5-2015

Influence of the Anti-HIV drug Elvitegravir on Chlamydial Development and the Characterization of Chlamydial Polymorphic Membrane Protein Expression in Herpes Simplex Virus (HSV)/C. trachomatis Co-infected Cells

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Influence of the Anti-HIV drug Elvitegravir on Chlamydial Development and the Characterization of Chlamydial Polymorphic Membrane Protein Expression in Herpes Simplex Virus (HSV)/*C. trachomatis* Co-infected Cells

Thesis submitted in partial fulfillment of the University Honors Scholars Program

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May 1, 2015

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ABSTRACT

Chlamydia trachomatis is the most common bacterial agent of sexually transmitted infections worldwide and a common co-infection in AIDS patients. Chlamydial genital tract infections are often asymptomatic; therefore many infections go untreated and result in complications like chronic inflammation, ectopic pregnancy, and pelvic inflammatory disease. *Chlamydia* share a unique developmental cycle and under stress, can enter a state known as persistence, in which the bacteria are noninfectious but still viable. Removal of the stressor allows the chlamydiae to re-enter and complete the developmental cycle. Exposure to low-dose quinolones can cause the chlamydiae to enter persistence and halt the developmental cycle. Notably, 1 in 20 people living with HIV/AIDS also suffers from chlamydial infections. Since the anti-HIV drug Elvitegravir (EVG) is a quinolone derivative, we hypothesized that EVG exposure would inhibit chlamydial development. To ascertain whether EVG affects chlamydial development, HeLa cells were infected with *C. trachomatis* or *C. muridarum* and then either mock treated or treated with EVG. The percent infectivity and production of infectious progeny were determined by immunofluorescence assay and chlamydial titer assay, respectively. Transmission electron microscopy (TEM) was used to examine chlamydial morphology and determine whether EVG caused *Chlamydia* to become persistent. Though percent infectivity and chlamydial morphology were similar between treated and untreated *Chlamydia*-infected cells, the production of infectious progeny was significantly decreased in EVG-exposed *Chlamydia*-infected cells. These data indicate that EVG is not a persistence-inducer, but does inhibit chlamydial development *in vitro*. In other studies, we tested chlamydial polymorphic membrane protein (PMP) expression in chlamydia/HSV co-infected cells by immunofluorescence staining. Since penicillin-induced persistence decreases the expression of some chlamydial PMPs, we

hypothesized that expression of PMP-A and PMP-B would be decreased by HSV-induced persistence. The results indicated that there was no significant difference in expression of PMP-A or PMP-B in co-infected versus *C. trachomatis* singly-infected cells. These data suggest that PMP expression is not a good indicator of chlamydial persistence when induced by HSV.

INTRODUCTION

Chlamydia trachomatis is the most common bacterial agent for sexually transmitted disease worldwide, causing approximately 2.86 million new infections annually in the United States alone (CDC, 2014). This number is likely an underestimate since chlamydia is largely a "silent", or asymptomatic, infection. Chlamydia can manifest itself in inflammatory conditions such as urethritis or cervicitis. However, its tendency to display no visible symptoms often leads to untreated infections which can cause complications such as pelvic inflammatory disease, ectopic pregnancy, and infertility (Engleberg et al., 2013). Although chlamydia is primarily transmitted through sexual contact, it can be spread from an infected pregnant woman to her child during childbirth, since the newborn can come into contact with the pathogen upon delivery (CDC, 2014). Infants infected with chlamydia can develop conjunctivitis or pneumonia, and chlamydia can also cause some adults to develop a rare complication known as reactive arthritis (Miller, 2006). As the causative agent of trachoma, the most common form of preventable visual impairment worldwide, *C. trachomatis* can also be spread through direct contact with poor hygiene conditions or flies (Mishori et al., 2012). Chronic chlamydial infections can lead to the buildup of dead epithelial cells from the continued inflammatory and healing responses of the host. This accumulation of necrotic cells can result in irreversible scarring in locations such as

the female genital tract or the eyelid, contributing to infertility and trachoma, respectively (Engleberg et al., 2013).

The genus *Chlamydia* contains nine species, all of which are obligate intracellular pathogens. Chlamydial organisms must be inside living host cells to replicate. Chlamydiae can infect a variety of hosts, ranging from humans to amoebae (Schoborg, 2011). *C. trachomatis*, the human strain of chlamydial pathogens, targets epithelial cells and can be found in the mucosal membranes of the genital tract, urinary tract, eyes, or lungs (Mishori et al., 2012). *C. trachomatis* is sensitive to macrolides, tetracyclines, and fluoroquinolones; common antibiotics used to treat the pathogen include erythromycin, doxycycline, and azithromycin (Engleberg et al., 2013). All chlamydia share a unique biphasic developmental cycle within host cells (**Figure 1**). After the epithelial host cell ingests the infectious but non-replicative elementary bodies (EB) of the pathogen, the vacuoles containing EB within the cell fuse to form a chlamydial inclusion. Within the inclusion, the EB differentiate into non-infectious but replicative reticulate bodies (RB). The RB use host cell metabolites for growth and replicate through binary fission. After several replication cycles, the RB transform back into infectious EB, which are then released by host cell lysis or inclusion extrusion to infect more cells (Schoborg, 2011).

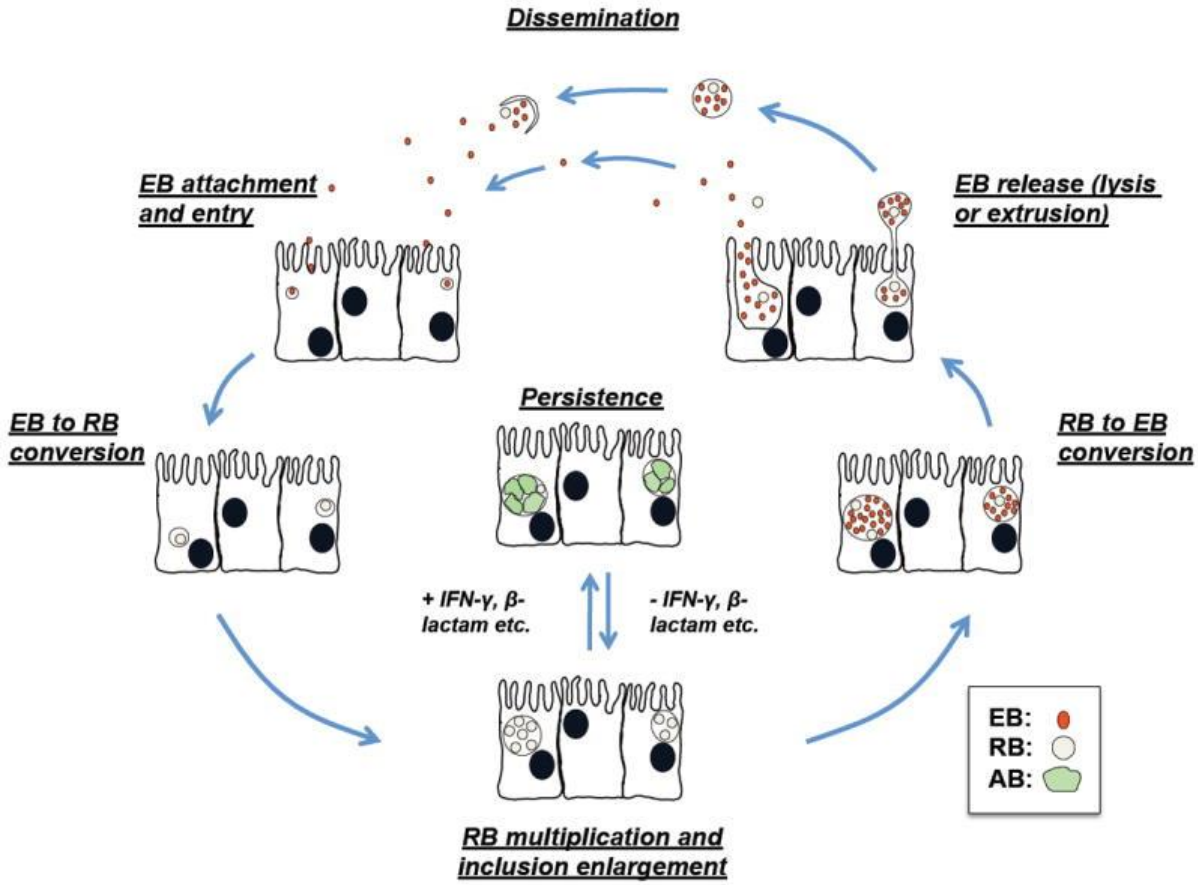


Figure 1. The chlamydial developmental cycle. Infectious elementary bodies (EB) attach to and are endocytosed by host epithelial cells to form a chlamydial inclusion. The EB within the inclusion transform into reticulate bodies (RB), the noninfectious but replicating form of the bacteria. After several rounds of replication, the RB transform back into EB that are then released by host cell lysis to infect other cells. If stressors such as IFN-λ or β-lactam antibiotics are introduced to chlamydia-infected cells, the chlamydia will enter a state known as persistence, in which the bacteria are noninfectious but still viable. Persistence is characterized by the enlargement of RB into aberrant bodies (AB) which no longer have the ability to replicate and transform into infectious EB. Removal of such stressors allows the re-entry of the bacteria into the normal infectious cycle (Schoborg, 2011).

When chlamydia-infected host cells are exposed to a stress such as nutrient deprivation or certain antibiotics, the chlamydia bacteria enter a state known as persistence. Persistence is characterized by the enlargement of RB in the inclusion and also the inhibition of their replication and differentiation into EB (Hogan et al., 2004). In persistence, the bacteria are non-infectious but still viable. It has been shown that persistent chlamydia can re-enter the normal

developmental cycle when stressors such as IFN-λ or β-lactam antibiotics are removed from infected cells (Beatty et al., 1995; Kintner et al., 2014). Because chlamydia commonly cause recurrent infection, some researchers have suspected a link between recurrence and persistence. Substantial data exists from *in vitro* studies into this link. Experiments conducted by Dean and Powers (2001) have shown that persistent chlamydia can alter host cell processes by resisting apoptosis, or programmed cell death. Persistent forms of chlamydia have also been linked to pro-inflammatory responses and serving as reservoirs for new infections, both of which contribute to chronic disease (Reveneau et al., 2005). Evidence also exists of a possible connection between persistence and the development of chlamydial resistance to antibiotic treatments such as azithromycin in cell culture (Dean, 2009).

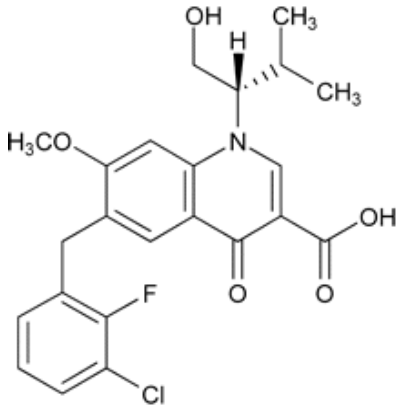
Fewer *in vivo* studies into chlamydial persistence exist due to the difficulties of obtaining live and controllable organismal models. Studies conducted by Nanagara et al. (1995) are one of few which support the contribution of persistent chlamydia to disease chronicity *in vivo*, since tissue samples from patients with chlamydia-associated arthritis were found to contain characteristically persistent chlamydia. Recent developments by the Department of Biomedical Sciences at the Quillen College of Medicine have led to the successful development of the first *tractable*, or easily controllable, animal model of chlamydial persistence. The breakthrough murine model has facilitated the *in vivo* discovery of persistent chlamydia in infected host mice stressed by amoxicillin, the most commonly prescribed antibiotic for bacterial infections in the United States and also the treatment of choice for pregnant women infected with chlamydia (Campbell et al., 2012). Recent studies with this animal model have also found that persistent chlamydiae *in vivo* are more resistant to azithromycin, supporting the similar results obtained *in vitro* (Phillips-Campbell et al., 2014; Dean, 2009).

Along with the biphasic developmental cycle, all chlamydial species share a unique family of genes encoding for polymorphic membrane proteins (PMPs). *C. trachomatis* expresses nine PMPs on the surface of its elementary bodies, designated PMP-A to PMP-I (Taylor, 2011). Although the role of PMPs in chlamydial pathogenesis is not fully understood, studies suggest that the proteins do play some role in virulence. Structural protein analysis reveals that each PMP subtype of *C. trachomatis* contains several GGAI amino acid motifs, which are associated with the adhesion of other organisms to host cells, and patients infected with *C. trachomatis* are known to produce antibodies for all PMPs (Gomes et al., 2006). Variance in PMP antibody profiles among patients infected with *C. trachomatis* suggests that the proteins are part of a mechanism of antigenic variation for the bacteria to evade the immune system, adjusting to different immunological pressures within infected hosts (Carrasco et al., 2011). In a study conducted among women with pelvic inflammatory disease, Taylor et al. (2011) found that those who expressed antibodies for PMP-A were less likely to achieve pregnancy or a live birth and those who expressed antibodies for PMP-I were more likely to have upper genital tract infections. Data from this study also revealed an increase of inflammatory markers in women with PMP-A antibodies, suggesting that PMP-A could serve as a potential biomarker for the increased risk of secondary sequelae from *C. trachomatis* infection (Taylor et al., 2011).

The expression of PMPs in relation to persistence has also been explored. A study by Carrasco et al. (2011) discovered that the transcription of some PMPs, such as PMP-B, is downregulated in *C. trachomatis*-infected cells under conditions of penicillin-induced persistence. Since *in vitro* studies by Deka et al. (2006) have found that chlamydial persistence can also be induced by co-infection of host cells with herpes simplex virus type-2 (HSV-2), we hypothesized that expression of certain PMPs, in this case PMP-A and PMP-B, would be

decreased under conditions of HSV-induced persistence. The question was explored to further determine whether PMPs could be used as biomarkers of chlamydial persistence *in vivo*.

In addition to being one of the most common sexually transmitted infections in the world, chlamydia is a common co-infection in human immunodeficiency virus/acquired immunodeficiency syndrome (HIV/AIDS) patients. A systematic review of 37 clinical and epidemiological studies of STI co-infections in HIV/AIDs patients across the world reports that 1 in 20 people living with HIV/AIDS also have chlamydia (Kalichman et al., 2011). One widely used class of anti-HIV drugs is comprised of integrase strand transfer inhibitors (INSTIs). INSTIs target the viral enzyme integrase, which is responsible for incorporating the retroviral DNA of HIV into the host genome. Some integrase inhibitors in clinical use today are raltegravir, dolutegravir, and elvitegravir (EVG), the drug of interest to our studies (Quashie, 2012). EVG, approved for use by the U.S. Food and Drug Administration in 2012, is a component of the pharmaceutical drug Stribild. Its mechanism of action consists of inhibiting integrase by preventing HIV-1 DNA strand transfer and integration (Lee et al., 2012). Studies have shown that EVG has broad antiretroviral activity, effectively inhibiting HIV replication, as well as that of simian immunodeficiency virus and murine leukemia virus (Shimura et al., 2008). Most INSTIs achieve their selectivity through the inclusion of a diketo acid moiety (DKA) in their structure, but EVG's structure (**Figure 2**)



elvitegravir (EVG)

Figure 2. The structure of Elvitegravir (Shimura, 2008).

actually contains a monoketo acid, a modified DKA, which maintains a high specificity for the strand transfer reaction (Quashie, 2012).

EVG is also a quinolone derivative. Although chlamydial infections can often be effectively treated by fluoroquinolones such as ofloxacin and levofloxacin, research shows that quinolones may also have a role in persistence. Subinhibitory or low dose concentrations of fluoroquinolones can produce fluoroquinolone-resistant mutants of *C. trachomatis* (Dessus-Babus et al., 1998). Testing conducted by Somani et al. (2000) on clinical isolates of *C. trachomatis*-infected patients who experienced treatment failure found that the chlamydiae exhibited multidrug resistance to common treatments such as ofloxacin. From the genotyping of the primary isolates and separate isolates obtained from infection relapses in those patients, it was found that recurrent disease was caused by the same resistant organisms and that many aberrant inclusions were present among the resistant strains. One theory of the researchers suggested that resistance may be linked to an alteration in the chlamydial life cycle resulting in some intermediate stage (i.e. persistence) which would make the bacteria more refractive to antimicrobial agents. Although other theories were also put forth as an explanation for the mechanism of resistance, it is possible that exposure to certain concentrations of antimicrobial agents such as ofloxacin induced the chlamydia to enter persistence (Somani et al., 2000). In another study by Patton et al. (1993), *C. trachomatis*-infected monkey models were treated with a quinolone analog therapy. Although chlamydial organisms were no longer recoverable in cell culture after completion of the therapy, chlamydial DNA was consistently present post-therapy, suggesting the presence of either dead organisms or viable organisms in an unculturable state (i.e. persistent organisms) (Patton et al., 1993). Since exposure to low-dose quinolones is implicated in chlamydial persistence and EVG is a quinolone derivative, we hypothesized that

EVG would inhibit chlamydial development. The question is relevant, considering the prevalence of chlamydial co-infections in HIV/AIDS patients and the fact that these patients must be on anti-HIV drugs for life. If EVG inhibits chlamydial replication, HIV patients on EVG would be expected to be more resistant to chlamydial infection.

MATERIALS AND METHODS

Chlamydia, HSV-2 and host cells:

HeLa cells, a human cervical adenocarcinoma epithelial cell line (ATCC CCL2) were used for both studies. A human urogenital isolate of *C. trachomatis* E/UW-5/CX was originally obtained from S.P. Wang and C. Kuo (University of Washington, Seattle, WA). *C. muridarum* Weiss strain was obtained from Dr. Kyle Ramsey. The same standardized inoculum of *C. trachomatis* serovar E or *C. muridarum* elementary bodies, propagated in Hec1B cells (human endometrial cell line ATCC#HTTB-113), was used for all experiments (Wyrick et al., 1996). HSV-2 strain 333 stocks were obtained from Mary K. Howett. Viral stocks were prepared in monolayers of Vero cells (African green monkey kidney cells ATCC No. CCL-81) using standard techniques (Duff and Rapp, 1971).

EVG studies:

Cell culture. HeLa cells were plated and infected with *C. trachomatis* serovar E or *C. muridarum* Weiss and exposed to either Elvitegravir (Selleckchem.com #S2001), amoxicillin, or tetracycline at physiologically relevant concentrations:

Drug	Class	Serum (1X) concentration	Diluent
Amoxicillin (AMX)	Penicillin	11 μg/mL	Water (ddH_2O)

Tetracycline (TET)	Tetracycline	4.3 μg/mL	Water (ddH_2O)
Elvitegravir (EVG)	Quinolone	1.7 μg/mL	Dimethyl sulfoxide (DMSO)

Table 1. Physiologically relevant drug concentrations.

Replicate cultures were either exposed to medium + diluent (DMSO or water, as appropriate), medium + amoxicillin, medium + tetracycline, or medium + EVG (1X [1.7 μg/mL] concentration) and incubated at 35°C. At 48 hours post infection, samples were collected for titer assay, immunofluorescence staining, and transmission electron microscopy.

Determining EVG concentration. The physiologically relevant concentration of EVG was set at 1.7 μg/mL (Ramanathan, 2008; "Stribild," 2013). That is the maximum concentration of EVG that can be found in human serum when ingested in the form of the trademark drug Stribild.

Immunofluorescence assay (IFA) and percent infectivity. At 48 hours post infection, cell monolayers were fixed and permeabilized with 1 mL cold methanol for 20 minutes. Glass coverslips for *C. trachomatis* and *C. muridarum* were stained with anti-chlamydial MOMP stain and LPS stain (Bio-Rad #30702 and #30701), respectively. All coverslips were counterstained with DAPI and mounted on glass slides. The number of inclusions and host cell nuclei in 10 fields/coverslip was determined at 630x magnification under oil immersion using an Axiovert S100 (Zeiss) microscope and Axiovert imaging software. Percent infectivity was calculated as the number of inclusions divided by the number of host cell nuclei per field on the coverslips. The data were analyzed statistically using a two-tailed t test with $p<0.05$ considered significant.

Infectious titer assay. Chlamydial titrations were conducted as previously described (Deka et al., 2006) using LPS and MOMP stains (Bio-Rad) to identify chlamydial inclusions

formed from subpassaged EB. The number of inclusion-forming units (IFU) in the undiluted inoculum was derived from triplicate counts and expressed as IFU/mL (Hall et al., 2014).

Transmission electron microscopy (TEM) processing. Cultured cells were scraped into gluteraldehyde/paraformaldehyde TEM fixative and incubated at 4°C for 1 hour before further processing. All samples were washed with cacodylate buffer and then incubated with 1% osmium tetroxide in cacodylate buffer for 1 hour at room temperature. Samples were washed again with cacodylate buffer and dehydrated with gradually increasing concentrations of ethanol before infiltration with Eponate 812 (Polysciences, Inc., Warrington, PA). Samples were finally embedded in fresh Epon, thin sectioned, and visualized using a Tecnai Philips transmission electron microscope at 80kV. All electron micrographs were taken at 12700x magnification.

PMP studies:

Cell culture. HeLa cells were divided into four groups for mock infection, chlamydial infection, HSV-2 infection, and *C. trachomatis*/HSV-2 double infection. Host cells were incubated with a dilution of crude EB stock calculated to infect greater than 80% of the cells. After one hour of adsorption, monolayers were refed with Minimal Essential Medium (MEM; Invitrogen #11095-098) and incubated at 35°C for 24 hours. Cultures were then infected with HSV-2 at a multiplicity of infection (MOI) of 10 plaque forming units (PFU) per cell. Mock-infected HeLa cells were treated similarly, except they were exposed to either 2SPG (0.2M sucrose, 6mM NaH_2PO_4, 15mM Na_2HPO_4, 5mM L-glutamine, pH 7.2; mock *C. trachomatis* infection) or growth medium (mock viral infection). After HSV adsorption, monolayers were refed with MEM and incubated at 35°C for 20 hours.

Flourescent staining and microscopy. The same staining procedures were used for PMP-A and PMP-B. Fluorescence analysis samples were prepared as described previously using formaldehyde fixation (Deka et al., 2006). Fixed cells were stained using the Carrasco et al. (2011) protocol. Anti-PMP primary antibodies were a kind gift from Dr. Patrick Bavoil (Tan, 2009).

To make a 1:25 initial dilution of anti-PMP primary antibody (guinea pig), 4 µL of anti-PMP primary antibody was added into a tube containing 96 µL of PBS (phosphate buffered saline) washing buffer. Fixed HeLa cells were washed 3 times in 1X PBS washing buffer, and then 100 µl of diluted primary antibody was added into each well of HeLa cells. The plate was then placed on a gyrator shaker at a speed of 3.5 for 30 minutes at room temperature to pre-adsorb the antisera. This process removes antibodies that cross-react to HeLa cell antigens (to reduce staining background), while anti-chlamydia PMP antibodies remain. The resulting pre-absorbed antibody was then used as the primary antibody for all subsequent fluorescence staining of chlamydia-infected cells. The secondary antibody (Alexa Fluor 488 goat anti-guinea pig (IgG)) (Invitrogen #A-11073) was diluted to a concentration of 1:500 with PBS washing buffer and DAPI solution.

For fluorescence staining, 10 µL drops of dilute primary antibody were pipetted onto parafilm. Sample coverslips were washed with PBS washing solution and inverted onto the primary antibody drops, cell monolayer down. Coverslips were incubated at 37°C for 1 hour and then washed with PBS washing buffer before secondary staining. Ten µL drops of secondary antibody were pipetted onto parafilm, and coverslips were inverted onto the drops and incubated at 37°C for 30 minutes. Coverslips were then washed with PBS washing buffer again before

being mounted (Fluoromount Fisher #OB100-01) on glass slides and stored in a dark drawer to dry.

Using an Axiovert S100 (Zeiss) microscope and Axiovert imaging software, the intensity of 50 inclusions from singly-infected cells and 50 chlamydial inclusions from co-infected cells were quantified and compared for each one of three replicates. Inclusion intensity measurements were divided by inclusion area measurements, and the resulting measurements for all three replicates of each sample were averaged to compare the expression of the PMP of interest in the chlamydial-infected cells versus the chlamydial/HSV-2 co-infected cells. Statistical analyses of the data were conducted using a two-tailed t test with $p<0.05$ considered significant.

RESULTS

EVG studies:

Several EVG studies were conducted. The following are representative data for the effects of EVG found on both the development of *C. trachomatis* and *C. muridarum*. The results encapsulate the percent infectivity obtained from IFA staining, production of infectious progeny from chlamydial titer assay, and chlamydial inclusion morphology from TEM analysis. Percent infectivity is equal to the number of chlamydial inclusions divided by the number of host cells counted per microscopic field on sample coverslips. Inclusion and cell counts were conducted on individual IFA images (**Figures 4 and 6**, for example) and average percent infectivity values were then calculated (**Figures 3 and 5**). Percent infectivity was used to determine if any great differences in infectivity, which could have affected experimental results, existed among the samples.

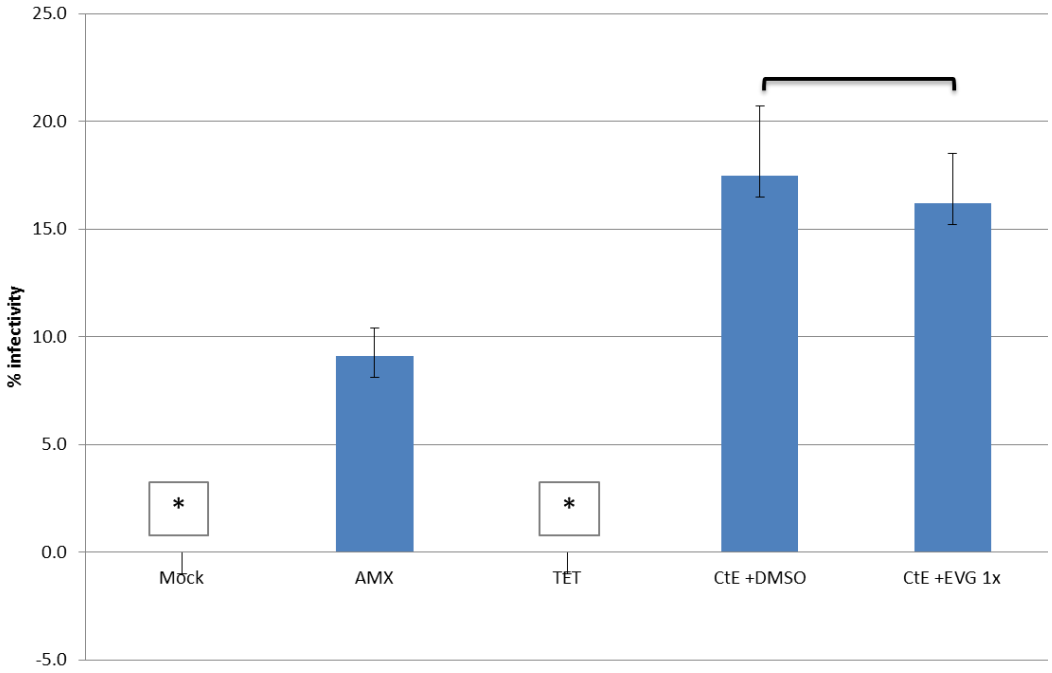


Figure 3. EVG Exposure Does Not Inhibit *C. trachomatis* Inclusion Formation. Percent infectivity (number of chlamydial inclusions divided by number of host cell nuclei) for three replicates (n=3) of *C. trachomatis* serovar E-infected samples stained using immunofluorescence assay (IFA) staining. An asterisk (*) indicates a statistically significant difference found after conducting a two-tailed t test using $p<0.05$ for significance. Mock, AMX, and TET samples were compared to CtE –DMSO positive control samples (data not shown). EVG samples were compared to the CtE +DMSO positive control samples. These data were obtained from study EVG.02.

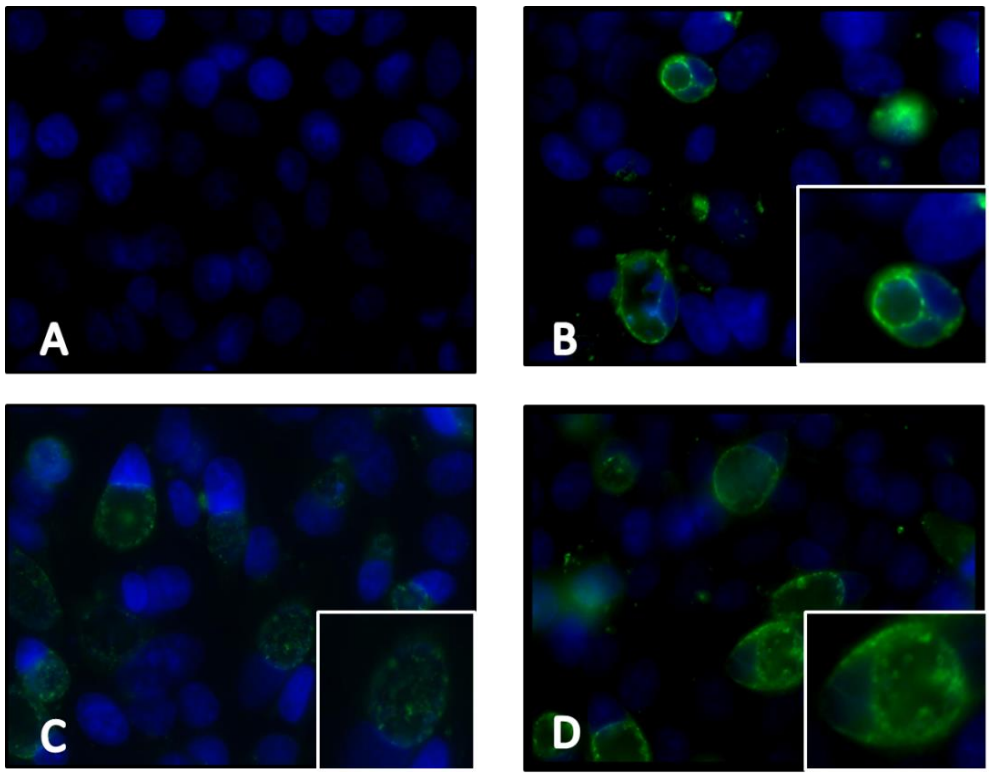


Figure 4. IFA-stained cells at magnification 630x under oil immersion: **A.** mock **B.** CtE +amoxicillin (AMX) **C.** CtE +DMSO **D.** CtE +EVG 1X concentration (1.7 µg/mL). Host cell nuclei are stained blue by DAPI stain and chlamydial inclusions are stained green by MOMP stain.

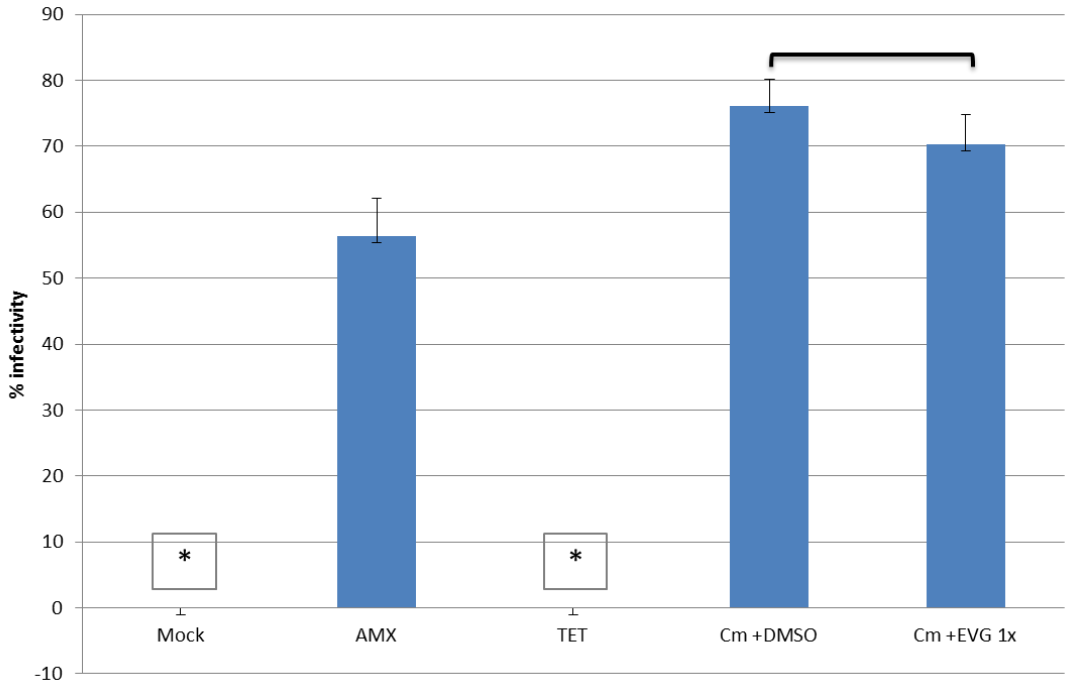


Figure 5. EVG Exposure Does Not Inhibit *C. muridarum* Inclusion Formation. Percent infectivity (number of chlamydial inclusions divided by number of host cell nuclei) for three replicates (n=3) of *C. muridarum*-infected samples stained using immunofluorescence assay (IFA) staining. An asterisk (*) indicates a statistically significant difference found after conducting a two-tailed t test using $p<0.05$ for significance. Mock, AMX, and TET samples were compared to Cm –DMSO positive control samples (data not shown). EVG samples were compared to the Cm +DMSO positive control samples. These data were obtained from study EVG.07.

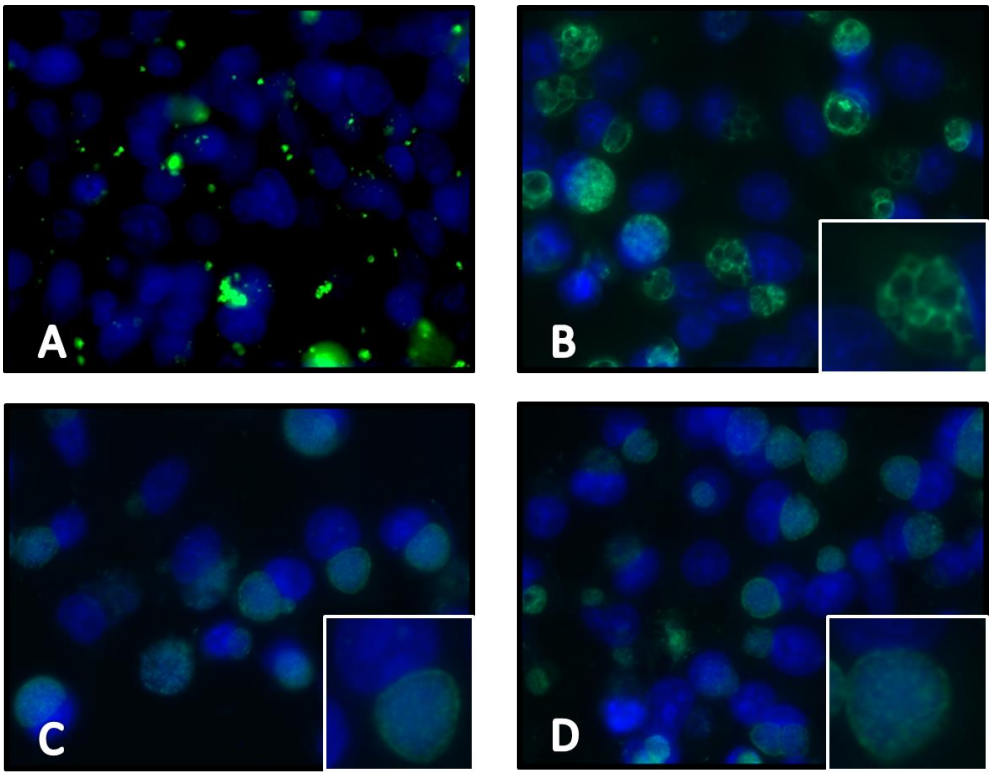


Figure 6. IFA-stained cells at magnification 630x under oil immersion: **A.** Cm +tetracycline (TET) **B.** Cm +AMX **C.** Cm +DMSO **D.** Cm +EVG 1X concentration (1.7 µg/mL). Host cell nuclei are stained blue by DAPI stain and chlamydial inclusions are stained green by LPS stain.

As expected, mock infected monolayers for both *C. trachomatis* (**Figure 3**) and *C. muridarum* (**Figure 5**) did not contain inclusions since those samples were not infected with chlamydia. Tetracycline-exposed *C. trachomatis* (**Figure 3**) and *C. muridarum* (**Figure 5**) did not display any inclusions, as was also expected, since tetracycline effectively kills chlamydia and prevents inclusions from developing. In contrast, amoxicillin-exposed samples for each species did contain inclusions. Amoxicillin served as a control for chlamydial persistence, so the drug did not inhibit the formation of inclusions. Diluent control cultures of chlamydia containing ddH_2O (the diluent used for TET and AMX) were also performed (data not shown). Chlamydial samples exposed to DMSO were used as a comparative reference for EVG samples since the EVG samples contained DMSO as the diluent for the drug. Statistical analysis for EVG-exposed versus unexposed chlamydia for both species showed that there was not a significant decrease in the formation of inclusions (**Figures 3 and 5**). It was thus determined that EVG did not significantly alter the percent infectivity and therefore did not pose a significant problem for interpreting other experimental results obtained for either EVG-exposed *C. trachomatis* or *C. muridarum*.

IFA images reveal a first look into inclusion morphology for the treated samples. Mock samples (**Figure 4A**) only exhibit blue host cell nuclei since no chlamydia are present to bind the FITC-labeled anti-LPS antibody (which fluoresces green). Residues of killed chlamydial inclusions are labeled by anti-LPS in tetracycline-exposed chlamydial cultures (**Figure 6A**). Amoxicillin-exposed chlamydia (**Figures 4B, 6B**) appeared persistent. Large, aberrant bodies can be seen within the insets of the inclusions. Chlamydia exposed to DMSO (**Figures 4C, 6C**) and EVG (**Figures 4D, 6D**) displayed very similar inclusion morphologies, which were clearly distinct from the amoxicillin-exposed chlamydia.

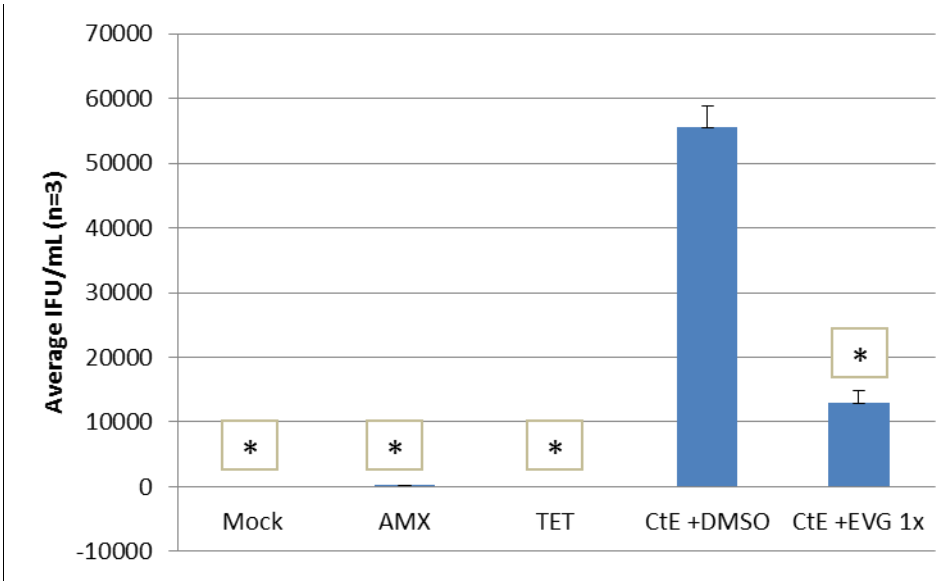


Figure 7. EVG Exposure Reduces Production of Infectious *C. trachomatis* Elementary Bodies. Titer assay results showing the number of infectious, recoverable EB counted from *C. trachomatis* serovar E-infected samples. An asterisk (*) indicates a statistically significant difference compared to the CtE +DMSO positive control samples found after conducting a two-tailed t test using $p<0.05$ for significance.

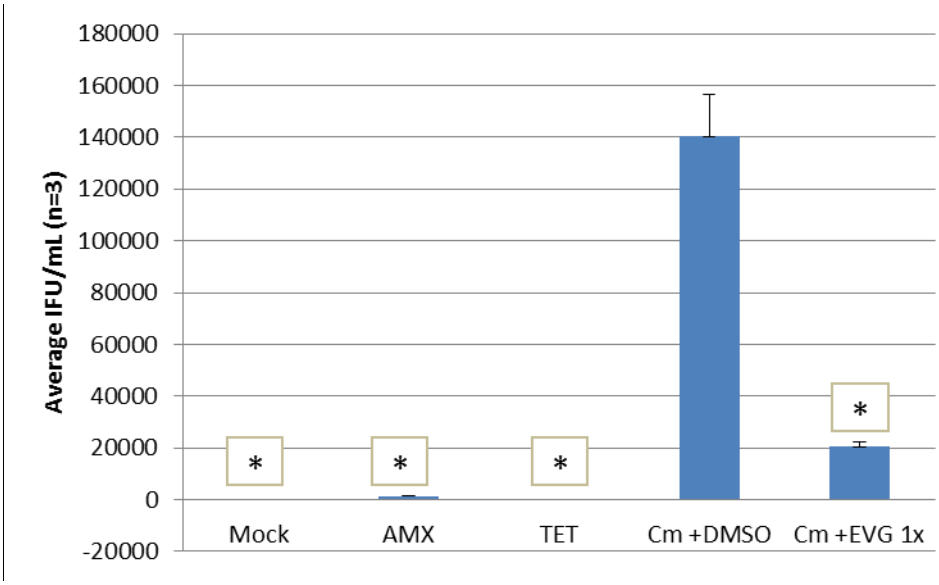


Figure 8. EVG Exposure Reduces Production of Infectious *C. muridarum* Elementary Bodies. Titer assay results showing the number of infectious, recoverable EB counted from *C. muridarum*-infected samples. An asterisk (*) indicates a statistically significant difference compared to the Cm +DMSO positive control samples found after conducting a two-tailed t test using $p<0.05$ for significance.

Titer results (**Figures 7 and 8**) for both *C. trachomatis* and *C. muridarum* showed that mock-, AMX-, and TET-exposed samples did not produce infectious EB, as expected. Although chlamydia can form inclusions when AMX exposed, the organisms are persistent (noninfectious) and thus not producing infectious EB. Titer data revealed a significant decrease in the production of viable infectious progeny from chlamydia exposed to EVG at the physiologically relevant (1X) concentration. Compared to the DMSO-exposed chlamydia, there was a much lower number of recoverable EB found from the EVG-exposed chlamydia. These results were consistently reproduced in several experimental replications and suggest that exposure to EVG causes a reduction in the production of infectious EB.

Transmission electron micrographs (TEMs) (**Figure 9** below) provided an up-close look into individual inclusions. As the figure shows, the morphology of *C. trachomatis* and *C. muridarum* inclusions appears very similar among samples exposed to DMSO and samples exposed to EVG. Both EB, indicated by yellow arrows, and RB, indicated by white arrows, are scattered throughout. This confirms the results of the IFA images, which also appeared to show morphologically similar inclusions between DMSO- and EVG-exposed samples. TEMs of characteristically persistent inclusions (images not shown) display very large, aberrant bodies. The aberrant bodies are few in number since persistent chlamydia cease replicating, and there is also an absence of the small, dark infectious EB since persistent chlamydia are also noninfectious.

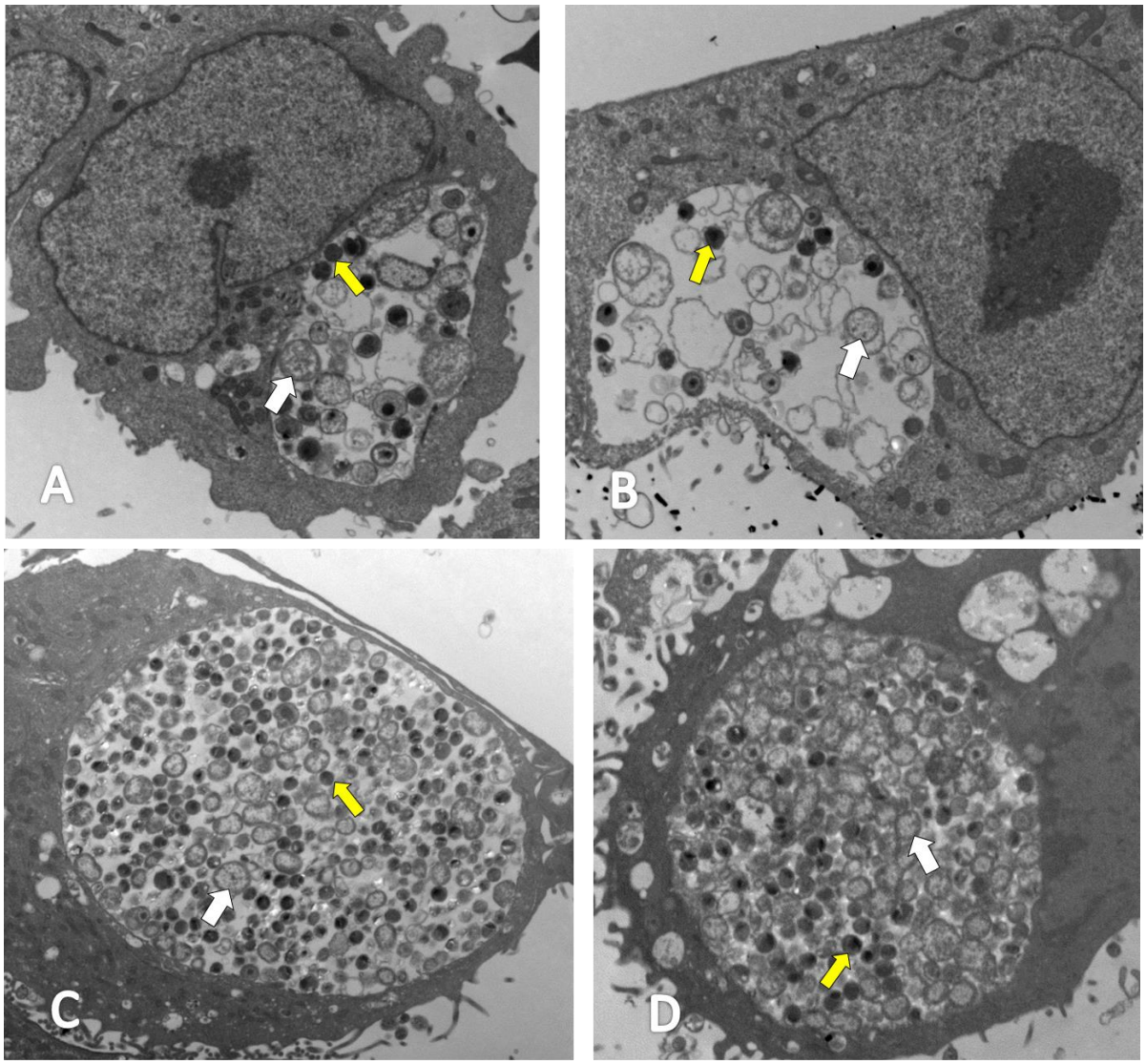


Figure 9. EVG Exposure Does Not Significantly Alter *C. trachomatis* or *C. muridarum* Morphology. Transmission electron micrographs at magnification 12700x: **A.** CtE +DMSO **B.** CtE +EVG 1X concentration (1.7 μg/mL) **C.** Cm +DMSO **D.** Cm + EVG 1X concentration**.** White arrows indicate RB and yellow arrows indicate EB within the chlamydial inclusions.

PMP studies:

The following are results from our studies of the expression of both PMP-B and PMP-A under conditions of HSV-induced persistence. Chlamydial PMP expression was measured by the amount of inclusion intensity from fluorescence staining divided by inclusion area.

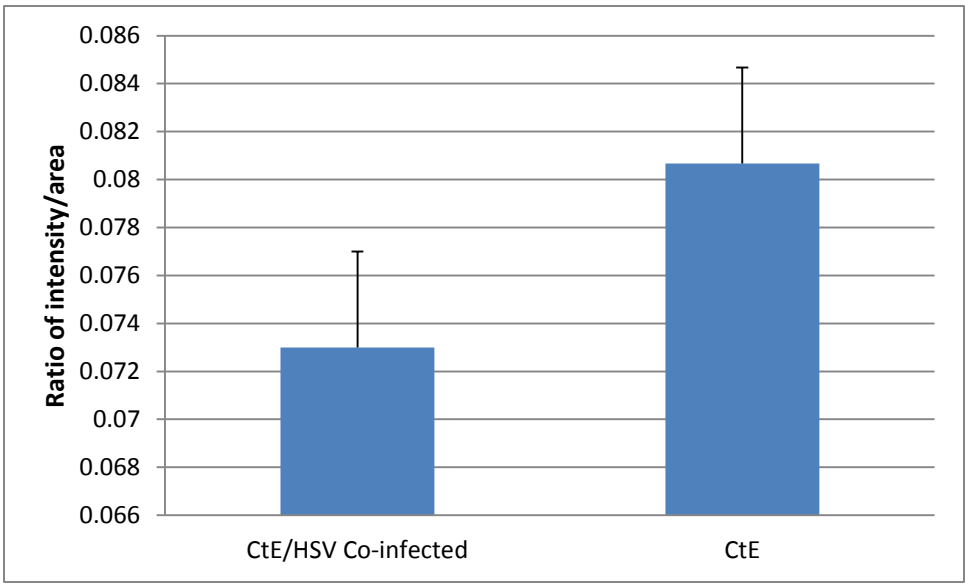


Figure 10. Co-infection Does Not Significantly Alter *C. trachomatis* PMP-B Accumulation. Average inclusion intensity/area measurements for three replicates (n=3) of PMP-B stained CtE/HSV co-infected versus CtE-infected cells. There was no significant difference between inclusion intensity staining with PMP-B antibody between co-infected and singly-infected samples ($p>0.05$).

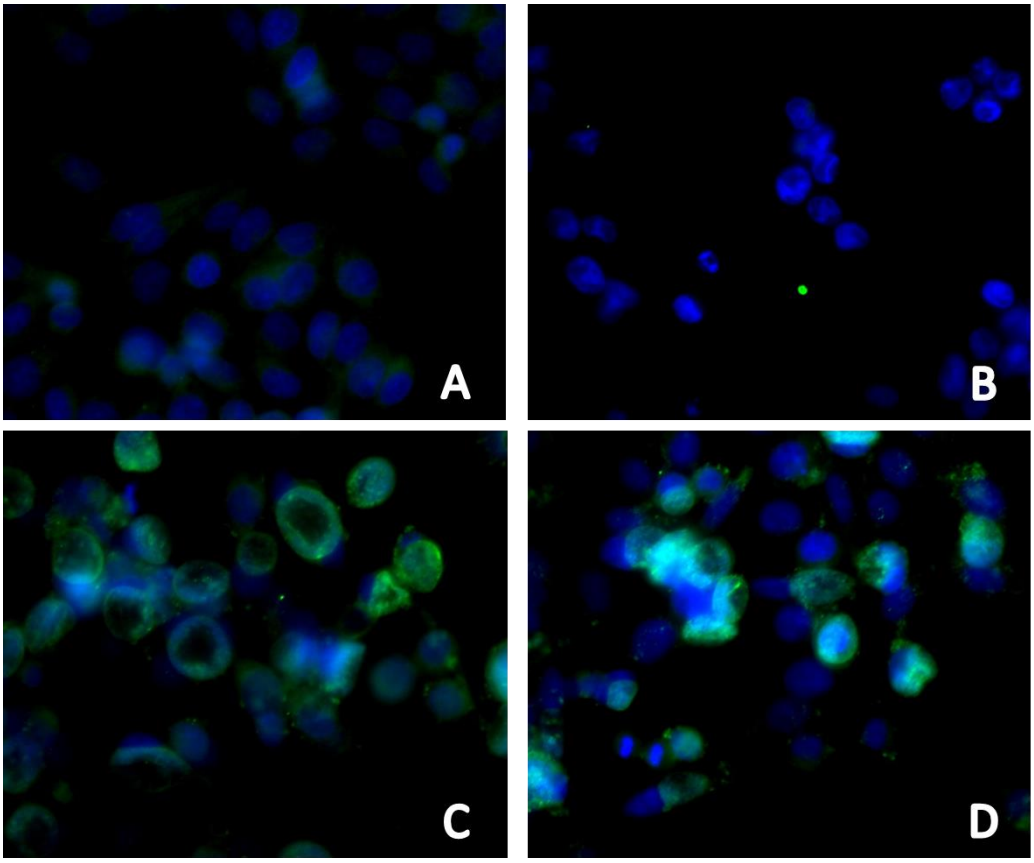


Figure 11. HeLa cells stained with PMP-B antibody at 400x magnification. **A.** mock **B.** HSV-infected **C.** CtE/HSV co-infected **D.** CtE-infected.

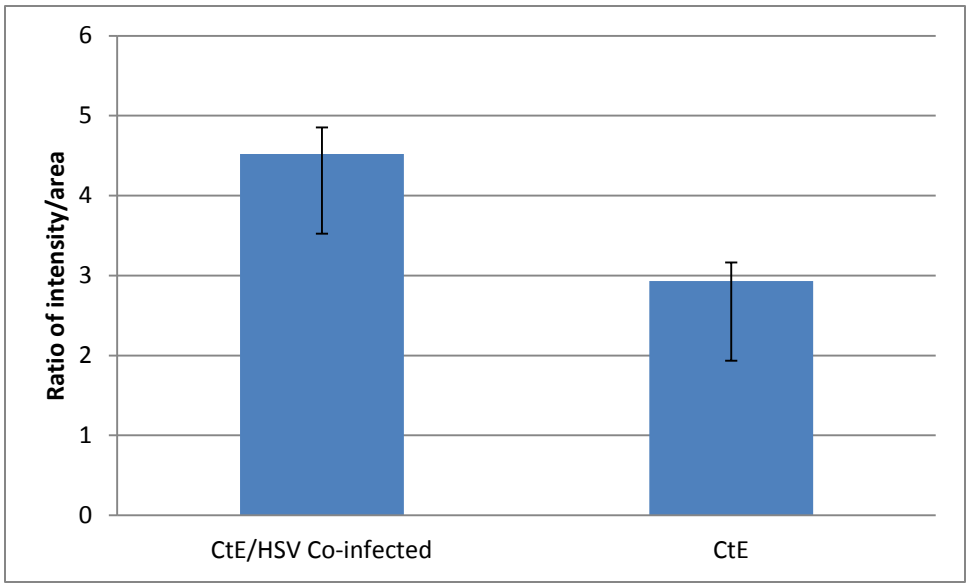


Figure 12. Co-infection Does Not Significantly Alter *C. trachomatis* PMP-A Accumulation. Average inclusion intensity/area measurements for three replicates (n=3) of PMP-A stained CtE/HSV co-infected versus CtE-infected cells. There was no significant difference between

inclusion intensity staining with PMP-A antibody between co-infected and singly-infected samples ($p>0.05$).

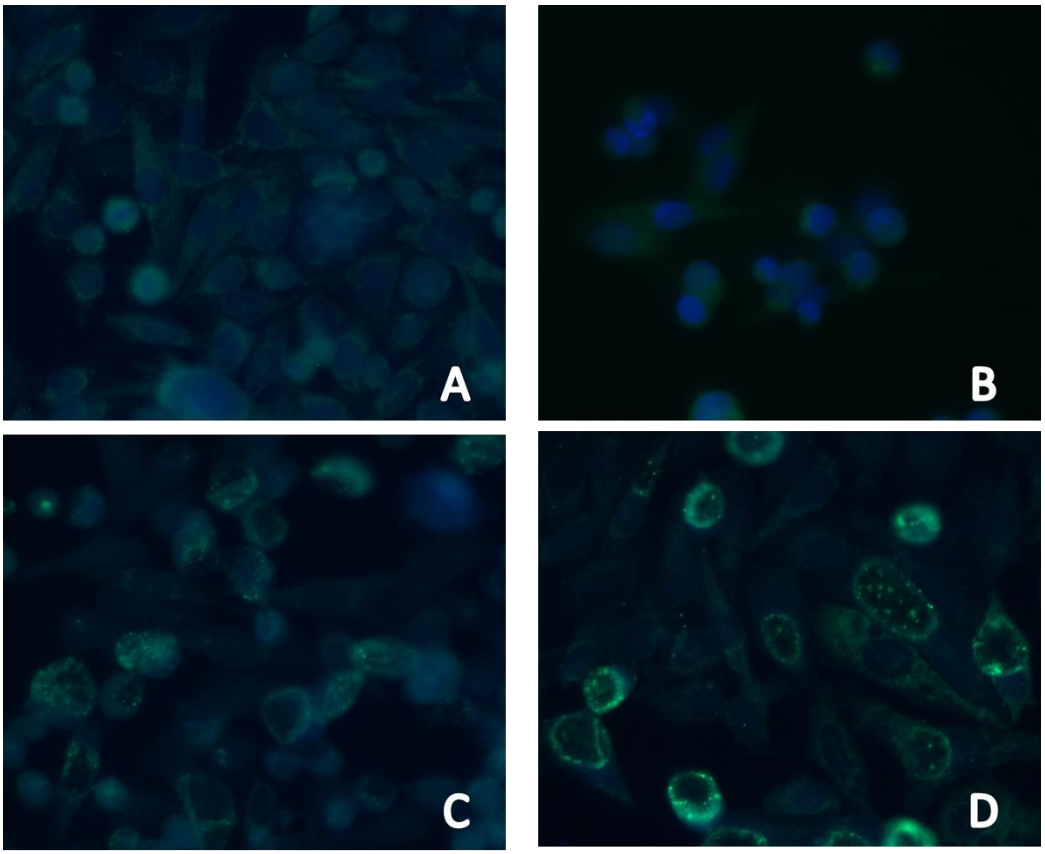


Figure 13. HeLa cells stained with PMP-A antibody at 400x magnification. **A.** mock **B.** HSV-infected **C.** CtE/HSV co-infected **D.** CtE-infected.

Results (**Figures 10 and 11**) revealed that there is no significant difference in expression of either PMP-B or PMP-A staining intensity between chlamydia-infected and chlamydia/HSV-2 co-infected cells. Representative images of PMP-B and PMP-A expression can be viewed in **Figure 11** and **Figure 13**, respectively.

DISCUSSION

Contrary to our initial hypothesis, the data from our PMP studies show that there was no significant difference in expression of PMP-A or PMP-B between unstressed chlamydia and chlamydia stressed by HSV-2. This suggests that neither PMP-B nor PMP-A play a special role

in chlamydial persistence when induced by HSV-2, leading to the conclusion that neither PMP would serve as a good biomarker of persistence when induced by HSV-2. Since Carrasco et al. (2011) did find some decreased levels of PMP expression among persistent chlamydia induced by penicillin, it seems that whether or not PMP expression changes during persistence may vary according to the type of stimulus that is used to induce persistence. Experimentation involving different inducers of persistence, such as IFN-λ or other β-lactams like amoxicillin, would be helpful in further exploring the question.

The results from EVG studies also lead to further questions. It can be directly observed from both IFA and TEM visuals that the appearance of EVG treated and untreated chlamydial inclusions is very similar. Exhibiting inclusions with EB and RB similar to the DMSO-exposed chlamydia, EVG exposure appears to not alter normal inclusion formation. It is clear by comparison to references of persistence such as the amoxicillin-exposed chlamydia that EVG is not inducing chlamydia into persistence. This would point to the conclusion that EVG has no effect on chlamydial development, but the results of infectious titer assays indicate otherwise. Since titer results revealed that EVG exposure significantly reduces the production of infectious EB in both *C. trachomatis* and *C. muridarum*, it appears that EVG does inhibit chlamydial development *in vitro* at physiologically relevant concentrations. Since this inhibition of the production of infectious progeny does not seem to be linked to persistence, it appears that another mechanism may be at work.

Although the mechanism of this inhibition of chlamydial development is currently unknown, there are several possibilities. Although TEMs indicate that EB are still present within chlamydia exposed to EVG, titer assays indicate that the EB are significantly less infectious. EVG could be reducing the infectious nature of EB after they are produced. It is possible, for

example, that the drug could be interacting with and partially inactivating EB surface proteins that are required for the EB to infect new cells. It is also possible that EVG could be delaying the release of EB from inclusions or that its activity could differ depending on the timing of the drug's addition to chlamydia infected cells. Further experimentation involving variations in timing of the drug's addition to infectious samples could shed more light on its activity.

Preliminary studies involving mice have been conducted in order to ascertain the effects of EVG *in vivo*. A group of 4 mice were administered with Stribild, the pharmaceutical drug form of EVG, to observe effects on chlamydial shedding. Compared to untreated mice, the mice administered with Stribild exhibited a shorter duration of chlamydial shedding by about 6 days. Stribild treated mice also seemed to show less gross pathology as well. Although an interesting finding, these results are interpreted with caution due to the very preliminary nature of the study. Only a small number of mice were used, so the reliability of the data is questionable until the experiment can be reproduced on a greater scale.

Although not serving as a persistence-inducer, EVG clearly has some inhibitory effect on the development of chlamydia *in vitro*. Further experiments involving animal models or other anti-HIV drugs similar to EVG may be used in the future to understand more about the mechanism at work.

ACKNOWLEDGEMENTS

I would like to take this opportunity to thank Dr. Robert Schoborg, Dr. Laraine Powers, and Dr. Frank Hagelberg for their reading and helpful discussion of this work. I would also like to thank Jennifer Kintner, Jessica Slade, Cheryl Moore, and Dr. Jennifer Hall for their continued guidance throughout this process. This research has been supported by funding from the National

Institutes of Health/National Institute of Allergy and Infectious Diseases grant R01 AI095637-04 and C06RR0306551, the Department of Biomedical Sciences at the James H. Quillen College of Medicine, and the Honors College at ETSU.

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