Molecular Techniques for the Detection of *Chlamydia trachomatis*

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A DNA probe assay (PACE; Gen-Probe, San Diego, Calif.) was compared with a culture reference method for the detection of *Chlamydia trachomatis*. Using stock isolates of each of the 15 serovars (A to K, Ba, L1, L2, and L3) of *C. trachomatis*, the lower limit of sensitivity for the DNA probe ranged between 1,086 inclusion-forming units (IFU) for serovar E (Bour) to 2,930 IFU for serovar L1 (440), with the only exception being serovar C (TW-3), with which 99 IFU was detected. There was no cross-reactivity with *Chlamydia psittaci* (Texas turkey) and *Chlamydia pneumoniae* (TWAR-183). Bacterial and fungal isolates representing 14 species of normal vaginal flora as well as *Neisseria gonorrhoeae* gave negative results with the DNA probe when tested at a level of 1.5 × 10^2 CFU/ml. In addition, the DNA probe, a direct fluorescent-antibody stain (DFA) (MicroTrak; Syva Corp., Palo Alto, Calif.), and an enzyme-linked immunosorbent assay (Chlamydiazyme; Abbott Laboratories, North Chicago, Ill.) were compared with culture for the detection of *C. trachomatis*, using 196 clinical cervical samples. Of the 196 samples, 20 (10%) were culture positive. Of the 176 culture-negative samples, 1 was not evaluated by DNA probe and 4, because of a lack of cellular material, were not evaluated by DFA. The sensitivities of the DNA probe, DFA, and enzyme-linked immunosorbent assay were 60, 75, and 85%, respectively, and specificities were 95, 99, and 97%, respectively. Of the false-positive direct results, there was only one specimen with which more than one direct method was positive, and with this specimen all three direct methods were positive. The majority of false-negative results by the direct methods were from specimens which by the culture method gave <100 IFU per culture.

*Chlamydia trachomatis* is one of the leading causes of sexually transmitted diseases (15). In addition to causing a localized urethritis or cervicitis, this organism has been implicated as a cause of salpingitis and as a contributing factor to infertility (2). Therefore, it is important to identify patients infected with this organism not only to reduce transmission but to minimize the risk of more serious infections and sequelae.

The "gold standard" for the laboratory detection of this organism is culture. However, because of the fastidious nature of the organism, there are several steps in the collection, transportation, and culture of this organism that may compromise viability (1, 10, 16). In addition, culture techniques on the average take 2 to 7 days for a final result. Therefore, in the past decade more rapid tests that use immunologic detection or DNA probe technology that does not rely on growth of *C. trachomatis* have been developed. Two of these methods, direct fluorescent-antibody staining (DFA) and enzyme-linked immunosorbent assay (ELISA), because of their commercial availability, have been widely compared with a reference culture (3, 5, 9, 12, 13). In the majority of studies that used an optimal culture system, neither assay was as sensitive as culture. DNA probes directed at the 7.4-kilobase plasmid in *C. trachomatis* have been used in research settings in solid-phase and in situ hybridizations and also have been found to be less sensitive than culture techniques with genital specimens (6, 7, 11). However, when used to detect *C. trachomatis* from eye specimens from trachoma cases, the plasmid probe appeared more sensitive than culture (17). As the authors of this work point out, this finding was with a very unique population, and results may not necessarily be able to be extrapolated to genital specimens or even specimens from cases of conjunctivitis. A nucleic acid hybridization assay which uses a nonisotopically labeled DNA probe to detect *C. trachomatis* rRNA in a liquid reaction mixture has recently become commercially available. In this study, the DNA probe assay was compared, along with DFA and ELISA, with culture for the detection of *C. trachomatis* in cervical samples. In addition, the sensitivity of the DNA probe assay to detect the 15 serovars of *C. trachomatis* was established.

**MATERIALS AND METHODS**

**Organisms.** The chlamydial isolates used in this study were raised in HeLa 229 and McCoy cells and frozen at −70°C in 0.2 M sucrose-0.02 M sodium phosphate (pH 7.2)–5 mM glutamic acid (2-SPG). The following *C. trachomatis* strains were used: L1 (440), L2 (434), L3 (404), A (G-17), B (HAR-36), Ba (Apache-2), C (TW-3), D (ICCal-8), E (Bour), F (UW-6), G (UW-57), H (UW-4), J (UW-36), and K (UW-31). *Chlamydia psittaci* (Texas turkey) and *Chlamydia pneumoniae* (TWAR-183) strains were also used.

Bacterial and fungal isolates were identified from clinical specimens submitted to the Medical Microbiology Laboratory at the University of California Irvine Medical Center. Organisms were identified by standard laboratory methods (8).

**Specimens.** Specimens in this study were obtained from female patients seen at the County of Orange Health Care Agency, Santa Ana, Calif. Upon cleansing the cervix, four swabs were taken and used for each of the four detection methods evaluated in this study (i.e., culture, probe, DFA, and ELISA). Cotton swabs (American Scientific Products, McGaw Park, Ill.) were used for the specimens for culture, and swabs supplied in the kits obtained from the manufacturer were used for the other assays. The order of culture,
DNA probe, DFA, and ELISA was rotated with every patient so that the swab taken first for one assay method from one patient became the last swab taken from the next patient. The specimens were transported at 4°C to the Department of Pathology at the University of California, Irvine, and were assayed within 48 h of collection.

**Tissue culture.** Specimens to be tested by tissue culture were stored and transported in 1 ml of 2-SPG containing amphotericin B (25 μg/ml) and gentamicin (50 μg/ml). Swabs were vortexed for 1 min, and 0.1 ml was used to inoculate McCoy cell monolayers in glass vials (15 by 45 mm) containing 12-mm-diameter cover slips. Vials were then centrifuged at 1,000 × g at room temperature for 1 h, after which 1 ml of Eagle minimum essential medium containing fetal calf serum (10%), gentamicin (50 μg/ml), and cycloheximide (1 μg/ml) was added, and the culture was then incubated at 37°C for 48 h. After incubation, the medium was discarded, and the cells were fixed for at least 10 min in methanol. The cover slip was removed and stained with a fluorescein-conjugated chlamydial monoclonal antibody (MicroTrak; Syva Corp., Palo Alto, Calif.) and read for chlamydial inclusions with a fluorescein microscope (Olympus) equipped with epillumination with a 100-W mercury vapor light source.

**DFA.** Specimens taken from the cervix were directly smeared onto a slide (8-mm wells) with a Dacron swab, fixed with acetone, and stained according to the instructions of the kit manufacturer. Slides were read with an Olympus fluorescence microscope and scored positive if more than 10 elementary or reticulate bodies were observed.

**Chlamydialysis.** Specimens tested by Chlamydialysis (Abbott Laboratories, North Chicago, Ill.) were collected, processed, and assayed according to the instructions of the manufacturer.

**Probe.** Upon collection, swabs were placed in transport medium (Gen-Probe, San Diego, Calif.), stored at 4°C, and processed according to the instructions of the manufacturer. Upon testing, specimens were allowed to reach room temperature and were vortexed for 5 s, and the swabs were discarded. Probe reagent (0.1 ml) was added to 0.1 ml of sample contained in assay tubes which were sealed and vortexed three times for 3 s each. The tubes were then incubated in a water bath at 1 h at 60°C. After the incubation, 2 ml of separation reagent was added to each tube. The tubes were sealed and vortexed by the same procedure and incubated again for 5 min at 60°C. The tubes were then placed on the rack of a magnetic separation unit for 2 min, the supernatants were decanted, and the tubes were blotted. Wash solution at 60°C (1 ml) was added to each tube, a sealing card was placed over the tubes, and the tubes were vortexed as before. The tubes were placed back into the magnetic separation unit for 2 min. The wash procedure was then repeated two more times. Elution reagent (0.3 ml) was added to each tube. The tubes were sealed, vortexed, and incubated for 5 min at 60°C. The sealing cards were removed, and disposable Pace-Mate Sleeves were inserted into all the tubes for 2 min. The sleeves were then withdrawn from the tubes and discarded. The sleeves were read on a luminometer (Leader 1; Gen-Probe), with the calculations based on the difference between the response in relative light units (RLU) of the specimen and the mean of the negative reference.

**Sensitivity and specificity of the probe.** The sensitivity of the probe was parallel tested with culture for the 15 serovars of *C. trachomatis*. For each serovar, McCoy monolayers were infected from frozen stocks in duplicate. After 48 h, one vial was fixed, stained, and read. To be used in sensitivity testing of the probe versus culture, monolayers needed to be 40 to 60% infected. Medium from the second vial was discarded, and the monolayer was washed with 1 ml of 2-SPG. 2-SPG (1 ml) was then added, and the vial was sonicated for 20 s. Sonicated inoculum (0.1 ml) was added to 0.9 ml of 2-SPG, and six 10-fold serial dilutions were made in 2-SPG. Subsequently, of these dilutions 0.1 ml was added to 0.9 ml of 2-SPG for culture and 0.1 ml was added to 0.9 ml of transport medium for the probe assay. Each culture dilution was used to inoculate a McCoy cell monolayer, which was centrifuged for 1 h at 1,000 × g and incubated for 48 h at 37°C. The six 10-fold dilutions of the *C. trachomatis* inoculum in transport medium were probed by the protocol described above. The results of the culture assay were calculated by determining the number of chlamydial inclusion-forming units (IFU) present per cover slip, and the results for the probe were calculated by determining the number of RLU. To determine the sensitivity of the probe, the negative reference plus 1,000 RLU was subtracted from the RLU value obtained for each serovar dilution. This is the calculation used by the manufacturer to determine whether a result with a clinical specimen is positive. These RLU values were plotted against the corresponding IFU. The best-fit linear line was drawn through the points, with the x intercept determining which value corresponded to the lowest IFU detected.

For specificity testing, Stock bacterial or fungal isolates were grown on 5% sheep blood agar or chocolate agar. Isolates tested included *Corynebacterium* spp., *Escherichia coli*, *Gardnerella vaginalis*, *Lactobacillus* spp., *Neisseria gonorrhoeae*, *Neisseria sicca*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus agalactiae*, *Streptococcus faecalis*, viridans group streptococci, *Bacteroides fragilis*, *Clostridium perfringens*, *Propionibacterium acnes*, and *Candida albicans*. Before testing, organisms were suspended in phosphate-buffered saline (0.01 M, pH 7.4) to a 0.5 McFarland standard (ca. 1.5 × 10^8 CFU/ml), and a 10-fold dilution of the suspension was made in transport medium. Subsequently, 0.1 ml of this suspension was used in the probe assay.

**Data analyses.** Sensitivities, specificities, and predictive values were calculated by the method of Ranshoff and Feinstein (14).

**RESULTS**

**Determination of sensitivity and specificity of the DNA probe, using stock isolates.** The lower limit of detection for the DNA probe assay with the 15 established serovars of *C. trachomatis* (A to K, Ba, L1, L2, and L3) can be seen in Fig. 1. Upon initial testing of the serovars, with the exception of serovars Ba, C, F, and G, all had a lower limit of detection that fell between 1,086 and 2,930 IFU. The four serovars that were not in this range fell below 1,000 IFU in sensitivity and were retested. The initial results with serovars Ba, C, F, and G were 743, 168, 687, and 911 IFU, respectively. Upon repeat of the assay, these values were 3,000, 30, 2,192, and 2,488 IFU for Ba, C, F, and G, respectively. The numbers of IFU in Fig. 1 for these four serovars represent the average of the two determinations. The data represented were derived by using as a positive cutoff 1,000 RLU above background. This was done to correspond to the calculation recommended by the manufacturer for use when testing clinical samples.

Microorganisms representing normal vaginal flora or pathogens found in the genital area were tested at a concen-
tation of 1.5 \times 10^7 CFU/ml with the DNA probe. In addition, C. psittaci (Texas turkey) and C. pneumoniae (TWAR-183) isolates at a concentration of 10^7 IFU/ml were included to test for cross-reactions with other Chlamydia species. There were no false-positive results with any of the organisms, including the C. psittaci and C. pneumoniae isolates tested.

Evaluation of the DNA probe, using clinical specimens. Of the 196 specimens included in the study, there were 20 (10%) positive by culture. Of the culture-positive specimens, 14 were cultured within 24 h of collection and the remaining 6 were cultured within 48 h. Of the 196 specimens in the study, 4 were not evaluated by DFA because insufficient cells were present on the smear, and 1 specimen was not evaluated by the DNA probe because of insufficient specimen. The overall results of the three direct methods relative to those of culture can be seen in Table 1. The most sensitive method was ELISA (85%, 17 of 20), followed by DFA (75%, 15 of 20) and DNA probe (60%, 12 of 20). However, DFA was the most specific (99%, 171 of 172), followed by ELISA (97%, 170 of 176) and DNA probe (95%, 167 of 175).

IFU were correlated with results of the direct methods (Fig. 2). Of the 20 culture-positive specimens, 7 had <100 IFU per culture, 5 had between 100 and 1,000 IFU per culture, and the other 8 had >1,000 IFU per culture. All three direct methods detected fewer specimens with <1,000 IFU per culture than specimens with >1,000 IFU per culture. The DNA probe detected only 29% (2 of 7) of the positive cultures that had <100 IFU per culture, while the other two direct assays both detected 71% (5 of 7) in this range.

Of the false-positive specimens, there were seven for which only the DNA probe was positive and five for which only ELISA was positive. There was one specimen for which all three direct methods were positive, yet the culture was negative. This latter specimen was cultured within 48 h of collection, and <50 elementary bodies were seen by DFA; thus, with this specimen the delay in culturing, along with the low number of elementary bodies present, may represent a culture failure. However, for data analyses this specimen was considered to be a true-negative by the reference method.

The order of swab collection was examined to see whether any correlation between discrepant results could be found. Of the 20 culture-positive specimens, 3, 8, 2, and 7 were from the first, second, third, and fourth swabs collected, respectively. False-negative and false-positive direct assay results showed no obvious correlation with swab order, with the exception of ELISA. All three false-negative specimens with ELISA were from the last swab collected.

**DISCUSSION**

In this study, all three direct methods tested were less sensitive than tissue culture for the detection of C. trachomatis. Of the direct methods, the sensitivities were 60, 75, and 85% for the DNA probe, DFA, and ELISA, respectively, while specificities were 95, 99, and 97%, respectively. Previously reported sensitivities and specificities for direct tests vary widely; however, DFA and ELISA values obtained in this study are very similar to those of several previously published reports (3, 5, 9, 13).

In our study population, the majority of tissue culture-positive specimens had <1,000 IFU per culture. As expected, it was in this range that the direct tests had the lowest sensitivities. This finding also correlates with previous studies we have done comparing the DFA with culture (12), as well as with those of others who have analyzed DFA and ELISA results compared with the IFU obtained by culture (18). Similarly, the majority of specimens not detected by DNA probe had <100 IFU by tissue culture. These findings correlated well with our data for stock isolates of the 13 serovars for which, with the exception of serovar C, the probe had a lower limit of detection of approximately 1 \times 10^3 to 3 \times 10^4 IFU.

Of the false-positive results, only one was positive by more than one direct method. With this specimen, since all three direct methods were positive, one could easily specu-
late that it was a tissue culture failure. With the other false-positive specimens (seven by DNA probe and five by ELISA), however, only one direct method was positive, making it more difficult to speculate that these were tissue culture failures. Since the DNA probe in our specificity studies with stock isolates used a relatively high organism concentration (1 x 10^7 per ml) and showed no cross-reactivity, these false-positive specimens were an unexpected finding. Whether the clinical material containing mucus and host cell debris actually trapped rather than cross hybridized with the probe remains to be determined. It is interesting that when all eight false-positive specimens by probe were repeated, two that were a very low positive reverted to negative while the other six remained positive. Likewise, a repeat ELISA of false-positive specimens showed only one reverting to negative.

In this study, the swab collection order was rotated with every patient. With the exception of ELISA, there was no obvious difference among the assays in the swab order as to whether the assay was positive or negative. With ELISA, however, all three false-negative specimens were with the fourth swab collected. However, because of the small number of samples in this study, these results need to be interpreted with caution. Hernandez et al. (4) reported a similar finding in a study of two female groups with a prevalence of C. trachomatis of 10 and 15%. They collected five cervical swabs from each patient and tested all five by ELISA (Chlamydiazyme; Abbott Laboratories). With 19 positive specimens of which the first and second swab were always positive, they found that the third, fourth, and fifth swabs were positive 86, 75, and 58% of the time, respectively.

This is the first report on the sensitivity of a DNA probe directed at rRNA, using the 15 serovars of C. trachomatis. We found that, in general, the probe was able to detect 1 x 10^3 to 3 x 10^3 IFU, which correlated well with the clinical specimens. Pao et al. (11), using a dot blot hybridization technique with a probe consisting of a nick-labeled C. trachomatis 7.4-kilobase plasmid, estimated that they could detect 3 x 10^2 C. trachomatis IFU. This number assumed that each genome was accompanied by 10 plasmid copies per C. trachomatis organism. We do not know how many copies of rRNA are contained in an IFU; however, if we assume that the liquid and solid-phase hybridization methods are comparable in sensitivity (19), then it would not be unreasonable to expect approximately 10^2 copies of rRNA per IFU. The majority of genital isolates had a lower limit of sensitivity of 1 x 10^2 to 3 x 10^3 IFU. Therefore, if the population under study had a high level (>1,000 IFU per culture) of C. trachomatis shedding, then the DNA probe could appear to be more sensitive than we found in our study.

It is interesting that with serovar C there was almost a 10-fold difference in the number of IFU detected with the DNA probe. Whether this result is due to more rRNA per IFU or a higher ratio of noninfectious C. trachomatis particles to viable elementary bodies with this serovar remains to be established. In an attempt to resolve this question, we performed particle counts and cultures on preparations of serovars C and L2, using the methodology that was used for preparing the samples for determining the sensitivity of the probe on stock swabs. In duplicate experiments on different days, we arrived at the same ratio of particles to IFU for both serovars. However, there are also inherent limitations in this type of comparison, for sonication was used to break the cells and this type of disruption could also break more fragile reticulate bodies that would then not be included in the particle counts.

Regardless of the reason for the ability of the DNA probe to detect lower numbers of serovar C, it must be kept in mind that serovar C is one of the least commonly found serovars isolated from genital infections (20).

In conclusion, in this population in which the majority of specimens yielded <1,000 IFU per culture, the DNA probe was the least sensitive method, followed by DFA and ELISA, while DFA was the most specific.

LITERATURE CITED