Detection and identification of bacterial DNA in semen

Ann A. Kiessling, Ph.D.,[a,c] *Bryan M. Desmarais, B.S.,*[a,c] *Hui-Zhong Yin, M.D.,*[a] *Joseph Loverde, M.S.,*[a,c] *and Robert C. Eyre, M.D.*[b,c]

[a] Bedford Research Foundation Laboratories, Somerville; [b] Faulkner Hospital; and [c] Harvard Institutes of Medicine, Boston, Massachusetts

Objective: To detect and identify bacteria in semen by sequencing polymerase chain reaction (PCR)–amplified ribosomal RNA gene regions (rDNAs).
Design: Bacterial rDNAs were detected by PCR amplification of semen DNA. Conditions were adjusted to detect only abundant organisms, no fewer than 20,000 bacteria/mL of semen.
Setting: Clinical andrology laboratory and academic research laboratories.
Patient(s): Men undergoing fertility evaluation (n = 29) or vasectomy (n = 5).
Intervention(s): None.
Main Outcome Meaure(s): Frequency of bacterial rDNA–positive specimens, relationship of rDNAs to bacteria in GenBank, and correlation with semen cells.
Result(s): Twenty-five (56%) of the specimens from 22 (65%) of the men were positive. A total of 141 bacterial rDNA sequences were compared with GenBank data for identification. The largest group matched gram-positive anaerobic cocci (*Peptoniphilis*, *Anaerococcus*, *Finegoldia*, *Peptostreptococcus* spp.) in 13 specimens, followed by *Corynebacterium* spp. in 10 specimens, *Staphylococcus*, *Lactobacillus*, and *Streptococcus* spp. in 7 specimens each, *Pseudomonas* spp. in 4 specimens, and *Haemophilus* and *Acinetobacter* spp. in 2 specimens each. The rDNA-positive specimens averaged 59 ± 13 million sperm/mL, 46 ± 5% of which were motile, not statistically different from the rDNA-negative specimens (77 ± 16 million/mL, 47 ± 5% motile). Normal sperm forms were lower in the rDNA-positive (10 ± 1.1%) than in the rDNA-negative specimens (22 ± 2%), and lymphocytes/monocytes were fivefold lower in the rDNA-positive specimens (0.4 ± 0.2 million/mL) than in the negative specimens (1.9 ± 0.7 million/mL).
Conclusion(s): Abundant bacteria in semen are not commensal, arise from infection in the male genitourinary tract, may influence fertility, and may reflect an inadequate cellular immune response. (Fertil Steril® 2008;90: 1744–56. ©2008 by American Society for Reproductive Medicine.)

Key Words: Semen, bacteria, leukocytospermia, semen infection, gram-positive anaerobic cocci, GPAC, sperm, vasectomy, male infertility, male genital tract infection

Ribosomal RNA gene sequences (rDNAs) are useful markers for the identification of bacteria, because they contain sequences that have evolved according to species. Comparisons of bacterial diversity by genotype and cultured phenotype in a variety of environmental samples has led to estimates that fewer than 1% of microorganisms have ever been cultured (1, 2). The diagnostic and treatment value of detecting and identifying bacteria in clinical specimens by genotype instead of phenotype is, therefore, becoming widely appreciated (2, 3).

One approach takes advantage of highly conserved regions of ribosomal genes that bracket species-specific regions. Polymerase chain reaction (PCR) amplification with primers to conserved regions results in PCR amplicons of the species-specific regions, which can be sequenced and compared with reference microbial gene banks for identification (3).

This genotypic diagnostic approach holds particular promise for clinical specimens such as semen, for which both aerobic and anaerobic microorganisms are of interest and may not be detected by routine culture methods. The PCR detection of fastidious anaerobic organisms in culture-negative urine specimens from renal transplant patients exemplifies this approach (4). Bacterial infection in male organs that contribute to an ejaculated semen specimen (testis, epididymis, vas deferens, ampulla, seminal vesicles, prostate, and urethra) may be important for a number of male diseases, including infertility and prostatitis. To be clinically useful, the PCR strategy should distinguish between common flora (commensals) and pathogenic infection.

To begin to understand the incidence and nature of abundant microorganisms in semen, we conducted a study of 45 semen specimens from 34 men. The five men undergoing vasectomy collected specimens before and after the procedure, providing an opportunity to compare a complete specimen with one minus contributions from the testis and epididymis. To avoid detection of commensals, we designed

Received February 28, 2007; revised and accepted August 31, 2007.
Funded by the New England Section of the American Urologic Association, the Bedford Research Foundation, and the National Institutes of Health (National Institute of Diabetes and Digestive and Kidney Diseases award no. DK52761).
Reprint requests: Ann A. Kiessling, Ph.D., Surgery, Harvard Medical School, 4 Blackfan Circle, Boston, MA 02115 (FAX: 781-275-5970; E-mail: info@bedfordresearch.org).

0015-0282/08/$34.00
doi:10.1016/j.fertnstert.2007.08.083

the PCR conditions to detect only abundant organisms, on the order of 200 per 10 μL of semen, the volume of a standard bacteriologic loop. The PCR products were sequenced directly; if the sequences indicated the presence of multiple organisms, the PCR was repeated and the products ligated and cloned for sequencing. The rDNA sequences were compared with GenBank by BLAST for identification of organisms. Bacterial rDNA remnants in laboratory reagents and plasticware were identified by repeat assays with reagents only, and those sequences eliminated from the patient database.

METHODS AND MATERIALS

Semen Specimens

Twenty-nine men undergoing infertility evaluation and five men undergoing vasectomy collected semen specimens for this study, which was reviewed and approved by the Human Subjects Committee, Faulkner Hospital. Fifteen men undergoing infertility evaluation collected specimens either in a fertility condom (Male Factor Pak), delivered to the laboratory within 1 hour, or collected at the laboratory by masturbation into a sterile urine container. Routine semen analyses were performed as described (6), including measurement of volume, viscosity, sperm count, round cell count, and percentage motility. The five men undergoing vasectomy collected specimens before and after vasectomy. Fourteen specimens were divided as described below and delivered for evaluation by overnight courier.

Isolation of DNA From Semen Specimens

Aliquots (0.5 mL) of the semen specimens were adjusted to 3.0 mol/L to 3.5 mol/L guanidium isothiocyanate, 5 mmol/L to 15 mmol/L EDTA, and 50 μg/mL to 200 μg/mL Poly A carrier (Sigma Aldrich, St. Louis, MO). The semen-guanidium mixture was centrifuged at 8,000*g* for 6 minutes to pellet unlysed sperm heads, and 200 μL of the supernatant was added directly to the AVL solution provided in the Qiagen QIAamp viral RNA mini-isolation kit (Valencia, CA) and stored at −80°C until use.

The DNA was isolated in a designated area of the lab, separate from all other PCR setup, amplification, cloning, and storage areas. Fifty microliters of the AVL mixture (equivalent to approximately 10 μL of the original specimen) were Qiagen column purified according to the manufacturer's instructions and eluted in 30 μL of mouse embryo–tested water (Sigma-Aldrich). The 30-μL DNA eluate was used directly for PCR amplification. For each experiment, a no-DNA PCR control was created by parallel column purification of AVL buffer without semen. Approximately 10% of such reaction blanks yielded detectable PCR products, which were routinely analyzed for rDNA sequences.

Bacterial DNA From Control Samples

Aliquots (one bacteria loopful from a culture plate) of bacterial cultures, kindly provided in a blinded fashion by Peter Al-Achi, Assistant Laboratory Supervisor of the Microbiology Teaching Laboratory, Northeastern University, were placed directly into 800 μL of Qiagen AVL buffer for transport and storage. The DNA was extracted from 50-μL aliquots of each bacteria-AVL solution as described in the preceding and used directly in the PCR.

The sensitivity of PCR detection of bacteria in the semen specimens was determined by end-point dilution of *Escherichia coli* into 50 μL aliquots of two specimens that tested negative. Fewer than 250 copies of *E. coli* DNA did not yield a visible PCR band in the PCR assay conditions described subsequently.

Immunostaining

Fixed semen cells (7) from 0.5 mL to 1.0 mL of each semen specimen were recovered by centrifugation, washed twice with phosphate-buffered saline (Sigma-Aldrich), plated onto slides, and immunostained with two commercially available monoclonal antibodies against leukocyte common antigen: CD45, HLe-1 (Becton Dickinson, San Jose, CA) and LCA (Dako, Carpinteria, CA). Total leukocytes in the semen specimen were calculated from percentage of immune-positive cells obtained by counting at least 200 nonsperm cells on the slides stained with HLe-1, and total lymphocytes/monocytes were calculated in the same way on the slides stained with LCA (7).

Polymerase Chain Reactions

Primers for the PCR amplifications were designed for specific regions of the bacterial genes encoding 16S ribosomal RNAs. The regions chosen contained two highly conserved sequence sites that bracket species-specific variable regions and have been used to detect a wide range of bacteria, including micoplasms and ureaplasms. The sequence for the forward primer, AACTGGAGGAAGGTGGGGAT (Invitrogen, Grand Island, NY), is analogous to *E. coli* (AE000474) bases 1446−1465; the sequence for the reverse primer, AGGAGGTGATC CAACCGCA, is analogous to *E. coli* bases 1798–1816. The PCR amplicon is approximately 365 bases long. A PCR strategy was developed to limit the sensitivity of detection to abundant organisms and to avoid detection of bacterial DNA remnants in laboratory reagents. The PCR cycling was carried out with *Taq* polymerase (Applied Biosystems, Roche, Branchburg, NJ), 0.75 U to 1.5 U; 50 pmol of each primer; 200 μmol eachof dATP, dCTP, dGTP, and TTP; 1.5 mmol/L $MgCl_2$; 20 mmol/L Tris-HCl (pH 8.3 at 25°C); 20 mmol/L KCl; 100 ug/mL bovine serum albumin; 0.05% NP40; and 3.5% ethylene glycol in a total volume of 100 μL. Cycling strategy was: 95°C for 4 minutes, followed by 30 cycles of 95°C, 55°C, 72°C, 30 seconds each, with a final 7-minute extension at 72°C. The PCR products were visualized by ethidium bromide (Continental Lab Products, Carlsbad, CA) staining of product DNAs after electrophoresis through 2.3% agarose gels in TBE buffer (BioRad). Products detected were purified through Qiagen PCR product purification

columns, and submitted to the Beth Israel Deaconess Medical Center gene sequencing core laboratory for sequencing in the forward, and sometimes the reverse, direction. Gene sequences containing more than 3% ambiguous bases were discarded. Sequences were edited with Sequencer for the Macintosh, trimmed to 320 bases (*E. coli* bases 1475–1795) and submitted to GenBank BLASTn search (www.ncbi.nih.gov) for comparison with known bacterial sequences. Gene sequences with fewer than 95% identical bases were not considered a match. Specimens yielding PCR products with >3% ambiguous bases were assayed again and the PCR products cloned into the TOPO-2 cloning kit according to the manufacturer's instructions before sequencing. At least 10 colonies were PCR amplified for sequencing. The sequences included in this report are GenBank accession numbers DQ859182 to DQ859258.

Statistical Analysis

Groups of data were compared by unpaired Student *t* test in Excel; *P* values greater than .02 were not considered to be statistically significant.

RESULTS

Assay Strategy

The assay strategy was designed to address three issues: 1) accurately identify bacteria; 2) avoid detection of commensals; and 3) distinguish laboratory reagent contaminants from bacterial rDNA in clinical specimens (8). The latter two problems arise because of the sensitivity of PCR amplification, which under standard assay conditions can amplify one DNA target sequence up to 10 million–fold in a single reaction (9).

An initial test of the assay strategy resulted in accurate identification from PCR products of five blinded test bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus epidermidis*, *Citrobacter freundii*, and *Streptococcus pyogenes*) with greater than 98% identity to their respective ribosomal gene sequences in GenBank. This outcome supported the possibility of identifying semen bacteria from direct sequencing of PCR products.

To decrease detection of reagent contaminants and commensals, the PCR reaction was adjusted to detect no fewer than 200 microorganisms in the 10 μL of semen. Because there are multiple copies of the rRNA gene in bacteria, the actual target number of sequences amplified ranged from 1,000 to 1,400. To control for laboratory reagent contaminants, multiple PCR reactions with no added DNA were carried out. Greater than 0.75 U of *Taq* polymerase in the PCR reaction resulted in a faint band in approximately 12% of the no-DNA controls. The cloned no-DNA control sequences (DQ859258, DQ859257) matched members of the genera *Acidovorax* and *Leptothrix* reported to be detectable DNA remnants in laboratory reagents (8).

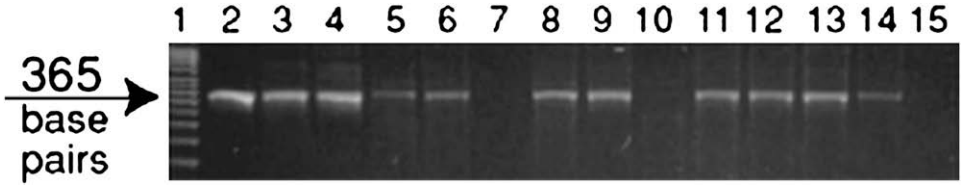
Semen Bacteria

Overall, 25 (56%) of the 45 semen specimens were positive for bacterial rDNAs from greater than 20,000 organisms/mL (examples in Fig. 1). The positive specimens were produced by 22 (65%) of the 34 men. Eleven of the 15 men contributing fresh specimens had at least one that tested positive for bacteria (Table 1). Similarly, 11 of the 19 men contributing specimens that were shipped to the laboratory had at least one positive for bacterial rDNA (Table 2). Interestingly, only two of the prevasectomy semen specimens, but all five of the postvasectomy specimens, were positive for bacteria (Table 2).

Only 7 of the 26 positive specimens yielded PCR products that were unambiguously sequenced and matched in GenBank without cloning. One was in a prevasectomy specimen (#28, DQ859243; Table 2) and matched to *Corynebacterium pseudogenitalium*; one was in a postvasectomy specimen (#31, DQ859218; Table 2) and matched to *Lactobacillus iners*. The other five were in infertility specimens and matched to: *Streptococcus mitis* (#1, DQ859216; Table 2), *Staphylococcus epidermidis/haemolyticus* (#2, DQ859231, #13, DQ 859246; Table 1), *Lactobacillus crispatus* (#19, DQ859217; Table 1), and *Alcaligenes faecalis* (#38, DQ859231; Table 2). That these organisms were identified directly from PCR products was taken to mean that they were the most abundant organisms in the specimen, not that they were the sole organisms; most were also present in other specimens with a mixture of bacteria whose identifies were resolved by cloning.

FIGURE 1

Polymerase chain reaction (PCR) detection of semen bacteria. Photo of ethidium bromide–stained agarose gel containing PCR products from 14 semen specimens. Lanes 7 and 10 were from specimens with undetectable bacteria; lane 15 is the no-DNA PCR control sample.



Kiessling. Bacteria in semen. Fertil Steril 2008.

TABLE 1

Semen cells and bacteria detected in fresh specimens.

Specimen no.	Sperm count[a]	% motile[b]	% normal forms[c]	Nonsperm cells[d]	Leukocytes[e]	Lymphocytes/ monocytes[f]	Bacterial rDNA[g]
1	44	21 (1)	5 (0)	0.5	0.1	<0.1	*Streptococcus mitis* 96, AF543291
2	101	28 (2)	18 (2)	3.8	1	<0.1	*Staphylococcus epidermidis* 95, CP000029
3	65	72 (2)	12 (1)	2.9	1.2	0.1	*Peptoniphilis harei* 93, Y07839 *Finegoldia magna* 98, Ab109771.1 *Anaerococcus prevoti* 96, AF542232
4	27	45 (20)	8 (0)	1.9	0.4	<0.1	*Anaerococcus vaginalis* 98, AF542229 *Pseudomonas putida* 98, Af131103 *Finegoldia magna* 98, Ab109771.1 *Corynebacterium tuberculostearicum* 98, AF438050
5	79	70 (2)	8 (0)	11	0.2	<0.1	*Finegoldia magna* 100, Ab109771.2 *Peptoniphilus harei* 98, Y07839
6	41	56 (2)	9 (0)	1	<0.1	<0.1	*Finegoldia magna* 100, Ab109771.2 *Peptoniphilus harei* 98, YO7839 *Peptostreptococcus anaerobius* 99, AY326462 *Streptococcus pneumoniae* 99, AE008552
7	25	72 (2)	21 (0)	1.0	0.9	<0.1	undet
8	91	73 (3)	12 (1)	0.9	0.3	<0.1	*Finegoldia magna* 96, Ab109771.2
9	168	70 (2)	20 (5)	1.1	0.2	<0.1	undet
10	100	20 (1)	10 (1)	24	14	31	undet
11	122	35 (2)	22 (2)	13	6	22	undet
12	62	47 (2)	26 (7)	9	3.5	2.7	undet
13	25	43 (2)	11 (0)	4.8	3.5	1.1	*Staphylococcus haemolyticus* 98, AP006716
14	30	44 (2)	13 (2)	3.5	1.5	0.4	*Finegoldia magna* 98, Ab109771.2 *Lactobacillus gasseri* 99, AF243156.1 *Lactobacillus crispatus* 97, AF257097 *Corynebacterium striatum* 97, X844442 *Anaerococcus prevoti* 98, AF542232 *Anaerococcus vaginalis* 98, AF542229
15	170	51 (3)	9 (1)	11	10	3.5	*Pseudomonas putida* 99, AF131103 *Acinetobacter junii* 99, AB10144
16	31	27 (2)	18 (8)	7	5	5.8	undet
17	113	35 (3)	27 (6)	12	7	5	undet
18	30	21 (2)	10 (3)	1.9	<0.1	0.4	*Finegoldia magna* 98, AB109771.1 *Corynebacterium tuberculostearicum* 96, AJ438050 *Anaerococcus prevoti* 97, AF542232 *Staphylococcus epidermidis* 98, CP000029
19	12	31 (2)	5 (0)	1	0.3	0.1	*Lactobacillus crispatus* 98, AF257097
20	33	67 (3)	39 (4)	0.5	0.4	<0.1	undet
21	42	50 (2)	17 (6)	3	9.5	0.3	undet

Note: Each of the specimen sets, nos. 5–7, 10–12, and 15–17 are from one man.

[a] Sperm/mL semen.

[b] Percentage of sperm moving; number in parentheses is speed of forward motion on a scale of 1 to 4.

[c] Sperm morphology according to World Health Organization criteria; number in parentheses is Kruger strict criteria.

[d] Round cells/mL of semen.

[e] Round cells staining red-brown with monoclonal antibody HLe-1 directed against all leukocytes.

[f] Round cells staining red-brown with anti-CD45 antibody LCA binding lymphocytes and monocytes.

[g] Bacteria species identified as highest match in GenBank followed by percentage identity to the accession number.

Kiessling. Bacteria in semen. Fertil Steril 2008.

TABLE 2

Semen cells and bacteria detected in shipped specimens.

Specimen no.[a]	Sperm/mL (mill)[b]	Nonsperm cells/mL[c]	Leukocytes[d]	Lymphocytes[e]	Bacterial rDNA
22	88	0.9	0.7	0.5	undet
23	<0.1	<0.1	<0.1	<0.1	*Finegoldia magna* 96, AB109771 *Streptococcus pneumoniae* 98, AE008532 *Aerococcus urinae* 99, AY422717
24	129	5.7	2.4	0.4	*Finegoldia magna* 96, AB109771 *Streptococcus pneumoniae* 98, AE008532 *Staphylococcus epidermidis* 99, AY728198 *Aerococcus urinae* 99, AY422717 *Corynebacterium pseudogenitalium* 99, AJ439348
25	<0.1	<0.1	<0.1	<0.1	*Anaerococcus prevoti* 98, AF542232 *Acinetobacter junii* 99, AB10144
26	184	10.8	5.6	4.4	undet
27	<0.1	2.5	1.1	<0.1	*Finegoldia magna* 96, AB109771 *Corynebacterium pseudogenitalium* 96, AJ439348 *Lactobacillus iners* 99, Y16329 *Neisseria weaveri* 97, L10738
28	215	5	2.5	1.0	*Corynebacterium pseudogenitalium* 96, AJ439348
29	0.1	2	1.2	0.1	*Corynebacterium pseudogenitalium* 96, AJ439348 *Streptococcus pneumoniae* 98, AE008552
30	147	21.5	6.7	3.1	undet
31	0	0.8	<0.1	<0.1	*Lactobacillus iners* 97, Y16329
32	23	4.6	2.3	2.1	undet
33	22	2.7	1.6	1.1	undet
34	8.2	1.6	1.6	1.2	undet
35	14	1.3	2.4	1.4	undet
36	75	1.8	0.5	0.4	*Haemophilus influenzae* 97, AF224308 *Streptococcus pneumoniae* 98, AE008552
37	18	1.3	0.5	0.4	undet
38	2.9	0.3	nd	nd	*Alcaligenes faecalis* 98, AF155147
39	48	5.3	0.7	0.3	*Streptococcus pneumoniae* 98, AE008552 *Staphylococcus epidermidis* 99, CP000029 *Pseudomonas putida* 99, AF131103
40	58	2.8	2.2	1.0	undet
41	0.7	0.5	nd	nd	undet
42	6.9	1.5	0.7	0.3	*Haemophilus parainfluenza* 100 M75081 *Lactobacillus iners* 97, Y16329 *Staphylococcus haemolyticus* 98, AP006716
43	8.1	2	1.3	0.5	undet
44	62	4	1.1	0.5	*Finegoldia magna* 96, AB109771 *Anaerococcus prevoti* 98, AF542232 *Peptoniphilus harei* 97, Y07839 *Corynebacterium pseudogenitalium* 96, AJ439348 *Pseudomonadales putida* 98, AF131103 *Staphylococcus epidermidis* 99, CP000029
45	27	3.2	0.5	<0.1	*Anaerococcus vaginalis* 98, AF542229 *Streptococcus pneumoniae* 98, AE008552

[a] Each of the specimen pairs nos. 22 and 23, 24 and 25, 26 and 27, 28 and 29, and 30 to 31 is from one man, with the first sample before and the second sample after vasectomy.
[b] Sperm/mL of semen specimen.
[c] Nonsperm cells/mL semen specimen.
[d,e] As in Table 1.

Kiessling. Bacteria in semen. Fertil Steril 2008.

Cloning the PCR products in the remaining 19 positive specimens introduced the possibility of detecting less abundant bacterial species, such as those present in assay reagents, as has been described earlier (8). We controlled for this by cloning and analyzing PCR products produced in multiple no-DNA assay controls. The two bacterial species consistently amplified in the positive assay reagent controls, *Acidovorax* and *Leptothrix*, were also cloned from 8 of the 19 bacterial rDNA–positive semen specimens and were eliminated from the patient data set. Other organisms, either not identified or possible chimeras of more than one organism, were also cloned from seven semen specimens and were also not included in the patient data set.

Overall, 149 rRNA gene sequences were analyzed, 78 of which (Table 3) were included in Tables 1 to 4. They fell into 14 groups according to GenBank matches. *Corynebacteria*, *Staphylococcus*, *Aerococcus*, *Lactobacillus*, *Streptococcus*, *Anaerococcus*, *Finegoldia*, *Peptoniphilus*, *Neisseria*, and *Acinetobacter* were genera identified in the vasectomy specimens (Tables 2 and 4). *Aerococcus* and *Neisseria* were genera not detected in the infertility specimens (Table 4), but several species not present in the vasectomy specimens were present in infertility specimens (Tables 1 and 4): *Lactobacillus gasseri/crispatus* (#14, #19), *Peptoniphilus harei* (#3, #5, #6, #44), *Alcaligenes faecalis* (#38), *Haemophilus influenza* (#36, #42; Table 2), and *Pseudomonas putida* (4, 15, 39, 44).

The boxshade analysis of the sequences referenced to *E. coli* (Table 3) reveals surprising homogeneity among the species detected in multiple semen specimens. Approximately one-third of the sequences identified were gram-positive anaerobic cocci (GPAC): *Peptoniphilis harei*, *Anaerococcus*, *Finegoldia magna*, and *Peptostreptococcus anaerobius*. Interestingly, the GPAC-positive vasectomy specimens contained one form each, either *Finegoldia magna* or *Anaerococcus*, whereas the GPAC-positive infertility specimens contained two to four species each, including *Finegoldia magna* in eight of the nine. It is important to note that although GPAC were the most abundant bacterial forms detected, none were amplified as a dominant species, all were present as part of a mixture of bacteria.

Although there were differences in the bacteria species detected in the vasectomy specimens relative to the infertility specimens, there were no quantitative differences in the average number of species, with a mean of 2.3 for the infertility specimens and 2.4 for the vasectomy specimens.

Semen Bacteria and Semen Analysis

There were no statistically significant differences in average sperm count, percentage motile sperm, or their forward progression between the bacterial rDNA–positive and –negative specimens delivered fresh to the laboratory (Table 1). There were, however, more normal sperm forms (Table 1) in the bacterial rDNA–negative specimens (22 $\pm$ 2%) than in the positive specimens (10 $\pm$ 1.2%; P=.01).

The bacterial rDNA–negative groups (Tables 1 and 2) contained more than twice as many nonsperm cells (NSCs) as the bacterial rDNA–positive group (6.3 million/mL and 3.2 million/mL, respectively). The immunostain results revealed that the increase in NSCs was principally due to leukocytes. The monoclonal antibodies used in this study against the pan-leukocyte marker CD45 distinguish the subset of lymphocytes/monocytes (LC positive) from all leukocytes, including polymorphonuclear cells (HLe-1 positive). There were threefold more HLe-1–positive leukocytes (3.4 $\pm$ .6 million/mL) in the bacterial rDNA–negative specimens than in the positive specimens (1.2 $\pm$ 4 million/mL) and fivefold more LC-positive leukocytes in the bacterial rDNA–negative specimens (1.9 $\pm$ 0.4 million/mL) than the positive specimens (0.4 $\pm$ 0.1 million/mL; P=.02). Importantly, some bacteria-positive specimens had elevated leukocytes (e.g., #15), whereas others had marked elevations in immature sperm forms (e.g., #5).

Response to Antibiotic Therapy

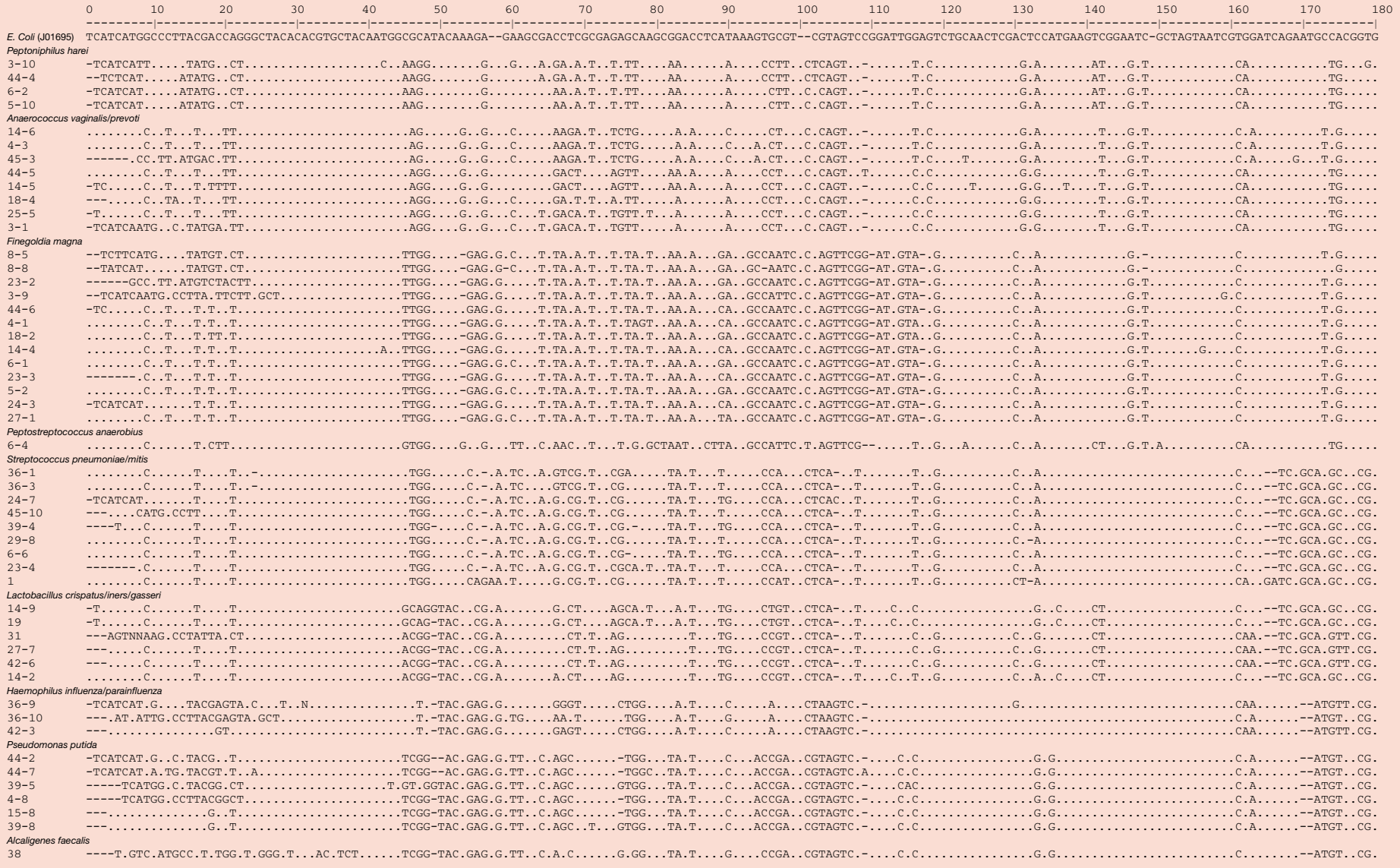
Three men were placed on antibiotic therapy after an initial semen analysis (specimens #5, #10, and #15; Table 1). Each man submitted repeat semen specimens (#6, #11, and #16, respectively; Table 1) after 2 weeks to 3 weeks of antibiotic therapy and a third follow-up specimen (#7, #12, and #17; Table 1) one month to two months after a second round of antibiotic therapy.

Two of the semen specimens from the first patient (#5–#7; Table 1) were positive for the same bacterial rDNA, indicating that 3 weeks of antibiotics were not sufficient to clear the bacteria; the third specimen tested negative. Direct sequencing of PCR products from the first two specimens revealed a mixture of bacteria. Cloning the PCR products resolved the mixture into *Streptococcus pneumoniae* and three GPAC (*Finegoldia magna*, *Peptoniphilus harei*, and *Peptostreptococcus anaerobius*; Table 1). Interestingly, most of the NSCs from this patient did not immunostain positively for leukocytes but exhibited characteristics of immature germ cells. The antibiotic therapy appeared to reduce the numbers of both sperm and NSCs, and the third, bacterial rDNA–negative, specimen had more morphologically normal sperm than the previous specimens (Table 1). This suggests that the bacteria may have been in the germ cell compartment and that they may have interfered with germ cell maturation, as has been suggested (10).

None of the semen specimens from the second man (#10–#12) were positive for bacteria in this study, although most of the NSCs immunostained positive for leukocytes and their numbers were markedly reduced after two rounds of antibiotics with some improvement in sperm motility. The PCR primers used in this study would detect mycoplasms and ureaplasms as well as most bacteria (3), but the negative findings suggest that this patient's semen contained either a lower concentration of bacteria or a microorganism whose rDNA was not amplified by these primers, such as yeast.

TABLE 3

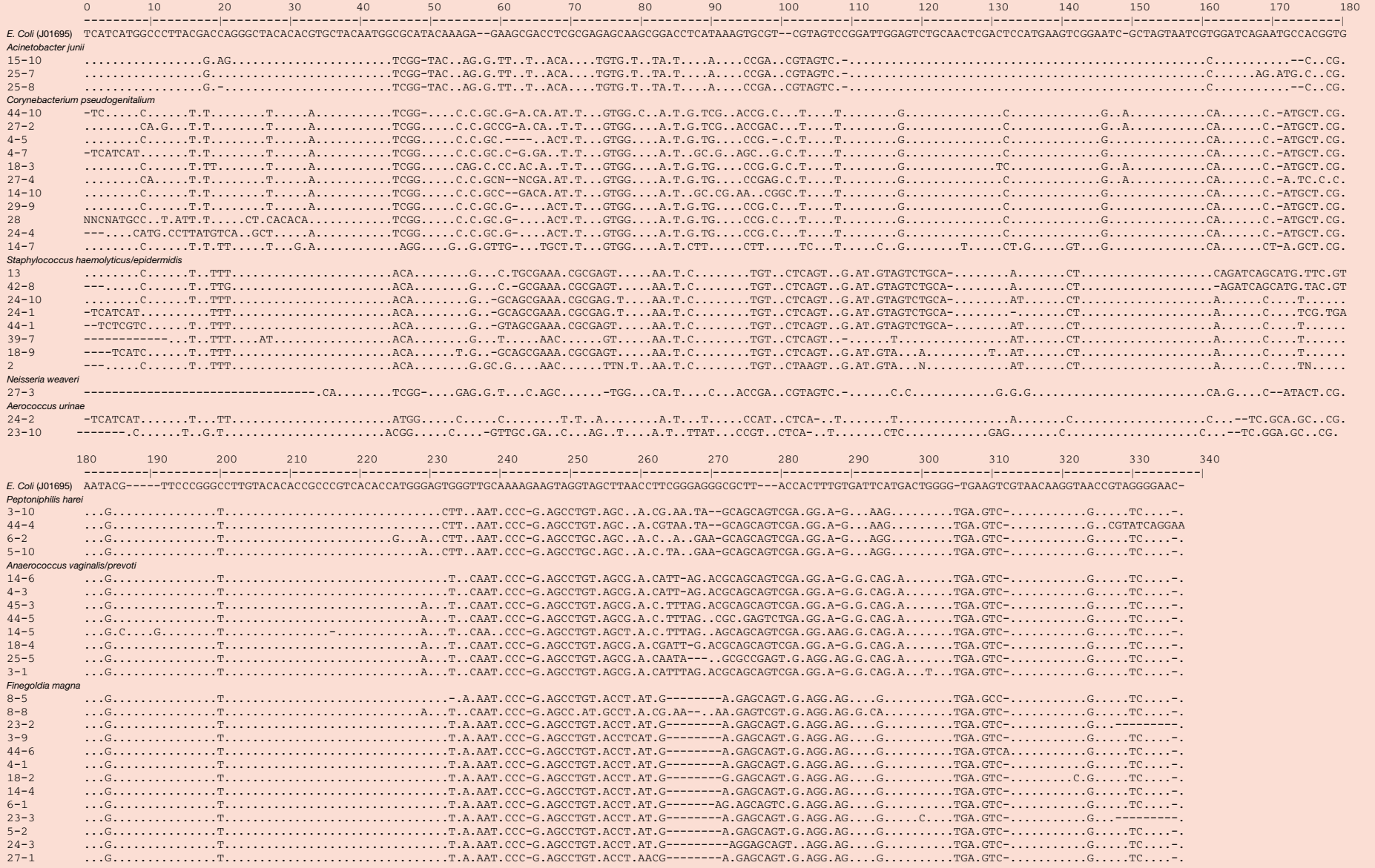
Boxshade of semen rDNA sequences relative to *Escherichia coli*.



Kiessling. Bacteria in semen. Fertil Steril 2008.

TABLE 3

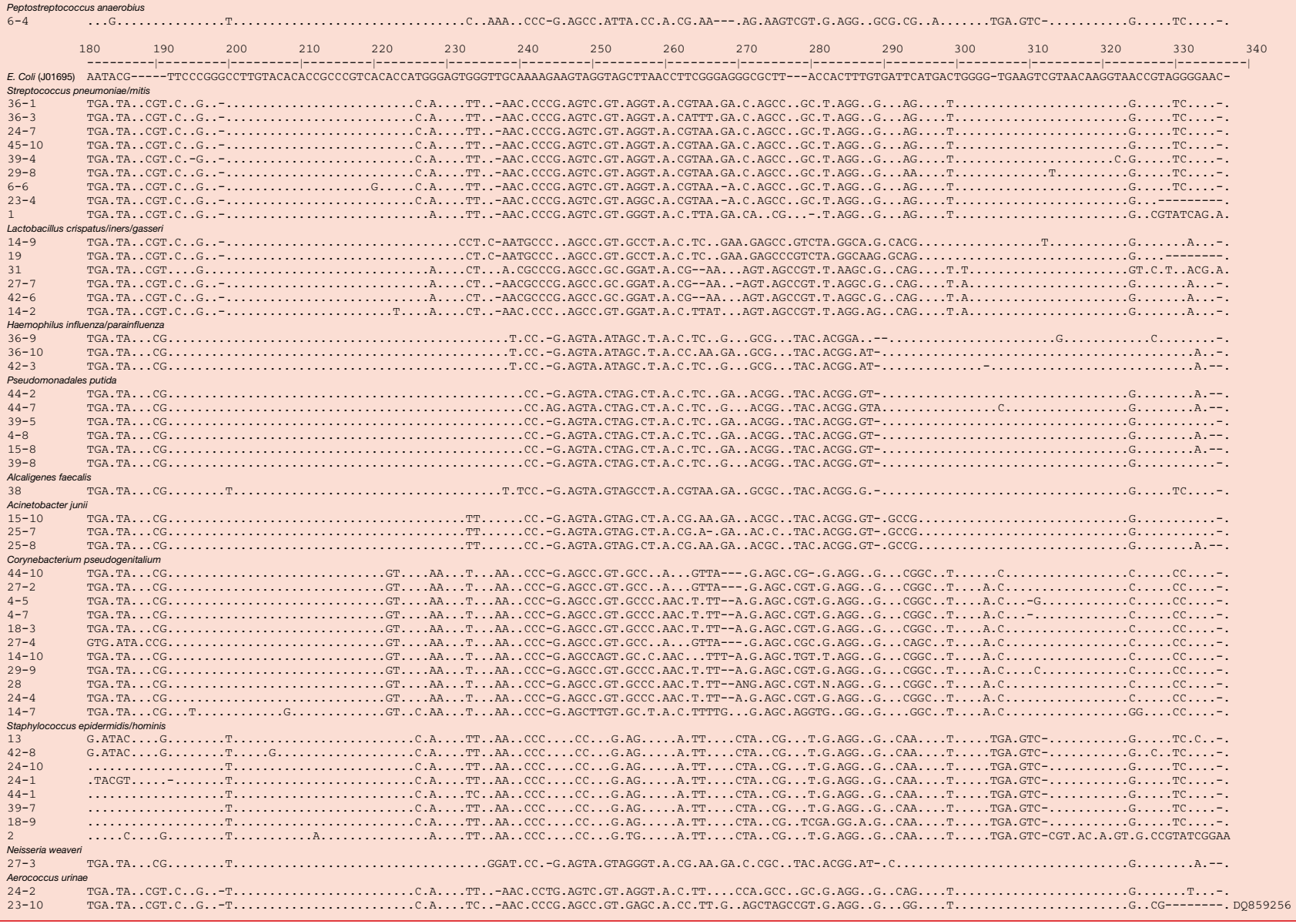
Continued.



Kiessling. Bacteria in semen. Fertil Steril 2008.

TABLE 3

Continued.



Kiessling. Bacteria in semen. Fertil Steril 2008.

TABLE 4

Distribution of bacteria among semen specimens testing positive.

		Infertility Group A											Vasectomy							Group B					
		1	2	3	4	5	6	8	13	14	15	18	19	23	24	25	27	28	29	31	36	38	39	42	44	45
Phylum:	**Actinobacteria**																									
Class:	Actinobacteria																									
Organism:	*Corynebacterium pseugogenitalium/ tuberculostearicum/ striatum*				X					X		X			X		X	X	X						X	
Phylum:	**Firmicutes**																									
Class:	Bacilli																									
Organism:	*Staphylococcus epidermidis/haemolyticus*		**X**						**X**			X			X								X	X	X	
	Aerococcus urinae													X	X											
	Lactobacillus iners																X			**X**				X		
	Lactobacillus gasseri									X																
	Lactobacillus crispatus									X			**X**													
	Streptococcus mitis/ pneumoniae	**X**					X							X	X				X		X		X			X
Class:	Clostridia																									
Organism:	*Anaerococcus prevoti/ vaginalis*			X	X					X		X				X									X	X
	Finegoldia magna			X	X	X	X	X		X		X		X	X		X								X	
	Peptoniphilus harei			X		X	X																		X	
	Peptostreptococcus anaerobius						X																			
Phylum:	**Proteobacteria**																									
Class:	Betaproteobacteria																									
Organism:	***Alcaligenes faecalis***																					**X**				
	Neisseria weaveri																X									
Class:	Gammaproteobacteria																									
Organism:	***Haemophilus influenzae***																				X			X		
	Acinetobacter calcoaceticus/junii										X					X										
	Pseudomonas putida				X						X												X		X	

Note: Infertility Group A refers to specimens by number as listed in Table 1. Vasectomy and Group B refer to specimens listed in Table 2. Bold X's indicate organisms identified by sequencing PCR products; organisms in bold were not present in vasectomy specimens.

Kiessling. Bacteria in semen. Fertil Steril 2008.

The first semen specimen from the third patient (#15; Table 1) was positive for bacteria and the two specimens (#16 and #17) after antibiotic therapy were negative. Two bacteria were identified, *Pseudomonas putida* and *Acinetobacter junii*. Antibiotic therapy did not bring about a significant change in the numbers of leukocytes in the semen specimens from this patient even though the bacterial signal was eliminated.

DISCUSSION

This study supports the potentially high clinical value of PCR amplification of bacterial rDNA in semen specimens to help monitor the health of male genitourinary organs. The PCR amplification of semen DNA to detect bacteria was previously reported by Jarvi et al. in 1996 (11), and a study of expressed prostatic secretions (EPS) was reported by Tanner et al. in 1999 (12). Those studies provided valuable background information which agrees in several important respects with the results of the present study.

First is the frequency of positive specimens: 66% of 39 men with no symptoms of urinary tract infection in the Jarvi study, 68% of EPS specimens from the 25 men in the Tanner study, and 65% of the 34 men in the present study. The PCR results were in contrast to the culture results: only 27% of the specimens from the infertile men and none of the sperm donor specimens were culture-positive in the Jarvi study, and only 41% of the 17 patients with chronic prostatitis had culture-positive EPS in the Tanner study.

Second is the nature of the bacteria detected by PCR. Importantly, *Escherichia coli* was not detected by PCR in

the Jarvi study nor in the present study, and in only 3 of 17 specimens in the Tanner study, although it is generally thought to be a common male genitourinary tract pathogen (13). This suggests that although *E. coli* may be present in semen, the numbers of organisms are too few to compete in the PCR reactions, but that they are more readily cultured than other organisms such as GPAC.

The PCR products from four specimens in the Jarvi study were sequenced and compared with GenBank for identification. An average of 9 (range 6 to 11) bacterial species were detected per specimen. At that time, the most common organisms did not have matches in GenBank, but were most closely related to *Peptostreptococcus* species, along with two species of *Streptococcus* and one *Corynebacterium*, in agreement with the results reported here. Multiple organisms, most commonly *Corynebacteria*, *Staphylococcus*, and *Streptococcus*, were also identified for each positive EPS specimen in the Tanner study, an average of 5 (range 2 to 12) for the prostatitis patients and an average of 2.5 (range 2 to 5) for the asymptomatic controls (12).

Third, there is agreement between the Jarvi study and the results reported here that the detection of bacteria does not correlate with elevations in semen leukocyte counts, an observation also reported by others, including a recent study published while the present work was in progress (14).

Several earlier studies designed to culture both aerobic and anaerobic organisms from semen of asymptomatic men have been reported. A 1979 study by Rehewy et al. (15) yielded bacterial growth from 54% of 26 specimens from fertile men and 73% of 83 infertile men. Five organisms were cultured from the fertile men: *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Corynebacterium*, *Mycoplasma hominis*, and *Ureaplasma urealyticum*. In contrast, the specimens from infertile men yielded the same five organisms, plus *E. coli: Klebsiella pneumoniae*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Candida albicans*, *Bacteroides fragilis*, *Peptostreptococcus*, *Eubacterium*, and three species of *Streptococcus*.

A 1990 study designed to culture both aerobic and anaerobic organisms from semen of asymptomatic men undergoing infertility evaluation, reported by Hillier et al. (16), detected organisms in 97% of 37 specimens. They initiated culture "within 5 minutes of sample collection." They observed an average of 5.2 organisms per ejaculate. They reported that microorganisms that "colonize the skin" (coagulase-negative staphylococci, "diphtheroids" (a term for *Corynebacteria*) were recovered from all positive semen specimens, suggesting to these authors that they commonly inhabit the distal urethra. *Lactobacillus*, *Gardnerella*, *Clostridium*, *Bacteroides*, *E. coli*, *Enterococcus*, and *Peptostreptococcus* also were cultured. Importantly, those authors also noted the lack of correlation between the presence of bacteria and elevations in semen leukocytes.

A more recent study cultured aerobic organisms from 299 semen specimens (14), of which 64% were positive. *Staphylococcus* spp., *Streptococcus viridans*, and *Enterococcus faecalis* were the most commonly cultured species, in basic agreement with the aerobic species detected by PCR in the present study. Importantly, that study also found no positive correlation between leukocytospermia and bacterial growth and no influence of bacteria on sperm count or motility; the researchers also found a negative correlation between normal sperm forms and the presence of bacteria.

Taken together, the data support new perspectives with respect to semen infection:

1. Leukocytospermia is not a reliable indicator of the presence of bacteria in semen. Whether or not the bacteria represent pathogenicity in male reproductive tract organs requires further studies, but it is clear that sperm may be exposed to significant populations of microorganisms in the absence of elevations in semen leukocytes. This observation is particularly important for sperm destined for assisted reproduction techniques such as intrauterine insemination or IVF. The lack of correlation between lymphocytes/monocytes and semen bacteria supports the somewhat controversial concept that at least some semen leukocytes are present in the ejaculate as a routine method of disposal from the epididymis (6). The decrease in semen leukocytes in the five post-vasectomy specimens included in this study supports this concept and agrees with data published over 20 years ago (17).

More recent studies of the mouse epididymis revealed populations of tissue-specific leukocytes within the gland that sometimes appear within the lumen of the epididymal tubules, but not in the interstitium surrounding the tubules (18). This supports the concept that the epididymis contains a population of leukocytes that can appear in the ejaculate but do not communicate with the lymphatic system. It is important to discover if a similar population of tissue-specific leukocytes exists in the human epididymis. It is tempting to speculate that tissue-specific leukocytes in the epididymis do not communicate with the lymphatic system to protect spermatozoa from immune recognition but are responsible for pathogen surveillance by mechanisms unique to the germ cell compartment. This suggests that semen lymphocytes/monocytes may be a measure of normal epididymal function, a notion in need of research. Infection within the epididymis or seminiferous tubules, perhaps subclinical, could have a profound effect on sperm production and maturation. Semen lymphocytes could be a measure of a robust immune response to infection in the germ cell compartment; the presence of bacteria in semen in the absence of lymphocytes could indicate the lack of immune response.

The increased incidence of bacteria in semen specimens after vasectomy could be spurious because of the small sample size, or it could be due to technical considerations, such as the sharp decrease in competitive DNA content in the semen specimen with the elimination of sperm. Alternatively, it could relate to the provocative epididymal-specific defensins recently described in the human epididymis (19, 20). Defensins are a class of antimicrobial proteins of emerging

importance in pathogen response. The epididymis of rodents and humans have been found to express relatively high levels of defensins, including some epididymal-specific members. Given the findings presented here, it is tempting to speculate that epididymal defensins may protect downstream tissues from bacterial infection and that abnormal sperm may reflect bacterial infection in the epididymis, perhaps GPAC, either innately resistant to defensins, or due to poor production of defensins. This is an area much in need of further research.

2. More than half of semen specimens contain bacteria in excess of 20,000 organisms/mL, but they are not the same organisms reported by the bacterial culture studies. More difficult-to-culture species, such as GPAC and *Corynebacteria*, appear to be the most common semen organisms. Although they are commensals in skin and mucosa, GPAC also account for about 25% to 30% of all anaerobic isolates from anaerobic infections in humans (5), supporting the possibility that the numbers of these organisms detected in the GPAC-positive specimens reflect infection (21, 22) and that more than one male organ may be involved. It is possible that at least some of the species of coryneforms reside in the distal urethra, but this is not the likely location of GPAC. The diversity and pathogenic importance of *Corynebacteria* has become appreciated in recent years; and it is possible that semen *Corynebacteria* arise from more than one male organ (23).

Streptococcus mitis, a known commensal in the oropharynx and genital tract, is also an important human pathogen (24–26). *Lactobacillus crispatus* is considered to be normal vaginal flora but of unknown significance in semen. *Aerococcus urinae*, detected in two vasectomy specimens, is generally associated with bladder infections in elderly patients but can also be the cause of serious soft tissue infection (27). *Staphylococcus* spp., known to be common skin commensals, are also known pathogens (28). *Acinetobacter junii* is an emerging nosocomial pathogen (29). *Pseudomonas putida* is a more common genitourinary tract infection than generally appreciated (30) and was detected only in infertility patients (n = 4) in the present study. *Alcaligenes faecalis* and *Neisseria weaveri* were each the only organism detected in a single semen specimen; *A. faecalis* is a water-borne motile rod of emerging importance in human infections (31, 32), and *N. weaveri* is normal flora in the mouths of dogs but known to cause human infections (33). *Haemophilus influenzae* and *H. parainfluenzae* are also human commensals known to cause infection (34).

Infection in the prostate is a common occurrence in men and is a likely source of semen bacteria. Although the presence of inflammatory cells in semen and/or EPS has generally been considered to be a significant clinical feature of prostatitis (12), the lack of correlation of semen bacteria and semen leukocytes in several studies suggests that this may not be the case. This notion is further supported by the profound programmed cell death initiated by prostatic factors on peripheral blood mononuclear cells (35), suggesting that factors produced by the gland may innately suppress a leukocyte response to infection. Studies to detect bacterial rDNAs in prostate tissues revealed positive signals in biopsies from men with benign prostatic hypertrophy and clinical prostatitis and an absence of bacteria in prostate biopsies from organ donors with no evidence of prostatic inflammation (36). A larger study had revealed bacterial rDNAs in the biopsies of 77% of patients with prostatitis (37). Most prostate bacterial sequences had no counterparts in GenBank at that time but some similarities to each other and some to known laboratory reagent contaminants. Taken together, the data indicate there are no "normal" flora in the prostate which may frequently be the site of bacterial infection. The role of bacterial infection in prostate disease is in need of further study. Similar studies of seminal vesicles are lacking.

In summary, this genetic approach to bacteria identification in semen provides new insights into the abundant microorganisms coming in contact with sperm. Both semen and prostate PCR studies have detected organisms related to GPAC and *Corynebacteria*, both bacterial groups of emerging pathogenic importance and not easily cultured. The presence of bacteria in specimens with elevations in immature sperm forms suggests the possibility that a bacterial infection in the germ-cell compartment, perhaps subclinical, may compromise normal spermatogenesis, a concept supported by a recent electron microscopy study that identified epididymal stereocilia in semen specimens with abnormal sperm forms (38). The authors suggested that the shed epididymal epithelial cell stereocilia was in response to unresolved epididymal infection which was influencing the production of normal spermatozoa. Infections in the prostate, seminal vesicles, and urethra may not compromise normal spermatogenesis but will add bacteria to the semen specimen, which may influence fertility and expose sexual partners to infection.

The present study raises many questions. Long-held views about the significance and role of leukocytospermia need to be reexamined in light of the many studies revealing a higher incidence of bacterial detection in the absence of elevations in semen leukocytes. Understanding the organ source of the organisms described in the present report is needed to further define their clinical significance and to design effective antibacterial treatment strategies if indicated. Distinguishing uncommon pathogens from bacterial DNA remnants in laboratory reagents will be a continuing challenge, particularly in tissue specimens handled in pathology laboratories. And the design of streamlined assays is essential to translating these new microbiology findings to routine clinical practice.

Acknowledgments: The authors are grateful for the cooperation of Peter Al-Achi, Northeastern University, who kindly provided the blinded control bacteria, and to Drs. Rob Kaufmann and Rodney Wade, for assisting in patient recruitment.

REFERENCES

1. Head IM, Saunders JR, Pickup RW. Microbial evolution, diversity, and ecology: a decade of ribosomal RNA analysis of uncultivated microorganisms. Microb Ecol 1998;35:1–21.

2. Hugenholtz P, Goebel BM, Pace NR. Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. J Bacteriol 1998;180:4765–74.
3. Relman DA. Detection and identification of previously unrecognized microbial pathogens. Emerg Infect Dis 1998;4:382–9.
4. Domann E, Hong G, Imirzalioglu C, Turschner S, Kuhle J, Watzel C, et al. Culture-independent identification of pathogenic bacteria and polymicrobial infections in the genitourinary tract of renal transplant recipients. J Clin Microbiol 2003;41:5500–10.
5. Song Y, Liu C, McTeague M, Finegold SM. 16S ribosomal DNA sequence-based analysis of clinically significant gram-positive anaerobic cocci. J Clin Microbiol 2003;41:1363–9.
6. Kiessling AA, Lamparelli N, Yin HZ, Seibel MM, Eyre RC. Semen leukocytes: friends or foes? Fertil Steril 1995;64:196–8.
7. Kiessling AA, Yin HZ, Purohit A, Kowal M, Wolf B. Formaldehyde-fixed semen is suitable and safer for leukocyte detection and DNA amplification. Fertil Steril 1993;60:576–81.
8. Tanner MA, Goebel BM, Dojka MA, Pace NR. Specific ribosomal DNA sequences from diverse environmental settings correlate with experimental contaminants. Appl Environ Microbiol 1998;64:3110–3.
9. Fitzgerald LM, Yin HZ, Kiessling AA. PCR amplification of HIV and cellular DNA sequences in formaldehyde-fixed, immunoreactive white blood cells. Biotechniques 1993;15:128–33.
10. Purvis K, Christiansen E. Infection in the male reproductive tract. Impact, diagnosis and treatment in relation to male infertility. Int J Androl 1993;16:1–13.
11. Jarvi K, Lacroix JM, Jain A, Dumitru I, Heritz D, Mittelman MW. Polymerase chain reaction–based detection of bacteria in semen. Fertil Steril 1996;66:463–7.
12. Tanner MA, Shoskes D, Shahed A, Pace NR. Prevalence of corynebacterial 16S rRNA sequences in patients with bacterial and "nonbacterial" prostatitis. J Clin Microbiol 1999;37:1863–70.
13. Vetrosky D, White G. Prostatitis. Lippincotts Prim Care Pract 1997;1: 437–41.
14. Rodin DM, Larone D, Goldstein M. Relationship between semen cultures, leukospermia, and semen analysis in men undergoing fertility evaluation. Fertil Steril 2003;79(Suppl 3):1555–8.
15. Rehewy MS, Hafez ES, Thomas A, Brown WJ. Aerobic and anaerobic bacterial flora in semen from fertile and infertile groups of men. Arch Androl 1979;2:263–8.
16. Hillier SL, Rabe LK, Muller CH, Zarutskie P, Kuzan FB, Stenchever MA. Relationship of bacteriologic characteristics to semen indices in men attending an infertility clinic. Obstet Gynecol 1990;75:800–4.
17. Olsen G, Shields J. Seminal lymphocytes, plasma and AIDS. Nature 1984;309:116–7.
18. Mullen TE Jr., Kiessling RL, Kiessling AA. Tissue-specific populations of leukocytes in semen-producing organs of the normal, hemicastrated, and vasectomized mouse. AIDS Res Hum Retroviruses 2003;19:235–43.
19. Yamaguchi Y, Nagase T, Makita R, Fukuhara S, Tomita T, Tominaga T, et al. Identification of multiple novel epididymis-specific beta-defensin isoforms in humans and mice. J Immunol 2002;169:2516–23.
20. Yenugu S, Hamil KG, French FS, Hall SH. Antimicrobial actions of the human epididymis 2 (HE2) protein isoforms, HE2alpha, HE2beta1 and HE2beta2. Reprod Biol Endocrinol 2004;2:61.
21. Cone LA, Battista BA, Shaeffer CW Jr. Endocarditis due to *Peptostreptococcus anaerobius*: case report and literature review of peptostreptococcal endocarditis. J Heart Valve Dis 2003;12:411–3.
22. Hill KE, Davies CE, Wilson MJ, Stephens P, Lewis MA, Hall V, et al. Heterogeneity within the gram-positive anaerobic cocci demonstrated by analysis of 16S–23S intergenic ribosomal RNA polymorphisms. J Med Microbiol 2002;51:949–57.
23. Funke G, von Graevenitz A, Clarridge JE 3rd, Bernard KA. Clinical microbiology of coryneform bacteria. Clin Microbiol Rev 1997;10: 125–59.
24. Catto BA, Jacobs MR, Shlaes DM. *Streptococcus mitis*. A cause of serious infection in adults. Arch Intern Med 1987;147:885–8.
25. Narikiyo M, Tanabe C, Yamada Y, Igaki H, Tachimori Y, Kato H, et al. Frequent and preferential infection of *Treponema denticola*, *Streptococcus mitis*, and *Streptococcus anginosus* in esophageal cancers. Cancer Sci 2004;95:569–74.
26. Ong G, Barr JG, Savage M. *Streptococcus mitis*: urinary tract infection in a renal transplant patient. J Infect 1998;37:91–2.
27. Schuur PM, Sabbe L, van der Wouw AJ, Montagne GJ, Buiting AG. Three cases of serious infection caused by *Aerococcus urinae*. Eur J Clin Microbiol Infect Dis 1999;18:368–71.
28. Arciola CR, Campoccia D, Gamberini S, Donati ME, Pirini V, Visai L, et al. Antibiotic resistance in exopolysaccharide-forming *Staphylococcus epidermidis* clinical isolates from orthopaedic implant infections. Biomaterials 2005;26:6530–5.
29. Linde HJ, Hahn J, Holler E, Reischl U, Lehn N. Septicemia due to *Acinetobacter junii*. J Clin Microbiol 2002;40:2696–7.
30. Yang CH, Young T, Peng MY, Weng MC. Clinical spectrum of *Pseudomonas putida* infection. J Formos Med Assoc 1996;95:754–61.
31. Ashwath ML, Katner HP. Pancreatic abscess secondary to *Alcaligenes faecalis*. Am J Med Sci 2005;329:54–5.
32. Bizet J, Bizet C. Strains of *Alcaligenes faecalis* from clinical material. J Infect 1997;35:167–9.
33. Panagea S, Bijoux R, Corkill JE, Al Rashidi F, Hart CA. A case of lower respiratory tract infection caused by *Neisseria weaveri* and review of the literature. J Infect 2002;44:96–8.
34. Matar GM, Chahwan R, Fuleihan N, Uwaydah M, Hadi U. PCR-based detection, restriction endonuclease analysis, and transcription of tonB in *Haemophilus influenzae* and *Haemophilus parainfluenzae* isolates obtained from children undergoing tonsillectomy and adenoidectomy. Clin Diagn Lab Immunol 2001;8:221–4.
35. Okamoto M, Byrn R, Eyre RC, Mullen T, Church P, Kiessling AA. Seminal plasma induces programmed cell death in cultured peripheral blood mononuclear cells. AIDS Res Hum Retroviruses 2002;18: 797–803.
36. Hochreiter WW, Duncan JL, Schaeffer AJ. Evaluation of the bacterial flora of the prostate using a 16S rRNA gene based polymerase chain reaction. J Urol 2000;163:127–30.
37. Riley DE, Berger RE, Miner DC, Krieger JN. Diverse and related 16S rRNA-encoding DNA sequences in prostate tissues of men with chronic prostatitis. J Clin Microbiol 1998;36:1646–52.
38. Gonzalez-Jimenez MA, Villanueva-Diaz CA. Epididymal stereocilia in semen of infertile men: evidence of chronic epididymitis? Andrologia 2006;38:26–30.