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Prokaryotic DNA Sequences in Patients with Chronic Idiopathic Prostatitis

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Half of all men experience symptoms of prostatitis at some time in their lives, but the etiology is unknown for more than 90% of patients. Optimal clinical and culture methods were used to select 135 men with chronic prostatitis refractory to multiple previous courses of antimicrobial therapy. The subjects had no evidence of structural or functional lower genitourinary tract abnormalities of bacteriuria or bacterial prostatitis by traditional clinical tests, or of urethritis or urethral pathogens by culture. Specific PCR assays detected *Mycoplasma genitalium*, *Chlamydia trachomatis*, or *Trichomonas vaginalis* in 10 patients (8%). Broad-spectrum PCR tests detected tetracycline resistance-encoding genes, *tetM-tetO-tetS*, in 25% of patients and 16S rRNA in 77% of subjects. The *tetM-tetO-tetS*-positive cases constituted a subset of the 16S rRNA-positive cases. Patients with 16S rRNA were more likely to have ≥1,000 leukocytes per mm^3 in their expressed prostatic secretions than men whose prostate biopsy specimens were negative for 16S rRNA ($P < 0.001$). Based on direct sequencing and repetitive cloning, multiple sources of 16S rRNA were observed in individual patients. Sequences of 29 cloned PCR products revealed 16S rRNAs distinct from those of common skin and gut flora. These findings suggest that the prostate can harbor microorganisms that are not detectable by traditional approaches. These organisms may be associated with inflammation in the expressed prostatic secretions. Molecular methods hold great promise for identifying culture-resistant microorganisms in patients with chronic prostatitis.

Accurate diagnosis of chronic prostatitis syndromes represents a major challenge for physicians and for clinical microbiology laboratories. The National Ambulatory Care Survey found that there were 20 office visits per 1,000 men per year for symptoms compatible with prostatitis (28). By one estimate, half of all men suffer from symptoms of prostatitis at some point in their lives (54). Lower urinary tract localization studies represent the "gold standard" for diagnostic evaluation of chronic bacterial prostatitis (33, 54). A lower urinary tract localization procedure involves a series of samples that includes first-void urine, midstream urine, expressed prostatic secretions, and postmassage urine specimens. The traditional criterion for diagnosis of chronic bacterial prostatitis is a 10-fold increase in the concentration of a uropathogen when comparing the postmassage urine or expressed prostatic secretion sample with the first-void urine (33, 40). This uropathogen should be identical to the organism documented previously as the cause of bacteriuria in the patient (22, 30, 39, 54). In contrast, the diagnosis of acute bacterial prostatitis presents few problems because patients present with acute cystitis and high counts of uropathogens in their voided urine and often have systemic symptoms. Unfortunately, men with acute or chronic bacterial prostatitis constitute only 5 to 10% of the large number of patients with prostatitis syndromes (13, 31, 33). The most common syndromes, termed chronic idiopathic prostatitis, are associated with negative urine cultures and classified further as nonbacterial prostatitis (for patients with purulent prostatic secretions) or prostatodynia (for patients with expressed prostatic secretions that are not purulent) (22, 30, 39, 54). Patients with these conditions may remain symptomatic for prolonged periods (31). Characteristic symptoms include perineal, penile, scrotal, suprapubic, and ejaculatory pain; voiding; or sexual dysfunction (7, 29, 31).

Some observations suggest that fastidious urogenital microorganisms may be important in the etiology of chronic idiopathic prostatitis. Patients frequently relate the onset of their disease to sexual activity, commonly to an episode of acute urethritis (6), and antimicrobial treatment is often transiently effective in relieving symptoms (11, 21, 29, 36). Some investigators report evidence for infection with *Chlamydia trachomatis* (1, 8, 9, 11, 53, 58), genital mycoplasmas (10, 56–58), *Trichomonas vaginalis* (23, 35, 36), staphylococci (41), or genital viruses (3, 16) in patients with chronic prostatitis. Other workers identified such organisms in remarkably few cases (4, 17, 52). In the great majority of studies, diagnosis depended on culture or antigen detection in samples obtained through the urethra, such as urine, urethral swabs, and expressed prostatic secretions. Interpretation of the findings is difficult because these samples may acquire organisms during passage through the urethra. Few investigators evaluated prostate tissue samples directly in patients with chronic idiopathic prostatitis.

Culture diagnosis of urogenital pathogens in men with chronic prostatitis represents an especially challenging clinical microbiology problem because of inhibitory substances (20, 34) and multiple previous courses of antimicrobial treatment (31). At present, many microorganisms are uncultivable under the most refined conditions (2). Environmental studies suggest that most bacteria in existence do not multiply on conventional media (37, 55, 59). Environmental organisms constitute a lower percentage of known bacteria than those infecting or inhabiting human tissues, but even this source contains a significant number of uncharacterized species (59). These observations suggest that molecular approaches to diagnosis might prove useful for evaluating patients with chronic idiopathic prostatitis.

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Specific PCR techniques are now available for pathogens implicated in chronic prostatitis. Broad-spectrum PCR techniques can also amplify genetic material that is distributed widely among bacteria. We developed a PCR test for common tetracycline resistance genes found in a wide variety of bacteria and used this test for characterization of urogenital specimens (51). Amplification and sequencing of bacterial 16S rRNA genes is another approach that has been used to document roles for microorganisms in other idiopathic diseases (15, 18, 19, 44, 47, 48). Amplifications are primed in conserved sequences, while the amplified region itself differs even among closely related species (24). The amplified variable-region sequences can then be compared with known 16S rRNA genes tabulated in the GenBank and EMBL databases, searching for matches and phylogenetic relationships with known sequences.

In this 5-year study, we used a combination of optimal clinical, cultural, and molecular methods to evaluate a well-defined population of 135 men with chronic idiopathic prostatitis. The clinical protocol included standardized examinations to exclude patients with chronic bacterial prostatitis, urethritis, or significant structural or functional abnormalities of the lower urinary tract. Microbiological studies included evaluation for fastidious microorganisms, lower urinary tract localization cultures, and counts of leukocytes in the expressed prostatic secretions. Prostate biopsy tissue was evaluated by PCRs for *C. trachomatis*, *T. vaginalis*, genital mycoplasmas, herpes simplex viruses, and cytomegalovirus as well as by broad-spectrum PCRs for both tetracycline resistance and 16S rRNA, followed by cloning and sequencing of the products. These studies provide evidence that bacteria may be important in the idiopathic inflammatory syndrome previously termed nonbacterial prostatitis.

MATERIALS AND METHODS

Patient population, clinical methods, and biopsy procedure. Subjects were recruited from patients attending a special prostatitis clinic at the University of Washington Medical Center following a protocol approved by the University of Washington Human Subjects Committee. Men who were over 18 years old were offered a standardized evaluation for infectious, inflammatory, structural, and functional conditions associated with prostatitis. No subject had taken antimicrobial agents for 6 weeks before the study, and none had documentation of bacteriuria. Following a standardized history and physical examination, we obtained specimens for urethral Gram stain and cultures for *Neisseria gonorrhoeae*, *C. trachomatis*, *Ureaplasma urealyticum*, and *Mycoplasma hominis* (4, 31). Urethral swab specimens were cultured for *T. vaginalis* in modified Diamond's medium at 37°C in a 5% CO_2 atmosphere (32).

During their second visit, 1 to 3 weeks later, patients underwent lower urinary tract localization cultures to evaluate the possibility of chronic bacterial prostatitis (33). Leukocyte concentrations in expressed prostatic secretions were determined by hemocytometer counts with a phase-contrast microscope in a laboratory independent from those performing the PCR assays.

During their third visit, each patient had uroflowimetry and residual urine determination by ultrasound to exclude the possibility of significant structural and functional abnormalities (31). During this visit, potential subjects who had prostate-localized uropathogens were excluded by the four-glass urine test (chronic bacterial prostatitis); also excluded were those with urethritis (more than five leukocytes per 400× field on the Gram-stained urethral smear) or positive urethral cultures for *C. trachomatis* or *T. vaginalis*. Subjects with positive results for *U. urealyticum* or *M. hominis* were included in the study if they remained symptomatic following antimicrobial treatment that cleared these infections, as determined by negative test-of-cure urethral cultures. Of the 260 men who met the entry criteria, 135 had urethral, urine, and expressed prostatic secretion evaluations and agreed to the prostate biopsy.

During the fourth visit, 1 to 3 weeks later, each subject underwent prostatic biopsy by a perineal (rather than a transrectal) approach to prevent microbial contamination by rectal flora. A double-needle method was used to limit the potential for contamination by skin flora. Following thorough skin preparation with povidone-iodine, the perineum was anesthetized with 30 to 60 ml of 1% lidocaine. A short 23-gauge needle was placed in the midline of the perineum. The biopsy needle was introduced through the first needle and into the prostate under the guidance of a finger in the rectum to ensure that the needle did not transverse the rectum. Cores of prostate tissue were obtained by using an Biopty gun (Radioplast AB, Uppsala, Sweden) with an 18-gauge biopsy needle.

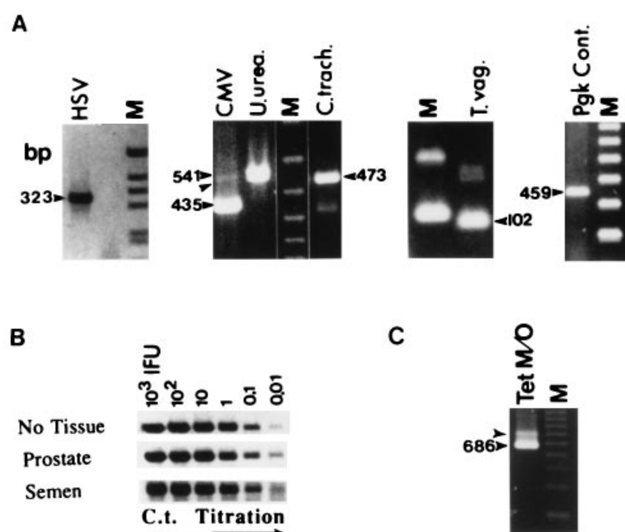

FIG. 1. PCR products from pathogen-specific DNA targets. (A) Left panel: the 323-bp herpes simplex virus (HSV) type 1 glycoprotein B gene DNA target, PCR amplified as described in Materials and Methods, then Southern blotted and probed as previously described (24). M, the 1-kb DNA sizing ladder (GIBCO-BRL, Gaithersburg, Md.). A product of identical size was obtained with herpes simplex virus type 2 DNA as expected. Second panel from left: the 435-bp major intermediate-early antigen cytomegalovirus (CMV) gene target. U.urea, PCR product of a 541-bp urease gene target of *U. urealyticum* (see Materials and Methods); M, the 123-bp ladder (GIBCO-BRL); C.trach, PCR product of a 473-bp *C. trachomatis* plasmid target sequence. Third panel from left: M, the 123-bp ladder showing the 123- and 246-bp markers; T.vag, the 102-bp product from the A6p sequence previously shown to be conserved among a wide variety of clinical *T. vaginalis* isolates (49). Right panel: Pgk Cont., PCR product of a conserved, 459-bp sequence from the 3′ flank of the human X-linked PGK gene (48). This target was used as a positive control, testing the PCR competence of all tissue biopsy samples in this study. M, the 123-bp ladder. (B) PCR products after amplifications of serially diluted titered stock preparations of *C. trachomatis* (C.t.) in the presence or absence of proteinase K-digested prostate tissue or proteinase K-digested whole semen. Because the prostate is the major source of seminal volume, this demonstrates that the high proteinase K conditions used overcome inhibition for semen as well as prostate tissue. The *C. trachomatis* stock was titrated by culture (IFU, inclusion-forming unit) and also counted microscopically (see Materials and Methods). (C) Products of amplification of a 686-bp target that occurs in the sequences of the *tetM-tetO-tetS* tetracycline resistance genes (TetM/O), which are commonly found in urogenital bacteria. The less intense band above the 686-bp product did not hybridize with the sequence specific probe. Negative controls were routinely run with all of the PCRs shown. M, the 123-kb ladder.

Periprostatic tissue samples were obtained with a separate biopsy needle. Control samples were obtained from the perineal skin surface (by using a sterile cotton swab) and deeper skin structures (by using a separate biopsy needle). Prostate biopsies were sent to the PCR laboratories in sterile containers within 30 min of collection. In each case, control specimens were also sent for histopathological evaluation to ensure that we had successfully sampled the prostatic parenchyma.

Tissue preparation. The molecular biologists instructed the surgeons and surgical staff in the particular methods of handling samples destined for the PCR laboratories. Initial PCR experiments with the phosphoglycerate kinase (PGK) gene control, described below, indicated that prostate tissue was either slightly inhibitory toward PCR when the tissue preparation protocol of Kawasaki (27) was used or that the tissue might be resistant to release of DNA. We determined that the inhibition or failure to release DNA occurred at the proteinase K step. Increasing the proteinase K concentration threefold, to 0.5 mg/ml, overcame the inhibition or ineffective release of DNA, resulting in sensitive detection of the PGK gene sequence (Fig. 1).

Prostate and skin biopsy specimens consisting of 10 to 50 μg of wet tissue were placed directly onto sterile physiological-saline-dampened Telfa pads (Kendall, Mansfield, Md.) in sterile covered cups. The samples were transported expeditiously to the PCR laboratory, and >80% were processed immediately by proteinase K treatment. Sample cups were opened one at a time in the PCR setup area, separate from all activities that generate amplicons, including thermocycling, cloning, gel electrophoresis, and PCR product storage. Biopsy specimens were lifted off the sterile Telfa pads with sterile pipette tips and then placed

individually into 200 μl of UV-treated K buffer (50 mM KCl, 20 mM Tris-HCl, 2.5 mM $MgCl_2$; pH 8.3) containing 0.5 mg of freshly added proteinase K per ml. Proteinase K treatment was for 2 h at 55°C, interrupted at 1 and 1.5 h by vortexing to disrupt the tissue completely. The proteinase K was then inactivated by heating at 95°C for 20 min.

Anticontamination procedures. The PCR laboratories meet or exceed the requirements for PCR research (42), including isolated setup areas with dedicated pipettors, small reagent and primer aliquots, and the use of aerosol-resistant pipette tips. Negative and positive controls were run with PCR assays to monitor for reagent and laboratory contamination. Freshly prepared 10% bleach was used to decontaminate bench tops and equipment. Frequent cleaning of the thermocycler with warm detergent helped eradicate contaminants. Results were rejected when the negative controls indicated contamination.

Of 10 separate PCR assays performed for this study, controls indicated that the 16S rRNA PCR was the most sensitive to contaminants. This likely reflects ubiquitous bacterial DNA that may even be present in some clinically sterile solutions. Initially, negative controls suggested the presence of 16S rRNA in commercial reagents that included Amplitaq polymerase (Perkin-Elmer Cetus, Norwalk, Conn.) and proteinase K (Bethesda Research Laboratories, Gaithersburg, Md.), although the contaminant wasn't traced specifically to either enzyme preparation.

Several changes to the protocol were initiated to counteract the 16S rRNA background. We used Deep-Vent polymerase (New England Biolabs, Beverly, Mass.) instead of Amplitaq for 16S rRNA PCR. Vent polymerase was cloned from deep-ocean hydrothermal-vent bacteria. K buffer, proteinase K, and all PCR reagents, including the oil overlay, were treated with UV light at 312 nm, at zero distance on a Fischer Biotech transilluminator, for 5 min to inactivate any reagent-associated bacterial DNA. UV treatment was performed immediately before addition of target DNA to the PCR sample tubes. Oil overlays were treated separately. These procedures eliminated contaminants reliably as evidenced by the absence of PCR products in negative controls run with every set of PCRs. All 16S rRNA results reported were obtained by the UV anticontamination procedure and monitored by the use of negative controls.

Specific PCR assays. PCR assays were performed for *C. trachomatis*, genital mycoplasmas (*M. hominis*, *U. urealyticum*, and *Mycoplasma genitalium*), *T. vaginalis*, and genital viruses (human cytomegalovirus and herpes simplex virus types 1 and 2). The GenBank database was searched with each primer sequence in an effort to obtain information on potential similarity to DNA sequences of heterologous species. Building block sequences were defined as 20-base sequences with ≥90% similarity to more than one GenBank sequence unrelated genetically to the intended DNA target. PCR reactions with the potential to amplify building block sequences were modified to exclude such sequences. In addition, we used the human PGK sequence as a positive control for each series of PCR assays.

(i) *C. trachomatis.* We modified the PCR assay of Østergaard et al. (43) for these studies. Specifically, primer IIB appeared to have been published in the 3′-to-5′ direction rather than the conventional 5′-to-3′ direction. Primer IIBC, 5′-GACCGGCCTCTAGCGCTGCG-3′, the inverse of the published sequence, was used because it corresponded to the *C. trachomatis* plasmid sequence in GenBank, release 73. Primers IIA (as published by Østergaard et al.) and IIBC were synthesized and tested on multiple *C. trachomatis* isolates, gifts from Lee Ann Campbell, University of Washington. These primers gave the anticipated 473-bp PCR product (43). The probe IA sequence disagreed at one position with the GenBank *C. trachomatis* plasmid DNA sequence, possibly because of isolate specific variation, so this probe was modified to be degenerate at the discordant position. The resulting probe, IA-M, 5′-GTTTAAGTGTTC(T/C)CATCATA-3′, was synthesized and used to probe Southern blots of products from the IIA-IIBC PCR assay. Thermal cycling conditions were 94°C for 4 min and then 42 cycles at 94, 52, and 72°C (30 s each), followed by extension at 72°C for 7 min.

(ii) Genital mycoplasmas. PCR assays were done to evaluate the presence of three genital mycoplasmas, *M. hominis*, *U. urealyticum*, and *M. genitalium*. A broad-spectrum nested mycoplasma PCR assay was modified from a published procedure (26) after discussions with the author. For this study, outer primers targeting a 504- to 519-bp sequence consisted of Myco9, 5′-(C/T)GCCTG(A/G)GTAGTA(C/T)(G/A)(T/C)(T/A)CGC-3′, and Myco3, 5′-GCGGTGTGTACAA(G/A)(T/C)(T/A)CGC-3′. Inner primers, targeting a 318- to 333-bp sequence, consisted of Myco 8, 5′-TGGTGCA(T/C)GGTTGTCGTCAG-3′, and Myco 5, 5′-GAACGTATTCACCGC(A/G)(A/G)C(G/A)T-3′. Thermal cycling conditions followed the published protocol. The *M. genitalium* PCR followed a published protocol, including thermal cycling conditions (26).

The *U. urealyticum* PCR was modified from a published protocol (5) because the product size overlapped other product sizes planned in the study. In addition, one of the published primers was similar to a building block sequence that was frequently matched in homology searches of the GenBank database. Therefore, primers UU1, 5′-AGAAGACGTTTAGCTAGAGG-3′, and UU2, 5′-ACGACGTCCATAAGCAACT-3′, were synthesized for this study. UU2 is from the previously published PCR, while UU1, avoiding the building block sequence and the size constraint, is unique to our study. The 541-bp target sequence is within the previously characterized region (5). Positive controls for this PCR were contributed by George Kenny (University of Washington). Thermal cycling conditions were 94°C for 5 min and then 46 cycles at 94, 50, and 72°C (1 min each), followed by extension at 72°C for 7 min.

(iii) *T. vaginalis.* The *T. vaginalis* PCR, including thermal cycling conditions, followed our published protocol (50). PCR products were subjected to Southern blotting, and the membranes were hybridized with the [^{32}P]dATP-labeled probe TVAP, 5′-AGACCTCTAGAAGAAGACTC-3′.

(iv) Genital viruses. PCR assays were employed to detect herpes simplex virus types 1 and 2 and human cytomegalovirus. The herpes virus PCR assay, including thermal cycling conditions, followed published methods (25). Control DNAs from herpes virus types 1 and 2 were gifts from Rhoda Ashley and Denise Galloway (University of Washington and the Fred Hutchinson Cancer Research Center, respectively). The PCR for cytomegalovirus was performed following a published procedure (14). Positive-control cytomegalovirus DNA was a gift from Rhoda Ashley. Verification of PCR products was achieved by Southern blotting or DNA sequencing. Thermal cycling conditions consisted of 94°C for 5 min, followed by 46 cycles at 94, 50, and 72°C (1 min each) and extension at 72°C for 7 min.

(v) PGK gene control. After preparation, all tissue biopsy specimens were tested for PCR competence by the PGK PCR. The target sequence is at the 3′ end of a highly conserved gene encoding the metabolic housekeeping enzyme PGK. This sequence is X linked and is present in a single copy on each X chromosome. Since the subjects were male, the PGK PCR detects a single-copy sequence per cell. Primers consisted of GK1, 5′-TATAAGAGCATTTATGTTCT-3′, and GK2, 5′-AGCATACACCTTTGCAATAT-3′, targeting a published 459-bp sequence (49). Thermal cycling conditions for the PGK PCR were 94°C for 5 min, followed by 32 cycles at 94, 51, and 72°C (1 min each) and extension at 72°C for 7 min. The PGK PCR was also used to maximize the efficiency of sample preparation at the outset of the study. PGK PCR was used routinely to monitor each biopsy sample for PCR inhibitors, which may vary from individual to individual and which could potentially lead to false negatives. No sample was scored as negative for any PCR assay if that sample wasn't proven to be PCR competent by the PGK PCR assay.

Broad-spectrum PCR assays. Two distinct PCR approaches were used to detect a broad spectrum of bacteria in patients with prostatitis: evaluation for common genes encoding tetracycline resistance and detection of 16S rRNA. These assays were done in two separate laboratories by personnel who were blinded to other PCR and clinical findings.

(i) Tetracycline resistance. PCR was used to detect common genes encoding resistance to tetracycline. We reasoned that if our patients harbored urogenital bacteria, then these organisms would likely be resistant to tetracycline because more than 70% of patients had received treatment with tetracycline drugs before entry in the study. The *tetM-tetO-tetS* PCR test for a common group of resistance genes was performed by our previously reported procedures (51).

(ii) Prokaryotic 16S rRNA. By PCR we amplified 16S rRNA encoding DNA whose sequence(s) constitutes molecular signatures of prokaryotes. The 16S rRNA nested PCR was described previously (46). Primers 91E and 13B from that study were synthesized in our laboratory and yielded PCR products of the correct size (475 bp) when we tested DNAs from *Escherichia coli* K-12, *Prevotella intermedia* (ATCC 25261 and ATCC 2536), *Prevotella disiens* (clinical strain Clt 75.5), and *Peptostreptococcus anaerobius* (clinical strain Clt 17.5).

Cloning and sequencing of PCR products. The 475-bp PCR products from the 16S rRNA PCR were amplified with primers 91E and 13B. After cloning into the PCRII vector (Stratagene, La Jolla, Calif.), the products were sequenced either by using Sequenase (United States Biochemical, Cleveland, Ohio) or by Cycle sequencing (Roche Molecular, Branchburg, N.J.) with [^{35}S]dATP. Sequencing was accomplished with wedge-gradient, 45-cm gels. Overlapping sequences were determined for both DNA strands, and the sequences were checked independently by two investigators.

Homology searches were done to compare the 16S rRNA sequences with both the GenBank and EMBL databases on CD-ROM using the program Genepro (Bainbridge Island, Wash.). Searches were also done with the BLAST feature at EMBL. The initial search window was 100 bp with Ktup and speed settings of 5 and 1, respectively. Searches employed both orientations of each sequence. Sequences in GenBank or EMBL that were determined to be the most similar to a patient sequence were then separately aligned with the unknown sequence. Pairwise alignments were performed with a window size of 100, a relative gap penalty of 10, and a maximum allowable gap of 10 bp. Using the same gap restraints, multiple alignments were performed with the multiple alignment feature of CLUSTALV.

Phylogenetic analyses. Sequence phylogenies were computed with and without removal of gaps and ambiguously aligned sequences. The programs SEQBOOT, FITCH, NEIGHBOR, and CONSENSE (Phylip Phylogeny Inference Package [21]) were used in the order given to infer bootstrapped phylogenetic relationships. Confidence estimates were determined from 1,000 bootstrap replicates. Distance matrices were calculated by Fitch–Margoliash–least-squares analyses using randomized data inputs (J option). The DNAML program was used separately to infer unrooted phylogenies, with similar results. Phylogenies were also inferred retrospectively by using the CLUSTAL V neighbor-joining method, with similar results.

RESULTS

Subjects, complications, and specimens analyzed. Overall, 135 subjects completed the study protocol. The mean age (±1 standard deviation) of study subjects was 38.3 ± 13.5 years

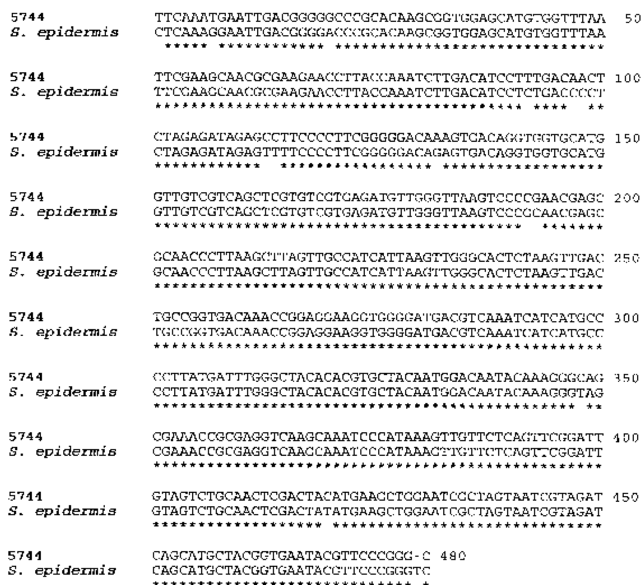

FIG. 2. Clustal alignment of the 16S rRNA-encoding DNA sequence of a cloned skin biopsy sample (5744) with *S. epidermidis* 16S rRNA. The similarity was 98%. The same patient's prostate biopsy specimen contained 16S rRNA-encoding DNAs that were <50% similar to known skin flora sequences but similar to 16S rRNA sequences in prostate biopsies from several other patients. Asterisks at the bottom of each row indicate identical nucleotides.

(median, 37 years). For each subject, at least two prostate biopsy specimens were evaluated by each molecular assay and two separate prostate biopsy samples were evaluated histologically (a minimum of six tissue cores). Histological studies confirmed the presence of prostatic parenchyma for all subjects (data not shown). Most subjects experienced some discomfort during the procedure, and most had transient hematospermia. One man developed a fever and bacteriuria that responded promptly to antimicrobial therapy. Four other men had hematospermia that persisted beyond 10 days, including one man who had blood clots in his semen for 3 weeks.

Environmental and patient controls. The operating room, clinical reagents, and instruments were evaluated by the broadest-spectrum PCR, the 16S rRNA assay. Swabs wetted with saline were used to sample fingertips of sterile glove lots, sterile Telfa pads, sterilized scissors, and the Biopty gun used to obtain biopsies. We also evaluated the physiological saline, lidocaine, syringes, and sterilized tabletops holding the biopsy equipment. Such environmental controls were run on two separate occasions. All of these controls were negative for 16S rRNA.

Patient perineal skin swab and biopsy specimens were also processed and PCR amplified along with prostate biopsy specimens from the same patients. Povidone-iodine-prepared skin from the perineum occasionally contained 16S rRNA. However, DNA sequencing revealed that these sequences represented typical skin flora sequences which were distinct from 16S rRNA sequences found in the same patients' prostate biopsy samples (Fig. 2).

Limits of the PCR assays for prostatic samples. *C. trachomatis* DNA from a titrated stock was used to monitor the molecular sensitivity of the *C. trachomatis* PCR assay in the presence and absence of prostate tissue and semen (Fig. 1). With appropriate levels of proteinase K, we detected as little as 0.01 culture inclusion-forming unit of *C. trachomatis*. This is consistent with the finding that the same stock of *C. trachomatis* contained >90% defective *C. trachomatis* particles after microscopic quantitation of gradient-purified *C. trachomatis* particles and culture titrations (35a). Thus, the PCR assay was more sensitive than culture for detecting *C. trachomatis* in prostate samples.

T. vaginalis was detected at the level of a single organism (less than 1 CFU), based on hemocytometer counts of cultures whose DNA was then purified and PCR amplified. With radioactive probes labeled to 10^9 dpm, the calculated sensitivity was at least 4 orders of magnitude greater than the sensitivity needed to detect a single organism in culture. Sensitivities of <10 organisms or tissue culture infecting units were also obtained for stock cultures of herpes simplex virus, cytomegalovirus, *M. genitalium*, and *U. urealyticum* DNAs, based on titrations by the laboratories that provided these samples (Fig. 3).

Specific PCR findings. Only 11 (0.5%) of 2,552 specific PCR assays (an average of 2.7 samples per subject × 135 subjects × 7 PCRs) were positive for specific pathogens. Of the 135 subjects evaluated, 10 (8%) had positive assays for one or more bacteria or protozoa, including *M. genitalium* (5 subjects), *C. trachomatis* (4 subjects), and *T. vaginalis* (2 subjects) (Table 1 includes one man who had positive tests for both *M. genitalium* and *C. trachomatis*).

None of these patients was positive for the general mycoplasma PCR assay or for the separate *U. urealyticum* assay. No patient had a positive PCR for the genital viruses (herpes simplex virus types 1 and 2 and cytomegalovirus). Each set of PCR was performed in parallel with known-positive samples for each virus and with human PGK gene controls as ongoing tests of both virus-specific DNA detection efficiency and general PCR competence of the tissue preparations.

Broad-spectrum PCR findings. The *tetM-tetO-tetS* assay was positive in 30 (25%) of 120 subjects tested (Table 1). The 16S rRNA PCR assay was positive in prostate or periprostate tissue from 103 (77%) of 134 subjects tested. Of the 30 subjects who were positive for *tetM-tetO-tetS*, 29 (97%) were also positive for the 16S rRNA while only 1 (3%) was negative for 16S rRNA.

Of the 90 subjects who were negative for *tetM-tetO-tetS*, 66 (73%) were positive for 16S rRNA while 24 (27%) were negative for 16S rRNA ($\chi^2 = 61.13$ [by McNemar's chi-square test], df = 1, $P < 0.001$). Thus, 16S RNA PCR-positive results were more common than *tetM-tetO-tetS* PCR-positive results, with the *tetM-tetO-tetS*-positive results constituting a subset of the 16S rRNA-positive patients. Presumably, the *tetM-tetO-tetS*-positive, 16S rRNA-negative subject either had a low level of microbial nucleic acid that proved difficult to detect consis-

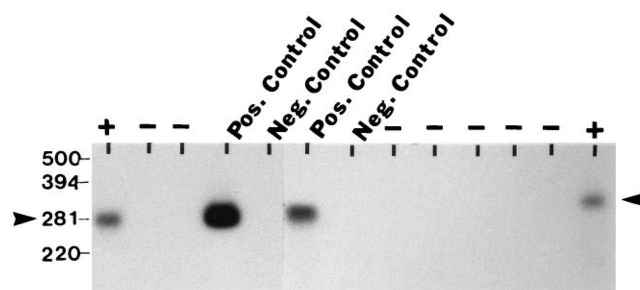

FIG. 3. *M. genitalium* PCR assays performed on proteinase K-digested prostate biopsy tissue. *M. genitalium* PCR was performed (see Materials and Methods); then the products were subjected to Southern blotting and probed with a ^{32}P-labeled oligonucleotide probe complementary to a sequence within the targeted 281-bp product sequence. Lanes representing positive and negative patients are indicated with arrowheads. Positive (Pos.) and negative (Neg.) controls are also shown.

TABLE 1. Molecular diagnosis of microorganisms in 135 men attending a chronic prostatitis clinic[a]

PCR assay	No. of subjects positive/no. of subjects assayed	Proportion positive (%)
Specific organism		
M. genitalium	5/135[b]	4
C. trachomatis	4/135[b]	3
T. vaginalis	2/135	2
U. urealyticum	0/135	0
Mycoplasma probe[c]		
Cytomegalovirus	0/135	0
Herpes simplex virus types 1 and 2	0/135	0
PGK[d]	135/135	100
Broad spectrum		
tetM-tetO-tetS[e]	30/120	25
16S rRNA[f]	103/134	77

[a] No subject demonstrated evidence of urethritis on Gram-stained urethral smear, and urethral cultures for *N. gonorrhoeae*, *C. trachomatis*, *U. urealyticum*, and *T. vaginalis* were negative. In addition, no subject had evidence of bacterial prostatitis by lower urinary tract localization studies.
[b] One man was positive for both *M. genitalium* and *C. trachomatis.*
[c] This general mycoplasma probe is described in Materials and Methods.
[d] The PGK PCR assay detects mammalian DNA. This is an internal positive control for the PCR reaction. Other positive controls include external standards for each organism that were included with each run. A negative control containing normal saline was also included with each run.
[e] *tetM-tetO-tetS* detects the prokaryotic gene encoding tetracycline resistance that is commonly found among urogenital bacteria.
[f] 16S rRNA detects the prokaryotic genetic material encoding rRNA.

tently or harbored a prokaryote that the 16S rRNA assay failed to detect.

We also compared the specific PCR results with the broad-spectrum PCR findings. Of the 10 men with specific organisms detected by PCR, the 16S rRNA PCR was positive in 8, including all men who had *M. genitalium* or *C. trachomatis* infections. In contrast, the *tetM-tetO-tetS* PCR was positive in only 2 of these 10 men. Both *tetM-tetO-tetS*-positive men had *C. trachomatis*. Neither 16S rRNA nor *tetM-tetO-tetS* was detected in the two men with *T. vaginalis*; *T. vaginalis* would not be expected result in a positive reaction in these PCR tests since the organism is a eukaryote.

Correlation of broad-spectrum PCR findings with inflammation in prostatic secretions. Of 84 subjects who had prostatic-secretion leukocyte counts and whose prostate biopsy specimens were evaluated by *tetM-tetO-tetS* PCR, 21 (25%) were *tetM-tetO-tetS* positive and 63 (75%) were *tetM-tetO-tetS* negative (Table 2). The median leukocyte count per cubic millimeter in expressed prostatic secretions was 80 for the *tetM-tetO-tetS*-positive men (range, 0 to 12,320), compared with a median of 160 for the *tetM-tetO-tetS*-negative men (range, 0 to 26,300; U = 654.5, [by Mann-Whitney test], $P = 0.94$). When inflammation was defined as the presence of ≥1,000 leukocytes per mm^3 in expressed prostatic secretions, 23 subjects were diagnosed with prostatic inflammation, including 8 (38%) of 21 men who were *tetM-tetO-tetS* positive and 15 (23%) of 63 men who were *tetM-tetO-tetS* negative ($\chi^2 = 0.036$ [by McNemar's chi-square test], df = 1, $P = 0.85$). Thus, men with inflammation in their prostatic secretions had a 15% higher prevalence of *tetM-tetO-tetS*-encoding sequences than men who had uninflamed prostatic secretions, but this difference was not statistically significant.

Of 95 subjects who had both prostate secretion leukocyte counts and prostate biopsy specimens evaluated by the 16S rRNA PCR assay, 69 (73%) were 16S rRNA positive and 26 (27%) were 16S rRNA negative (Table 2). The median leukocyte count per cubic millimeter in expressed prostatic secretions was 240 for the 16S rRNA-positive men (range, 0 to 26,300), compared with a median of 15 for the 16S rRNA-negative men (range, 0 to 1,010; U = 527.0 [by Mann-Whitney test], $P = 0.002$) (Fig. 4). When inflammation was defined as the presence of ≥1,000 leukocytes per mm^3 in expressed prostatic secretions, 23 subjects had diagnoses of prostatic inflammation, including 22 (32%) of 69 men who were 16S rRNA positive and 1 (4%) of 26 men who were 16S rRNA negative ($\chi^2 = 42.18$, df = 1, [by McNemar's chi-square test], $P < 0.001$). Thus, the presence of 16S rRNA correlated strongly with inflammation in the expressed prostatic secretions.

DNA sequencing of cloned PCR products from patients with 16S rRNA. We tried direct sequencing of uncloned PCR products from 16S rRNA PCR-positive men. This approach revealed heterogeneous 16S rRNA sequences, suggesting the presence of more than one species per patient. Thus, sequence identification of the 16S rRNA PCR products required cloning to isolate individual 16S rRNA-encoding DNA sequences. The 16S rRNA PCR products of positive biopsy specimens were inserted into the TA cloning vector to isolate plasmid clones for DNA sequencing.

A total of 29 clones from 10 patients were sequenced completely or partially. Figure 5 shows two independent 16S rRNA-encoding DNA clones from the same patient aligned with the closest matching sequence from GenBank. The two clones were 86% similar to each other (i.e., 86% identical bases). Clones 5725 and 5735 were 93 and 86% similar to *Vibrio furnissii* 16S rRNA. A 16S rRNA-encoding PCR product clone from the perineal skin of the same patient was 97% similar to *Staphylococcus aureus* 16S rRNA. Another patient, for whom we sequenced both skin and prostate biopsy sample PCR clones, had skin-associated 16S rRNA-encoding DNA that was 98% similar to *Staphylococcus epidermidis* and two prostate clones that were <50% similar to skin flora and <81% similar to any previously reported 16S rRNAs. Five independent clones from the prostate of a third patient were

TABLE 2. Broad-spectrum PCR findings and inflammation in expressed prostatic secretions (EPS) of patients with chronic prostatitis

PCR assay and result	Median no. (range) of leukocytes/mm^3 in EPS[a]	No. of men (%) with ≥1,000 leukocytes/mm^3 in EPS[a]
tetM-tetO-tetS ($n = 84$)[b]		
Positive ($n = 21$; 25%)	80 (0–12,320)	8 (38%)
Negative ($n = 63$; 75%)	160 (0–12,320)	15 (23%)
Significance[c]	NS[d]	NS
16S rRNA ($n = 95$)[e]		
Positive ($n = 69$; 73%)	240 (0–26,300)	22 (32%)
Negative ($n = 26$; 27%)	15 (0–1,010)	1 (4%)
Significance[c]	$P = 0.002$	$P < 0.001$

[a] Leukocyte concentrations in the EPS were determined by hemocytometer counts.
[b] The *tetM-tetO-tetS* PCR assay detects common bacterial genes for tetracycline resistance.
[c] The Mann-Whitney U test was used to assess differences in absolute counts of leukocytes in the EPS between men with positive and negative PCR assays. McNemar's chi-square test was used to evaluate differences in the prevalence of inflammation between men with positive and negative PCR assays, when inflammation was defined as the presence of ≥1,000 leukocytes per mm^3 in the EPS.
[d] NS, no statistically significant difference.
[e] The 16S rRNA PCR assay detects prokaryotic ribosomal gene sequences.

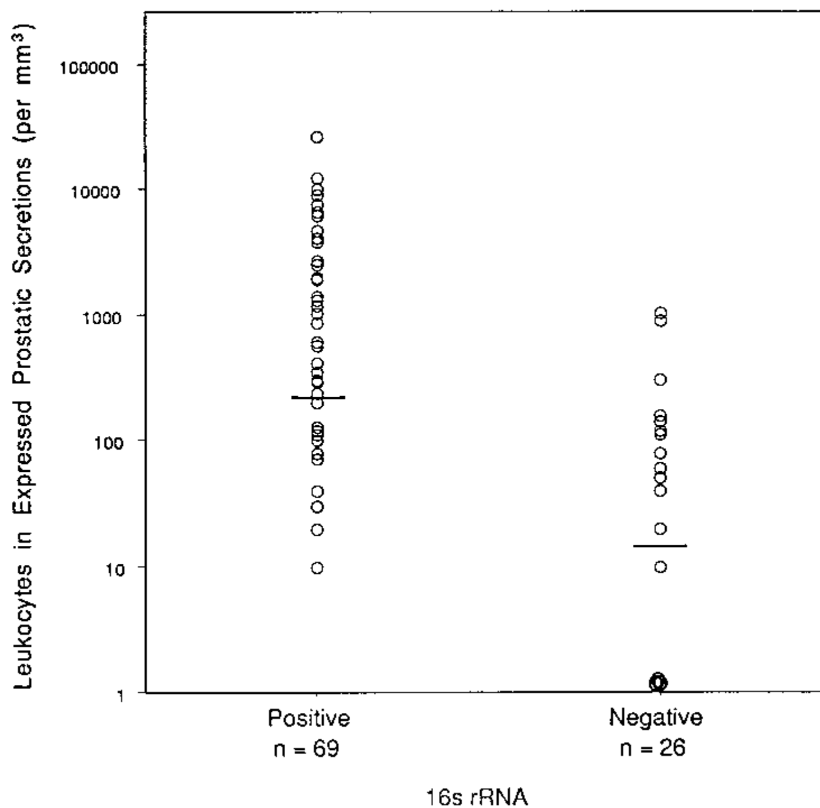

FIG. 4. Scatter plots of leukocyte counts in expressed prostatic secretions of patients whose prostate biopsies were positive or negative for 16S rRNA. The horizontal lines indicate median values for each group. Note the logarithmic scale. Positivity for 16S rRNA-encoding sequences correlated with significantly higher leukocyte counts (Table 2). The nonconcentric circles at the lower right indicate multiple patients who were negative for leukocytes in their prostatic secretions and also negative for 16S rRNA-encoding DNA sequences.

sequenced. Three of the five clones were identical to each other but were ≤70% similar to other sequences in GenBank. The 70% match was to a 16S rRNA sequence from an uncharacterized proteobacterium. The two other clones from this patient were over 90% similar to each other and 90% similar to *Aeromonas allosaccharophila*. None of the clones sequenced was from a patient positive for the chlamydia or mycoplasma PCR assay.

Of the 29 clones sequenced, 28 16S rRNA-encoding DNA clones were distinct from normal gut flora sequences and from 16S rRNA sequences found on the perineal skin of the same patients whose prostates were biopsied. The remaining clone was >95% similar to *E. coli*. Overall, 28 (97%) of 29 16S rRNA sequences from the prostate were defined as having <95% similarity to known sequences in GenBank and EMBL. The inferred sequence phylogenies of prostate and skin 16S rRNAs from a single patient are shown in Fig. 6 along with other patient and known sequences. Phylogenetic inference involving 1,000 bootstrap replicates strongly suggested grouping of 16S rRNA-encoding sequences from several patients (clones I5725, C5752, I5751, and possibly EcCom). Other patients had 16S rRNAs that branched outside this group. Topologies of phylogenetic trees were relatively insensitive to changes in alignment method. Thus, our results demonstrate multiple and, in some patients, related 16S rRNAs (Fig. 5 and 6).

DISCUSSION

Physicians and clinical microbiologists become discouraged with the prospect of providing accurate diagnoses for patients with chronic prostatitis. Although many organisms have been associated with these syndromes, it can be exceedingly difficult for the most sophisticated laboratories to provide conclusive results, even if they are provided with optimal clinical samples. Because of the low yield of diagnostic evaluations, many clinicians treat patients empirically with a wide constellation of antimicrobial agents without trying to make an etiological diagnosis. A survey of urologists at the 1996 Annual Meeting of the American Urological Association revealed that more than 95% felt that microbiological evaluation is not cost-effective in their practice settings (30a).

This study evaluated a most difficult population of men with chronic prostatitis. Participants were recruited from patients attending a special clinic for evaluation of long-standing symptoms that had persisted despite multiple courses of antimicrobial therapy. Each subject was evaluated by a standardized protocol for infectious, inflammatory, structural, and functional conditions associated with prostatitis.

In earlier reports, *N. gonorrhoeae*, *C. trachomatis*, *U. urealyticum*, *M. hominis*, *T. vaginalis*, and Gram-positive bacteria were implicated as etiological agents in prostatitis (1, 3, 8–12, 16, 22, 23, 30, 35, 36, 38, 41, 53, 56–58). However, most studies included patients whose urethral specimens were found to have organisms. In this study, potential subjects were excluded if they exhibited bacteriuria, bacterial prostatitis, or urethritis or had a urethral culture that was positive for urogenital pathogens. Thus, we evaluated prostate biopsy tissues obtained from a population of men who could not be diagnosed by optimal clinical and microbiological methods. This clinical protocol

FIG. 5. CLUSTAL V multiple sequence alignment and reverse contrast map of two 16S rRNA-encoding cloned DNA sequences from separate patients (INV5725 and INV5735) and the 16S rRNA of *Vibrio furnissii* (Vfurnis; referenced in Genbank). White letters on a dark background indicate nucleotides that are identical in two or more sequences. Asterisks at the bottom of each row indicate bases that are identical in all three sequences. Gap penalties were set at 10 (fixed) and 10 (floating). The weighted option of the transition toggle was selected. The *V. furnissii* 16S rRNA sequence was chosen for this alignment because of the 6,343 16S rRNA sequences in GenBank release 75, it was the most similar to the patient sequences, INV5725 and INV5735, with 86 and 93% similarities, respectively, and was also similar to other patient sequences (data not shown). Similarity levels of <95% are presently insufficient to classify the patient sequences at either the genus or species level. However, many known bacteria, including common flora, appear unlikely to occur in these prostate biopsy samples because their sequences were <80% similar to the sequences found to date.

included over 1,000 physician visits, representing four or more visits for each of the 260 men evaluated to select the 135 subjects.

In this population, we reasoned that any organisms would be culture resistant, drug resistant, or present in low concentrations. Multiple prior courses of antimicrobial agents would increase the chance of defective or noncultivable organisms. Inhibitory substances in prostate tissue and secretions, such as high concentrations of zinc and prostatic antibacterial factor (20, 34), would further reduce the chance for positive cultures. We developed or adapted PCR techniques for each of the specific pathogens that might be present in prostate tissue. In control studies using cultured organisms in prostate tissue or semen, the sensitivity of these PCR methods proved to be substantially higher than optimal culture methods for detecting each agent.

Despite the sensitivity of the technical methods, we were surprised to find that any of the specific PCR tests was positive in this highly selected population. Of the 135 subjects evaluated, 10 (8%) had positive PCR assays for one or more specific agents, including *M. genitalium* (4 subjects), *C. trachomatis* (3 subjects), and *T. vaginalis* (2 subjects), and one man was positive for both *M. genitalium* and *C. trachomatis*. These findings are in agreement with previous observations that both *C. trachomatis* (1, 45, 53) and *T. vaginalis* (23) may be identified in prostate tissue. To our knowledge, this is the first demonstration of *M. genitalium* in prostate tissue. These observations suggest that *C. trachomatis*, *T. vaginalis*, and *M. genitalium* may be associated with some cases of chronic prostatitis, even among men who have no evidence of urethritis and men who have negative urethral cultures.

Overall, 2,541 (99.5%) of 2,552 specific PCR assays were negative. There were no positives for either the general mycoplasma assay or the *U. urealyticum* assay. Thus, *M. genitalium* was the only genital *Mycoplasma* species associated with chronic prostatitis in our population. Previous reports suggesting an important role for *M. hominis* and *U. urealyticum* may reflect contamination of specimens with urethral organisms in less selected populations (10, 13, 56, 58). No patient had a positive PCR for herpes simplex virus types 1 and 2 or cytomegalovirus.

Broad-spectrum PCR assays provided the most provocative findings. Sequences encoding tetracycline resistance were detected in 25% of subjects, and 16S rRNAs were detected in 77% of the subjects tested. The *tetM-tetO-tetS* and 16S rRNA assays were done in different laboratories by personnel who were blinded to other clinical and microbiological findings, but the results were highly correlated, with the *tetM-tetO-tetS*-pos-

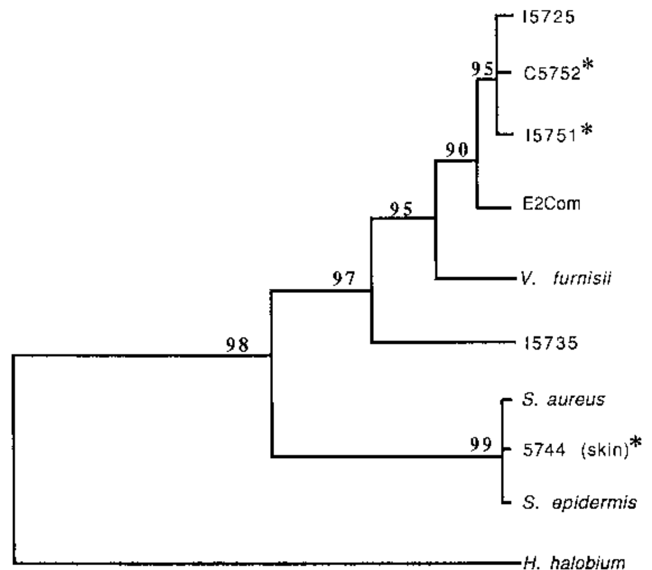

FIG. 6. Unrooted dendrogram obtained by using the Phylip phylogenetic inference program. Sequences aligned by CLUSTAL V were input into the SEQBOOT program of Phylip to generate 1,000 bootstrap replicates consisting of 1,000 sequence alignments, each with randomly selected portions of the sequences rendered inactive. The numbers to the left of the nodes indicate the percentages of resulting phylogenetic trees with the nodal topologies shown. These topologies also agreed with the best tree found by using the maximum likelihood program DNAML. *Halobacterium halobium* was input as the outgroup species, although the phylogeny is unrooted. Horizontal-line lengths are proportional to inferred evolutionary distances. Italicized entries to the right indicate previously known 16S rRNA sequences (referenced in GenBank), while unitalicized entries represent patient prostatic sequences. The separation of the group consisting of *S. aureus*, *S. epidermidis*, and 5744 (skin biopsy), from the grouping of patient prostate biopsy sample sequences, is highly significant (98% confidence). Asterisks indicate prostate (C5752 and I5751) and skin (5744) clones from a single patient who had two distinct but similar sources of 16S rRNA-encoding DNA in his prostate tissue. The prostate clones from this patient were also similar (97%) to 16S rRNA-encoding DNA from another patient, represented by clone I5725.

itive results constituting a subset of the 16S rRNA-positive patients. There was a strong correlation between inflammation in the expressed prostatic secretions and detection of 16S rRNA in prostatic tissue ($P < 0.001$). While there was no statistical correlation between inflammation in the prostatic secretions and *tetM-tetO-tetS* in prostatic tissue, we found a 15% higher rate of inflammation among the men whose prostate biopsy samples contained *tetM-tetO-tetS*-encoding sequences. Lack of statistical significance may reflect the relatively low number of subjects with positive *tetM-tetO-tetS* assays. While it is impossible to determine cause and effect with a case control design, these findings suggest that fastidious or noncultivable microorganisms may be important in the etiology of chronic inflammatory prostatitis.

A number of observations suggest that the 16S rRNA PCR findings do not represent contaminants. First, the great majority of PCR tests in this study were negative, including 99.5% of 2,552 specific-organism PCRs. These negative assays employed high numbers of cycles, radioactive hybridization probes labeled to high specific activity, and dilute positive controls with each set of reactions. Second, extreme care was taken in procuring and handling the clinical samples, including the use of a double-needle biopsy method to limit skin contamination. Multiple environmental controls were negative. As an additional control, we evaluated skin biopsy or swab samples from 14 patients after standard skin preparation with povidone-iodine solution. Of these 14 subjects, 12 (86%) were positive for 16S rRNA. However, DNA sequencing revealed that the positive skin samples contained normal flora, such as *S. aureus* and *S. epidermidis*, that were not detected in prostate biopsy specimens from the same patients. Third, the 16S rRNA data were supported by the *tetM-tetO-tetS* data obtained in an independent laboratory. Finally, patients with 16S rRNA-encoding DNA sequences in their prostate biopsy samples were significantly more likely to have inflammation in their expressed prostatic secretions, i.e., to have inflammatory prostatitis, according to the new consensus definition (reference 40a and unpublished data).

DNA cloning and sequencing indicated that prostate tissue from patients with chronic prostatitis harbored multiple sources of 16S rRNA-encoding DNA. Data from 10 subjects, with an average of 2.5 cloned sequences per patient, suggested that many 16S rRNA-encoding sequences were novel, defined as <95% related to known bacterial rRNA sequences. A similarity of 90 to 95% in the highly variable region of the 16S rRNA DNA studied is not sufficient to identify a single species, or even genus, because separate genera in GenBank may share ≥90% similarity (unpublished observation). Such similarities may be influenced by differing standards of classification, including morphological, biochemical, and sequence-based phylogenetic criteria.

Additional sequencing is necessary to define the microbiology of the prostate gland and to determine the relationship of these microorganisms to chronic prostatitis. Such studies should include many more patients with chronic prostatitis as well as other populations. Sequencing PCR products from the chlamydia- and mycoplasma-positive biopsy specimens would provide further confidence in the broad-spectrum PCR approach as a diagnostic tool and might render some of the more specific PCR tests obsolete as sequencing becomes less time-consuming in the future. Despite these limitations, the cloning and sequencing data suggest that prostate tissue from our patients contained previously unsequenced 16S rRNA-encoding sequences that in some distinct patients were similar enough to be grouped phylogenetically.

The finding of 16S rRNA and tetracycline resistance-encoding DNA sequences in prostate tissue from patients with chronic prostatitis is not sufficient to establish that bacteria cause this syndrome. However, none of Koch's postulates holds for every known infectious agent (59). The evidence supporting pathogenic roles of microorganisms varies from one disease to another. Establishing a role for bacteria in the etiology of chronic prostatitis will likely involve multiple steps. Detection of bacteria in prostate tissue represents an important step in determining the etiology of these syndromes. Our DNA sequence data strongly suggest that the 16S rRNA PCR positives were from a source separate from known gut, skin, and laboratory contaminants. The correlation between 16S rRNA-positive and *tetM-tetO-tetS*-positive results is also consistent with the clinical observation that antibiotic therapy provides transitory, if any, relief of symptoms for many patients.

In summary, we used optimal clinical and culture methods to select a population of 135 men with refractory chronic prostatitis. Subjects had no evidence of structural or functional abnormalities, bacteriuria, urethritis, or bacterial prostatitis by standard criteria. Specific PCR assays detected *M. genitalium*, *C. trachomatis*, or *T. vaginalis* in prostate tissue from 10 patients (8%). PCRs detected sequences encoding tetracycline resistance in 25% of patients and sequences encoding 16S rRNA in 77% of patients. Subjects with 16S rRNA-encoding sequences in their prostate biopsy samples were more likely to have inflammation in their prostatic secretions. Cloning and sequencing of 16S rRNA PCR products showed multiple bacterial species colonizing or invading the prostatic parenchyma of individual patients. These observations suggest that identification, cloning, and sequencing of prokaryotic DNA in prostate tissue may help elucidate chronic prostatitis syndromes.

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