Bioluminescence Imaging of *Chlamydia muridarum* Ascending Infection in Mice

Jessica Campbell\(^2\), Yumeng Huang\(^1\), Yuanjun Liu\(^1\), Robert Schenken\(^2\), Bernard Arulanandam\(^3\), Guangming Zhong\(^1\)*

1 Department of Microbiology and Immunology, The University of Texas Health Science Center at San Antonio, San Antonio, Texas, United States of America, 2 Department of Obstetrics and Gynecology, The University of Texas Health Science Center at San Antonio, San Antonio, Texas, United States of America, 3 Department of Biology, University of Texas at San Antonio, San Antonio, Texas, United States of America

**Abstract**

Chlamydial pathogenicity in the upper genital tract relies on chlamydia ascending from the lower genital tract. To monitor chlamydial ascension, we engineered a luciferase-expressing *C. muridarum*. In cells infected with the luciferase-expressing *C. muridarum*, luciferase gene expression and enzymatic activity (measured as bioluminescence intensity) correlated well along the infection course, suggesting that bioluminescence can be used for monitoring chlamydial replication. Following an intravaginal inoculation with the luciferase-expressing *C. muridarum*, 8 of 10 mice displayed bioluminescence signal in the lower with 4 also in the upper genital tracts on day 3 after infection. By day 7, all 10 mice developed bioluminescence signal in the upper genital tracts. The bioluminescence signal was maintained in the upper genital tract in 6 and 2 mice by days 14 and 21, respectively. The bioluminescence signal was no longer detectable in any of the mice by day 28. The whole body imaging approach also revealed an unexpected airway infection following the intravaginal inoculation. Although the concomitant airway infection was transient and did not significantly alter the genital tract infection time courses, caution should be taken during data interpretation. The above observations have demonstrated that *C. muridarum* can not only achieve rapid ascending infection in the genital tract but also cause airway infection following a genital tract inoculation. These findings have laid a foundation for further optimizing the *C. muridarum* intravaginal infection murine model for understanding chlamydial pathogenic mechanisms.

**Introduction**

Pelvic Inflammatory Diseases can be caused by ascending infections from sexually transmitted microorganisms, including *Chlamydia trachomatis* [1,2]. However, it remains unclear how *C. trachomatis* organisms ascend to the upper genital tract. Although live chlamydial organisms or chlamydial nucleic acids can be monitored from women’s vaginal/cervical swab samples [3], these approaches only measure the lower genital tract infection. Due to the difficulty in routinely detecting upper genital tract infection in women, it is not possible to study *C. trachomatis* ascension mechanisms in humans. *Chlamydia muridarum* organisms have been extensively used to model the mechanisms of *C. trachomatis* pathogenesis [4]. This is because intravaginal inoculation with *C. muridarum* in mice can lead to ascending infection, resulting in upper genital tract pathology that is similar to what is induced by *C. trachomatis* in humans [5]. During mouse model studies, live organism recovery from vaginal swabs is often used for monitoring chlamydial infection in the lower genital tract [6,7]. Some have monitored ascending infection in mice by euthanizing mice for examining infection status in the upper genital tract tissues at multiple time points after infection [6,8,9]. This approach not only requires euthanizing many mice but also fails to follow the same animals for progression of ascending infection and eventual development of upper genital tract pathology.

Bioluminescence imaging with firefly luciferase has been used to monitor viral and bacterial infections in live animals [10,11,12]. This technology allows one to continuously follow live microbial infection in the same animals over time since luciferase is expressed by the organisms that are being followed. Transformation of chlamydial organisms has become possible [13,14,15]. We recently developed a protocol for transforming *C. muridarum* [16], which has made it possible for utilizing the luciferase-catalyzed bioluminescence imaging technology for monitoring *C. muridarum* infection in mice.

In the current study, we engineered a luciferase-expressing *C. muridarum* strain and found that the luciferase enzymatic activity measured as bioluminescence intensity correlated well with *C. muridarum* replication in cell culture system. When the luciferase-expressing *C. muridarum* were inoculated intravaginally into mice, bioluminescence signal was detected from the same mice along the entire course of infection. This technology has enabled us to monitor *C. muridarum* spreading from the lower to upper genital track.
tracts in real time, which has laid a foundation for further understanding chlamydial pathogenic mechanisms.

**Materials and Methods**

1. **Construction of recombinant plasmid for transformation**

   A DNA sequence from *C. muridarum* plasmid pgp4 promoter region was linked to the firefly luciferase gene *luc* by PCR. The firefly luciferase gene was amplified from pGME-luc vector purchased from Promega (Madison, WI). The transcription termination signal sequence stemming from ORF CT579 of *Chlamydia trachomatis* serovar D was conjoined to the 3 prime of the luciferase gene. In this way, an independent operon consisting of pgp4 promoter, *luc* gene and termination signal was established and inserted in front of the GFP-CAT gene of the pGFP::CM plasmid shuttle vector using an infusion cloning technique as described previously [16]. The newly created recombinant plasmid pGFP-luc-CM was used for transforming *C. muridarum* as described below.

2. **Transforming plasmid-free *C. muridarum* organisms**

   The pGFP-luc-CM plasmid was introduced into a plasmid-free *C. muridarum* strain designated as CMUT3 [9] in the form of a purified elementary body (EB) by following the protocol published previously [15,16]. The luciferase-expressing *C. muridarum* organisms were plaque-purified as described previously [17,18] for both in vitro characterization and in vivo imaging as described below.

3. **Detecting luciferase activity by measuring bioluminescence generated from luciferase-catalyzed luciferin**

   HeLa cells grown in 24-well plate were infected with luciferase-expressing *C. muridarum* organisms at an MOI of 2.5. At 6 h, 9 h, 12 h, 15 h, 18 h, 21 h, 24 h, 27 h & 30 h after infection, D-luciferin (cat#P1043, Promega) was added to each well at the final concentration of 1 mg/ml/well. The bioluminescence intensity from each well was detected and analyzed using Spectra MAX GEMINXS and SOFTmax Pro software (Molecular Devices, Sunnyvale, CA). After bioluminescence measurement, the infected cells were harvested using TRIzol reagent (Life Technologies, Grand Island, NY) for quantitating luciferase mRNA using quantitative RT-PCR as described below.

4. **Quantitative real-time RT-PCR**

   The culture samples harvested in TRIzol reagent as described above were extracted for total RNA using a Direct-zol RNA Miniprep kit (Zymo Research, Irvine, CA). The total RNA was used as template for reverse transcription to produce cDNAs with random hexamer primers with a ThermoScript reverse transcription-PCR (RT-PCR) system (Life Technologies, Grand Island, NY). The luciferase cDNA copy numbers were quantitated using a TaqMan RT-PCR assay carried out on a CFX96 Touch Deep Well real-time PCR detection system (Bio-Rad, Hercules, CA) with iQ Supermix (Bio-Rad). The following primers were used: *luc* forward 5'-GAACATCACGTACGCGGAATA-3' and reverse 5'-CCAACACCGGCATAAAGAATT-3'. The probe sequence used was 5'/-56-FAM/TCG GTT GGC/ZEN/AGA AGC TAT GAA ACGA/3IbKFQ/-3'. The qPCR conditions included an initial denaturation step at 95°C for 3 minutes, followed by 40 cycles of amplification at 95°C for 15 seconds and 60°C for 1 minute.

5. **Animal infection**

   The luciferase-expressing *C. muridarum* used in the current study were propagated in HeLa cells (human cervical carcinoma epithelial cells, ATCC cat# CCL2.1), purified, aliquoted and stored as described previously [19]. Female Balb/cj (#000651) were purchased at the age of 5 to 6 weeks old from Jackson Laboratories (Bar Harbor, Maine). Each mouse was inoculated intravaginally with 2×10⁵ IFUs of live luciferase-expressing *C. muridarum* in 20 μl of SPG (sucrose-phosphate-glutamate buffer). The animal experiments were carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Laboratory Animal Experiments of the University of Texas Health Science Center at San Antonio.

6. **Monitoring live *C. muridarum* organism recovery from vaginal swabs**

   To monitor live organism shedding, vaginal swabs were taken on different days after infection. Each swab was suspended in 500 μl of ice-cold SPG followed by vortexing with glass beads, and the released organisms were titrated on HeLa cell monolayers in duplicates as described previously [20]. The total number of IFUs per swab was calculated based on the number of IFUs per field, number of fields per coverslip, dilution factors and inoculation and total sample volumes. An average was taken from the serially diluted and duplicate samples for any given swab. The calculated total number of IFUs/swab was converted into log10 and the log10 IFUs were used to calculate means and standard deviations for each group at each time point.

7. **In vivo imaging**

   Mice infected intravaginally infected with luciferase-expressing *C. muridarum* organisms were imaged using the Xenogen IVIS imaging system (PerkinElmer, Hopkinton, MA) on days 3, 7, 10, 14, 21 and 28 after infection when vaginal swabs were taken. Prior to imaging, 500 μl of D-luciferin (40 mg/ml in sterile PBS) were intraperitoneally injected into each mouse. Twenty-five minutes after injection, mice were anesthetized in an induction chamber with 2% isofluorane. Bioluminescence images of the whole mouse bodies were captured. To acquire high quality images, the exposure time was set as 1 minute for each image. To measure photon radiance quantitatively, regions of interest (ROI) were selected on the surface of the mouse bodies using the automatic ROI. Intensity of bioluminescence was analyzed by using a Living Image software from PerkinElmer.

8. **Statistical analyses**

   The Spearman Correlation was used to analyze relationships between the different parameters, including luciferase gene expression versus luciferase activity (bioluminescence intensity) along the infection course in cell culture and live organisms recovered from the lower genital tract versus bioluminescence intensity taken from either the lower or upper genital tract areas. The statistical significance of these correlations was analyzed using Student’s t-Test.

**Results**

1. **Characterization of luciferase-expressing *C. muridarum***

   The luciferase-expressing *C. muridarum* were used to infect HeLa cells for monitoring both the luciferase gene expression and luciferase activity measured as bioluminescence intensity (Fig. 1). The luciferase expression level correlated well with the luciferase...
activity. Both became detectable at 12 hours (h) after infection. By 15 h after infection, both reached peak levels. Significant decrease was noted for both by 24 hours after infection. This time course is consistent with the rapid *C. muridarum* replication cycle in which the reticulate body (RB) replication peaks between 15 and 18 h with most RB differentiating into elementary body (EB) by 24 h after infection. These observations suggest that luciferase activity can be used for monitoring *C. muridarum* replication.

2. The in vivo imaging reveals rapid *C. muridarum* ascending infection with peak levels of ascension within the first 10 days after intravaginal inoculation

To monitor ascending infection, 10 female Balb/cJ mice were intravaginally infected with luciferase-expressing *C. muridarum* and monitored by both titrating live organism recovery from the lower genital tract and imaging whole mice for bioluminescence signals. Significant amounts of live organisms were recovered from the lower genital tracts of all 10 mice during the first 10 days after infection (Fig. 2). Starting day 14, both the level of live organisms and number of mice with positive shedding significantly decreased. By day 28, all but one mouse cleared infection. This time course is consistent with those of genital tract infection with wild type *C. muridarum* [9,21], suggesting that luciferase expression did not significantly alter *C. muridarum* infection in the mouse genital tract.

As shown in Fig. 3, the simultaneously monitoring for luciferase-generated bioluminescence using the Xenogen IVIS imaging system revealed that as early as 3 days after infection, bioluminescence signal became detectable in the upper genital tract area of 4 mice, suggesting rapid ascension by *C. muridarum* organisms upon intravaginal inoculation. By day 7, all 10 mice displayed bioluminescence signal in the upper genital tract area with peak levels of the signal in 6 mice. The remaining 4 mice developed peak levels of bioluminescence signal in their upper genital tract by day 10. These observations indicated that all mice developed peak ascending infection within the first 10 days after intravaginal inoculation. The ascending infection was maintained in the upper genital tract in 6 and 2 mice by days 14 and 21, respectively. No more bioluminescence signal was detected in any mice by day 28. When the relationships between live organisms recovered from the lower genital tract and bioluminescence intensity measured from either the lower or upper genital tract areas were analyzed (Fig. 4), significant correlations were found. Interestingly, the live organism recovery from the lower genital tract displayed a stronger correlation with the lower than upper genital bioluminescence signals.

Figure 2. Live *C. muridarum* organism recovery from vaginal swabs along the infection time course. After the intravaginal infection with the luciferase-expressing *C. muridarum* organisms, vaginal swabs were taken at different time points as indicated along the X-axis for monitoring live chlamydial organism shedding from the lower genital tract. The number of live organisms recovered from the vagina/cervix swabs expressed as log$_{10}$ IFUs per swab (top panel) and percent of mice that remained positive for shedding live organisms (bottom panel) were displayed along the Y-axis. Note that high levels of live organisms were recovered from the lower genital tract during the first 10 days after infection and the shedding dropped significantly by day 14 after infection, which is consistent with the time courses of genital tract infection with wild type *C. muridarum*. doi:10.1371/journal.pone.0101634.g002
have also acquired airway infection. Infection by days 7 or 10 after intravaginal infection while some mice developed a similar genital tract infection course regardless of their airway infection status. As negative controls, mice k and l that were infected with wild type C. muridarum and 7 days after infection, bioluminescence imaging was taken after injection with luciferin. Note a similar genital tract infection time course between mice c and h and lack of any significant bioluminescence signals in mice k and l.

doi:10.1371/journal.pone.0101634.g005

Discussion

By imaging bioluminescence signals catalyzed by Chlamydia muridarum-expressed luciferase, we have successfully visualized Chlamydia muridarum organisms in mouse genital tract without euthanizing any mice. This non-invasive approach has allowed us to conclude that C. muridarum organisms can start ascension as early as day 3 and maximize ascension on days 7 or 10 after intravaginal inoculation in female Balb/cJ mice. Please also note a
considerable variation in both chlamydial ascending time courses and ascending patterns among the mice of the same inbred strain. For example, mouse “a” developed a very significant ascending infection on day 3 and the ascending peaked in the left uterine horn and oviduct on day 7 while mouse “b” in the same cage did not start ascension until day 7 and the chlamydial organisms reached a peak level of ascending in the right side of the genital tract by day 10 after infection. The delayed ascending infection in mouse “b” seemed to enable mouse “b” to develop longer time course of bioluminescence signal in the genital tract. Mice “f” & “g” with delayed ascending peaks on day 10 also developed prolonged genital tract infection. The question is whether these variations can lead to differences in development of upper genital pathologies.

Unexpectedly, the whole body imaging also revealed significant bioluminescence signals in the chest area, indicating airway infection. Although all mice were only intravaginally inoculated with C. muridarum organisms, significant lung infection developed. All mice with lung infection on day 3 after intravaginal inoculation were housed in the same cage. We speculated that the airway infection was caused by inhaling luciferase-expressing C. muridarum leaked from mouse vagina. We did not independently detect live organisms from the lungs that displayed bioluminescence signal. This is because we wanted to keep the mice alive and monitor the same mice over time. Nevertheless, we expect to detect live organisms from these lungs. This speculation is consistent with previous findings that live organisms were recovered from lung tissues of mice intravaginally infected with C. muridarum [22,23]. It is also possible that the lung bioluminescence signal might come from nonviable materials containing luciferase activity. This hypothesis is consistent with the observation that the bioluminescence signal from the chest area was often transient. The airway signal detected on day 3 resolved by day 7 in most mice and residual airway signal was visible only in one mouse by day 7. More importantly, mice with lung bioluminescence signal did not show any significant alteration in the genital tract infection course. Mice with or without airway signal all developed peak ascending infection in the genital tract on either day 7 or 10 after infection. Nevertheless, a prolonged airway infection may significantly alter infection courses in the genital tract. For example, mouse “j” displayed airway infection on both days 3 & 7, which correlated with the shortened time course of genital tract infection. Mouse “j” cleared genital tract infection by day 14 when bioluminescence signals were still detectable in the genital tract of most other mice. This observation is consistent with the hypothesis that active lung infection can induce robust adaptive immunity that may shorten the infection course in the genital tract. This hypothesis is supported by a recent report that an intranasal inoculation with 50 IFUs of live C. muridarum induced sterile immunity in the genital tract against a challenge infection with $2 \times 10^8$ IFUs [24]. Thus, the airway bioluminescence signals should be carefully examined and properly interpreted.

Interestingly, we did not detect any significant bioluminescence signal from the mouse gastrointestinal tract despite a previous report that live organisms were recovered from the gastrointestinal tract following an intravaginal inoculation with C. muridarum [25]. In any case, we will be looking out the possibility using the whole body imaging technology since it was recently reported that C. muridarum could survive in the gastrointestinal tract for long periods of time, which could serve as a reservoir for persistent infection [25].

We are aware that the bioluminescence signals detected may not always correlate with chlamydial biosynthesis and replication in genital tract tissues. This is because bioluminescence is generated by luciferase-mediated catalysis of D-luciferin and luciferase expression controlled by pgp4 promoter may not always be proportional to chlamydial replication. Many factors independent of chlamydial replication may affect the luciferase-catalyzed bioluminescence intensity, including the expression level of luciferase and plasmid copy number. Although we have demonstrated a strong association of bioluminescence intensity with luciferase expression and chlamydial replication in cell culture system, it is not known whether such an association can be maintained during C. muridarum infection in vivo. We have previously shown that the plasmid copy number of the C. muridarum transformants is 2 to 3 fold higher than that of wild type C. muridarum [16]. The luciferase-expressing C. muridarum is no exception and its plasmid copy number is also 2.5 fold higher than that of wild type C. muridarum (data not shown). However, it is not known whether the plasmid copy number can be maintained at a constant level during C. muridarum infection in mice. Nevertheless, the plasmid copy number in C. trachomatis infecting human ocular tissues seemed to be relatively stable [26]. More importantly, although it is certain that lack of plasmid can significantly attenuate the pathogenicity of both C. muridarum and C. trachomatis [9,27], the effect of plasmid copy number on chlamydial pathogenicity remains unclear [26]. In any case, direct detection of chlamydial organisms in the upper genital tract may still be necessary for validating the infection status. The fact that a stronger correlation of the lower genital tract live organism recovery was established with the lower than the upper genital tract bioluminescence signals suggests that bioluminescence intensity is able to be used to both track live chlamydial organisms and more accurately detect upper genital tract infection. Thus, the luciferase imaging technology may have provided a convenient means for both investigating chlamydial pathogenic mechanisms and evaluating efficacies of intervention and prevention strategies.

**Author Contributions**

Conceived and designed the experiments: GZ RS BA. Performed the experiments: JC YH YL. Analyzed the data: GZ JC YH. Contributed to the writing of the manuscript: GZ JC YH BA.

---

**References**

1. Price MJ, Ades AE, De Angelis D, Welton NJ, Macleod J, et al. (2013) Risk of pelvic inflammatory disease following Chlamydia trachomatis infection: analysis of prospective studies with a multistate model. Am J Epidemiol 178: 230–234.

2. Davies B, Turner K, Ward H (2013) Risk of pelvic inflammatory disease after Chlamydia infection in a prospective cohort of sex workers. Sex Transm Dis 40: 239–243.

3. Schachtner J, McCormack WM, Chernesky MA, Marin DH, Van Der Pol B, et al. (2003) Vaginal swabs are appropriate specimens for diagnosis of genital tract infection with Chlamydia trachomatis. J Clin Microbiol 41: 3784–3789.

4. Cotter TW, Merg Q, Shen ZL, Zhang YX, Su H, et al. (1995) Protective efficacy of major outer membrane protein-specific immunoglobulin A (IgA) and IgG monoclonal antibodies in a murine model of Chlamydia trachomatis genital tract infection. Infect Immun 63: 4704–4714.

5. Shah AA, Schepensma JR, Imitaz MT, Sagar IM, Kasimov J, et al. (2005) Histopathologic changes related to fibrotic oviduct occlusion after genital tract infection of mice with Chlamydia muridarum. Sex Transm Dis 32: 49–56.

6. Tang L, Zhang H, Lei L, Gong S, Zhou Z, et al. (2013) Oviduct infection and hydrosalpinx in DBA/1j mice is induced by intravaginal but not intravaginal inoculation with Chlamydia muridarum. PLoS One 8: e61649.

7. Tang L, Yang Z, Zhang H, Zhou Z, Arulanandamb M, et al. (2014) Induction of protective immunity against Chlamydia muridarum intravaginal infection in DBA/1j mice. Vaccine 32: 1407–1413.
8. Chen L, Lei L, Chang X, Li Z, Lu C, et al. (2010) Mice deficient in MyD88 Develop a Th2-dominant response and severe pathology in the upper genital tract following Chlamydia muridarum infection. J Immunol 184: 2602–2610.

9. Lei L, Chen J, Hou S, Ding Y, Yang Z, et al. (2014) Reduced live organism recovery and lack of hydrosalpinx in mice infected with plasmid-free Chlamydia muridarum. Infect Immun 82: 983–992.

10. Gonzalez RJ, Weening EH, Frothingham R, Sempowski GD, Miller VL. (2012) Bioluminescence imaging to track bacterial dissemination of Yersinia pestis using different routes of infection in mice. BMC Microbiol 12: 147.

11. Kong Y, Shi Y, Chang M, Akin AR, Francis KP, et al. (2011) Whole-body imaging of infection using bioluminescence. Curr Protoc Microbiol Chapter 2: Unit 2C 4.

12. Barry MA, May S, Weaver EA (2012) Imaging luciferase-expressing viruses. Methods Mol Biol 797: 79–87.

13. Wang Y, Kahane S, Cutcliffe LT, Skilton RJ, Lambden PR, et al. (2011) Development of a transformation system for Chlamydia trachomatis: restoration of glycogen biosynthesis by acquisition of a plasmid shuttle vector. PLoS Pathog 7: e1002358.

14. Song L, Carlson JH, Whitmire WM, Kari L, Virtaneva K, et al. (2013) Chlamydia trachomatis plasmid-encoded PepR is a transcriptional regulator of virulence-associated genes. Infect Immun 81: 636–644.

15. Gong S, Yang Z, Lei L, Shen L, Zhong G (2013) Characterization of Chlamydia trachomatis plasmid-encoded open reading frames. J Bacteriol 195: 3819–3826.

16. Liu Y, Chen C, Gong S, Hou S, Qi M, et al. (2014) Transformation of Chlamydia muridarum reveals a role for Pgp5 in suppression of plasmid-dependent gene expression. J Bacteriol 196: 969–980.

17. Banks J, Eddie B, Schachter J, Meyer KF (1970) Plaque formation by Chlamydia in L cells. Infect Immun 1: 259–262.

18. Carlson JH, Whitmire WM, Crane DD, Wicke L, Virtaneva K, et al. (2008) The Chlamydia trachomatis plasmid is a transcriptional regulator of chromosomal genes and a virulence factor. Infect Immun 76: 2273–2283.

19. Cheng W, Shivshankar P, Li Z, Chen L, Yeh IT, et al. (2008) Caspase-1 contributes to Chlamydia trachomatis-induced upper urogenital tract inflammatory pathologies without affecting the course of infection. Infect Immun 76: 515–522.

20. Dong X, Liu Y, Chang X, Lei L, Zhong G (2014) Signaling via TNFR1 but not TLR2 contributes significantly to hydrosalpinx development following Chlamydia muridarum infection. Infect Immun 82: 1833–1839.

21. Tang L, Yang Z, Zhang H, Zhou Z, Arulanandam B, et al. (2013) Induction of protective immunity against Chlamydia muridarum intracervical infection in DBA/1j mice. Vaccine 32: 1407–1413.

22. Cotter TW, Ramsey KH, Miranpuri GS, Poulsen CE, Byrne GI (1997) Dissemination of Chlamydia trachomatis chronic genital tract infection in gamma interferon gene knockout mice. Infect Immun 65: 2143–2152.

23. Perry LL, Hughes S (1999) Chlamydial colonization of multiple mucosae following infection by any mucosal route. Infect Immun 67: 3686–3689.

24. Lu C, Zeng H, Li Z, Lei L, Yeh IT, et al. (2012) Protective immunity against mouse upper genital tract pathology correlates with high IFNgamma but low IL-17 T cell and anti-secretion protein antibody responses induced by replicating chlamydial organisms in the airway. Vaccine 30: 475–485.

25. Yeruva L, Spencer N, Bowlin AK, Wang Y, Rank RG (2013) Chlamydial infection of the gastrointestinal tract: a reservoir for persistent infection. Pathog Dis 68: 85–95.

26. Last AR, Roberts C, Cassanna E, Nabicsa M, Molina-Gonzalez S, et al. (2014) Plasmid copy number and disease severity in naturally occurring ocul Chlamydia trachomatis infection. J Clin Microbiol 52: 324–327.

27. Sagar IM, Schripsema JH, Wang Y, Clarke IN, Cutcliffe LT, et al. (2014) Plasmid deficiency in urogenital isolates of Chlamydia trachomatis reduces infectivity and virulence in a mouse model. Pathog Dis 70: 61–69.