Touchdown Enzyme Time Release-PCR for Detection and Identification of *Chlamydia trachomatis*, *C. pneumoniae*, and *C. psittaci* Using the 16S and 16S-23S Spacer rRNA Genes

GUILLERMO MADICO,1,2 THOMAS C. QUINN,1,2 JENS BOMAN,3 AND CHARLOTTE A. GAYDOS1*

Division of Infectious Diseases, The Johns Hopkins University, Baltimore, Maryland; Laboratory of Immunoregulation, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland; and Umeå University, Umeå, Sweden

Received 21 July 1999/Returned for modification 22 October 1999/Accepted 8 December 1999

Three touchdown enzyme time release (TETR)-PCR assays were used to amplify different DNA sequences in the variable regions of the 16S and 16S-23S spacer rRNA genes specific for *Chlamydia trachomatis*, *Chlamydia pneumoniae*, and *Chlamydia psittaci* as improved tests for sensitive diagnosis and rapid species differentiation. The TETR-PCR protocol used 60 cycles of amplification, which provided improved analytical sensitivity (0.004 to 0.063 inclusion-forming unit of *Chlamydia* species per PCR). The sensitivity of TETR-PCR with primer set CTR 70-CTR 71 was 96.7%, and the specificity was 99.6%, compared to those of the AMPLICOR PCR for the detection of *C. trachomatis* in vaginal swab samples. TETR-PCR for *C. pneumoniae* with primer set CPN 90-CPN 91 was 90% sensitive and 93.3% specific compared with a nested PCR with primer set CP1/2-CPC/D for clinical respiratory samples. TETR-PCR for *C. psittaci* with primer set CPS 100-CPS 101 showed substantial agreement with cell culturing (κ, 0.78) for animal tissue samples. Primer sets were then combined into a single multiplex TETR-PCR test. The respective 315-, 195-, and 111-bp DNA target products were precisely amplified when DNA from each of the respective *Chlamydia* species or combinations of them was used. Multiplex chlamydia TETR-PCR correctly identified one strain of each of the 15 serovars of *C. trachomatis*, 22 isolates of *C. pneumoniae*, and 20 isolates of *C. psittaci*. The primer sets were specific for each species. No target products were amplified when DNA from *C. pecorum* or a variety of other microorganisms was tested for specificity. TETR-PCR with primers selected for specific sequences in the 16S and 16S-23S spacer rRNA genes is a valuable test that could be used either with individual primers or in a multiplex assay for the identification and differentiation of *Chlamydia* species from culture isolates or for the detection of chlamydiae in clinical samples.

Three species in the genus *Chlamydia* can cause disease in humans: *C. pneumoniae*, *C. trachomatis*, and *C. psittaci*. Diseases caused by chlamydiae include trachoma; respiratory infection, including pneumonia; and sexually transmitted infection of reproductive organs, including cervicitis, pelvic inflammatory disease, urethritis, and epididymitis (24, 34, 38). Pneumonia in adults can be caused by both *C. pneumoniae* and *C. psittaci* (psittacosis), and that in newborn children can be caused by *C. trachomatis* (19). Recent isolations of *Chlamydia* species in cardiovascular tissue have also been reported, suggesting a broader range of diseases and syndromes (36).

*Chlamydia* species are intracellular bacteria that require tissue culture techniques to be isolated (16, 28). Identification of species depends on phenotype differences and immunoreactivity to species-specific monoclonal antibodies (37). Also, molecular amplification techniques based on genomic sequences have been used for the differentiation of *Chlamydia* species (32, 40). In addition, various techniques to detect and differentiate *Chlamydia* species have been developed; they include DNA hybridization with genomic DNA probes (3), restriction fragment polymorphism analysis of PCR-amplified products (20, 31, 43), and nested PCR (5, 32). Recently, molecular amplification techniques have been demonstrated to have improved sensitivity compared to culturing and other diagnostic assays for the detection of chlamydia infection in urine, endo-, cervix, throat and urethral swabs, sputa, bronchoalveolar lavage fluids, and eye secretions (4, 5, 27, 35, 40, 41).

In this study, three primer sets targeting the 16S and 16S-23S spacer rRNA genes were designed for the detection of *C. trachomatis*, *C. pneumoniae*, and *C. psittaci*. Primers were designed to target DNA sequences that were most heterogeneous in the 16S and 16S-23S spacer rRNA genes of the three *Chlamydia* species (11, 15, 33). The three amplified products were designed to be sufficiently different in size so that they could be easily discriminated when detected by gel electrophoresis. An improved PCR protocol was also developed. Four characteristics distinguish our touchdown enzyme time release (TETR)-PCR: a hot start polymerase enzyme to avoid artifacts before amplification, a touchdown protocol for annealing temperatures to improve the specificity of primer binding, an enzyme time release protocol to allow 60 cycles of amplification for improved analytical sensitivity, and multiplex target amplification capabilities.

The sensitivity, specificity, and agreement of TETR-PCR with various primer sets used to detect DNA of *C. trachomatis* in vaginal swab samples, DNA of *C. pneumoniae* in clinical respiratory specimens, and DNA of *C. psittaci* in animal tissue samples were measured. TETR-PCR with primer set CTR 70-CTR 71 (CTR 70/71) for the detection of *C. trachomatis* was compared to a commercial PCR test (AMPLICOR *Chlamydia trachomatis* test; Roche Diagnostics Systems, Branchburg, N.J.). TETR-PCR with primer set CPN 90-CPN 91 (CPN 90/91) for the detection of *C. pneumoniae* was compared to a nested PCR with primer sets CP1-CP2 and CPC-CPD (CP1/2-CPC/D) (5, 40). TETR-PCR with primer set CPS 100-CPS

---

*Corresponding author. Mailing address: Division of Infectious Disease, The Johns Hopkins University, 1151 Ross Research Bldg., 720 Rutland Ave., Baltimore, MD 21205. Phone: (410) 614-0932. Fax: (410) 614-9775. E-mail: cgaydos@welch.jhu.edu.
Pneumoniae, C. psittaci, 20 strains of (pH 8.2), and 50 obtained from our freezer repository to be tested by multiplex TETR-PCR respiratory infection in northern Sweden (5). These included sputum laboratory. (AMPLICOR) and kept at 4°C to prevent DNA degradation until arrival at the collected by clinicians were placed in 1 ml of specimen transport medium were invited to participate in the study from March to September 1997. During Disease Control Clinic at the Womack Army Medical Center, Fort Bragg, N.C., were inoculated, incubated, fixed, and stained as described above in duplicate wells. (Behring Diagnostics Inc., Cupertino, Calif.) or for C. trachomatis for 60 min at 35°C. Following aspiration of the supernatant, 1 ml of inoculation replicate into shell vials containing monolayers of buffalo green monkey kidney (9, 16). Chlamydia strains (200 g) of incubation at 35°C, a second passage was performed. The DNAs from chlamydia cultures, processed respiratory samples were combined into a multiplex TETR-PCR to detect and identify C. pneumoniae, C. trachomatis, or C. psittaci tissue culture isolates in a single test.

MATERIALS AND METHODS

Clinical samples. Vaginal samples for the detection of C. trachomatis were obtained as part of another study designed to assess the utility of a vaginal sample in the outbreak detection of sexually transmitted diseases (29). This study was approved by the institutional review boards of The Johns Hopkins University and Fort Bragg Womack Army Medical Center. Consecutive active-duty military women, 18 to 59 years old and attending the Epidemiology and Disease Control Clinic at the Womack Army Medical Center, Fort Bragg, N.C., were invited to participate in the study from March to September 1997. During enrollment, 89% (265 of 298) consented to participate. Vaginal swab samples collected by clinicians were placed in 1 ml of specimen transport medium (AMPLICOR) and kept at 4°C to prevent DNA degradation until arrival at the laboratory. Fifty respiratory specimens were obtained in 1994 during an outbreak of C. pneumoniae respiratory infection in northern Sweden (5). These included sputum (3), and 2 strains of (5, 39, 40). The optimal concentration of primers was obtained by checkerboard combination of primer sets and nested CP1/2-CPC/D PCR were determined by extracting psittaci stock culture DNA preparations (CPS 100/101). Oligonucleotides were synthesized and purified after synthesis on a DNA synthesizer (380; Applied Biosystems, Norwalk, Conn.) at the Johns Hopkins Genetic Core Laboratory. The sequences were as follows: for C. trachomatis (D85722), Escherichia coli (M25588), Haemophilus ducreyi (M75864), Neisseria gonorrhoeae (X07714), Gardnerella vaginalis (M8744), Treponema pallidum (M88762), and Trichomonas vaginalis (U17350) (Fig. 1). The targeted DNA sequence of primer set CPS 90/91 was aligned with the 16S rRNA sequences of other bacteria present in respiratory samples (GenBank accession number): C. pneumoniae (L06108), Staphylococcus aureus (L37597), Streptococcus pneumoniae (Z22807), Legionella pneumophila (M59157), Klebsiella pneumoniae (X87276), Haemophilus influenzae (X79796), and E. coli (M25588) (Fig. 2). In the same way, the targeted DNA sequence of primer set CPS 100/101 was aligned with the 16S and 16S-23S rRNA sequences of other chlamydia species. The optimal concentration of primers was determined by the manufacturer of the polymerase (Perkin-Elmer, Branchburg, N.J.). Since 1.0 mM MgCl2 is a component of AMPLICOR specimen buffers, 1.5 mM MgCl2 was added to the PCR mixtures when vaginal swabs were tested. Otherwise, 2.5 mM MgCl2 was used when cultures or tissue or respiratory samples were tested as recommended by the manufacturer of the polymerase (Perkin-Elmer). A touchdown method for thermal cycling (10) combined with an enzyme time release protocol was used with a DNA thermal cycler (480; Perkin-Elmer Cetus, Norwalk, Conn.). Cycling times were 75 s at 95°C (to activate a small fraction of the heat-activated DNA polymerase), followed by 60 cycles of denaturation at 94°C for 45 s, annealing beginning at 62°C and ending at 52°C for 45 s, and extension at 72°C for 45 s. Following a 15 min initial annealing temperature. The analytical sensitivities of TETR-PCR with the various primer sets and nested CPI/2-CPC/D PCR were determined by extracting DNA from fourfold serial dilutions of stock cultures of C. trachomatis VR 348 serovar E, C. pneumoniae AO3, and C. psittaci SM006. Chlamydia stock cultures of known titer were diluted in culture media containing tissue culture cells to keep the level of background DNA constant. The analytical sensitivities of primer sets CTR 70/71, CTR 70/71, and CPS 100/101 were also determined with a previously described PCR protocol (14) which did not use a TETR-PCR protocol. In addition, analytical sensitivity with spiked clinical specimens were tested by use of the TETR-PCR protocol described above but with an alternative DNA polymerase (2 U of HotStarTaq DNA polymerase; Qiagen, Valencia, Calif.) and 1.5 mM MgCl2.

Multiplex TETR-PCR. Multiplex TETR-PCR was performed with 5-μl chlamydia culture DNA preparations (~25 IFU/PCR) processed by the Chelex method. The three primer sets, CTR 70/71, CTR 70/71, and CPS 100/101, were combined for the simultaneous detection and identification of the three Chlamydia species. The optimal concentration of primers was obtained by checkerboard combination with 1 IFU each of C. trachomatis, C. pneumoniae, and C. psittaci per PCR. Concentrations of primers ranging from 10 to 30 pmol were tested. Optimal primer concentrations were 10 pmol for primers CTR 70, CTR 71, and CPS 100, 25 pmol for CPS 90, 25 pmol for CPR 90, and 30 pmol for CPS 100 and CPS 101. The analytical sensitivities of primers were determined by the multiplex TETR-PCR described above except for the amount of primers (a total of 115 pmol of primers per reaction in the multiplex TETR-PCR).
<table>
<thead>
<tr>
<th>Isolates</th>
<th>Result obtained with the following primer set (PCR product, bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. trachomatis isolate</td>
<td>CTR 70/71 (315) CPN 90/91 (195) CPS 100/101 (111)</td>
</tr>
<tr>
<td>C. pneumoniae AR39</td>
<td>21 2 VR 571 ser. A</td>
</tr>
<tr>
<td></td>
<td>22 1 6BC</td>
</tr>
<tr>
<td></td>
<td>21 2 VR 347 ser. Ba</td>
</tr>
<tr>
<td></td>
<td>22 1 AW-17-9-6</td>
</tr>
<tr>
<td></td>
<td>21 2 VR 572 ser. C</td>
</tr>
<tr>
<td></td>
<td>22 1 B577-2</td>
</tr>
<tr>
<td></td>
<td>21 2 VR 885 ser. D</td>
</tr>
<tr>
<td></td>
<td>22 1 FC-GRW#97-5</td>
</tr>
<tr>
<td></td>
<td>22 1 VR 878 ser. G</td>
</tr>
<tr>
<td></td>
<td>22 1 LW613-11</td>
</tr>
<tr>
<td></td>
<td>22 1 VR 879 ser. H</td>
</tr>
<tr>
<td></td>
<td>22 1 LW646-4</td>
</tr>
<tr>
<td></td>
<td>22 1 T2364</td>
</tr>
<tr>
<td></td>
<td>21 2 VR 886 ser. J</td>
</tr>
<tr>
<td></td>
<td>22 1 11245 PA</td>
</tr>
<tr>
<td></td>
<td>22 1 VR 887 ser. K</td>
</tr>
<tr>
<td></td>
<td>22 1 18331 C</td>
</tr>
<tr>
<td></td>
<td>22 1 AO3</td>
</tr>
<tr>
<td></td>
<td>22 1 VR 902 ser. L2</td>
</tr>
<tr>
<td></td>
<td>22 1 21339</td>
</tr>
<tr>
<td></td>
<td>22 1 AL-1</td>
</tr>
<tr>
<td></td>
<td>22 1 VR 903 ser. L3</td>
</tr>
<tr>
<td></td>
<td>22 1 95-28189 Nebraska</td>
</tr>
<tr>
<td></td>
<td>22 1 IOL 1515</td>
</tr>
<tr>
<td></td>
<td>22 1 95-42776 #3</td>
</tr>
<tr>
<td></td>
<td>22 1 T4 59 5 3</td>
</tr>
<tr>
<td></td>
<td>22 1 95-42776 #9</td>
</tr>
<tr>
<td></td>
<td>22 1 U1092</td>
</tr>
<tr>
<td></td>
<td>22 1 U127</td>
</tr>
<tr>
<td></td>
<td>22 1 AKRON</td>
</tr>
<tr>
<td></td>
<td>22 1 U1271</td>
</tr>
</tbody>
</table>

**Notes:**
- Positive: +
- Negative: −
- Results for C. pecorum VR 628 and VR 629 (from the American Type Culture Collection) were negative.
- Provided by Margaret R. Hammerschlag (SUNY Health Science Center, Brooklyn, N.Y.).
- Provided by Carolyn M. Black (Centers for Disease Control and Prevention, Atlanta, Ga.).
- Provided by Jim T. Summersgill (University of Louisville, Louisville, Ky.).
- Provided by Jens Boman (University Hospital of Northern Sweden).
- Isolated in the Chlamydia Laboratory, The Johns Hopkins University.
- Provided by J. Storz (Louisiana State University, Baton Rouge).
- Provided by Trudy O. Messmer (Centers for Disease Control and Prevention).
To avoid product carryover, PCRs were set up in an area physically separate from all activities involving amplified target sequences, including thermal cycling, PCR product storage, and the running of gels. A separate set of pipettes was devoted to the setup of PCRs, and aerosol barrier pipette tips were used. Negative controls, including uninoculated cell cultures, were used throughout the specimen preparation, DNA extraction, and PCR processes. A low concentration of DNA (from 10 IFU of Chlamydia per PCR) was used as a positive control and was included in every PCR run.

C. trachomatis comparison commercial PCR test. The AMPLICOR Chlamydia trachomatis test was performed by following the manufacturer's instructions. Briefly, 50 μl of processed vaginal samples or controls was added to a PCR tube containing 50 μl of master mix, including biotin-labeled primers targeting a

FIG. 1. Alignment of the target sequences of primer set CTR 70/71 in the 16S rRNA gene of C. trachomatis with the 16S rRNA genes of other vaginal pathogens. A dot indicates the same base, and a letter indicates a base different from that in C. trachomatis.

FIG. 2. Alignment of the target sequences of primer set CPN 90/91 in the 16S rRNA gene of C. pneumoniae with the 16S rRNA genes of other respiratory pathogens. A dot indicates the same base, and a letter indicates a base different from that in C. pneumoniae. A dash indicates a deletion.
Respiratory samples with discrepant results between the TETR-PCR with primer set CTR 70/71 and the AMPLICOR PCR for C. pneumoniae, and culturing for C. psittaci was calculated with the kappa test (13). Agreement was qualified with the following kappa values: <0, poor; 0 to 0.2, slight; 0.21 to 0.40, fair; 0.41 to 0.60, moderate; 0.61 to 0.80, substantial; and >0.80, excellent (12, 30).

RESULTS

The predicted 315-, 195-, and 111-bp targeted DNA products were successfully amplified by TETR-PCR with each primer set and the corresponding Chlamydia species DNA (CTR 70/71 for C. trachomatis, CTR 90/91 for C. pneumoniae, and CPS 100/101 for C. psittaci DNAs, respectively). No amplified DNA products were detected when DNA from 29 other microorganisms were tested for specificity (Acinetobacter baumanii 10292, Bordetella pertussis, Candida albicans 71, Chlamydia trachomatis 10562, Cryptococcus neoformans 116, Enterococcus cloacae 10174, Enterococcus faecalis 9385, E. coli, Giardia intestinalis ATCC 30588, K. pneumoniae 10288, K. pneumoniae, Macrococcus caseolyticus 3534, Mycobacterium tuberculosis 14323, Mycoplasma pneumoniae, N. gonorrhoeae ATCC 19424, Porphyromonas gingivalis ATCC 53878, Proteus mirabilis 10954, Pseudomonas aeruginosa 10173, S. aureus 3513, Staphylococcus epidermidis 3578, Staphylococcus hominis 4887, Streptococcus agalactiae 9385, Streptococcus faecalis 9385, V. parahemolyticus ATCC 14323, V. vulnificus ATCC 19424, V. vulnificus ATCC 19424, Yersinia enterocolitica 10288, and Y. pestis 10288).
Chlamydia trachomatis test was 100% (30 of 30) sensitive and 99.6% (234 of 235) specific.

**C. pneumoniae.** Fifty respiratory clinical samples were tested by TETR-PCR with primer set CPN 90/91 and by nested PCR with primer set CP1/2-CPC/D. Sixteen specimens were found positive and 28 were found negative by both PCR methods. Six samples had discrepant results (Table 3). Four samples with discrepant results (two TETR-PCR positive and two nested CP1/2-CPC/D PCR positive) were judged true positive by an additional nested PCR with primer set CPN A/B-pTW 50/51 (14, 17). Two additional TETR-PCR-positive discrepant specimens were not confirmed as true positive. After resolution of discrepant results, 40% (20 of 50) specimens were judged true positive. The sensitivity of TETR-PCR with primer set CPN 90/91 was 90% (18 of 20), and the specificity was 93.3% (28 of 30). Nested CP1/2-CPC/D PCR was 90% (18 of 20) sensitive and 100% (30 of 30) specific.

**C. psittaci.** Thirty-five percent (7 of 20) of the animal tissue samples had a previously positive culture reported by the reference laboratory (National Animal Disease Center). TETR-PCR with primer set CPS 100/101 detected 7 of 20 (35%) positive samples. However, one sample was culture positive and TETR-PCR negative, and one sample was TETR-PCR positive and culture negative. The agreement between the two

**TABLE 2.** Comparison of TETR-PCR with primer set CTR 70/71 and AMPLICOR PCR for the detection of *C. trachomatis* in 265 vaginal swabs

<table>
<thead>
<tr>
<th>No. of samples</th>
<th>CTR 70/71 TETR-PCR</th>
<th>AMPLICOR PCR</th>
<th>omp1 PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>29</td>
<td>+</td>
<td>+</td>
<td>NT</td>
</tr>
<tr>
<td>1</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>1</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>1</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>233</td>
<td>–</td>
<td>–</td>
<td>NT</td>
</tr>
</tbody>
</table>

* Discrepant results were resolved by a third PCR targeting the *omp1* gene of *C. trachomatis* (4). +, positive; −, negative; NT, not tested.
tests was substantial (κ, 0.78). No resolution of discrepant results was performed.

**Multiplex TETR-PCR.** Primer sets CTR 70/71, CPN 90/91, and CPS 100/101 were combined into a multiplex TETR-PCR for the identification of chlamydia tissue culture isolates in a single test. Titration of the primer concentration was performed to achieve the simultaneous amplification of all three target products. Only predicted target products were amplified, and no negative results were obtained when DNA equivalent to 1 IFU of a *Chlamydia* species was tested alone or in combinations of two or three *Chlamydia* species by multiplex TETR-PCR (Fig. 5). Multiplex TETR-PCR correctly identified all 15 different serovars of *C. trachomatis* (315-bp DNA product), 22 of 22 isolates of *C. pneumoniae* (195-bp DNA product), and 20 of 20 isolates of *C. psittaci* (111-bp DNA product). The primer sets were specific for the respective species, only predicted DNA products were amplified, and no targeted products were amplified when DNA from two *C. pecorum* strains was tested (Table 1).

**DISCUSSION**

Species-specific TETR-PCR for the detection of chlamydia DNA in the 16S and 16S-23S spacer rRNA genes is a practical and useful method that could be easily implemented in laboratories currently performing molecular amplification techniques with basic laboratory reagents and infrastructure. The three sets of primers used individually or in a multiplex assay detected and differentiated *C. trachomatis*, *C. pneumoniae*, and *C. psittaci*.

With the use of a touchdown method, TETR-PCR showed improved annealing conditions for the primers, especially in the multiplex TETR-PCR, where three set of primers with different optimal annealing temperatures were used simultaneously. Successful coamplification of multiple targeted DNA products was achieved when this protocol was used with the three primer sets, CTR 70/71, CPN 90/91, and CPS 100/101. The targeted DNA products were of different sizes and were easy to identify by means of gel electrophoresis (315 bp for *C. trachomatis*, 197 bp for *C. pneumoniae*, and 111 bp for *C. psittaci*). In addition, multiplex TETR-PCR did not require dimethyl sulfoxide, which has been previously used for coamplification of multiple DNA targets (7) but is reported to inhibit Taq polymerase (18). The enzyme time release protocol of TETR-PCR allowed 60 cycles of amplification, which improved the analytical sensitivity of the test, allowing detection of 0.004 to 0.063 IFU of *Chlamydia* species per PCR compared to 0.1 to 4 IFU when a conventional PCR protocol with 35 cycles was used (14). These levels of analytical sensitivity are better than those reported for conventional PCR protocols (6, 14, 32) and PCR-enzyme immunoassays (3, 17, 21, 41) and are equivalent to those of nested PCR assays (0.063 IFU for nested CP1/2-CPC/D PCR) (2, 40). No reduction in the analytical sensitivity was noted when the TETR-PCRs were tested with spiked clinical samples (0.004 to 0.063 IFU). In addition, similar analytical sensitivities (0.004 to 0.063 IFU) were obtained when HotStarTaq DNA polymerase was used in place of AmpliTaq Gold DNA polymerase, suggesting that HotStarTaq DNA polymerase could be used as an alternative enzyme for TETR-PCR.

The DNA sequences of primer sets CTR 70/71 and CPN 90/91 and of primer CPS 100 were selected from the 16S rRNA gene. Since the 16S rRNA gene is very conserved within *Chlamydia* species (15, 33), very few regions are distinctive for *C. psittaci* (11). The correct primer selection provided specificity for the TETR-PCRs. One primer tar-

### TABLE 3. Comparison of TETR-PCR with primer set CPN 90/91 and nested CP1/2-CPC/D PCR (40) for the detection of *C. pneumoniae* in 50 respiratory samples

<table>
<thead>
<tr>
<th>No. of samples</th>
<th>Result obtained by:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CPN 90/91 TETR-PCR</td>
</tr>
<tr>
<td>16</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
</tr>
<tr>
<td>28</td>
<td>-</td>
</tr>
</tbody>
</table>

*a* Discrepant results were resolved by a third nested PCR (14, 17). +, positive; −, negative; NT, not tested.
getting a unique chlamydia DNA sequence was sufficient to confer specificity to a primer set. Adequate specificity of the primer sets was supported by the lack of amplified products when DNAs from *C. pecorum* and other microorganisms were tested. The alignment of the target sequences of primer sets CTR 70/71, CPN 90/91, and CPS 100/101 with the 16S rRNA gene and/or 16S-23S spacer rRNA gene sequences from a selection of other pathogens and normal flora that could be present in clinical samples demonstrated that the selected primer sets, CTR 70/71, CPN 90/91, and CPS 100/101, were significantly different from the 16S rRNA gene sequences of the other organisms, thus confirming their specificity.

The 96.7% sensitivity and 99.6% specificity achieved by TETR-PCR with primer set CTR 70/71 for the detection of *C. trachomatis* in vaginal specimens were comparable to the sensitivity and specificity of the commercially available AMPLICOR *Chlamydia trachomatis* test, which includes a colorimetric hybridization assay for the detection of amplified PCR products and has been reported to have high sensitivity and specificity compared with culturing (23, 35). The agreement between the two PCR tests in this study was excellent (κ, 0.94). In addition, TETR-PCR with primer set CPN 90/91 for the detection of *C. pneumoniae* in respiratory samples was as sensitive (90%) as the nested PCR described by Tong and Silliss, which uses two consecutive PCRs and has been reported to have a high sensitivity for detecting *C. pneumoniae* DNA in respiratory specimens (40). TETR-PCR with primer set CPS 100/101 for the detection of *C. psittaci* showed substantial agreement (κ, 0.78) with culturing when evaluated with animal tissue samples.

We evaluated our set of primers combined into a multiplex TETR-PCR for the identification of *Chlamydia* species from tissue culture isolates only and not from clinical specimens. The 16S rRNA and the 16S-23S rRNA spacer genes are highly conserved (11, 15, 33), and primers based on these regions provided for the correct identification of all 57 chlamydia isolates tested by the multiplex TETR-PCR. The actual testing of the many strains of *C. pneumoniae*, *C. psittaci*, and *C. trachomatis* corroborated that the differences between strains in the 16S rRNA genes where the primers were targeted were indeed conserved differences, as there were no strains that failed to be amplified by multiplex TETR-PCR with their respective primer sets. Multiplex TETR-PCR is more difficult to perform than single-target PCR. To avoid competition, purification of primer sets was supported by the lack of amplified products of the first amplification tested with a small number of targeted DNA copies are required to prevent reduction of the analytical sensitivity.

Multiplex TETR-PCR can potentially be used for clinical samples. Messmer et al. (32) described a nested multiplex PCR targeting a different region of the 16S rRNA gene. Messmer et al. (32) were able to detect 13 specimens positive for *C. psittaci* among 75 bird tissue or feces samples (4 confirmed by culturing). One human tissue sample detected by nested multiplex PCR for *C. psittaci* was also reported (32). Multiplex TETR-PCR may also be useful for the identification of tissue culture isolates of chlamydiae from respiratory specimens, where any of the three species of chlamydiae may cause disease. The analytical sensitivity can be reduced slightly when multiple primer sets are combined in a single PCR. Competition of the primers for dNTPs and DNA polymerase enzyme, the increased probability of amplification of spurious DNA products, and the increased chance for primer-dimer formation are the problems to circumvent with good primer design and PCR techniques.

We extracted the DNA by boiling the cultures with a suspension containing chelating resin as described previously (42). This method is simple, inexpensive, and easy to perform and requires minimal sample manipulation, which may reduce the chances for contamination (29). TETR-PCR being a single amplification assay provided fewer chances for carryover contamination than nested PCR, in which amplified DNA products of the first amplification are transferred to a second PCR amplification.

In conclusion, we have developed a sensitive and specific TETR-PCR method for the identification of all *Chlamydia* species that can cause disease in humans by using the 16S rRNA and 16S-23S spacer rRNA genes. This single-step TETR-PCR method combines (i) a DNA polymerase activated at 95°C, simulating a hot start to prevent DNA synthesis before thermal cycling; (ii) a touchdown protocol that, by reducing the annealing temperature from high to low, improved the specificity of the primers; (iii) the gradual activation of the DNA polymerase enzyme during the thermal cycling, allowing 60 amplification cycles for improved analytical sensitivity; and (iv) a range of annealing temperatures (62 to 52°C) that allowed specific coamplification of multiple DNA targets with primers with different annealing temperatures. These four characteristics provided for sensitivities and specificities comparable to those of the AMPLICOR *Chlamydia trachomatis* test with vaginal swab samples and the nested PCR for *C. pneumoniae* with respiratory samples and substantial agreement with animal tissue culturing for *C. psittaci*.

ACKNOWLEDGMENTS

This study was partially supported by grant DAMD17-96-1-6309, U. S. Army Medical Research and Material Command, Fort Detrick, Md.

We thank Arthur Andersen, Anne Rompalo, Kelly T. McKee, Jr., Marie Tenant, J. Storz, and Katherine Kacena for collaboration and assistance; and M. Atenas for assistance with the preparation of the manuscript.

REFERENCES

1093  

DETECTION OF CHLAMYDIA BY TETR-PCR  

and domain I of the 23S rRNA gene are phylogenetic markers for Chlamydia  

delphia, Pa.  

Wiley & Sons, Inc., New York, N.Y.  

30:796–800.  

Phylogenetic relationship of Chlamydia psittaci to Chlamydia trachomatis as  
43:610–612.  

Quinn. 1996. Replication of Chlamydia pneumoniae in vitro in human macro-  
phages, endothelial cells, and aortic artery smooth muscle cells. Infect.  
Immun. 64:1614–1620.  

Detection of Chlamydia pneumoniae by polymerase chain reaction-enzyme  
17:718–725.  

PCR protocols, a guide to methods and applications. Academic Press, Inc.,  
San Diego, Calif.  

23:549–553.  

entiation of Chlamydia trachomatis, Chlamydia psittaci, and Chlamydia pneu-  

1998. Rapid detection of Chlamydia pneumoniae by PCR-enzyme immuno-  

detection of Chlamydia pneumoniae in urine specimens from symptomatic  
31:1209–1212.  

Evaluation of commercial polymerase chain reaction assay for the detection  


G. Leckie, and W. Stamm. 1995. Diagnosis of Chlamydia trachomatis geni-  
tourinary infection in women by ligase chain reaction assay of urine. Lancet  
345:213–216.  

glycosylase to control carry-over contamination in polymerase chain reac-  

ods for detection of Chlamydia pneumoniae in bronchoalveolar lavage fluid  

1798.  

1998. Diagnosis of Trichomonas vaginalis infection by PCR using vaginal  

Phylogenetic analysis of the genus Chlamydia based on 16S rRNA gene  

31. Quinn, T. C. 1994. Recent advances in the diagnosis of sexually transmitted  

32. Quinn, T. C., L. Welsh, A. Lentz, K. Crotchfeld, J. Zenilman, J. Newhall,  
and C. A. Gaydos. 1996. Diagnosis by AMPLICOR PCR of Chlamydia trach-  
matis infection in urine samples from women and men attending sexually  

1996. Isolation of Chlamydia pneumoniae from the coronary artery of a  

labaratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold  
Spring Harbor, N.Y.  

Mandell, J. E. Bennett, and R. Dolin (ed.), Principles and practice of infec-

tious diseases. Churchill Livingstone, New York, N.Y.  

differentiation of Chlamydia species by antigen detection in sputum speci-

mens from patients with community-acquired acute respiratory infections.  
J. Infect. 25:77–86.  


Diagnosis of Chlamydia trachomatis infections in asymptomatic men and  

for simple extraction of DNA for PCR-based typing from forensic material.  

of a simplified polymerase chain reaction-enzyme immunoassay for the  