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Polymorphic membrane protein expression in Chlamydia/HSV co-infected cells

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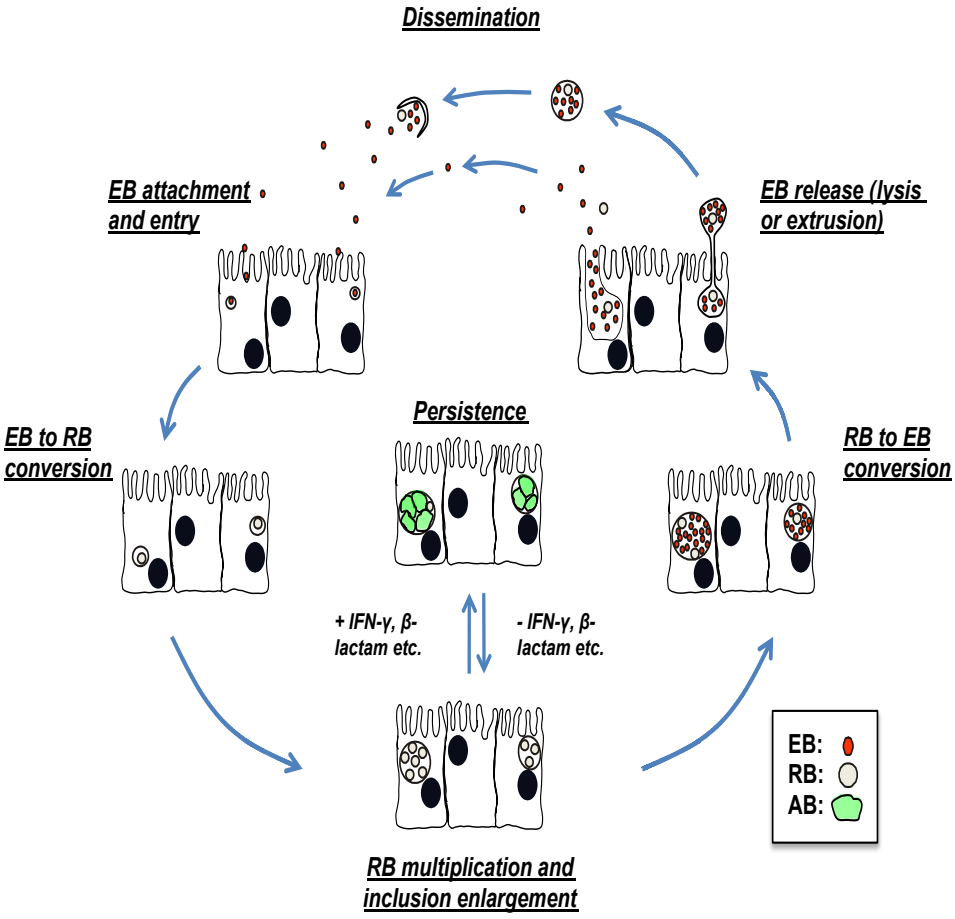
Introduction

The *Chlamydiaceae* are a bacterial family that contains a single genus: Chlamydia. The genus Chlamydia consists of 9 species that are obligate, intracellular pathogens. The 9 species are: *C. abortus, C. caviae, C. felis, C. muridarum, C. pecorum, C. pneumoniae, C .psittaci, C. suis,* and *C. trachomatis.* Recently, there has been a new, potential species discovered called *Chlamydia ibidis*, which results in 10 total species within the genus Chlamydia (1). These bacteria are characterized as Gram negative organisms and possess a coccoid appearance. The Chlamydia species are obligate aerobes and utilize oxygen to thrive by deriving the necessary energy from the host (2).

The *Chlamydiae* also possesses a unique genome containing nine polymorphic membrane proteins (PMP) genes (3). These specialized proteins are utilized as chlamydia-specific autotransporters, which are secreted proteins assembled in the outer membrane of bacteria. The autotransporters' passenger domains are vital for bacterial pathogenesis, with some released from the outer membrane by proteolysis while others remain attached to the bacterial surface (4). Some of these surface-exposed, polymorphic proteins are known to function as logical mediators of bacterial/host cell attachment (5). Polymorphic proteins may also provide an advantage to these bacterial species by aiding in infection and transmission (3).

Additionally, Chlamydia has an unusual life cycle that involves a non-infectious, replicating reticulate body (RB) and an infectious, non-replicating elementary body (EB). Upon interaction with host cells, the bacteria enter the cell by inducing endocytosis of the elementary body (6). Now inside a phagosome, the elementary body is protected from the host defenses and differentiates into the reticulate body form of Chlamydia (7). The reticulate body then replicates within an inclusion, a membrane-bound vacuole unique to Chlamydia. Inc proteins of Chlamydia

obtain nutrients and lipids from the host's organelles and modify the inclusion membrane through interactions with the host cell's components. The bacteria replicate and survive by secreting specific bacterial proteins into the host's cytosol, which thus, allows the use of the host innate immune response and other molecular signals to its advantage (8). The reticulate bodies proliferate every 2-3 hours, and the infectious cycle can span from 30 – 72 hours (7, 9). After propagation, the reticulate bodies revert back into EBs and are then either excreted from the host cell through exocytosis or released by host cell lysis. The now-released elementary bodies interact with proximal cells, and the process starts all over again (Fig. 1) (7).



Schoborg, R.V., 2011, *Microbes Infect* 13, p649

Figure 1. The life cycle of Chlamydia involves an elementary body and a reticulate body.

Of the 10 Chlamydia species, only *Chlamydia pneumoniae, Chlamydia psittaci,* and *Chlamydia trachomatis* are pathogenic to humans. *Chlamydia pneumoniae* causes bronchitis or mild pneumonia in children and young adults, but the pathogen can become more severe and cause reoccurring infections in older adults (10). *Chlamydia psittaci* is an avian infection that causes respiratory psittacosis or ornithosis in humans (11). Most cases of *C. psittaci* present with fever and pneumonia that can be fatal, however, some patients experience asymptomatic infections. *Chlamydia trachomatis* serovars A-C are the bacterial agents that cause trachoma (6). According to the Center of Disease Control and Prevention, trachoma is the world's leading cause of preventable blindness. Nearly 8 million people are visually impaired world-wide due to recurring infections of trachoma, which causes the inside of the eyelid to be severely scarred. This terrible affliction can lead to trichiasis, the scratching of the cornea by the eyelashes, resulting in permanent blindness (12).

C. trachomatis serovars D-K have been reported to be the most frequent sexually transmitted disease caused by a bacterial infection in the United States. Annually, over 2 million chlamydial infections occur and yet only half of the chlamydia-related cases are reported nation-wide to the CDC (13). The causation of over 1 million unreported cases is due to the fact that most chlamydial infections are asymptomatic; hence the reason Chlamydia is called the 'silent' infection (13). Untreated *C. trachomatis* infections can lead to serious health ramifications, such as ectopic pregnancy, tubal factor infertility, pelvic inflammatory disease, and long-term pelvic pain. Pelvic inflammatory disease can result from an acute or subclinical infection, where the bacteria advances into the uterus or fallopian tubes leading to permanent damage, which can result in the inability to become pregnant, chronic pelvic pain, and possibly fatal ectopic pregnancy. *Chlamydia trachomatis* has been identified to be associated with pre-term delivery

and newborn pneumonia and conjunctivitis. The best method for preventing neonatal chlamydial infections is treatment and screening of pregnant women. Additionally, Chlamydia can be treated easily with antibiotics and a 3 month screening after treatment, which should (but does not always) prevent a reoccurring infection. (13).

Chlamydial persistence occurs when developing chlamydiae are exposed to unfavorable, environmental conditions, such as deprivation of essential nutrients, or exposure to penicillin G, or interferon-γ. Under these conditions, the normal chlamydial-developmental cycle deviates into a persistent state, where RBs continue to grow but do not divide. The abnormally, enlarged reticulate bodies are no longer infectious in the persistent state; however, they can re-enter the normal developmental cycle and become infectious again when the environmental stressor is removed. Co-infection of *Chlamydia trachomatis/*herpes simplex virus type-2 also stimulates persistent chlamydiae formation. Previous studies have hypothesized that viral attachment and/or invasion triggers a unique host pathway that restricts the development of *Chlamydia trachomatis* via stimulation of chlamydial persistence in mucosal epithelial cells (9).

According to the manuscript, "*Altered developmental expression of polymorphic membrane proteins in penicillin-stressed Chlamydia trachomatis,"* by Carrasco, et al, penicillin exposure induces chlamydial persistence *in vitro* and provides an effective model to study bacterial persistence in human chlamydial infection (6). Concerning the nine PMP proteins of *C. trachomatis*, each protein (Pmp subtypes A – I) was variably expressed in individual inclusions at the chlamydial surface. The results from the paper suggest that various *pmp* genes are differentially expressed during development. *Pmp* genes regulation occurs at multiple levels of regulation, which include transcriptional and post-transcriptional mechanisms. It was found that expression of six of the nine *Pmp* proteins (Pmp-B, Pmp-C, Pmp-E, Pmp-F, Pmp-G, and Pmp-H)

are strongly down-regulated in persistent cultures exposed to penicillin stress. In contrast, levels of Pmp-A, Pmp-D, and Pmp-I proteins are unchanged during penicillin-stress/persistence. Since HSV co-infection also induces persistence, we hypothesized that HSV co-infection would decrease expression of Pmp-B and Pmp-C, but not that of Pmp-A and Pmp-F. Persistent chlamydiae, due to penicillin-stressed conditions, are also characterized by up-regulation of heat-shock chaperonins expression, which results in inhibition of cell division and generates abnormally, enlarged RBs that are present for a long period of time (6).

Under stressful conditions, the *pmp* gene family's expression profile was noticeably altered, and when infected cells were exposed to penicillin, the transcription of *pmpBC, pmpFE,* and *pmpGH* was significantly down-regulated. On the other hand, the transcription of Pmp-A, Pmp-D, and Pmp-I, the most conserved *pmp* genes in *C. trachomatis*, was uniquely unaffected by stress. This suggests that Pmp-A, Pmp-D, and Pmp-I proteins play a crucial role in chlamydial pathogenesis, especially under unfavorable conditions for growth. In addition, polymorphic proteins were expressed differentially at various developmental stages with some constant and others exponentially different at specific hpi. It is hypothesized that under stressed conditions continued expression of Pmp-A, Pmp-D, and Pmp-I proteins may provide an advantage to the bacteria and possibly reflects the significance for chlamydial survival in cellular and mucosal sites, which are least hospitable for growth of chlamydiae (6).

Materials and Methods

Reagents:

- Fixative – 100% Methanol or Formaldehyde

- 1 X PBS Washing buffer – 0.1% Triton X-100 and 1 mg/ml BSA in 1X PBS

  For 200 ml, mix

20 ml	10X PBS
2 ml	10% Triton X-100
2 ml	100 mg/ml BSA
188 ml	Water

- Appropriate primary antibody (guinea pig) diluted in washing buffer to a concentration of 1:400 dilution.
- Appropriate conjugated secondary antibody (Alexa Fluor 488 goat anti-guinea pig (IgG)) diluted in washing buffer to a concentration of 1:500 dilution.

Methods:

Host cells, chlamydia, and co-infection *Deka, et al*:

HeLa cells (human cervical carcinoma epithelial cells) were grown and infected essentially as described in Deka, *et al*. (14). Briefly, HeLa cells were plated at 1 x 10^5 cells/well in standard culture medium + gentamycin on 25mm glass coverslips. The next morning, cultures were either mock-infected (with 2SPG diluent) or infected with *C. trachomatis* serovar E diluted in 2SPG at 1 MOI (multiplicity of infection of 1 infectious EB/cell). After 1 hour of infection at 37°C, the infection medium was removed and the cells were refed with culture medium without antibiotics. After 24 hours of incubation at 37°C, the medium was aspirated and the cells were either mock-infected with culture medium alone or infected with Herpes Simplex Virus type 2 (HSV-2) at 10 MOI (multiplicity of infection of 10 infectious HSV/cell). After 1 hour of infection, the infection medium was removed and the cells refed with culture medium without antibiotics for an additional 20 hours at 37°C. At the end of the experiment, cells were fixed as described below and subjected to immunoflourescent staining as described below.

Fixation:

Methanol-fixed cells were exposed to ice-cold 100% MeOH for 10 minutes, washed with 1X PBS washing buffer and stored wet in 1X PBS washing buffer at 4°C until use. Formaldehyde-fixed cells were fixed and permiabilized as described in Deka et al 2006 (ref). Briefly, cells were fixed in 3.7% formaldehyde in PBS for 30 minutes at room temperature, and washed 3 times with 1X PBS washing buffer. Formaldehyde-fixed cells were then incubated in 0.1% Nonidet NP40 (a detergent) in PBS for 10 minutes to punch holes in the cell membrane, washed 3 times with 1X PBS washing buffer, and stored wet in 1X PBS washing buffer at 4°C until use.

Pre-absorb antisera with HeLa cells for immunofluorescent staining from *Carrasco, et al*:

- 100 µl of diluted anti-PMP antibody with a final dilution of 1:400
- Antibodies: PMP-A, PMP-B, PMP-C, PMP-F

To make a 1:200 initial dilution of anti-PMP antibody, 1 µl of anti-PMP primary antibody was added into a labeled tube containing 199 µl of PBS washing buffer and briefly mixed by centrifugation. The fixed HeLa cells were washed 3 times in 1X PBS washing buffer, and then 100 µl of diluted primary antibody was added into each washed well of HeLa cells. The plate was then placed on a gyrator shaker at a speed of 3.5 for 30 minutes. The pre-absorbed antibody from each well was transferred into a labeled tube, where the volume was measured accordingly. To create a final dilution of 1:400, an equal volume of PBS washing buffer to the diluted primary antibody was added and placed on ice until needed.

To dilute secondary antibody with a concentration of 1:500 dilution, 978 µl of PBS washing buffer, 20 µl of DAPI working solution, and 2 µl of secondary antibody were added into a tube, mixed, and placed in ice until needed.

Immunofluorescent staining protocol derived from *Carrasco, et al*:

A sheet of Parafilm was laid out in a plastic petri plate and labeled appropriately (PMP#: Mock, HSV, C+, C+/HSV), and 15 µl of diluted primary antibody was pipetted onto the surface of the parafilm next to the appropriate label. Then, the appropriate coverslips were washed in PBS washing solution and inverted onto the drop of primary antibody on the Parafilm, with the cell monoloayer down (ie. in contact with the primary antibody). The staining plate was placed in the incubator at 37ºC for 1 hour. For secondary staining, another Parafilm sheet was labeled appropriately, placed in a petri plate, and 15 µl drops of diluted secondary antibody were pipetted onto the parafilm surface as described above. The appropriate coverslips from the primary antibody tray were washed in PBS washing buffer and transferred onto the drop of secondary antibody on the Parafilm. The staining plate was placed in the incubator at 37ºC for 30 minutes.

Microscopic glass slides were labeled appropriately, and 10 µl of mounting media was pipetted onto slide. The coverslips from the secondary antibody tray were washed in PBS washing buffer and transferred onto the drop of mounting media on the glass slide under each labeled category with the cell monolayers oriented down (ie. in contact with the mounting medium). The slides were placed in a dark drawer for 2 hours and after drying, the slide box with the stained samples was stored in the dark at 4ºC in the refrigerator.

Note: All four PMP were stained at a primary dilution of 1:400 and a secondary dilution of 1:500 as derived from Carrasco, et al. protocol. However, the 1:400 primary antibody dilution was only optimal for Pmp-C, while the optimal primary antibody dilution for the other PMP were found by trial and error. Additionally, it was found that Pmp-A, Pmp-B, and Pmp-F stained better when the HeLa cells were fixed with formaldehyde rather than with methanol.

Quantifying data:

A total of 3 slides were stained for each PMP-C and PMP-F containing coverslips of Mock, HSV, C+, and C+/HSV. Using AxioVision 4.7 software, 60 pictures of Pmp-C and 60 pictures of Pmp-F were taken from the 6 slides and quantified by circling the chlamydial inclusions vs. chlamydial co-infected inclusions. The intensity of the inclusions was divided by the area of the inclusions in Microsoft Excel software, and the average value was compared to averages of each of the 3 slides for the polymorphic protein of interest. The data was graphed appropriately and p-values were calculated and compared using a t-test.

Results

Concerning optimization, it is important to have optimal fixation conditions and antibody dilution for staining, because it will result in low background and high signal that creates a clear image. Therefore, we first determined optimal antibody dilution and fixation conditions for each antibody of interest (Table 1). For Pmp-C, the optimal primary antibody dilution was 1:400, and the optimal secondary antibody dilution was 1:500. For Pmp-F, the optimal primary antibody dilution was 1:100, with an optimal secondary antibody dilution of 1:500. For Pmp-A, the optimal primary antibody was 1:25, and the optimal secondary antibody dilution was 1:500. For Pmp-B, the optimal primary antibody dilution was 1:50, with an optimal secondary antibody dilution of 1:500. Additionally, it was found that formaldehyde-fixed HeLa cells stained better than methanol-fixed cells for Pmp-F, Pmp-A, and Pmp-B proteins. Alternatively, anti-Pmp-C had the best results with the use of methanol-fixed HeLa cells (Table 1).

In the next experiment, Pmp-C and Pmp-F staining intensity in Chlamydia infected and Chlamydia/HSV infected cells were analyzed by comparing averages of the intensity/area ratio for significance (Fig. 2, 4). Based on 3 independent experiments for Pmp-C and Pmp-F, the

results showed no significant difference between the 2 variables (Fig. 3, 5). This indicates that PMP expression did not significantly alter in a normal vs. stressful environment.

Table 1. The optimal fixation and optimal dilution for PMP.

PMP	Optimal fixation	Optimal 1° Ab dilution	Optimal 2° Ab dilution
Pmp-C	Methanol	1:400	1:500
Pmp-F	Formaldehyde	1:100	1:500
Pmp-A	Formaldehyde	1:25	1:500
Pmp-B	Formaldehyde	1:50	1:500

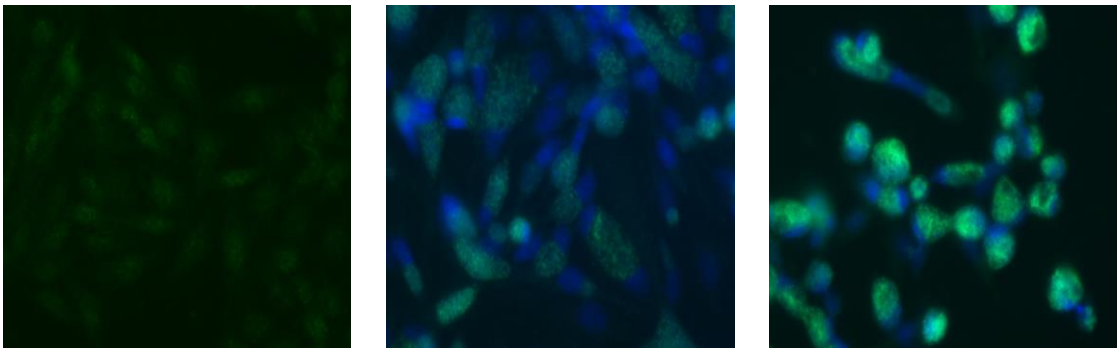


Figure 2. PMP-C (from left to right): Mock, CTE, CTE-Co

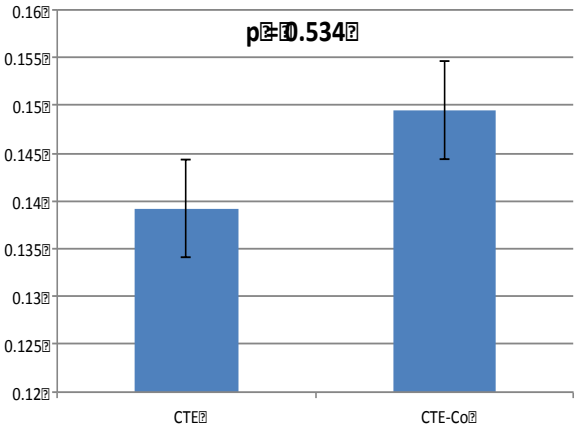


Figure 3. PMP-C: The graph represents the averages of 3 independent experiments comparing the intensity/area ratio of Chlamydia vs. Chlamydia/HSV infected cells. The calculated *p* value shows that there was no significant difference between the two variables.

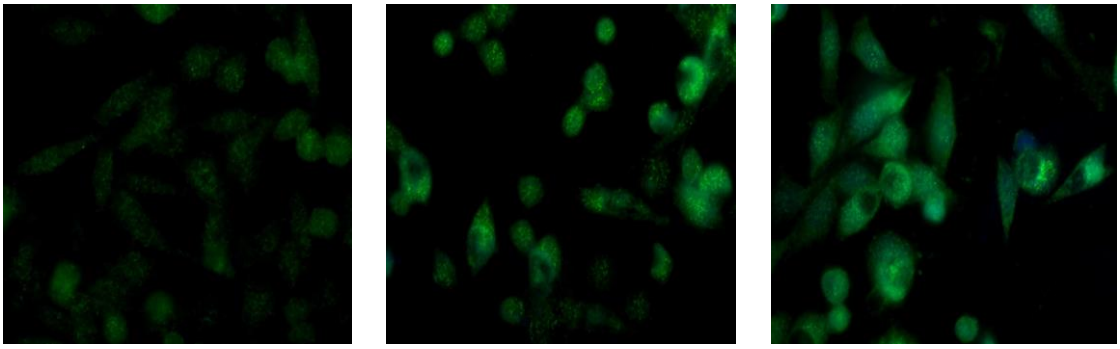


Figure 4. PMP-F (from left to right): Mock, CTE, CTE-Co

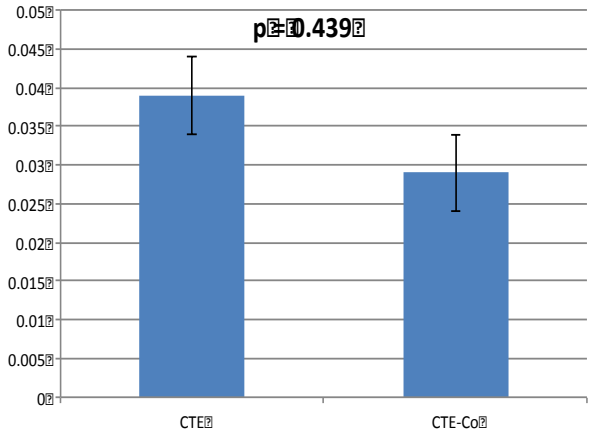


Figure 5. PMP-F: The graph represents the averages of 3 independent experiments comparing the intensity/area ratio of Chlamydia vs. Chlamydia/HSV infected cells. The calculated *p* value shows that there was no significant difference between the two variables.

Discussion

Concerning the best fixation and antibody dilution conditions for PMP immunostaining, it was found that a primary antibody dilution of 1:400 using methanol fixed HeLA cells, as derived from Carrasco, et al. protocol, was only optimal for PMP-C staining. Pmp-A, Pmp-B, and Pmp-F were found to stain brighter with formaldehyde fixed, infected HeLa cells and using different primary antibody dilutions. The optimal primary antibody dilution for Pmp-F was 1:100 instead of the initial 1:400 dilution described in Carrasco et al. For Pmp-A, the optimal primary antibody dilution was 1:25, while the optimal primary antibody dilution for Pmp-B was determined to be 1:50. Although we determined the optimal fixation and dilutions for Pmp-A

and Pmp-B, these two PMP proteins were not compared between singly- and co-infected cells in this study.

Based on the manuscript by Carrasco, et al., chlamydial persistence caused by penicillin-stressed conditions showed a decrease in Pmp-B and Pmp-C protein expression between 24-48 hpi, while Pmp-A and Pmp-F expression stayed the same under the stressful conditions (6). We hypothesized that under HSV-induced persistence, the same results would be obtained. However, the data acquired from Pmp-C and Pmp-F experiments show that there was no significant difference in PMP expression between a chlamydial infection and a viral co-infection induced persistence. These data indicates that the chlamydial response to stressful conditions is not the same among persistence-inducers and implies that various inducers of persistence may affect PMP expression differently.

Initially, we also hypothesized that PMP expression could be utilized as an indicator to determine whether an infected individual has a productive or persistent chlamydial infection. Due to the experiments' results, PMP expression is most likely not a good marker to identify the type of chlamydial infection (*ie.* productive or persistent) in the host. For future experiments, it would be interesting to see if other polymorphic membrane proteins (*ie.* Pmp-A, Pmp-B, Pmp-D, Pmp-E, Pmp-G, Pmp-H, or Pmp-I) have the same or different results depending upon the persistent-inducer. This research provides information in finding an excellent marker to identify regular or persistent chlamydial infections, which will eventually aid in developing a vaccine.

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