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Infection of Mammalian Hepatocytes by Francisella tularensis LVS

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Infection of Mammalian Hepatocytes by *Francisella tularensis*

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Abstract

Francisella tularensis, the causative agent of Tularemia, is a facultative intracellular parasite. The goal of this project is to examine how *F. tularensis* infects and replicates within mammalian cells, and the impact of such an infection upon the signaling pathways within the host cell. The murine embryonic hepatocyte cell line TIB-73 serves as our model system for infection by *F. tularensis* live vaccine strain (LVS). The number of bacteria associated with hepatocytes was quantified via adhesion, invasion and intracellular replication assays. We have determined that a large multiplicity of infection (MOI) of *F. tularensis* LVS is required to infect the hepatocyte cells. The infective capacity of *F. tularensis* LVS in hepatocytes was found to be ineffective below an MOI of 0.1. Western blotting was used to determine the presence of various kinases in hepatocytes before infection with *F. tularensis* LVS.

Introduction

Francisella tularensis, the causative agent of tularemia, is a naturally occurring disease of rabbits that causes disease in humans through accidental contact with bacteria via infected material or insect vectors (1). Tularemia is considered to be a likely agent for use in a bioweapons attack and is a Category A biothreat agent (along with smallpox, plague and anthrax), due to its high infectivity when released as an aerosol (3, 5). Aerosolized *F. tularensis* has an ID_{50} with the inhalation of as few as 10 bacteria (1, 7). *F. tularensis* live vaccine strain (LVS) is a useful laboratory model for understanding the infectious process of this organism as it is generally considered avirulent for humans, but can be lethal to mice and rats (2, 4).

F. tularensis is a facultative intracellular pathogen known to infect and multiply inside macrophages (4, 5, 10). Once it is established in the cells of the host, *F. tularensis* replicates, disseminates and continues the infection (5).

In lethal cases of tularemia in mice, pathological deterioration of the spleen and more notably the liver has been observed (5). Histological studies of livers from infected mice demonstrate extensive necrosis and bacteria-laden hepatocytes (6).

Our hypothesis is that one cause of pathogenesis of tularemia is that *F. tularensis* LVS attacks and infects causing failure of the liver, although the mechanism by which this is accomplished has not yet been identified.

Based on many published reports of liver pathology and bacterial burden during *Francisella* infection, we propose to examine the ability of hepatocytes to be infected. There are multiple cell types within the liver, including Kupffer cells, stellate cells and hepatocytes (8). In order to examine hepatocyes in isolation, we have used a murine embryonic cell line, TIB-73. TIB-73 hepatocyte cells have been proven to be a useful non-phagocytic cell infection model for the study of *Salmonella typhimurium infection* (9).

To better understand the consequences of infection of mammalian cells by *F. tularensis* LVS, we will examine activation of several important and shared protein phosphorylation pathways. This will help to elucidate the disease process and may reveal new drug targets for novel treatments.

The purpose of this study was to demonstrate that *F. tularensis* LVS is able to adhere to, invade and replicate within hepatocyte cells and to establish the presence of important kinases in hepatocytes . This work sets the stage for our future studies.

Materials and Methods

Bacterial strains and growth conditions. *F. tularensis* LVS was obtained from the American Type Culture Collection (Manassas, VA). Bacteria were grown in TSB-C (Trypticase Soy Broth, supplemented with 0.1% cysteine HCl).

Tissue culture cells. All experiments were carried out using murine embryonic TIB-73 hepatocyte cells (ATCC, BNL.CL.2). Cells were cultured in T-25 flasks and maintained in Dulbecco's modified Eagle medium (DMEM), supplemented with 10% Fetal Bovine Serum (FBS).

Adhesion Assay. Hepatocytes were grown to confluency and trypsinized with 5% Trypsin-EDTA. The cells were counted using a hemocytometer, added to a flat bottom 96-well tissue culture plate to yield approximately $1x10^5$ cells per well, and were allowed to adhere overnight. An overnight culture (approximately 18 hours) of *F. tularensis* LVS was grown to an OD_{600} of about 0.6 yielding approximately $1x10^9$ CFU/ml and was diluted to approximately $1x10^8$ CFU/ml in TSB-C (an aliquot was removed for CFU determination by colony counting). Tissue culture wells were washed twice with fresh DMEM and 100µl of DMEM was placed in each well. A bacterial inoculum with a multiplicity of infection (MOI) of 100 (approximately 100 µl of $1x10^8$ CFU/ml) was added to each experimental well; TSB-C was added to each negative control well. The plate was centrifuged for 5 min at 500 g to encourage contact of bacterial cells with hepatocytes. Infected cells were incubated at 37ºC in 5% CO_2. At various time intervals, cells were washed three times with fresh DMEM to remove free bacteria. Cells were lysed in 200 µl of sterile deionized water followed by vigorous pipetting. Serial dilutions of collected lysate were performed (using sterile deionized water), plated onto Columbia Blood Agar supplemented with 5% defibrinated horse blood (CBA-H 5%), incubated at 37ºC and counted after a 72 hour growth period.

Invasion Assay. To determine the number of invading bacteria, a hepatocyte monolayer was infected with bacterial inocula (as above) and incubated for 120 min. Cells were washed 3 times with DMEM and gentamicin sulfate was added to each experimental well at a concentration of 20 µg/ml in DMEM for 10 min. Then, cells were washed 3 times with DMEM to remove excess gentamicin. Cells were lysed and processed (as above).

Intracellular replication of *F. tularensis* LVS in hepatocytes. To study intracellular growth, a replication assay was performed at the same time and set up in the same manner as the invasion assay. After treating infected cells with 20 µg/ml of gentamicin, cells were washed three times with fresh DMEM. The cells were incubated at 37ºC in 5% CO_2 for a total of 48 hours. Cells were lysed and processed (as above) at various time intervals.

Antibodies. Antibodies to kinase molecules that were used in this study include: Mouse monoclonal: IRAK-1 (Santa Cruz Biotechnology #sc-5288, directed to amino acids 440-712). Rabbit polyclonals: p38 MAP Kinase (Cell Signaling Technology #9212); p65 NFκB (Cell Signaling Technology #3034); MEK1/2 (Cell Signaling Technology #9122); IKKα (Cell Signaling Technology #2682); NIK (Santa Cruz Biotechnology #7211).

Western immunoblotting. TIB-73 hepatocyte cells were lysed in RIPA detergent buffer (10mM Tris-HCl pH 7.2, 1% Triton-X, 0.5% sodium deoxycholate, 150mM NaCl, 1mM EDTA, 1mM sodium orthovanadate, 50 µg/ml leupeptin and 0.5% aprotinin). All procedures were performed at 4-10^{0}C. Lysates were cleared by centrifugation for 15 min at 15,000 x g in a microcentrifuge, and protein concentration was determined by BioRad protein reagent. Samples were diluted in 2X sample buffer (125mM Tris-HCl, 4% SDS, 10% glycerol, 0.02% bromophenol blue, 4% β-mercaptoethanol) and boiled for 5 min. Lysate proteins were separated by electrophoresis through a 7.5% SDS-polyacrylamide gel and transferred to Immobilon-P membranes (Millipore, Bedford, MA). (50 µg whole-cell lysate was assayed per lane.) Membranes were

blocked with either 5% non-fat dry milk or 3% bovine serum albumin in Tris-buffered saline + Tween solution for 1 hour-overnight, and incubated with primary antibody over night at 4ºC. Binding of primary antibodies was visualized by enhanced chemiluminescence (ECL) (Amersham, Buckinghamshire, England), using Kodak BioMax Light Film.

Immunofluorescence Antibodies. Antibodies to *F. tularensis* used in this study include: Mouse monoclonal: anti-*Francisella* LPS antibody, blend of FB11 and T14 (Chemicon International #MAB8267).From the manufacturer: Antibody "isotype: IgG2a (FB11) and IgG3 (T14). Clones FB11 and T14 were derived from hybridization of Sp2/0 myeloma cells with spleen." Specificity: This antibody "recognizes LPS of virulent and vaccine strains of *F. tularensis*. There is no cross-reactivity with: *F. novicida, Br. abortus, Br. suis, Br. melitensis, Br. ovis, Y. pestis, Y. entercolitica, Y. pseudotuberculosis, E. coli, V. cholerae.* The binding site for clone FB11 is located on the O-antigen polysaccharide chain". Secondary polyclonal: FITC anti-mouse IgG (H+L) (Biomeda #F63).

Immunofluorescence. TIB-73 cells were plated in a slide chamber at a cell concentration of 5×10^5 cells per chamber. The cells were allowed to adhere overnight and were infected with *F. tularensis* LVS at an MOI of 100 and incubated at 37ºC in 5% CO_2 for 12 hours. The cells were then washed with 4ºC phosphate buffered saline (PBS), fixed in a 2% Paraformaldehyde (PFA) fixative (4% PFA, 3x PBS, 2N NaOH). The cells were treated with a 10% bovine serum album (BSA) blocking buffer (10% BSA, 1x PBS, 0.3% Triton X-100) for 20 min. Cells were incubated with primary anti-*Francisella* LPS antibody (5 μg/ml) for 60 min at room temperature. Cells were washed and incubated with secondary anti-mouse antibody with fluorescein (1 μg/ml) for 45 min. Binding of antibodies was visualized by fluorescence microscopy, Canon digital camera and Adobe Photoshop 6.0.

Results

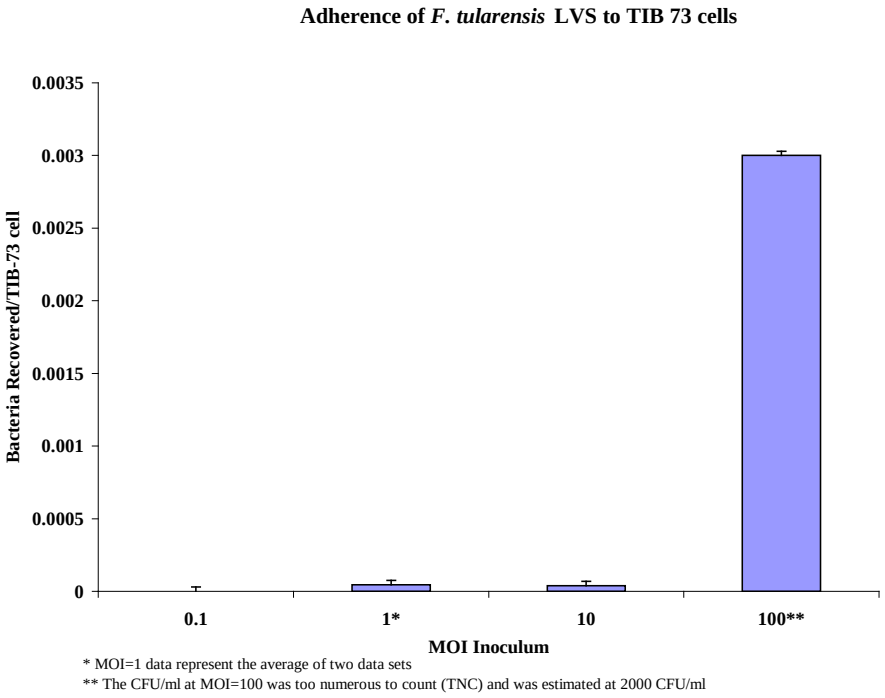


Figure 1: Adhesion assay demonstrating optimal MOI of *F. tularensis* in TIB-73 hepatocytes. Increasing amounts of bacteria were added to 1 x 10^5 TIB-73 cells for 1 hour. Data are expressed as mean +/- standard error mean (SEM).

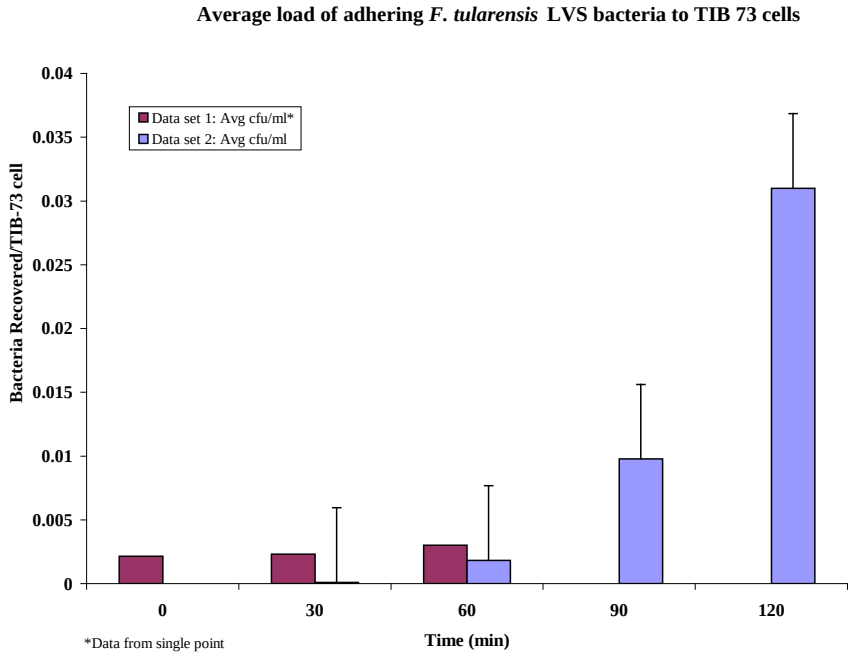


Figure 2: Adhesion assay demonstrating optimal incubation times at an MOI of 100. Data shown are from two separate data sets, but the number of bacteria per cell is consistent between trials. The number of adhering bacteria increased over time and yielded 0.035 bacteria per TIB-73 cell.

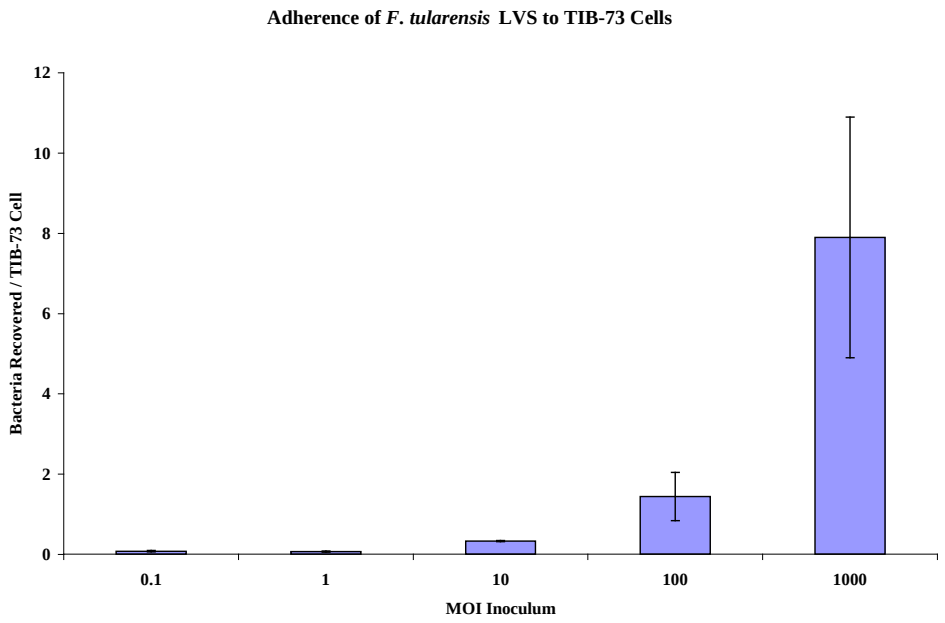


Figure 3: Adhesion assay demonstrating optimal MOI to 1000 MOI. An increasing number of bacteria were recovered with an increase in MOI inoculum.

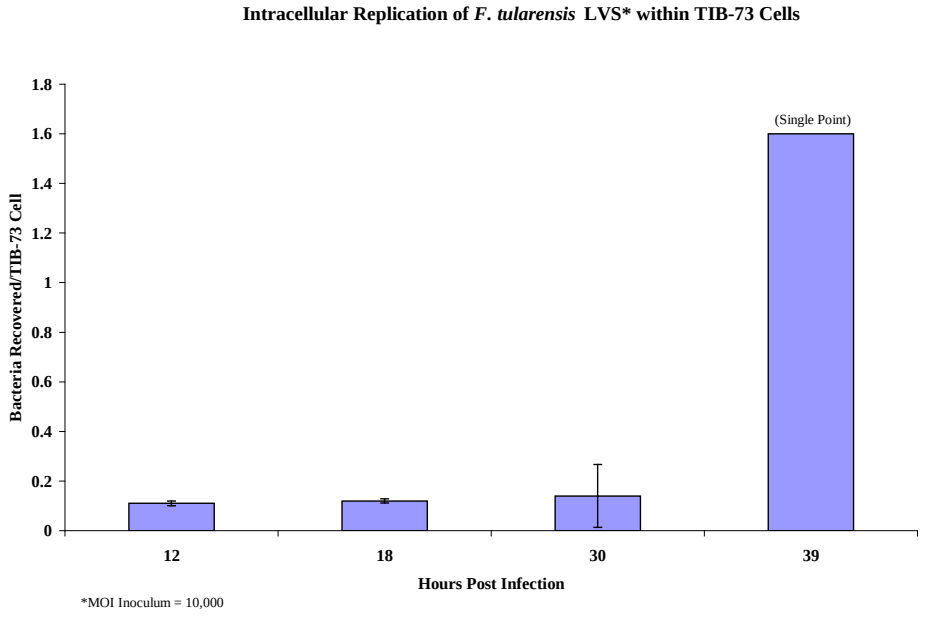


Figure 4: Intracellular replication assay demonstrating increasing amounts of recovered bacteria following TIB-73 infection.

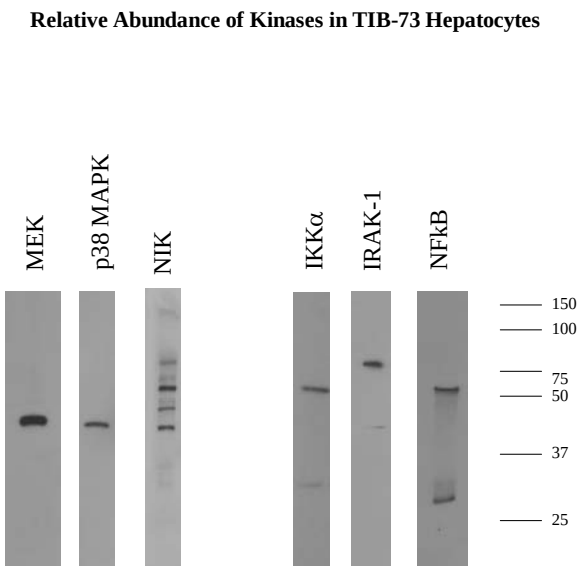
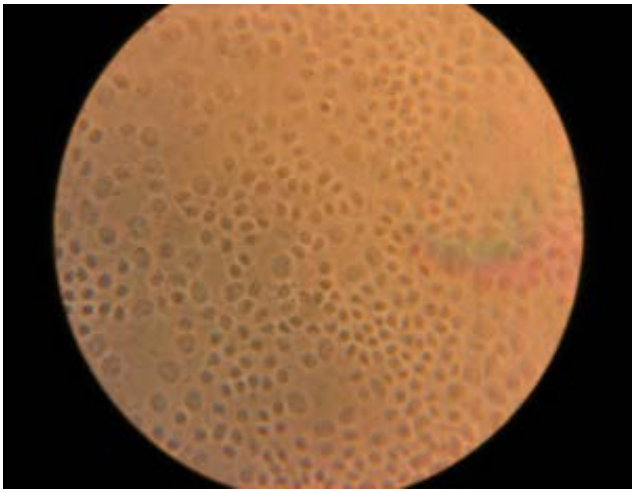
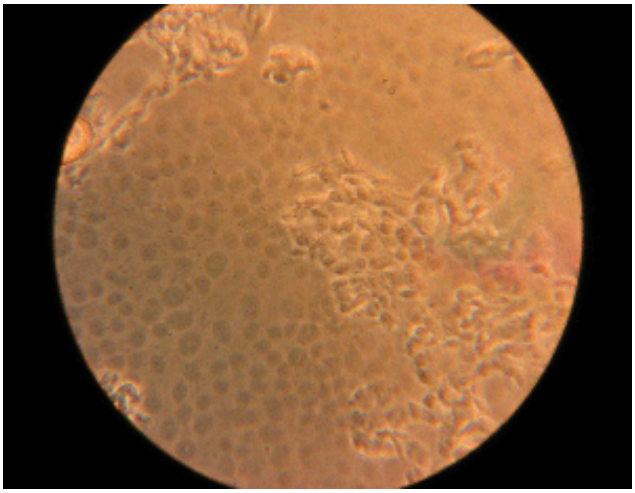


Figure 5. Relative abundance by Western Blotting of various kinases believed to be active in the TLR signaling pathway in hepatocytes.



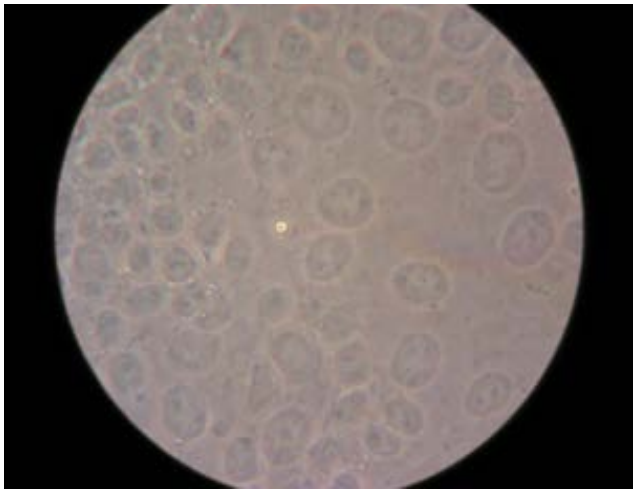
Panel (A) **Panel (B)**

Panel (A)

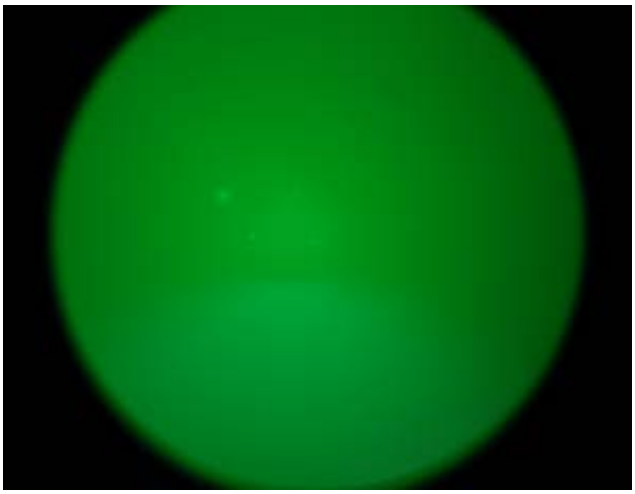


Panel (B)

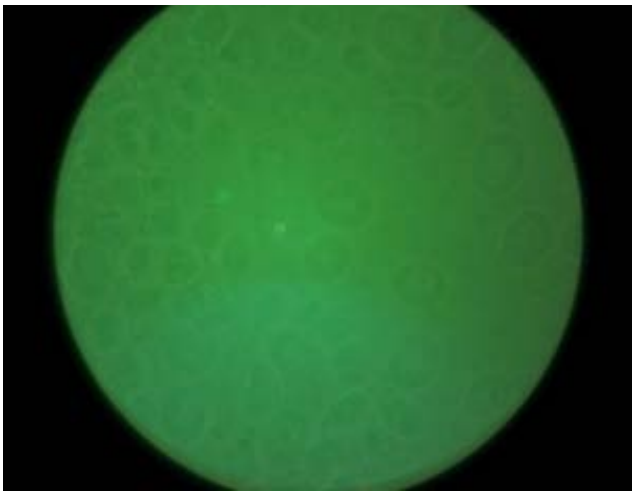
Figure 6: Light Microscopy of Uninfected and Infected TIB-73 cells. TIB-73 cells were examined under 40X light microscopy. Panel (A) represents uninfected cells. Panel (B) are cells infected for 12 hours, illustrating some cytopathic effects.



Panel (A)



Panel (B)



Panel (C)

Figure 7: Immunofluorescence of *Francisella tularensis* LVS infected TIB-73 cells. TIB-73 cells were examined under 100X light and fluorescence microscopy. Panel (A) shows immuno-fluorescence image with bright small dots representing bacteria. Primary: anti-*Francisella* LPS Mouse monoclonal antibody, Blend of FB11 and T14. Secondary: FITC anti-mouse IgG Polyclonal. Panel (B) is the same field under light

microscopy illustrating the TIB-73 cells. Panel (C) shows an overlay of the images and bacteria are seen to be associated with the margins of the TIB-73 cells.

Conclusions

We present evidence to support our hypothesis that *F. tularensis* is able to effectively adhere, invade, and multiply within hepatocyte cells. Our data is consistent with other studies of intracellular pathogens such as *S. typhimurium* (9), which are able to adhere, invade and replicate within non-phagocytic hepatocyte cells, as well as with studies of *Francisella* replication in macrophages (10).

At an MOI of 100 about 0.003 bacteria adhered per TIB-73 cell after 60 minutes of incubation. Lajarin et. al. demonstrated that S. typhimurium infected TIB-73 cells with a maximum of 15 cell-associated bacteria per cell and an invasion capacity of 0.003 bacteria per hepatocyte. The number of adhering bacteria increased 10 fold following an additional 60 minutes of incubation.

Golovliov et. al. used an MOI of 200 to produce 1.7 bacteria per macrophage (11) and is thought to be correlated to hepatocytes as well. An MOI of 500 has been used to infect macrophages but has not demonstrated a linear relationship between MOI and the number of bacteria per macrophage (12).

Hepatocytes have been found to be comparably susceptible to infection by *Francisella* with respect to macrophage infection. It is unclear where in the liver invasion occurs, but our aim is to investigate whether pathogenesis occurs in hepatocyte cells.

Acknowledgements
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