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Infection with a Plasmid-Free Variant Chlamydia Related to *Chlamydia trachomatis* Identified by Using Multiple Assays for Nucleic Acid Detection

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Clinical samples in transport media from 40 patients exhibiting pathologies potentially caused by *Chlamydia trachomatis* infection were analyzed for chlamydial nucleic acid, and the results were compared with those of culture. Chlamydial culture was performed by a shell vial centrifugation method with HeLa 229 host cells. Polymerase chain reaction (PCR) assays were used to detect either regions on a 7.5-kb plasmid characteristic of *C. trachomatis* (plasmid-PCR) or a segment of the 16S rRNA genes (rRNA-PCR). All PCR results were confirmed by hybridization with probes for the specific amplified products in either a Southern or a dot blot format. An RNase protection (RNP) assay was used to detect genus-specific chlamydial 16S rRNA directly from the clinical samples. The PCR assays detected *C. trachomatis* but not other bacteria, including *Chlamydia* spp. *C. trachomatis* was isolated from six samples which were positive by the rDNA-PCR and plasmid-PCR assays. Five of the culture-positive specimens were positive by the RNP assay. Twenty-two samples were negative by all criteria. Surprisingly, nine samples were positive by rRNA-PCR and RNP assays only. Nucleic acid sequencing of the rRNA-PCR-amplified products indicated a close relationship between the variants and *C. trachomatis*. The data may indicate an unrecognized process in *C. trachomatis* infection or that these patients were infected by a variant strain of *C. trachomatis* which lacks the *C. trachomatis*-specific plasmid.

Chlamydia trachomatis is one of the most common causes of sexually transmitted disease in the United States today (4). Clinical symptoms can include urethritis, cervicitis, endometriosis, salpingitis, and perihepatitis (26). Chlamydial infections are often asymptomatic or nonspecific in their clinical presentation (2), and this, combined with difficulties in isolation, propagation, and identification of the organisms, has complicated the development of rapid, simple laboratory diagnostic methods.

Currently, diagnosis of chlamydial infections is based primarily on isolation of the organisms in either HeLa 229 or McCoy cells. The specificities of cell culture methods are high; however, the sensitivities of such assays are typically 90 to 95% compared with those of newer methods of detection (6, 10, 14, 18). The sensitivity of culture depends on factors such as the status of patient symptomology, specimen collection techniques, the number of sites from which samples are obtained, the specific sites from which samples are obtained, specimen transport and storage, and cell culture methods. In order to circumvent the need for culture and reduce the time required for diagnosis, several alternative methods have been developed for the rapid, specific detection of *C. trachomatis*. Direct fluorescent-antibody tests as well as enzyme immunoassays with monoclonal antibodies have been described and compared with culture methods for the detection and identification of *C. trachomatis* (2, 26). However, none of the current immunoassays have been shown to have high levels of either sensitivity or specificity (2, 26).

Because of their potential ability to detect and identify *Chlamydia* species with high levels of sensitivity and specificity, several types of nucleic acid hybridization assays have been developed. A nonisotopic DNA probe assay (Gen-Probe PACE; Gen-Probe Inc., San Diego, Calif.) for detection of *C. trachomatis* in urogenital specimens is commercially available and has been compared with both cell culture and enzyme immunoassays (13, 15, 30).

More recently, polymerase chain reaction (PCR) assays for the detection of *C. trachomatis* genes encoding 16S rRNA (15), the major outer membrane protein (12), or a cryptic plasmid reported to be found in all *C. trachomatis* serovars (15, 29) have been developed and compared with DNA probe assays as well as enzyme immunoassays and culture. The *C. trachomatis* cryptic plasmid has been the most frequently used target for PCR. From a total of 375 isolates representing all 15 serovars of *C. trachomatis* (5, 22, 23), only one isolate has been shown to lack this plasmid (23). Generally, PCR assays have shown high sensitivities and specificities (12, 15, 29) and have allowed the simultaneous differentiation of the three species of *Chlamydia* (29).

In addition, a potential advantage of nucleic acid detection as a method of diagnosing infections is the ability to detect nonculturable microorganisms. Nucleic acid hybridization and PCR assays have frequently been reported to detect the presence of *C. trachomatis* in culture-negative specimens (5, 15, 19, 24). These results are usually attributed to either the presence of nonviable organisms or, in some cases of PCR, a false-positive reaction. The cause of the nonviability is assumed to be due to problems inherent in handling patient specimens, the insensitivity of the cell cultures used, and the low infectivity of *C. trachomatis* in culture. However, in many cases, the actual reasons for the discrepancies between PCR assay and culture results remain unknown.

In an examination of a series of urogenital specimens obtained from patients with suspected *C. trachomatis* infec-

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tion, we also noted discrepancies between PCR assay results and culture as well as between PCR assays directed against different nucleic acid targets. In the study described here, a comparison of a PCR assay targeted against the 16S rRNA gene of *C. trachomatis* (rRNA-PCR) yielded more than twice as many positive results as did culture or PCR assays targeting the *C. trachomatis*-specific plasmid (plasmid-PCR). Multiple primers used for amplification of the plasmid sequences did not increase the frequency of positive results. The presence of chlamydial 16S rRNA was confirmed in the samples positive in the rRNA-PCR assay by use of an RNase protection (RNP) assay directed against a different target region of the 16S rRNA.

Therefore, the discrepancies between the two PCR assays cannot be explained on the basis of nucleotide sequence variability or PCR contamination. We present evidence here for the possibility that this pattern of results (culture negative, plasmid-PCR negative, rRNA-PCR positive, RNP positive) from urogenital specimens may identify either a previously unrecognized stage in the *C. trachomatis* infection process or, alternatively, a novel strain of *Chlamydia*.

MATERIALS AND METHODS

Patient specimens and cell culture. Coded samples were submitted to our laboratory from the University of Massachusetts Medical Center. The patients were from a family medicine clinic or a gynecology clinic associated with the hospital or were patients admitted to the University of Massachusetts Hospital. The samples were obtained from patients with symptoms consistent with a sexually transmitted disease and prior to the administration of antibiotics. For female urogenital samples, mucus was removed from the cervix with a swab and the swab was discarded. A calcium alginate swab was inserted into the endocervical canal and was gently rotated 180 degrees. For male urethral specimens, a swab was inserted 4 cm into the urethra and was rotated similarly. Rectal swabs were inserted against the mucosal wall of the rectum and rotated vigorously. For ocular specimens, swabs were taken from as much of the involved conjuctival surface as possible without touching the skin, external eyelid, or eyelashes. Swabs were placed immediately into 1 ml of 0.2 M sucrose-phosphate medium (2-SP) containing gentamicin (5 μg/ml), vancomycin (12.5 μg/ml), and nystatin (12.5 μg/ml) and were stored in the medium at −70°C. Aspirates were combined with 0.5 to 1.0 ml of 2-SP and were stored similarly. Specimens were thawed, and 100-μl aliquots were inoculated in duplicate onto cultures of HeLa 229 cells seeded on coverslips (12 mm) in glass vials (Viromed Inc., Minetonka, Minn.). The HeLa 229 cell cultures were pretreated with DEAE-dextran (30 μg/ml) in phosphate-buffered saline (PBS) for 30 min at 37°C prior to inoculation. The cultures were centrifuged at 1,000 × *g* at room temperature for 1 h, after which 1 ml of Eagle's minimal essential medium containing 10% fetal calf serum was added and the cultures were incubated at 37°C for 48 h. After incubation, the medium was removed and the cells were fixed in methanol. Coverslips were removed and stained with either a genus- or species-specific fluorescein-conjugated monoclonal antibody (Kallestadt Diagnostics, Chaska, Minn.) and were read for chlamydial inclusions. A culture exhibiting one or more fluorescent inclusions was considered a positive isolation result.

Bovine cervical mucus. Pooled bovine cervical mucus was obtained from a local breeding farm. Mucus was screened for the presence of chlamydiae by cell culture, PCR, and immunostaining. Mucus certified as negative was used as a clinical matrix for simulating clinical conditions. The mucus was diluted 1:4 with 2-SP immediately prior to use to achieve a viscosity comparable to that of human mucus.

TABLE 1. Inclusivity and exclusivity of *Chlamydia* PCR assays

Organism	ATCC strain no.[a]	PCR results	
		Plasmid-PCR	rRNA-PCR
Candida albicans	18804	−	−
Mycobacterium smegmatis	14468	−	−
Pseudomonas cepacia	Clinical isolate[b]	−	−
Pseudomonas aeruginosa	10145	−	−
Pseudomonas alcaligenes	9886	−	−
Clostridium perfringens	3624	−	−
Proteus rettgeri	29944	−	−
Proteus stuarti	29914	−	−
Yersinia enterocolitica	9610	−	−
Streptococcus pneumoniae	6303	−	−
Streptococcus agalactiae	13813	−	−
Streptococcus pyogenes	19615	−	−
Escherichia coli	Clinical isolate	−	−
Klebsiella pneumoniae	13883	−	−
Staphylococcus aureus	12600	−	−
Staphylococcus epidermidis	14990	−	−
Haemophilus influenzae	19418	−	−
Bacillus fragilis	23745	−	−
Neisseria gonorrhoeae	Clinical isolate	−	−
Chlamydia psittaci	VR125	−	−
Chlamydia pneumoniae	TW183[c]	−	−
Chlamydia trachomatis	VR887	+	+

[a] ATCC, American Type Culture Collection.
[b] The strain was isolated from a clinical sample.
[c] The strain was obtained from the Washington Research Foundation, University of Washington, Seattle.

Bacterial strains and growth of *C. trachomatis*. The strains used to verify the specificities of the PCR and RNP assays are listed in Table 1. All bacteria were either clinical isolates or were obtained from the American Type Culture Collection (Rockville, Md.). Bacterial strains were grown and stored at −70°C according to standard clinical microbiological procedures (20).

C. trachomatis elementary bodies (EBs) were prepared from infected McCoy cell cultures. McCoy cells (Whittaker Bioproducts, Walkersville, Md.) were cultured in McCoy cell growth medium (Eagle's minimal essential medium with Earle's salts, 1% L-glutamine, 10% fetal calf serum, and 10% μg of gentamicin sulfate per ml; GIBCO Bethesda Research Laboratories, Grand Island, N.Y.). For infection, 20 T175 flasks containing confluent monolayers of McCoy cells were washed with 5 ml of DEAE-dextran (30 μg/ml; Pharmacia Inc., Milwaukee, Wis.) in Hanks' balanced salt solution (GIBCO Bethesda Research Laboratories). Each flask was incubated for 20 min at room temperature with an additional 5 ml of DEAE-dextran in Hanks' salts solution. The DEAE-dextran solution was removed, and 1 ml of an inoculum of *Chlamydia trachomatis* serovar K (American Type Culture Collection) containing 5.0×10^8 EBs per ml was added to each flask. The flasks were incubated for 2 h at room temperature with gentle agitation. Following the incubation period, 60 ml of chlamydial growth medium (McCoy cell growth medium supplemented with 4 mg of glucose per ml and 1 μg of cycloheximide per ml; Sigma Chemical Co., St. Louis, Mo.) was added and the monolayers were incubated

at 37°C for 3 days in a 5% CO_2 atmosphere. Chlamydiae were harvested when the monolayers had 15 to 30 inclusions per high-power microscopic field. Approximately 30 to 50% of the cell monolayer appeared to be infected. The medium was removed from the monolayers, and EBs were recovered from the supernatant by centrifugation at 10,000 × *g*. Flasks containing infected monolayers were placed in a water bath and were subjected to ultrasonic treatment in the presence of glass beads for 5 min. Lysed cells were pooled and clarified by centrifugation at 750 × *g* for 5 min to remove cellular debris. Pellets from the original supernatant were pooled with the clarified supernatant and were treated with DNase (Sigma Chemical Co.) at a final concentration of 20 μg/ml for 30 min at 37°C. DNase-treated EBs were pelleted by centrifugation at 10,000 × *g* and were resuspended in 2 ml of 2-SP containing gentamicin only. Concentrated EBs were layered onto a step gradient of Renograffin-76 (E. R. Squibb & Sons, Princeton, N.J.) at concentrations of 29, 34, and 40% (wt/vol) and were centrifuged at 103,000 × *g* for 1 h in an SW28 rotor (Beckman Instruments, Fullerton, Calif.). The visible band at the interface of the 34 and 40% layers was collected, washed with PBS, and quantitated by direct fluorescent-antibody staining (Kallestadt Diagnostics, Chaska, Minn.).

Extraction and quantitation of chlamydial nucleic acid. Chlamydial rRNA was purified by a modification of the procedure described by Barns et al. (3). A 0.5-ml portion of a stock of purified chlamydia EBs (1.0×10^{11} EBs per ml) was lysed by vortexing for 4 min in a 2.0-ml microcentrifuge tube containing 400 μl of acid-washed, 0.5-mm-diameter baked glass beads (Biospec Products, Bartlesville, Okla.), 750 μl of buffer (50 mM sodium acetate, 10 mM EDTA [pH 5.1]), 50 μl of 20% sodium dodecyl sulfate (SDS), and 500 μl of buffer-saturated phenol. Beads were pelleted by centrifugation for 10 min at low speed in a Speed Vac Concentrator (Savant, Farmingdale, N.Y.). The aqueous phase was reextracted two to three times with 400 μl of buffer-saturated phenol and 100 μl of chloroform-isoamyl alcohol (24:1 [vol/vol]). Total nucleic acids were precipitated by the addition of 0.1 volume of 3.0 M sodium acetate and 2 volumes of ethanol; this was followed by incubation on dry ice for 10 min. Following centrifugation for 5 min at 15,000 × *g*, the pellets were dissolved in 1.45 ml of TMK buffer (10 mM Tris-HCl [pH 7.4], 5 mM $MgCl_2$, 30 mM KCl) and were incubated for 5 min at room temperature with 20 U of RQ1 DNase (Promega Inc., Madison, Wis.). The samples were mixed with 2.1 ml of a 2.01-g/ml concentration of cesium trifluoroacetate (Pharmacia Inc.) and were loaded into a 3-ml Quick Seal tube (Beckman Instruments). High-molecular-weight RNA was pelleted by equilibrium density centrifugation (>4 h at 150,000 × *g* at 4°C) in a TLA 100.3 rotor with a Beckman TL100 ultracentrifuge. Pellets were suspended in 200 μl of TMK buffer, precipitated with sodium acetate-ethanol, dried, and dissolved in RNase-free water. The RNA was quantitated by spectrophotometry.

Oligonucleotides and nucleic acid probes. Oligonucleotides were synthesized on a model 381A automated DNA synthesizer (Applied Biosystems Inc., Foster City, Calif.) by using β-cyanoethylphosphoramidite chemistry. Two sets of primers were used for PCR amplification of the *C. trachomatis*-specific plasmid. Two forward primers, P_fp-1 (5′-GGACAA ATCGTATCTCGG-3′) and P_fp-2 (5′-CAAGCTTAGATCCG TTTCTC-3′), and two reverse primers, P_rp-1 (5′-GAAACC AACTCTACGCTG-3′) and P_rp-2 (5′-CCTATAGATGGTC TAGCTGC-3′), were used for amplification. The probe for detecting both PCR products was 5′-CGCAGCGCTAGAGG CCGGTATATTTATGAT-3′. The primers P_fp-1 and P_rp-1 have been described previously (15). P_fp-1 and P_rp-1 generate a 517-bp product, while P_fp-2 and P_rp-2 generate a 403-bp product. For amplification of the *C. trachomatis* 16S rRNA gene, the forward primer was P_fRNA (5′-GAAGGCGGT TAATACCCGCTG-3′), and the reverse primer was P_rRNA (5′-GATGGGGTTGAGACCATCC-3′). These primers generate a 398-bp product following amplification of the *C. trachomatis* 16S rRNA gene. The probe used to detect this PCR product was a T7-generated riboprobe transcribed from a fragment of the *C. trachomatis* 16S rRNA gene cloned into the plasmid vector pGEM-1 (Promega Inc.). The regions analyzed by PCR and RNP assays are illustrated in Fig. 1.

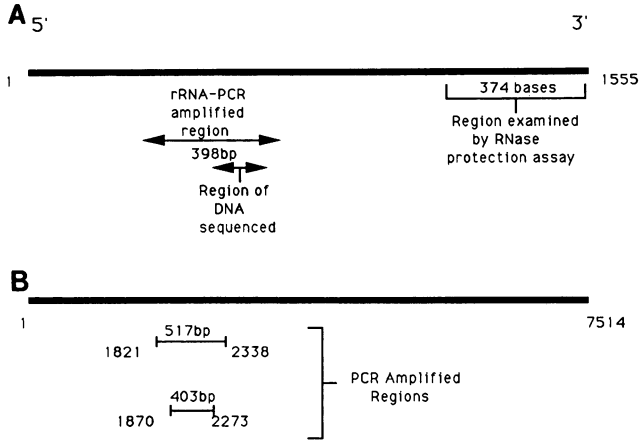

FIG. 1. Schematic representation of the regions of the chlamydial 16S rRNA gene (A) and *C. trachomatis* cryptic plasmid (B) analyzed. The rRNA-PCR assay amplified a 398-bp segment of the 16S rRNA gene, of which a portion was sequenced. The sequence numbers for the 16S rRNA gene are those of Weisburg et al. (28). The RNP assay detected 374 bases of the 3′ end of the 16S rRNA molecule. The plasmid PCR assays amplified overlapping regions of the *C. trachomatis*-specific plasmid. Sequence numbers for the plasmid are those of Comanducci et al. (8).

The riboprobe used for the RNP assay was generated by transcription of a DNA fragment containing 374 bases of the 3′ end of the *C. trachomatis* 16S rRNA gene cloned into pGEM-1. The vector was linearized with *Hpa*I and was transcribed with SP6 RNA polymerase by using a commercially available transcription kit (Promega Inc.) according to the manufacturer's instructions. The radiolabeled transcript was purified by electrophoresis on an 8% acrylamide gel. The band containing the riboprobe was cut from the gel following autoradiography and was eluted by crushing the acrylamide with a sterile glass rod, adding elution buffer (0.5 M ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA [pH 8.0], 0.1% SDS), and incubating the solution at 37°C for 6 h. The riboprobe was recovered following removal of the acrylamide by centrifugation at 15,000 × *g* for 2 min and ethanol precipitation of the nucleic acid from the remaining aqueous solution. The 425-base transcript contains 374 bases complementary to the *C. trachomatis* 16S rRNA gene and 51 bases complementary to plasmid DNA.

PCR. All PCRs were carried out under recommended containment conditions (16). PCR assays for both the 16S rRNA gene and plasmid targets were optimized for primer concentration, temperature of primer annealing, and Mg^{2+} concentration. The number of cycles yielding optimal amplification was 40. For PCR amplification of clinical samples, a 50-μl portion from a swab in 2-SP was amplified directly in a

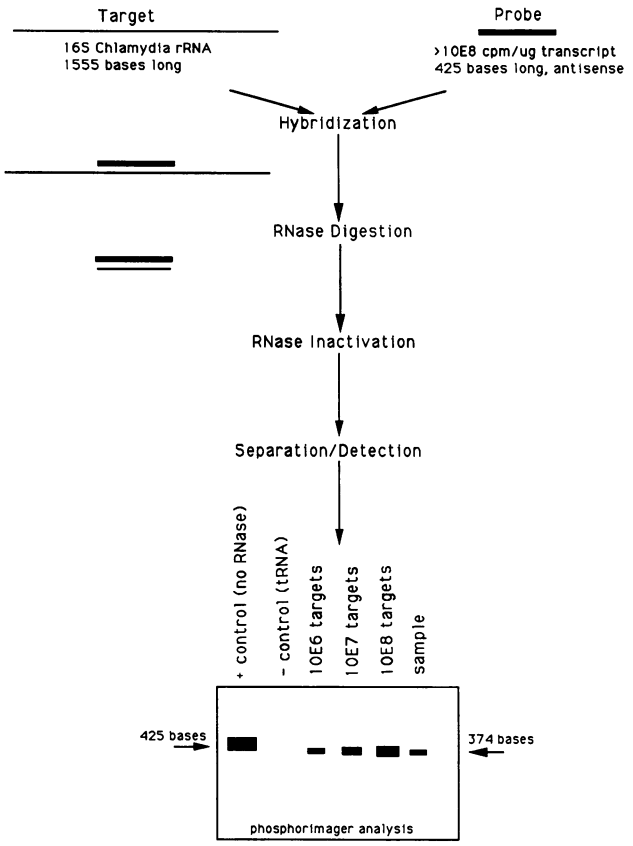

FIG. 2. Outline of the principle and methodology used for the RNP assay.

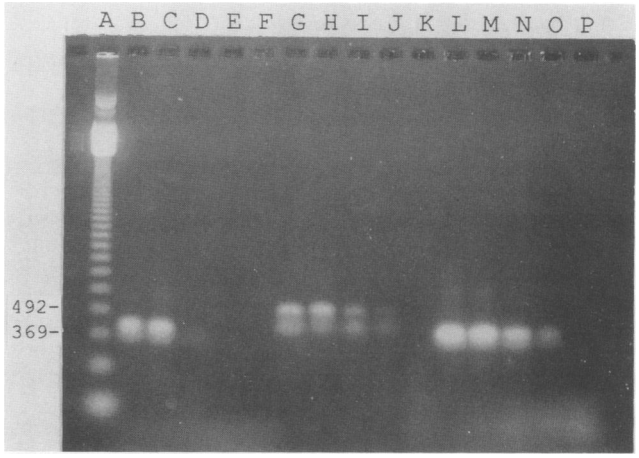

FIG. 3. Characterization of the products of the PCR assays for *C. trachomatis*. A 123-bp DNA fragment ladder was used as a marker (lane A). The expected products were 398 bp for the rRNA-PCR assay (lanes B to F), 517 bp for the plasmid-PCR assay large product (lanes G to K), and 403 bp for the plasmid-PCR assay small product (lanes L to P). The limits of detection for each PCR were determined by amplifying various numbers of EBs. The samples contained 5.0×10^4 EBs (lanes B, G, and L), 5.0×10^3 EBs (lanes C, H, and M), 5.0×10^2 EBs (lanes D, I, and N), 5.0×10^1 EBs (lanes E, J, and O), and 5.0 EBs (lanes F, K, and P).

total reaction volume of 100 μl. Alternatively a 100-μl portion was extracted with phenol-chloroform (1:1 [vol/vol]). The aqueous phase was retained, and sodium acetate (pH 7.0) was added to a final concentration of 0.3 M. The nucleic acid was precipitated with 2 volumes of ethanol and was recovered by centrifugation for 5 min at 15,000 × *g* in a microcentrifuge. The pelleted nucleic acid was washed once with 70% ethanol, dried briefly, and dissolved in 1× amplification buffer (Perkin-Elmer Cetus Inc., Norwalk, Conn.) containing 2.5 mM $MgCl_2$ and 2.5 U of Amplitaq polymerase (Perkin-Elmer Cetus Inc.). Amplifications were performed in a thermocycler (Perkin-Elmer Cetus Inc.) and consisted initially of 10 cycles of denaturation at 94°C for 1.5 min, renaturation at 42°C for 1.5 min, and polymerization at 72°C for 1.5 min. This was followed by 30 cycles of 30 s at each temperature. The products of the PCR amplifications were subjected to electrophoresis through 1.0% agarose gels and were visualized by ethidium bromide staining. The identities of the PCR products were confirmed by either Southern blot or dot blot hybridization by using radiolabeled probes specific for each PCR product (1). A radioactive signal was detected and quantitated by using a phosphorimager (Molecular Dynamics Inc., Sunnyvale, Calif.).

RNP assay. The RNP assay (1, 21) was performed by using the RPA II kit (Ambion Inc., Austin, Tex.). The protocol is outlined in Fig. 2. Briefly, the 425-base radiolabeled probe (>1.0×10^8 cpm/μg) was combined with purified chlamydial RNA, EBs, or the pellet obtained after centrifugation of 100 μl of a clinical specimen at 15,000 × *g* for 30 min. The hybridization was done in 4.0 M guanidine HCl–0.1 M Tris-HCl (pH 7.8)–20 mM EDTA for 2 h at 37°C in a total volume of 20 μl. RNase A and RNase T1 were added to final concentrations of 1 and 4,000 U/ml, respectively, and the mixtures were incubated at 37°C for 1 h. The reaction was terminated and the nucleic acid was precipitated by adding the precipitation solution included in the kit. The pellet was washed with 70% ethanol, dried, and dissolved in 8 μl of loading buffer (80% formamide, 0.1% xylene cyanol, 0.1% bromophenol blue, 2 mM EDTA). Samples were heated at 90°C for 4 min prior to loading onto a 1.4% agarose gel. Following electrophoresis, the gels were dried and analyzed by using a phosphorimager to detect and quantitate the radioactive signal.

Nucleic acid sequencing. The products obtained from the amplification of 16S rRNA genes were purified by using a MagicPrep PCR purification kit (Promega Inc.). PCR products were sequenced by using a commercially available ^{35}S-sequencing kit (Sequenase; United States Biochemicals Inc., Cleveland, Ohio). Sequencing was performed by using one of two primers: forward (5′-GAATTTGAGCGTACCAG GTAA-3′) or reverse (5′-ATGGTCCCATAGATTAGG-3′). Sequences were read manually and were analyzed by using the University of Wisconsin Genetics Computer Group sequence analysis package (9).

RESULTS

Characterization of PCRs. As shown in Fig. 3, the PCR assay directed against the *C. trachomatis* 16S rRNA gene gave rise to the predicted 398-bp product, while the PCR assays directed against the *C. trachomatis*-specific plasmid generated the predicted 517- and 403-bp products. The sensitivities of the PCR assays were 500 EBs for the 16S rRNA gene target and 50 EBs for the plasmid target.

Each PCR was examined for its specificity by amplifying the nucleic acids from a broad range of microorganisms. As shown in Table 1, both PCR assays were specific for *C.*

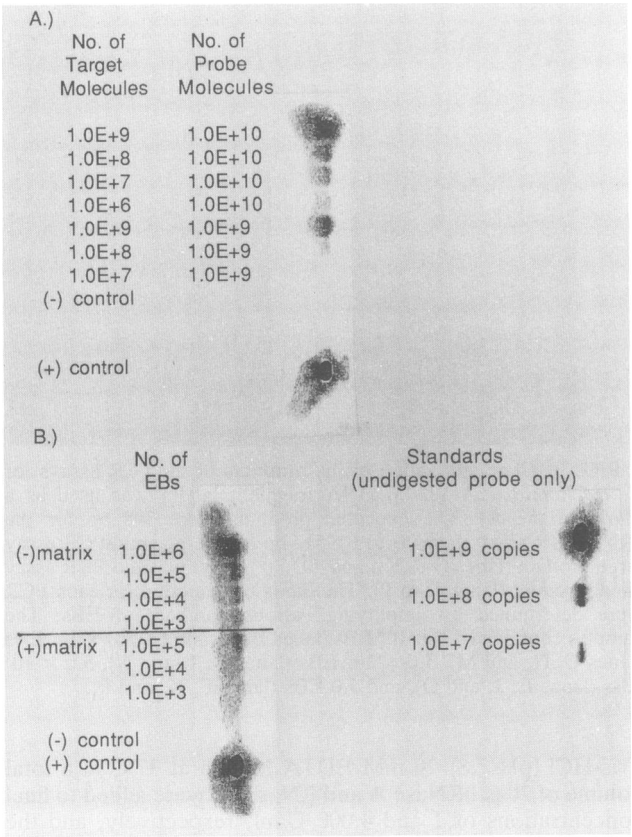

FIG. 4. Characterization of the RNP assay by phosphorimager analysis. (A) Optimization of the signal-to-noise ratio. Either 1.0×10^{10} or 1.0×10^{9} probe molecules were hybridized with various numbers of target molecules. The negative control was 1.0×10^{10} probe molecules hybridized with 25 μg of yeast RNA. The positive control was 1.0×10^{9} probe molecules used as a size marker for the agarose gel analysis. (B) Detection limits of the RNP assay on purified *C. trachomatis* EBs in the presence or absence of bovine cervical mucus. The controls were as described above. The standards were various numbers of undigested probe molecules used for signal standardization of the phosphorimager.

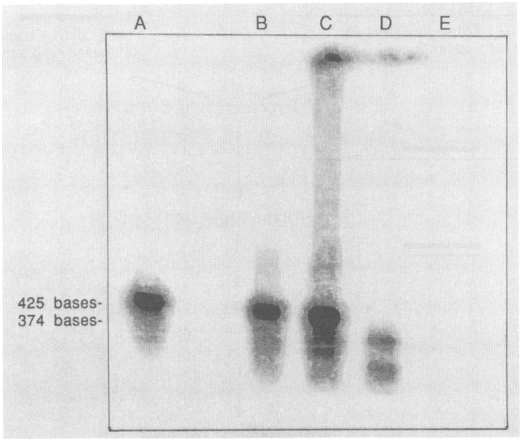

FIG. 5. Reactivity of chlamydial 16S rRNA in the RNP assay. Purified total RNA (approximately 1.0×10^{9} molecules of 16S rRNA) from *C. trachomatis* (lane B), *C. psittaci* (lane C), or *C. pneumoniae* (lane D) or 25 μg of yeast tRNA (lane E) was hybridized with 1.0×10^{10} molecules of the *C. trachomatis* probe and analyzed by the RNP assay. The radioactive signal was detected by using a phosphorimager. Lane A, 1.0×10^{9} molecules of undigested probe as a control.

trachomatis. In addition, a computer search of nucleic acid sequences deposited in GenBank revealed no sequence homology with other organisms, including those either commonly associated with urogenital pathologies, such as *Ureaplasma urealyticum* and *Gardnerella vaginalis*, or those found in the normal flora of the urogenital tract.

Characterization of the RNP assay. The methodology and theoretical results from the RNP assay are shown in Fig. 2. The 425-base riboprobe should protect a region of the 16S rRNA 374 bases in length. The probe is homologous to the 3′ end of the 16S rRNA of *C. trachomatis* (Fig. 1). Although DNA may also be protected in this assay, the copy number of 16S rRNA is considerably greater than that of the DNA (17). Therefore, the assay products observed are the result of protection of 16S rRNA rather than the gene itself.

Initially, the probe concentration was optimized to give the greatest sensitivity and least amount of noise. As shown in Fig. 4A, the signal-to-noise ratio was greatest at an input of 1.0×10^{10} copies of the probe. At that concentration of probe, the assay had a lower detection limit of 1.0×10^{6} copies of 16S rRNA target. The sensitivity of the assay was determined for *C. trachomatis* EBs, and as shown in Fig. 4B, the sensitivity was determined to be approximately 1.0×10^{3} EBs. A phosphorimager was used to quantitate the signal generated from the RNP assay products for purified nucleic acid and for whole EBs. The signal comparison indicated that 1 EB contains approximately 1.0×10^{3} copies of 16S rRNA. This result was confirmed by using three separate preparations of *C. trachomatis* EBs. To estimate the amount of 16S rRNA found in clinical specimens, a scale of 0 to 4+ was used, where 1+ is equal to 1.0×10^{6} targets, 2+ is equal to 1.0×10^{7} targets, 3+ is equal to 1.0×10^{8} targets, and 4+ is equal to or greater than 1.0×10^{9} targets present in a 100-μl specimen volume analyzed. Each assay rating had a range of ±0.05 log unit.

The RNP assay was repeated on EBs in bovine cervical mucus to examine the effects of a clinical matrix on assay sensitivity. Figure 4B shows that the sensitivity of the RNP assay in a simulated clinical matrix was approximately the same as the result obtained for purified EBs.

It has been reported that RNP assays can distinguish between nucleic acid molecules that differ by a single base change (21). When the RNP assay was applied to representative samples of all three *Chlamydia* species, *C. trachomatis* and *C. psittaci* showed similar reaction patterns, whereas *C. pneumoniae* had a significantly different reaction pattern (Fig. 5). The two faint bands below the major 374-bp band generated by RNP assay of *C. psittaci* (Fig. 5, lane C) probably represent partial cleavage products of the *C. psittaci* 16S rRNA-riboprobe hybrid complex. Within the region of the 16S rRNA protected by the riboprobe, the nucleotide sequence of *C. psittaci* 16S rRNA differs from that of *C. trachomatis* by two base changes located internally 56 and 96 nucleotides from the 5′ end of the probe (27, 28). If these sites were partially cleaved, we would expect the generation of additional fragments of 318 and 278 bp. The reason that these sites were not recognized 100% of the time is unclear, but it may be related to the high degree of secondary structure in this region of the 16S rRNA that renders it partially resistant to RNase cleavage. The RNP assay did not detect other microorganisms.

TABLE 2. Analysis of clinical samples for the presence of *Chlamydia* spp.

GTS no.[a]	Sample source[b]	Sex[c]	Chlamydial assay results			
			Culture[d]	RNP	Plasmid-PCR	rRNA-PCR
1	Urethral	M	−	−	−	−
2	Cervical	F	−	−	−	−
3	NI[e]	F	−	4+	−	+
4	NI	F	−	−	−	−
5	NI	F	−	−	−	−
6	NI	F	−	−	−	−
7	Cervical	F	−	−	−	−
8	NI	F	+	3+	+	+
9	Cervical	F	−	−	−	−
10	Cervical	F	−	1+	−	+
11	Cervical	F	−	−	−	−
12	Rectal	M	−	−	−	−
13	Cervical	F	−	3+	+	+
14	Cervical	F	−	−	−	−
15	Cervical	F	+	1+	+	+
16	Cervical	F	−	−	−	−
17	Cervical	F	−	4+	−	+
18	Rectal	F	−	4+	−	+
19	Vaginal	F	−	−	−	−
20	Urethral	M	+	−	+	+
21	Cervical	F	−	2+	−	+
22	Vaginal	F	−	−	−	−
23	Cervical	F	−	2+	−	+
24	NI	F	−	−	−	−
25	Cervical	F	−	−	−	−
26	Cervical	F	−	−	−	−
27	Cervical	F	−	−	−	−
28	Bronchial[f]	M	−	1+	−	ND[g]
29	Eye[h]	M	+	1+	+	+
30	Cervical	F	+	1+	+	+
31	Cervical	F	−	1+	−	+
32	Cervical	F	−	−	−	−
33	NI	M	−	−	−	−
34	Cervical	F	−	−	−	−
35	Cervical	F	+	1+	+	+
36	Rectal	F	−	1+	−	+
37	Uretha	M	−	−	−	−
38	Endotrach[i]	F	−	−	−	−
39	Culdesac[j]	F	−	1+	−	+
40	NI	NI	−	−	−	−

[a] GTS, GENE-TRAK Systems.
[b] Samples consisted of swabs placed in 2-SP, except where indicated otherwise.
[c] M, male; F, female.
[d] A positive culture result was defined as the detection of one or more fluorescent inclusions following staining of infected cultures with either a genus- or a species-specific fluorescein-conjugated monoclonal antibody.
[e] NI, not indicated.
[f] Bronchial wash.
[g] ND, not done because of an insufficient sample.
[h] Eye swab.
[i] Endotracheal aspirate.
[j] Cul-de-sac fluid.

Analysis of clinical specimens. Culture and PCR and RNP assay results with 40 clinical specimens are given in Table 2. All culture and immunofluroescent assay characterizations were initially done by the hospital in the course of routine analysis. Each specimen was subjected to PCR assays against the 16S rRNA gene and the *C. trachomatis*-specific plasmid as well as the RNP assay. A total of 22 specimens were negative by all criteria for the presence of chlamydiae. *C. trachomatis* was isolated from six of the specimens. HeLa 229 cells rather than McCoy cells were used for culture because HeLa 229 cells support the growth of both *C. trachomatis* and *C. pneumoniae*.

All six of the *C. trachomatis* culture-positive samples were positive by the rRNA-PCR and the plasmid-PCR assays. The RNP assay was positive for five of the culture-positive specimens. The negative result in the remaining culture-positive specimen (specimen 20) was probably due to the presence of *C. trachomatis* below the detection limits of the RNP assay. One specimen (specimen 13) was positive by all criteria except culture. We considered this a false-negative culture result. Specimen 28 was weakly positive by the RNP assay. Because this specimen was negative by the other criteria used, we considered this to be a false-positive RNP result, the cause of which is unknown.

Surprisingly, nine samples were positive in the rRNA-PCR assay but negative by culture and the plasmid-PCR assay. Minor sequence variations have been reported for the *C. trachomatis*-specific plasmid (7); these sequence variations might have contributed to a false-negative result. However, when a second region of the plasmid was amplified (Fig. 1), the results were the same. Amplification of plasmid sequences occurred only in those specimens which were culture positive. Because of the precautions taken during amplification, the positive rRNA-PCR assay results on plasmid-PCR-negative samples did not appear to be false positives because of PCR carryover contamination. These samples appeared to be unique in that the presence of the *C. trachomatis* 16S rRNA gene was detected in the absence of the *C. trachomatis* cryptic plasmid.

In order to verify the presence of the *C. trachomatis* 16S rRNA gene in these samples, RNP assays were carried out on each sample to detect the rRNA product of the gene. In all cases, various levels of chlamydial 16S rRNA were detected. The RNP assay was targeted to sequences which did not overlap the rRNA-PCR-amplified region, such that contamination with rRNA-PCR product, if it occurred, would not generate a false-positive result by the RNP assay. Therefore, we concluded that these samples were true positives containing the *C. trachomatis* 16S rRNA gene and its rRNA product.

The rRNA-PCR and RNP assay results indicating the presence of a chlamydial infection were further supported by patient pathology. Four of the nine patients with positive rRNA-PCR and RNP results (patients 10, 17, 21, and 31) had a clinical diagnosis of pelvic inflammatory disease. Therefore, the molecular detection of chlamydial nucleic acid in these samples was consistent with the pathology.

DNA sequence analysis of PCR products. Although we tested both the PCR assays and the RNP assays for their specificities, the final proof that these samples contained *C. trachomatis* was confirmed by nucleic acid sequencing of the PCR products. The PCR products from four of the variant samples were purified and subjected to partial DNA sequence analysis. As shown in Fig. 6, all four of the isolates were completely homologous to *C. trachomatis* over the region sequenced. A summary of the characteristics of the chlamydiae found in the variant specimens in comparison with the characteristics of known *Chlamydia* species is shown in Table 3. The data suggest that these samples contain a variant form of *C. trachomatis* which lacks the *C. trachomatis*-specific plasmid.

DISCUSSION

We tested samples submitted for diagnosis of chlamydial infection using several different nucleic acid-based tests. To

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Nucleotide
   No.      624                                                    670
C.trach.   TAGGCGGAAA  GGTAAGTTAG  TTGTCAAAGA  TCGGGGCTCA  ACCCCGA
GTS#17     ..........  ..........  ..........  ..........  .......
GTS#18     ..........  ..........  ..........  ..........  .......
GTS#21     ..........  ..........  ..........  ..........  .......
GTS#23     ..........  ..........  ..........  ..........  .......
C.psittaci ..........  ..A...A...  ....T...TC  .T........  .....A.
C.pneumo.  ..........  ..A...A...  ....T...TC  .T........  .....A.
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FIG. 6. Comparison of the partial nucleotide sequence of the rRNA-PCR assay-amplified products from four chlamydial variants, *C. trachomatis*, *C. psittaci*, and *C. pneumoniae*. GTS, GENE-TRAK Systems. Nucleotide sequence numbers are those of Weisburg et al. (27, 28).

our knowledge, this is the first application of a quantitative test such as the RNP assay to analysis of chlamydiae. The RNP assay was used for several reasons. First, we initially screened urogenital samples using a PCR directed against a region of the DNA of the 16S rRNA gene. In contrast, the RNP assay detects a nonoverlapping region of the RNA product of the 16S rRNA gene. The presence of the actual 16S rRNA is independent confirmation of the presence of the actual 16S rRNA gene by using a criterion different from detection of the DNA sequence itself. This is analogous to detecting a gene encoding a structural protein and confirming the presence of the gene by detecting either the mRNA or protein product of the gene.

Second, RNP assays generally show greater sensitivity than Northern blotting for detection of RNA (21) and, in the present study, were useful in obtaining a quantitative measure of chlamydial infection. We showed in the study described here that the copy number of 16S rRNA molecules in an EB is approximately 1.0×10^3. On the basis of this estimate, the RNP assay had a sensitivity of 1,000 EBs, whereas the PCR directed against the 16S rRNA gene or the *C. trachomatis*-specific plasmid had a sensitivity of 500 or 50 EBs, respectively. The number of molecules of chlamydial 16S rRNA detected in the clinical specimens varied greatly, ranging from below 1.0×10^6 to greater than 1.0×10^9 per 100-μl sample. However, no correlation was seen between the target concentration and pathology. For example, patients with a diagnosis of pelvic inflammatory disease had chlamydial 16S rRNA concentrations encompassing the entire detectable range.

Third, the RNP assay is extremely specific, potentially much more so than a PCR. It can potentially differentiate RNA-RNA hybrids that differ by only a single-base mismatch. Therefore, it allowed us to quickly recognize similarities and differences between organisms prior to DNA sequencing. While the RNP assay described here could clearly differentiate *C. trachomatis* from *C. pneumoniae* and other bacteria, it had difficulty differentiating *C. trachomatis* from *C. psittaci*. This was not surprising, since the 16S rRNA genes of *C. trachomatis* and *C. psittaci* are less than 5% divergent (27).

TABLE 3. Summary of the characteristics of *Chlamydia* spp.

Organism	Isolation in HeLa cells	Assay result			Sequence identity
		rRNA-PCR	Plasmid-PCR	RNP	
Chlamydial variants	−	+	−	+	*C. trachomatis*
C. trachomatis	+	+	+	+	*C. trachomatis*
C. pneumoniae	+	−	−	−	NA[a]
C. psittaci	+	−	−	+	NA

[a] NA, not amplified by the rRNA-PCR assay.

Isolation of chlamydiae in tissue culture remains the "gold standard" for diagnosis. However, discrepant results have been frequently reported when culture is compared with newer methodologies (12, 15, 29). PCR assays are routinely reported as possessing greater sensitivity than culture methods for chlamydiae (12, 15, 29) and are relatively unaffected by variability in patient sampling. During our studies we noted discordance between culture results and PCR results in the form of significantly more positive rRNA-PCR results than positive culture results. We also noted discordance between the results of a PCR assay directed against the *C. trachomatis* 16S rRNA gene and one directed against an endogenous plasmid reported to be conserved in *C. trachomatis*. Since both PCR assays were performed simultaneously under identical, carefully controlled conditions, it was not possible to attribute the discordance to either PCR carryover contamination in the assays or to a PCR-inhibitory substance present in the samples.

In the present study of patients with suspected *C. trachomatis* infections, the plasmid-PCR and the rRNA-PCR assays were in 100% agreement on culture-positive specimens. None of the samples tested positive by the plasmid-PCR assay only. However, nine samples yielded positive results with the rRNA-PCR assay and negative results by the plasmid-PCR assay. Two separate PCR assays were used to analyze a highly conserved region of the *C. trachomatis*-specific plasmid. Although the regions of the plasmid amplified in each assay overlap, the primer sites are unique. Thus, these are, in fact, two distinct PCR assays. The presence of a substance inhibitory to the plasmid-PCR assay was ruled out because of the positive results obtained by the rRNA-PCR assay on the same samples. Therefore, these samples are true negatives by the plasmid-PCR assay.

As we showed in the study described here, the rRNA-PCR assay is specific for *C. trachomatis* on the basis of its inability to amplify other organisms (Table 1). The RNase protection assay independently verified the presence of the RNA product of a chlamydial 16S rRNA gene in the variant specimens. Furthermore, the sequence obtained for the variant chlamydia rRNA-PCR amplification products was identical to the *C. trachomatis* sequence found in GenBank (29). Therefore, the discordant results were not due to nonspecific detection of other bacteria.

Although the partial sequence analysis of the products of the rRNA-PCR establishes that the putative chlamydiae present in these specimens are closely related to *C. trachomatis*, we cannot say that they are definitively *C. trachomatis*, since the sequence data are limited. However, the *C. trachomatis*-like variant contained in these samples differed significantly from typical *C. trachomatis* serovars in their lack of the cryptic plasmid. We are trying to amplify the remainder of the 16S rRNA gene for further sequence analysis and identification.

The role of the *C. trachomatis*-specific plasmid is unclear. One of the open reading frames found in the plasmid bears a striking resemblance in its coding potential to the *dnaB* gene of *Escherichia coli* and gene 12 of bacteriophage P22 (8, 11, 25). Because of this similarity it has been proposed that a plasmid-encoded protein may play a role in DNA binding or replication. Because of its high degree of conservation, it has also been proposed that it may play a role in the growth, development, or pathogenesis of *C. trachomatis* (8, 11, 25). One isolate of *C. trachomatis* serovar L1 has been reported to lack this plasmid, thus calling into question the idea that it is necessary for *C. trachomatis* growth (23). Our data support this conclusion since, in nine specimens, *C. trachomatis*-like organisms were detected, all of which lacked the cryptic plasmid. However, none of these isolates grew in tissue culture. In contrast, all isolates in which plasmid sequences were detected were culture positive.

There are at least two explanations for these results. The analysis reported here may have identified a *C. trachomatis*-like strain which lacks the cryptic plasmid, or alternatively, the plasmid may have been lost during the infection process. Further work will be necessary to resolve this question as well as to determine the frequency with which this pattern of results is observed. It is clear, however, that caution must be used when targeting the cryptic plasmid as a means of detecting chlamydial infections. Since the organisms described here could be neither isolated by standard tissue culture techniques and detected with *C. trachomatis*-specific immunoassays nor amplified by using the plasmid as a target, a significant number of chlamydial infections might be missed. The patients in the present study had pathologies consistent with chlamydial infections, yet culture and plasmid-PCR assay results were negative. The rRNA-PCR and RNP assay information was more consistent with the pathologies.

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