria when placed in a nutrient poor environment. The bacteria were observed to fill the nucleus of the *A. polyphaga* hosts within 12 hours after infection. After filling the nucleus for 24-48 hours, the bacteria eventually lyse the amoeba host cell. The bacteria remain motile for about 12-24 hours after lysing the cell, after which they become non-motile and adhere to the bottom surface of the culture flask. CC99 was found to be Gram-stain-negative and coccoid in shape with diameters of 0.30-0.60 μm (Mehari *et al*, 2016).

The first aim of this study was to determine the level of infectivity of CC99 for another protozoan host. The host used was *Dictyostelium discoideum* which is an amoeboid protozoan, and an excellent model organism for studying pathogenesis (Steinert and Heuner, 2005). Studies using *Legionella* strains, a genus related to CC99, show that the bacteria are taken up into the *D. discoideum* organisms by way of phagocytosis (Steinert and Heuner, 2005). The bacteria are retained within a vacuole in the host where they replicate and utilize host-produced cell factors to increase their growth rate. Eventually, the bacteria exit the host, killing it in the process. In the experiment, two different strains

of *D. discoideum* were infected with CC99, and the level of infectivity within the host was assessed. This study also used two strains of *D. discoideum* to determine whether bacterium CC99 could infect these host cells. *D. discoideum* is a model organism for host-pathogen relationships because it shares many genes with humans. The availability of genetic mutants for this organism will also facilitate a better understanding of the intracellular and intranuclear lifestyle of CC99 (Bozzaro, 2013).

The second aim of this study was to examine the infectivity of CC99 from one host cell type to another. This gives insight on the possibilities of transfer between different cell types or different hosts. For this part of the experiment, A. *polyphaga* and U937 human macrophage-like cells were used. U937 cells are human monocyte cells that act as macrophages when they are triggered, and readily phagocytize bacteria (Swanson et al. 2000). The process of infection of U937 macrophage-like cells with intracellular bacteria is similar to that of amoebae. The host cells phagocytize (engulf) the bacteria by extending pseudopods around the bacteria. These bacteria resist digestion by the cell and instead replicate within the host vacuole. The host is killed as the large number of bacteria exit (Swanson et al. 2000). The effectiveness of invasion between different eukaryotic hosts, such as amoebae (*A. polyphaga*) to macrophage (U937 cells) and macrophage to macrophage were investigated to better understand how the bacterium may be passed between different hosts or cell types.

MATERIALS AND METHODS

Culture of Protozoan Cells

Cultures of *A. polyphaga* were grown in tryptic soy broth (TSB; Becton-Dickinson, Franklin Lakes, NJ) growth medium in 25 cm^3 culture flasks (Corning, Corning, NY) at 25°C. Cells in the flasks were passaged every 5-8 days by adding 1 mL of *A. polyphaga* from the previous flask to 4 mL of fresh TSB medium in a new flask. Growth of DH1 and AX3 strains of *D. discoideum* were in HL5-Maltose growth medium [14.3 g bacto peptone and 7.15 g yeast extract (both form Becton-Dickinson), 18 g maltose, 0.641 g $Na_2HPO_4{*}2H_2O$, 0.490 g KH_2PO_4 (all from Sigma-Aldrich, St. Louis, MO) 1000 mL H_2O, pH to 6.6-6.7] in 25 cm^3 flasks at 25°C. Passage of *D. discoideum* occurred every 5-8 days by adding 0.5 mL of *D. discoideum* to 3.5 mL of HL-Maltose in a new flask.

Maintenance of Bacteria

As the bacteria do not grow on traditional growth media, they had to be grown within the amoeba host, *A. polyphaga.* For the passage of bacteria, TSB was removed from flasks of *A. polyphaga,* the adhered cells were washed with 5 mL of sterile spring water, and then replaced with 5 mL of sterile spring water and 0.1 mL of CC99 bacteria. New flasks were stored at 25°C until lysis. Once amoeba-CC99 co-cultures had lysed, bacteria were passaged into a new culture of *A. polyphaga* within one week.

Culture of Mammalian Cells

U937 cells were maintained in Tissue Culture Medium (TCM; RPMI 1640 culture medium supplemented with 10% fetal bovine serum; Fisher Scientific, Pittsburgh, PA). Cells were maintained in 75 mL tissue-culture flasks at 37°C in a humidified incubator

with 5% carbon dioxide. Cells were passed by combining 1.5 mL of cells from the older flask with 13.5 mL of fresh TCM medium every 3-5 days.

Infection of Cells

To infect *D. discoideum,* the HL5-Maltose media was removed from the *D. discoideum* flask and replaced with 4 mL of sterile spring water and 1 mL of CC99 bacteria. CC99 bacteria were harvested from *A. polyphaga* following a 4-5-day infection of *A. polyphaga* at which time the bacteria had completely lysed the amoebae. The bacterial lysate was centrifuged at 2000 rpm for 5 minutes in a ThermoFisher XT centrifuge to remove any amoebal debris and then filtered through a 5 μm syringe filter to ensure removal of any intact *A. polyphaga* cells. A volume of 1 mL of these bacteria was used to infect *D. discoideum.* Flasks were stored at 25°C and monitored for infection by Giemsa staining.

To infect U937 cells, the cells were differentiated into macrophage-like cells by treatment with 12 μL of 1 mg/mL phorbol myristic acid (PMA; Sigma-Aldrich) in TCM and incubated in a 75 mL flask at 37°C and 5% CO_2 for 3 days prior to infection. For treatment of U937 cells, TCM growth medium was removed from the cells and replaced with TCM containing 0.3 mL of CC99 bacteria from a cell lysate. Incubations were continued for up to 6 days and infections were monitored by Giemsa staining.

Giemsa Staining

Infections in cells were monitored using Giemsa staining. At the appropriate time points after infection, cells (*D. discoideum, A. polyphaga,* or U937) were harvested from treatment flasks or multiwall plates using a cell scraper. Volumes of 0.1 mL of the harvested cells were pelleted onto glass slides at 800 rpm for 5 minutes using a Shandon Cytospin (Fisher Scientific) and the cells were then fixed by immersion in absolute

methanol for 1-2 min. Slides were removed from methanol, briefly air dried, and immersed into Giemsa stain solution. The Giemsa stain is based on taffinity of acidophilic and basophilic dyes for cell structures. The cytoplasm of eukaryotic cells stains bluish-purple, the nuclei stain pink, and the CC99 bacteria stain a deep purple color. Giemsa stain was prepared by mixing 0.5 mL of stain stock (Fluka Chemical, Morris Plains, NJ) with 19.5 mL of distilled water and one drop of Triton X-100 (Sigma-Aldrich). Using a light microscope at 100X magnification, ten fields were chosen at random and the numbers of uninfected and infected cells in each field counted. The infected cells were also scored as to whether bacteria appeared in the nucleus or only in the cytoplasm.

Counting Bacteria

After infecting a flask of *A. polyphaga* cells or U937 cells as described above, a cell scraper was used to remove the bacteria from the bottom of the flask. The bacterial lysate was then spun down at 2000 rpm for 10 minutes in a Thermo-Fisher XTR centrifuge to remove any cellular debris. The supernatant containing the bacteria was then passed through a 5 μm filter syringe and diluted to 1:1000 in sterile spring water in a microcentrifuge tube. The bacteria were then stained by adding 3 μL of 4', 6-Diamidino-2-Phenylindole Dihydrochloride (DAPI; Invitrogen, Carlsbad, CA) stain to the tube, waiting 5 minutes to ensure that the bacteria had taken up the stain. A vacuum pump was then used to pull 1 mL of DAPI stained bacteria onto a 0.2 μm black nucleopore filter (GE Osmonics, Minnetonka, MN). The filter was placed on a glass slide with a drop of mounting oil. The slide was viewed on 1000X oil immersion using an Olympus BX-60 microscope equipped with epifluorescence and a DAPI ultraviolet filter (Center Valley,

PA). Ten fields of bacteria were counted on each of three slides from triplicate treatments and the number of bacteria per milliliter calculated from this value.

Infection between Cell Lines

In order to determine the infectivity of the CC99 bacteria between *A. polyphaga* cells, infections were performed with a multiplicity of infection of 1:1. Cells from a confluent flask were counted using a 1:1 ratio of cells to Trypan blue stain (Sigma-Aldrich) on a hemocytometer. Cells were added to a 6-well plate (Corning) with 1×10^6 cells/well in TSB and infected after one hour with an equal number of bacteria in sterile spring water, counted by DAPI-staining as described above. Infections were harvested in triplicate and stained after 24 and 48-hour time points according to the cytospin and Giemsa staining procedures outlined above. Ten fields of cells were counted at 1000X magnification using an Olympus BX-60 light microscope and the percentage of infection calculated from these counts. The procedures for infection from *A. polyphaga* to U937 cells and between U937 cells were similar with the following exceptions. For these procedures, a 1:2 ratio of cells to Trypan blue stain was counted, and the number of cells and bacteria per well was changed to 2×10^6 cells. Cells were harvested for staining and counting at 48 and 72 hours following infection due to the delayed infection that has been observed in the mammalian cells compared to the amoeba cell lines (Mehari et al., 2016). TCM was also used throughout the experiment instead of TSB and sterile spring water.

Statistical Analysis

Experiments were repeated at least two times with triplicate samples to ensure reproducibility and consistency of results. Data were analyzed for statistical significance using t-tests with statistical significance determined at $p \leq 0.05$. (Microsoft Office Excel).

RESULTS

The purpose of this study was to determine the infectivity of the CC99 bacterium for the model organism, *Dictyostelium discoideum*, as well as to investigate the ability of the bacteria to pass between different host cell types. The results of the infections of *D. discoideum* with CC99 showed that by 24 hours, 97-100% of the cells already had infection in the nucleus with 100% of cells showing infection either in the cytosol alone or both the cytosol and nucleus. By 48 hours post-infection, 100% of all cells had infected nuclei (Table 1). Figure 1 depicts CC99 infection in *D. discoideum* nuclei at both 24 (A) and 48 (B) hours after infection. These results showed that the amoeba, *D. discoideum*, could serve as a suitable amoeba host for CC99.

Table 1. Number of cells infected with CC99 for two strains of *Dictyostelium discoideum* at 24 and 48-hour time points post-infection.

Strain	**AX3**		**DH1**	
Time after infection (hours)	**24**	**48**	**24**	**48**
Number of cells with infection both in cytosol and nucleus	297	134	161	113
Number of cells with infection in cytosol only	7	0	0	0
Total	304	134	161	113
Total percent infected	100	100	100	100
Percent infected nuclei	97.7	100	100	100

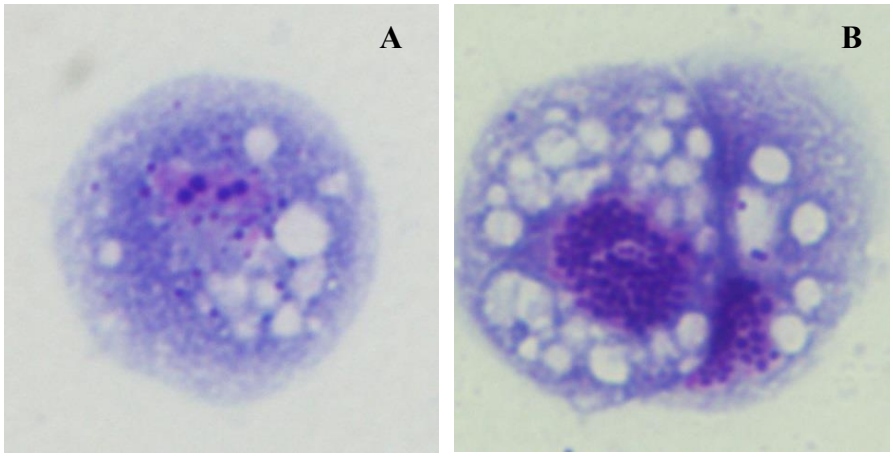

Figure 1. *D. discoideum* infected with bacterium CC99. A) Infection of amoeba at 24 hours shows dark purple circular bacteria within the nucleus (pink). Bacteria also appear within the cytoplasm (blue). B) At 48 hours post-infection, increased numbers of CC99 are visible within the nuclei although some bacteria are also visible within the cytoplasm.

The amoeba, *Acanthamoeba polyphaga*, is the host cell line typically used to culture bacterium CC99. To confirm the levels of infection in the nuclei and cytosol, *A. polyphaga* were infected with CC99 harvested from *A. polyphaga*. Two replicate trials showed that the passage of CC99 bacteria between *A. polyphaga* amoebal cells resulted in greater than 99% of the nuclei becoming infected within 24 hours, and 100% infection of nuclei by 48 hours after infection, as shown in Figure 2. The differences between the levels of infection in the nucleus at 24 and 48 hours was not significant (Trial 1: p = 0.098, N= 6; Trial 2: p = 0.211, N= 6). Figure 3 shows *A. polyphaga* infected with CC99. By 48 hours, bacteria fill the nucleus of the amoebal cell.

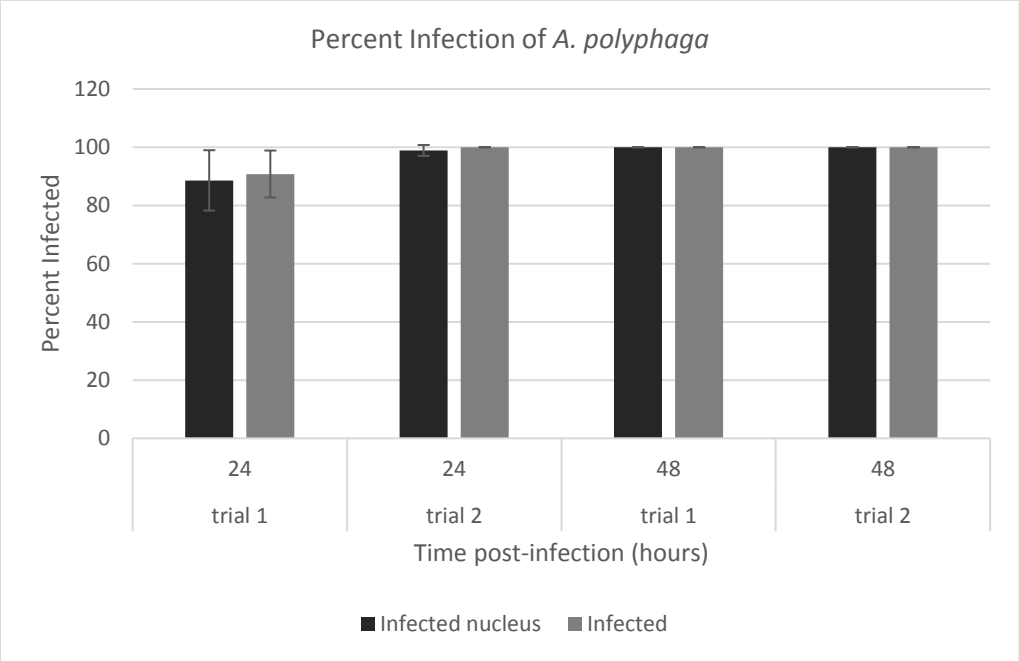

Figure 2. Percent infection of *A. polyphaga* with bacterium CC99 harvested from *A. polyphaga*. Two trials showing percent infection of *A. polyphaga* at 24 and 48-hour time points post-infection. The infected category (gray bar) indicates the total percentage of cells infected either within the cytosol or the cytosol and nucleus. The infected nucleus category (black bar) indicates the percentage of cells with visible infection of the nucleus). Error bars indicate the calculated standard deviation between triplicate values. Two replicated trials are shown.

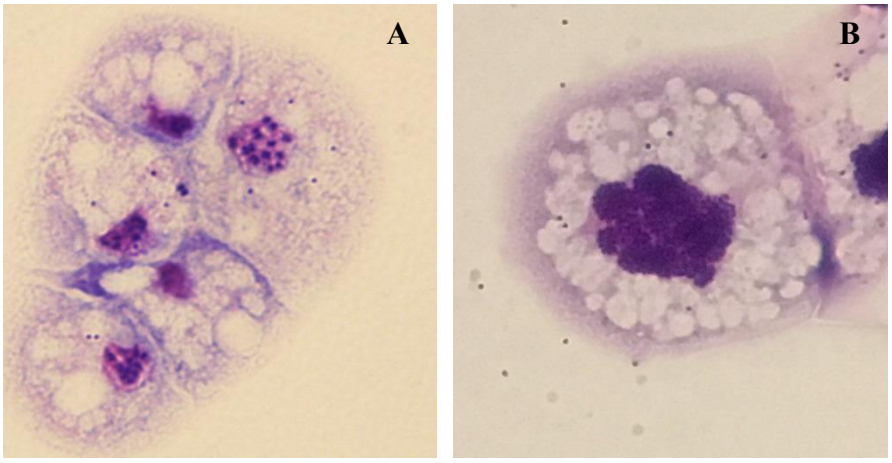

Figure 3. *A. polyphaga* infected with bacterium CC99. A) *A. polyphaga* infected with CC99 for 24 hours shows dark purple, circular bacteria within nuclei (pink). B) By 48 hours, dark purple bacteria fill the nucleus with very few bacteria visible in the cytoplasm.

Passaging of CC99 bacteria into human U937 macrophages used 48 hour and 72 hour time points, because infection is often delayed in human cells compared with amoeba hosts. The results of passing CC99 between *A. polyphaga* and U937 cells showed that the bacteria were taken up into 87.3-100% of the cells by 48 hours (Figure 4). However, less than 3% of nuclei were infected at 48 hours. By 72 hours, total percentages of infected cells had decreased, most likely due to cell death, although percentages of infected nuclei significantly increased when compared to 48 hours (Trial 1: $p = 0.027$, N= 6; Trial 2: $p = 0.001$, N= 6). Cytoplasmic and nuclear CC99 bacteria can be seen in Figure 5.

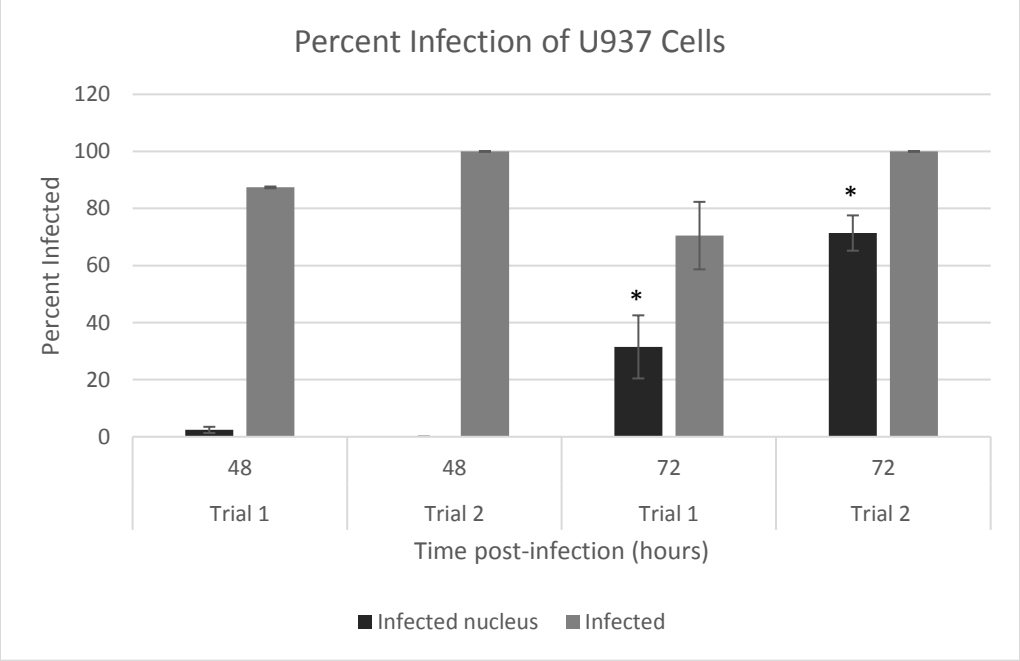

Figure 4. Percent infection of U937 cells with bacterium CC99 harvested from *A. polyphaga.* Two trials showing percent infection of U937 cells at 48 and 72-hour time points post-infection. The bacteria used in these trials were passed from *A. polyphaga*. The infected category indicates the percentage of cells infected either within the cytosol or the cytosol and nucleus. The infected nucleus category indicates the percentage of cells with visible infection of the nucleus. Error bars indicate the calculated standard deviation between triplicate values. (*) Indicate statistical significance between 48 and 72-hour time points.

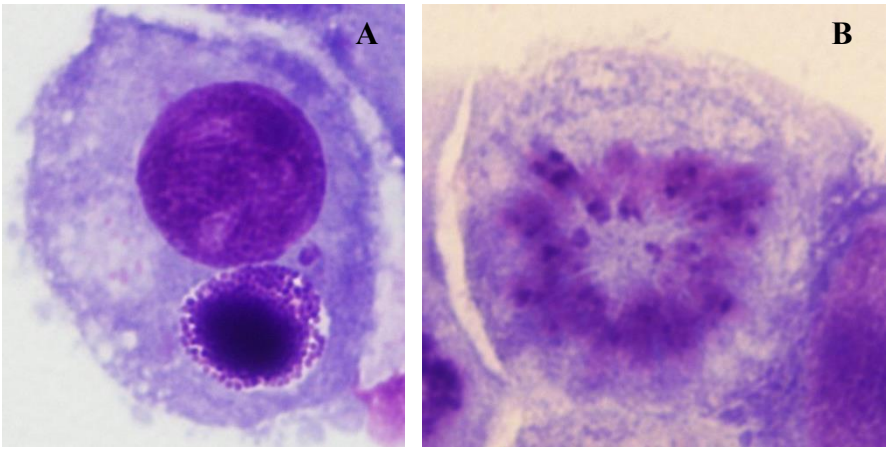

Figure 5. U937 cells infected with bacterium CC99. A) U937 cells infected with CC99 for 48 hours show a large cluster of CC99 bacteria contained within a vesicle in the cytoplasm. B) U937 cells infected with CC99 for 72 hours show CC99 (dark purple circles) in the nucleus (pink).

The passage of bacteria between U937 cells resulted in 84-90% infection in the cytoplasm with 18.2-59.1% infection in the nuclei as early as 48 hours post-infection. By 72 hours, infection of the nuclei rose to 75.4-92.5% (Figure 6). The only significant differences were between the level of infection in the cytoplasm and nucleus at 48 hours post-infection (Trial 1: $p = 0.001$, N= 6; Trial 2: $p = 0.018$, N= 6).

When making comparisons between transfer of infection between hosts, at the early time point of 24 hours, *A. polyphaga* exhibits > 93% infection of nuclei with 100% nuclear infection by 48 hours. When U937 human macrophages are infected by CC99 harvested from *A. polyphaga*, at the early time point of 48 hours, < 2% of nuclei are infected and infection levels rise to less than 80% by 72 hours. These levels of infection are significantly lower than the infection between amoebae ($p < 0.001$ at early time point and

p = 0.002 at the second time point). However, when the infection is transferred between U937 cells, infection of the nucleus significantly increases (p = 0.008 at 48 hours and p = 0.037 at 72 hours). These results suggest that infection between hosts of the same cell type results in greater infection of the nucleus than when the infection is transferred from an amoeba to a human cell.

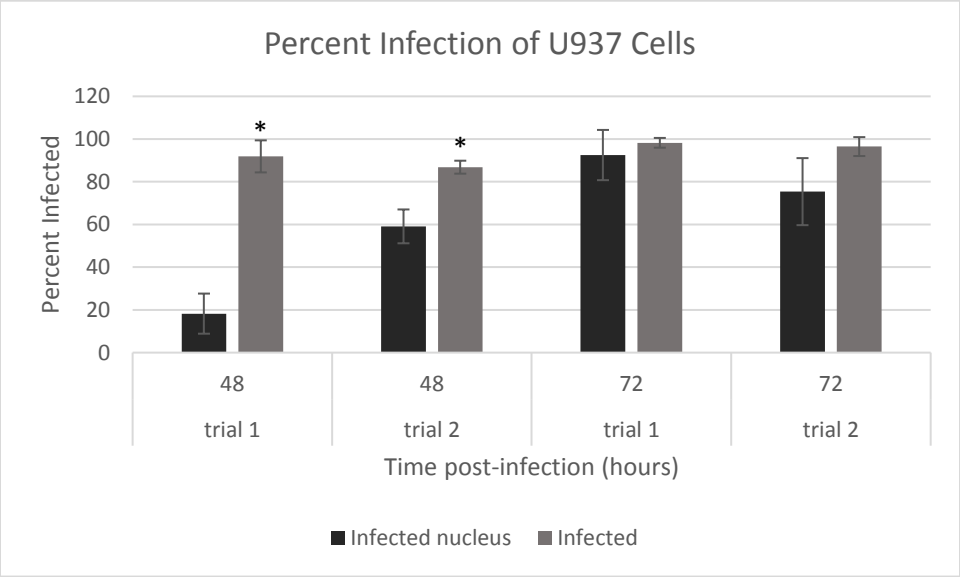

Figure 6. Two trials showing percent infection of U937 cells at 48 and 72-hour time points post-infection. The bacteria used in these trials were passed from U937 cells. The infected category indicates the percentage of cells infected either within the cytosol or the cytosol and nucleus. The infected nucleus category indicates the percentage of cells with visible infection of the nucleus. Error bars indicate the calculated standard deviation between triplicate values. (*) indicate significant differences between infected nuclei and total infection at 48 hours.

DISCUSSION

The results of the first portion of the study show that the CC99 bacteria are readily taken up into the model organism, *Dictyostelium discoideum*, resulting in complete infection of the nucleus by 48 hours after infection (Table 1). For this study, two different strains of *D. disoideum* were used, which represent two parent strains commonly used for the development of genetic mutants (Fey *et al.*, 2013). While not the most complex of experiments, the knowledge that CC99 is able to infect *D. discoideum* is nonetheless beneficial. As a model organism, *D. discoideum* has been studied in great depth. With the advantage of a fully sequenced genome, knowledge of signaling pathways, and other pathways that relate to pathogenesis, using *D. discoideum* as a model for the CC99 bacteria will allow us to look at the mechanisms of infection that are used by the bacteria in future studies.

The results of the final portion of the study show that CC99 easily passes between *A. polyphaga* cells and is prevalent in the nuclei by 24 hours post-infection (Figures 2-3). There were no significant differences between the levels of infection in the nucleus at the 24-hour and 48-hour time points, which is likely due to the rapidity of infection. The results for the passage of CC99 from *A. polyphaga* to U937 cells, showed a significant number of the nuclei becoming infected by 72 hours after infection (Figure 4). The uptake of bacteria into mammalian cells such as the U937 cells typically takes longer than in amoebae, and the data points reflect this trend. The fact that CC99 is able to enter and infect the nucleus of these mammalian, macrophage-like cells shows that the bacteria could potentially infect cells within the human body. The ability for the bacteria to pass from *A. polyphaga* cells into U937 cells demonstrates the possibility of infection in

humans who have been exposed to infected amoebae. The bacteria were also capable of passing between U937 cells, resulting in a significant majority of nuclei becoming infected by 72 hours post-infection. The passage of CC99 between U937 cells showed significantly higher levels of infection in the nucleus at 72 hours than in the passage of CC99 between *A. polyphaga* and U937 cells. This demonstrates the potential ability of the bacteria to pass between macrophage cells within the human body, or between human hosts. This pattern of infection is in contrast to the related bacterium, *Legionella pneumophila.* *L. pneumophila* also infects both amoebae and human macrophages, including the U937 cell line. Although cases of person-to-person spread have been reported, much evidence indicates that person-to-person spread is rare (Correia *et al*., 2016; Katz *et al*., 1982). Because bacterium CC99 has increased transmission when spread from U937 to U937 cells, it may potentially be transmitted between human hosts. Further research is needed to better understand not only the mechanism of how CC99 can infect the nucleus but also its role in human disease.

REFERENCES

Alberts, B. (2008). *Molecular Biology of the Cell* (Sixth ed.). New York: Garland Science Taylor & Francis.

Anacarso, I., de Niederhäusern, S., Messi, P., Guerrieri, E., Iseppi, R., Sabia, C., & Bondi, M. (2012) *Acanthamoeba polyphaga*, a potential environmental vector for the transmission of food-borne and opportunistic pathogens. J Basic Microbiol. 52: 261-268.

Bozzaro S. 2013. The model organism Dictyostelium discoideum. Methods Mol Biol. 983: 17-37.

Cirillo J. D., Tompkins L. S., Falkow S. (1994) Growth of *Legionella pneumophila* in *Acanthamoeba castellanii* enhances invasion. Infect Immun. 62: 3254–3261.

Coleman, S. A., Fischer, E. R., Howe, D., Mead, D. J., & Heinzen, R. A. (2004) Temporal analysis of *Coxiella burnetii* morphological differentiation. J Bacteriol. 186: 7344–7352.

Correia, A.M., Ferreira, J.S., Borges, V., Nunes, A., Gomes, B., Capucho, R., et al. (2016) Probable person-to-person transmission of Legionnaires' disease. N Engl J Med. 374: 497-498.

Fajardo, M., Schleicher, M., Noegel, A., Bozzaro, S., Killinger, S., Heuner, K., & ... Steinert, M. (2004) Calnexin, calreticulin and cytoskeleton-associated proteins modulate uptake and growth of *Legionella pneumophila* in *Dictyostelium discoideum*. Microbiology 9: 2825.

Fey, P., Dodson, R., Basu, S., Chisholm, R. L. (2013). One stop shop for everything Dictyostelium: dictyBase and the Dicty Stock Center. Methods Mol. Biol. 983: 59-92.

Gao, L. Y., Harb, O. S., & Abu Kwaik, Y. (1997). Utilization of similar mechanisms by Legionella pneumophila to parasitize two evolutionarily distant host cells, mammalian macrophages and protozoa. *Infection And Immunity*, *65*(11), 4738-4746.

Hilbi, H., Jarraud, S., Hartland, E., & Buchrieser, C. (2010) Update on Legionnaires' disease: pathogenesis, epidemiology, detection and control. Molec Microbiol. 76: 1–11.

Horwitz, M. A., & Silverstein, S. C. (1980). Legionnaires' Disease Bacterium (*Legionella pneumophila*) multiplies intracellularly in human monocytes. J Clin Invest. 66: 441–450.

Katz, S. M., Habib, W. A, Hammel, J. M., Nash, P. (1982) Lack of airborne spread of infection by *Legionella pneumophila* among guinea pigs. Infect Immun. 38: 620-622.

Molmeret, M., Horn, M., Wagner, M., Santic, M., Abu Kwaik, Y. (2005) Amoebae as training grounds for intracellular bacterial pathogens. Appl Environ Micro. 71: 20–28.

Mehari, Y. T., Hayes, B. J., Redding, K. S., Mariappan, P. G., Gunderson, J. H., Farone, A. L., & Farone, M. B. (2015) Description of '*Candidatus* Berkiella aquae' and '*Candidatus* Berkiella cookevillensis', two intranuclear bacteria of freshwater amoebae. Intl J System Evol Microbiol. 66: 536-541.

Steinert, M., Heuner, K. (2005). Dictyostelium as host model for pathogenesis. Cell Microbiol. 7: 307-314.

Swanson, M. S., Hammer, B. K. (2000). *Legionella pneumophila* pathogenesis: a fateful journey from amoebae to macrophages. *Annual Review Of Microbiology*,

Takemura, H., Yamamoto, H., Kunishima, H., Ikejima, H., Hara, T., Kanemitsu, K., & ... Shimada, J. (2000) Evaluation of a human monocytic cell line THP-1 model for assay of the intracellular activities of antimicrobial agents against *Legionella pneumophila*. J Antimicrob Chemother. 46: 589-594.