Microtest Procedure for Isolation of Chlamydia trachomatis

BRUCE L. YODER,† WALTER E. STAMM,1,2* C. MARK KOESTER,1 AND E. RUSSELL ALEXANDER1†

Department of Epidemiology, School of Public Health and Community Medicine,1 and Department of Medicine, School of Medicine,2 University of Washington, Seattle, Washington 98104

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Management of patients potentially infected with Chlamydia trachomatis has been hampered by the cost and time required to perform Chlamydia cultures. To isolate C. trachomatis, we developed a microtiter method that exhibited equal sensitivity but less frequent contamination than our previously used vial-cover slip culture system. In addition, costs and technician time were substantially reduced with the microttest method. Subsequent studies showed that cycloheximide-treated cells were superior to 5-ido-2-deoxyuridine-treated cells in the microtest method and that a subpassage significantly enhanced the sensitivity of the method. The microttest method appears to be a sensitive, rapid, and economical method for isolating C. trachomatis.

Within the last 10 years, Chlamydia trachomatis has been implicated as an important causative agent in several syndromes whose etiologies were previously obscure: nongonococcal urethritis, epididymitis, acute proctitis, and Reiter's disease in men; cervicitis, urethral syndrome, salpingitis, bartholinitis, and perhaps postpartum endometritis in women; and inclusion conjunctivitis and a distinctive pneumonia syndrome in newborns (8). In addition, C. trachomatis infection may result in third-trimester abortion, fetal wastage, and cervical dysplasia. The frequency and potential sequelae of these infections indicate a clear need for routine diagnostic isolation of C. trachomatis (and perhaps eventually for screening of pregnant women), but most laboratories cannot process large numbers of specimens quickly and economically. Currently available isolation procedures generally represent modifications of the in vitro cell culture method introduced by Gordon et al. (2), which requires irradiation of tissue culture cells, or the subsequent method of Wentworth and Alexander, which uses 5-ido-2-deoxyuridine (IUDR)-treated cells (12). These methods utilize cell monolayers grown on cover slips in individual 1-dram (3.697-ml) vials.

To facilitate testing of numerous specimens, McComb and Puzniak evaluated a microisolation procedure by using irradiated baby hamster kidney cells on cover slips in microtiter plates (4). Infectivity titrations comparing this system with yolk sac inoculations showed the microsystem to be more sensitive, but the procedure has not been widely used. In 1977, Smith employed a similar microisolation procedure which used IUDR-treated McCoy cells (9), but found that only 3 of 43 previously positive urethral specimens stored frozen yielded positive results in the microtiter assay, whereas 29 of 43 were positive in a conventional vial assay. Owing to the increasing number of specimens submitted to our laboratory for C. trachomatis isolation, we reevaluated a microisolation method and found that it compared favorably with the vial procedure and was less expensive and time consuming.

MATERIALS AND METHODS

Cells and media. Mycoplasma-free McCoy heteroploid cells were grown in Eagle minimal essential medium supplemented with 6.6 mM sodium bicarbonate, 10% fetal calf serum, 2 mM glutamine, 12.5 U of nystatin per ml, 5 μg of gentamicin per ml (11), and 12.5 μg of vancomycin per ml (CMA medium). Cells were seeded in CMA with double concentrations of antibiotics and 0.4% additional glucose (CMGA medium) (2). Inoculated cells were overlaid with CMGA containing 20 mM N-2-hydroxyethylpiperezine-N'-2-ethanesulfonic acid (HEPES) buffer (pH 7.5; CMGA-HEPES medium) (13). Specimens were transported in 1.5 ml of 0.2 M sucrose-phosphate buffer, described by Gordon et al. (2), containing 25 U of nystatin per ml and 50 μg of streptomycin per ml (2SPA medium). Midway through the comparison of IUDR with cycloheximide, the streptomycin in 2SPA was replaced with 10 μg of gentamicin per ml and 25 μg vancomycin per ml.

Chemicals for cell treatment. IUDR (Stoxil; Smith, Kline and French Laboratories, Philadelphia, Pa.) was used at a concentration of 10 μg/ml in CMGA. Cycloheximide (Sigma Chemical Co., St. Louis, Mo.) was diluted to 1 mg/ml in CMA to prepare a stock
solution and was stored frozen at -20°C. It was further diluted in CMGA-HEPES to a working dilution of 1.5 µg/ml. The most effective cycloheximide concentration was determined by inoculating 10² inclusion-forming units per ml in 10-fold dilutions and then calculating endpoints (5).

Treatment of cells. Cells to be treated with IUdR were suspended in CMGA containing 10 µg of the drug per ml and were incubated at 35°C for 3 to 7 days before being used. Cells to be treated with cycloheximide after inoculation were overlaid with CMGA-HEPES containing 1.5 µg of the drug per ml.

Isolation technique. (i) Vials. Our method for isolating C. trachomatis by using 1-dram vials has been described in detail previously (2, 12). Briefly, 7-day-old McCoy cells were harvested with trypsin and seeded 1 ml per vial into 1-dram shell vials, each containing one cover slip (12-mm diameter), at a concentration of 1.5 x 10⁵/ml with 10 µg of IUdR per ml (13). Three- to seven-day-old monolayers pretreated for 30 min with 30 µg of diethylaminoethyl-dextran per ml (3) were inoculated with 0.2 ml of specimen material, centrifuged at 2,400 x g, and incubated at 35°C for 40 to 72 h.

(ii) Microtest. Seven-day-old McCoy cells were harvested with trypsin from 32-oz (0.946-liter) bottles and were suspended in CMGA at a final concentration of 3.0 x 10⁵ cells per ml. Plastic tissue culture plates containing 96 5-mm wells (Falcon 3040, Falcon Plastics, Oxnard, Calif.; Microtest II, Becton, Dickinson & Co., Cockeysville, Md.) were seeded with 0.2 ml of the cell suspension per well. After the plates were sealed with adhesive film (Falcon 3044), they were incubated for 2 to 7 days at 35°C in a dry-air incubator. Immediately before inoculation, all but a thin layer (ca. 0.05 ml) of the CMGA was aspirated from each of the wells to prevent the monolayer from drying, whereas the specimens to be inoculated thawed in a 37°C water bath. One-tenth milliliter of specimen material was inoculated per well. The inoculated plates were covered with plastic lids (Falcon 3041) and centrifuged at 1,100 x g and 30°C for 1 h, using carriers designed specifically for 96-well tissue culture plates (Dynatech Laboratories Inc., Alexandria, Va.). After centrifugation, the plates were incubated for 30 min at 35°C, and all but approximately 0.05 ml of the specimen material was aspirated. Each well was overlaid with 0.2 ml of CMGA-HEPES plus 1.5 µg of cycloheximide per ml, unless IUdR was used at the time the cells were seeded. The plates were sealed with adhesive film and incubated at 35°C for 40 to 72 h. To keep the exposure to the air minimal, the plates were kept covered with the plastic lids as much as possible during the procedure.

Staining of inclusions. Cover slips from inoculated vials and from each well of the microtest plates were fixed with alcohol-Formalin and stained with iodine by the method of Gordon et al. (2). Cover slips were examined at x400, and microtest wells were examined at x250 for inclusions. We found that a 5-mm cover slip (Belco Glass, Inc., Vineland, N. J.) placed in each well after being stained enhanced the appearance of the stained monolayers. Microtest plates were examined upside down on a specially designed microscope stage that accommodated the entire microtest plate (Scientific Instrument Division, Academic Support Services, University of Washington, Seattle).

Subpassage. Specimens to be subpassaged 40 to 72 h after inoculation were freeze-thawed immediately before subpassage. Cell material and media from the vials or microtest wells were aspirated and reincubated into the respective system (see above).

Source of specimens. Ninety percent of the specimens used in the microtest-vial comparison were urethral and cervical cultures from women and urethral cultures from men attending a sexually transmitted disease clinic. Many of these patients had complaints other than urethral or vaginal discharge, and they were cultured as part of a screening project. The remaining 10% of cultures were obtained from pregnant women seen in a prenatal clinic. Cervical specimens were collected with sterile type III plastic-shaft calcium alginate swabs (Inolex Corp., Park Forest South, Ill.), and urethral specimens were collected with type I aluminum-shaft calcium alginate swabs (Inolex). Swabs were placed immediately into 1.5 ml of 2SPA, frozen at -70°C within 15 min of collection, and transported to the laboratory on dry ice.

Statistical comparisons. Statistical comparisons were made by the Fisher exact test, the chi-square test with the Yates correction, or the McNemar test for comparison of matched-pair data.

RESULTS

Microtest versus vials. Of 892 clinical specimens inoculated in parallel with IUdR-treated cells, 131 (14.7%) in the vial system and 29 (3.3%) in the microtest system were unreadable owing to contamination or debris (P < 0.001). Of the readable specimens, 67 (8.8%) of 761 grew C. trachomatis in the vial system, compared with 75 (8.7%) of 863 in the microtest system. Of specimens that were readable in both systems (717 pairs), 66 (9.2%) were positive in the vial system, and 65 (9.1%) were positive in the microtest system. Of the 66 specimens positive in at least one system, 13 were positive only in the vial system, 12 were positive only in the microtest system, and 53 were positive in both.

Cycloheximide versus IUdR. With the microtest system, 445 clinical specimens from the cervix and urethra of women seen in a sexually transmitted disease clinic were inoculated onto McCoy cells treated with either IUdR or cycloheximide. Twenty-six (5.8%) of 445 cultures in the IUdR group and 17 (3.5%) of 445 in the cycloheximide group were unreadable owing to contamination or debris. Of the readable specimens, 28 (6.7%) of 419 cultured with IUdR and 35 (8.4%) of 428 cultured with cycloheximide grew C. trachomatis (P < 0.40). Of 415 specimen pairs uncontaminated in both systems, 28 (6.7%) were positive in IUdR-treated cells, compared
with 35 (8.4%) in the cycloheximide group ($P < 0.40$). No specimens were positive only in the IUdR group, whereas seven were positive only in the cycloheximide group ($P < 0.01$).

In the microtest method, chlamydial inclusions were more easily detected in cycloheximide-treated cells, because the cells overlaid with cycloheximide remained distinct from each other, and their inclusions were generally larger and brighter than those produced in IUdR-treated cells.

**Subpassage versus no subpassage.** The value of a subpassage in the microtest system was evaluated in 1,269 specimens inoculated in parallel into cycloheximide-treated McCoy cells. Forty-six (3.6%) of 1,269 cultures without a subpassage and 53 (4.2%) of 1,269 with a subpassage were unreadable owing to contamination or debris ($P < 0.20$). Of the 1,200 readable specimen pairs, 133 (11.1%) with a subpassage and 103 (8.6%) without a subpassage were positive. ($P < 0.01$). Forty-five (3.8%) specimens were positive only with a subpassage, and 15 (1.3%) were positive only without a subpassage ($P < 0.001$).

Specimens positive without a blind pass generally increased a log in titer after a subpassage. However, the quality of the monolayer decreased slightly after the subpassage, owing to additional cell debris.

**DISCUSSION**

Currently, the most common isolation procedure for *C. trachomatis* involves inoculating specimens in flat-bottom vials containing cover slips with an appropriate cell monlayer. Owing to the tedious task of preparing and handling each individual vial, four major disadvantages emerge when this system is used for processing large numbers of specimens: time, expense, space, and contamination. The microtiter isolation system has enabled us to triple the number of specimens processed per week and to decrease by at least one-half the time, expense, space requirements, and contamination rate. Our procedure replaces 96 vials, caps, and cover slips with one microtest plate and adhesive film cover, and it differs from the microsystems described by McComb and Puzniak (4) and Smith (9), which both used cells grown on 5-mm cover slips. We found that the most cumbersome step, handling the cover slips, could be eliminated by growing the cells directly on the plate surface, a procedure which greatly simplifies the staining and reading procedures.

The vial and microtest systems appeared comparable with respect to rates of isolation of *C. trachomatis* from clinical specimens, despite the fact that equipment and procedure changes necessary to implement the microtest system probably favored isolation in the vials. The centrifuge carriers for the plates, for example, are limited to a relative centrifugal force of 1,500 x $g$, and therefore they were spun at 1,100 x $g$, whereas the vials were spun at 2,400 x $g$ (10). The restricted volume of the microtiter wells also limits the inoculum to half that used in the vials and, during this study, pretreatment of cells with diethylaminoethyl-dextran (3, 7) was carried out in the vials but not in the plates.

A major concern in using the microtiter plates was the possibility of well-to-well cross-contamination and, thus, false-positive results. To evaluate this possibility, we inoculated *Escherichia coli* into every other well in plates containing nutrient broth and then carried out our usual isolation procedures. Every inoculated well supported bacterial growth, and uninoculated wells remained sterile. In addition, we placed higher positive and negative controls adjacent to each other on every plate during routine isolation, and we did not observe cross-contamination with *C. trachomatis*. In the vial-microtiter comparison, 53 of 66 positive results were found in both systems, and the percent positive only in the microtiter system was approximately the same as that found in the vials. Finally, we routinely inoculated each specimen on duplicate plates and found both the positive-negative pattern and the pattern of contaminated specimens to be generally consistent on each plate.

Ripa and March (6) as well as others (1) have reported that cycloheximide treatment of McCoy cells enhances the sensitivity for *Chlamydia* isolation. To determine the efficacy of cycloheximide in the microtest isolation system, we compared *C. trachomatis* recovery in cycloheximide-treated cells with IUdR-treated cells in vitro and found that in both vials and microtest plates, cycloheximide resulted in a more sensitive system, especially with a blind passage. Cycloheximide-treated cells also contained larger and brighter inclusions, which were easier to read. In clinical comparisons using the microtest system, cycloheximide treatment resulted in 25% more positive results than did IUdR treatment. Despite the fact that it requires extra time and effort, the blind passage procedures also resulted in a substantially increased yield in the microtest procedure, and we routinely use it in processing all specimens.

**LITERATURE CITED**


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