Evaluation of Antimicrobial Resistance and Treatment Failures for Chlamydia trachomatis: A Meeting Report

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Each year, Chlamydia trachomatis causes ~3 million new infections and results in more than $1 billion in medical costs in the United States. Repeat or persistent infection occurs in 10%–15% of women who are treated for C. trachomatis infection. However, the role played by antimicrobial resistance in C. trachomatis treatment failures or persistent infection is unclear. With researchers in the field, we reviewed current knowledge and available approaches for evaluating antimicrobial resistance and potential clinical treatment failures for C. trachomatis. We identified key research questions that require further investigation. To date, there have been no reports of clinical C. trachomatis isolates displaying in vitro homotypic resistance to antimicrobials, but in vitro heterotypic resistance in C. trachomatis has been described. Correlation between the results of existing in vitro antimicrobial susceptibility tests and clinical outcome after treatment for C. trachomatis infection is unknown. Animal models may provide insight into chlamydial persistence, since homotypic resistance against tetracycline has been described for Chlamydia suis in pigs. Evaluating C. trachomatis clinical treatment failures, interpreting laboratory findings, and correlating the 2 clearly remain extremely challenging undertakings.

Chlamydia trachomatis infection is the most prevalent bacterial sexually transmitted infection in the United States. Every year, ~3 million new genital C. trachomatis infections occur in this country [1]. The annual medical cost of C. trachomatis infection—including direct costs of sequelae, such as pelvic inflammatory disease and infertility—was estimated to be $1.5 billion in the United States in 1994 [2].

Given the substantial morbidity and economic cost caused by this organism, several key pathophysiological questions about C. trachomatis infections merit further thought and attention. It would be valuable to understand why recurrent or persistent C. trachomatis infection occurs in 10%–15% of women treated for C. trachomatis infection [3]. In particular, the potential for C. trachomatis to develop antimicrobial resistance has not been well studied, despite some published case reports [4–7] that suggest resistance as a cause for clinical treatment failures. There have been no descriptions either of isolation of C. trachomatis strains that display stable resistance to antimicrobial agents recommended for therapy or of mechanisms of putative antimicrobial resistance for isolates obtained from patients with treatment failures. However, in vitro genetically mediated fluoroquinolone- and rifampin-resistant variants have been described [8–12], and, recently, 4 clinical isolates that demonstrated in vitro resistance to macrolides were shown to carry mutations in the 23S rRNA gene [13]. Major impediments to investigating antimicrobial resistance among Chlamydia species include the lack of a standardized in vitro assay and the lack of an understanding of the relationship between results of existing in vitro tests and clinical outcome after treatment.
In October 2001, a meeting in Atlanta, Georgia, was convened by the Centers for Disease Control and Prevention (CDC), to review the state of knowledge on antimicrobial susceptibility testing for *C. trachomatis* and possible approaches to evaluating the role of antimicrobial resistance in causing apparent *C. trachomatis* clinical treatment failures. The meeting was divided into 3 sessions: Test Methodologies, Comparison of Test Methodologies, and Clinical Applications. The following is a summary of the discussions and the research questions that were identified.

**C. TRACHOMATIS ANTIMICROBIAL SUSCEPTIBILITY TEST METHODOLOGIES**

**Cell culture.** Chlamydiae are obligate intracellular bacteria that have a strict requirement for ATP produced by the host eukaryotic cell [14]. Established epithelial cell lines have long been used to cultivate chlamydiae in vitro and are the most likely candidates for cell culture when determining the effects of antimicrobials on the growth of the organism.

A number of epithelial cell lines are permissive to *C. trachomatis* infection and support intracellular growth of the organism [15]. Most laboratories adopt a particular cell line for the growth of *C. trachomatis*, on the basis of availability of the cell line and technical experience or impression. When McCoy, HeLa, BGMK, HEp-2, HL, and Vero cell lines were used for in vitro antimicrobial susceptibility testing of *C. trachomatis* against tetracycline, doxycycline, and ofloxacin, similar results were observed [16]. However, in BGMK cells incubated with concentrations of azithromycin and erythromycin shown to be inhibitory in other cell lines, there was continued growth of *C. trachomatis*. With in vitro antimicrobial susceptibility testing of *C. trachomatis*, there are cell-line–dependent differences in the concentrations of antimicrobials necessary to inhibit chlamydial growth [17]. Although these differences suggest that the types of cell lines used for susceptibility testing should be standardized among laboratories, there is scant research on the most appropriate cell type for this procedure.

Serial subcultivation of cells in culture may affect their ability to become infected with and support the growth of chlamydiae. The passage number is the number of times cells have been serially subcultivated. For growth of *C. trachomatis*, some laboratories limit the number of serial passages to 50 and/or start a fresh cell line of low passage number every 2 months. However, there has been no formal attempt to correlate chlamydial growth rate with number of passages for various cell lines. In addition, cells must be grown in media free of antimicrobial agents known to be active against chlamydiae before being used for susceptibility testing.

Inoculation of cells with chlamydiae requires attachment of the organism to the cell membrane. Different techniques can be employed to facilitate this primary interaction, but centrifugation at ~2500 g for 1 h is the most commonly used method. Intracellular chlamydial growth is aided by the addition of 1 µg/mL cycloheximide to the culture medium, to inhibit eukaryotic protein synthesis; cycloheximide is used in most chlamydial antimicrobial susceptibility tests. However, since cycloheximide inhibits the synthesis of DNA and proteins in eukaryotic cells, it may interfere with antimicrobial uptake by the host cell. Cellular infection by most strains of *C. trachomatis* results in the formation of an intracellular inclusion that develops from each infectious organism. Inclusions can be stained and counted to standardize the inoculum level as inclusion-forming units (IFUs).

Uptake of antimicrobials by cells varies from the basal to the apical end of the cell, with enhanced uptake at the basal end. Ideally, cultured cell monolayers should be polarized so that their basal ends are uniformly arranged, to allow for equal uptake of an antimicrobial. Although polarized cells may reduce variability between cells in intracellular accumulation of antimicrobials and have been used in antimicrobial research with *C. trachomatis* [18], their use in routine antimicrobial susceptibility testing is limited, since the generation of polarized cells requires a great deal of time and technical expertise. The result of inconsistent antimicrobial uptake by cultured eukaryotic cells may be an undefined variability in test results.

Other cell types, such as macrophages or monocytes, can be infected with *C. trachomatis* after in vitro exposure [19]. Under certain conditions, inflammatory cells may not kill chlamydiae, allowing the organism to remain viable within the cell [19]. There are no data on the efficacy of antimicrobial treatment of inflammatory cells infected with chlamydiae. The use of these cell types in assessing in vitro resistance of *C. trachomatis* is limited, because they do not support robust chlamydial growth in tissue culture. The potential of inflammatory cells to act as in vivo reservoirs of *C. trachomatis* in patients undergoing antimicrobial treatment has not been elucidated.

**Determination of resistance.** Laboratory assessment of chlamydial susceptibility to antimicrobials requires growing the organism in eukaryotic cells cultured in medium containing sequential dilutions of relevant antimicrobials. After incubation, cells are examined for typical chlamydial inclusions, and the level of antimicrobial that inhibits inclusion formation is reported as the MIC. Continued culture of apparent inclusion-negative cells, after replacement of antimicrobial-containing medium with antimicrobial-free medium, is suggested as a method of determining chlamydicidal activity. The end point used for determining the MIC can prove to be challenging, since some inclusions will appear atypical or aberrant and their classification as being dead or alive may skew the MIC. Recently, a transition-point MIC (MIC<sub>TP</sub>) has been used to define the
drug concentration in which 90% of the inclusions appear aberrant [16]. The MIC<sub>FP</sub> was found to be more consistent than the MIC for end-point assessment. The minimum chlamydial concentration (MCC) has conventionally been reported as the amount of antimicrobial that prevents inclusion formation after antimicrobial removal and continued culture for 1 passage. However, the number of passages required for MCC determination varies by researcher and has yet to be formally defined [6, 16, 20].

The term “heterotypic resistance” refers to the replication of a heterogeneous population of resistant and susceptible bacteria from a subculture of a single resistant organism propagated on antimicrobial-containing medium. In contrast, “homotypic resistance” refers to replication of a homogeneous, clonal population of resistant organisms from a subculture of a single resistant organism [21, 22]. There has been no detection of or selection for homotypic resistance among C. trachomatis isolates, although homotypic resistance has been detected among Chlamydia suis isolated from pigs [23]. Heterotypic resistance in C. trachomatis can be observed when cells are inoculated with a large number of organisms and then grown in the presence of antimicrobials. In one study, C. trachomatis serovar D was shown to become heterotypically resistant to doxycycline when >10,000–50,000 IFUs/culture well was used to infect McCoy and BGMK cells [16]. Another study, in which 5000–20,000 IFUs/culture well was used [24], has shown variability in detecting heterotypic resistance. Results of one study of isolates from women with recurrent C. trachomatis infection suggested that treatment failures were more common in women infected with heterotypic resistant strains, as determined by McCoy and BGMK cells infected with 5000–20,000 IFUs/culture well, than in women infected with susceptible isolates [25]. These data highlight the difficulty of interpreting in vitro susceptibility test results for C. trachomatis and the need for clinical correlation with in vitro findings.

Antimicrobial dilutions should be prepared just before addition to infected cells, to ensure drug stability. The time at which an antimicrobial agent is added to the cell culture medium has varied by laboratory, from immediately after cell inoculation to 48 h afterward. This interval can have profound effects on the development of chlamydial inclusions and, thus, on the interpretation of susceptibility tests. For example, the addition of fluoroquinolones 20 h after inoculation, at concentrations as high as 64 μg/mL, was insufficient to inhibit C. trachomatis growth, whereas similar concentrations added sooner did inhibit growth [26]. Recent studies have confirmed that MICs typically increase when addition of the antimicrobial is delayed >8 h after inoculation [16].

The in vitro chlamydial developmental cycle has been estimated to vary from 24 to 72 h. The ratio of cells with visible chlamydial inclusions to those without apparent inclusions is greatest at ~48 h of incubation. Typically, cells are examined for chlamydial inclusions after 48 h of incubation [15]. A sufficient number of assays should be started in parallel, to allow MCC determination after 48 h of incubation in antimicrobial-containing medium and to allow MCC determination after an additional 48 h of incubation in antimicrobial-free medium.

Chlamydial inclusions are typically identified by microscopic examination after reaction with fluorescence-conjugated monoclonal antibodies directed against cell-wall components of the organism. Monoclonal antibodies against the major outer membrane protein are specific for C. trachomatis, whereas those against the lipopolysaccharide react with all chlamydial species. The appearance of characteristic inclusions after the addition of fluorescence-conjugated monoclonal antibodies is typically used in determining antimicrobial susceptibility, but the intracellular growth of chlamydiae can be slowed or arrested by various growth conditions that result in the development of aberrant inclusions. As discussed above, the determination of MICs and MCCs may be affected by the viability of organisms within these aberrant inclusions. A flow-cytometric antibiotic susceptibility assay using genus-specific fluorescence-conjugated monoclonal antibodies compared favorably to microscopic reading of MICs [27]. However, no data were presented on the sensitivity of the flow-cytometric method in detecting aberrant inclusions. Molecular techniques for identifying infected cells (e.g., real-time polymerase chain reaction or detection of messenger RNA) may be more useful than methods relying on inclusion staining [28].

The few C. trachomatis isolates shown to have some degree of in vitro antimicrobial resistance were difficult to propagate and were eventually lost through continued cell culture. These observations suggest the possibility that antimicrobial-resistant C. trachomatis may not survive as well as nonresistant organisms in cell culture, suggesting that they may be “less fit.”

Key meeting summary points and research needs are identified in Appendix A for C. trachomatis antimicrobial susceptibility testing methods and in Appendix B for understanding the nature of resistance in C. trachomatis.

**COMPARISON OF ANTIMICROBIAL SUSCEPTIBILITY TEST METHODOLOGIES**

**Laboratory methods.** The vast differences in antimicrobial susceptibility results obtained in the past by use of different methodologies have highlighted the need for the development of a standardized test. One comparative study, in which the same C. trachomatis isolates and antimicrobial agents were provided to individual laboratories proficient in the cultivation of chlamydiae, demonstrated substantial differences in MICs and MCCs among laboratories [29, 30]. Adoption of a standardized
antimicrobial susceptibility test procedure for *C. trachomatis* could reduce this variability, but standardization may be premature until there is better understanding as to how well the results obtained by use of different methods do or do not correlate with treatment outcome.

**Animal models.** Animal models of human chlamydial infections have contributed to understanding the pathogenesis of reproductive tract disease and related immunobiological factors. Surrogate mouse models for *C. trachomatis* infection of the reproductive tract are difficult to adapt to the study of antimicrobial susceptibility, because such infections are of shorter duration than *C. trachomatis* infections in humans [31]. The monkey model for *C. trachomatis* infection may be more relevant but is expensive [32]. Therefore, more useful information on the potential for chlamydiae to become resistant to antimicrobials would require the study of animals such as birds, ruminants, and pigs, in which chlamydiae are natural pathogens. *C. suis* infection in pigs is widespread and not often associated with clinical disease. Pig farmers routinely administer tetracycline to prevent neonatal diarrhea and pneumonia caused by bacteria other than *C. suis*. When *C. suis* has been recovered from pigs, most—but not all—isolates have been shown to have homotypic resistance to tetracyclines [23, 33], perhaps as a result of repeated tetracycline exposure. Investigation of these homotypically resistant *C. suis* isolates could provide a model for the examination of mechanisms of antimicrobial resistance [34], which may provide insight into the potential for the development of resistance in human chlamydiae isolates.

**CLINICAL APPLICATIONS**

**Defining and diagnosing C. trachomatis treatment failures.** Apparent posttreatment persistence or recurrence of *C. trachomatis* infection after CDC-recommended antimicrobial therapy may represent (1) reexposure to an untreated, infected sex partner and, thus, reinfection; (2) treatment failure due to noncompliance with treatment, antimicrobial resistance, or poor absorption of the drug; or (3) persistence due to host factors such as immune response or other undefined host factors. It is difficult to differentiate between *C. trachomatis* reinfection and treatment failure with certainty, because the possibility of reexposure and reinfection cannot always be confidently ruled out and because the correlation between treatment failure and in vitro chlamydial antimicrobial resistance is still inconclusive. A possible definition of *C. trachomatis* treatment failure that may be due to antimicrobial resistance is as follows:

persistent infection as determined by a positive *C. trachomatis* culture (or other *C. trachomatis* diagnostic test) in a patient who has completed treatment and who (a) has not had sexual exposure since receiving treatment, (b) has had sex only with a treated partner after the partner completed treatment, or (c) has used condoms for every sexual encounter since treatment.

Depending on the specific design of a *C. trachomatis* treatment-failure study, a stricter definition might permit only option (a) and might exclude the possibilities of (b) and (c). Confirmation that the *C. trachomatis* found after treatment is the same serovar as the *C. trachomatis* found before treatment is consistent with—but does not prove—persistent infection.

The optimal time frame for the posttreatment interval within which a persistent *C. trachomatis* infection could most likely be attributed to antimicrobial resistance is unknown. Reasonable start times for defining posttreatment intervals may vary with different diagnostic tests and may range from immediately after treatment to 7 days after treatment to 14 days after treatment. Reasonable end times of posttreatment intervals range from 1 to 3 months after treatment. Different time intervals may need to be considered, depending on whether doxycycline or azithromycin was used for treatment. Defining the posttreatment interval as a longer period would provide greater sensitivity for detecting treatment failure, but a shorter interval might decrease the likelihood of reexposure and reinfection, thus providing greater specificity.

Characteristics of *C. trachomatis* diagnostic tests create challenges for the evaluation of treatment failure. Culture methods are less susceptible to the false-positive results that might be produced by nonculture methods, particularly if shorter posttreatment time intervals are used and dead organisms are present. However, culture methods are also less sensitive in detecting infection than are nonculture methods. Nucleic acid amplification tests are very sensitive and might be used with a transport medium that would permit culture isolation, if the amplification test results were positive. Use of amplification tests, however, would require that the start time of the posttreatment interval begin 14 days after treatment, to avoid detection of residual DNA.

**Monitoring C. trachomatis treatment failures.** Systematic monitoring of *C. trachomatis* treatment failures in 1 or more centers could permit (1) the tracking of treatment-failure trends, (2) the clinical-epidemiologic study of *C. trachomatis* treatment failures and their potential association with antimicrobial resistance and/or the host immune response, and (3) the microbiological evaluation of the characteristics and mechanisms of antimicrobial resistance, if resistance is identified. Possible approaches to monitoring *C. trachomatis* treatment failures include performing passive surveillance, such as establishing a treatment-failure registry and collecting *C. trachomatis* isolates from patients with treatment failures for susceptibility testing, and performing active surveillance by implementing a prospective test-of-cure protocol.
Evaluating C. trachomatis treatment failures. The value of investigating and understanding why C. trachomatis treatment failures occur is supported by evidence that treatment failures may not be rare events. A multicenter cohort study of determinants of persistent and recurrent C. trachomatis infection among young women [3] and a multicenter randomized trial of patient-delivered partner treatment with active follow-up of women with C. trachomatis infection who were treated [35] demonstrated that 10%–15% of women in these studies were infected with C. trachomatis when retested ~4 months after C. trachomatis treatment, regardless of treatment compliance, condom use, partner treatment, or having a steady or new partner.

Developing an understanding of both the clinical significance and the causes of C. trachomatis treatment failures are crucial research priorities. Do patients with treatment failures have more frequent adverse outcomes (i.e., more pelvic inflammatory disease, more infertility, etc.) than those without treatment failures? Do patients with treatment failures contribute more to transmission of C. trachomatis than patients without treatment failures? What are the causes of C. trachomatis treatment failures? Chlamydial persistence has been observed when C. trachomatis cultures are incubated with folic acid inhibitors, β-lactams, and fluoroquinolones in vitro [36–39]; in the presence of the antimicrobials, bacteria were noncultivable, but withdrawal of the antimicrobial resulted in resumption of overt chlamydial growth. What are the clinical implications of these in vitro findings?

CONCLUSION

Appendix C summarizes the key clinical research questions regarding antimicrobial resistance and posttreatment persistence of C. trachomatis that were identified at the meeting. Evaluating C. trachomatis clinical treatment failures, interpreting laboratory findings, and correlating the 2 clearly remain extremely challenging undertakings. Such challenges suggest that we still have much to learn about human C. trachomatis infection [40].

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APPENDIX A

CHLAMYDIA TRACHOMATIS ANTIMICROBIAL SUSCEPTIBILITY TESTING

Meeting summary points

• Epithelial cell lines commonly used to grow chlamydiae are most practical for antimicrobial susceptibility testing.
• Scientific data are not sufficient to recommend a single cell line for antimicrobial susceptibility testing of chlamydiae.
• Cell lines of low pass number should be used to reduce variability in results. As with all cell cultures, routine cell culture assessments for quality assurance should be maintained.
• Well-defined methods of enhancing cellular infection and intracellular chlamydial growth (i.e., centrifugation, addition of cycloheximide, etc.) should be used when assessing in vitro antimicrobial susceptibility.
• Antimicrobial dilutions should be prepared in a manner that ensures antimicrobial drug potency (e.g., they should be prepared immediately before addition to infected cells and added immediately after cell inoculation).
• The MIC and transition-point MIC should be determined after 48 h of incubation in medium containing antimicrobials. The minimum chlamydical concentration (MCC) has conventionally been determined after a single passage from antimicrobial-containing medium to antimicrobial-free medium, with 48 h of incubation in each medium.
• Cell cultures grown in a uniform orientation—that is, polarized—may be more consistent in antimicrobial uptake but are technically difficult to generate.
• Inflammatory cells currently have limited use for routine testing of antimicrobial resistance but may act as a source of persistent infection in vivo.

Research needs

• Define a list of suitable and unsuitable cell lines for antimicrobial susceptibility testing.
• Investigate the growth and clinical significance of chlamydiae within inflammatory cells.
• Consider developing methods of chlamydial growth that are not reliant on staining inclusions (e.g., molecular methods).
• Clarify the factors contributing to cell-line–dependent differences in MIC/MCC results.

APPENDIX B

DETERMINING THE PRESENCE, ABSENCE, AND NATURE OF RESISTANCE IN CHLAMYDIA TRACHOMATIS

Meeting summary points

• In vitro resistance of C. trachomatis to antimicrobials is expressed in a heterotypic fashion—that is, not all progeny from a single resistant bacterium will be equally resistant to antimicrobials.
• Because the level of infectious chlamydiae used to inoculate cells will affect antimicrobial susceptibility results (i.e., inoculating cells with a high infectious titer will increase the likelihood of finding heterotypic resistance), a range of inoculum sizes should be evaluated.
• To date, there have been no reports of C. trachomatis displaying in vitro homotypic resistance to antimicrobials.
• In most animal models, C. trachomatis infection is self-limiting.
• In vivo isolates of Chlamydia suis, a natural, potentially pathogenic chlamydial infection in pigs, have been shown to be homotypically resistant to tetracyclines, which are commonly used in the porcine industry.

Research needs

• Compare the results from at least 3 inclusion-forming unit (IFU) levels of chlamydial infectivity—that is, low, medium, and high (e.g., 10^4, 10^5, and 10^6 IFUs, etc.)—to determine the level at which heterotypic resistance develops.
• Determine whether inoculum size and identification of heterotypic resistance varies with different clinical isolates.
• Compare the fitness of homotypically resistant and susceptible C. suis isolates in pigs, in an attempt to elucidate in vivo tetracycline resistance.
• Investigate other animal species naturally infected with chlamydiae and compare the in vivo outcome of antimicrobial treatment with different in vitro measures of antimicrobial susceptibility.

APPENDIX C

KEY CLINICAL RESEARCH QUESTIONS REGARDING ANTIMICROBIAL RESISTANCE AND POSTTREATMENT PERSISTENCE OF CHLAMYDIA TRACHOMATIS

• What are the clinical implications of heterotypic resistance in C. trachomatis?
• Does homotypic resistance exist in C. trachomatis?
• Are C. trachomatis infections resistant or susceptible to antimicrobials, or, rather, are they suppressible or non-suppressible infections, depending on conditions?
• What is the significance of C. trachomatis persistence in vitro and in vivo? Are persistent isolates less fit? Is there a relationship between heterotypic resistance to antimicrobials and persistence secondary to other stimuli? Do antimicrobials help induce persistence?
• Do patients with C. trachomatis treatment failures or persistence have more frequent adverse outcomes (i.e., more pelvic inflammatory disease, more infertility, etc.) than patients without treatment failures or persistence? Do patients with treatment failures or persistence contribute more to transmission of C. trachomatis than patients for whom C. trachomatis eradication appears to be successful?

References


