Micro Direct Inoculation Method for the Isolation and Identification of Chlamydia trachomatis

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Current standard procedures for the isolation and identification of Chlamydia trachomatis are laborious. A more rapid method for the isolation and identification of C. trachomatis has been developed. This method features the use of a multiwell microplate to which trypsinized McCoy cells and specimens are added simultaneously, followed by centrifugation. A simplified one-step iodine-glycerol staining procedure is used to detect the chlamydial inclusions. The requirements of the conventional macro method for preseeding cell monolayers and for the use of cumbersome cover slips in glass vials are eliminated. Studies on 345 clinical specimens showed that the micro method is as sensitive as the conventional method but can be performed more conveniently, rapidly, and economically.

Chlamydia trachomatis causes a wide variety of human diseases including trachoma, inclusion conjunctivitis, neonatal pneumonia, and genital tract infections such as male nongonococcal urethritis and female cervicitis (2, 8-12). Published reports state that as many as 40 to 70% of cases of nongonococcal urethritis are caused by C. trachomatis (5, 7, 8, 11, 13, 14). Therefore C. trachomatis rivals, or even exceeds, Neisseria gonorrhoeae in importance.

The current accepted methods of isolation of C. trachomatis are generally modifications of the in vitro cell culture method first introduced by Gordon et al. (4). The conventional procedure is laborious and cumbersome and requires a lengthy incubation period before and after inoculation of the McCoy cell monolayers, making it impractical for routine testing of large numbers of specimens (1).

To facilitate testing a larger number of specimens, some laboratories have adopted the use of microplates either with micro-cover slips (10, 15) or without cover slips (9, 17, 19). These modifications have simplified the techniques; however, they still require the preparation of cell monolayers 1 to 3 days before the inoculation of the specimens.

In this study we report the development and clinical evaluation of a micro direct inoculation (MDI) method in which the McCoy cells and specimens are added simultaneously to microtiter plates. The method is simple and rapid and obviates the need for preparing cell monolayers before inoculation of specimens.

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MATERIALS AND METHODS

Cultures. A total of 345 clinical specimens submitted by the Infectious Diseases Clinic and private physicians over a period of 1 year were examined with both the conventional macro (CM) and the MDI methods. One stock culture of C. trachomatis, obtained from the Central Public Health Laboratories in Toronto, was also used as a reference strain.

CM method. The CM method makes use of McCoy cells grown on cover glasses in glass vials, pretreatment of cells with iododeoxyuridine, and Giemsa staining of infected monolayers as described by Schachter (15).

MDI method. Three tissue culture media are required for the MDI method, namely, (i) wash medium for use in preparation of the cell suspension, (ii) inoculation medium, and (iii) postinoculation medium.

Media. The media used are variations of a single base medium prepared as follows. The base medium (for the MDI method) consists of the following: Eagle minimal essential medium, 500 ml; fetal calf serum, 50 ml; vitamins 100× (Hyland Co.), 5 ml; glutamine (200 mM; GIBCO Laboratories), 5 ml; streptomycin (5,000 µg/ml; Pfizer Inc.), 5 ml; vancomycin (10,000 µg/ml; Eli Lilly & Co.), 5 ml; amphotericin B (Fungizone; 3.125 mg/ml; Flow Laboratories, Inc.), 0.5 ml; glucose (21.2 g/200 ml of Eagle medium; Difco Laboratories), 25 ml; N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid buffer (1 M), pH 7.4 (Sigma Chemical Co.), 10 ml; 7.5% sodium bicarbonate (Fisher), 4 ml. The wash medium consists of base medium containing DEAE Dextran (Sigma) at 30 µg/ml (final concentration). The inoculation medium is the unmodified base medium. The postinoculation medium consists of the base medium containing cycloheximide (Aldrich Chemical Co.) at 1.0 µg/ml (final concentration).

Preparation of cell suspension and inoculation of specimens. Cells obtained from trypsinization of a McCoy cell monolayer were suspended in MDI wash medium and centrifuged three times. During the last wash step, the cell suspension was incubated for 1 h at room temperature to allow for the action of DEAE-dextran on the McCoy cells. The cell pellet after the last centrifugation was suspended in inoculation medium to obtain a cell suspension containing 600,000 cells per ml. Approximately 0.1 ml (4 drops from a Pasteur pipette) of the cell suspension was transferred to each well of a 96-well microtiter tissue culture plate. The 345 clinical specimens were tested over a period of 1 year, with about 12 specimens per run.

Clinical specimens were received as swabs in transport medium. They were stored at 4°C if testing was to be done within 24 h or were snap frozen and stored at −70°C if testing was to be delayed for more than 24 h. Specimens that were grossly contaminated with bacteria or erythrocytes were diluted 1:2 with inoculation medium before inoculation.

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Specimens were shaken on a Vortex mixer for about 5 s before inoculation. These specimens and positive and negative controls were tested in duplicate. Two drops of specimen were added to one well and mixed five times with the same Pasteur pipette, and one drop from the first well was transferred to a second well. This dilution step was found to be essential for specimens that were toxic to McCoy cells in the first wells. The microplates were then sealed with tape (Dynatech Laboratories, Inc.) and centrifuged for 1 h. After centrifugation the plates were allowed to remain in the centrifuge for 1 h or placed in a 37°C incubator. The fluid from each well was withdrawn with a Pasteur pipette and then replaced with 5 drops of postincubation medium. The microplate was then resealed with sealing tape (Flow; catalog no. 76-401-05) and incubated at 37°C for 72 h. Proper sealing of the plate is critical to maintain the pH at 7.0 during incubation. When precisely controlled CO₂ incubators are available, the pH can be maintained without sealing the plates.

After 72 h of incubation, the tissue culture fluid was removed with a Pasteur pipette, and the cells were fixed, stained, and preserved in a single step by the addition of 6 drops of a 1:1 mixture of 5% iodine in absolute methanol and glycerol. After a staining period of approximately 30 min, the iodine stain was withdrawn from the wells. The stained cells were allowed to stand at room temperature for at least 1 h before examination. This was shown to enhance the intensity of staining of the chlamydia inclusions. (The stock iodine stain consists of the following: potassium iodide, 5 g; iodine crystals, 5 g; absolute methanol, 100 ml. The working iodine stain is made by mixing equal volumes of glycerol and iodine stain.) The stained cells were examined with an inverted microscope (10× objective) using bright-field illumination. Chlamydia inclusions appear as brown-mahogany granular bodies and are typically in the cell cytoplasm, adjacent to the cell nucleus.

RESULTS

Agreement between the two methods was obtained for 52 positive and 287 negative specimens; 5 specimens were positive with the MDI method but negative using the CM method, whereas 1 specimen was positive in the CM method but negative by the MDI method.

Samples which gave discrepant results were repeated, where possible. Only three of the five samples which were positive by MDI and negative by CM had sufficient material for retesting. Identical results were obtained on retesting. The one sample which was negative by MDI and positive by CM was positive by the MDI method when the test was repeated on the diluted specimen.

The two methods were also compared from the standpoint of technical time required to test 48 specimens. A detailed comparison of the time required to test 48 samples in duplicate by each method is presented in Table 1. The MDI method required 3.75 h of actual work time to complete, as compared with the CM method, which required 16.75 h.

DISCUSSION

Some researchers have modified the conventional method for the isolation and laboratory identification of C. trachomatis by replacing vials with plastic microplates, with or without cover slips (10, 16, 19), but still preseeded the plates several days (10, 16, 19) or hours (3, 6) before inoculation of specimens. Since it is difficult to predict the number of specimens which will be received ahead of time, the requirement for preseeding monolayers is impractical. If more specimens arrive than anticipated, testing will be delayed until more cell monolayers are prepared, whereas if too few specimens are received, time and materials are wasted in preparing excess wells or vials.

We agree with Yoder et al. (19) that the use of a preseeded microtiter plate by itself will reduce the time and expense to perform this test when compared with preseeded cover slips in vials, since our original approach also used a 96-well microplate preseeded 24 h in advance. The MDI method, however, has a distinct advantage in that it eliminates the need to prepare monolayers in advance. The introduction of cycloheximide-treated cells as described by Ripa and Mardh (14) makes this approach possible. Hipp et al. (7) have also reported success in recovering C. trachomatis by inoculation of a McCoy cell suspension but by using the traditional procedure of cover slips in plastic tubes and cyclochalsin B-treated McCoy cells. In contrast to our findings, they described difficulty in recovering chlamydiae in cells treated with cycloheximide. This may have been a result of the particular cycloheximide used, since some batches have been shown to be toxic to McCoy cells. They also observed that bacteria present in clinical specimens had a more deleterious effect on McCoy cells which were in suspension compared with those in monolayers.

Our experience with the MDI method, which we have been using since 1980 for the examination of over 3,000 clinical specimens, has shown that the rate of bacterial and fungal contamination is not significantly different from that of the CM method, which we used for 2 years before. This may be due to our practice of diluting specimens from the first wells to the second wells to reduce toxicity and contamination. In addition, the formulation of the MDI medium, which contains high concentrations of vancomycin and streptomycin, may be a factor. We observed 5 of the 52 positive results in the second wells only, since the specimens were toxic to the McCoy cells in the undiluted samples in the first wells.

A further simplification of the procedure is the use of a one-step iodine staining procedure, which has eliminated the usual initial fixation or preservation step previously described (18). The modified staining procedure results in chlamydia inclusions which are dark brown and are easily distinguishable from colorless uninfected cells and pale-stained cell debris introduced by the specimen. The McCoy cell retain their "normal" morphology; the iodine-stained inclusions are visible within the cell cytoplasm but lack the

<table>
<thead>
<tr>
<th>Step</th>
<th>Time required (h)</th>
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<tbody>
<tr>
<td>CM method</td>
<td>MDI method</td>
</tr>
<tr>
<td>Prepare and sterilize vials with cover slip</td>
<td>1.5</td>
</tr>
<tr>
<td>Prepare and seed McCoy cells (1 to 3 days before specimen inoculation)</td>
<td>1</td>
</tr>
<tr>
<td>Specimen inoculation</td>
<td>2</td>
</tr>
<tr>
<td>Staining (fixation and stain)</td>
<td>3.25h</td>
</tr>
<tr>
<td>Mount cover slips</td>
<td>2</td>
</tr>
<tr>
<td>Microscopic examination</td>
<td>8h</td>
</tr>
<tr>
<td>Actual work time</td>
<td>16.75</td>
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</tbody>
</table>

* Includes time for trypsinizing, counting, and dispensing cell suspension on day of inoculation.
* Processing 48 specimens in duplicate (96 vials with cover slips for CM method and 96 wells in the microplate method).
“halo” effect described by others. The clear halo is likely an artifact which is produced by the harsh effect of fixation with absolute methanol, a step eliminated in the MDI staining procedure.

In comparing the CM and MDI methods as reported here, our results indicate that these two methods are equally sensitive in recovering C. trachomatis isolates from clinical specimens despite the fact that the microplate procedure appears to be less favored. The saving in time is not offset by a reduction in test sensitivity, which is in agreement with the report by Yoder et al. (19).

In conclusion, the MDI method is fast, sensitive, convenient, and economical for the isolation and identification of C. trachomatis and should prove to be a valuable diagnostic tool for some laboratories which have to process large numbers of specimens, thus improving the access to facilities for the culture of C. trachomatis.

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LITERATURE CITED