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Repeated *Chlamydia trachomatis* infections are associated with lower bacterial loads

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Short Paper

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Abstract

Chlamydia trachomatis (CT) infections remain highly prevalent. CT reinfection occurs frequently within months after treatment, likely contributing to sustaining the high CT infection prevalence. Sparse studies have suggested CT reinfection is associated with a lower organism load, but it is unclear whether CT load at the time of treatment influences CT reinfection risk. In this study, women presenting for treatment of a positive CT screening test were enrolled, treated and returned for 3- and 6-month follow-up visits. CT organism loads were quantified at each visit. We evaluated for an association of CT bacterial load at initial infection with reinfection risk and investigated factors influencing the CT load at baseline and follow-up in those with CT reinfection. We found no association of initial CT load with reinfection risk. We found a significant decrease in the median $\log_{10}$ CT load from baseline to follow-up in those with reinfection (5.6 CT/ml *vs.* 4.5 CT/ml; $P = 0.015$). Upon stratification of reinfected subjects based upon presence or absence of a history of CT infections prior to their infection at the baseline visit, we found a significant decline in the CT load from baseline to follow-up (5.7 CT/ml *vs.* 4.3 CT/ml; $P = 0.021$) exclusively in patients with a history of CT infections prior to our study. Our findings suggest repeated CT infections may lead to possible development of partial immunity against CT.

Chlamydia trachomatis (CT) causes the most frequently reported bacterial sexually transmitted infection in the world [1], and about 3 million CT infections occur in the USA alone every year [2]. Adolescent and young adult females are disproportionately affected by CT infection, which may lead to severe reproductive sequelae such as pelvic inflammatory disease, which can be further complicated by chronic pelvic pain, tubal factor infertility or increased risk for ectopic pregnancy. One of the challenges in CT control efforts is that CT infection often goes unnoticed, in part because most CT-infected women are asymptomatic. Another challenge with controlling CT infection is that reinfection occurs in about 10–20% of CT-infected individuals within months of treatment [3], which suggests a lack of complete protective immunity in some cases. Conversely, some patients naturally clear CT infection in the lower urogenital tract prior to receiving treatment, suggesting some individuals develop some degree of protective immunity against CT [4].

One potential way to demonstrate protective immunity to CT is to show a lower bacterial load with subsequent infections, as has been shown in a murine chlamydia model [5]. Although there have been several previous studies evaluating the relationship of CT load with patient characteristics, findings from these studies have been inconsistent [6]. Sparse studies that have investigated differences in CT bacterial load between initial infection and repeat infection have found that the CT load was lower for repeat infection, which would imply that initial infection imparts some protective immunity to subsequent infection. However, these studies were limited by the small number of reinfections evaluated ($\leqslant$11) [7, 8]. In order to elucidate whether past CT infection confers some degree of protection, a larger longitudinal study with detailed demographic data and serial sampling is required. In this study, we evaluated CT load in CT-infected women at a baseline visit and 3- and 6-month follow-up visits after treatment. We had two objectives: (1) to determine whether the CT load prior to treatment was associated with risk for reinfection, and (2) to investigate differences in the CT load at the time of baseline *vs.* follow-up in those with reinfection and what clinical factors may influence differences in the CT load. We hypothesised that the individuals with a lower baseline CT load would be at lower risk for reinfection due to having a stronger adaptive immune response against CT and CT load would be lower at the time of reinfection, reflecting partial immunity following a recent CT infection.

Women $\geqslant$16 years of age presenting to the Jefferson County Department of Health (JCDH) STD Clinic in Birmingham, Alabama, for treatment of a recent positive screening CT nucleic

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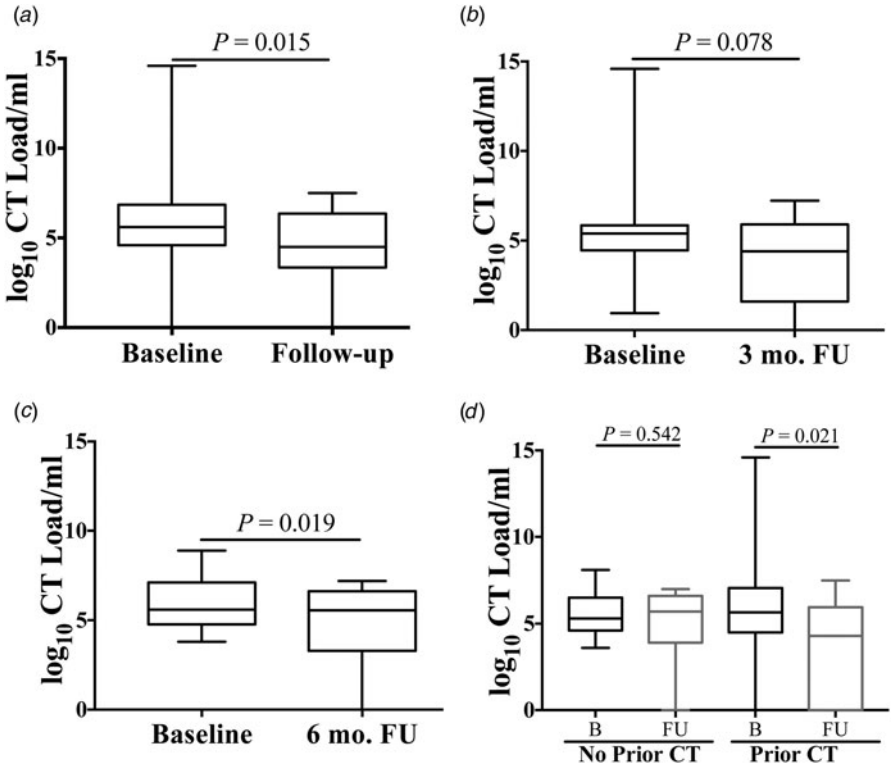

Fig. 1. *Chlamydia trachomatis* (CT) organism load measured by real-time PCR at baseline and follow-up visits in women with CT reinfection after treatment. Box and whisker plots compare the CT load at (a) baseline and follow-up (3- or 6-month) visit ($n = 37$); 3-month CT load was plotted if women were infected at both the follow-up visits, (b) baseline and 3-month follow-up visit ($n = 25$), (c) baseline and 6-month follow-up visit ($n = 18$) and (d) baseline (B) and follow-up (FU) visit upon stratification into No Prior CT infection ($n = 13$) *vs.* Prior CT infection ($n = 24$). The box and whiskers denote interquartile ranges with the whiskers denoting the 5th and 95th percentiles. The median is shown as the horizontal line. Significance between CT loads was determined by the Wilcoxon signed-ranked test.

acid amplification test (NAAT) (Hologic Aptima Combo 2 (AC2); Hologic, Inc., Marlborough, MA, USA) were enrolled after providing written consent, treated with azithromycin 1 g single-dose therapy given as directly observed, and returned for 3- and 6-month follow-up visits. Women who were pregnant, had a prior hysterectomy, were co-infected with HIV or gonorrhoea (tested at screening), or had received antibiotics with anti-CT activity in the prior 30 days were excluded. At each visit, participants were interviewed and data were collected on demographics, sexual history, hormonal contraceptive use, antibiotic use, clinical findings and reported partner treatment. A pelvic examination was performed to obtain a vaginal swab specimen for a wet mount and an endocervical swab specimen for CT and gonorrhoea testing by AC2. The study was approved by the University of Alabama at Birmingham (UAB) Institutional Review Board (IRB) and JCDH. The Centers for Disease Control and Prevention (CDC) determined that CDC involvement did not constitute engagement in human subjects research, and CDC IRB review was therefore not required.

CT bacterial load quantification was performed using the Cobas CT/*Neisseria gonorrhoeae* assay (Roche Diagnostics, Indianapolis, IN, USA). The assay uses amplification targets on both the CT cryptic plasmid and on the CT genome. To estimate bacterial load, a CT calibrator was run with each testing lot using well-characterised stock CT reference strains with known organism counts (determined in the Van Der Pol laboratory). This allowed creation of cycle threshold standard curves for comparison with clinical samples, providing reliable and reproducible results that allowed for relative quantification on a log scale.

Reinfection was defined as a positive CT NAAT at the 3- and/or 6-month follow-up visit. The $\log_{10}$ CT load is presented as median and interquartile range (IQR). The relationship of baseline $\log_{10}$ CT load with patient characteristics and subsequent reinfection was evaluated with the Kruskal–Wallis test, and differences between CT load at baseline and the time of reinfection were evaluated with the Wilcoxon signed-rank test. Associations of participant characteristics with reinfection were evaluated with the Fisher's exact test. Analyses were performed using SAS 9.3 (SAS Institute, Cary, NC, USA).

Of 239 women that tested positive for CT at enrolment (i.e. the baseline visit), 200 (83.7%) returned for follow-up visits. The study population predominantly consisted of African Americans (95%), with a median age of 22 years (range 16–50). There were 44.8% of women on hormonal contraceptives and 49% were symptomatic. About half of the women (53%) had a history of prior CT infection based on self-report or laboratory test results documentation. Bacterial vaginosis (BV) was the most frequent co-infection at the baseline (22.5%), followed by vulvovaginal candidiasis (14%) and trichomoniasis (8%). The median (IQR) $\log_{10}$ CT bacterial load at the baseline visit was 5.7 (4.8–6.9) CT/ml. No correlation was found between baseline CT load and age, symptoms, hormonal therapy or prior CT. There was a trend towards a higher baseline CT load (CT/ml) in those who were African American *vs.* non-African American race (median (IQR) 5.8 (4.8–6.9) *vs.* 5.0 (4.2–6.2); $P = 0.08$) and in those with a baseline visit diagnosis of cervicitis (6.4 (5.1–7.1) *vs.* 5.7 (4.7–6.8); $P = 0.06$) and BV (6.1 (5.2–7.0) *vs.* 5.7 (4.6–6.8); $P = 0.09$).

CT reinfection occurred in a total of 37 (18.5%) participants, with a median time to detection of reinfection of 92 days (range 54–204). Of the 37 reinfected participants, 19 (51.4%) were CT-positive at the 3-month follow-up visit only, 12 (32.4%) at the 6-month follow-up visit only and six (16.2%) at both the 3- and 6-month follow-up visits; participants that were CT-positive at a follow-up visit were provided azithromycin 1 g for CT treatment. There was no association of participant characteristics with CT reinfection. We also found no significant association between baseline CT bacterial load and subsequent reinfection risk: median (IQR) $\log_{10}$ baseline load: 5.8 (4.8–6.9)

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CT/ml in those without reinfection *vs.* 5.6 (4.7–6.8) CT/ml in those with reinfection ($P = 0.44$). The findings are consistent with no predictive effect of CT load at the time of treatment on reinfection risk, and do not support our hypothesis that individuals with lower initial CT load prior to treatment have a stronger protective response and are therefore at a lower risk to get reinfected.

We next evaluated the changes in the CT bacterial load between baseline and follow-up in those who had CT reinfection at follow-up. There was a significant decrease in the median (IQR) $\log_{10}$ CT load from the baseline to follow-up visit in women with reinfection (5.6 (4.7–6.8) *vs.* 4.5 (3.5–6.3) CT/ml; $P = 0.015$) (Fig. 1a). We found similar differences in CT load between the baseline and follow-up visits upon stratifying the follow-up visits into 3-month (5.4 (4.5–5.8) *vs.* 4.4 (3.2–5.8); $P = 0.078$) and 6-month (5.6 (4.8–7.1) *vs.* 5.55 (3.5–6.6); $P = 0.019$) visits (Fig. 1b and c). Prior CT infection before the treatment visit did not predict bacterial load at the follow-up visit. There were six (3%) participants found to be CT-infected at both 3- and 6-month visits and their CT loads did not significantly differ between their 3- and 6-month visits.

Next, we investigated whether having had a prior CT infection (before the baseline visit) had an impact on the differences in the CT bacterial load from the baseline to follow-up visit. Upon stratification of women based upon the presence or absence of a CT infection prior to the study, we found that there was a significant decrease in the CT load between visits of reinfected women in only those with a CT infection prior to the baseline visit (5.7 *vs.* 4.3 CT/ml; $P = 0.021$), whereas there was no evidence of a change seen in those without any CT infection prior to the baseline visit (5.3 *vs.* 5.7 CT/ml; $P = 0.542$) (Fig. 1d). These observations suggest repeated CT infections could lead to the development of partial protective immunity to CT, as reflected in the lower CT loads with subsequent infection.

Timing of CT reinfections likely influences the degree of protective immunity. A prior study showed lower CT reinfection rates when the index (i.e. initial) infection was <6 months *vs.* more than 6 months earlier [9], indicating that prior CT infections may confer only short-lived partial adaptive immunity in some individuals. This is also consistent with a murine model of genital CT infection that demonstrated insufficient, short-lived adaptive immunity [5]. The lack of development of long-lasting 'complete' protective immunity may in part explain why the magnitude of CT bacterial load in an individual did not affect the susceptibility to a subsequent infection, rather it is repeated infections that likely provide some degree of partial immunity that perhaps helps to clear the subsequent infections quicker.

Our study population consisted of only women and was predominantly African American, which may limit the generalisability of our findings. In contrast to our study showing a higher CT bacterial load in African Americans, limited prior studies evaluating the relationship of demographics with CT load have reported a higher CT load in Caucasians compared with African Americans [6]; however, the sample size of Caucasians in our study was very small. We did not know the timing of the prior CT infection in about half of participants, the duration of the CT infection at baseline for all participants, or the timing of reinfection in the affected participants, which may have potentially affected the CT load. Our prior CT infection data were based upon self-reporting and medical record review of laboratory testing results, which may underestimate the proportion of subjects with prior CT infections since all individuals may not be aware of a previous infection or may have been diagnosed with CT infection at another clinic; for those subjects in whom we did have data on prior CT infection, most had the infection more than 6 months prior to baseline. We also cannot rule out the rare possibility that a subject failed their initial treatment and had persisting CT infection rather than reinfection; however, based on a recent randomised controlled CT treatment trial reporting an azithromycin cure rate of 98% in CT-infected women [10], the frequency of treatment failure was likely very low. Our future studies will evaluate how cellular immune responses influence the risk for CT reinfection and will correlate immune response data with CT load.

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Author contributions. KG, RKB and WMG designed the laboratory experiments. CGP and WMG conducted clinical procedures. GD and LB conducted the laboratory experiments. JYL performed statistical analyses. KG and WMG primarily interpreted the results and wrote the manuscript, with additional feedback and manuscript editing from RKB, BVDP, RG and JP.

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Conflict of interest. None.

Disclaimer. The findings and conclusions in this report are those of the authors, and do not necessarily represent the official position of the Centers for Disease Control and Prevention.

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