Chlamydia trachomatis Laboratory Strains vs. Recent Clinical Isolates:

Implications for Routine Microbicide Testing

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ABSTRACT

A topical microbicide that women can use to prevent sexually transmitted diseases (STDs) is essential and many microbicide candidates are being tested for activity against HIV and other STDs, including *Chlamydia trachomatis*. Screening assays for assessing the activity of microbicides against *C. trachomatis* are typically done with laboratory adapted strains, but it is possible that recent clinical isolates may have different susceptibilities to microbicides, as has been seen with *Neisseria gonorrhoeae* and *Lactobacillus* (17). We utilized three types of microbicides to help define this aspect of our assay to test microbicides against *C. trachomatis in-vitro*. To simulate conditions of transmission, we used an assay that we previously developed in which we exposed chlamydial elementary bodies (EBs) to microbicides prior to contact with epithelial cells. We first determined the toxicity of microbicides to the cells used to culture *Chlamydia* in the assay, and if necessary, modified the assay to eliminate toxicity at the concentrations tested. We compared sensitivities of recent clinical isolates of *Chlamydia* vs. laboratory strains of the same serovar, and found major differences in sensitivity to non-9, but only minor differences were seen with the other microbicides. We thus conclude that when assessing activity of potential topical microbicides vs. the obligate intracellular bacteria *C. trachomatis*, using recent clinical isolates may not be necessary to draw a conclusion about a microbicide’s effectiveness. However, it is important to keep in mind that differences (like those seen with non-9) are possible and clinical isolates could be included in later stages of testing.
INTRODUCTION

Topical microbicides are a promising method for prevention of sexually transmitted disease (STD) in women who are not able to persuade their partners to use condoms. Interest in the antimicrobial capabilities of spermicides began with products containing Nonoxynol-9 (non-9), a non-ionic detergent that is the active ingredient in many existing spermicides. Non-9 has been shown to inhibit HIV (12), Chlamydia trachomatis (2, 5, 14, 20), Neisseria gonorrhoeae (23), HSV-2 (3, 24), and Trichomonas vaginalis (22) both in-vitro and in-vivo. Its potential value as a microbicide is controversial, however, because results of testing have not been consistent and because it is toxic to cells used in in-vitro testing (1, 7, 10, 13, 15). It can increase susceptibility to HPV in mice (21) and with frequent use, non-9 appears to actually increase a person’s chance of contracting HIV because of the epithelial lesions it causes (8, 26). For these reasons, attention has recently turned from surfactants (like non-9) to other compounds, such as lipids and peptides, as possible topical microbicide candidates (11, 16, 18, 19, 27, 28).

The ideal topical microbicide would prevent multiple STDs, but the experiments reported here have focused on C. trachomatis specifically because it is the most commonly reported STD in the United States. In 2006, more than one million new Chlamydia infections were reported to the CDC, which is almost three times the number of new cases of Neisseria gonorrhoeae reported in the same year (6). Many Chlamydia infections are asymptomatic and not reported, but if left untreated, can cause infertility, ectopic pregnancy, and pelvic inflammatory disease (6). Further, C. trachomatis is an obligate intracellular parasite with a unique bi-phasic developmental cycle. The
infectious form, the elementary body (EB), is found in genital secretions and would be exposed to microbicides during transmission. Topical microbicides are intended to prevent infection, and thus we previously developed the Minimum Cidal Concentration (MCC) assay, which focused on microbicide action against the extracellular EBs (16).

This assay mimics what would happen in the human vagina during exposure to C. trachomatis because EBs would come in contact with the microbicide before reaching the target cells. Whichever microbicide is being tested, careful consideration must be given to the design of the in-vitro assay used in order to produce reliable results that are relevant to infection in humans.

It is difficult and time-consuming to grow high titers of C. trachomatis in the laboratory. Clinical isolates are particularly difficult to propagate because they have not yet adapted to growth in-vitro and most research facilities do not have easy access to patient samples. For this reason, most anti-chlamydial testing is done with strains that have been passaged for decades in the laboratory and have adapted to in vitro growth. Although convenient, we hypothesized that such laboratory passaged strains may not be the most relevant model of clinical infections. Thus, in these experiments we tested whether recent clinical isolates of C. trachomatis had different sensitivities to microbicides than laboratory adapted strains of the same serovars.

MATERIALS AND METHODS

Cell Culture. McCoy mouse fibroblast cells (ATCC CRL 1696) were maintained in antibiotic free Eagles’ minimal essential medium supplemented with 10% fetal calf serum, 0.017M glucose, 0.02M HEPES, 0.084% sodium bicarbonate, and 2mM L-
glutamine (CMGH). The McCoy cells were tested once per month for mycoplasma contamination by PCR.

**Inoculum.** Laboratory prototype strains of *C. trachomatis* serovars D(UW-3/Cx), E(UW-5/Cx), F(UW-6/Cx), J(UW-36/Cx), and L2(434/Bu), and recent clinical isolates of the same serovars were purified from McCoy cells as previously described (4). Clinical strains used were provided by the *Chlamydia* clinical laboratory at the University of Washington and they were passaged as few times as possible in order to obtain the high titers required for testing. We were able to achieve the appropriate titers for all clinical isolates in less than or equal to 17 passages in our laboratory. The laboratory strains have all been passaged hundreds or thousands of times over decades in the laboratory. Table 1 describes the passage number(s) of each isolate and which compound(s) they were tested against. Immediately before use, purified organisms were thawed and diluted to the appropriate concentration in SPG. All purified chlamydial isolates were tested for mycoplasma contamination by PCR. The serotype of each isolate was confirmed with a plate typing method (25).

**Microbicides.** Pure nonoxynol-9 (Spectrum #N1217, lot# PSO145) was donated to us by Dr. Lisa Rohan (Magee Women’s institute, Pittsburgh, PA) and stored at room temperature. Novispirin G10 (G10) and SMAP-29 peptides were synthesized and provided to us by Dr. Robert Lehrer (UCLA school of medicine, Los Angeles, CA). All peptides were re-hydrated in sterile water, aliquoted, and stored at -20°C until use. 1-O-octyl-sn-glycerol (1-OG) lipid was synthesized and provided to us by Dr. Charles Isaacs (University of Pittsburgh, PA) reconstituted in 100% ethanol and stored at 4°C until use. Surgilube, a sterile surgical lubricant gel containing 0.25% chlorhexidine, was purchased...
from E Fougera & CO (a division of Altana Inc., Melville, New York 11747) and stored at room temperature until use.

**Controls.** Penicillin G (Sigma 61K1045) and polymyxin B (Sigma P4932) were used as negative and positive inhibition controls respectively in the MCC assay. An inoculum control, in which no drug was added, was included for each *C. trachomatis* strain at each time-point to monitor normal inclusion formation. Percent inhibition of inclusion formation was calculated using this inoculum control. A cell control (no drug, no inoculum) was included in order to monitor McCoy cell morphology and possible cross-contamination. SPG and 10% [vol/vol] triton X-100 (Sigma T8787) were used as negative and positive toxicity controls respectively in the Alamar Blue™ cytotoxicity assays (see below).

**Minimum Cidal Concentration (MCC) Assay.** We used our previously published pre-inoculation assay (16) to test the anti-chlamydial activity of the microbicides. The day prior to the assay, 96-well TC plates were seeded with 5 X 10⁴ mycoplasma free McCoy cells in 0.2ml CMGH per well. Plates were incubated at 37°C in 5% CO₂ overnight. On the day of the assay, between five and ten two-fold dilutions of each test compound (starting at a concentration of 50% [vol/vol] for surgilube, 200µM for G10 and SMAP-29 peptides, 50mM for 1-OG lipid, 1.44mM for polymyxin B, and 5.37mM for penicillin G) were made in SPG. The remainder of the assay was performed as previously published. At the conclusion of the assay, McCoy cell monolayers were fixed with methanol and stained with a primary antibody to *C. trachomatis* LPS, E6-H1 (provided by Harlan Caldwell). Secondary staining was done with an anti-mouse IgG FITC conjugated antibody (Sigma F-9006) diluted 1:250 in Evans Blue counter stain.
(0.5% Evans Blue, 5% sodium azide, 94.5% phosphate buffered saline). Each concentration was plated in triplicate, three fields per well were counted, and the inclusion forming unit (IFU) counts between replicates were averaged. Percent inhibition of inclusion formation was calculated with the following formula: 
\[
\text{percent inhibition of } C. \text{ trachomatis IFU formation} = \left( \frac{(\text{average IFU in the inoculum control}) - (\text{average IFU in the test})}{(\text{average IFU in the inoculum control})} \right) \times 100
\]
Assays were performed twice, on different days. The results of the two independent assays were averaged and the standard deviations between the two assays at each concentration were calculated. Results were reported as percent inhibition of \textit{C. trachomatis} inclusion formation compared to a no drug control. The MCC was defined as the lowest concentration of test compound that completely inhibited \textit{C. trachomatis} inclusion formation.

**Minimum Cidal Concentration (MCC) Assay adapted for Non-9.** We slightly modified our previously published pre-inoculation assay (16) to adjust for the cytotoxicity of non-9 as follows. McCoy cell 96-well plates were set up as described in the previous section. On the day of the assay, ten four-fold dilutions of non-9 (starting at a concentration of 2% [vol/vol]), polymyxin B (starting at a concentration of 1.44mM), and penicillin G (starting at a concentration of 5.37mM) were made in SPG. \textit{C. trachomatis} inoculum was added to the samples in the same manner as before except at a higher concentration (1 X 10^7 IFU). After incubation at room temperature for 120 minutes, the reaction mixture was diluted 1:1600 in SPG to effectively eliminate the non-9. The remainder of the assay was performed as described previously except that 0.2ml of the inoculum was added to each McCoy cell monolayer instead of 0.1ml.
**Alamar Blue™ cytotoxicity assay.** Prior to the MCC assay, we measured the cytotoxicity of the dilutions of all test microbicides to McCoy cells in the MCC assay. The MCC assay procedure, as described above, was followed except that no inoculum was added to the test compounds. The compounds were instead diluted by half with SPG to imitate the dilution when an inoculum was added. After incubating cells at 37°C for 48 hours, the CMGH in each well was replaced with fresh CMGH containing 10% Alamar Blue™ reagent. Plates were incubated for an additional four hours, and cytotoxicity was determined spectrophotometrically according to the manufacturer’s instructions.

**RESULTS**

**Cytotoxicity of microbicides to McCoy cells as determined with the AlamarBlue™ assay.** In our standard MCC assay, all compounds were exposed to EBs and then compound-organism mixtures were diluted 1:40 in SPG before addition to McCoy cells, resulting in exposure of cells to maximum concentrations of 0.625%, 2.5µM, and 0.625mM for surgilube, the peptides, and 1-OG respectively. In our toxicity-adapted MCC assay, EBs were exposed to maximum non-9 concentrations of 1% and non-9-organism mixtures were diluted 1:1600 before addition to McCoy cells, resulting in exposure of the cells to a maximum non-9 concentration of 0.000625%. We tested whether any residual microbicide in the dilution was toxic to McCoy cells used to culture viable organisms in this assay system. None of the concentrations of the microbicides were toxic to McCoy cells in our assay conditions. Any reduction of *C. trachomatis*
inclusion formation was therefore due to the action of the tested microbicides on the chlamydial EBs, not the host cells.

**MCC of microbicides against *C. trachomatis* serovars D, E, F, J, and L2**

**Clinical isolates and laboratory prototype strains.** To determine whether laboratory strains of *C. trachomatis* had different sensitivities to treatment with surfactants (surgilube, non-9), peptides (G10, SMAP-29), and lipids (1-OG) than recently isolated clinical strains, we tested two or more of a variety of laboratory prototype serovars D, E, F, J, and L2 and at least one clinical isolate from each serovar in parallel. The serovars chosen are representative of the three *C. trachomatis* serological groups (9). For surgilube and 1-OG, we tested serovars J and D clinical and laboratory strains. For G10 and SMAP-29, we compared the sensitivity of clinical and laboratory strains of serovars D and E. We tested non-9 against serovars D, E, F, J, and L2 clinical and laboratory strains. Two different clinical isolates were included in the non-9 assays against serovars D, E, and J. In each assay, we included Polymyxin B as an active control and Penicillin G as a minimally active control. Both of these control compounds behaved as expected in all assays.

1-OG was 100% active for concentrations greater than or equal to 3.13mM and then activity rapidly dropped below 50% inhibition (figure 1). The MCC for 1-OG was statistically the same between clinical and prototype strains of serovars J and D (3.13mM). However, at concentrations below 3.13mM, there was a statistically significant difference between clinical and prototype activity for serovar J, with the prototype strain being more sensitive to the lipid than the clinical strain (figure 1).
Prototype D was also significantly more sensitive than clinical D to 1-OG at 1.56mM (data not shown).

The G10 and SMAP-29 peptides showed more of a classical dose response curve; activity was high for the higher test concentrations and then gradually fell off as the dilutions decreased in concentration (figures 2-5). G10 and SMAP-29 activity was similar against serovars D and E, but G10 was slightly more active. There were very few differences when comparing sensitivities of clinical and prototype strains to both peptides.

Surgilube was very active (>75% inhibition) against serovars D and J at concentrations ≥ 3.13% after a 120 minute exposure. The only differences between clinical and prototype isolates of the same serovar were seen with serovar J after exposure to 1.56%, 0.78%, and 0.39% surgilube, when the prototype isolate was statistically significantly more sensitive to treatment (figure 6).

However, we observed marked differences between the clinical and prototype isolates of the same serovar in some but not all serovars when tested against another surfactant, non-9. For serovars D and J, the differences between clinical and prototype strains were large, highly significant, and present over a large range of concentrations, with the clinical strain showing much more resistance to the non-9 treatment (figures 7, 8). For example, when exposed to 0.25% non-9, the serovar D laboratory prototype strain infectivity was reduced by 79.1% (SD 5.8) when compared to the no-drug control, and one recent clinical isolate of serovar D (designation 9427) was inhibited 63.1% (SD 8.2) (figure 7). However, a second clinical isolate (designation 9939) was only inhibited by 21.4% (SD 11.4) when exposed to the same concentration of non-9. When exposed to
0.0625% non-9, *C. trachomatis* serovar J prototype strain infectivity was reduced by 94.2% (SD 2.2), whereas a recently isolated clinical strain of the same serovar (designation 1178) was only reduced by 16.4% (SD 3.9) (figure 8). A second J clinical isolate (designation 9379) was inhibited by 16.8% (SD 5.9) when exposed to the same concentration of non-9. The clinical isolates were not always less sensitive to non-9 than the laboratory strains. Prototype F was found to be statistically significantly more resistant to the non-9 treatment than a clinical F strain at some concentrations, though the difference was not as large as seen in serovars J and D (figure 9). The clinical and laboratory prototype serovar L2 had no significant difference in sensitivity to non-9 (data not shown). The clinical and laboratory prototype E strains, had minor differences in susceptibility at some of the lower concentrations, but in general, the activity was the same (data not shown).

**DISCUSSION**

It is challenging to test microbicidal activity against *C. trachomatis* in the laboratory because many compounds are toxic to the McCoy cells used to grow the test organisms. Since *C. trachomatis* is an obligate intracellular parasite, measuring the health of host cells is crucial in determining the number of surviving EBs and test concentrations must thus be limited to those which will produce reliable data. In our MCC pre-inoculation assay, the compound is not required to enter the host cells, which makes it the ideal assay for testing compounds whose ultimate purpose will be to act on chlamydial EBs before they infect host cells. This assay is versatile and can be employed
to test numerous chlamydial strains against multiple concentrations of compound at any
reasonable length of exposure.

Using our MCC assay, we found major differences between *C. trachomatis*
laboratory prototype strains and recent clinical isolates of the same serovar in
susceptibility to non-9. We also found minor differences between prototype and clinical
strains after exposure to some concentrations of another surfactant (surgilube), to two
peptides, and to a lipid, although these differences were not as dramatic as with non-9.
The mode of action of a surfactant (like non-9) is on the lipid component of the outer
membrane. Our results with non-9 suggest that the outer surface of prototype strains may
be different from that of clinical isolates. The membrane components of prototype strains
could have been altered during passages in the laboratory, resulting in a change in
sensitivity to non-9. An alternative explanation is that the clinical isolates may have
altered membrane components in order to survive repeated exposure to non-9 in the
environment. The latter explanation may be more plausible because surgilube, another
surfactant, did not affect clinical and prototype isolates in the same way. Surgilube has
been used in the medical environment for a long time, but it is used as a sterile surgical
lubricant, not as a method of birth control. This hypothesis is strengthened by the results
of Hillier and Moncla (2005), showing that *N. gonorrhoeae* and *Lactobacillus* laboratory
and clinical strains have varying susceptibility to non-9, indicating development of
resistance (17).

In our experiments, when exposed to varying concentrations of microbicidal
compounds, recent clinical isolates of *C. trachomatis* did not have markedly different
sensitivities than laboratory adapted strains of the same serovar. The exception was non-
9 (0.000004% to 1% [vol/vol]); when EBs were exposed for 120 minutes, recent clinical isolates of *C. trachomatis* often behaved significantly differently than laboratory strains of the same serovar. Though more extensive testing is needed in order to draw a definitive conclusion, these results suggest that extensive testing of *C. trachomatis* clinical isolates may not be necessary when screening microbicides for anti-chlamydial activity, especially when testing those compounds which have not been present in the environment in another form (such as a spermicide). However, microbicides that have shown promising activity could be tested against clinical isolates to fully characterize the potency of the microbicide.

Testing of clinical isolates could potentially be used as an additional step between *in-vitro* screening with prototype strains and human or animal trials. Additional studies should be undertaken to determine whether clinical strains behave similarly when exposed to other microbicides. In summary, while it is true that some major differences were seen between some *C. trachomatis* clinical and prototype isolates’ susceptibility to non-9, major differences in susceptibility to other compounds have not yet been demonstrated. Testing topical microbicide candidates against clinical isolates as well as laboratory adapted strains could lead to a better understanding of the range of activity of the compounds *in vitro*, but it may not always result in drawing a different conclusion about the overall usefulness of a compound.

ACKNOWLEDGMENTS
The authors would like to thank Robert Suchland for generously providing the recent clinical isolates used in this study. We also thank Robert Lehrer for providing us with the novispirin G10 and SMAP-29 peptides, Charles Isaacs for providing the 1-O-octyl-sn-glycerol lipid, Lisa Rohan for providing the nonoxynol-9, and Harlan Caldwell for providing the E6-H1 anti-chlamydial LPS antibody.

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REFERENCES


Effectiveness of COL-1492, a nonoxynol-9 vaginal gel, on HIV-1 transmission in female sex workers: a randomized controlled trial. Lancet. 360:971-977.


FIGURE LEGENDS

Table 1. Designation, number of passages in the laboratory, and list of compounds tested against each C. trachomatis isolate used in the MCC assays.

Figure 1. The MCC pre-inoculation assay was used to compare the activity of 1-OG against C. trachomatis serovar J laboratory prototype strain and clinical isolate 9379 after 120 minutes of exposure. Two-fold dilutions of the negative and positive controls (penicillin G and polymyxin B respectively) were also run against the prototype J strain. The highest test concentrations of penicillin G and polymyxin B were 2.69mM and 0.72mM respectively. Percent inhibition of inclusion formation was calculated based on the number of inclusions in the no-drug control using the following formula: \([\frac{\text{mean IFU no-drug control} - \text{mean IFU test}}{\text{mean IFU no-drug control}}] \times 100\). Each test was performed twice, on different days, and the reported results are the average of those two tests. The standard deviations of the results are indicated with error bars.
Figure 2. The MCC pre-inoculation assay was used to compare the activity of G10 peptide against *C. trachomatis* serovar D laboratory prototype strain and clinical isolate 9939 after 120 minutes of exposure. All assay details described in the legend to Figure 1 were used.

Figure 3. The MCC pre-inoculation assay was used to compare the activity of G10 peptide against *C. trachomatis* serovar E laboratory prototype strain and clinical isolate 89 after 120 minutes of exposure. All assay details described in the legend to Figure 1 were used.

Figure 4. The MCC pre-inoculation assay was used to compare the activity of SMAP-29 peptide against *C. trachomatis* serovar D laboratory prototype strain and clinical isolate 9939 after 120 minutes of exposure. All assay details described in the legend to Figure 1 were used.

Figure 5. The MCC pre-inoculation assay was used to compare the activity of SMAP-29 peptide against *C. trachomatis* serovar E laboratory prototype strain and clinical isolate 89 after 120 minutes of exposure. All assay details described in the legend to Figure 1 were used.

Figure 6. The MCC pre-inoculation assay was used to compare the activity of surgilube against *C. trachomatis* serovar J laboratory prototype strain and clinical isolate 1178 after
120 minutes of exposure. All assay details described in the legend to Figure 1. were
used.

Figure 7. The MCC pre-inoculation assay was used to compare the activity of non-9
against *C. trachomatis* serovar D laboratory prototype strain and clinical isolates 9427
and 9939 after 120 minutes of exposure. All assay details described in the legend to
Figure 1. were used. No data is available for clinical isolate #1 against 1% non-9 due to
partial cytotoxicity.

Figure 8. The MCC pre-inoculation assay was used to compare the activity of non-9
against *C. trachomatis* serovar J laboratory prototype strain and clinical isolates 1178 and
9379 after 120 minutes of exposure. All assay details described in the legend to Figure 1.
were used. There is no standard deviation data available for isolate #2 against 1% non-9
due to partial cytotoxicity in one of the experiments.

Figure 9. The MCC pre-inoculation assay was used to compare the activity of non-9
against *C. trachomatis* serovar F laboratory prototype strain and clinical isolate 9397
after 120 minutes of exposure. All assay details described in the legend to Figure 1. were
used.
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Percent inhibition of C. trachomatis serovar D inclusion formation compared to a no drug control.

- **Non-9 Prototype D**
- **Non-9 Clinical D isolate #1 (9427)**
- **Non-9 Clinical D isolate #2 (9939)**
- **Polymyxin B Prototype D**
- **Penicillin G Prototype D**

**Percent Non-9**: 1.0000, 0.2500, 0.0625, 0.0156, 0.0039, 0.0010