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Diagnosis of *Chlamydia trachomatis* Cervical Infection by Detection of Amplified DNA with an Enzyme Immunoassay

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A sensitive and specific system for detection of amplified *Chlamydia trachomatis* DNA from cervical specimens by fluorometric quantitation in an enzyme immunoassay (EIA) format (polymerase chain reaction [PCR]-EIA) is described. The primers selected for PCR-amplified DNA were from the 15 serovars of *C. trachomatis* and two strains of *Chlamydia pneumoniae* (TWAR). One strain of *Chlamydia psittaci* (Borg) was not amplified. One hundred four previously cultured cervical specimens were evaluated. Forty-six culture-positive specimens containing from 1+ to 4+ inclusion bodies were all positive by PCR-EIA. Of 58 culture-negative specimens, 2 were repeatedly positive and were nonreactive with control probes. This assay system represents a sensitive and specific combination of technologies for the quantitative detection of *C. trachomatis* DNA directly from a body fluid.

Chlamydia trachomatis is an obligate, intracellular bacterium which infects mucosal surfaces of the cervix, urethra, rectum, nasopharynx, or conjunctiva. Cervical infections can ascend into the endometrium and fallopian tubes and cause pelvic inflammatory disease, perihepatitis, infertility, or ectopic pregnancy. Infection during pregnancy may adversely affect the newborn, leading to neonatal conjunctivitis or infant pneumonia. In males, this organism causes 40 to 50% (16) of the cases of nongonococcal urethritis, which is one of the most common sexually transmitted diseases in heterosexual males. Ascending infections from the urethra can result in acute epididymitis. In homosexual males, proctitis may occur following receptive anal intercourse. The L1 to L3 serovars of *C. trachomatis* cause lymphogranuloma venereum, a sexually transmitted infection that involves regional lymph nodes and produces systemic manifestations. In addition to genital infections, *C. trachomatis* is the etiologic agent of trachoma, the leading cause of blindness in developing countries. The rapid identification of chlamydial infections is, therefore, essential for the proper treatment of infected patients and for the prevention of transmission to susceptible individuals.

Recently, it has been shown that small quantities of DNA can be specifically amplified by repeated reactions with DNA polymerase and oligonucleotide primers flanking the 3′ and 5′ ends of the target DNA (13). This reaction system, generally known as the polymerase chain reaction (PCR), has been used for the characterization of human genomic DNA (7) as well as for the detection of viral DNA from human retroviruses (8), hepatitis B virus (18), papillomaviruses (5), toxigenic *Escherichia coli* (12), and *C. trachomatis* serovars by using primers in the major outer membrane protein (MOMP) gene (6). One factor limiting the application of PCR techniques for the diagnosis of infectious diseases has been the lack of a sensitive, nonisotopic system for quantitation of DNA generated by the amplification reactions. While such DNA has been detected by hybridization to ^{32}P-labeled probes or by visualization of bands following gel electrophoresis, such systems are difficult to use in a reproducible and quantitative fashion for the testing of large numbers of specimens in a clinical laboratory environment.

We have recently devised a DNA detection system in which a monoclonal antibody to DNA-RNA duplexes is used to quantify hybrids formed between amplified DNA and complementary RNA probes (PCR-enzyme immunoassay [EIA]) (3). We report here the application of this method for the diagnosis of chlamydial infections of the cervix.

MATERIALS AND METHODS

Clinical specimens. Specimens were taken from the endocervix with one Dacron swab and placed in transport medium containing sucrose-phosphate buffer with 2% fetal bovine serum and antibiotics. They were stored at −70°C until they were processed for DNA amplification. Specimens were obtained from patients seen at a sexually transmitted disease clinic in Baltimore, Md. A total of 46 culture-positive and 58 culture-negative specimens were randomly selected from a collection of stored samples. They were coded, and the person performing the amplification-DNA detection assays was unaware of the chlamydial culture results.

Bacterial strains. Chlamydial isolates were obtained from the American Type Culture Collection (Rockville, Md.) and included the following serovars (strain designations are given in parentheses): *C. psittaci* (Borg); *C. trachomatis* L1 (440), L2 (434), L3, A (Har 13), B (Har 36), Ba, C (Har 32), D (UW-3/Cx), E (Bour), F (IC-Cal-3), G (UW-57/Cx), H (UW-43/Cx), I, J, and K (UW-31/Cx); and *C. pneumoniae* (CDC/CWL-029). The Seattle strain of *C. pneumoniae* (TW 183E5 H16) was obtained from the Washington Research Foundation. Clinical isolates of *Neisseria gonorrhoeae*, *Trichomonas vaginalis*, *Ureaplasma urealyticum*, and *Mycoplasma pneumoniae* were obtained from the Johns Hopkins Hospital Microbiology Laboratory.

***C. trachomatis* culture.** Chlamydia cultures were performed within 24 h of specimen collection. Specimens were

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tested in duplicate in 96-well microtiter plates by using McCoy cell monolayers which were pretreated with 30 μg of DEAE-dextran per ml for 30 min at 37°C. Inoculated cultures were centrifuged at 800 × *g* for 60 min at 35°C and incubated for 30 min at 35°C. Following aspiration of the supernatant, 0.2 ml of inoculation medium containing 0.5 mg of cycloheximide per ml was added to each well. After 48 h of incubation, cultures were fixed and stained with monoclonal antibody directed against the MOMP protein (Syva, Palo Alto, Calif.) and examined for the presence of inclusion bodies by using an epifluorescence microscope (Zeiss, Oberkochen, Federal Republic of Germany). Cultures were scored as follows: 1+, 1 to 9 inclusions per well; 2+, 10 to 20 inclusions per well; 3+, 1 to 10 inclusions per high-power field; and 4+, >10 inclusions per high-power field. All negative and toxic cultures as well as those positive cultures containing fewer than three inclusions were passed for an additional 48 h of in vitro cultivation. The portion of patient samples in the 1+ to 4+ categories was 28, 24, 26, and 22%, respectively. Of the 46 culture-positive samples, 2 were negative in primary culture but positive in the second passage.

Lysis and amplification. Portions of 200 μl of the clinical specimens were treated with Nonidet P-40–Tween 20 and proteinase K (Sigma Chemical Co., St. Louis, Mo.) at final concentrations of 0.5 and 0.5% (vol/vol) and 100 μg/ml, respectively. They were incubated at 60°C for 1 h, boiled for 3 min, and cooled quickly on ice. Tissue culture specimens of the 15 chlamydia serovars, *C. pneumoniae*, and *C. psittaci* were standardized to 2,000 infectious units/ml and lysed as described above. A culture suspension of *N. gonorrhoeae* (10^9 CFU/ml); and scrapes from agar plates containing an abundant growth of *U. urealyticum*, *T. vaginalis*, or *M. pneumoniae* were prepared for amplifications in the same fashion. The PCR was performed in a 100-μl volume containing 50 μl of sample, 0.5 μM primers, 0.2 mM deoxynucleoside triphosphates, 1× PCR buffer (10 mM Tris [pH 8.3], 50 mM KCl, 2.5 mM $MgCl_2$, 0.01% gelatin), and 1.5 U of *Taq* polymerase (Amersham Corp., Arlington Heights, Ill.). The reaction proceeded in an automated, programmable thermocycler (Perkins-Elmer Cetus, Norwalk, Conn.) with 30 cycles of denaturation (94°C, 1 min), annealing (55°C, 1 min), and extension (72°C, 1 min).

The assay system used a set of primers that was complementary to sequences in the MOMP of *C. trachomatis* L2. Oligonucleotides were synthesized by the phosphoramidite method (MilliGen/Biosearch, Bedford, Mass.) and separated by reverse-phase high-pressure liquid chromatography (5500; Varian Instruments, Sunnyvale, Calif.). The sequences of the primers from 5′ to 3′ are as follows:

Ct. 0005 GATAGCCAGCACAAAGAGAGCTAA
Ct. 06 CTTTGTTTTCGACCGTGTTTTGCAAACAGATGTGAA

RNA probe preparation. A second primer set was internal or nested to the set given above and was used exclusively to prepare a probe. The sequences of the primers from 5′ to 3′ are as follows:

Ct. 03T7 TTAATACGACTCACTATAGGGTCTGCTTCCTCCTTGCAAGCAAGTCTGCC
T7 promoter
Ct. 04 CAGCATGCGTATGGGTTACTATGG (17)

A stock strain of *C. trachomatis* L2 was lysed and amplified for 30 cycles with the nested set of primers to yield a 150-base-pair (bp) DNA fragment. Approximately 500 ng of the DNA fragment, which contained the T7 RNA polymerase promoter at the 5′ end, was transcribed with T7 RNA polymerase in the presence of biotin 11-UTP (Enzo, New York, N.Y.). The DNA template and primers were digested for 30 min at 37°C with RQ1 DNase (Promega, Madison, Wis.), and then the enzyme was heat inactivated for 3 min at 100°C. Unincorporated biotin 11-UTP was separated from labeled RNA by chromatography on a Sephadex G-25 column (NAP-5; Pharmacia, Uppsala, Sweden). The probe was divided into equal portions and stored at −70°C.

Solution hybridization and EIA. Fifty microliters of the amplified target DNA was mixed with 50 μl of a 1:200 dilution of the biotinylated RNA probe in 2× hybridization buffer (600 mM NaCl, 60 mM sodium citrate [pH 7], 20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 7.4], 4 mM EDTA, 0.5% sodium dodecyl sulfate). The nucleic acid mixtures were boiled for 3 min and hybridized at 78°C for 1 h. Samples were cooled to room temperature, and 10 μl of 10% Triton X-100 was added. Fifty-microliter portions were inoculated into duplicate wells of a black microtiter plate (Microfluor "B" 964-microplate; Dynatech Laboratories, Inc., Chantilly, Va.). The plate was previously coated overnight at 4°C with 50 μl of polyclonal goat anti-biotin antibody (Sigma Chemical Co.) per well at 1 μg/ml in 0.06 M carbonate buffer (pH 9.6). Before the addition of samples, the plate was washed six times with phosphate-buffered saline (10 mM phosphate buffer [pH 7.2], 150 mM NaCl) containing 0.05% Tween 20. After addition of the samples, the plate was incubated at 37°C for 1 h and then washed. The Fab′ fragment of a monoclonal antibody that recognized DNA-RNA hybrids and conjugated to β-D-galactosidase (1) (gift of Robert Carrico, Miles Laboratory, Inc., Elkhart, Ind.) was diluted to 184 ng/ml in phosphate-buffered saline–Tween 20–0.5% gelatin containing 0.5% mouse serum, and 50 μl was added to each well. After incubation at 37°C for 1 h, the plate was again washed. Fifty microliters of substrate solution containing 1 mM 4-methylumbelliferyl β-D-galactoside and 10 mM phosphate buffer (pH 7), 100 mM NaCl, 1 mM $MgCl_2$, and 50 μg of bovine serum albumin per ml was added to each well. Incubation was allowed to proceed for 2 h at room temperature. The fluorogenic end product, methylumbelliferone, was measured by using a fluorometer (detection wavelength, 365 nm; and emission wavelength, 450 nm; Microfluor; Dynatech Laboratories). A sample was considered positive if its mean fluorescence value exceeded that of amplified, uninoculated McCoy cells plus 3 standard deviations.

Electrophoresis. Electrophoresis was performed through a horizontal 2% agarose gel in a Tris acetate EDTA running buffer at 5 V/cm of gel length or in a 7.5% polyacrylamide gel (vertical). ϕX174 *Hae*III fragments (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) were used as molecular weight markers. Gels were stained with ethidium bromide (0.5 μg/ml), and photography was performed with coaterless film (667; Polaroid, Needham, Mass.) through a red Wratten filter while the gels were transilluminated at 265 nm.

Control procedures. Transfer and extraction of specimens was done in a biosafety hood. Several precautions were taken to minimize contamination by amplified molecules: use of positive displacement pipettors (Gilson, Rainin, Woburn, Mass.), use of several negative controls in each amplification run, frequent changing of gloves when handling specimens, and swabbing of equipment and the hood surface with 1 N HCl. Each amplification run included moderate- and low-positive tissue cultures as positive controls and uninoculated McCoy cells in distilled water as negative controls. An additional control, consisting of the RNA probe

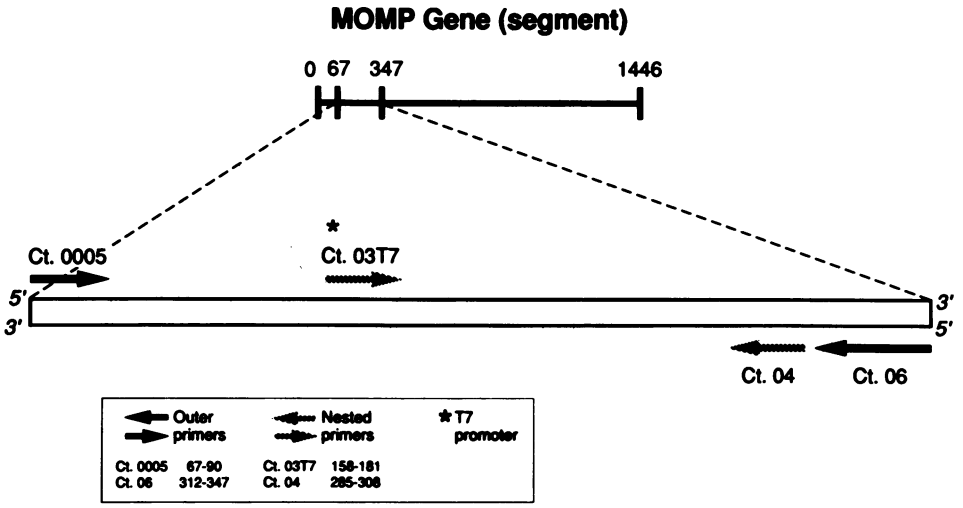

FIG. 1. Locations of the outer and nested sets of primers within the MOMP gene. The numbering system is derived from sequence information from GenBank accession no. M14738.

alone, was included in the hybridization and immunodetection assays.

RESULTS

Initial studies were aimed at the development of a reliable and objective method to measure chlamydial DNA amplified by PCR. Two sets of primers were selected from known sequences of the major outer membrane gene of *C. trachomatis* serovar L2. The designation of outer and nested was assigned to each primer pair and was based on their positions in the MOMP gene (Fig. 1). The outer set, Ct. 0005 and Ct. 06, is located at bp positions 67 to 90 and 312 to 347, respectively. The nested set, Ct. 03T7 and Ct. 04, resides at bp 158 to 181 and 285 to 308, respectively (GenBank accession no. M14738). The design of the method is depicted in Fig. 2. The outer set of primers was used in amplification reactions with clinical samples or stock strains, and PCR yielded a 280-bp DNA fragment when chlamydial DNA was present in the sample. The sequences of these primers are conserved among *C. trachomatis* serovars. Chlamydial DNA amplified by the outer set was measured in a procedure that involved two steps. First, amplified DNA was hybridized in solution with a biotinylated RNA probe. Second, hybrids between target DNA and the RNA probe were captured in wells of a microtiter plate coated with antibiotin antibody. Bound hybrids were reacted with a β-D-galactosidase-labeled monoclonal antibody that recognized DNA-RNA hybrids. The hybrids labeled in this way could be quantitated by measurement of the fluorescence signal generated after reactivity with β-D-galactoside methylumbelliferone.

The nested set of primers was used solely to prepare an RNA probe. Amplification of *C. trachomatis* serovar L2 DNA with these primers yielded a 150-bp fragment composed of sequences between the outer primers. One primer of the nested set contained the T7 RNA polymerase promoter at its 5′ end, which allowed a biotinylated RNA probe to be prepared from the fragment in a transcription reaction with T7 RNA polymerase in the presence of biotin-11-UTP.

Following definition of optimal levels of reagents and reaction conditions, the sensitivity of the amplification-DNA detection system (PCR-EIA) was determined by testing

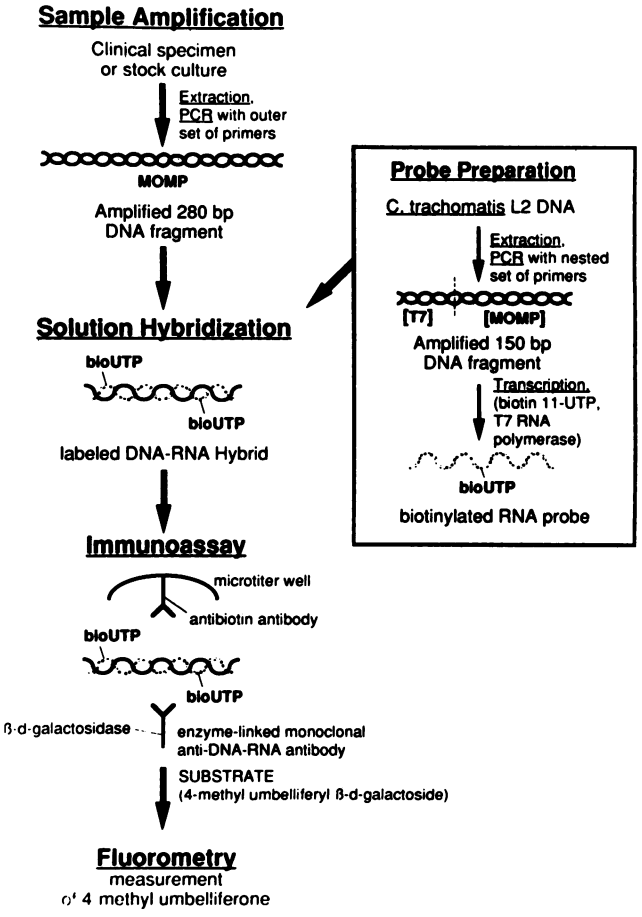

FIG. 2. Schematic of PCR-EIA amplification detection system. The DNA target from a clinical sample and the biotinylated RNA probe were generated in two separate procedures which converged to yield duplexes in a solution hybridization reaction. Biotinylated RNA-DNA hybrids captured in wells of microtiter plates coated with goat anti-biotin antibody were reacted to a β-D-galactosidase-linked monoclonal antibody that recognizes DNA-RNA hybrids. The fluorogenic substrate was cleaved by the enzyme to yield a quantifiable product, 4-methylumbelliferone.

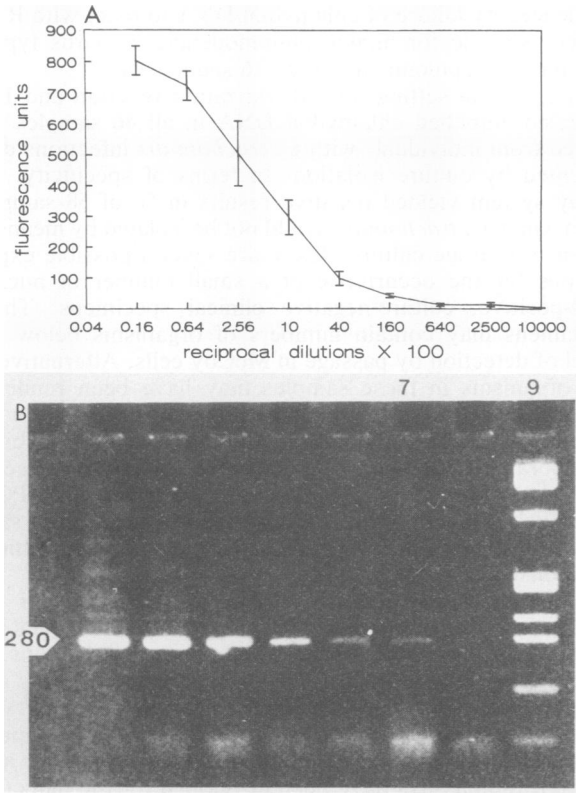

FIG. 3. (A) Titration of *C. trachomatis* serovar L2 dilutions. Serial fourfold dilutions were amplified, hybridized, and quantified in the immunoassay. The datum points represent the mean of three fluorescence values plus 1 standard deviation. (B) The amplified material depicted in panel A was electrophoresed in a polyacrylamide gel and stained with ethidium bromide. Lanes 2 to 6 and 8 (unmarked), Serial fourfold dilutions from 1:4 to 1:16,000 dilutions; lane 7, the last dilution showing a visible 280-bp DNA band; lane 9, ϕX174 *Hae*III fragments used as molecular size markers.

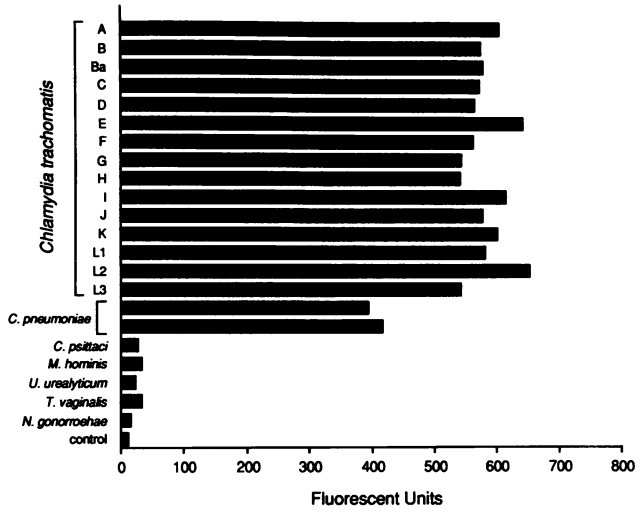

FIG. 4. Reactivity of bacterial strains. Each bar represents a mean of two fluorescence values for each organism. Two thousand infectious units of the chlamydial species per ml and heavy growth from nonchlamydial organisms were amplified.

serial dilutions of a lysate of a *C. trachomatis* L2 culture. It was possible to detect chlamydial DNA from a 5-μl portion of a 1:16,000 dilution of starting material, and there was a linear relationship between fluorescence units and dilutions of the sample (Fig. 3A). In contrast, when L2 DNA was amplified by the same primers but visualized with ethidium bromide-stained polyacrylamide gels, the minimum detectable level was a 1:4,000 dilution (Fig. 3B). Viable organisms could be recovered in tissue culture from a 100-μl portion of a 1:10,000 dilution. Titration of three clinical samples also showed a linear relationship between fluorescence units and sample dilution.

Next, the specificity and reproducibility of the PCR-EIA was determined. The PCR-EIA system was evaluated for its ability to detect the 15 known serovars of *C. trachomatis*, *C. psittaci*, and *C. pneumoniae*. Strong signals of 560 to 680 fluorescence units were obtained by using 2,000 infectious units of *C. trachomatis* serovars per ml (Fig. 4). Two strains of *C. pneumoniae* reacted in the assay (400 and 425 fluorescence units), but *C. psittaci* did not. In addition, signals above the background were not generated by reactions with a wide range of nonchlamydial microbial agents, including *T. vaginalis*, *U. urealyticum*, *M. pneumoniae*, and *N. gonorrhoeae*. Control probes were used to determine the specificity of the hybridization reaction. Significant signals were not detected when a 1:2,000 dilution of the *C. trachomatis* L2 lysate was amplified by the chlamydia primers and reacted with RNA probes for either a segment of the *gag* gene of human immunodeficiency virus type 1 or a segment of the E7-E1a gene of human papillomarivus type 16. These values were 6 and 12 fluorescence units for the human immunodeficiency virus type 1 and human papillomavirus type 16 probes, respectively, compared with 400 fluorescence units for the *C. trachomatis* target plus its homologous probe. Additional experiments were performed to determine the reproducibility of the PCR. Portions from 1:4,000, 1:16,000, and 1:64,000 dilutions of a *C. trachomatis* L2 lysate were amplified on 4 separate days. The hybridization reactions and EIAs were performed on the same day. The overall interday coefficient of variation was 4%.

The PCR-EIA was then used to determine the presence of *C. trachomatis* DNA in 104 endocervical samples. All 46 samples from which *C. trachomatis* could be cultivated were also positive in the nucleic acid detection method (Fig. 5). The signals ranged from 396 to 946 fluorescent units. Of the 58 specimens which did not yield *C. trachomatis* in tissue culture, 56 were negative for chlamydial DNA. The mean signal for the negative clinical samples was 14 fluorescence units, and the range was 4 to 47 fluorescence units. There were two samples in which *C. trachomatis* DNA could be detected, even though viable organisms could not be recovered following cultivation. These two samples yielded signals of 298 and 165 fluorescence units. Positive results were also obtained on reextraction of the initial samples and retesting under identical conditions. These samples did not react with a control probe represented by human papillomavirus type 16. Elementary bodies were not seen when the two samples were examined microscopically by using fluorescent antibody to the MOMP gene.

DISCUSSION

An assay system combining a liquid hybridization reaction and EIA for biotinylated DNA-RNA hybrids was developed for the detection of amplified chlamydial DNA. PCR-EIA displayed high degrees of reactivity with all reference strains of *C. trachomatis* and two strains of *C. pneumoniae* (TWAR) but not with one *C. psittaci* strain or nonchlamydial

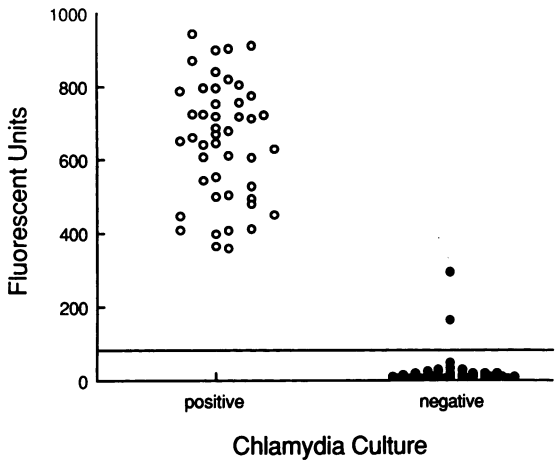

FIG. 5. Scatter plot of fluorescence values for clinical samples. Cervical samples from 104 patients were tested (46 and 58 culture-positive and -negative, respectively). Each datum point represents the mean of duplicate fluorescence values. The negative cutoff value was determined by the signal generated from uninoculated McCoy cells plus 3 standard deviations.

organisms that are likely to be present in genital samples. Although the overall homology between *C. trachomatis* and *C. pneumoniae* chromosomal DNA is 10% (4), homology within the MOMP gene may be greater than 10%. For example, a cloned portion of the MOMP gene of *C. trachomatis* serovar L2 was shown to hybridize under stringent conditions with a 1.1-kbp DNA fragment from *C. pneumoniae* (2). It is likely that the outer primer sequences chosen from the MOMP gene of *C. trachomatis* L2 are complementary to regions in the MOMP gene of *C. pneumoniae*. The amplification and detection of *C. pneumoniae* is unlikely to pose a problem in the analysis of genital samples, since TWAR is not considered a sexually transmitted pathogen (9). As nucleotide sequence information for the MOMP gene of *C. pneumoniae* becomes available, other primer pairs could be used to specifically distinguish *C. trachomatis* from *C. pneumoniae*. Although the nucleotide sequence of the MOMP gene of the Borg strain of *C. psittaci* was not available for comparison, the failure of our PCR to amplify DNA from this strain is likely to reflect low homology for the primers. Nucleotide sequences of the MOMP gene from two other strains of *C. psittaci* (GPIC and Mn) were recently published and shown to have 80% homology with each other and 68% with *C. trachomatis* L2 (19). Despite the overall homology of the MOMP gene in the two species, one of our *C. trachomatis* primers shared only 37% homology with the corresponding region from GPIC and Mn strains of *C. psittaci*, and the PCR would not be expected to amplify these strains. Given the genetic heterogeneity of chlamydial species and the poorly defined relationship between primer homology and the efficiency of amplification reactions, our findings may not apply to all wild-type strains of either *C. psittaci* or *C. pneumoniae* (10, 14, 15).

PCR-EIA was more sensitive than ethidium bromide-stained gel analysis for the detection of amplified DNA. The level of sensitivity in the latter test could not be improved by additional cycles of amplification. Chlamydial DNA could be detected by PCR-EIA in dilutions of a stock culture from which viable organisms could not be recovered. This suggests that the assay may be more sensitive than tissue culture. The specificity of the hybridization reaction was evidenced by failure of chlamydial DNA to react with RNA probes specific for human immunodeficiency virus type 1 and human papillomavirus type 16 sequences.

In a clinical setting, the hybridization reaction and EIA detected amplified chlamydial DNA in all 46 samples obtained from individuals with *C. trachomatis* infections documented by culture isolation. In terms of specificity, the assay system yielded negative results in 56 of 58 samples from which *C. trachomatis* could not be isolated by means of passage in tissue culture. There are several possible explanations for the occurrence of a small number of nucleic acid-positive, culture-negative clinical specimens. These specimens may contain numbers of organisms below the level of detection by passage in McCoy cells. Alternatively, the organisms in these samples may have been rendered nonviable by the process of collection and storage or by administration of antibiotics to the patients. The possibility of laboratory cross-contamination, which has been recognized as a potential problem in other systems that use PCR, is unlikely because of the repeated positivity of these samples after repeat lysis and amplification of separate portions and consistent negativity of appropriate controls.

While there are a number of assays available for the diagnosis of *C. trachomatis* infections, none are ideal for acute management of patients with suspected infections. In vitro cultivation can take up to 4 to 7 days to complete and requires access to cell culture facilities and trained personnel. While antigen detection assays, such as immunofluorescence microscopy and EIAs, and conventional nucleic acid hybridization assays have been developed for the detection of *C. trachomatis*, these assays generally do not display adequate degrees of sensitivity, especially for the diagnosis of infections in asymptomatic individuals (11). The PCR-EIA described here may find widespread use for the diagnosis of chlamydial infections. However, additional studies should test larger numbers of specimens and samples from different body sites and should compare PCR-EIA with antigen detection assays.

The data presented in this study indicate that the combination of a solution hybridization reaction and EIA for biotinylated DNA-RNA hybrids represents a practical method for indicating the presence of DNA amplified from complex body fluid samples. The PCR-EIA format offers a number of potential advantages for infectious disease diagnosis, including sensitivity, specificity, reproducibility, and avoidance of radioactive isotopes. In addition, the objective nature of the assay and its low degree of intertest variation make it amenable to quantitation, which could be used for following the course of infectious diseases and responses to antimicrobial chemotherapy. Application of this assay system might substantially improve the medical capabilities for diagnosis and management of patients with infectious diseases and facilitate epidemiological surveillance for the prevention of disease transmission within susceptible populations.

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