

## Methodologies and Cell Lines Used for Antimicrobial Susceptibility Testing of *Chlamydia* spp.

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**In vitro** susceptibility testing was performed on strains of *Chlamydia trachomatis*, *Chlamydia pneumoniae*, and *Chlamydia psittaci* under various conditions, including the cell line utilized, the time between infection and the addition of an antimicrobial, the concentration of inoculum, and the effect of multiple passage on the minimal chlamydicidal concentrations for the antibiotics doxycycline, azithromycin, erythromycin, ofloxacin, and tetracycline. With macrolides, the MIC varied depending upon the cell line utilized. With all antimicrobials, the MIC was related to the time at which the antimicrobial was added after infection. By an optimized cell culture passage method, all strains of chlamydia tested demonstrated survival after exposure to high levels (>100 times the MIC) of antimicrobials. Furthermore, upon retest, these surviving organisms did not demonstrate increased MICs. Thus, this phenomenon does not reflect selection of antimicrobial-resistant mutants but rather survival of some organisms in high antimicrobial concentrations (heterotypic survival). An additional 44 clinical isolates of *C. trachomatis* from patients with single-incident infections were tested against those from patients with recurrent or persistent infections, and heterotypic survival was seen in all isolates tested; hence, *in vitro* resistance did not correlate with the patient's apparent clinical outcome.

*Chlamydia trachomatis*, an obligate intracellular bacterium, causes a broad spectrum of clinical syndromes ranging from urethritis and epididymitis in men to cervicitis, endometritis, pelvic inflammatory disease, and ectopic pregnancy in women. *C. trachomatis* infection is the most commonly reported bacterial sexually transmitted disease in the United States (7). Despite a decline in prevalence in geographic areas with screening and treatment programs, an estimated three million individuals still contract chlamydia each year in the United States (6). The estimated annual cost attributable to chlamydia and its complications is approximately two billion dollars (14).

In an era of increasing resistance to antimicrobials in many bacterial species, resistance has not been of great concern in the treatment of chlamydial infections. This may be due in part to the facts that very few patients have chlamydia cultures obtained, that tests of cure evaluations are not routinely done, and that even if cultured, few isolates are tested for antimicrobial susceptibilities due to the laborious and unstandardized methodology. Recently, however, clinical treatment failures have been reported and attributed to multidrug-resistant *C. trachomatis* strains (15, 27; G. Schmid, B. Van Der Pol, R. B. Jones, and R. Johnson, Abstr. Int. Congr. Sex. Transm. Infect., Int. J. Sex. Transm. Dis. AIDS 12:41, 2001). These strains were reported to demonstrate heterotypic resistance, a pattern in which small numbers of organisms survive antimicrobial concentrations well above the MIC (15, 27; G. Schmid et al., Abstr. Int. Congr. Sex. Transm. Infect.). *C. trachomatis* strains exhibiting homotypic resistance, a pattern in which the majority of organisms survive antimicrobial concentrations well above the MIC, have not been reported in isolates from humans.

Despite its apparent rarity, there are reasons to suspect that antimicrobial resistance does indeed occur in urogenital chlamydia. For example, *C. trachomatis* may be repeatedly cultured from some patients despite antimicrobial therapy (15, 18, 27; G. Schmid et al., Abstr. Int. Congr. Sex. Transm. Infect.), suggesting resistance. Same-serovar recurrences of chlamydia are frequent (2, 4, 9, 23), which would also be consistent with resistant strains persisting after therapy (but could be due to reinfection as well). Finally, selection for resistance in the laboratory has been demonstrated for a variety of antimicrobials (10, 17), and recent *C. trachomatis* isolates from swine (termed *Chlamydia suis*) chronically exposed to tetracycline demonstrate homotypic resistance (1, 19). *Chlamydia pneumoniae* responds to treatment with macrolides, tetracyclines and fluoroquinolones, and to date naturally occurring antimicrobial resistance has not been reported in *C. pneumoniae* or in other *Chlamydia* species (28).

Although many research groups perform antimicrobial susceptibility testing of *Chlamydia* organisms worldwide, there is not a standardized methodology or uniformly accepted interpretation of results. Because of the potential impact of antimicrobial resistance on the prevention and treatment of *Chlamydia* infections, we investigated differences in methodologies, cell lines utilized, and interpretation of antimicrobial susceptibility testing results in order to provide a basis for standardizing the methodologic approach and interpretation of antimicrobial susceptibility testing in the *Chlamydia* species. As the association of *in vitro* antimicrobial resistance with treatment failures or recurrences of *C. trachomatis* infection in humans has not been extensively studied, we also assessed the MIC and minimal chlamydicidal concentration (MCC) of *C. trachomatis* isolates associated with either treatment failure or same-serovar recurrence in comparison with those of strains associated with apparently successful therapy. As an example of homotypic resistance in our studies, we utilized a *C. suis* strain for

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TABLE 1. Effect of cell line on MIC, MCC, and MCC3 for *C. trachomatis* serovar D<sup>a</sup>

Cell type (origin)	Doxycycline			Azithromycin			Erythromycin			Ofloxacin			Tetracycline		
	MIC	MCC	MCC3	MIC	MCC	MCC3	MIC	MCC	MCC3	MIC	MCC	MCC3	MIC	MCC	MCC3
McCoy (human)	0.064	>8	64	0.125	>8	64	0.25	>8	128	1	>8	128	0.25	>8	64
HeLa (human)	0.064	1	4	0.016	1	8	0.032	2	4	1	4	16	0.25	2	16
BGMK (simian)	0.064	>8	64	1.0	>8	64	2	>8	128	1	>8	128	0.25	>8	64
HEp-2 (human)	0.064	1	4	0.064	4	32	0.125	4	8	1	4	64	0.25	4	32
HL (human)	0.064	2	32	0.008	4	32	0.016	4	0.5	1	8	32	0.25	4	32
Vero (simian)	0.064	2	8	0.25	2	4	0.5	4	16	1	8	32	0.25	2	16

<sup>a</sup> All values are in micrograms per milliliter. The MIC was defined as one twofold dilution more than the MIC<sub>TP</sub>; MCC, minimal chlamydicidal concentration following one freeze-thaw passage in microtiter plates; MCC, minimal chlamydicidal concentration following one passage in shell vials; MCC3, minimal chlamydicidal concentration following three passages in shell vials.

which the tetracycline and doxycycline MICs were elevated (8.0 and 1.0 µg/ml, respectively).

#### MATERIALS AND METHODS

**Organisms.** Laboratory reference strains for *C. trachomatis* (serovars B, D, E, F, G, H, I, Ia, H, J, K, and L<sub>2</sub>), *C. pneumoniae* (TW-183 and CWL-029), and *C. psittaci* (6BC and GPIC) are maintained and routinely used in our laboratory for research. The tetracycline-resistant *C. suis* strain R-19 was provided by Arthur A. Andersen, National Animal Disease Center, Ames, Iowa. Clinical strains were selected from a large archive of serotyped clinical isolates obtained from patients who had a culture-documented *C. trachomatis* genital infection at any of the Seattle-King County Health Department sexually transmitted disease clinics. Single-episode *C. trachomatis* isolates were selected from patients who had no record in our database of having an additional previous or subsequent chlamydial infection. Persistent isolates were chosen from patients who had three or more same-serovar recurrent *C. trachomatis* infections over a period of more than 2 years with no intervening infections by a different chlamydial serovar. Treatment failure isolates were selected from patients who had two or more visits with a positive *C. trachomatis* culture after receiving documented treatment. Four unknown strains without identifiers were provided by the Centers for Disease Control and Prevention (CDC) for susceptibility testing; two isolates were from patients diagnosed as treatment failures and had been determined to be multi-drug resistant by CDC laboratories, and two isolates were controls without antimicrobial resistance.

**Antimicrobial susceptibility testing.** Antimicrobial susceptibility testing of *C. trachomatis*, *C. pneumoniae*, and *C. psittaci* strains in six cell lines (HeLa, McCoy, BGMK, HEp-2, HL, and Vero) without passage (MIC testing) on 96-well microtiter plates and by multiple passage (MCC testing) in 48-well microtiter plates and 12-mm glass shell vials was undertaken with doxycycline, azithromycin, erythromycin, ofloxacin, and tetracycline. Antimicrobial susceptibility testing in 48-well microtiter plates and 12-mm glass shell vials with doxycycline, azithromycin, and ofloxacin was undertaken in McCoy cells for the *C. suis* strain R-19, the reference *C. trachomatis* and *C. psittaci* strains, and the clinical isolates of *C. trachomatis* and in HEp-2 cells for the reference *C. pneumoniae* strains. Cells were maintained in antimicrobial-free growth medium consisting of minimal essential medium with 10% fetal bovine serum and 220 µg of L-glutamine/liter added. The inoculum size of infectious chlamydial forms for all MIC and MCC comparisons was 10,000 to 50,000 inclusion-forming units (IFU) in each well for 48-well microtiter plates or on 12-mm coverslips in shell vials, resulting in the infection of approximately 10 to 50% of the cells in the monolayers.

MIC determinations were performed on monolayers in 96-well plates. For all centrifugation procedures, a Beckman model J-6 M centrifuge was utilized. Each chlamydial strain was inoculated into three rows of 12 wells and centrifuged at 1,200 × g for 1 h at 37°C, and then supernatants were aspirated. Antimicrobial agents were obtained in powder form, weighed and adjusted for purity, and reconstituted according to the manufacturer's instructions. Dilution schemes were prepared by using twofold dilutions in antimicrobial-free growth medium containing cycloheximide (1.0 µg/ml). For each antimicrobial, 100 µl of each dilution (300 µl for 48-well plates and shell vials) was added to the appropriate wells to give a final concentration range of 0.008 to 128 µg/ml. Passage of tissue culture plates was done by a freeze-thaw method where monolayers were frozen at -70°C, thawed in a 37°C water bath, disrupted with a pipette tip, passed onto fresh monolayers, and centrifuged at 1,200 × g for 1 h at 37°C. Wells were then aspirated and overlaid with appropriate twofold drug dilutions made in antimicrobial-free growth medium with cycloheximide added. Cells were incubated at

37°C in 4% CO<sub>2</sub> for 24 to 72 h (48 h for *C. trachomatis*, 72 h for *C. pneumoniae*, and 24 h for *C. psittaci*) and fixed with methanol. Chlamydial inclusions were detected by fluorescence by using a genus-specific monoclonal antibody CF-2 (Washington Research Foundation, Seattle).

The passage of monolayers in shell vials differed from that on culture plates in that after incubation, the medium containing the antimicrobial was aspirated and the cells were rinsed twice with Hanks balanced salt solution. The cells were then disrupted by sonication by using an ultrasonic processor, and the resulting suspension was centrifuged at 300 × g for 10 min at 4°C to remove debris. The supernatant was inoculated onto fresh monolayers in shell vials, centrifuged at 1,200 × g for 1 h at 37°C, aspirated, and overlaid and incubated as described previously. For each passage, 100 µl of each passage vial was added to corresponding wells on a 96-well microtiter plate for observation of inclusions. A laboratory reference strain, serovar D/uw-3/cx, which is antimicrobial sensitive, was used in all comparisons and in experiments comparing the MIC, MCC, and MCC3 (the MCC after three passages in shell vials; see below).

The transition point MIC (MIC<sub>TP</sub>) was defined as the concentration of drug in which 90% or more of the inclusions were altered in size and morphology. The MIC was defined as the concentration of drug that is one twofold dilution more concentrated than the MIC<sub>TP</sub>. The MCC was defined as the lowest concentration of drug that produced no morphologically normal inclusions by one freeze-thaw passage in microtiter plates (microtiter plate method) or by one passage in shell vials (shell vial method) in antimicrobial-free medium. The MCC3 was determined to be the MCC after three passages in shell vials (shell vial method) in antimicrobial-free medium.

To determine the effect of cell line variation on MCC3 levels, several representative *Chlamydia* strains were passed three times in McCoy, BGMK, HeLa and HEp-2 cells. To test for selection of antimicrobial-resistant organisms, chlamydiae surviving the highest MCC3 were retested against the respective antimicrobial and the MIC was determined.

A twofold dilution scheme with *C. trachomatis* strain UW-3 (serovar D) inocula ranging from ~320,000 to 300 IFU/well (48-well microtiter plate) was used to determine the effect of inoculum size on the MIC, MCC, and MCC3. The effect on MICs of the time that elapsed between infection and the addition of a given antimicrobial was tested by adding the drug at 2-h intervals up to 24 h after infection.

#### RESULTS

**Antimicrobial susceptibility testing of *Chlamydia* species in different cell lines.** The MICs of tetracycline, doxycycline, and ofloxacin were comparable in all cell lines for serovar D (Table 1). Interestingly, there was considerable MIC variation when *C. trachomatis* was tested against azithromycin and erythromycin, with the lowest MICs being those for isolates tested in HeLa and HL cell lines and with the MICs for those tested in the BGMK cell line being significantly higher (Table 1). The MCC and MCC3 for *C. trachomatis* (Table 1) were highest in McCoy and BGMK cells.

MICs for *C. pneumoniae* strain TW-183 and *C. psittaci* strain 6BC were comparable for each antimicrobial tested (not shown). The MCC and MCC3 for *C. pneumoniae* were highest

TABLE 2. Effect of inoculum size on doxycycline MIC, MCC, and MCC3 for *C. trachomatis* serovar D<sup>a</sup>

Inoculum (10 <sup>3</sup> IFU/well)	MIC	MCC	MCC3
320	0.064	>8	64
160	0.064	>8	64
80	0.064	>8	64
40	0.064	>8	64
20	0.064	>8	64
10	0.064	>8	64
5	0.064	>8	64
2.5	0.064	0.064	0.064
1.25	0.064	0.064	0.064
0.625	0.064	0.064	0.064
0.3125	0.064	0.064	0.064

<sup>a</sup> All values are in micrograms per milliliter. The MIC was defined as one twofold dilution more than the MIC<sub>TP</sub>; MCC, minimal chlamydicidal concentration following one freeze-thaw passage in microtiter plates; MCC, minimal chlamydicidal concentration following one passage in shell vials; MCC3, minimal chlamydicidal concentration following three passages in shell vials.

in HEp-2 and HL cells (not shown), while the corresponding values for *C. psittaci* were highest in McCoy and BGMK cells (not shown).

**Effect of inoculum size on antimicrobial susceptibility testing in *C. trachomatis*.** There was no observed difference in MICs when the inoculum size of viable chlamydia was varied over the range of 300 to 300,000 IFU/well. However, when an inoculum of less than 300 IFU/well was used, it was difficult to find a sufficient number of inclusions to read accurately. Variation of inoculum size did influence MCCs by shell vial passage, with elevation of MCCs and MCC3s above the MIC (Table 2). However, when the inoculum fell below 5,000 IFU/well, there was no observed difference between the MIC and MCC. This suggests that the survival rate of *Chlamydia* exposed to an effective antimicrobial is approximately one bacterium per 5,000 infected cells. Inclusions produced from surviving bacterium by definition of the chlamydial developmental cycle must have been produced from viable elementary bodies generated from aberrant inclusions, since no normal inclusions are observed. Independent incubation death studies done in our laboratory showed that elementary bodies could not survive in cell-free growth medium at an incubation temperature of 37°C for periods of longer than 24 h (unpublished data).

**Effect of the time between infection and the addition of antimicrobial agents on antimicrobial susceptibility testing in *C. trachomatis*.** There were no observable differences in MICs when an antimicrobial was added at 2-h intervals from 0 to 8 h after infection, but the MIC increased steadily after 8 h, suggesting that the drug had less of an effect on chlamydial development after 8 h postinfection (data not shown).

**Antimicrobial susceptibility testing of reference strains of *Chlamydia* species and clinical isolates of *C. trachomatis*.** Table 3 shows the results of in vitro susceptibility testing of the *C. trachomatis*, *C. pneumoniae*, and *C. psittaci* reference strains against doxycycline, azithromycin, and ofloxacin. For the tetracycline-resistant *C. suis* strain, the doxycycline MICs were considerably higher than those for the other reference strains tested, but the azithromycin and ofloxacin MICs were comparable to those for the other reference strains. Figure 1 illustrates the elevated doxycycline MIC (homotypic resistance) demonstrated by the *C. suis* strain R-19 relative to that for the sensitive *C. trachomatis* strain serovar D. The MCCs determined by microtiter plate passage were consistently lower than those determined by shell vial passage for all of the *Chlamydia* reference strains. Further, the MCCs determined by shell vial passage were consistently lower than the MCC3s. The MCC3s were consistently >100 times the MICs, demonstrating the survival of some organisms at high drug concentrations. Normal inclusions formed from surviving chlamydia at higher MICs after one passage (MCC) are illustrated in Fig. 2A. When *Chlamydia* strains reflecting the highest MCC3s were retested, there was no homotypic elevation of MICs, but rather a repeat of the initial MIC pattern with the heterotypic survival of a small number of organisms at higher MICs of a given antimicrobial (Fig. 2B and C). For some strains, the retesting of surviving organisms reflecting the highest MCC3s was repeated up to five times, still with no apparent elevation of the MIC for this select group of organisms. When MCC3s were determined for isolates in different cell lines, those for isolates cultured in the McCoy and BGMK cell lines were comparable. For *Chlamydia* in HeLa cell lines, the MCC3s were elevated (>10 times the MIC), but never to the higher degree seen in the McCoy and BGMK cell lines, exemplifying the various degrees of infectivity that different cell lines have for *Chla-*

TABLE 3. Antimicrobial susceptibilities of reference strains of *Chlamydia* species<sup>a</sup>

<i>Chlamydia</i> reference strain(s)	Doxycycline				Azithromycin				Ofloxacin			
	Microtiter		Shell vial		Microtiter		Shell vial		Microtiter		Shell vial	
	MIC	MCC	MCC	MCC3	MIC	MCC	MCC	MCC3	MIC	MCC	MCC	MCC3
<i>C. trachomatis</i> B, D, E, F, G, H, I, Ia, J, and K (human genital serovars)	0.064	0.125	>8	16–32	0.125	0.25	>8	64	0.5	1.0	>8	>128
<i>C. trachomatis</i> L2 (LGV serovar)	0.064	0.125	>8	32	0.125	0.25	>8	64	0.5	1.0	>8	>128
<i>C. trachomatis</i> MoPn (mouse pneumonitis strain)	0.064	0.5	>8	64	0.125	0.5	>8	128	0.5	1.0	>8	>128
<i>C. suis</i> R-19 (porcine strain) <sup>b</sup>	1.0	2.0	>8	>128	0.125	0.25	>8	>128	0.5	1.0	>8	>128
<i>C. pneumoniae</i> TW-183, CWL-029 <sup>c</sup>	0.064	0.125	4–8	32–64	0.125	0.25	4–8	64	0.5	1.0	>8	>128
<i>C. psittaci</i> 6BC, GPIC	0.064	0.250	>8	>128	0.125	0.50	>8	>128	0.5	2.0	>8	>128

<sup>a</sup> All values are in micrograms per milliliter. The MIC was defined as one twofold dilution more than the MIC<sub>TP</sub>; MCC, minimal chlamydicidal concentration following one freeze-thaw passage in microtiter plates; MCC, minimal chlamydicidal concentration following one passage in shell vials; MCC3, minimal chlamydicidal concentration following three passages in shell vials.

<sup>b</sup> Tetracycline-resistant (MIC, >4 µg/ml) porcine strain provided by Arthur A. Andersen, National Animal Disease Center, Ames, Iowa.

<sup>c</sup> *C. pneumoniae* strains grown in HEp-2 cells.

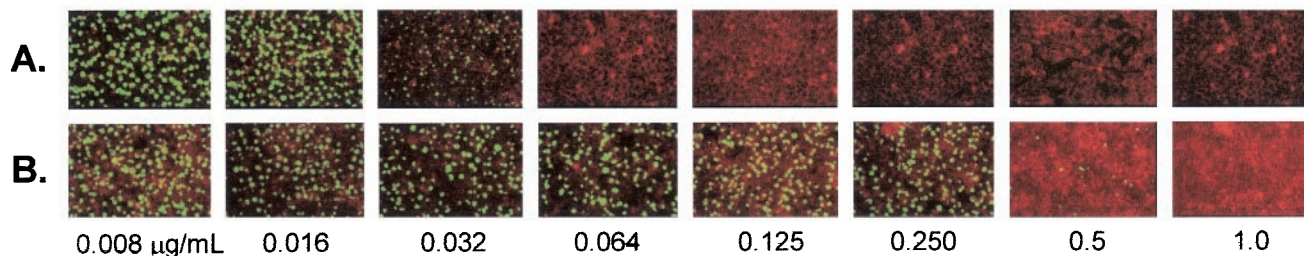


FIG. 1. Sequential photomicrographs of MIC growth patterns of *C. trachomatis* serovar D (A) and *C. suis* strain R-19 (B) at various concentrations of doxycycline. The *C. suis* strain demonstrates homotypic resistance, in which most of the organisms survive at concentrations well above MIC for the *C. trachomatis* strain. Also shown is an example of the MIC<sub>TP</sub>, the transition point where the concentration of antimicrobial first noticeably influences chlamydial inclusion growth (MIC<sub>TP</sub> is 0.032 µg/ml for *C. trachomatis* and 0.5 µg/ml for *C. suis*).

*mydia*. For *C. pneumoniae* strains, the MCC3s were found to reach those seen in McCoy cells when HEp-2 cells were used.

**Antimicrobial susceptibility testing of clinical isolates of *C. trachomatis*.** Susceptibility testing of the different clinical isolates of *C. trachomatis* showed only slight variation (by one twofold dilution) of the doxycycline, azithromycin, and ofloxacin MICs (Table 4). There was no apparent difference in the MIC, MCC, or MCC3 when strains from individuals with treatment failure or persistence were compared with those from persons with single-episode isolates.

## DISCUSSION

Cell culture systems using immunofluorescence staining to identify chlamydial inclusions are the most common methodology employed in antimicrobial susceptibility testing of *Chlamydia*. Such systems are not completely physiologic in that during natural infection, chlamydiae are usually exposed to antimicrobials long after the establishment of intracellular infection and the induction of a host inflammatory response. In contrast, with the *in vitro* systems, many investigators add antimicrobials simultaneously with or shortly after the infection (28). The failure to detect infectious chlamydiae in cell cultures *in vitro* does not exclude a viable state that could recur after antibiotics are removed (3). Dreses-Werringloer et al. used a reverse transcriptase PCR (RT-PCR) assay to demonstrate the presence of primary 16S rRNA transcripts in chlamydia-infected cells treated with 0.5 µg of azithromycin per ml over a period of 16 days (12). These results support our finding of survival of small numbers of organisms at concentrations well above the MIC for all antimicrobials tested. Hence, it is possible that *in vivo*, chlamydial organisms could survive in patients treated with a single dose of azithromycin despite being susceptible *in vitro*.

However, *in vitro* culture systems do not allow for the role of immune clearance mechanisms in a host. For example, Rasmussen et al. demonstrated that despite the effect of antimicrobials on chlamydia growth and development, the infected cells continued to be recognized and killed by cytotoxic T cells (24). The importance of the host response is further supported by findings by Parks et al. demonstrating possible spontaneous clearance of chlamydial infection in a minority of untreated patients. Resolution of infection was associated with increasing duration of clinical follow-up (22). Hence, heterotypic survival of small numbers of infectious elementary bodies may occur *in*

*vitro*, but in natural infections, host factors may eradicate the remaining surviving chlamydial organisms.

We found that when *Chlamydia* isolates were tested in the presence of tetracycline, doxycycline, and ofloxacin, antimicrobial susceptibilities were comparable with all cell lines utilized. However, the MICs and MCCs varied significantly by cell line when tested with azithromycin and erythromycin, particularly being increased in BGMK cells. Many laboratory conditions, such as pH, temperature, nutrients present in the media, polarity of the cell type infected, and secretion of cytokines by infected cells, likely differ from natural infection and may affect the ability of a particular antimicrobial to penetrate intracellularly and exert its action (13, 16, 25, 26, 29, 30). Rota demonstrated that McCoy cells in media with a high glucose concentration, a pH closer to neutrality, and a higher temperature during centrifugation (33 to 35°C being more effective than 20 to 25°C) produced higher numbers of *C. trachomatis* inclusions upon infection (26). These conditions could be more variable *in vivo*, and therefore the *in vitro* MIC may not necessarily correlate with that *in vivo*. Wyrick et al. reported that use of polarized host cells, rather than nonpolarized cells such as laboratory-adapted HeLa or McCoy cell monolayers, resulted in more efficient transport and internal concentration of azithromycin within host cells and that *C. trachomatis* appeared more susceptible to the lethal action of azithromycin in polarized cells (29). They postulated that basolateral uptake of antibiotics was compromised when cells were cultured *in vitro* on nonporous surfaces, compared with internalization of antibiotics *in vivo* by mucosal epithelial cells via basolateral membranes. The polarity of the various cell lines tested with azithromycin may have influenced its uptake, which may explain the differences in azithromycin MICs in different cell lines. Thus, the type of cell line used influences the MIC. In addition, extensive quality control and monitoring of a given cell line is necessary to achieve optimal sensitivity.

Additional variables that may influence results of susceptibility testing in *Chlamydia*, but which have been incompletely studied, include the inoculum size, the interval between the establishment of infection and the administration of an antimicrobial, and the timing of antimicrobial removal (21). We observed no differences in MICs when antimicrobials were added from 0 to 8 h after infection, while the MIC increased steadily after 8 h. Notomi et al. reported that the addition of ofloxacin or sparflaxacin even at concentrations up to 64 µg/ml

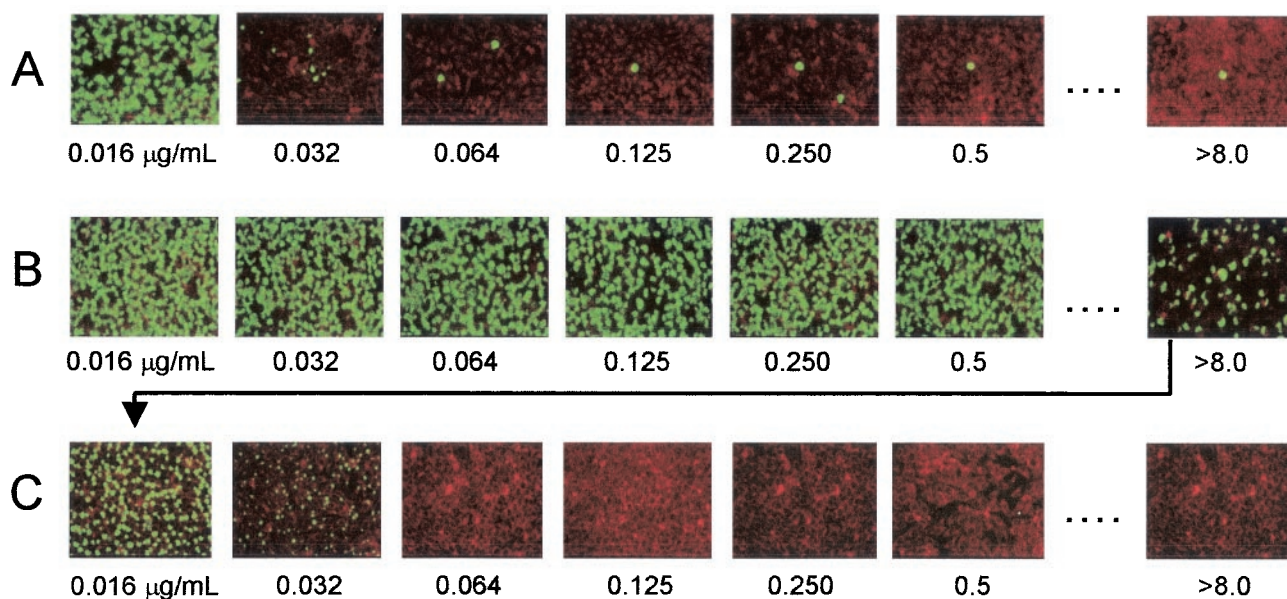


FIG. 2. Sequential photomicrographs of growth *C. trachomatis* serovar D at three different passage levels after initial exposure to given concentrations of doxycycline. (A) Illustration of MCC with heterotypic survival at concentrations well above the MICs after one passage in antimicrobial-free medium. (B) Illustration of MCC3 with complete growth of surviving chlamydiae after three passages in antimicrobial-free medium. (C) Illustration of the MIC pattern seen after retesting chlamydiae from the highest drug concentration in which organisms survived (arrow).

could not inhibit the formation of *C. trachomatis* inclusions when added 20 h after the start of infection (21). These findings suggest that addition of an antimicrobial after a certain time interval following infection may lessen the effect of the antimicrobial on chlamydial development, but more studies are needed to clarify this time interval for various antimicrobials.

A critical methodological aspect of susceptibility testing in *Chlamydia* is the endpoint utilized for defining the MIC, since distinguishing aberrant from normal inclusions can be tedious, and subjective interpretation may lead to severalfold variation in the MICs. At the MIC<sub>TP</sub>, there is a dramatic alteration in the morphology and size of the inclusions, leading to nearly every inclusion being morphologically aberrant. At further serial dilutions, occasional inclusions are seen and it becomes

difficult to determine what can be considered an inclusion of typical morphology. Hence, the MIC<sub>TP</sub> provides a consistent end point where almost all chlamydial inclusions are inhibited, and one twofold concentration above the MIC<sub>TP</sub> would seem to provide a reasonable standardized endpoint to define the MIC. By using this definition, different groups performing antimicrobial susceptibility testing of *Chlamydia* organisms would achieve reproducible and comparable MIC results. While small numbers of chlamydiae survive at concentrations well above the MIC, the use of these organisms to define the MIC is problematic. It is likely that differences in interpretation of inclusion morphology at higher antimicrobial concentrations may explain the differences in MICs that can arise when the same isolates are tested by different laboratories. This, for

TABLE 4. Antimicrobial susceptibilities of clinical isolates of *Chlamydia trachomatis*<sup>a</sup>

<i>C. trachomatis</i> clinical isolate(s)	Doxycycline				Azithromycin				Ofloxacin			
	Microtiter		Shell vial		Microtiter		Shell vial		Microtiter		Shell vial	
	MIC	MCC	MCC	MCC3	MIC	MCC	MCC	MCC3	MIC	MCC	MCC	MCC3
Single isolates ( <i>n</i> = 30)	0.064–0.125	0.125–0.250	>8	16–64	0.125–0.250	0.25–0.50	>8	64	0.5–1.0	1–2	>8	>128
Treatment failure isolates ( <i>n</i> = 6)	0.064–0.125	0.125–0.250	>8	16–64	0.125–0.250	0.25–0.50	>8	64	0.5–1.0	1–2	>8	>128
Recurrent same-serovar isolates ( <i>n</i> = 6)	0.064–0.125	0.125–0.250	>8	16–64	0.125–0.250	0.25–0.50	>8	64	0.5–1.0	1–2	>8	>128
CDC strains												
Treatment failures ( <i>n</i> = 2) <sup>b</sup>	0.064–0.125	0.125–0.250	>8	16–64	0.125–0.250	0.25–0.50	>8	64	0.5–1.0	1–2	>8	>128
Sensitive controls ( <i>n</i> = 2) <sup>c</sup>	0.064–0.125	0.125–0.250	>8	16–64	0.125–0.250	0.25–0.50	>8	64	0.5–1.0	1–2	>8	>128

<sup>a</sup> All values are in micrograms per milliliter. The MIC was defined as one twofold dilution more than the MIC<sub>TP</sub>; MCC, minimal chlamydical concentration following one freeze-thaw passage in microtiter plates; MCC, minimal chlamydical concentration following one passage in shell vials; MCC3, minimal chlamydical concentration following three passages in shell vials.

<sup>b</sup> Strains from two treatment failures provided by the CDC and determined at CDC laboratories to be multidrug resistant.

<sup>c</sup> Strains from two controls provided by the CDC and determined at CDC laboratories to be sensitive isolates.

example, may explain why our MIC results on the *C. trachomatis* strains obtained from the Centers for Disease Control differ from the Centers' findings. Many other research groups have demonstrated their reproducibility of determining the MIC<sub>TP</sub> (5, 8, 11, 18, 27, 29).

Recently, an RT-PCR-based method for antimicrobial susceptibility testing in *C. trachomatis* has been described which may be more sensitive and specific than standard cell culture methods, since it has the ability to detect viable chlamydiae that otherwise may be unrecognizable upon immunofluorescence staining of chlamydial inclusions (8). Cross et al. reported RT-PCR to be consistently more sensitive than conventional cell culture methods and demonstrated MICs that were 1.6-fold higher for erythromycin by this method than by conventional methods (8). RT-PCR may certainly be a useful method to validate the results of *Chlamydia* susceptibility tests that utilize cell culture methods; further studies of this type are needed.

Relatively few reports have described antimicrobial resistance in clinical isolates of *C. trachomatis*. One explanation for the apparent lack of resistance in *C. trachomatis* may be the organism's unique developmental cycle. Since gene replication occurs isolated within an intracellular inclusion in an infected epithelial cell, acquisition of antibiotic resistance genes from other organisms would be difficult (28). However, it is plausible that point mutations leading to antimicrobial resistance may occur under selective pressure from exposure to subinhibitory concentrations of antimicrobials. Selection for antimicrobial resistance has been clearly demonstrated in the laboratory by serial passage of *C. trachomatis* strains in subinhibitory concentrations of sulfonamides, penicillins, rifampin, and fluoroquinolones (10, 17). Dessus-Babus et al. exposed L2 strains of *C. trachomatis* to subinhibitory concentrations of ofloxacin (0.5 µg/ml) and sparflaxacin (0.015 µg/ml) and after four passages isolated two mutant strains that demonstrated high-level resistance to various fluoroquinolones, particularly sparflaxacin (1,000-fold increase in the MIC) (10). The MICs of doxycycline and erythromycin for the mutant strains were identical to those for the reference strain. A point mutation was found in the *gyrA* quinolone resistance-determining region of both mutant strains. Similar results were recently reported by Morrissey and coworkers (20). The ability to select for antimicrobial resistance in the laboratory by exposure to subinhibitory antimicrobial concentrations suggests that a similar mechanism could lead to the development of resistant chlamydial organisms in humans (or animals) exposed to antimicrobial therapy.

Despite selection for antimicrobial resistance to *C. trachomatis* in the laboratory, it is unclear whether continuous exposure to antimicrobials promotes resistance in vivo. The finding of antimicrobial resistance in swine *C. suis* isolates supports this possibility. Andersen and Rogers reported stable high-level resistance to tetracycline (MIC, ≥5 µg/ml) in eight swine *C. suis* isolates that had undergone 10 to 15 passages in tetracycline-free medium before testing (1). Since tetracycline congeners were used as swine feed additives, it seems plausible that the selective pressure of continuous exposure to this antimicrobial may have selected for more resistant swine *C. trachomatis* strains.

Relatively few clinical isolates of *C. trachomatis* with antimicrobial resistance have been described. In 1990, Jones et al.

reported five patients who were infected with *C. trachomatis* isolates that were resistant (MIC, ≥8 µg/ml) to tetracycline, doxycycline, and erythromycin but were sensitive to ofloxacin (15). Resistance was demonstrated only when a relatively large inoculum was used, and <1% of a population of organisms showed high-level resistance. Further, the fully resistant phenotypes either died or lost their resistance on serial passage in antibiotic-free medium. Treatment failure was suspected in four of the five patients. In 1997, Lefèvre et al. reported a single *C. trachomatis* strain that was isolated from a symptomatic patient following tetracycline therapy as having resistance to tetracycline (MIC and MCC, >64 µg/ml) (18). Similar to the report by Jones et al. (15), fewer than 1% of organisms demonstrated high-level resistance. In 2000, Somani et al. reported three patients who were infected with strains of *C. trachomatis* that exhibited resistance (MCC, >4 µg/ml) to doxycycline, ofloxacin, and azithromycin (27). A heterotypic pattern of resistance, with only a small population of organisms demonstrating resistance, was also noted, similar to the reports of Jones et al. (15) and Lefèvre et al. (18). Two of the three patients had clinical evidence of treatment failure, and the third patient was the wife of one of the treatment failures. In the 44 clinical isolates tested in our study, survivability of chlamydial organisms in high concentrations of antimicrobials was seen in all isolates tested and hence did not correlate with their apparent clinical outcome (i.e., success or failure of therapy). It is possible that this phenomenon, i.e., survivability of small numbers of chlamydial organisms in the presence of high levels of antimicrobials, has evolved due to selective pressure from frequent exposure to antimicrobials, or this may be an innate characteristic that is only now being detected due to heightened sensitivity of tissue culture methods.

In conclusion, susceptibility testing for *Chlamydia* requires careful attention to conditions which may affect both the ability of chlamydial organisms to infect cells in culture and the efficacy of a given antimicrobial through mechanisms such as intracellular uptake. Since MICs and MCCs varied by cell line when tested against azithromycin and erythromycin, one cell line should be consistently used for *Chlamydia* susceptibility testing. Our study suggests that using McCoy cells for susceptibility testing for *C. trachomatis* and HEp-2 cells for *C. pneumoniae* testing offers the most reliable and consistent results. Since a common endpoint is needed for defining the MIC, we suggest defining the MIC as the concentration of antimicrobial that is one twofold concentration more than the MIC<sub>TP</sub>. Survivability of chlamydial organisms at antimicrobial concentrations well above the MIC, heterotypic survival, appears to be a common phenomenon, but studies with larger numbers of patients with treatment failures or same-serovar recurrences are needed to meaningfully assess the correlation of heterotypic survival with clinical outcome. To our knowledge, homotypic resistance, as demonstrated by the tetracycline-resistant *C. suis* strain, has not been detected in any human *Chlamydia* species.

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