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# Probe Directed at a Segment of *Rickettsia rickettsii* rRNA Amplified with Polymerase Chain Reaction

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**In an effort to explore a sensitive taxon-specific detection system for bacteria, we sequenced the 16S rRNA from two strains of *Rickettsia rickettsii*, five other rickettsiae, and *Coxiella burnetii* to find a probe site unique to *R. rickettsii*. We then synthesized a 16-mer that hybridized only to the rRNA of *R. rickettsii*. Using a primer complementary to a sequence found only in rickettsial rRNA, we then generated a cDNA. We amplified the probe site in a 180-base segment of the cDNA by using the cDNA primer and a second primer also unique to rickettsiae in a polymerase chain reaction. The segments of rRNA from each of the rickettsiae were amplified $10^6$- to $10^7$-fold, and the *R. rickettsii* probe hybridized only to the amplified segment from *R. rickettsii*. The rRNAs from *Staphylococcus aureus*, *C. burnetii*, and *Neisseria meningitidis* were not amplified and did not hybridize with the probe. The approach detailed below may prove clinically useful in the direct detection of pathogens that are difficult to cultivate.**

Several investigators have shown that taxon-specific oligonucleotide probes can be directed successfully at 16 or 18S rRNA (3–5, 15). The high copy number of rRNA makes it appealing from the standpoint of sensitivity. However, even when labeled with $^{32}P$ and directed at a relatively large organism, such as a clostridium, these probes can detect no fewer than $10^3$ cells (15). Oligonucleotide probes directed at rRNA are therefore not sensitive enough to detect small populations of bacteria. For instance, the numbers of bacteria typically found in blood during bacteremia could not be detected. To improve the sensitivity of rRNA probes, we have taken the approach of synthesizing a cDNA from bacterial rRNA and then using the polymerase chain reaction (PCR) (10) to amplify a segment containing a probe site. Because PCR is able to increase the amount of a nucleic acid sequence by a factor of $10^6$ or more, the procedure should make diagnostic probing highly sensitive.

We have initially directed this approach toward improving the detection of *Rickettsia rickettsii*. During the summer months, Rocky Mountain spotted fever is prevalent in several geographic areas of the United States (1). Because of the high mortality rate of the infection and its nonspecific manifestations, it is necessary in endemic areas to treat many febrile patients empirically in order not to miss treating the few who actually have Rocky Mountain spotted fever (17). Currently, diagnostic tests lack adequate sensitivity. Cultivation of the causative agent, *R. rickettsii*, presents a significant laboratory hazard, and at any rate the organism multiplies too slowly to be detected in time to affect treatment. Antibodies to *R. rickettsii* do not appear early enough to affect management, and the fluorescent-antibody test used to detect the organisms directly in tissues lacks adequate sensitivity (12).

In applying PCR to *R. rickettsii*, we amplified a segment of rickettsial rRNA generically by priming each end of the segment at sites containing sequences that were specific for rickettsial rRNA. The amplified segment was then hybridized to a probe specific for *R. rickettsii*.

## MATERIALS AND METHODS

**Bacterial strains.** *R. rickettsii* Morgan and Sheila Smith, *Rickettsia montana*, *Rickettsia conorii*, *Rickettsia typhi*, and *Rickettsia prowazekii* were grown in embryonated eggs and purified at the Rocky Mountain Laboratory as described previously (11, 13). *Coxiella burnetii*, Nine Mile strain, phase 1 (307GP/1TC/1EP, clone 7) was inoculated into 6-day-old embryonated White Leghorn chicken eggs (H and N Hatchery, Redmond, Wash.) and incubated at 38°C. The infected yolk sacs of viable embryos were harvested 8 days after inoculation. *C. burnetii* was purified from pooled frozen yolk sacs, as described by Williams et al. (14) and modified by Hendrix and Mallavia (7). The purified organisms were stored as a pellet at −80°C. *Staphylococcus aureus* (ATCC 25923) and *Neisseria meningitidis* (ATCC 13090) were obtained from the American Type Culture Collection, Rockville, Md.

**Extraction of rRNA.** rRNA was extracted from the rickettsiae and *C. burnetii* as described previously (15). *S. aureus* and *N. meningitidis* were extracted in a modified protocol as follows. Cells were grown overnight and then centrifuged at 2,000 × *g* at 4°C for 20 min, and the pellet was suspended in 0.5 ml of LETS buffer (100 mM LiCl–10 mM EDTA–10 mM Tris [pH 7.8]–1% sodium dodecyl sulfate). An equal volume of phenol-chloroform-isoamyl alcohol (50:48:2) and 1/20 of a volume of 25- to 50-μm-diameter glass beads were added. The mixture was agitated in a 1.5-ml Eppendorf tube on a minibeadbeater (Biospec Products) for 1 min at room temperature. The sample was centrifuged for 1 min at 10,000 rpm in an Eppendorf centrifuge, and then the aqueous phase was extracted again in phenol-chloroform-isoamyl alcohol. The aqueous phase was then transferred to a clean Eppendorf tube, and the nucleic acids were precipitated with 1/10 of a volume of 2 M LiCl and 2.5 volumes of ethanol for 15 min on ice. The tubes were centrifuged for 20 min at 15,000 rpm at 4°C in an Eppendorf centrifuge, and the

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TABLE 1. Alignment of rickettsial 16S rRNA sequences at the probe site for *R. rickettsii*

| Organism | Sequence[a] |
|---|---|
| *R. prowazekii* | CUGGGCUACaACUGAnG |
| *R. montana* | CUGGGCUgCaACUGACG |
| *R. rickettsii* Morgan | CUGGGCUACCACUGACG |
| *R. rickettsii* Sheila Smith | CUGGGCUACCACUGACG |
| *R. typhi* | CUGGGCUACaACUGACG |
| *R. conorii* | CUGGGCUACaACUGACG |
| *C. burnetii* | CUGGaCcAauACUGACa |

[a] Sites where sequences differ from that of *R. rickettsii* are indicated by lowercase letters.

pellet was washed with 95% ethanol. The pellet was then dried in vacuo and dissolved in 50 μl of $H_2O$. Nucleic acids were extracted from human fibroblasts after the cells were washed twice with 5 ml of phosphate-buffered saline and suspended in 3 ml of digestion buffer (100 mM NaCl, 10 mM Tris [pH 8.0], 25 mM EDTA, 0.5% sodium dodecyl sulfate). Proteinase K was added to a final concentration of 0.1 mg/ml. The slurry was pipetted through a 20-gauge needle four times, and the sheared lysate was extracted twice in phenol and then once in chloroform-isoamyl alcohol. Nucleic acids were precipitated with sodium acetate and ethanol and then suspended in $H_2O$.

**rRNA sequencing.** The sequencing protocol has been described previously (9, 15). The three oligonucleotide primers used are complementary to highly conserved regions of rRNA and are thus useful for sequencing 16S rRNA from any source.

**cDNA reaction.** Because *Taq* polymerase does not use RNA as a substrate, we generated a cDNA from the 16S rRNA template. The oligonucleotide primer used for the reverse transcriptase reaction (GAAACCGAAAGAGAATC TTCCGAT) was chosen because it was theoretically not highly involved in secondary structure. Also, a computer search of GenBank and the European Molecular Biology Laboratory (Heidelberg, Federal Republic of Germany) through the SEQ program of Intelligenetics showed it not to be complementary to any previously reported rRNA. This primer was complementary to all rickettsial rRNAs that we sequenced but not to rRNA from *C. burnetii*. Bacterial rRNA in 4 μl of distilled water (DW) was added to 10 μl of 2× reverse transcriptase buffer (100 mM Tris [pH 8.3]–20 mM $MgCl_2$–100 mM KCl) and 8 ng of primer. The mixture was brought to a volume of 20 μl, then heated to 65°C for 5 min, and slow-cooled to room temperature. A total of 5 U of reverse transcriptase (Seikagaku America, St. Petersburg, Fla.), 1 μl of 200 mM dithiothreitol, and 2.5 μl of a deoxynucleoside triphosphate mix containing each deoxynucleoside triphosphate at 10 mM were then added. The reaction mixture was incubated at room temperature for 2 min and then at 50°C for 30 min. A 1/10 volume of 2 M sodium acetate and 3 volumes of ethanol were added, and the nucleic acids were precipitated for 15 min on ice. The Eppendorf tubes were then centrifuged at 4°C in a microcentrifuge, and the supernatant was decanted.

**PCR.** The cDNA reaction products were suspended in 50 μl of DW. A 10-μl volume of PCR buffer (100 mM Tris [pH 8.3]–500 mM KCl–9 mM $MgCl_2$–0.1% gelatin) (Cetus Corp.), PCR primers to give a final concentration of 1 μM each, deoxynucleoside triphosphates to give a final concentration of 200 μM, and 2.5 U of *Taq* polymerase (Cetus) were added. DW was added to bring the volume to 100 μl. The mixture was placed in a thermal cycler (Coy Laboratory Products, Ann Arbor, Mich.), and the temperature was cycled to 94°C for 1 min 10 s, 43°C for 2 min 30 s, and then 72°C for 3 min. The cycle was repeated a total of 25 times. One of the primers used for this reaction was the same as the primer used for generation of the cDNA. The second primer (TCCTAGTGTAGAGGTGAAATTCTTA) was 180 bases downstream from the first primer and was complementary to the cDNA. Several criteria were used for the choice of priming sites. As mentioned above, the sequences used were conserved among rickettsiae. Further, a computer search showed that the primers were not within 4 bases of being complementary to rRNA from any other organisms for which sequences are found in GenBank or the European Molecular Biology Laboratory. The priming sites were as free of secondary structure as possible so that the rRNA molecule itself would not compete with the primer. This consideration may be especially important at the 3′ end of the primer, where polymerases bind. Because initial studies suggested that the shorter the segment, the more efficient the amplification (data not shown), we chose priming sites as close as possible to the probe site. We made both primers of lengths such that the calculated hybridization temperatures were as close as possible. We did this because of the theoretical concern that if the hybridization temperatures were too different, the primer with the higher hybridization

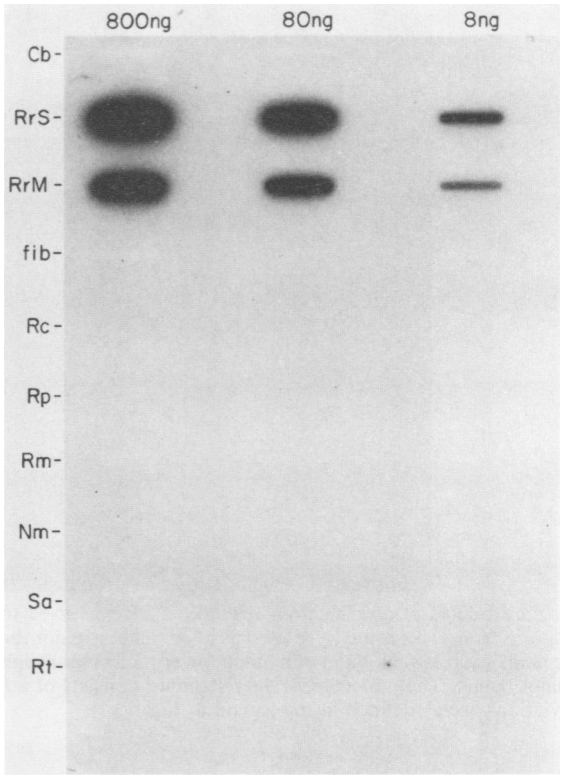


FIG. 1. Hybridization of an oligonucleotide probe complementary to 16S rRNA from *R. rickettsii* to nucleic acids extracted from *R. rickettsii*, four other rickettsiae, *C. burnetii*, *S. aureus*, and *N. meningitidis*. Various amounts of rRNA from each of these organisms were applied directly to a nylon membrane and hybridized to $^{32}P$-labeled probe. While detecting as little as 8 ng of rRNA from *R. rickettsii*, the probe did not hybridize detectably to rRNA from the other organisms. Cb, *C. burnetii*; RrS, *R. rickettsii* Sheila Smith; RrM, *R. rickettsii* Morgan; fib, human fibroblasts; Rc, *R. conorii*; Rp, *R. prowazekii*; Rm, *R. montana*; Nm, *N. meningitidis*; Sa, *S. aureus*; Rt, *R. typhi*.

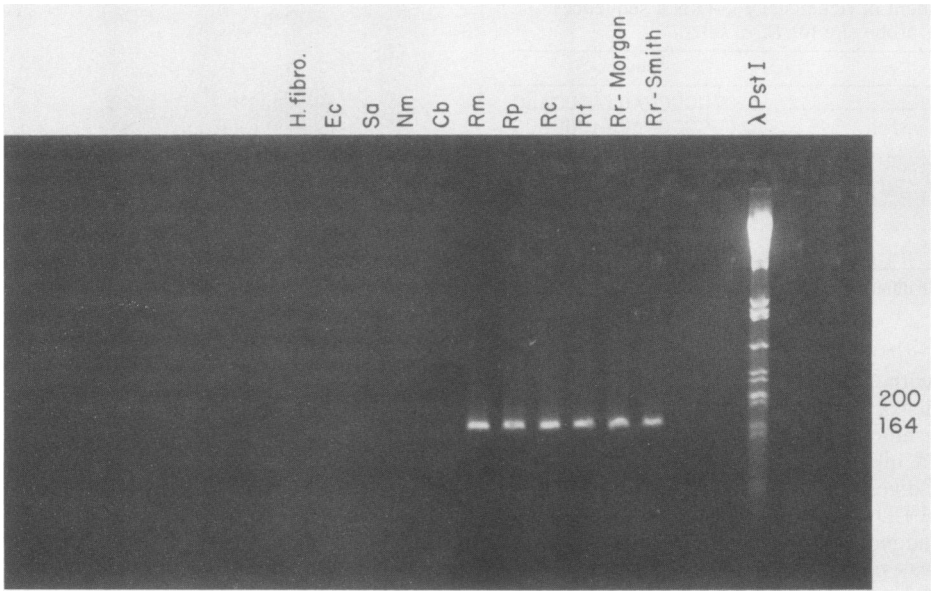


FIG. 2. Products of amplification reaction. PCR was used to amplify a 180-base segment of rickettsial 16S rRNA. When nucleic acid preparations from rickettsiae were used as a template, a major reaction product of the expected molecular weight was seen. Human fibroblast nucleic acids gave a weak band of a much lower molecular weight; *C. burnetii*, *Escherichia coli* (Ec), *S. aureus*, and *N. meningitidis* did not give major bands. The molecular weight standard consists of a *Pst*I digest of lambda phage; fragments 164 and 200 bases long are marked. Abbreviations are as defined in the legend to Fig. 1.

temperature might hybridize nonspecifically when the temperature was lowered to allow hybridization of the other primer. The primer with the higher hybridization temperature might then prime more or less randomly, resulting in amplification of unwanted segments.

Four microliters of the reaction products underwent electrophoresis on 3% Nusieve agarose gels containing 0.5 μg of ethidium bromide per ml, and bands were transferred to a nylon membrane (GeneScreen; Dupont, NEN Research Products, Boston, Mass.) in a Miniprotean 2 electrotransfer apparatus (Bio-Rad Laboratories, Richmond, Calif.). rRNA and the reaction products of the PCR were probed as described previously (15) with an oligonucleotide complementary only to the rRNA of *R. rickettsii*.

## RESULTS

**Sequence analysis.** The universal 16S rRNA primer C of Lane et al. (9) was less efficient than the other primers for sequencing reactions involving rickettsial 16S rRNA. The data, therefore, are based on two sequencing primers, A and B. Sequence analysis using a primer directed at a conserved segment near the 5′ end of the 16S rRNA molecule showed that rickettsial rRNA mismatched primer C. The sequence of primer C is ACGGGCGGTGTGTRC, where R = A or G (9). In rickettsiae, the complementary base at position 7 was T, causing a G-T mismatch. In all, there were 465 bases of common sequence that could be used for comparison of all organisms. Both strains of *R. rickettsii* gave the same rRNA sequence. With *R. rickettsii* as a reference, the sequence similarities were as follows: *R. montana*, 99.4%; *R. typhi*, 99.1%; *R. prowazekii*, 98.5%; *R. conorii*, 98.5%; and *C. burnetii*, 79%. At one site, the sequence of *R. rickettsii* differed from the sequence of *R. montana* by 2 bases, differed from the sequences of the other three rickettsiae by 1 base, and differed from the sequence of *C. burnetii* by 4 bases (Table 1). A computer search of GenBank and the European data base (European Molecular Biology Laboratory), using the Bionet computer system, showed that a 17-base segment of *R. rickettsii* rRNA containing this site was not within 3 bases of matching any previously reported rRNA sequence or sequence of rRNA from *C. burnetii*. An oligonucleotide probe complementary to this segment was synthesized (CGTCAGTGGTAGCCCAG).

**Probe for *R. rickettsii*.** Nucleic acid extracts from *R. rickettsii*, each of the other rickettsiae studied, *C. burnetii*, *S. aureus*, *N. meningitidis*, and human fibroblasts were applied to a nylon membrane in amounts ranging from 800 to 8 ng and probed with the *R. rickettsii* probe. The probe hybridized to 8 ng of *R. rickettsii* nucleic acid extract but not to 800 ng from any of the other organisms (Fig. 1).

**Amplification.** Amounts of nucleic acid extracts ranging down to 0.008 pg were amplified by first constructing a cDNA and then performing PCR. A 0.08-pg amount from each of the rickettsiae yielded a visible band consistent with a 180-base fragment in agarose gels stained with ethidium bromide (Fig. 2). Nucleic acid extracts from human fibroblasts, *S. aureus*, *C. burnetii*, and *N. meningitidis* did not give significant reaction products. Figure 3 shows the results of Southern blot hybridization with the $^{32}$P-labeled oligonucleotide probe complementary to *R. rickettsii* rRNA. While hybridizing to the 180-base segment amplified from *R. rickettsii* nucleic acids, the probe did not hybridize to PCR products from the other rickettsiae or human fibroblasts. DNase digestion of the nucleic acid extracts had little effect on the sensitivity of the method (Fig. 4). However, omitting the step in which a cDNA was generated markedly decreased the efficiency.

## DISCUSSION

Compared with culturing, the use of nucleotide probes for the detection of microbes has been insensitive. Even using a

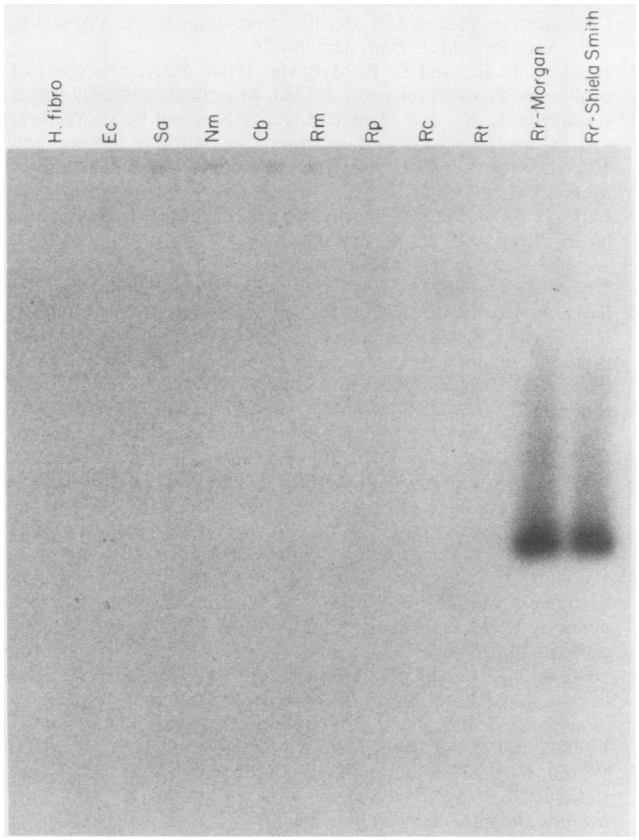


FIG. 3. Hybridization of the *R. rickettsii* probe to the reaction products of PCR. Bands on the gel shown in Fig. 2 were transferred to a nylon membrane and probed with the *R. rickettsii*-specific probe. The probe hybridized to the PCR products of only the two strains of *R. rickettsii*. Ec, *E. coli*; other abbreviations are as defined in the legend to Fig. 1.

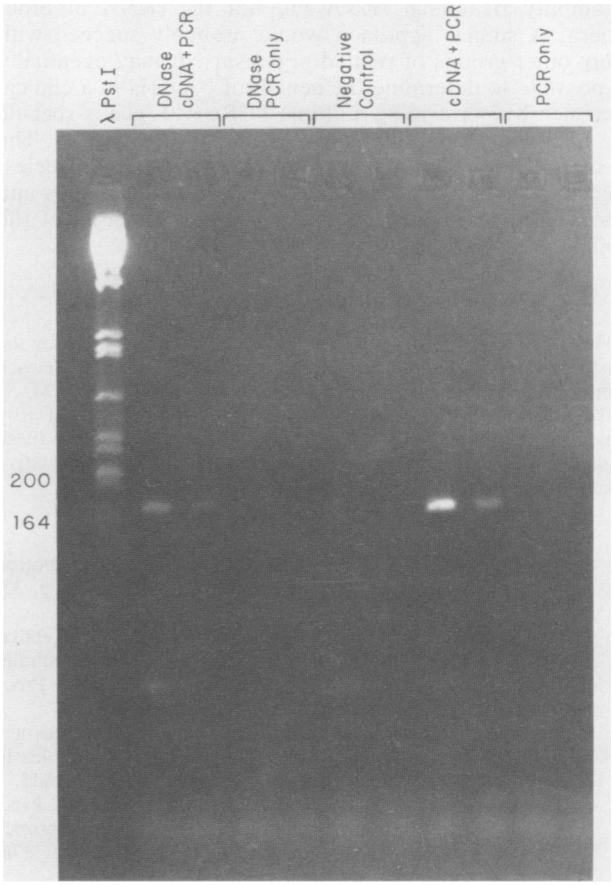


FIG. 4. Effect of the cDNA reaction on the sensitivity of PCR. Two amounts of nucleic acid extract, 8 pg (left lanes) and 0.8 pg (right lanes) were used under each condition shown. Results of the usual method of cDNA generation and PCR are shown (lanes cDNA + PCR). Bands were still visible when DNase digestion was followed by cDNA generation and PCR but were lost if the cDNA step was left out (lanes DNase PCR only and PCR only). Molecular lengths in bases are indicated at the left.

$^{32}$P-labeled probe directed at rRNA, which has a copy number of around 10,000 per cell, we were able to detect no fewer than $10^3$ *Clostridium difficile* cells (15) after exposing autoradiographs for 2 weeks. Given the large size and rapid growth rate of *Clostridium difficile* compared with *R. rickettsii*, the latter organism undoubtedly has far fewer ribosomes and would therefore be more difficult to detect. Assuming that all of the nucleic acid present in our extracts was 16S rRNA, we amplified around $10^{-18}$ mol of the target sequence over 100,000-fold. However, 23S rRNA, 5S rRNA, tRNA, mRNA, and genomic DNA were also in the extracts, so the amplification factor probably exceeded $10^6$-fold. This degree of amplification should be adequate to allow detection of 1 to 10 rickettsial cells by using a nonradiolabeled probe. Even given the small numbers of organisms that are thought to be circulating in Rocky Mountain spotted fever (8), it should be feasible to detect the organisms in a 10-ml blood sample.

The *R. rickettsii* probe promises to be specific in the appropriate clinical setting. Diseases very similar to Rocky Mountain spotted fever are caused by other rickettsiae, *N. meningitidis*, *S. aureus*, and several viruses. Our data show that the bacterial pathogens that may mimic spotted fever are not detected by using this methodology. Because viruses do not have ribosomes, they would be unlikely to contain any of the three sequences required to give a positive result. Furthermore, it is likely that the probe site we chose for *R. rickettsii* is present in nearly all members of the species. Sequence analysis showed that, overall, 16S rRNA sequences of rickettsiae varied little, and in particular, a strain of *R. rickettsii* from Montana and one from North Carolina had identical sequences. Given the similarity of the rickettsial 16S rRNAs, probing with oligonucleotides is probably the best method for distinguishing among them. When hybridized under stringent conditions, oligonucleotide probes give excellent specificity, failing to hybridize to sequences that have only one base mismatching the probe (2, 15).

Besides a high copy number, rRNA has other qualities that make it especially useful for the approach described above. Its sequences vary in an orderly manner across phylogenetic lines so that closely related organisms have closely related rRNA sequences. This quality has made rRNA very useful in the study of molecular evolution and systematics (16) and means that taxa can be identified by their rRNA sequences. rRNA sequences do not appear to change over time at a constant rate at all locations in the molecule. Some sites are highly conserved, while others change more rapidly (6). Thus, it is possible to identify sequences that are specific for any taxonomic level. This fact made it possible to identify priming sites that could be used

to amplify rickettsial rRNA but not the rRNA of other genera. A similar approach would probably succeed with many other groups of related organisms. It may eventually be possible to determine the genera of bacteria in a clinical specimen by performing multiple PCRs with genus-specific sets of primers and looking for the amplified segments. The species of bacteria could then be determined with species-specific probes. Given improvements in biotechniques and automation, this approach could simplify the tasks of the clinical microbiologist.

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## LITERATURE CITED

1. **Centers for Disease Control.** 1988. Rocky Mountain spotted fever—United States, 1987. Morbid. Mortal. Weekly Rep. **37:** 388–389.
2. **Conner, B. J., A. A. Reyes, C. Morin, K. Itakura, R. L. Teplitz, and R. B. Wallace.** 1983. Detection of sickle cell beta-s-globulin allele by hybridization with synthetic oligonucleotides. Proc. Natl. Acad. Sci. USA **80:**278–282.
3. **Drake, T. A., J. A. Hindler, G. W. Berlin, and D. A. Bruckner.** 1987. Rapid identification of *Mycobacterium avium* complex in culture using DNA probes. J. Clin. Microbiol. **25:**1442–1445.
4. **Giovannoni, S. L., E. F. DeLong, G. J. Olsen, and N. R. Pace.** 1988. Phylogenetic group-specific oligodeoxynucleotide probes for identification of single microbial cells. J. Bacteriol. **170:** 720–726.
5. **Gobel, U., R. Maas, G. Havn, C. Vinge-Martins, and E. J. Stanbridge.** 1987. Synthetic oligonucleotide probes complementary to rRNA for group and species-specific detection of mycoplasmas. Isr. J. Med. Sci. **23:**742–746.
6. **Gutell, R. R., B. Weiser, C. R. Woese, and H. F. Noller.** 1985. Comparative anatomy of 16S-like ribosomal RNA. Prog. Nucleic Acid Res. Mol. Biol. **32:**155–216.
7. **Hendrix, L. R., and L. P. Mallavia.** 1984. Active transport of proline by *Coxiella burnetii*. J. Gen. Microbiol. **130:**2857–2863.
8. **Kaplowitz, L. G., J. V. Lange, J. J. Fischer, and D. M. Walker.** 1981. Correlation of rickettsial titers, circulating endotoxin, and clinical features in Rocky Mountain spotted fever. Arch. Intern. Med. **143:**1149–1151.
9. **Lane, D. J., B. Pace, G. J. Olsen, D. A. Stahl, M. L. Sogin, and N. R. Pace.** 1985. Rapid determination of 16S ribosomal RNA sequences for phylogenetic analysis. Proc. Natl. Acad. Sci. USA **82:**6955–6959.
10. **Saiki, R. K., S. Scharf, F. Faloona, K. B. Mullis, G. T. Horn, H. A. Erlich, and N. Arnheim.** 1986. Enzymatic amplification of β-globin genomic sequence and restriction site analysis for diagnosis of sickle-cell anemia. Science **230:**1350–1354.
11. **Stonner, H. G., D. B. Lackman, and E. J. Bell.** 1962. Factors affecting the growth of rickettsiae of the spotted fever group in fertile hen's eggs. J. Infect. Dis. **110:**121–128.
12. **Walker, D. H., B. G. Cain, and P. M. Olmstead.** 1978. Laboratory diagnosis of Rocky Mountain spotted fever by immunofluorescent demonstration of *Rickettsia rickettsii* in cutaneous lesions. Am. J. Clin. Pathol. **69:**619–623.
13. **Weiss, E., J. C. Coolbaugh, and J. C. Williams.** 1975. Separation of viable *Rickettsia typhi* from yolk sac and L-cell host components by Renografin density gradient centrifugation. Appl. Microbiol. **30:**456–463.
14. **Williams, J. C., M. G. Peacock, and T. F. McCaul.** 1981. Immunological and biological characterization of *Coxiella burnetii* phase I and phase II, separated from host components. Infect. Immun. **32:**840–851.
15. **Wilson, K. H., R. Blitchington, B. Hindenach, and R. C. Greene.** 1988. Species-specific oligonucleotide probes for rRNA of *Clostridium difficile* and related species. J. Clin. Microbiol. **26:** 2484–2488.
16. **Woese, C. R.** 1987. Bacterial evolution. Microbiol. Rev. **51:** 221–271.
17. **Woodward, T. E.** 1984. Rocky Mountain spotted fever: epidemiological and early clinical signs are keys to treatment and reduced mortality. J. Infect. Dis. **150:**465–468.