16S rRNA Gene Sequencing for Bacterial Pathogen Identification in the Clinical Laboratory

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For many years, sequencing of the 16S ribosomal RNA (rRNA) gene has served as an important tool for determining phylogenetic relationships between bacteria. The features of this molecular target that make it a useful phylogenetic tool also make it useful for bacterial detection and identification in the clinical laboratory. Sequence analysis of the 16S rRNA gene is a powerful mechanism for identifying new pathogens in patients with suspected bacterial disease, and more recently this technology is being applied in the clinical laboratory for routine identification of bacterial isolates. Several studies have shown that sequence identification is useful for slow-growing, unusual, and fastidious bacteria as well as for bacteria that are poorly differentiated by conventional methods. The technical resources necessary for sequence identification are significant. This method requires reagents and instrumentation for amplification and sequencing, a database of known sequences, and software for sequence editing and database comparison. Commercial reagents are available, and laboratory-developed assays for amplification and sequencing have been reported. Likewise, there are an increasing number of commercial and public databases. Despite the availability of resources, sequence-based identification is still relatively expensive. The cost is significantly reduced only by the introduction of more automated methods. As the cost decreases, this technology is likely to be more widely applied in the clinical setting.

Key words: 16S rRNA, bacteria identification, sequence identification.

Most clinical bacterial isolates are quickly and accurately identified by conventional techniques used in the microbiology laboratory. For most isolates, a final identification is available less than 24 hours after isolation of a discrete colony. Because conventional identification techniques are relatively inexpensive, there is no need to use more expensive molecular techniques for identification of such isolates. However, there are several situations in the clinical laboratory in which molecular identification would significantly improve both the time to and accuracy of identification. For example, *Mycobacterium* spp. are slow-growing bacteria that can take up to 6 to 8 weeks to grow in culture and another 4 to 6 weeks or more to identify by biochemical profiling. In addition, mycobacterial identification requires significant technical expertise, which can take years to develop. Many clinical laboratories, facing an ever-decreasing work force, are struggling to maintain this expertise. Molecular identification offers an attractive alternative method of identifying mycobacteria because it is rapid, and a single technique can be used to identify a variety of different species. In addition to slow-growing bacteria, molecular techniques are useful for the

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identification of fastidious bacteria, unusual isolates, and bacteria that are poorly differentiated using conventional techniques.

Once the need for molecular identification is established, the method of identification needs to be determined. Gen-Probe (San Diego, CA) offers a number of culture identification tests (the Accu-Probe Assays) that are based on hybridization of a probe to RNA extracted from a culture isolate [1–6]. Each kit contains one probe for a specific organism. These kits perform very well and are useful for specific applications, but their use is limited because an unknown isolate can be tested with only one probe at a time, and a limited number of probes are available. High-density probe arrays will allow for hybridization to many probes at the same time [7–9]. This technology will be a valuable tool for bacterial identification, especially in clinical situations in which the types of bacteria present in a specimen are predictable. However, identification by hybridization only allows a microbiologist to ask whether an unknown isolate is one of these organisms. There will always be unpredictable or novel isolates. Sequencing is a more powerful molecular identification method because it answers the question "What is the unknown isolate?"

Bacterial identification by sequencing of the 16S ribosomal RNA (rRNA) gene is a universal bacterial identification method. Bacterial taxonomists have used this technology for a number of years as a measure of DNA similarity between isolates. More recently, 16S rRNA gene amplification and sequencing has been used to detect and identify fastidious bacterial pathogens such as *Tropheryma whippelii*, the agent of Whipple disease, and *Bartonella quintana*, the agent of bacillary angiomatosis, in clinical specimens [10,11]. It was only a matter of time before 16S rRNA gene sequencing became an identification tool in the clinical laboratory. This article reviews the technical aspects of sequence identification and how this technique is being applied in the clinical laboratory.

The 16S rRNA Gene

Several features of the 16S rRNA gene make it an important phylogenetic tool and hence a useful target for clinical identification. One important feature is that this gene is present in all bacteria; thus it is a universal target for bacterial identification. Second, the function of 16S rRNA has remained constant over a long period, so sequence changes are more likely to reflect random changes than selected changes that would alter the molecule's function [12]. Random sequence changes are a more accurate measure of time. Finally, the 16S rRNA gene is large enough (approximately 1,500 bp) to contain statistically relevant sequence information, but more importantly the molecule consists of approximately 50 functional domains (Fig. 1). The number of domains is important because the introduction of selected changes in one domain does not greatly affect sequences in other domains. As the number of domains increases, the less impact selected changes have on phylogenetic relationships. These characteristics are among the reasons Woese referred to rRNA as the "ultimate molecular chronometer" [12].

Unfortunately, 16S rRNA gene sequence is not a perfect measure of overall sequence divergence between bacteria. Sequence diversity between strains is more accurately measured by a DNA–DNA reassociation assay. Taxonomists define a species as strains that have 70% or greater DNA similarity by reassociation [13]. Species with this level of relatedness typically have 97% or greater sequence identity of the 16S rRNA gene [14]. Strains with less than 97.5% 16S rRNA gene sequence identity are unlikely to be related at the species level. However, there are a number of strains that share less than 50% DNA similarity by reassociation and therefore are classified as distinct species but share 99% to 100% 16S rRNA gene sequence identity. For example, *Mycobacterium chelonae* and *Mycobacterium abscessus* have more than 99% 16S rRNA gene sequence identity, but their DNA similarity by reassociation is only 35% [15,16]. For the taxonomist, this means species-level classification based on 16S rRNA gene sequence alone is not valid, and bacterial classification must be based instead on a polyphasic approach. For the clinical microbiologist, this means that 16S rRNA gene sequence will not always provide a definitive species-level identification.

With today's technology, sequencing of the entire 16S rRNA gene is not a practical approach for routine bacterial identification in the clinical laboratory. Automated sequencing can generate approximately 500 bp of sequence data, so several sequencing reactions are required to generate 1,500 bp of sequence data. The cost of reagents and labor necessary to sequence the entire gene is beyond the means of most clinical laboratories. Fortunately, the

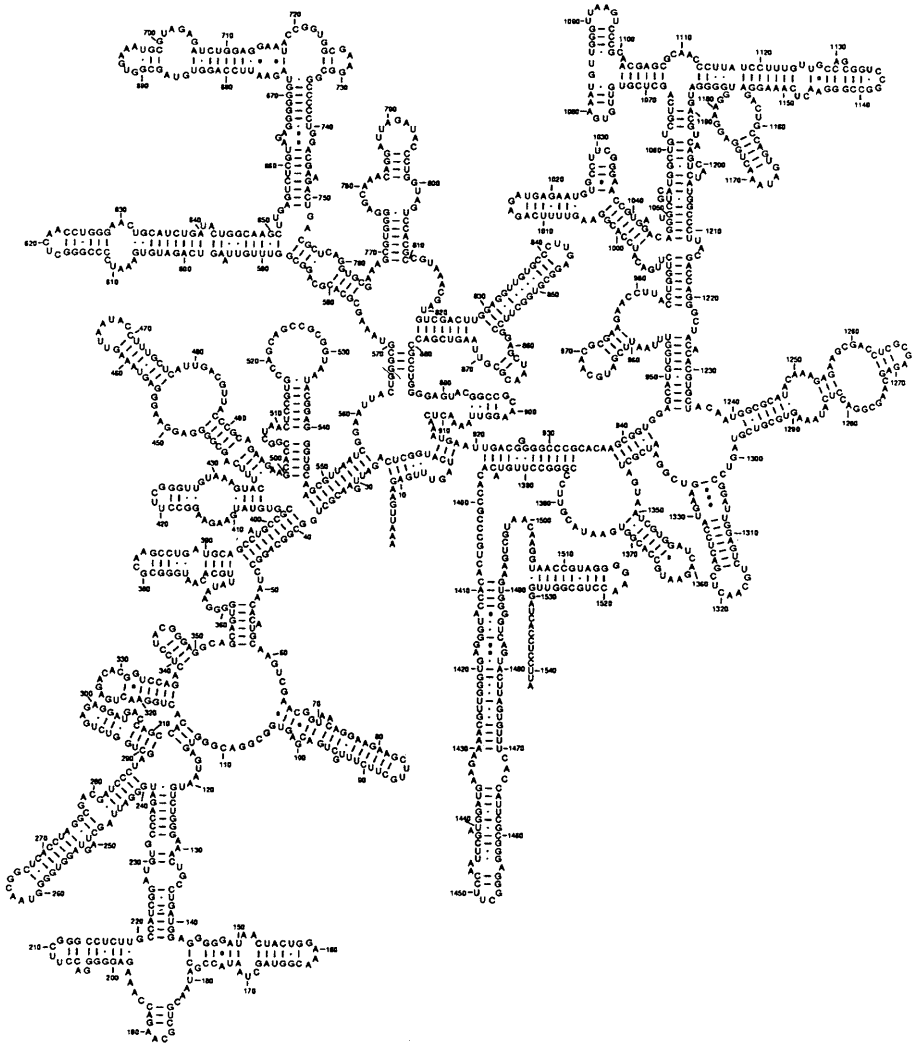

Fig. 1. Secondary structure of 16S rRNA (used with the permission of Applied Biosystems, Forest City, CA).

entire gene does not have to be sequenced to achieve a species-level identification for most bacteria. Although phylogenetically informative positions occur throughout the entire gene, the region of greatest heterogeneity occurs in approximately the first 500 bases of the 5' end [17–19]. Therefore, sequencing of only these first 500 bases is usually sufficient for identification of a clinical isolate, whereas a full gene sequence is more accurate for characterization of a novel isolate.

Technical Aspects of Sequencing

Sequencing procedures can vary depending on which manufacturer's reagents and equipment are used, but the basic steps remain the same (Fig. 2). The first steps are growth and isolation of a potentially significant bacterium from a patient's specimen, followed by extraction of DNA from the isolate. There are many options for extraction, including Chelex extraction, alkaline lysis, phenol-chloroform extraction, and mechanical disruption [20,21]. Not all methods are equally effective for all types of bacteria. After extraction, the target is amplified by PCR. At this point it may be desirable to check the product on an agarose gel to ensure that a single fragment of the appropriate size was amplified. Cycle sequencing reactions are performed next, and the products of these reactions are analyzed by electrophoresis. If both strands of the amplicon are sequenced, the sequences must be assembled and edited for discrepancies. The final step is to compare the unknown sequence to a database. Depending on the type of sequencing, it

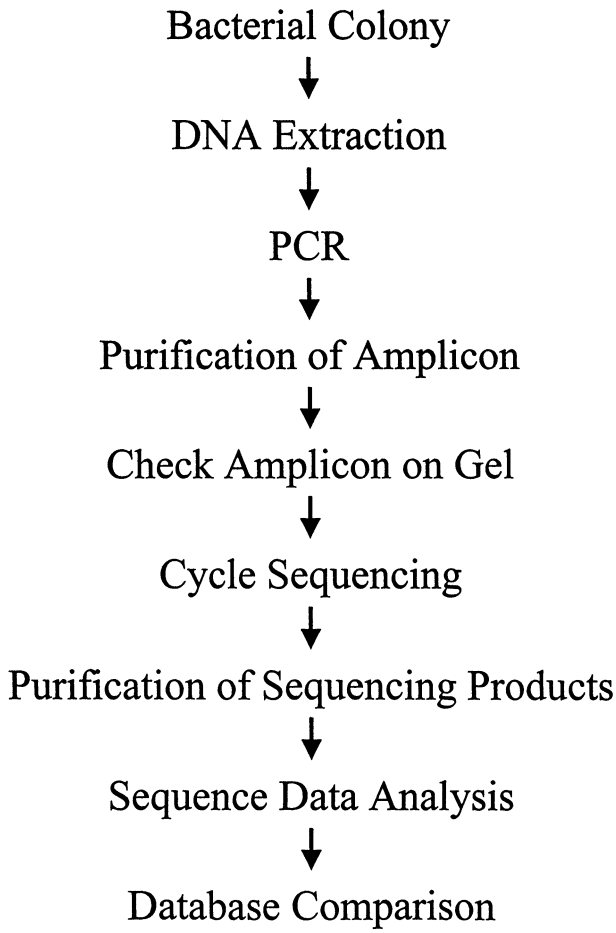

Fig. 2. Steps of sequence identification.

may be necessary to purify the PCR amplicon before sequencing and to purify the sequencing products before electrophoresis.

Bacterial identification by 16S rRNA gene sequence analysis has been greatly facilitated by the availability of a commercial system. The MicroSeq system (Applied Biosystems, Forest City, CA) consists of reagents, software, and a database of sequences. There are two versions of reagent kits, a full gene version in which the entire gene is sequenced by one amplification reaction and 12 sequencing reactions (6 forward and 6 reverse), and the 500-bp kit for sequencing of the gene's 5' end with one amplification reaction and two sequencing reactions (1 forward and 1 reverse). These kits are designed for universal bacterial identification (i.e., the primers hybridize to sequences conserved among all bacteria). The MicroSeq software contains tools for sequence assembly, editing, database comparison, file management, and other tasks. One of the most valuable features of the MicroSeq system is the database. It contains more than 1,100 different sequences. Nearly all of the sequences are from "type strains" (i.e., the reference specimen for the name), assuring that these sequences are assigned to the correct species. There are full-gene and 500-bp versions of the database. An important feature of both databases is that all of the sequences have defined ends (i.e., the flanking primers used for amplification). This allows for uniform calculation of sequence divergence between an unknown sequence and the database entries.

Although MicroSeq is the only commercial system for 16S rRNA gene sequencing, a laboratory may choose to develop its own assay. The amplification and sequencing reactions can be performed using any amplification enzymes and sequencing reagents. In addition, there are multiple options for software that can analyze sequence data. Probably the biggest decision for laboratories that develop their own assay is which database to use or whether a new database should be created. There are several databases to consider (Table 1). GenBank, a collection of all publicly available DNA sequences, is the most extensive database. The Ribosomal Database Project (RDP) is a database of only ribosomal sequences. This database was created for the purpose of better understanding phylogenetic relationships between organisms. The database found on the Ribosomal Differentiation of Medical Microorganisms (RIDOM) server is also limited to ribosomal sequences, but the entries are of only medically important bacteria [22]. This database is still

Table 1. Databases of 16S rRNA Gene Sequences

	Database	Web Address
Public	GenBank	www.ncbi.nlm.nih.gov
	Ribosomal Database Project	www.cme.meu.edu/RDP/html/index.html
	Ribosomal Differentiation of Medical Micro-organisms	www.ridom.de
Commercial	MicroSeq	www.appliedbiosystems.com
	SmartGene IDNS	www.smartgene.ch

being developed, but the list of *Mycobacterium* sequences is extensive. Finally, *SmartGene* IDNS offers a variety of services for sequence-based identification of bacteria, including a "cleaned up" version of ribosomal sequences from GenBank.

In considering any database, the primary concerns are whether the sequence data are accurate and whether the genus and species assignment of a database entry is valid. With a public database like GenBank, anyone can deposit a sequence and attach a name to that sequence. Using such a sequence for clinical identification is potentially dangerous because it could lead to an inaccurate identification, and often significant clinical decisions such as antimicrobial therapy are based upon the isolate's identity. This does not necessarily eliminate GenBank as a resource. It simply means that a sequence should be backed up by a reliable reference before it is used for clinical purposes. Many laboratories find that the best solution is a combination of a commercial or public database and a database that is developed within the laboratory. For example, our laboratory has collected a number of sequences from clinical isolates as a result of our validation experiments and application of sequencing for routine mycobacterial identification. As a result, we use both the MicroSeq database and our own database for routine identification.

Sequencing of the 16S rRNA gene from a single isolate usually generates clear and unambiguous sequence data. Rarely, the sequence data can appear to be "messy" (i.e., multiple overlapping peaks on the electropherogram). Messy sequence data may be the result of a mixed culture; however, it can also occur when a single isolate contains multiple copies of the ribosomal gene and these copies have unique sequences. The number of 16S rRNA gene copies in an isolate varies greatly between genera. For example, *Escherichia coli* isolates characteristically have seven copies of the gene, whereas the slow-growing *Mycobacterium* spp. typically have one to two copies of the gene. It is unclear how frequently sequence heterogeneity between copies of a single isolate occurs. Several reports in the literature describe isolates with 16S rRNA heterogeneity [23–27], but only two of these reports are of clinical isolates, *Mycobacterium terrae* complex [28] and *Mycobacterium celatum* [29]. A possible explanation for the lack of reports in other bacteria is that sequencing of an amplified product minimizes the potential for detecting sequences that represent a minority of the population. When heterogeneity is detected, it can be resolved by first cloning the genes before sequencing. This step is useful for academic purposes, but cloning is labor-intensive, so for clinical purposes it may be best to consider an alternative identification method. Reports of heterogeneity within a single isolate may increase as sequence-based identification is more widely applied.

Applications of 16S rRNA Gene Sequence-based Identification

Sequence-based identification is used in the clinical laboratory primarily to identify isolates that are either slow-growing bacteria or bacteria that are difficult to identify using conventional techniques. The first application of 16S rRNA gene sequencing in the clinical laboratory was for identification of mycobacterial isolates. Less often, this technology is used for identification of bacteria directly from specimens of normally sterile body sites.

Rogall et al. [18] were the first to show that sequence analysis of regions within the 5' portion of the 16S rRNA gene is sufficient for species-level identification of most clinically relevant *Mycobacterium* isolates. Krischner et al. [30] subsequently described an assay that used genus-specific primers to amplify and sequence DNA from *Mycobacterium* isolates in clinical cultures, demonstrating that sequencing could be readily adapted to the clinical laboratory. Our laboratory validated sequence-based mycobacterial identification for clinical isolates using the MicroSeq 500 16S rDNA Bacterial Sequencing Kit, software, and database [21].

Compared with phenotypic identification, sequence-based identification offers several advantages. Besides some of the more practical advantages discussed earlier, sequencing is more accurate than phenotypic identification, and it allows for more rapid recognition of novel isolates [21,30,31]. For example, our laboratory found several isolates of mycobacteria that were misidentified by phenotypic methods but were correctly identified by sequencing. We could trace these errors back to phenotypic variability within a species and phenotypic assay variability. We also sequenced two isolates that were originally sent to two different reference laboratories for identification. One isolate was identified as *Mycobacterium alcapulcensis,* and the other was re-

ported as *Mycobacterium szulgai*. However, both isolates had the same 5' 500-bp sequence, and this sequence is unique from any other sequences in GenBank [21]. These two isolates are likely related to each other at the species-level and probably represent a novel species, yet identification by nonsequence methods provides no information that these isolates are closely related to each other. Increased use of sequence-based identification has led to a significant increase in newly described, medically important *Mycobacterium* spp. (Table 2).

Sequencing was also evaluated as an identification method for groups of bacteria that either are poorly differentiated using conventional methods or require more than 48 hours to identify. Tang et al. [17] compared three rapid identification methods with conventional phenotypic identification for unusual aerobic Gram-negative bacteria. The three rapid methods were based on 16S rRNA gene sequencing (MicroSeq), cellular fatty acid profiles, and carbon source use. MicroSeq identification matched both the genus and species reference identity more often than did the identifications produced by either of the other methods. Additionally, their data showed that a 5' 500-bp MicroSeq identity was comparable to a full gene identity. The full gene and 500-bp 16S rRNA gene identities always agreed at the genus level, and 93.1% of the 500-bp species assignments were the same as the species assignments by the full gene method. In another study by Tang et al. [32], the MicroSeq 5' 500-bp identification proved a useful tool for the identification of *Corynebacterium* and *Corynebacterium*-related isolates. Sequence identification provided the same genus identity as conventional and supplemented phenotypic methods for all isolates tested. The species-level identity was the same for 66.7% of all isolates. Sequence identification was most reliable (100% concordance) for the two most clinically significant species, *Corynebacterium diptheriae* and *Corynebacterium jeikeium*. 16S rRNA gene sequencing is a particularly important method for identification of fastidious bacteria both from culture and from clinical specimens. Our laboratory was able to identify a blood culture isolate that failed to grow on conventional media as *Leptotrichia* sp. only after an aliquot of the positive blood culture bottle was submitted for sequence analysis [33]. Sequencing also indicated that this isolate is probably a novel species of *Leptotrichia*. The literature contains several similar reports. For example, the uncommon isolate *Anaerobiospirillum succiniciproducens* was identified as a cause of bacteremia in three patients by sequence identification [34]. In another report, an isolate from joint fluid of a patient with septic arthritis was identified as a novel species of *Helicobacter* [35]. Sequence identification will undoubtedly be applied to the identification of other groups of bacterial isolates. Our laboratory is currently evaluating sequencing as a means of identifying isolates of aerobic and anaerobic actinomycetes.

Table 2. Medically Important *Mycobacterium* spp. Recently Recognized by 16S rRNA Gene Sequencing

New Species	Reference
M. genavense	39
M. branderi	40
M. interjectum	41
M. conspicuum	42
M. lentiflavum	43
M. novocastrense	44
M. triplex	45
M. bohemicum	13
M. heidelbergense	46
M. tusciae	47
M. wolinskyi	48
M. heckeshornense	49

More often, clinical laboratories are using sequence identification to detect and identify pathogens directly from clinical specimens that should otherwise be sterile. *Neisseria meningitidis* was identified in brain pus from a patient with culture-negative meningitis [36]. The isolate presumably failed to grow because of prior antibiotic use. A novel *Helicobacter* species was identified directly from drainage of an abdominal abscess in a patient with X-linked hypogammaglobulinemia [37]. Our laboratory used 16S rRNA gene sequencing to detect and identify *Mycoplasma orale* in the tissue of a patient with hypogammaglobulinaemia who was suffering from a persistently culture-negative inflammatory arthritis (M. Paessler, M. Shuster, J.B. Patel, I. Nachamkin, unpublished data). Although sequence-based identification directly from a clinical specimen is a potentially powerful technique, this technology lacks some of the advantages that culture provides. For example, sequence-based identification does not easily allow for the detection of multiple pathogens, and there is no way to measure the relative abundance of different organisms. Likewise, without culture there is no isolate

for further characterization such as susceptibility testing. For these reasons, sequence-based identification directly from a clinical specimen is primarily used only when culture has failed or is not possible.

Is Sequence-based Identification Cost-effective?

Sequencing is a relatively expensive method of identification. One laboratory estimated their cost at approximately $84.25 per test, and another laboratory calculated a cost of $40.00 to $85.00 per test [32,38]. We have estimated our cost to perform a single identification, which includes a negative amplification control, to be $144.00 (J.B. Patel, unpublished data). This number includes the cost of extraction, disposables, reagents, database use, and labor. Most of the cost is labor, so the total cost drops to $87.00 per identification if two isolates are sequenced at the same time. These figures do not include the cost of purchasing instrumentation.

One justification for the expense of sequence-based identification is that the improved accuracy and speed of this method will have a positive impact on clinical care. However, there are no studies evaluating the impact of sequence-based identification on the quality of patient care or the cost of treating a patient. Despite this lack of data, it may not be necessary to look beyond a laboratory's own budget to justify the expense of sequencing. For example, a laboratory may not have the capability of performing mycobacterial identification in-house, but the cost of sending the specimen to a reference laboratory far exceeds the cost of a sequence-based identification. Such a laboratory may consider sequencing, especially in a situation in which instruments for amplification and sequence analysis are already available or can be shared.

The introduction of more automated methods undoubtedly will have the biggest impact on decreasing the cost of sequence identification and will result in increased use of this technology in hospital and reference laboratories.

Conclusions

Sequencing of the 16S rRNA gene is a powerful identification method in the clinical laboratory. This technique is applicable for routine identification of several groups of bacteria as well as for identification of novel isolates. As the technical resources for sequence identification become more abundant and less expensive, more clinical microbiologists will consider using this method in their laboratories' work flow.

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References

1. Lebrun L, Espinasse F, Poveda JD, Vincent-Levy-Frebault V: Evaluation of nonradioactive DNA probes for identification of mycobacteria. J Clin Microbiol 1992;30:2476–2478
2. Bourbeau PP, Heiter BJ, Figdore M: Use of Gen-Probe AccuProbe Group B streptococcus test to detect group B streptococci in broth cultures of vaginal-anorectal specimens from pregnant women: Comparison with traditional culture method. J Clin Microbiol 1997;35:144–147
3. Denys GA, Carey RB: Identification of *Streptococcus pneumoniae* with a DNA probe. J Clin Microbiol 1992;30:2725–2727
4. Young H, Moyes A: Comparative evaluation of AccuProbe culture identification test for *Neisseria gonorrhoeae* and other rapid methods. J Clin Microbiol 1993;31:1996–1999
5. Padhye AA, Smith G, McLaughlin D, Standard PG, Kaufman L: Comparative evaluation of a chemiluminescent DNA probe and an exoantigen test for rapid identification of *Histoplasma capsulatum*. J Clin Microbiol 1992;30:3108–3111
6. Padhye AA, Smith G, Standard PG, McLaughlin D, Kaufman L: Comparative evaluation of chemiluminescent DNA probe assays and exoantigen tests for rapid identification of *Blastomyces dermatitidis* and *Coccidioides immitis*. J Clin Microbiol 1994;32: 867–870
7. Westin L, Miller C, Vollmer D, Canter D, Radtkey R, Nerenberg M, O'Connell JP: Antimicrobial resistance and bacterial identification utilizing a microelectronic chip array. J Clin Microbiol 2001;39: 1097–1104
8. Anthony RM, Brown TJ, French GL: Rapid diagnosis of bacteremia by universal amplification of 23S ribosomal DNA followed by hybridization to an oligonucleotide array. J Clin Microbiol 2000;38: 781–788

9. Troesch A, Nguyen H, Miyada CG, Desvarenne S, Gingeras TR, Kaplan PM, Cros P, Mabilat C: *Mycobacterium* species identification and rifampin resistance testing with high-density DNA probe arrays. J Clin Microbiol 1999;37:49–55
10. Relman DA, Loutit JS, Schmidt TM, Falkow S, Tompkins LS: The agent of bacillary angiomatosis. An approach to the identification of uncultured pathogens. N Engl J Med 1990;323:1573–1580
11. Relman DA, Schmidt TM, MacDermott RP, Falkow S: Identification of the uncultured bacillus of Whipple's disease. N Engl J Med 1992;327:293–301
12. Woese CR: Bacterial evolution. Microbiol Rev 1987;51:221–271
13. Reischl U, Emler S, Horak Z, Kaustova J, Kroppenstedt RM, Lehn N, Naumann L: *Mycobacterium bohemicum* sp. nov., a new slow-growing scotochromogenic mycobacterium. Int J Syst Bacteriol 1998;48 Pt 4:1349–1355
14. Stackebrant E, Goebel BM: Taxonomic note: A place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. Int J Syst Bacteriol 1994;44:846–849
15. Kusunoki S, Ezaki T: Proposal of *Mycobacterium peregrinum* sp. nov., nom. rev., and elevation of *Mycobacterium chelonae* subsp. *abscessus* (Kubica et al.) to species status: *Mycobacterium abscessus* comb. nov. Int J Syst Bacteriol 1992;42:240–245
16. Springer B, Bottger EC, Kirschner P, Wallace RJ Jr.: Phylogeny of the *Mycobacterium chelonae*-like organism based on partial sequencing of the 16S rRNA gene and proposal of *Mycobacterium mucogenicum* sp. nov. Int J Syst Bacteriol 1995;45:262–267
17. Tang YW, Ellis NM, Hopkins MK, Smith DH, Dodge DE, Persing DH: Comparison of phenotypic and genotypic techniques for identification of unusual aerobic pathogenic gram-negative bacilli. J Clin Microbiol 1998;36:3674–3679
18. Rogall T, Flohr T, Bottger EC: Differentiation of *Mycobacterium* species by direct sequencing of amplified DNA. J Gen Microbiol 1990;136:1915–1920
19. Woese CR, Gutell R, Gupta R, Noller HF: Detailed analysis of the higher-order structure of 16S-like ribosomal ribonucleic acids. Microbiol Rev 1983;47:621–669
20. MicroSeq 16S rRNA gene kit protocol. Applied Biosystems, Forest City, CA, 2000 (www.appliedbiosystems.com)
21. Patel JB, Leonard DG, Pan X, Musser JM, Berman RE, Nachamkin I: Sequence-based identification of *Mycobacterium* species using the MicroSeq 500 16S rDNA bacterial identification system. J Clin Microbiol 2000;38:246–251
22. Harmsen D, Rothganger J, Singer C, Albert J, Frosch M: Intuitive hypertext-based molecular identification of micro-organisms. Lancet 1999;353:291
23. Liefting LW, Andersen MT, Beever RE, Gardner RC, Forster RL: Sequence heterogeneity in the two 16S rRNA genes of *Phormium* yellow leaf phytoplasma. Appl Environ Microbiol 1996;62:3133–3139
24. Mylvaganam S, Dennis PP: Sequence heterogeneity between the two genes encoding 16S rRNA from the halophilic archaebacterium *Haloarcula marismortui*. Genetics 1992;130:399–410
25. Nubel U, Engelen B, Felske A, et al.: Sequence heterogeneities of genes encoding 16S rRNAs in *Paenibacillus polymyxa* detected by temperature gradient gel electrophoresis. J Bacteriol 1996;178:5636–5643
26. Wang Y, Zhang Z, Ramanan N: The actinomycete *Thermobispora bispora* contains two distinct types of transcriptionally active 16S rRNA genes. J Bacteriol 1997;179:3270–3276
27. Ueda K, Seki T, Kudo T, Yoshida T, Kataoka M: Two distinct mechanisms cause heterogeneity of 16S rRNA. J Bacteriol 1999;181:78–82
28. Ninet B, Monod M, Emler S, et al.: Two different 16S rRNA genes in a mycobacterial strain. J Clin Microbiol 1996;34:2531–2536
29. Reischl U, Feldmann K, Naumann L, et al.: 16S rRNA sequence diversity in *Mycobacterium celatum* strains caused by presence of two different copies of 16S rRNA gene. J Clin Microbiol 1998;36:1761–1764
30. Kirschner P, Springer B, Vogel U, et al.: Genotypic identification of mycobacteria by nucleic acid sequence determination: Report of a 2-year experience in a clinical laboratory. J Clin Microbiol 1993;31:2882–2889
31. Springer B, Stockman L, Teschner K, Roberts GD, Bottger EC: Two-laboratory collaborative study on identification of mycobacteria: Molecular versus phenotypic methods. J Clin Microbiol 1996;34:296–303
32. Tang YW, Von Graevenitz A, Waddington MG, et al.: Identification of coryneform bacterial isolates by ribosomal DNA sequence analysis. J Clin Microbiol 2000;38:1676–1678
33. Patel JB, Clarridge J, Schuster MS, Waddington M, Osborne J, Nachamkin I: Bacteremia caused by a novel isolate resembling *Leptotrichia* species in a neutropenic patient. J Clin Microbiol 1999;37:2064–2067
34. Tee W, Korman TM, Waters MJ, et al.: Three cases of *Anaerobiospirillum succiniciproducens* bacter-

emia confirmed by 16S rRNA gene sequencing. J Clin Microbiol 1998;36:1209–1213
35. Husmann M, Gries C, Jehnichen P, et al.: *Helicobacter* sp. strain Mainz isolated from an AIDS patient with septic arthritis: Case report and nonradioactive analysis of 16S rRNA sequence. J Clin Microbiol 1994;32:3037–3039
36. Logan JM, Orange GV, Maggs AF: Identification of the cause of a brain abscess by direct 16S ribosomal DNA sequencing. J Infect 1999;38:45–47
37. Han S, Schindel C, Genitsariotis R, Marker-Hermann E, Bhakdi S, Maeurer MJ: Identification of a unique *Helicobacter* species by 16S rRNA gene analysis in an abdominal abscess from a patient with X-linked hypogammaglobulinemia. J Clin Microbiol 2000;38:2740–2742
38. Clarridge JE, Zhang Q, Heward S: 16S rDNA sequence analysis as a real-time addition to the clinical microbiology laboratory. General Meeting of the American Society for Microbiology, Orlando, FL, May 20-24, 2001 (abstr)
39. Haas WH, Kirschner P, Ziesing S, Bremer HJ, Bottger EC: Cervical lymphadenitis in a child caused by a previously unknown mycobacterium. J Infect Dis 1993;167:237–240
40. Koukila-Kahkola P, Springer B, Bottger EC, Paulin L, Jantzen E, Katila ML: *Mycobacterium branderi* sp. nov., a new potential human pathogen. Int J Syst Bacteriol 1995;45:549–553
41. Springer B, Kirschner P, Rost-Meyer G, Schroder KH, Kroppenstedt RM, Bottger EC: *Mycobacterium interjectum*, a new species isolated from a patient with chronic lymphadenitis. J Clin Microbiol 1993;31:3083–3089 (erratum J Clin Microbiol 1994;32:1417)
42. Springer B, Tortoli E, Richter I, et al.: *Mycobacterium conspicuum* sp. nov., a new species isolated from patients with disseminated infections. J Clin Microbiol 1995;33:2805–2811
43. Springer B, Wu WK, Bodmer T, et al.: Isolation and characterization of a unique group of slowly growing mycobacteria: Description of *Mycobacterium lentiflavum* sp. nov. J Clin Microbiol 1996;34:1100–1107
44. Shojaei H, Goodfellow M, Magee JG, Freeman R, Gould FK, Brignall CG: *Mycobacterium novocastrense* sp. nov., a rapidly growing photochromogenic mycobacterium. Int J Syst Bacteriol 1997;47:1205–1207
45. Floyd MM, Guthertz LS, Silcox VA, et al.: Characterization of an SAV organism and proposal of *Mycobacterium triplex* sp. nov. J Clin Microbiol 1996;34:2963–2967
46. Haas WH, Butler WR, Kirschner P, et al.: A new agent of mycobacterial lymphadenitis in children: *Mycobacterium heidelbergense* sp. nov. J Clin Microbiol 1997;35:3203–3209
47. Tortoli E, Kroppenstedt RM, Bartoloni A, et al.: *Mycobacterium tusciae* sp. nov. Int J Syst Bacteriol 1999;49 Pt 4:1839–1844
48. Brown BA, Springer B, Steingrube VA, et al.: *Mycobacterium wolinskyi* sp. nov. and *Mycobacterium goodii* sp. nov., two new rapidly growing species related to *Mycobacterium smegmatis* and associated with human wound infections: A cooperative study from the International Working Group on Mycobacterial Taxonomy. Int J Syst Bacteriol 1999;49:1493–1511
49. Roth A, Reischl U, Schonfeld N, et al.: *Mycobacterium heckeshornense* sp. nov., a new pathogenic slowly growing *Mycobacterium* sp. causing cavitary lung disease in an immunocompetent patient. J Clin Microbiol 2000;38:4102–4107