JOURNAL OF CLINICAL MICROBIOLOGY, Feb. 2004, p. 734–740 Vol. 42, No. 2
0095-1137/04/$08.00+0 DOI: 10.1128/JCM.42.2.734–740.2004

Prospective Study of Use of PCR Amplification and Sequencing of 16S Ribosomal DNA from Cerebrospinal Fluid for Diagnosis of Bacterial Meningitis in a Clinical Setting

Tim Schuurman,* Richard F. de Boer, Anna M. D. Kooistra-Smid, and Anton A. van Zwet

Department of Research and Development, Regional Public Health Laboratory for Groningen and Drenthe, Groningen, The Netherlands

Received 3 July 2003/Returned for modification 7 September 2003/Accepted 27 October 2003

We have evaluated the use of a broad-range PCR aimed at the 16S rRNA gene in detecting bacterial meningitis in a clinical setting. To achieve a uniform DNA extraction procedure for both gram-positive and gram-negative organisms, a combination of physical disruption (bead beating) and a silica-guanidiniumthiocyanate procedure was used for nucleic acid preparation. To diminish the risk of contamination as much as possible, we chose to amplify almost the entire 16S rRNA gene. The analytical sensitivity of the assay was approximately 1×10^2 to 2×10^2 CFU/ml of cerebrospinal fluid (CSF) for both gram-negative and gram-positive bacteria. In a prospective study of 227 CSF samples, broad-range PCR proved to be superior to conventional methods in detecting bacterial meningitis when antimicrobial therapy had already started. Overall, our assay showed a sensitivity of 86%, a specificity of 97%, a positive predictive value of 80%, and a negative predictive value of 98% compared to culture. We are currently adapting the standard procedures in our laboratory for detecting bacterial meningitis; broad-range 16S ribosomal DNA PCR detection is indicated when antimicrobial therapy has already started at time of lumbar puncture or when cultures remain negative, although the suspicion of bacterial meningitis remains.

Bacterial meningitis is a serious disease with high morbidity and mortality. To reduce death or permanent neurological sequelae as much as possible, a fast and correct diagnosis is of the utmost importance. The current standard for the diagnosis of bacterial meningitis is microscopic examination and subsequent culture of cerebrospinal fluid (CSF). However, this approach might have some disadvantages with regard to the desired rapidity and sensitivity.

Results of culture may only be available after 24 to 48 h and sometimes, for instance, when the number of viable organisms in the CSF is low, it may take even longer. Moreover, the sensitivity of microscopic examination and culture of CSF can be debated.

First, bacterial concentration in the CSF has a profound effect on the results of microscopy. Regardless of the type of organism in the CSF, the percentage of positive microscopic results is only 25% with $<10^3$ CFU/ml and 60% in the range of 10^3 to 10^5 CFU/ml (32). Second, in an extensive study over a period of 27 years, it appeared that culture might miss the diagnosis of bacterial meningitis in at least 13% of cases (16, 55). Acknowledged reasons for this lack in sensitivity are CSF obtained after the start of antibiotic treatment and meningitis due to fastidious or slow-growing microorganisms.

Recently, PCR-based assays have become available to provide an early and accurate diagnosis of bacterial meningitis (2, 10, 12, 14, 22, 30, 33, 35, 39, 42, 45, 46, 51, 54, 60).

Some of these assays are aimed at specific pathogens of bacterial meningitis, such as *Neisseria meningitidis*, *Streptococcus pneumoniae*, and *Haemophilus influenzae* (10, 12, 46, 51, 54), whereas others use broad-range bacterial PCR (2, 14, 22, 30, 33, 35, 39, 42, 45, 60). The use of broad-range bacterial PCR has a great advantage in that it also detects microorganisms that are found less frequently or even unknown causative agents of bacterial origin. Even though broad-range PCR has been available since the early 1990s (5, 58), most clinical laboratories have not yet implemented this technique in their daily clinical practice because its use is hampered by two major problems.

First, this method is more vulnerable to contamination than a species-specific PCR. *Taq* DNA polymerases are frequently reported as a major source of contaminating bacterial DNA (6, 13, 27, 40, 47). Several approaches overcoming this problem have been reported with success in agarose gel electrophoresis detection systems, such as UV irradiation, 8-MOP treatment, DNase treatment, or restriction endonuclease treatment (9, 21, 25, 26, 28, 44, 52). However, it has been clearly demonstrated that most decontamination procedures also affect the sensitivity of the broad-range PCR when a sensitive detection system, such as TaqMan, is used (13). Amplicon size might have an effect on contamination, since most contaminating DNA derives from nonviable organisms. This DNA is most probably unprotected and therefore susceptible to DNA degradation. This could implicate that bigger amplicons are less sensitive for this kind of contamination than are small amplicons. In fact, for cytomegalovirus DNA it has been demonstrated that amplicon size does have a great effect on final PCR results (4). This would implicate that amplifying the entire 16S rRNA gene would be less contamination sensitive compared to a smaller part of the gene.

Second, at this time no DNA extraction protocol is available

* Corresponding author. Mailing address: Department of Research and Development, Regional Public Health Laboratory for Groningen and Drenthe, Van Ketwich Verschuurlaan 92, 9721 SW Groningen, The Netherlands. Phone: 31-50-5215247. Fax: 31-50-5271488. E-mail: t.schuurman@slgd.nl.

that has the same effectiveness for both gram-positive and gram-negative bacteria. Most studies use enzyme treatment (e.g., proteinase K or lysozyme), boiling, phenol-chloroform extraction, ethanol precipitation, commercial kits, or some combination of these methods (12, 14, 30, 33, 42, 45). Bead beating in a guanidinium thiocyanate buffer, followed by organic extraction, has been shown to be effective for extracting nucleic acids from clinical samples containing mycobacteria (29). However, organic extraction procedures that use phenol and chloroform are a hazard for laboratory staff because of the toxic nature of both compounds. Most organic extraction procedures are also relatively laborious and therefore not suitable for processing samples in a clinical laboratory. Recently, it was shown that physical disruption in combination with the use of a commercial extraction kit has the same quality as standard phenol-chloroform extraction (41).

To tackle these problems, we have chosen to combine a widely used silica-guanidinium thiocyanate procedure for extracting nucleic acids (3, 7, 15, 18, 20, 23, 24, 34, 38, 43, 48, 49, 50, 53, 56, 57, 61) with bead beating in order to achieve a uniform extraction procedure. Moreover, to diminish contamination problems as much as possible, primers were chosen so that almost the entire 16S rRNA gene was amplified.

Here we describe the outcome of a prospective study of the performance of our broad-range bacterial PCR with subsequent sequencing on CSF in comparison with conventional procedures in the diagnosis of bacterial meningitis. After amplification with universal bacterial primers, positive amplicons were sequenced directly, and the obtained sequences were compared to database sequences for bacterial determination.

MATERIALS AND METHODS

Bacterial strains. The following bacterial strains were used in reconstruction experiments: *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923. Bacteria were grown on 5% sheep blood agar plates overnight at 37°C and harvested ($\sim 10^9$ CFU) into 1 ml of saline.

CSF samples and patients. Between January 2002 and May 2003, 227 CSF samples from 222 patients were collected at the decentralized units of the Regional Public Health Laboratory for Groningen and Drenthe, the Laboratory for Pathology and Medical Microbiology of the Isala Clinics in Zwolle, and the Laboratory for Medical Microbiology of the Academic Hospital of Groningen. All patients included had meningitis as part of their differential diagnosis. These patients were characterized with the following features: mean age, 24.5 years (range, 0 to 87.9 years), and 125 males (56.3%) and 97 females (43.7%). Upon arrival at the laboratory, 200 to 400 μl of each CSF sample was removed under sterile conditions and stored at −20°C until further processing for DNA extraction and PCR. The remaining CSF was used for standard bacterial culture and Gram and methylene blue staining. All included samples had sufficient volume, ensuring that both methods would produce reliable results.

Bacterial culture and direct microscopic examination. After removal and storage of 200 to 400 μl of CSF for DNA extraction, the remaining CSF was centrifuged for 15 min at 1,500 × *g*. The supernatant was discarded and the sediment was resuspended in the remaining liquid by vortexing. From this suspension Gram and methylene blue stains were made, and bacterial culture was inoculated. The bacterial culture was inoculated into 5% sheep blood and chocolate agar plates and a thioglycolate enrichment broth with XV-factor and then incubated in a CO_2 incubator at 35°C.

DNA extraction from CSF. DNA was extracted from CSF samples by a modification of the procedure described by Boom et al. (3). Briefly, 200 μl of CSF was added to 1,000 μl of lysis buffer L6 (5.25 M guanidinium thiocyanate, 50 mM Tris-HCl [pH 6.4], 20 mM EDTA, 1.3% [wt/vol] Triton X-100) and 800 mg of acid washed 0.1-mm-diameter zirconia-silica beads (BioSpec Products, Inc., Bartlesville, Okla.). This mixture was shaken in a Mini-Beadbeater-8 (BioSpec Products, Inc.) for 1 min at 3,200 rpm. After bead beating, the sample was centrifuged for 1 min at 12,000 × *g*. Then, 1,000 μl of supernatant was transferred to a new screw-cap reaction tube containing 20 μl of size-fractionated silica particles prepared as described previously (3) (Silica, SiO_2, S-5631; Sigma-Aldrich Chemie, Zwijndrecht, The Netherlands). After being vortexed, the sample was left at ambient temperature for 10 min. After this 10-min binding step, the silica-DNA pellets were washed and dried as described previously (3). DNA was eluted in 100 μl of TE buffer (10 mM Tris, 1 mM EDTA [pH 8.0]). If the DNA was not used immediately, it was stored at −20°C until further use.

PCR and sequencing primers. All primers were obtained from Eurogentec (Eurogentec Nederland b.v., Maastricht, The Netherlands). The primer pair used for amplification consisted of 27F (5′-AGA GTT TGA TC[A/C] TGG CTC AG-3′) and 1492R (5′-G[C/T]T ACC TTG TTA CGA CTT-3′). This primer pair amplifies an ~1,500-bp fragment of the 16S rRNA gene between positions 8 and 1509 of the *E. coli* 16S rRNA gene and is considered to be universal for the domain *Bacteria* (31). For reamplification of samples that yielded insufficient product for a successful sequencing reaction, the following primer pair was used: 357F (5′-CCT ACG GGA GGC AGC AG-3′) and 1221R (5′-CAT TGT AGC ACG TGT GTA GCC-3′). Sequencing was done with the following primers: 27F (5′-AGA GTT TGA TC[A/C] TGG CTC AG-3′) and 515R (5′-TAC CGC GGC TGC TGG CAC-3′). For reamplified samples, the sequencing primers used were: 357F (5′-CCT ACG GGA GGC AGC AG-3′), 797F (5′-CAA AC[A/G] GGA TTA GAT ACC C-3′), 907R (5′-CCG TCA ATT C[A/C]T TTG AGT TT-3′), and 1221R (5′-CAT TGT AGC ACG TGT GTA GCC-3′).

16S ribosomal DNA (rDNA) PCR. Amplification of the bacterial 16S rRNA gene was carried out in a GeneAmp 9600 thermocycler (Applied Biosystems, Nieuwekerk a/d IJssel, The Netherlands). The final reaction mixture (50 μl) contained 25 μl of DNA eluate; 400 ng of each primer; 2.5 U of AmpliTaq Gold DNA polymerase (Applied Biosystems); 0.5 U of uracil-*N*-glycosylase (Applied Biosystems); 5 μg of bovine serum albumin (Roche Diagnostics Nederland B.V., Almere, The Netherlands); 20 μg of alpha-casein (C-6780, Sigma-Aldrich Chemie); 10 mM Tris-HCl (pH 8.3); 50 mM KCl; 3.5 mM $MgCl_2$; dATP, dGTP, and dCTP at concentrations of 200 μM each; and 400 μM dUTP (Applied Biosystems). The thermal cycling profile used was as follows: 2 min at 50°C and 10 min at 95°C, followed by 30 cycles of 20 s at 95°C, 20 s at 50°C, and 3 min at 72°C, followed finally by 10 min at 72°C.

Amplicon detection was carried out by agarose gel electrophoresis of 12 μl of amplification product through horizontal 1% agarose (Agarose MP; Roche Diagnostics Nederland B.V.) gels containing 1 μg of ethidium bromide in 0.5× Tris-borate-EDTA buffer. After electrophoresis, amplicons were made visible by UV light irradiation.

Reamplification of samples that yielded insufficient amounts of product for direct sequencing was carried out in the same reaction mixture of the first-round PCR, except for the presence of uracil-*N*-glycosylase, which was not included in reamplification reactions. Template DNA was purified with the QIAquick PCR purification kit (Westburg, Leusden, The Netherlands) according to the manufacturer's instructions and subsequently diluted 1,000 times. The thermal cycling profile was also the same as for first-round PCR.

Precautions to prevent false positives and false negatives. In order to prevent false-positive and false-negative results in the broad-range PCR, our laboratory workflow was organized in the way described by Millar et al. (36), with a few minor exceptions. These exceptions were as follows: (i) all reagents were molecular grade, but not UV irradiated before use, and (ii) separate pipettes were used in sample preparation, PCR setup, and post-PCR handling, but pipettes were not UV irradiated before use. Extra measures used to prevent false positives were as follows: (i) use of the uracil-*N*-glycosylase system to prevent amplicon carry-over contamination, (ii) a negative extraction control was included for every five clinical samples to check for cross-contamination during extraction and for quality control of extraction chemicals, and (iii) a negative PCR control was included in each run to quality control the PCR chemicals. To prevent false negatives, all PCRs were done in duplicate—one reaction without a spike and one reaction with 40 pg of purified *E. coli* DNA as a spike to control for PCR inhibition.

Sequencing. Amplicons were purified with the QIAquick PCR purification kit (Westburg) according to the manufacturer's instructions. The sequencing reactions were performed with the Big Dye terminator cycle sequencing kit v1.0 or v1.1 (Applied Biosystems) according to the manufacturer's instructions. Cycle reactions were purified by either manual sodium acetate-ethanol precipitation or by the use of the DyeEx 2.0 spin kit (Westburg) according to the manufacturer's instructions. The manual procedure was as follows: 20 μl of cycle sequencing product was added to 50 μl of 96% (vol/vol) ethanol and 2 μl of 3 M sodium acetate (pH 6.4), and this mixture was left at ambient temperature for 15 min. The mixture was centrifuged for 30 min at 12,000 × *g*, and the supernatant was discarded by pipetting. A portion (250 μl) of 70% (vol/vol) ethanol was added, and the mixture was vortexed and centrifuged for 15 min at 12,000 × *g*. The

TABLE 1. Overall results obtained by 16S rDNA PCR compared to culture

Method	Finding	No. of CSF samples found to be culture:		Total no. of specimens
		Positive	Negative	
16S rDNA PCR	Positive	24	6	30
	Negative	13	184	197
Total		37	190	227

supernatant was again discarded by pipetting, and the pellet was centrifuged for 5 min at 12,000 × *g*. After the small amount of supernatant was removed by pipetting, the pellet was dried for 15 min at 60°C under vacuum conditions. This drying step is part of both the manual and the DyeEx procedures. The dried cycle sequencing products were then dissolved in 25 μl of template suppression reagent (Applied Biosystems) and analyzed on an ABI Prism 310 genetic analyzer (Applied Biosystems) according to the manufacturer's instructions.

Data analysis. Raw data was analyzed with the Sequence Analysis software v 3.3 and the Factura software v 2.2 (Applied Biosystems). Sequences from both strands were aligned by using the AutoAssembler software v 2.1 (Applied Biosystems) and edited, to resolve discrepancies, by evaluation of the electropherograms. The resulting consensus sequence, created from the double-stranded part of the alignment, was used for comparison with sequences stored in GenBank, EMBL, and DDJB by using the basic local alignment search tool (BLAST; http://www.ncbi.nlm.nih.gov/BLAST/) and the similarity matrix tool from the Ribosomal Database Project II (RDP-II; http://rdp.cme.msu.edu/html/analyses.html) (11). For both BLAST and RDP-II analyses, the highest-scoring species was reported. Sequences identified in the present study are deposited in GenBank under accession numbers AY325737 to AY325764, AY332613 to AY332615, and AY429260.

RESULTS

Lower limit of detection of the procedure. To assess the lower limit of detection, the procedure was performed on 10-fold serial dilutions of *E. coli* and *S. aureus* ranging from 10^7 to 10^0 CFU/ml of pooled CSF, which were previously proven to be both culture and broad-range 16S rDNA PCR negative. Bacteria used for spiking were quantified by agar dilution. Agarose gel electrophoresis of the amplified products showed a lower limit of detection of 100 and 200 CFU/ml of CSF for *E. coli* and *S. aureus*, respectively. Amplicons obtained from the dilutions that contained 100 and 1,000 CFU/ml needed reamplification for a successful sequencing identification. All amplicons yielded the correct identification of the spiked organism after sequencing (data not shown).

Laboratory findings for CSF samples from conventional procedures. A total of 227 CSF samples were analyzed with both conventional and broad-range 16S rDNA PCR and the results are summarized in Table 1. Microbial growth in bacterial culture was detected for 37 CSF samples. In 21 samples the direct microscopic examination was confirmed by culture, but for the remaining 16 culture-positive samples no bacteria were seen. None of the culture-negative samples had any evidence of bacteria in the direct microscopic examination, but in four samples yeasts were seen. Bacterial species isolated during culture were the following: *N. meningitidis* (14 isolates), coagulase-negative staphylococci (CNS) (8 isolates), *S. pneumoniae* (8 isolates), *Haemophilus influenzae* (2 isolates), *E. coli* (2 isolates), viridans group streptococci (1 isolate), *Listeria monocytogenes* (1 isolate), and a *Pantoea* species. (1 isolate).

Laboratory findings from broad-range 16S rDNA PCR. Broad-range 16S rDNA PCR resulted in a total of 30 positive and 197 negative specimens. Sequencing of these positive amplicons resulted in the following identifications: *S. pneumoniae* (11 times), *N. meningitidis* (10 times), *H. influenzae* (2 times), *E. coli* (2 times), *Streptococcus salivarius* (1 time), *Streptococcus agalactiae* (1 time), *Prevotella bivia* (1 time), and *Fusobacterium nucleatum* (1 time). One sample resulted in a mixed sequence.

Concordant PCR-positive and culture-positive samples. Table 2 shows the results for 22 CSF samples for which conventional and molecular methods were in complete concordance. Pathogens identified by both methods included most of the common causative agents of bacterial meningitis. *N. meningitidis* was identified most often, with a total of nine cases, followed by *S. pneumoniae* (eight cases), *E. coli* and *H. influenzae* (two cases each), and finally *S. salivarius* (viridans group streptococci in culture) with one episode. In this group the broad-range 16S rDNA PCR showed supplementary value in one sample. This sample yielded a strain of *S. pneumoniae* with very poor growth characteristics, making biochemical identification not possible, even after 8 days. Identification of the isolated strain, resulting in *S. pneumoniae*, was only possible by 16S rRNA gene sequencing. Broad-range 16S rDNA PCR, followed by sequencing of the original CSF sample, resulted in *S. pneumoniae* within 48 h.

Concordant culture-negative and PCR-negative samples. Bacteria were not detectable in the vast majority, i.e., 81.1% (184 of 227 specimens), of analyzed CSF samples by both conventional and molecular methods, as illustrated in Table 1. Retrospective analysis of these 184 CSF samples revealed a virus or yeast by culture, microscopy, or specific viral PCR in 7 samples: enterovirus RNA and herpes simplex virus DNA, respectively, were detected in 1 sample each. In the other five samples yeasts were detected by microscopy and/or culture. The low number of viral agents that were finally detected is due to the fact that only a minority of the samples was analyzed for the presence of viruses.

PCR-negative and culture-positive discrepant samples. For 13 CSF samples the positive culture result obtained could not be confirmed by the broad-range 16S rDNA PCR. Eight of these CSF samples resulted in the isolation of CNS, as is shown in Table 3. From the remaining five samples, *N. meningitidis* was isolated three times, and *L. monocytogenes* and a *Pantoea* sp. were both isolated once each. The CNS and *Pantoea* sp. culture results were regarded as contamination of bacterial culture and therefore as false positives. In the other four patients the diagnosis of bacterial meningitis was confirmed clinically.

PCR-positive and culture-negative discrepant samples. For six culture-negative CSF samples the broad-range 16S rDNA PCR did yield a positive result. In three of these samples *S. pneumoniae* was identified, and in the other three *N. meningitidis*, *S. agalactiae*, and *P. bivia* were detected once each (Table 3). For five of these CSF samples antimicrobial therapy had already started at the time of lumbar puncture.

PCR-positive and culture-positive discrepant samples. For two samples sequence data did not match the organism isolated by culture (Table 3). The first sample, from which *N. meningitidis* was isolated in culture, showed clear signs of a mixed sequence in its electropherograms obtained after broad-

TABLE 2. Results of concordant PCR-positive and culture-positive specimens

Lab no.	Broad-range 16S rDNA method			Conventional method	
	PCR[a]	BLAST (% identity)	RDP-II (% similarity)	Culture result[b]	Microscopy result[c]
1489	+	*E. coli* (100)	*E. coli* (1.000)	*E. coli* (+)	(−)
1495	+++	*S. salivarius* (99.9)	*S. salivarius* (1.000)	Viridans group streptococci (+++)	GPC (++++)
1530	+++	*S. pneumoniae* (100)	*S. pneumoniae* (1.000)	*S. pneumoniae* (+++)	GPC (++++)
1532	+++	*N. meningitidis* (100)	*N. meningitidis* (0.998)	*N. meningitidis* (+++)	GNC (++++)
1563	+++	*S. pneumoniae* (100)	*S. pneumoniae* (0.998)	*S. pneumoniae* (+++)	GPC (++++)
1577	++	*N. meningitidis* (100)	*N. meningitidis* (1.000)	*N. meningitidis* (++)	GNC (+++)
1682	+++	*N. meningitidis* (99.8)	*N. meningitidis* (0.995)	*N. meningitidis* (+++)	GNC (+++)
1713	++	*N. meningitidis* (100)	*N. meningitidis* (0.998)	*N. meningitidis* (++)	GNC (++)
1826	+++	*N. meningitidis* (99.8)	*N. meningitidis* (0.993)	*N. meningitidis* (+)	GNC (+)
1835	+++	*S. pneumoniae* (99.8)	*S. pneumoniae* (0.998)	*S. pneumoniae* (++)	GPC (++++)
1836	+	*S. pneumoniae* (99.9)	*S. pneumoniae* (0.999)	*S. pneumoniae* (+)	GPC (+)
1863	+	*E. coli* (99.5)	*E. coli* (0.998)	*E. coli* (+++)	GNR (++)
1898	+++	*N. meningitidis* (99.8)	*N. meningitidis* (0.993)	*N. meningitidis* (++)	GNC (++++)
1908	+++	*N. meningitidis* (99.8)	*N. meningitidis* (0.993)	*N. meningitidis* (+++)	GNC (++)
1963	+	*H. influenzae* (99.7)	*H. influenzae* (0.999)	*H. influenzae* (++)	(−)
2055	+++	*S. pneumoniae* (99.6)	*S. pneumoniae* (0.994)	*S. pneumoniae* (+)[d]	(−)
2108	++	*H. influenzae* (99.7)	*H. influenzae* (0.997)	*H. influenzae* (+++)	GNR (++++)
2109	++	*S. pneumoniae* (99.8)	*S. pneumoniae* (0.998)	*S. pneumoniae* (+++)	GPC (++++)
2302	++	*N. meningitidis* (100)	*N. meningitidis* (0.999)	*N. meningitidis* (++)	GNC (+)
2452	++	*S. pneumoniae* (100)	*S. pneumoniae* (0.998)	*S. pneumoniae* (+)	GPC (++)
2457	+++	*S. pneumoniae* (99.8)	*S. pneumoniae* (0.998)	*S. pneumoniae* (+++)	GPC (++++)
2471	++	*N. meningitidis* (100)	*N. meningitidis* (1.000)	*N. meningitidis* (+++)	GNC (++)

[a] The PCR result is reported as follows: +, weakly positive; ++, moderately positive; and +++, strongly positive.
[b] Symbols in parentheses are relative numbers of colonies on the primary plate: +, few or enrichment broth positive; ++, moderate; and +++, many.
[c] GPC, gram-positive cocci; GNC, gram-negative cocci; GNR, gram-negative rods. Symbols in parentheses are relative numbers of bacterial cells seen in direct microscopy: −, none; +, sporadic; ++, few; +++, moderate; and ++++, many.
[d] Enrichment broth yielded a positive result; however, subculturing of the strain did not yield enough growth for identification procedures. The strain was identified by 16S rDNA sequencing.

range 16S rDNA PCR. When this sequence was analyzed by using the BLAST algorithm, the sequence was most related to a *Lactococcus* species. However, when the sequence was analyzed with the similarity matrix algorithm from RDP-II it resulted in a *Neisseria* species. Another sample, from which also a *N. meningitidis* strain was isolated in culture, resulted in a sequence identification of *F. nucleatum*.

Overall performance of the broad-range PCR assay compared to culture. For calculation of the overall performance of the assay compared to bacterial culture, the data from Table 1 were used, with the exception that the nine false-positive culture results were excluded from the analysis. This results in an overall sensitivity of 86%, a specificity of 97%, a positive predictive value (PPV) of 80%, and a negative predictive value (NPV) of 98%.

DISCUSSION

In the present study we evaluated broad-range 16S rDNA PCR and subsequent sequencing in diagnosis of bacterial meningitis in a clinical setting. Our results show that the molecular findings were in good agreement with those obtained from direct microscopy and bacterial culture. The results were in complete concordance for 206 CSF specimens (91%), including 184 culture-negative and PCR-negative samples and 22 culture-positive and PCR-positive samples.

Our broad-range 16S rDNA PCR proved to be extremely valuable in detecting bacterial meningitis when antibiotics had been started prior to lumbar puncture. For five CSF samples obtained after the start of antimicrobial therapy, a significant bacterial pathogen (*S. pneumoniae* [three times], *S. agalactiae* [one time], and *N. meningitidis* [one time]) was identified by broad-range 16S rDNA PCR, whereas the culture results remained negative. These data confirm the findings of earlier studies (10, 30, 42, 45).

Our broad-range 16S rDNA PCR also proved of value in the case of a 2.5-month-old boy with clinical symptoms of sepsis and meningitis. *P. bivia* was found in the CSF of this child, while conventional methods remained negative, since anaerobic culture is not part of the standard operation procedure for detecting bacterial meningitis in most microbiological laboratories. The boy was treated empirically with ampicillin and ceftazidime and recovered completely. Recently, isolation of *P. bivia* from CSF in a critical ill young patient has been described (8).

In two samples, both PCR and culture results were positive, but the microorganism found was different for each method. The first discrepant identification involved a CSF sample from which *N. meningitidis* was isolated. The sequence obtained after broad-range bacterial PCR showed clear signs of a mixed sequence in its electropherograms. This sequence was analyzed with the algorithms from the both BLAST and RDP-II databases. The BLAST search resulted in a *Lactococcus* species, most closely related to *L. lactis* (87.8%), with *N. meningitidis* (86.5%) as the next possible match, whereas the RDP-II search resulted in a *Neisseria* species (percent similarity values, 0.925 to 0.923). Upon manual comparison, >99% of the sequences from both *L. lactis* and *N. meningitidis* could be retrieved within the electropherogram. Finally, to confirm the previous findings, the original sequence was also subjected to a third search algorithm, SSEARCH from the Software and Tools for

TABLE 3. Results of discordant PCR and culture specimens

Discrepancy type and lab no.	Broad-range 16S rDNA method			Conventional method		Comment(s)[g]
	PCR result[a]	BLAST (% identity)	RDP-II (% similarity)	Microscopy result[b]	Culture result[c]	
PCR-negative and culture-positive discrepancies						
1572	−	ND[f]	ND	(−)	CNS (+)	Contamination*
1705	−	ND	ND	(−)	CNS (+)	Contamination*
1751	−	ND	ND	(−)	CNS (+)	Contamination*
1818	−	ND	ND	(−)	*N. meningitidis* (+)	Bacterial meningitis*
1824	−	ND	ND	(−)	CNS (+)	Contamination*
1961	−	ND	ND	(−)	*N. meningitidis* (+)	Bacterial meningitis*
2061	−	ND	ND	(−)	*L. monocytogenes* (+)	Bacterial meningitis*
2106	−	ND	ND	(−)	CNS (+)	Contamination*
2225	−	ND	ND	(−)	CNS (+)	Contamination*
2228	−	ND	ND	(−)	CNS (+)	Contamination*
2251	−	ND	ND	(−)	*Pantoea* species (+)	Contamination*
2263	−	ND	ND	(−)	CNS (+)	Contamination*
2266	−	ND	ND	GNC (+)	*N. meningitidis* (+)	Bacterial meningitis*
PCR-positive and culture-negative discrepancies						
1750	+	*S. pneumoniae* (99.9)	*S. pneumoniae* (0.999)	(−)	(−)	Antibiotic treatment†
2125	+++	*S. agalactiae* (99.7)	*S. agalactiae* (0.997)	(−)	(−)	Antibiotic treatment†
2145	+++	*P. bivia* (98.5)	*P. bivia* (0.998)	(−)	(−)	Anaerobic culture not performed†
2264	++	*N. meningitidis* (99.8)	*N. meningitidis* (1.000)	(−)	(−)	Antibiotic treatment†
2304	+++	*S. pneumoniae* (100)	*S. pneumoniae* (0.999)	(−)	(−)	Antibiotic treatment†
2403	+++	*S. pneumoniae* (100)	*S. pneumoniae* (0.999)	(−)	(−)	Antibiotic treatment†
PCR and culture identification discrepancies						
1936[d]	+	*Lactococcus* sp. (87.8)	*Neisseria* sp. (0.925)	(−)	*N. meningitidis* (+)	SSEARCH result *N. meningitidis*
2253[e]	+++	*F. nucleatum* (99)	*F. nucleatum* (0.998)	GNC (+)	*N. meningitidis* (+)	PCR contamination

[a] The PCR result is reported as follows: +, weakly positive; ++, moderately positive; and +++, strongly positive. Numbers in parentheses are identity percentages.
[b] GNC, gram-negative cocci. Symbols in parentheses reflect relative numbers of bacterial cells seen in direct microscopy: −, none; and +, sporadic.
[c] Symbols in parentheses reflect relative numbers of colonies on the primary plate: +, few or enrichment broth positive; −, none.
[d] An electropherogram showed clear signs of a mixed sequence and resulted in discrepant sequence results between BLAST and RDP-II. A third analysis with the SSEARCH algorithm identified *N. meningitidis.*
[e] The sequencing result of *F. nucleatum* did not match the clinical presentation of the patient, although all controls were valid. Reanalysis of the original CSF and the original DNA isolate identified both *N. meningitidis.* The first result was therefore reported as contamination of the PCR reaction.
[f] ND, not done.
[g] *, Clinical interpretation; †, possible reason for negative culture.

Genome Analysis website (http://www-btls.jst.go.jp/), resulting in *N. meningitidis.* Although lactococci have recently been associated with human disease (1, 17, 19, 37, 59), we concluded that, based upon the clinical evaluation, the lactococcal DNA was most likely a contaminant introduced at the time of lumbar puncture. The second discrepant result was the detection of *F. nucleatum* in the CSF of a 13-year-old girl presenting with mild symptoms of bacterial meningitis. Culture of the CSF yielded *N. meningitidis*, and the child was treated accordantly. The detection of *F. nucleatum* did not match the clinical presentation of the patient, even though all controls included were valid. Therefore, the original CSF sample, as well as the original DNA isolate, was retested, resulting in the identification of *N. meningitidis* for both samples. Resequencing the original PCR product again yielded *F. nucleatum* as in the first analysis, confirming that the first PCR had to be contaminated. This case emphasizes that all laboratory findings should always be placed in perspective with the clinical symptoms, even when all controls to prevent false-positive results make a run valid.

One of the shortcomings of our approach seems to be the sensitivity in the lower range of bacterial loads encountered in bacterial meningitis. A total of 13 culture-positive CSF samples remained negative by broad-range 16S rDNA PCR. In four of these samples it was evident that a bacterial pathogen was present, whereas the others were considered to be contaminants of bacterial culture on clinical grounds. All of these samples, which had low numbers of colonies on primary plates, were estimated to have bacterial loads below our limit of detection. However, it should be emphasized that the sensitivity of our assay (100 CFU/ml of CSF) should be sufficient for detecting the majority of bacterial meningitis cases, since La Scolea et al. showed that only 16% of culture-positive CSF samples in bacterial meningitis have bacterial loads of <1,000 CFU/ml (32).

The overall performance of our assay, compared to bacterial culture, is in line with other published broad-range PCR methods (30, 33, 42), with an overall sensitivity of 86%, a specificity of 97%, a PPV of 80%, and an NPV of 98%. It should be emphasized that the relatively low PPV is at least partly explained by the inadequacy of bacterial culture: in six PCR-positive, culture-negative CSF samples a significant bacterial pathogen was identified in a patient with all of the signs of bacterial meningitis (Table 3).

The molecular method validated here is not only suitable for the detection of bacteria in CSF but can also be used for the detection of bacteria in many other clinical samples from anatomic sites that are considered to be sterile in healthy subjects. We have demonstrated that we can detect clinically sig-

nificant bacteria in other clinical samples, such as blood culture fluids, a brain abscess, a liver abscess, pus from a vertebra, synovial fluids, and pericardial fluid, for which conventional methods could not (data not shown).

In conclusion, our broad-range 16S rDNA PCR with subsequent sequencing has proven to be a valuable supplementary test in daily clinical practice. Bacterial culture of CSF remains the cornerstone in the diagnosis of bacterial meningitis. However, especially when antimicrobial therapy has already started at the time of lumbar puncture, conventional methods should always be accompanied by molecular detection, since the sensitivity of direct microscopic examination and bacterial culture drops substantially when therapy has started. We are currently adapting the standard procedures in our laboratory for the detection of bacterial meningitis; broad-range 16S rDNA detection is indicated when antimicrobial therapy has already been started at the time of lumbar puncture or when cultures remain negative, although the suspicion of bacterial meningitis remains.

ACKNOWLEDGMENTS

We thank all of the members of the decentralized units of the Regional Public Health Laboratory for Groningen and Drenthe, the members of the Laboratory for Pathology and Medical Microbiology of the Isala Clinics in Zwolle, and the members of the Laboratory for Medical Microbiology of the Academic Hospital of Groningen for their contributions to this study.

REFERENCES

1. **Akhaddar, A., B. El Mostarchid, M. Gazzaz, and M. Boucetta.** 2002. Cerebellar abscess due to *Lactococcus lactis*: a new pathogen. Acta Neurochir. **144:**305–306.
2. **Bäckman, A., P. G. Lantz, P. Rädström, and P Olcén.** 1999. Evaluation of an extended diagnostic PCR assay for detection and verification of the common causes of bacterial meningitis in CSF and other biological samples. Mol. Cel. Probes **13:**49–60.
3. **Boom, R., C. J. A. Sol, M. M. M. Salimans, C. L. Jansen, P. M. Wertheim-van Dillen, and J. van der Noordaa.** 1990. Rapid and simple method for purification of nucleic acids. J. Clin. Microbiol. **28:**495–503.
4. **Boom, R., C. J. A. Sol, T. Schuurman, A. van Breda, J. F. L. Weel, M. Beld, I. J. M. ten Berge, P. M. E. Wertheim-van Dillen, and M. D. de Jong.** 2002. Human cytomegalovirus DNA in plasma and serum specimens of renal transplant recipients is highly fragmented. J. Clin. Microbiol. **40:**4105–4113.
5. **Böttger, E. C.** 1989. Rapid determination of bacterial rRNA sequences by direct sequencing of enzymatically amplified DNA. FEMS Microbiol. Lett. **53:**171–176.
6. **Böttger, E. C.** 1990. Frequent contamination of *Taq* polymerase with DNA. Clin. Chem. **36:**1258–1259.
7. **Brisson-Noël, A., C. Aznar, C Chureau, S. Nguyen, C. Pierre, M. Bartoli, R. Bonete, G. Pialoux, B. Gicquel, and G. Garrique.** 1991. Diagnosis of tuberculosis by DNA amplification in clinical practice evaluation. Lancet **338:**364–366.
8. **Busch, N., P. R. Mertens, T. Schonfelder, H. Nguyen, H. U. Marschall, H. Kierdorf, G. Haase, R. Urhahn, H. G. Sieberth, and S. Matern.** 1996. Lemierre's post-tonsillitis sepsis with meningitis and intravascular consumption coagulopathy as complication of infectious mononucleosis with pansinusitis. Dtsch. Med. Wochenschr. **121:**94–98.
9. **Carroll, N. M., P. Adamson, and N. Okhravi.** 1999. Elimination of bacterial DNA from *Taq*DNA polymerases by restriction endonuclease digestion. J. Clin. Microbiol. **37:**3402–3404.
10. **Cherian, T., M. K. Lalitha, A. Manoharan, K. Thomas, R. H. Yolken, and M. C. Steinhoff.** 1998. PCR-enzyme immunoassay for detection of *Streptococcus pneumoniae* DNA in cerebrospinal fluid samples from patients with culture-negative meningitis. J. Clin. Microbiol. **36:**3605–3608.
11. **Cole, J. R., B. Chai, T. L. Marsh, R. J. Farris, Q. Wang, S. A. Kulam, S. Chandra, D. M. McGarrell, T. M. Schmidt, G. M. Garrity, and J. M. Tiedje.** 2003. The Ribosomal Database Project (RDP-II): previewing a new autoaligner that allows regular updates and the new prokaryotic taxynomy. Nucleic Acids Res. **31:**442–443.
12. **Corless, C. E., M. Guiver, R. Borrow, V. Edwards-Jones, A. J. Fox, and E. B. Kaczmarski.** 2001. Simultaneous detection of *Neisseria meningitidis*, *Haemophilus influenzae*, and *Streptococcus pneumoniae* in suspected cases of meningitis and septicemia using real-time PCR. J. Clin. Microbiol. **39:**1553–1558.
13. **Corless, C. E., M. Guiver, R. Borrow, V. Edwards-Jones, E. B. Kaczmarski, and A. J. Fox.** 2000. Contamination and sensitivity issues with a real-time universal 16S rRNA PCR. J. Clin. Microbiol. **38:**1747–1752.
14. **Dicuonzo, G., G. Lorino, D. Lilli, D. Rivanera, P. Guarino, S. Angeletti, G. Gherardi, and F. Filadoro.** 1999. Polymerase chain reaction, with sequencing, as a diagnostic tool in culture-negative bacterial meningitis. Clin. Microbiol. Infect. **5:**92–96.
15. **Dorigo-Zetsma, J. W., R. P. Verkooyen, H. P. van Helden, H. van der Nat, and J. M. van den Bosch.** 2001. Molecular detection of *Mycoplasma pneumoniae* in adults with community-acquired pneumonia requiring hospitalization. J. Clin. Microbiol. **39:**1184–1186.
16. **Durand, M. L., S. B. Calderwood, D. J. Weber, S. I. Miller, F. S. Southwick, V. S. Caviness, Jr., and M. N. Schwartz.** 1993. Acute bacterial meningitis in adults: a review of 493 episodes. N. Engl. J. Med. **328:**21–28.
17. **Durand, J. M., M. C. Rousseau, J. M. Gandois, G. Kaplanski, M. N. Mallet, and J. Soubeyrand.** 1995. *Streptococcus lactis* septicemia in a patient with chronic lymphocytic leukemia. Am. J. Hematol. **50:**64–65.
18. **Dyer, J. R., B. L. Gilliam, J. J. Eron, Jr., L. Grosso, M. S. Cohen, and S. A. Fiscus.** 1996. Quantitation of human immunodeficiency virus type 1 RNA in cell free seminal plasma: comparison of NASBA™ with Amplicor™ reverse transcription-PCR amplification and correlation with quantitative culture. J. Virol. Methods **60:**161–170.
19. **Facklam, R., and J. A. Elliott.** 1995. Identification, classification and clinical relevance of catalase-negative, gram-positive cocci, excluding the streptococci and enterococci. Clin. Microbiol. Rev. **8:**479–495.
20. **Fidler, H. M., G. A. Rook, N. M. Johnson, and J. McFadden.** 1993. *Mycobacterium tuberculosis* DNA in tissue affected by sarcoidosis. BMJ **306:**546–549.
21. **Frothingham, R., R. B. Blitchington, D. H. Lee, R. C. Greene, and K. H. Wilson.** 1992. UV absorption complicates PCR decontamination. BioTechniques **13:**208–210.
22. **Hall, L. M. C., B. Duke, and G. Urwin.** 1995. An approach to the identification of the pathogens of bacterial meningitis by the polymerase chain reaction. Eur. J. Clin. Microbiol. Infect. Dis. **14:**1090–1094.
23. **Hale, A. D., J. Green, and D. W. G. Brown.** 1996. Comparison of four RNA extraction methods for the detection of small round viruses in faecal specimens. J. Virol. Methods **57:**195–201.
24. **Hand, O., M. Höss, M. Krings, and S. Pääbo.** 1994. Ancient DNA: methodological challenges. Experientia **50:**524–529.
25. **Heininger, A., M. Binder, A. Ellinger, K. Botzenhart, K. Unertl, and G. Döring.** 2003. DNase pretreatment of master mix reagents improves the validity of universal 16S rRNA gene PCR results. J. Clin. Microbiol. **41:**1763–1765.
26. **Hilali, F., P. Saulnier, E. Chachaty, and A. Andremont.** 1997. Decontamination of polymerase chain reaction reagents for detection of low concentration of 16S rRNA genes. Mol. Biotechnol. **7:**207–216.
27. **Hughes, M. S., L. A. Beck, and R. A. Skuce.** 1994. Identification and elimination of DNA sequences in *Taq*DNA polymerase. J. Clin. Microbiol. **32:**2007–2008.
28. **Jinno, Y., K. Yoshiura, and N. Niikawa.** 1990. Use of psoralen as extinguisher of contaminating DNA in PCR. Nucleic Acids Res. **18:**6739.
29. **Kirschner, P., J. Rosenau, B. Springer, K. Teschner, K. Feldmann, and E. C. Böttger.** 1996. Diagnosis of mycobacterial infections by nucleic acid amplification: 18-month prospective study. J. Clin. Microbiol. **34:**304–312.
30. **Kotilainen, P., J. Jalava, O. Meurman, O. Lehtonen, E. Rintala, O. Seppälä, E. Eerola, and S. Nikkari.** 1998. Diagnosis of meningococcal meningitis by broad-range bacterial PCR with cerebrospinal fluid. J. Clin. Microbiol. **36:**2205–2209.
31. **Lane, D. J.** 1991. 16S/23S rRNA sequencing, p. 115–175. *In* E. Stackebrandt and M Goodfellow (ed.), Nucleic acid techniques in bacterial systematics. Wiley & Sons, Chichester, United Kingdom.
32. **La Scolea, L. J., Jr., and D. Dryja.** 1984. Quantitation of bacteria in cerebrospinal fluid and blood of children with meningitis and its diagnostic significance. J. Clin. Microbiol. **19:**187–190.
33. **Lu, J.-J., C.-L. Perng, S.-Y. Lee, and C.-C. Wan.** 2000. Use of PCR with universal primers and restriction endonuclease digestions for detection and identification of common bacterial pathogens in cerebrospinal fluid. J. Clin. Microbiol. **38:**2076–2080.
34. **Mahony, J. B., X. Song, S. Chong, M. Faught, T. Salonga, and J. Kapala.** 2001. Evaluation of the NucliSens basic kit for detection of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* in genital tract specimens using nucleic acid sequence-based amplification of 16S rRNA. J. Clin. Microbiol. **39:**1429–1435.
35. **Margall Coscojuela, N., M. Majó Moreno, C. Latorre Otín, D. Fontanals Aymerich, À. Domínquez García, and G. Prats Pastor.** 2002. Use of universal PCR on cerebrospinal fluid to diagnose bacterial meningitis in culture-negative patients. Eur. J. Clin. Microbiol. Infect. Dis. **21:**67–69.
36. **Millar, B. C., J. Xu, and J. E. Moore.** 2002. Risk assessment models and contamination management: implications for broad-range ribosomal DNA

PCR as a diagnostic tool in medical bacteriology. J. Clin. Microbiol. **40:**1575–1580.
37. **Nakarai, T., K. Morita, Y. Nojiri, J. Nei, and Y. Kawamori.** 2000. Liver abscess due to *Lactococcus lactis cremoris.* Pediatr. Int. **42:**699–701.
38. **Nijhuis, M., N. van Maarseveen, R. Schuurman, S. Verkuijlen, M. de Vos, K. Hendriksen, and A. M. van Loon.** 2002. Rapid and sensitive routine detection of all members of the genus *Enterovirus* in different clinical specimens by real-time PCR. J. Clin. Microbiol. **40:**3666–3670.
39. **Nikkari, S., F. A. Lopez, P. W. Lepp, P. R. Cieslak, S. Ladd-Wilson, D. Passaro, R. Danila, and D. A. Relman.** 2002. Broad-range bacterial detection and the analysis of unexplained death and critical illness. Emerg. Infect. Dis. **8:**188–193.
40. **Rand, K. H., and H. Houck.** 1990. *Taq* polymerase contains bacterial DNA of unknown origin. Mol. Cell. Probes **4:**445–450.
41. **Rantakokko-Jalava, K., and J. Jalava.** 2002. Optimal DNA isolation method for detection of bacteria in clinical specimens by broad-range PCR. J. Clin. Microbiol. **40:**4211–4217.
42. **Rantakokko-Jalava, K., S. Nikkari, J. Jalava, E. Eerola, M. Skurnik, O. Meurman, O. Ruuskanen, A. Alanen, E. Kotilainen, P. Toivanen, and P. Kotilainen.** 2000. Direct amplification of rRNA genes in diagnosis of bacterial infections. J. Clin. Microbiol. **38:**32–39.
43. **Revello, M. G., F. Baldanti, A. Sarasini, M. Zavattoni, M. Torsellini, and G. Gerna.** 1997. Prenatal diagnosis of rubella virus infection by direct detection and semiquantitation of viral RNA in clinical samples by reverse transcriptase-PCR. J. Clin. Microbiol. **35:**708–713.
44. **Rochelle, P. A., A. J. Weightman, and J. C. Fry.** 1992. DNase I treatment of *Taq*DNA polymerase for complete PCR decontamination. BioTechniques **13:**520.
45. **Saravolatz, L. D., O. Manzor, N. VanderVelde, J. Pawlak, and B. Belian.** 2003. Broad-range bacterial polymerase chain reaction for early detection of bacterial meningitis. Clin. Infect. Dis. **36:**40–45.
46. **Saunders, N. B., D. R. Shoemaker, B. L. Brandt, and W. D. Zollinger.** 1997. Confirmation of suspicious cases of meningococcal meningitis by PCR and enzyme-linked immunosorbent assay. J. Clin. Microbiol. **35:**3215–3219.
47. **Schmidt, T. M., B. Pace, and N. R. Pace.** 1991. Detection of DNA contamination in *Taq* polymerase. BioTechniques **11:**176–177.
48. **Schee, C. van der, A. van Belkum, L. Zwijgers, E. van der Brugge, E. L. O'neill, A. Luijendijk, T. van Rijsoort-Vos, W. I. van der Meijden, H. Verbrugh, and H. J. F. Sluiters.** 1999. Improved diagnosis of *Trichomonas vaginalis* infection by PCR using vaginal swabs and urine specimens compared to diagnosis by wet mount microscopy, culture, and fluorescent staining. J. Clin. Microbiol. **37:**4127–4130.
49. **Schoone, G. J., L. Oskam, N. C. M. Kroon, H. D. F. H. Schallig, and S. A. Omar.** 2000. Detection and quantification of *Plasmodium falciparum* in blood samples using quantitative nucleic acid sequence-based amplification. J. Clin. Microbiol. **38:**4040–4072.
50. **Secchiero, P., D. R. Carrigan, Y. Asano, L. Benedetti, R. W. Crowley, A. L. Komaroff, R. C. Gallo, and P. Lusso.** 1995. Detection of human herpesvirus 6 in plasma of children with primary infection and immunosuppressed patients by polymerase chain reaction. J. Infect. Dis. **171:**273–280.
51. **Seward, R. J., and K. J. Towner.** 2000. Use of an automated DNA analysis system (DARAS®) for sequence-specific recognition of *Neisseria meningitidis* DNA. Clin. Microbiol. Infect. **6:**29–33.
52. **Sharma, J. K., V. Gopalkrishna, and B. C. Das.** 1992. A simple method for elimination of unspecific amplifications in polymerase chain reaction. Nucleic Acids Res. **20:**6117–6118.
53. **Solmone, M., E. Girardi, F. Costa, L. Pucillo, G. Ippolito, and M. R. Capobianchi.** 2002. Simple and reliable method for detection and genotyping of hepatitis C virus RNA in dried blood spots stored at room temperature. J. Clin. Microbiol. **40:**3512–3514.
54. **Taha, M.-K.** 2000. Simultaneous approach for nonculture PCR-based identification and serogroup prediction of *Neisseria meningitidis.* J. Clin. Microbiol. **38:**855–857.
55. **Tunkel, A. R., and W. M. Scheld.** 1995. Acute bacterial meningitis. Lancet **346:**1675–1680.
56. **Vernazza, P. L., J. R. Dyer, S. A. Fiscus, J. J. Eron, and M. S. Cohen.** 1997. HIV-1 viral load in blood, semen and saliva. AIDS **11:**1058–1059.
57. **Wall, S., Z. M. Kunze, S. Saboor, J. Soufleri, P. Seechurn, R. Chiodini, and J. J. McFadden.** 1993. Identification of sheroplast-like agents isolated from tissues of patients with Crohn's disease and control tissues by polymerase chain reaction. J. Clin. Microbiol. **31:**1241–1245.
58. **Wilson, K., R. Blitchington, and R. Greene.** 1990. Amplification of bacterial 16S ribosomal DNA with polymerase chain reaction. J. Clin. Microbiol. **28:**1942–1946.
59. **Wood, H. F., K. Jacobs, and M. McCarthy.** 1985. *Streptococcus lactis* isolated from a patient with subacute bacterial endocarditis. Am. J. Med. **18:**345–347.
60. **Xu, J., B. C. Millar, J. E. Moore, K. Murphy, H. Webb, A. J. Fox, M. Cafferkey, and M. J. Crowe.** 2003. Employment of broad-range 16S rRNA PCR to detect etiological agents of infection from clinical specimens in patients with acute meningitis: rapid separation of 16S rRNA PCR amplicons without the need for cloning. J. Appl. Microbiol. **94:**197–206.
61. **Zipeto, D., F. Baldanti, D. Zella, M. Furione, A. Cavicchini, G. Milanesi, and G. Gerna.** 1993. Quantification of human cytomegalovirus DNA in peripheral blood polymorphonuclear leukocytes of immunocompromised patients by the polymerase chain reaction. J. Virol. Methods **44:**45–46.