Detection and identification of bacteria directly from clinical samples by broad-range PCR targeting the 16S rRNA gene and DNA sequencing (direct 16S rRNA gene sequencing) make up a well-established method in many laboratories. This method gives the possibility to identify bacteria that died during transportation or as a consequence of antibiotic treatment and to uncover bacteria with special growth requirements. The latest advances in PCR and sequencing technology also offer a more rapid identification than that obtained with standard phenotypic methods that depend on bacterial growth.

Because of difficulties in the interpretation of DNA chromatograms resulting from direct sequencing of polybacterial samples, the use of this diagnostic tool has been limited to infections that are predominantly monobacterial. We earlier described RipSeq (iSentio, Bergen, Norway), a web-based application for the analysis of mixed DNA chromatograms (12). In the same article, we presented the RipSeq performance on a number of mixed DNA chromatograms obtained by direct 16S rRNA gene sequencing from polybacterial human clinical samples. In this study, we used direct 16S rRNA gene sequencing to investigate 264 samples from a wide range of suspected human bacterial infections. The sequence-based identification was compared with the results from routine culture-based identification. A total of 151 samples were positive by the first PCR, producing 85 pure and 66 mixed DNA chromatograms. All mixed chromatograms were analyzed by RipSeq, although seven were so complex that only the dominant bacterial sequences could be identified. In general, sequence-based identification detected a larger number of species than did culture for samples from patients who had received antibiotics prior to sample collection and for samples containing anaerobic bacteria. RipSeq made it possible to apply this supplementary diagnostic tool to typical polybacterial specimens, such as internal abscesses, pleural fluids, and bile.

Detection and identification of bacteria directly from clinical samples by broad-range PCR targeting the 16S rRNA gene and DNA sequencing (direct 16S rRNA gene sequencing) make up a well-established method in many laboratories. This method gives the possibility to identify bacteria that died during transportation or as a consequence of antibiotic treatment and to uncover bacteria with special growth requirements. The latest advances in PCR and sequencing technology also offer a more rapid identification than that obtained with standard phenotypic methods that depend on bacterial growth.

Because of difficulties in the interpretation of DNA chromatograms resulting from direct sequencing of polybacterial samples, the use of this diagnostic tool has been limited to infections that are predominantly monobacterial. We earlier described RipSeq (iSentio, Bergen, Norway), a web-based application for the analysis of mixed DNA chromatograms (12). In the same article, we presented the RipSeq performance on a number of mixed DNA chromatograms obtained by direct sequencing from saline suspensions containing two and three different bacterial species and discussed the possible benefits and limitations one could experience if the method was to be applied to human clinical samples. In this study, direct 16S rRNA gene sequencing was used to investigate 264 human clinical samples from a wide range of locations, including typical polybacterial specimens such as abscesses and pleural fluids. All mixed DNA chromatograms were analyzed with the RipSeq program, and the sequence-based results were compared to routine culture-based diagnostics in our hospital laboratory.

We also discuss special concerns in the selection of a lysis procedure and primers for use on polymicrobial samples as well as the establishment of a reliable negative control.
volumes; for other samples, 400 μl was used, if available. Two hundred microliters was the smallest volume that would still provide 400 μl of supernatant for the subsequent DNA purification and was the smallest volume accepted for all specimens. A negative control containing lysate buffer and 400 μl of PCR-grade water was included in every batch of samples. The samples were run twice for 45 s each in a FastPrep machine (Cepheid) at speed 6.5. After a short spin, 400 μl of supernatant was transferred to a MagNa Pure Compact automated extractor (Roche), and DNA was extracted and purified using the “total nucleic acid” program according to the manufacturer’s instructions. Of the resulting 50 μl of eluate, 2 μl was used as a template in the broad-range PCR. An amplification control to detect possible remaining inhibitory substances in the samples was not included in this assay.

Primer. The following primers were used for the first PCR as well as the cycle sequencing reactions: forward primer, 5'–CGG-CCC-AGA-CTC-CTG-TAG-GAG-GCG-TGG-ACT-ACC-AGG-GTA-TCA-AAG-AAC-AGG-GCA-GCA-3'; and reverse primer, 5'-GGG-TTG-ACT-ACC-AGG-GTA-TCA-AAG-AAC-AGG-GCA-GCA-3'.

The PCR product obtained with these primers has a size of approximately 460 bp, covering the variable areas V3 and V4 of the 16S rRNA gene (3). By use of the RipSeq program, these primers were found to bind more poorly to *Chlamydia trachomatis* (R4), *mydia trachomatis* (R3), *Coxiella burnetii* (R4), *Dermabacter hominis* (R3), *Leucosristoe* spp. (F4), and *Microbacterium* spp. (R3), and *Propionibacterium* spp. (R3). The PCR thermal profile included an initial polymerase activation step of 10 s at 95°C followed by 40 cycles of 15 s at 95°C, 10 s at 70°C, and 20 s at 72°C. The PCR products were spun out of the Smart Cycler reaction tube on a Smart Cycler real-time apparatus (Cepheid). The PCR mixture consisted of 12.5 μl Perfect master mix (TaKaRa, Japan), 0.4 μM of each primer, 8.5 μl PCR-grade water, and 2 μl extracted DNA. The PCR thermal profile included an initial polymerase activation step of 10 s at 95°C followed by 40 cycles of 15 s at 95°C, 10 s at 70°C, and 20 s at 72°C.

Positive samples were run without SYBR green, using the same protocol as that described above, but replacing the ExTaq SYBR master mix with ExTaq Perfect master mix (TaKaRa, Japan). The number of cycles in this PCR was adjusted based on the results from the SYBR green reaction to make sure that all positive samples reached the reaction plateau level.

Definition of a positive sample. A positive sample was defined as a sample reaching the fluorescence threshold value (C_T) ≥ 3 cycles before the negative control did. A sample was also defined as positive if it reached the C_T fewer than three cycles before the negative control if the subsequent melting curve analysis showed a single distinct peak clearly different from that for the negative control.

Sequencing. The PCR products were run on agarose gel and purified using an ExoSAP-IT enzymatic degradation kit (Affymetrix). Sequencing was performed in a core facility using an ABI Prism 1.1 Big Dye sequencing kit and an ABI 3730 DNA analyzer (Applied Biosystems).

**RESULTS**

A total of 264 samples were included in the study. Among these, 13 were abscesses from patients with appendicitis, diverticulitis, or recent colonic surgery located close to or in direct connection with the colon or appendix. For these samples, sequencing contributed to the detection of a total of 18 anaerobic species not found by culture, but 18 others were discovered uniquely by culture. Most of the chromatograms were so complex that only the dominant peaks could be interpreted. Even though they contributed to additional findings, we concluded that these samples in general were not suitable for direct sequencing and excluded them from further analysis and presentations of results.

Of the remaining 251 samples, a total of 160 were positive by one or both methods. One hundred fifty-one were positive by the broad-range PCR, and 125 were positive by culture. An overall comparison of broad-range PCR and culture is shown in Fig. 1. Eighty-five samples produced pure chromatograms containing a single bacterial sequence, whereas 66 were mixed and could not have been interpreted without the RipSeq program. Four samples would have been defined as negative based on the C_T value alone but were included as positive based on...
This resulted in the sequence-based detection of a distinct melting peak, as described in Materials and Methods. A total of 3564 Staphylococcus aureus, Staphylococcus epidermidis, Mycobacterium tuberculosis (all confirmed by culture), and Francisella tularensis (confirmed serologically). An overview of positivity rates, concordance, and percentages of samples affected by antibiotics for the different specimen categories is given in Table 1.

Thirty-five of the PCR-positive samples were completely negative by culture, with 28 presenting pure DNA chromatograms and 7 being mixed. All but one were from patients who had been on antibiotic treatment at the time of sampling. The exception was an ovarian abscess containing DNA from Chlamydia trachomatis. Sixty-nine samples, 50 monobacterial and 19 polybacterial, gave the same answer by both culture and direct sequencing. For the remaining 47 PCR-positive samples, there was partial concordance between culture and sequencing in 37 and no concordance in 10.

For patients not on antibiotic treatment at the time of sample collection, concordance with culture was seen in 41 of 55 PCR-positive samples (75%). For patients who had been on antibiotic treatment for more than 1 day prior to sample collection, concordant results were found in 28 of 97 PCR-positive samples (29%).

Nine of the PCR-negative samples were positive by culture and had or might have had clinical relevance. These were two samples from aorta grafts, growing Propionibacterium acnes (BH = growth in enrichment culture only) and one colony of a Micrococcus sp.; two biopsies from osteomyelitic lesions, with Staphylococcus aureus and Staphylococcus lugdunensis (BH); three samples from prosthetic joints, one with Enterococcus faecalis, one with S. aureus, and one with S. epidermidis (BH); one spleen bed abscess with P. acnes; and one tissue sample from an incisional surgical site infection growing P. acnes (BH) and Streptococcus sanguinis (BH). None of these nine samples were affected by antibiotics. In eight of the samples, growth was scarce or intermediate and the number of genome copies was probably below the analytical sensitivity of the assay. For the sample from the spleen bed, abscess growth was rich. The reason for the negative PCR for this sample is most likely the reverse primer mismatch against P. acnes mentioned earlier.

Previously, the use of 16S rRNA amplification and sequencing has been limited to specimens expected to be monobacterial. It has been shown to be useful in the microbiological diagnosis of brain abscesses (16), endocarditis (13), infected prosthetic joints (20), meningitis (2, 5, 18), osteomyelitis (9, 21), septic arthritis (17), spondylodiscitis (8), and vascular graft infections (19). In our material, samples from these conditions comprised 27% (70 samples) of the total, and 85% of those positive by the broad-range PCR produced pure chromatograms. The exceptions consisted of one biopsy from an osteomyelitic lesion (S. aureus and S. epidermidis, one paraprosthetic vascular graft abscess (Corynebacterium tuberculosis and Staphylococcus haemolyticus), and four brain abscesses (Table 2). The largest proportions of mixed chromatograms were derived from abscesses in internal organs/ spaces, bile, and pleural fluids. These specimens frequently contain anaerobic bacteria, and the majority of patients (74 to 85%) had been treated with antimicrobial agents prior to sample collection. Concordance between culture and sequencing was low (Table 1). The total number of positive samples in these three specimen categories was 66. From these 66 samples, the sum of bacteria recovered by direct 16S rRNA gene sequencing exclusively was 64 (39 aerobes, 24 anaerobes, and 1 atypical bacterium). For 40 of the 43 samples where sequencing gave additional information, the patient had already started on antibiotic treatment when the sample was taken. The most interesting findings were seen among the abscesses and pleural fluids. The detailed results for these samples are given in Tables 2 and 3.

In addition to a pelvic abscess with Chlamydia trachomatis, two culture-negative samples contained DNAs from atypical bacteria. One was a sternum biopsy from a child with acute lymphatic leukemia and suspected osteomyelitis, from which abundant DNA from Mycoplasma hominis was isolated (be-
TABLE 2. Overview of results for PCR- and/or culture-positive abscesses in internal organs/spaces\(^d\)

<table>
<thead>
<tr>
<th>Abscess location</th>
<th>Sample ID</th>
<th>Culture result</th>
<th>Sequencing result</th>
<th>Antimicrobial treatment(^d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aorta</td>
<td>1A</td>
<td>No growth</td>
<td>Streptococcus pneumoniae/pseudopneumonia(^b)</td>
<td>DX</td>
</tr>
<tr>
<td>Brain</td>
<td>2A</td>
<td>Streptococcus intermedius</td>
<td>Aggregatibacter aphrophilus, Streptococcus intermedius</td>
<td>--</td>
</tr>
<tr>
<td>Brain</td>
<td>3A</td>
<td>Streptococcus intermedius</td>
<td>Streptococcus intermedius</td>
<td>--</td>
</tr>
<tr>
<td>Brain</td>
<td>4A</td>
<td>Streptococcus milleri group</td>
<td>Streptococcus intermedius</td>
<td>--</td>
</tr>
<tr>
<td>Brain</td>
<td>5A</td>
<td>No growth</td>
<td>Streptococcus proteus</td>
<td>--</td>
</tr>
<tr>
<td>Brain</td>
<td>6A</td>
<td>Peptostreptococcus sp., Streptococcus milleri group</td>
<td>Fusobacterium/nucleatum, Parvimonas micra, Streptococcus intermedius</td>
<td>--</td>
</tr>
<tr>
<td>Brain</td>
<td>7A</td>
<td>No growth</td>
<td>Campylobacter gracilis, Fusobacterium nucleatum, Parvimonas micra</td>
<td>MZ, PC</td>
</tr>
<tr>
<td>Brain</td>
<td>8A</td>
<td>Actionomyces meyeri (Seq), Fusobacterium nucleatum</td>
<td>Fusobacterium nucleatum</td>
<td>--</td>
</tr>
<tr>
<td>Brain</td>
<td>9A</td>
<td>Enterococcus sp., Fusobacterium nucleatum, Peptostreptococcus sp.</td>
<td>Enterococcus cacaee, Gemella hemolyzans, Streptococcus mitis group</td>
<td>CT, MZ</td>
</tr>
<tr>
<td>Brain</td>
<td>10A</td>
<td>No growth</td>
<td>Campylobacter gracilis, Fusobacterium nucleatum/noviforme, Streptococcus intermedius</td>
<td>CT, MZ</td>
</tr>
<tr>
<td>Kidney</td>
<td>11A</td>
<td>Escherichia coli</td>
<td>Escherichia coli/Strigella spp.</td>
<td>--</td>
</tr>
<tr>
<td>Kidney</td>
<td>12A</td>
<td>Escherichia coli</td>
<td>Escherichia coli/Strigella spp.</td>
<td>--</td>
</tr>
<tr>
<td>Liver</td>
<td>13A</td>
<td>No growth</td>
<td>Clostridium perfringens, Escherichia coli/Strigella spp.</td>
<td>CT</td>
</tr>
<tr>
<td>Liver</td>
<td>14A</td>
<td>Clostridobacter yonguei</td>
<td>Clostridobacter gililieni</td>
<td>--</td>
</tr>
<tr>
<td>Liver</td>
<td>15A</td>
<td>Enterococcus faecium, Escherichia coli, Klebsiella sp., Staphylococcus haemolyticus</td>
<td>Enterococcus durans/faecium, Klebsiella sp./Strigella spp.</td>
<td>AM</td>
</tr>
<tr>
<td>Liver</td>
<td>16A(^*)</td>
<td>Staphylococcus haemolyticus</td>
<td>Enterococcus cacaee, Gemella hemolyzans, Streptococcus mitis group</td>
<td>PT</td>
</tr>
<tr>
<td>Lung</td>
<td>17A</td>
<td>Lactobacillus sp.</td>
<td>Lactobacillus gassi</td>
<td>CF, CL</td>
</tr>
<tr>
<td>Ovary</td>
<td>18A</td>
<td>Fusobacterium sp., Peptostreptococcus sp.</td>
<td>Fusobacterium naviforme, Parvimonas micra</td>
<td>--</td>
</tr>
<tr>
<td>Ovary</td>
<td>19A</td>
<td>No growth</td>
<td>Chlamydia trachomatis</td>
<td>--</td>
</tr>
<tr>
<td>Pancreas</td>
<td>20A</td>
<td>Bacteroides fragilis group, Enterococcus sp.</td>
<td>Bacteroides fragilis, Enterococcus durans/faecium</td>
<td>IP</td>
</tr>
<tr>
<td>Pancreas</td>
<td>21A</td>
<td>Mycobacterium tuberculosis</td>
<td>Mycobacterium tuberculosis complex</td>
<td>--</td>
</tr>
<tr>
<td>Pancreas</td>
<td>22A</td>
<td>No growth</td>
<td>Neisseria subflava</td>
<td>PT</td>
</tr>
<tr>
<td>Pancreas</td>
<td>23A</td>
<td>Coagulase-negative staphylococci</td>
<td>Campylobacter concis/mucosalis, Prevotella melitogenica/hist, Staphylococcus capitis/caprae/epidermidis</td>
<td>IP</td>
</tr>
<tr>
<td>Spleen</td>
<td>24A</td>
<td>Enterococcus faecalis, Staphylococcus epidermidis</td>
<td>Enterococcus faecalis, Streptococcus anginosus, Lactobacillus lactis</td>
<td>--</td>
</tr>
<tr>
<td>Spleen</td>
<td>25A</td>
<td>No growth</td>
<td>Fusobacterium nucleatum</td>
<td>PC, MP</td>
</tr>
<tr>
<td>Pelvic</td>
<td>26A</td>
<td>Enterococcus faecalis, Escherichia coli</td>
<td>Enterococcus faecalis, Escherichia coli</td>
<td>CL, MZ</td>
</tr>
<tr>
<td>Psoas</td>
<td>27A</td>
<td>No growth</td>
<td>Enterobacter hormaechei, Enterococcus durans/faecium</td>
<td>VA, MZ, CI</td>
</tr>
<tr>
<td>Psoas</td>
<td>28A</td>
<td>No growth</td>
<td>Enterococcus durans/faecium, Klebsiella pneumoniae</td>
<td>MP</td>
</tr>
<tr>
<td>Psoas</td>
<td>29A</td>
<td>Staphylococcus aureus</td>
<td>Staphylococcus aureus</td>
<td>OX, CL</td>
</tr>
<tr>
<td>Retroperitoneal</td>
<td>30A</td>
<td>Enterococcus faecium</td>
<td>Enterococcus durans/faecium, Klebsiella pneumoniae</td>
<td>MP</td>
</tr>
<tr>
<td>Retroperitoneal</td>
<td>31A(^*)</td>
<td>Bacteroides fragilis group, Enterococcus faecium</td>
<td>Bacillus subtilis, Enterococcus sp.</td>
<td>CI, MZ, LZ</td>
</tr>
<tr>
<td>Subphrenic</td>
<td>32A</td>
<td>Enterococcus durans</td>
<td>Enterococcus durans/faecium, Streptococcus salivarius/therphilis</td>
<td>--</td>
</tr>
<tr>
<td>Subphrenic</td>
<td>33A</td>
<td>Enterococcus sp.</td>
<td>Enterococcus faecalis</td>
<td>--</td>
</tr>
<tr>
<td>Subphrenic</td>
<td>34A</td>
<td>Enterococcus faecalis, Klebsiella sp.</td>
<td>Enterococcus durans/faecium, Klebsiella pneumoniae</td>
<td>GE, MZ</td>
</tr>
<tr>
<td>SSI abdominal (Bilroth I)</td>
<td>35A</td>
<td>No growth</td>
<td>Haemophilus haemolyticus</td>
<td>CT, MZ</td>
</tr>
<tr>
<td>SSI spleen bed (splenectomy)</td>
<td>36A</td>
<td>Propionibacterium acnes</td>
<td>Propionibacterium acnes</td>
<td>--</td>
</tr>
<tr>
<td>SSI kidney bed (nephrectomy)</td>
<td>37A</td>
<td>Citrobacter koseri</td>
<td>Citrobacter koseri</td>
<td>--</td>
</tr>
<tr>
<td>SSI liver (liver resection)</td>
<td>38A</td>
<td>Klebsiella pneumoniae</td>
<td>Haemophilus parainfluenzae, Klebsiella pneumoniae</td>
<td>CT</td>
</tr>
<tr>
<td>SSI liver (liver resection)</td>
<td>39A</td>
<td>No growth</td>
<td>Staphylococcus capitis/caprae/epidermidis</td>
<td>--</td>
</tr>
<tr>
<td>SSI liver (liver resection)</td>
<td>40A</td>
<td>No growth</td>
<td>Haemophilus parainfluenzae, Streptococcus agalactiae</td>
<td>--</td>
</tr>
<tr>
<td>SSI retroperitoneal (Whipples)</td>
<td>41A</td>
<td>Enterococcus sp.</td>
<td>Enterococcus durans/faecium, Streptococcus agalactiae</td>
<td>CT, MZ</td>
</tr>
<tr>
<td>SSI retroperitoneal (Whipples)</td>
<td>42A</td>
<td>Enterococcus sp., Staphylococcus sp., Peptostreptococcus sp., Coagulase-negative staphylococci, Serratia sp., Stenotrophomonas maltophilia</td>
<td>Enterococcus sp., Staphylococcus sp., Peptostreptococcus sp., Coagulase-negative staphylococci, Serratia sp., Stenotrophomonas maltophilia</td>
<td>MP</td>
</tr>
<tr>
<td>SSI subphrenic (ventricular resection)</td>
<td>43A</td>
<td>Coagulase-negative staphylococci, Serratia sp., Stenotrophomonas maltophilia</td>
<td>Pseudomonas aeruginosa</td>
<td>--</td>
</tr>
</tbody>
</table>

\(a\), chromatogram was too complex to allow for complete analysis. Only dominant peaks were included.

\(b\), separation from several other members of the Streptococcus mitis group by only 1 bp (<0.3\%).

\(c\), --, no treatment; AM, amoxicillin; CI, ciprofloxacin; CF, cefuroxime; CT, cefotaxime; CX, ceftiraxone; DX, doxycycline; GE, gentamicin; IP, imipenem; LZ, linezolid; MP, meropenem; MZ, metronidazole; OX, oxacillin; PC, penicillin G; PT, piperacillin-tazobactam; RI, rifampin; VA, vancomycin.

\(d\), Seq, identification was confirmed/formed by 16S rRNA gene sequencing from pure culture; SSI, surgical site infection (with procedure in parenthesis). Results in bold indicate discordant findings.

coming positive 12 cycles before the negative control did). The other was from a young male without known immunodeficiency who was diagnosed with a low-grade spondylodiscitis. The PCR became positive four cycles before the negative control did and showed a distinct peak in the melting curve analysis. The resulting pure chromatogram gave a 99.9% match against “Flexispira rappini.” Other unusual findings comprised a nonmixed DNA chromatogram from a subhepatic abscess.
with 100% similarity to the type strain of Haemophilus haemolyticus (EU909671) and the discovery of Campylobacter spp. in six polymicrobial samples (two brain abscesses, one pancreatic abscess, and three pleural fluids), among which only one was also found by culture.

The results for the positive blood culture bottles are listed in Table 4. These are representatives of samples where the comparison between culture-based isolation and sequence-based detection was not biased by antibiotic treatment or sample collection and transportation procedures. Still, the results were not in concordance for 3 of 10 samples (samples 2, 4, and 8).

**DISCUSSION**

The main purpose of this article was to investigate the usefulness of mixed DNA chromatogram analysis of human clinical specimens. The majority of polybacterial samples were from various abscesses, pleural fluids, and bile, but occasional mixed chromatograms were also derived from typically monobacterial specimens. Many bacteria were recovered exclusively by sequencing, especially for patients who had been treated with antibiotics prior to sample collection. Because our hospital does not utilize dedicated transport containers for anaerobic bacteria, additional anaerobic species were found by sequencing in samples from both antibiotic-treated and untreated patients.

For all specimen categories, most bacteria found are widely accepted to have a pathogenic potential in humans, but a small selection mentioned in Results are more unusual and have a less definite status. Mycoplasma hominis has previously been reported as the probable cause of a range of extragenital infections. An overview with a focus on sternal infections and mediasinitis is given by Mattila et al. (14). Fewer reports exist of human infections with “Flexispira rappini”-like organisms, and there are still unsolved matters concerning their taxonomy. The reference that gave the closest match with our sequence

### TABLE 3. Overview of results for PCR- and/or culture-positive pleural fluids

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Culture result</th>
<th>Sequencing result</th>
<th>Antimicrobial treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1P</td>
<td>Streptococcus intermedia</td>
<td>Campylobacter gracilis, Fusobacterium nucleatum/campylophilum/Streptococcus intermedius/anginosus</td>
<td>CT, GE, PC</td>
</tr>
<tr>
<td>2P*</td>
<td>Coagulase-negative staphylococci, diphtheroids, Streptococcus constellatus</td>
<td>Campylobacter gracilis, Fusobacterium nucleatum</td>
<td>CI, CL</td>
</tr>
<tr>
<td>3P</td>
<td>Anaerobic gram-positive rod, Prevotella sp.</td>
<td>Dialister pneumosintes, Peptostreptococcus stomatis</td>
<td>CT</td>
</tr>
<tr>
<td>4P</td>
<td>Streptococcus intermedius</td>
<td>Fusobacterium navorforme, Parvimonas micra, Streptococcus intermedius</td>
<td>PC, CT</td>
</tr>
<tr>
<td>5P</td>
<td>Escherichia coli</td>
<td>Clostridium tertium, Enterococcus faecalis</td>
<td>PT</td>
</tr>
<tr>
<td>6P</td>
<td>No growth</td>
<td>Campylobacter gracilis, Fusobacterium nucleatum, Parvimonas micra, Prevotella pleuritidis</td>
<td>PC</td>
</tr>
<tr>
<td>7P</td>
<td>No growth</td>
<td>Streptococcus pogenes</td>
<td>CT, MZ</td>
</tr>
<tr>
<td>8P*</td>
<td><em>Campylobacter gracilis (Seq), Eikenella corrdenis</em> (Seq), Streptococcus parasanguinis</td>
<td>Clostridium tertium, Enterococcus faecalis</td>
<td>PC</td>
</tr>
<tr>
<td>9P</td>
<td>No growth</td>
<td>Parvimonas micra, Peptostreptococcus stomatis, Streptococcus anginosus</td>
<td>PT</td>
</tr>
<tr>
<td>10P</td>
<td>No growth</td>
<td>Streptococcus pogenes</td>
<td>PT</td>
</tr>
<tr>
<td>11P*</td>
<td><em>Neisseria sp., Streptococcus sp.</em></td>
<td>Granulicatella adjacens/para-adiacens</td>
<td>CT</td>
</tr>
<tr>
<td>12P</td>
<td>Prevotella sp., Streptococcus mitis group</td>
<td>Peptostreptococcus nucleatum, Prevotella histicola, Streptococcus constellatus</td>
<td>PC, GA</td>
</tr>
<tr>
<td>13P</td>
<td>No growth</td>
<td>Streptococcus intermedius</td>
<td>CT, VA</td>
</tr>
</tbody>
</table>

*See the footnotes to Table 2 for further explanations.

### TABLE 4. Comparison between culture and direct sequencing results for positive blood culture bottles

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Culture result</th>
<th>Sequencing result</th>
<th>Antimicrobial treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Bacteroides fragilis group, Clostridium boltae (Seq)</td>
<td>Bacteroides fragilis, Clostridium boltae</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Bacteroides fragilis group, <em>Eubacterium lentum</em> (Seq), <em>Pseudomonas aeruginosa, Sutterella wadsworthensis</em> (Seq)</td>
<td>Bacteroides ovatus/dorei, <em>Dialister pigra</em>, Sutterella wadsworthensis</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Bacteroides fragilis group, Clostridium boltae (Seq), Peptostreptococcus sp.</td>
<td>Bacteroides fragilis, Clostridium boltae, <em>Ruminococcus gnavus</em></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td><em>Parvimonas micra</em> (Seq), <em>Dialister pneumosintes</em> (Seq)</td>
<td><em>Catonella sp.</em>, <em>Dialister pneumosintes</em>, <em>Parvimonas micra</em>, <em>Porphyromonas asaccharolytica</em></td>
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</tr>
<tr>
<td>5</td>
<td><em>Enterococcus casseliflavus, Escherichia coli</em></td>
<td><em>Enterococcus casseliflavus/gallinarum, Escherichia coli</em></td>
<td></td>
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<tr>
<td>6</td>
<td><em>Escherichia coli, Streptococcus galactolyticus</em></td>
<td><em>Escherichia coli, Streptococcus galactolyticus</em></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td><em>Escherichia coli, Streptococcus agalactiae</em></td>
<td><em>Escherichia coli, Streptococcus agalactiae</em></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td><em>Bacteroides fragilis group, Clostridium sp., Escherichia coli</em></td>
<td><em>Clostridium ramosum, Escherichia coli</em></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td><em>Escherichia coli, Pseudomonas aeruginosa</em></td>
<td><em>Escherichia coli, Pseudomonas aeruginosa</em></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td><em>Enterococcus faecalis, Staphylococcus hominis</em></td>
<td><em>Enterococcus faecalis, Staphylococcus hominis/lugdunensis</em></td>
<td></td>
</tr>
</tbody>
</table>

*See the footnotes to Table 2 for further explanations.
was a blood culture isolate from a patient with diarrhea (GenBank accession no. AF286053). Two case reports of bone infections with “Flexispira rappini”-like bacteria have been published (4, 9). Our patient recovered on treatment with doxycycline, gentamicin, and penicillin. G. H. haemolyticus is part of the normal flora of the upper respiratory tract and is considered to be a nonpathogen (15). Our sample was taken from a subhepatic abscess in a 67-year-old woman 9 days after a Billroth I operation. She presented with fever and an elevated C-reactive protein level and neutrophil count and had been treated with antibiotics for 4 days before specimen collection. She recovered upon abscess drainage and treatment with cefotaxime. A review of the literature confirms the role of *Campylobacter gracilis* in certain extra-oro-intestinal abscesses and empyemata (6, 11). Most recently, it was discovered by broad-range amplification and cloning in three polybacterial brain abscesses (1).

The blood culture results were included mainly to get a comparison between culture and sequencing, unaffected by antibiotics, sample collection procedures, or transportation. Some of the cultured bacteria were not found by sequencing. These are examples of one of the limitations attached to sequencing directly from mixed clinical samples. Because all bacteria in a sample will be competing for the same reagents, those present at the lowest concentrations might be outcompeted in the PCR and not visible in the resulting DNA chromatogram. We have shown experimentally that this is likely to occur when the molar ratio exceeds 1:10. In two of the blood cultures, some of the anaerobic bacteria were detected solely by sequencing. This may be due to bacterial lysis in the bottles, unsuccessful subcultivation, or difficulties in differentiating between colonies on the agar plates.

The RipSeq algorithm has been validated for samples containing up to three different species of bacteria. Occasionally, four bacterial species can be accepted, e.g., if variations in primer affinities lead to the detection of different bacteria with the forward and reverse chromatograms. For two abscesses, two bile samples, and three pleural fluids, the chromatograms were so complex that all peaks could not be included without exceeding this limitation of the RipSeq algorithm (Tables 2 and 3). A high y-axis cutoff was used to include only the most dominant peaks and to make the RipSeq analysis valid. All seven samples had been exposed to antibiotics, and despite ignoring the lower portions of the chromatograms, additional bacteria not found by culture were still detected in all of them.

The limitation in how complex a chromatogram can be before specificity becomes too low and the competition for reagents in mixed samples are the two major challenges for the use of broad-range PCR and DNA sequencing directly from polybacterial samples. This was clearly illustrated with abscesses originating from the colon and appendix, but also with the other samples where a proportion of the involved bacteria were found by culture only. One possible way to reduce these problems is to use Gram stain type-specific broad-range primers and to amplify gram-positive and gram-negative bacteria in different tubes. In a recent publication, these problems were omitted by the use of cloning and high-throughput pyrosequencing in the investigation of brain abscesses (1). A surprising level of complexity was demonstrated in some of the samples. Unfortunately, this sort of diagnostics is currently not within reach for the routine laboratory. A less costly alternative is the use of denaturing gradient gel electrophoresis followed by DNA sequencing of the different fragments. This approach is labor-intensive and technically challenging, and although it will not in theory have a limitation when it comes to the number of different species in a sample, it will have shortcomings in detecting the minor populations in samples with large differences in the relative concentrations of the different participants. In addition to denaturing gradient gel electrophoresis, a number of methods have been developed that work by the principle of separating the different DNA fragments based on their physical properties. The WAVE system (Transgenomic, Omaha, NE) uses denaturing high-performance liquid chromatography to separate the different DNA fragments in combination with an automated fragment collector and has been shown to be useful in the analysis of complex human clinical samples (7). The automatic fragment collection makes it easier to further analyze the fragments with DNA sequencing. A proportion of the fragments will not be separable by denaturing high-performance liquid chromatography, but DNA chromatograms for these will typically contain only two or three different species and should be suitable for RipSeq analysis.

The limited sensitivity is the main challenge for direct 16S rRNA gene sequencing in general (10). For our assay, we found a sensitivity of 2,000 to 4,000 genome copies per ml of sample material. Based on the chosen pre-PCR treatment, the amount of template used in the PCR, and the definition of a positive sample, this is very close to the theoretical detection limit. The consequence of a low sensitivity is that a negative broad-range PCR can never exclude the presence of bacterial DNA in a sample, and the laboratory should always make this clear to the physician in charge of the patient. In addition, in this study, because we did not include an amplification control, some of the investigated samples might have produced false-negative results due to unrecognized inhibitory substances.

The choice of the most relevant specimens could be optimized further. As already mentioned, sequencing from abscesses in direct connection to the colon/appendix was judged not to be clinically useful. Peritoneal fluids had a low positivity rate, and sequencing gave little added value. Sequencing from osteomyelitic lesions in adults and from prosthetic joints gave additional information for a number of samples where the patients had been treated with antibiotics but had a lower sensitivity than culture with nontreated samples. The inclusion criteria used for pleural fluids led to the investigation of a high proportion of negative samples. Retrospectively, we found that a pulmonary infection was suspected in about 60% of the cases, including all of the positive cases. The remaining 40% were chiefly thought to be caused by cardiovascular conditions or cancer. If we had access to this information in the first place, the positivity rate would have improved from 30 to 48%.

The use of real-time PCR with SYBR green detection has important advantages compared to traditional PCR with gel-based detection. With gel-based detection, the number of cycles in the first PCR has to be limited to ensure that no visible band appears in the negative control. The amplification process with positive samples containing lower levels of DNA will then be aborted before it reaches the plateau level, resulting in large intersample variations in the amount of DNA used in the cycle sequencing reactions. With real-time PCR, all samples...
can be run to the plateau level, since a positive sample is defined relative to when the negative control reaches its $C_T$ value. More importantly, it gives a semiquantitative measure of how much bacterial DNA a sample contains. A sample that reaches its $C_T$ value early contains more bacterial DNA, and the final result is less likely to represent contamination than if it was from a sample that reaches its $C_T$ value just before the cutoff, even if both are positive by definition. Finally, we found that with the possibility of doing mixed chromatogram analysis, the definition of a positive sample had to be modified. For some of the samples becoming positive three to six cycles before the negative control did, contaminant bacterial DNAs from the reagents were still detectable as lower peaks in the chromatograms. Consequently, only the dominant peaks were considered to be relevant, and these samples were analyzed using a higher cutoff on the $y$ axis in the RipSeq program.

When DNAs from multiple species are going to compete for the same primers, the primers not only must be universal but also must bind equally efficiently to the respective bacteria. A bacterium with a single mismatch close to the 3′ end of one of the primers will be outcompeted in the amplification process by a bacterium with a perfect match. It is also crucial that the lysis procedure is equally efficient for all relevant bacteria. If these two matters are not addressed properly, the DNA chromatograms may give a false impression of the number of bacteria in the sample (12).

The single strongest reason to consider direct 16S rRNA gene sequencing as a supplement to culture should be the administration of antibiotics prior to sample collection. In hospitals that do not have dedicated transport systems for anaerobic bacteria, it should also be considered for important samples such as brain abscesses, regardless of antimicrobial status. There has been a tendency in our hospital to await culture results and eventually to proceed to direct sequencing only with culture-negative samples. This policy fails to take into consideration that in samples affected by antibiotics some species can still be able to grow, whereas others are not. An incomplete microbiological answer can be misleading and result in insufficient antimicrobial coverage, especially in patients where standard empirical therapy cannot be used or in patients that are transferred to oral treatment. This article shows that with the RipSeq program, the use of direct 16S rRNA gene sequencing can be expanded to a number of typical polymicrobial specimens. It was found to be of particular value for internal abscesses, pleural fluids, and bile. The program allowed for partial or complete interpretation of all 66 mixed DNA chromatograms in the study.

ACKNOWLEDGMENTS

This work was supported by The Research Council of Norway and Innovation Norway. A patent application has been filed for several aspects of the RipSeq algorithm. The RipSeq program is accessible through a commercial web service owned by iSentio AS. The first author is a coowner and the CMO of this company.

REFERENCES


