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ChAT Expression in *Chlamydia muridarum*-infected Female Murine Genital
Tract

Thesis submitted in partial fulfillment of the University Honors Scholars
Program

By

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ABSTRACT

Chlamydia trachomatis is the most prevalent agent of bacterial sexually transmitted infections in the world. However, a profuse number of cases are unreported, as the infection is often asymptomatic. Sequelae such as pelvic inflammatory disease, an increased risk of cervical cancer, premature birth, and perinatal infections in pregnant women can occur. Inflammation occurs in the body in response to infection or injury. Although inflammation can lead to some unwanted secondary effects, such as pain, it serves to return the body to homeostasis by restoring injured tissues and eliminating pathogens. One recently identified connection between the central nervous system and the immune system that regulates inflammation is the cholinergic anti-inflammatory pathway (CAP). In the CAP, pathogen-associated molecular patterns stimulate the vagus nerve to activate the pathway, which ultimately results in acetylcholine (ACh) release, which down regulates inflammation. We hypothesized that genital chlamydial infection would increase the expression of choline acetyltransferase (ChAT), the enzyme that synthesizes ACh, in the female murine genital tract, therefore down regulating inflammation and promoting chlamydial infection. Transgenic female mice carrying a ChAT-promoter driven GFP reporter gene were vaginally infected with *C. muridarum*. Mice were sacrificed on days 3, 9, 15, and 21 post infection; cervical, uterine horn, and ovarian tissues were removed and embedded in paraffin. Small sections of each tissue were cut and mounted onto slides. The tissue sections were then stained for the expression of ChAT using immunohistochemical techniques. Finally, tissue sections were viewed under a microscope for positive staining and the data was analyzed. The results

indicated that there is a significant increase in the number of cells that express ChAT in genital tract of chlamydia-infected mice versus non-infected mice.

INTRODUCTION

Chlamydia trachomatis is the most prevalent agent of bacterial sexually transmitted infections in the world. One hundred one million infections are caused by this bacterium each year, as reported by the World Health Organization (Malhotra et al., 2013). However, a substantial number of cases are unreported, as the infection is asymptomatic in 10% of men and 5-30% of women. A sexually transmitted infection of *Chlamydia trachomatis* can manifest as cervicitis, urethritis, and, or, proctitis (CDC, 2016). Sexually transmitted infections caused by this agent are more common in women; sequelae such as pelvic inflammatory disease, an increased risk of cervical cancer, premature birth, and perinatal infections in pregnant women can occur. In fact, in the United States about 20%-30% of cases of pelvic inflammatory disease are accounted for by *Chlamydia trachomatis*. Reinfections or chronic infections with *C. trachomatis* can occur, in which delayed hypersensitivity reactions can develop. The immune response that ensues in these chronic infections can cause damage such as the formation of scar tissue and fibrosis in the genital tract – which can lead to infertility or life-threatening ectopic pregnancy (Malhotra et al., 2013). *C. trachomatis* can also be transmitted from mother to child in the birth canal, which can result in pneumonia and, or conjunctivitis in the neonate (Hogan et al., 2004).

C. trachomatis is a obligate intracellular Gram-negative pathogen with a biphasic developmental cycle (**Figure 1**). First, elementary bodies enter host

genital epithelial cells. Elementary bodies are infectious, but are less metabolically active and unable to reproduce. Once inside the cell, within a membrane bound vesicle called an inclusion, elementary bodies differentiate into reticulate bodies. Reticulate bodies are more metabolically active than EB and divide inside of the inclusion, but are unable to infect other cells. An unknown cue causes the reticulate bodies to differentiate back into elementary bodies. These progeny elementary bodies are released from the cell by either host cell rupture or inclusion extrusion and can go on to infect other cells and repeat this developmental cycle (Wolf et al., 2006).

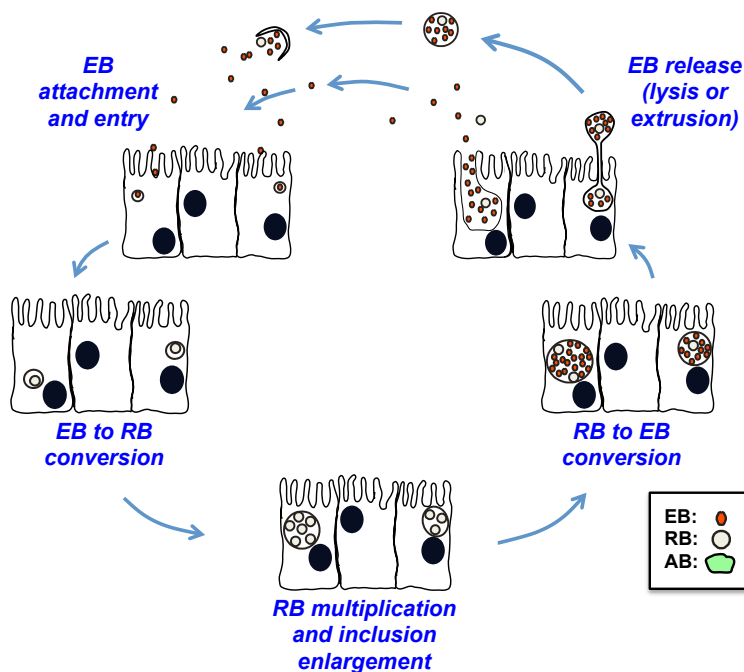


Figure 1. Chlamydial Developmental Cycle

Cholinergic neurotransmission is one of the central means by which the body maintains homeostasis, and therefore life (Wessler and Kirkpatrick, 2008). In animals, acetylcholine (ACh) is synthesized by the enzyme choline acetyltransferase (ChAT) (Wessler et al., 1998). Cholinergic neurons release ACh into the synaptic cleft; these neurotransmitters act on nicotinic and muscarinic receptors to carry out neurotransmission. Not only is ACh an important neurotransmitter, but it also has other important signaling functions. Even in organisms like bacteria, fungi, algae, and protozoa, that do not have neurons, cholinergic communication has been found (Wessler and Kirkpatrick, 2008). Thus, it is not surprising that the ChAT enzyme, ACh, and nicotinic and muscarinic receptors for ACh have also been found in non-neuronal cells in mammals. Many non-neuronal cells produce ACh, and most of these cell types also express nicotinic and muscarinic receptors that play a role in the autocrine and paracrine cholinergic regulation of non-neuronal cells (Wessler and Kirkpatrick, 2008). These autocrine and paracrine loops regulate cellular functions such as proliferation, differentiation, and migration (Wessler and Kirkpatrick, 2008). It has been determined that ACh can also alter immune responses. For example, release of proinflammatory molecules TNF and IL1- β from macrophages that express $\alpha 7$ nicotinic receptors can be inhibited when these cells are stimulated by ACh. This neuroinflammatory reflex is known as the cholinergic anti-inflammatory pathway (CAP) (Wessler and Kirkpatrick, 2008).

Inflammation occurs in the body in response to infection or injury. Although inflammation can lead to some unwanted secondary effects, such as pain, it serves to return the body to homeostasis by promoting healing within injured tissues and eliminating pathogens (Pavlov et al., 2003). The CAP is a recently identified connection between the central nervous system and the immune system that suppresses inflammation. In this response, pro-inflammatory cytokines, substances from necrotic tissues, and pathogen-associated molecular patterns, which are substances unique to microbes, activate afferent fibers of the vagus nerve (Reardon, 2016). This vagal sensory information is directed to the nucleus tractus solitarius in the brainstem. Some neurons in the nucleus tractus solitarius synapse with neurons in the rostral ventrolateral medulla. Neurons in the rostral ventrolateral medulla project to the locus coeruleus, which then projects to sympathetic preganglionic neurons in the spinal cord (Pavlov et al., 2003). Efferent vagal nerve fibers are also stimulated, which stimulate postganglionic sympathetic fibers in the celiac ganglion. These activated neurons release norepinephrine (NE) into the spleen, and likely into other organs as well. A particular group of CD4+ T-cells have β -adrenergic receptors that bind NE, which, upon NE binding, express the ChAT enzyme and release ACh (Reardon, 2016). Immune cells that produce cytokines express nicotinic acetylcholine receptors (nAChR); ACh binds to the $\alpha 7$ subunit of these nAChR. As a result of this binding, decreased NF- κ B activation, pro-inflammatory cytokine and innate immunity activity occurs (Tracey, 2009).

The $\alpha 7$ nAChR has been found to be expressed on macrophages, which are the primary immune cells that initiate inflammatory responses. TNF produced by LPS-activated macrophages can be dose-dependently decreased by ACh (Pavlov et al., 2003). Gene knockout mice for the $\alpha 7$ nAChR gene have been found to be more sensitive to stimuli that induce inflammation. During endotoxemia a considerable increase in IL-1, IL-6, and TNF was found in the serum of the mice deficient in the $\alpha 7$ nAChR versus the wild type mice. Although vagus nerve stimulation reduces pro-inflammatory cytokines in wild type mice with endotoxemia, mice deficient in the $\alpha 7$ nAChR are unable to produce this same response. Experiments have also been performed on rats to demonstrate the role of the CAP in localized peripheral inflammation. In a rat with paw edema, electrical stimulation of the distal vagus nerve reduced local inflammation. Additionally, when the paw was treated with ACh, muscarine, or nicotine (all of which are agonists that bind to and activate ACh receptors) prior to electrical stimulation the local inflammation of the paw was inhibited. These experiments demonstrate that vagal nerve stimulation or stimulation of $\alpha 7$ nAChR downregulates inflammation, which provides supporting evidence of CAP function (Pavlov et al., 2003).

Research also indicates that stimulation of nAChR promotes pathogenesis in some infections. In a mouse model of *Staphylococcus pneumoniae* infection, inflammation and bacterial load in the lungs was significantly increased in mice treated with nicotine (Giebelen et al., 2009). In another study, the bacterial load and mortality in mice infected with *Francisella tularensis* was increased in groups

treated with either ACh or an acetylcholinesterase inhibitor, neostigmine (Pohanka et al., 2012). This data suggests that ACh can enhance pathogenesis during infection, perhaps by reducing the host inflammatory response to infection and, thereby, allowing the pathogen to evade host immune responses.

Therefore, we hypothesized that genital tract infection with *C. muridarum* would increase the number of ChAT-expressing cells in the genital tract. If this hypothesis is correct, we would also predict that this chlamydia-induced production of ACh would reduce local inflammation and help the bacteria evade the host immune response.

MATERIALS AND METHODS

Animal Infections

All protocols were approved by the University Committees on Animal Care, Biosafety, and Hazardous Materials and followed by the applicable state and federal guidelines. ChAT (BAC) eGFP female mice from Jackson Laboratory (strain B.6Cg-Tg (RP23-268L19-EGFP) 2Mik/J; #007902) were injected subcutaneously with 2.5mg/100µL medroxyprogesterone acetate suspension from Greenstone Pharmaceuticals 7 days before vaginal infection with *C. muridarum*. Vaginal swabs were taken for titers. The mice were then sacrificed by cervical dislocation on the appropriate day post infection. Immediately upon being sacrificed, the appropriate tissue was removed and fixed in formalin (10% formaldehyde). Two to 3 days later the tissue was moved to 70% ethanol (EtOH). Tissue was then embedded in paraffin.

Cutting and mounting embedded samples

Tissue samples embedded in paraffin were kept in the freezer or on ice until immediately before they were cut. Imbedded tissues in blocks were cut into 5 μm sections using a microtome. Several sections were cut at once to form a ribbon of tissue sections, which was transferred to a piece of cardboard. A razor blade was then used to cut the strip into smaller, three to four section, strips. These smaller strips were then transferred to a 43°C water bath. Sections were then mounted onto glass microscope slides by lifting tissue sections out of the water bath with a microscope slide. These slides were then set upright in a tray to dry at room temperature for at least one day.

De-paraffinization and Tissue Rehydration

Slide-mounted sections were placed in an oven at 60°C for 1-3 hours to melt the paraffin. After removing the slides and letting them cool to room temperature, they were placed in glass boats and rehydrated in a series of baths. Slides were first incubated in xylene baths twice for five minutes each. Slides were then placed in 100% EtOH baths twice for two minutes each, then 95% EtOH baths twice for two minutes each. Then slides were placed in 75% EtOH baths for 2 minutes followed by a 50% EtOH bath for an additional 2 minutes. Slides were then placed in a distilled water bath twice for 5 minutes each. Finally slides were placed in 0.1M phosphate buffered saline (PBS) for 5 minutes. Antigen retrieval proceeded immediately.

Antigen Retrieval

Rehydrated slides were spaced evenly in a tissue tech slide holder and placed in its container. The container was then filled with Antigen Retrieval

Solution (300mL dH₂O + 0.882g sodium citrate, pH: 7). This was placed inside of a microwave for 3.5 minutes at full power and then for 6 minutes set at defrost. The container was removed from the microwave and allowed to cool to room temperature. The slides were then washed in distilled water 3 times for 5 minutes each. The Immunostaining Day 1 protocol started immediately or the slides were placed in IHC PBS overnight.

Immunostaining Day 1

If completed the same day as the antigen retrieval protocol was completed, slides were rinsed in IHC PBS (29.6g Na₂HPO₄ + 8.6g KH₂PO₄ + 14.4g NaCl + dH₂O to make total volume 2L, pH 7.3) 4 times for 5 minutes each. If the slides were left overnight in IHC PBS, slides were rinsed only 2 times for 5 minutes each. Slides were then rinsed with 0.5% bovine serum albumin (BSA) solution made in IHC PBS. Slides were then rinsed with a 1% hydrogen peroxide solution made in IHC PBS for 15 minutes. Then slides were rinsed with IHC PBS 4 times for 5 minutes each, followed by a 10 minute rinse in 0.5% BSA solution. The slides were then removed from the tissue tech holder and then dried using a kimwipe. A PAP pen was then used to draw a water barrier block around each tissue section. The barrier was left to dry for up to a minute. Slides were then placed into a humidity box and tissue sections were covered with a blocking buffer made with 150 µl of goat serum (Vector Laboratories Catalog S-1000) and 10 mL of 1% BSA solution. The slides were left covered in the blocking buffer in the humidity box for 2 hours at room temperature. The blocking buffer was then poured off of the slides and replaced with the primary antibody solution of 1:1000

dilution made of 3 μ l of antibody (Anti-GFP antibody-ChIP Grade, Abcam ab290) and 3 ml of blocking buffer or 25 μ l of Rabbit IgG (Santa Cruz sc-2027) and 2 ml of blocking buffer for control slides. The slides with the primary antibody covering the tissue sections were incubated overnight in a humidity box at room temperature.

Immunostaining Day 2

As each slide was removed from the humidity box it was rinsed with IHC PBS. Then the slides were placed in the tissue tech holder and rinsed with IHC PBS 4 times for 5 minutes each. Slides were then rinsed with 0.5% BSA solution for 10 minutes. The slides were placed back in the humidity box and covered with biotinylated secondary antibody solution. The biotinylated secondary antibody solution was made using 10 mL of the blocking buffer used during Immunostaining Day 1 and 1 drop of biotinylated secondary antibody from the vectastain elite ABC kit (Vector Laboratory #PK-6101). The slides were left for 2 hours at room temperature in the humidity box. The secondary antibody solution was then poured off and slides were rinsed with IHC PBS as they were removed from the humidity box after the incubation. The slides were rinsed in IHC PBS 4 times for 4 minutes each, then rinsed with 0.5% BSA for 10 minutes. The slides were then placed back in the humidity box and the tissue was covered with the ABC reagent. The ABC reagent was made at least 30 minutes before use and was made using 1 drop of solution A and 1 drop of solution B from the vectastain elite ABC kit and 2.5 mL of 1.0% BSA solution. The slides were left in the humidity box for 1.5 hours. The ABC reagent was then poured off of the tissue;

each slide was rinsed with tris buffered saline (TBS) (6.35g Tris-HCl + 1.18g Tris Base + 8.77g NaCl + 1L dH₂O, pH 7.5) solution as it was removed from the humidity box. The slides were rinsed with TBS 4 times for 5 minutes each. During the last rinse the VIP substrate was prepared; it was made using 1.67 mL of IHC PBS and 1 drop of solutions 1, 2, and 3 and hydrogen peroxide from Vector VIP Peroxidase Kit (Vector Laboratories #SK-4100). The slides were placed onto a white background. The tissue sections were covered with the VIP solution for 2 minutes to allow for color development.

After color development, each slide was rinsed with distilled water before placing them back into the tissue tech holder. The slides were rinsed twice for 2 minutes each with distilled water to stop color development. The slides were then again placed on the white background. The tissue sections were covered with methyl green for 1 minute. The slides were then rinsed thoroughly with distilled water. The slides were then placed into glass boats to undergo the dehydration process, which is the reverse of the hydration process used during the De-paraffinization and tissue re-hydration step, starting with a 50% EtOH wash for 2 minutes and ending with two 5 minute xylene washes. The slides were then wiped dry with a kimwipe, avoiding the tissue sections. Coverslips were then attached using cyto seal. The slides were allowed to air dry in a hood overnight.

Quantification

After the slides were allowed to dry for at least one night, the tissue sections were examined underneath a microscope at 630x magnification using an oil immersion lens. Twelve fields were examined for each tissue section. The

fields were taken starting at the top left corner of the tissue, moving right across the tissue, then down at least one field, then moving back left across the tissue. This zig-zag like technique was used across the entire tissue, randomly selecting non-overlapping fields from all areas of the tissue section. A picture was taken of each field under 630x oil immersion lens. The picture was then examined to determine the number of positively stained cells. Dark cells with somewhat defined edges that were also visible when directly looking through the microscope were regarded as positively stained cells. The data was then analyzed to determine the average number of positive cells per field in each tissue section, microscope slide, mouse, infected mice samples, and mock mice samples. Statistical significance was determined for the average number of positively stained cells in mice infected with *C. muridarum* versus the non-infected mice using a two-tailed t test; $p < 0.05$ was used for significance.

RESULTS

Previous preliminary work in the Schoborg lab determined that the number of ChAT expressing cells increased in Chlamydia-infected cervical tissue compared to mock-infected (uninfected) control on days 3-21 post infection (**Figures 2, 3**). This preliminary data lead to further experiments, first confirming significance in cervical tissue on day 9 post infection (**Figure 4**), then performing staining experiments on both uterine horn and ovarian tissue on days 3-21 post infection, labeled Chat 1 experiments (**Figures 5, 6, 8, 9**). Repeat experiments were also performed on the day 9 post infection uterine horn, labeled Chat 2

experiments (Figure 7) and ovarian tissue (Figure 10) to provide more data to support or dispute the preliminary findings.

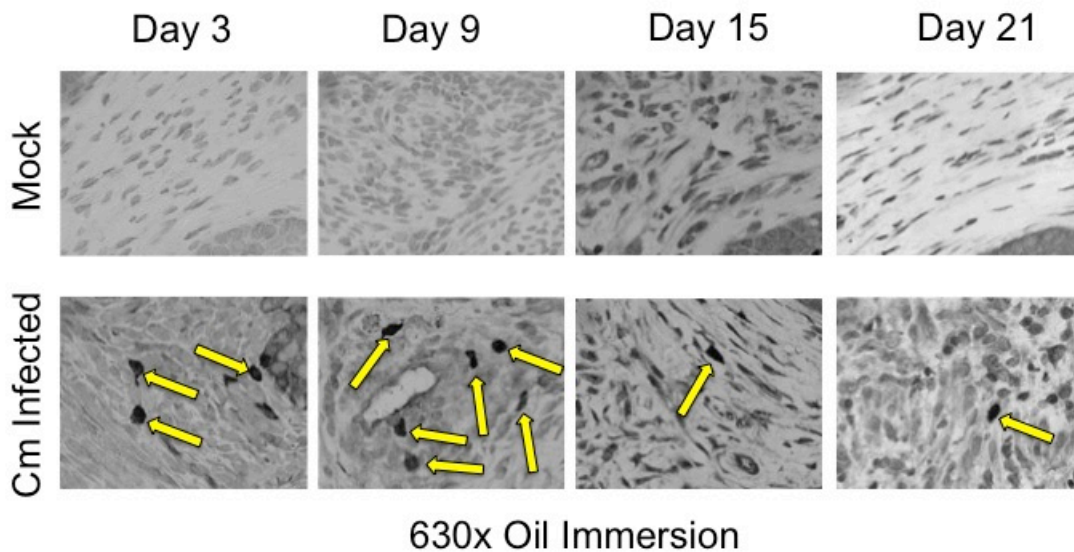


Figure 2. *C. muridarum* Infection Increases The Number of ChAT-expressing Cells in Cervical Tissue Days 3-21 Post Infection. Cervical tissue was immunostained for ChAT expression using the IHC technique. ChAT positive cells were counted in random fields at magnification 630x under oil immersion. The pictures are representative of stained sections that were counted for the indicated day post infection. Arrows indicate the presence of positive staining for the expression of ChAT. The day post infection that the mock tissue was harvested is shown above each column of photographs. Mock indicates sections from a mock-infected mouse; Cm infected indicates sections from a *C. muridarum* infected mouse.

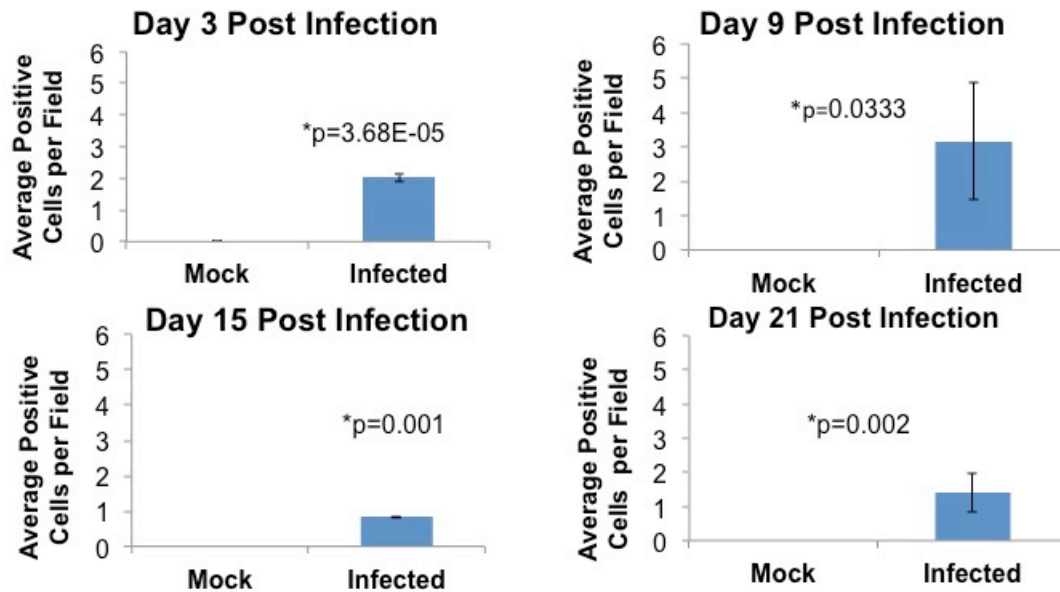


Figure 3. *C. muridarum* Infection Increases the Number of ChAT-expressing cells in Cervical Tissue Days 3-21 Post Infection. The cervical sections stained for the experiment shown in Figure 2 were quantified and the average number of cells expressing ChAT per 630x microscope field was calculated for random fields, as described in the Methods section. The average number of ChAT positive cells per field in cervical tissue is shown by the blue bars, in either a mock-infected mouse (mock) and *C. muridarum* infected mouse (infected). The time post infection that the tissue was harvested is shown above each graph. Mock and infected ChAT positive cell counts were compared using a two-tailed t test; $p < 0.05$ was considered significantly different. P values obtained for each comparison are shown in each graph. A statistically significant difference was found in the number of cells expressing ChAT on days 3, 9, 15 and 21 post infection with *C. muridarum* versus those that were not infected.

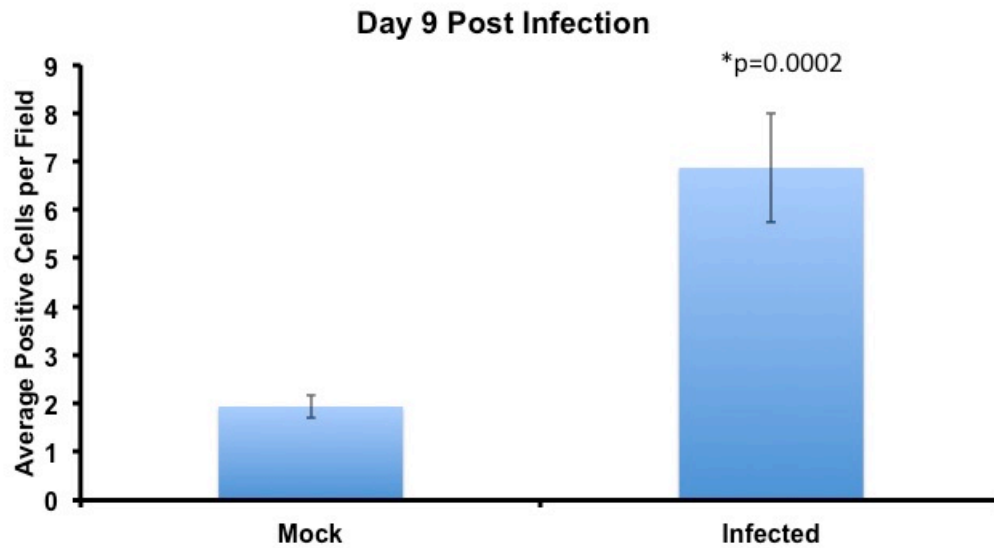


Figure 4. *C. muridarum* Infection Increases Expression of ChAT in Cervical Tissue on Day 9 Post Infection. The average number of cells expressing ChAT per 630x microscope field was calculated using day 9 post infection cervical tissue sections from 3 mice infected with *C. muridarum* as well as 3 non-infected mice (mock) as a control. The asterisk signifies a statistically significant increase determined using a two-tailed t test; $p < 0.05$ was used for significance. The p value for the indicated comparison is shown on the graph. These data were obtained from the ChAT 2 experimental repeat.

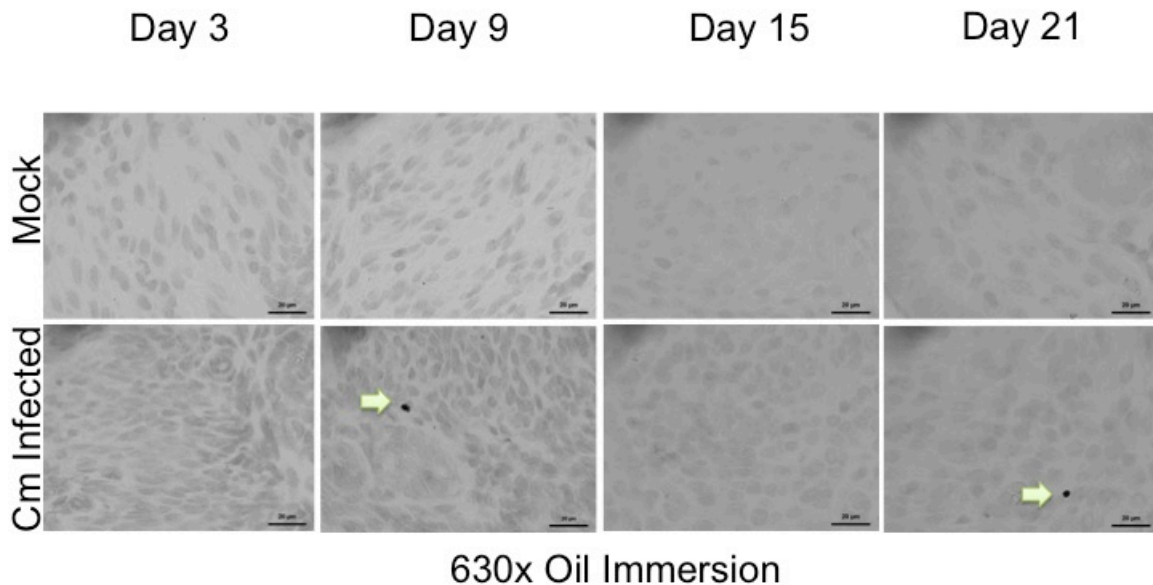


Figure 5. *C. muridarum* Infection Increases the Number of Chat-expressing Cells in Uterine Horn Tissue Day 9 Post Infection. Uterine horn tissue was immunostained for ChAT expression using the IHC technique. ChAT positive cells were counted in random fields at magnification 630x under oil immersion. The pictures are representative of stained sections that were counted for the indicated day post infection. Arrows indicate the presence of positive staining for the expression of ChAT. The day post infection that the tissue was harvested is shown above each column of photographs. Mock indicates sections from a mock-infected mouse; Cm infected indicates sections from a *C. muridarum* infected mouse.

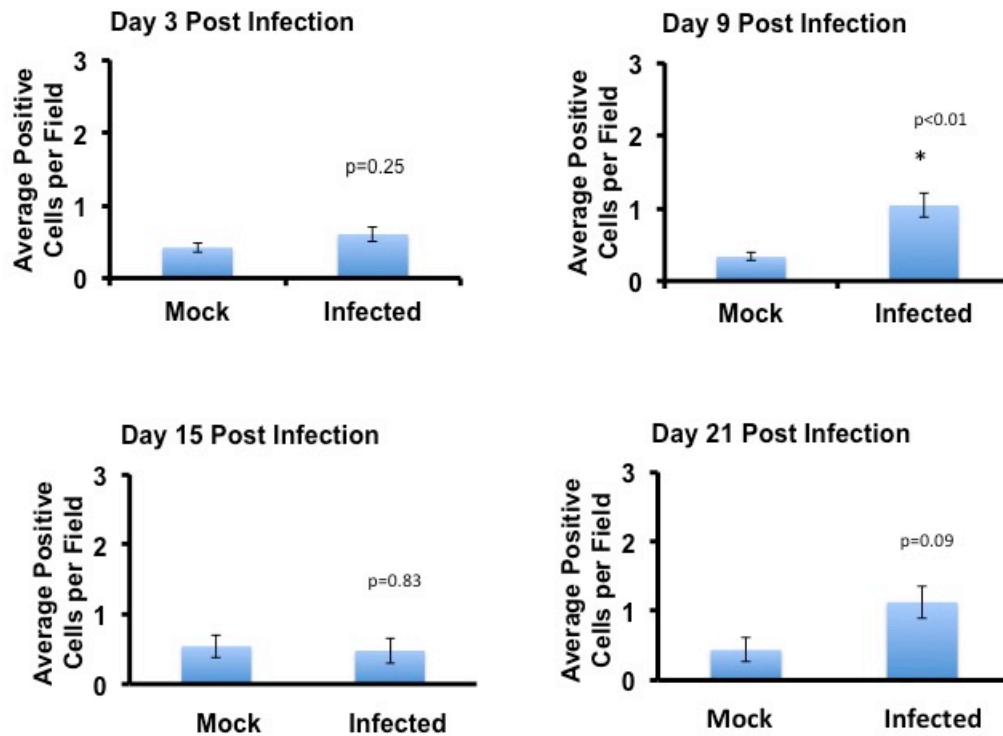


Figure 6. *C. muridarum* Infection Increases the Number of ChAT-expressing cells in Uterine Horn Tissue on Day 9 Post Infection. The uterine horn sections stained for the experiment shown in Figure 5 were quantified and the average number of expressing ChAT per 630x microscope field was calculated as described in the Methods section. The average number of ChAT positive cells per field in uterine horn tissue is shown by the blue bars, in either a mock-infected mouse (mock) and *C. muridarum* infected mouse (infected). The time post infection that the tissue was harvested is shown above each graph. Mock and infected ChAT positive cells counts were compared using a two-tailed t test; $p < 0.05$ was considered significantly different. P values obtained for each comparison are shown in each graph. A statistically significant difference was found in the number of cells expressing ChAT on day 9 post infection with *C. muridarum* versus those that were not infected.

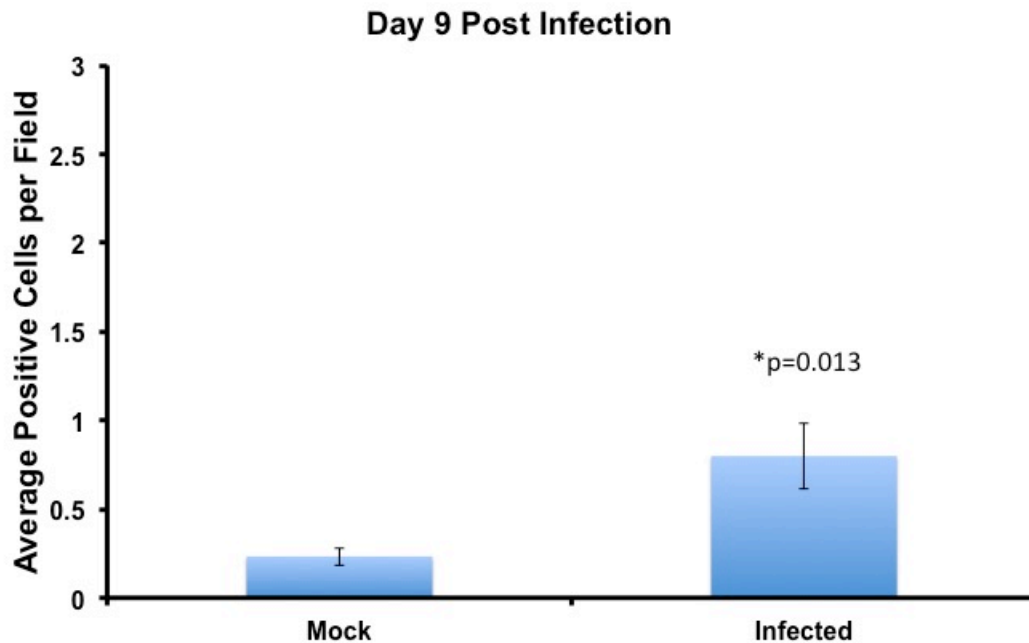


FIGURE 7. *C. muridarum* Infection Increases Expression of ChAT in Uterine Horn Tissue Day 9 Post Infection. The average number of cells expressing ChAT per 630x microscope field was calculated using day 9 post infection uterine horn tissue sections from 3 mice infected with *C. muridarum* as well as 3 non-infected mice (mock) as a control. The asterisk signifies a statistically significant increase determined using a two-tailed t test; $p < 0.05$ was used for significance. The p value for the indicated comparison is shown on the graph. These data were obtained from the ChAT 2 experimental repeat.

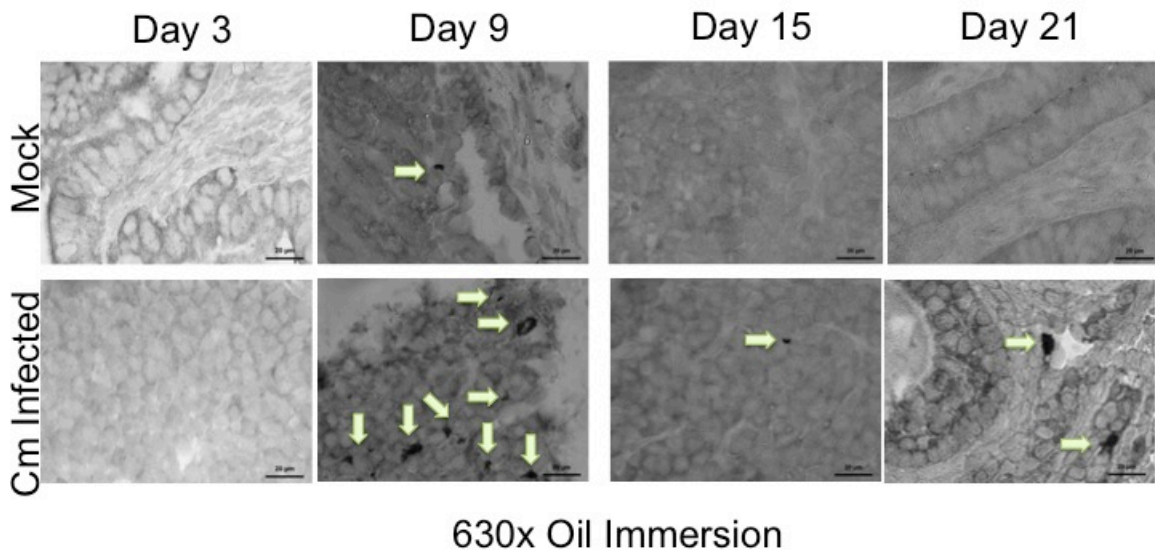


Figure 8. *C. muridarum* Infection Increases the Number of ChAT-expressing Cells in Ovarian Tissue Days 9-21 Post Infection. Ovarian tissue was immunostained for ChAT expression using the IHC technique. ChAT positive cells were counted in random fields at magnification 630x under oil immersion. The pictures are representative of stained sections that were counted for the indicated day post infection. Arrows indicate the presence of positive staining for the expression of ChAT. The day post infection that the tissue was harvested is shown above each column of photographs. Mock indicates sections from a mock-infected mouse; Cm infected indicates sections from a *C. muridarum* infected mouse.

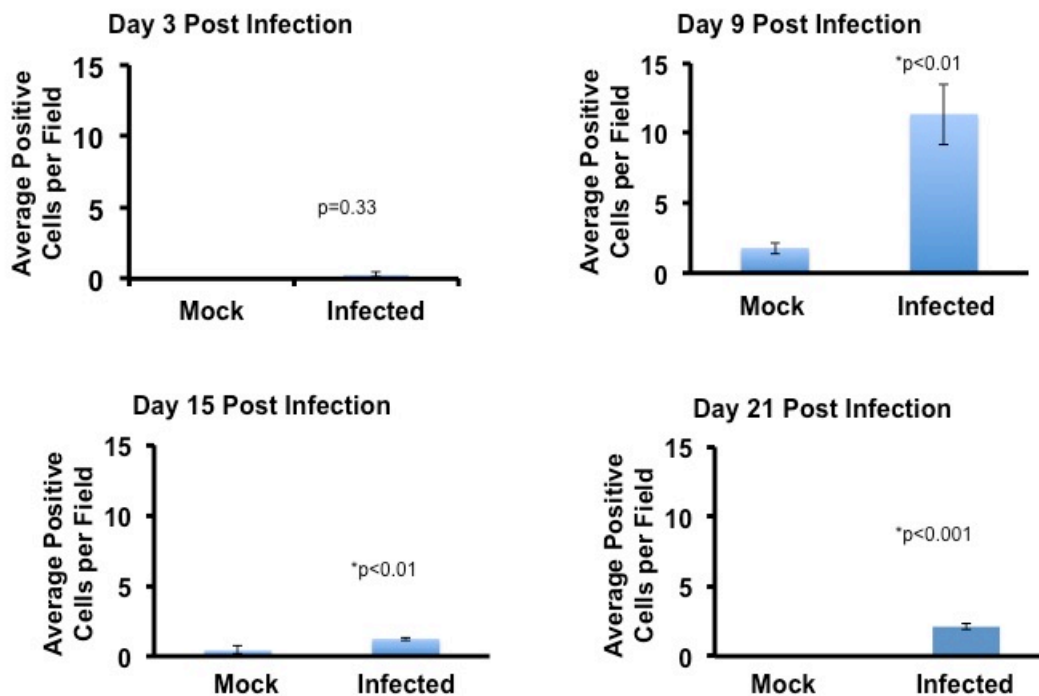


Figure 9. *C. muridarum* Infection Increases the Number of ChAT-expressing cells in Ovarian Tissue Days 9-21 Post Infection. The ovarian sections stained for the experiment shown in Figure 8 were quantified and the average number of cells expressing ChAT per 630x microscope field was calculated for random fields, as described in the Methods section. The average number of ChAT positive cells per field in ovarian tissue is shown by the blue bars, in either a mock-infected mouse (mock) and *C. muridarum* infected mouse (infected). The time post infection that the tissue was harvested is shown above each graph. Mock and infected ChAT positive cell counts were compared using a two-tailed t test; $p < 0.05$ was considered significantly different. P values obtained for each comparison are shown in each graph. A statistically significant increase was found in the number of cells expressing ChAT on days 9, 15, and 21 post infection with *C. muridarum* versus that that were not infected.

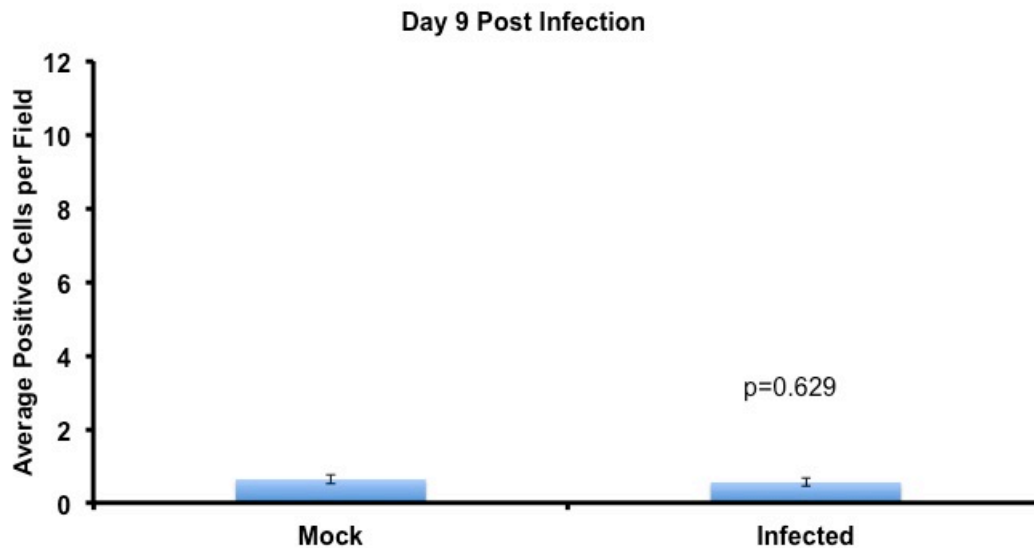


FIGURE 10. *C. muridarum* Infection Does Not Increase the Number of ChAT-Expressing Cells in Ovarian Tissue on Day 9 Post Infection. The average number of cells expressing ChAT per 630x microscope field was calculated using day 9 post infection ovarian tissue sections from 3 mice infected with *C. muridarum* as well as 3 non-infected mice (mock) as a control. It was determined that there was not a significant increase in the number of ChAT-expressing cells using a two t-tailed t test; $p < 0.05$ was used for significance. The p value for the indicate comparison is shown on the graph. These data were obtained from the ChAT 2 experimental repeat.

Repeated staining experiments exhibited a significant increase in the number of ChAT-expressing cells on day 9 post infection in the cervix (**Figure 4**), supporting previous preliminary findings in the Schoborg lab. As expected, ChAT expression was increased in Chlamydia-infected uterine horn on days 3, 9, and 21 post infection, with a statistically significant increase in the number of ChAT-

expressing cells on day 9 (**Figure 6**). Repeated staining experiments on day 9 post infection uterine horn tissue supports the statistical significance found in the preliminary experiments (**Figure 7**). Initial experiments on ovarian tissue found an significant increase in ChAT-expressing cells on days 3, 9, 15, and 21 post infection in infected tissue versus non-infected tissue (**Figure 9**). Repeated experiments on day 9 post infection ovarian tissue did not find a significant increase in the expression of ChAT between infected and non-infected tissue (**Figure 10**).

DISCUSSION

Supporting our original hypothesis, data from repeated IHC staining experiments on day 9 post infection cervical tissue exhibits a significant increase in the number of cells expressing ChAT in *C. muridarum* infected tissue. Data from uterine horn tissue exhibited a significant increase of ChAT expression on day 9 post infection. Although initial experiments with ovarian tissue indicated a significant increase of ChAT expression on day 9 post infection, repeated experiments did not identify a significant difference. This may be the result of an unevenly distributed infection of the ovarian tissue, which could have caused an uneven distribution of ChAT-expressing cells in the ovarian tissue, as the tissue sections used were very small portions of the entire ovary. More experiments need to be carried out to determine if this disparity was due to experimental error, or if there is truly no significant difference in ChAT expression in ovarian tissue on day 9 post infection. Further experiments are also needed on all days post infection to substantiate or refute the general initial findings of an increase in

ChAT-expressing cells in Chlamydia-infected genital tract tissue versus non-infected tissue.

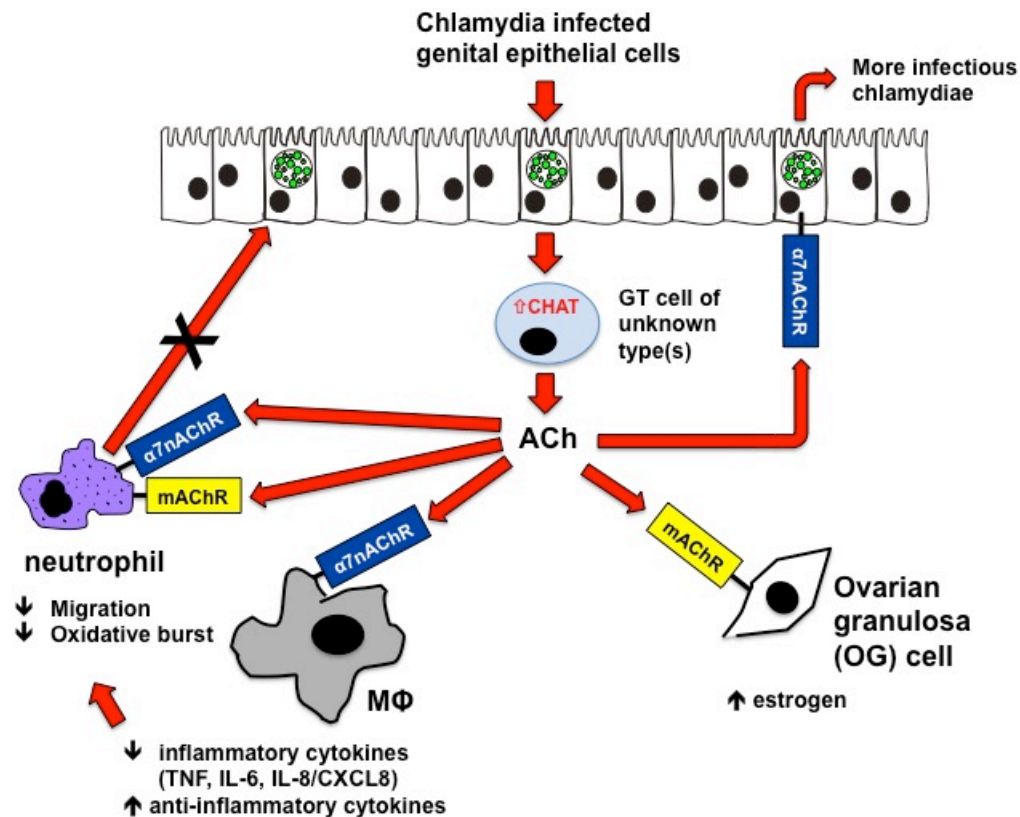


Figure 11. Possible Mechanisms of Enhancement of Chlamydial Infection

This increase in ChAT, and therefore ACh, in the genital tract could, in fact, promote chlamydial infection through several different mechanisms (**Figure 11**). Direct stimulation of nAChRs by nAChR agonists, including ACh, has been shown to increase the growth of *C. pneumoniae* in human epithelial cells *in vivo* (Yamaguchi et al., 2003). Stimulation of α7nAChR by its agonists has also shown to decrease chemokines produced by macrophages resulting in decreased migration of neutrophils to the area of infection (Su et al., 2010). ACh treatment

also decreases expression of cellular adhesion molecules on endothelial cells, preventing migration of neutrophils, and therefore destruction of microbes, in infected tissues (Reardon et al., 2013). Human ovarian granulosa cells treated with acetylcholine have increased estrogen secretion (Kornya, 2001). Increased estrogen has been shown in animal models to increase both the severity and longevity of genital chlamydial infection (Rank et al., 1982). Therefore, an increase in production of acetylcholine in the genital tract could promote chlamydial infection through any or several of these mechanisms.

As stated above, this data indicates that *C. murdarium* infection does increase the expression of ChAT in the genital tract for at least days 3-21 post infection. Although the mechanism is unknown, this could occur by activation of CAP or a CAP-independent mechanism that causes the expression of ChAT in genital cells. Therefore, the presence of ACh in the genital tract, or activation of CAP, could promote genital chlamydial infections. These findings could be applied to find new or improve current treatments for genital chlamydial infections using pre-existing acetylcholinesterase inhibitors or AChR agonists or antagonists to treat these infections, and, or their sequelae.

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