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NOTES

DNA Probes for Detection and Identification of *Mycoplasma pneumoniae* and *Mycoplasma genitalium*

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DNA probes specific for *Mycoplasma pneumoniae* and *Mycoplasma genitalium* were selected from genomic libraries prepared in pUC13. The ^{32}P-labeled probes could detect, by dot blot hybridization, down to about 0.1 ng of the specific mycoplasma DNA or 10^5 CFU. Biotinylation of probe decreased the sensitivity of detection and produced nonspecific background reactions with nonhomologous DNAs. Sulfonation of probe yielded a similar level of sensitivity with less background.

Present techniques for laboratory diagnosis of *Mycoplasma pneumoniae* infections are of little use to the clinician. Primary isolation of the organism from clinical specimens may take weeks (12). Serological tests become meaningful only on demonstration of a rise in antibody titer, requiring the testing of at least two serum samples, again causing a delay in delivery of laboratory results to the physician. Furthermore, routine serological methods for the diagnosis of *M. pneumoniae* infection suffer from a significant degree of variability and nonspecificity (2). Recent studies also revealed that *M. pneumoniae* shares antigenic determinants of protein and lipid nature with *Mycoplasma genitalium*, a newly discovered mycoplasma isolated from cases of nongonococcal urethritis in humans (3, 4, 6). The discovery of *M. genitalium* has raised another diagnostic problem, even more difficult than that encountered in *M. pneumoniae* infections. *M. genitalium* grows extremely slowly on primary isolation, and, in fact, very few strains have been cultivated in vitro. Consequently, information on the occurrence and possible pathogenic role of this mycoplasma in humans is very scarce (11). Serological cross-reactivity between *M. genitalium* and *M. pneumoniae* must be taken into account in the evaluation of serological data, because a rise in antibody titer to *M. genitalium* could be the result of concurrent respiratory infection by *M. pneumoniae*.

The development of DNA probes specific to *M. pneumoniae* and *M. genitalium* can be expected to provide a solution to the diagnostic problems by overcoming the need for cultivating the agents and the difficulties resulting from the serological cross-reactions. DNA probes should enable rapid detection of the pathogen directly within the clinical specimen in sufficient time to have an impact upon the care and treatment of the patient. To apply the simple and fast dot blot DNA hybridization technique, probes containing DNA sequences specific for each of the mycoplasmas are needed. This report deals with the selection from genomic libraries of DNA probes for *M. pneumoniae* and *M. genitalium* suitable for detecting small numbers of the organisms by dot blot hybridization.

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The human mycoplasma strains used in this study included *M. pneumoniae* FH and eight other *M. pneumoniae* strains isolated from pneumonia patients in the Seattle, Wash., area during 1964 to 1974, *M. genitalium* M30 and G-37, *Mycoplasma salivarium* PG20, *Mycoplasma orale* CH19299, *Mycoplasma buccale* CH20247, *Mycoplasma hominis* PG-21, PG25, DC63, and Bottle, *Mycoplasma fermentans* PG18, and *Ureaplasma urealyticum* serotypes 7 and 8. *M. genitalium* and *M. pneumoniae* were grown in a modified SP-4 medium (15). *U. urealyticum* was grown in a urea-containing medium, and all other mycoplasmas were cultivated in a modified Edward medium (9). Mycoplasmal DNA was extracted by the method of Marmur (8).

Total DNA of *M. pneumoniae* FH and of *M. genitalium* M30 was digested to completion with *Hin*dIII (New England BioLabs, Inc., Beverly, Mass.). The resulting fragments were separated on a 0.7% low-gelling-temperature agarose gel (Sigma Chemical Co., St. Louis, Mo.). DNA fragments in the 4- to 10-kilobase range were isolated from the gel and ligated into the unique *Hin*dIII site of the pUC13 plasmid multilinker (14). The resulting clones were digested with *Hin*dIII and tested in Southern hybridization (10) to total *M. genitalium* DNA labeled radioactively by nick translation (7). Southern hybridizations were performed under nonstringent conditions at 56°C in 6 × SSC (1 × SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.01 M EDTA–5 × Denhardt solution–0.5% sodium dodecyl sulfate (SDS)–100 μg of denatured salmon sperm DNA per ml (7). After hybridization, the filters were washed at 56°C in 2 × SSC–0.1% SDS, followed by 0.5 × SSC–0.1% SDS and then 0.2 × SSC–0.1% SDS. This procedure allowed the selection of clones containing DNA fragments specific for *M. pneumoniae* from the *M. pneumoniae* library and *M. genitalium*-specific clones from its library. The autoradiographs showed that 2 of the 40 *M. pneumoniae* clones tested hybridized strongly with the *M. genitalium* probe. Five of the remaining clones gave a weak hybridization signal with this probe. Clones that showed no hybridization at all, even upon extended exposure of the autoradiogram, were chosen for further testing as species-specific *M. pneumoniae* probes.

The *M. genitalium* DNA library, consisting of about 1,500 colonies, was screened by in situ colony hybridization (7) for

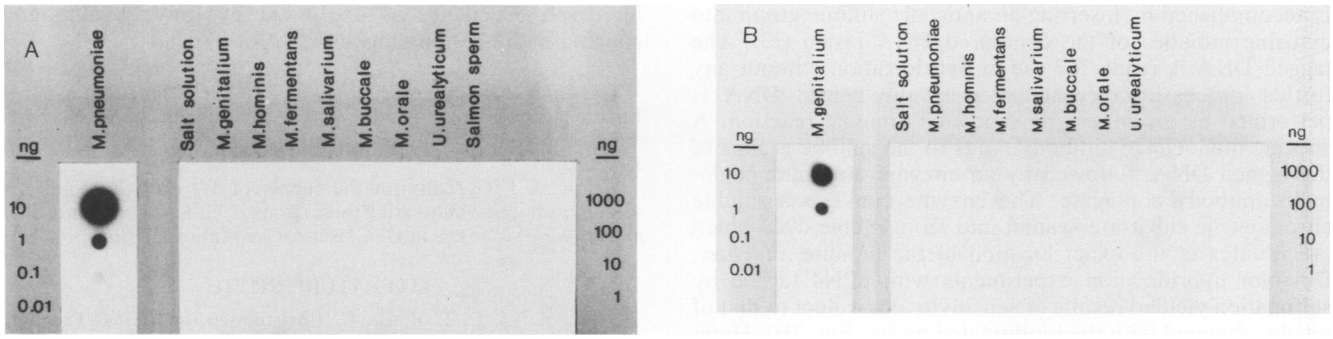

FIG. 1. Dot blot hybridization of DNA of human mycoplasmas with (A) ^{32}P-labeled *M. pneumoniae* DNA probe (pPN4); the *M. pneumoniae* target DNA was from strain FH; and (B) ^{32}P-labeled *M. genitalium* DNA probe (pGN3). The *M. genitalium* target DNA was from strain M30. Note that the initial concentration of the nonhomologous DNAs tested was 100-fold higher than that of the homologous DNA.

M. genitalium DNA inserts hybridizing with nick-translated total *M. pneumoniae* DNA. Of the 160 clones which reacted positively, 9 produced a strong hybridization signal. Some of the clones were also subjected to Southern blot hybridization to nick-translated total *M. pneumoniae* DNA. One of the clones which did not hybridize with *M. pneumoniae* DNA, designated pGN3, was selected for further testing as a specific *M. genitalium* probe. Our finding that about 15% of the *M. pneumoniae* genomic library clones yielded a positive hybridization signal with *M. genitalium* DNA, and vice versa, is in accord with our previous data. Southern blot hybridization of digested total DNA of the pair *M. pneumoniae-M. genitalium*, in which one served as the probe and the other served as the target, revealed a significant number of hybridization bands additional to those containing rRNA gene sequences, identifiable by specific rRNA gene probes (15).

Clone pGN3 of *M. genitalium* and a clone of *M. pneumoniae* (designated pPN4) were tested for specificity by dot blot hybridization against purified DNAs of seven different human *Mycoplasma* and *Ureaplasma* species. A mixture of DNA, 8% formaldehyde, and 7 × SSC was heated at 60°C for 10 min to denature the DNA. A total of 1.7 volumes of 11 × SSC was then added, and aliquots were deposited under vacuum on a presoaked (in 20 × SSC) nitrocellulose filter placed in a dot blot apparatus (Bethesda Research Laboratories, Inc., Gaithersburg, Md.). The filter was baked for 2 h at 80°C under vacuum. Dot blot prehybridization and hybridizations were performed at 42°C in 50% formamide–5 × SSC–1 × Denhardt solution–0.05 M sodium phosphate (pH 7.0)–0.001 M EDTA–0.1% SDS–50 μg of denatured salmon sperm DNA per ml. After hybridization, the filters were washed first in 2 × SSC, then in 0.1 × SSC–0.1% SDS for 1 h at 50°C, and again in this solution at room temperature.

^{32}P-labeled pPN4 hybridized to *M. pneumoniae* DNA only, whereas pGN3 hybridized to *M. genitalium* DNA only (Fig. 1). The *M. pneumoniae* probe produced a positive, although weak, hybridization signal with 0.1 ng of the specific homologous DNA and no or a barely visible hybridization reaction with 1,000 ng of the nonhomologous DNAs of the other mycoplasmas (Fig. 1). The probe reacted at the same level of specificity and sensitivity with DNAs of the eight *M. pneumoniae* strains isolated from patients.

Two nonradioactive methods of labeling DNA probes were used in this study, biotinylation (5) and sulfonation (13). We tested several biotinylation methods developed by ENZO Biochem, Inc., New York, N.Y. Of these, the Biobridge labeling system appeared to be the most sensitive. The biotinylation system enabled detection of as little as 1 ng of *M. pneumoniae* DNA (Fig. 2). The reaction of the probe with DNAs of the other mycoplasmas produced weakly colored dots of about the same intensity as that obtained with dots containing salmon sperm DNA (data not shown). Another system tested for nonradioactive labeling and detection of the mycoplasmal DNA probe consisted of the Chemiprobe kit (Orgenics, Ltd., Yavne, Israel). The tagging

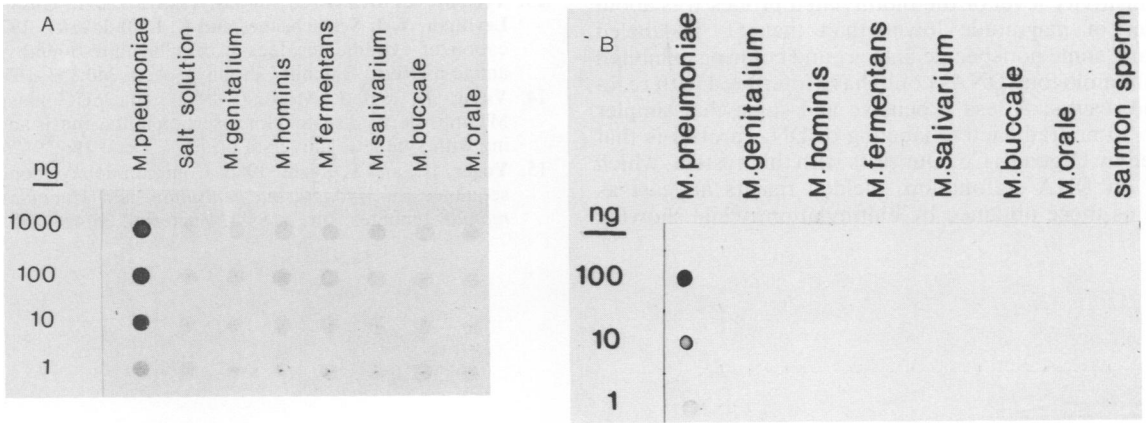

FIG. 2. Dot blot hybridization of DNA of human mycoplasmas with the *M. pneumoniae* DNA probe (pPN4) labeled and detected by the ENZO Biobridge labeling system (A) or the Orgenics Chemiprobe kit (B). The dot blot in panel B was prepared without any apparatus. For this blot, DNAs were mixed 1:1 with 10 × SSC, and 2 μl was placed on the dry filter. Note that the highest quantity of DNA in a dot was 100 ng.

is accomplished by inserting an antigenic sulfone group into cytosine moieties of the denatured DNA probe (13). The tagged DNA is ready for use in hybridization without any further processing. Visualization of the tagged DNA is performed by an enzymatic sandwich immune reaction. A specific monoclonal antibody binds to the sulfone groups of the tagged DNA, followed by an enzyme–anti-immunoglobulin antibody conjugate. The enzyme converts a soluble chromogenic substrate system into an insoluble dye, which precipitates at the exact location of the immune reaction. Dot blot hybridization experiments with pPN4 tagged by sulfonation yielded results of sensitivity equivalent to that of results obtained with the biotinylated probe (Fig. 2B). However, unlike in the biotinylation system, nonspecific background coloring of the heterologous DNAs was almost negligible when 100-ng DNA dots were tested. Sensitivity of detection by the sulfonated probe increased by about 10-fold (down to 0.1 ng of *M. pneumoniae* DNA) when the potency of the conjugate was improved by affinity chromatography (results not shown).

The clones selected in our study to serve as specific DNA probes for *M. pneumoniae* and *M. genitalium* yielded no or extremely weak hybridization signals in dot blots with nonhomologous DNAs at concentrations 1,000-fold higher than the minimal concentration of homologous DNA exhibiting a hybridization signal. The nonhomologous DNAs included those of other human mycoplasmas present in the respiratory or urogenital tract either as part of the normal flora or as pathogens. The *M. pneumoniae* pPN4 probe, carrying a 9-kilobase-pair fragment of the *M. pneumoniae* genome, produced a visible hybridization signal with 0.1 ng of *M. pneumoniae* DNA. Given that the *M. pneumoniae* genome is 480 megadaltons in size (1), 0.1 ng of *M. pneumoniae* DNA would be expected to be contained in about 10^5 organisms. In fact, our hybridization experiments with *M. pneumoniae* cells placed directly and lysed on the nitrocellulose filter appeared to confirm this estimate. By lowering the amount of the tested homologous DNA, sensitivity could be increased to some extent by ensuring excess of probe during hybridization. Sensitivity could also be increased by the addition of other *M. pneumoniae*-specific clones to pPN4, thereby increasing the segment of the *M. pneumoniae* genome recognized by the probe. Thus, a composite probe made of nine recombinant pUC13 plasmids containing different *M. penumoniae* genome inserts totaling 37 to 52 kilobase pairs increased the sensitivity of dot blot detection of *M. pneumoniae* DNA by two- to fivefold (results not shown).

The sensitivity level of the biotinylated probes was about one order of magnitude lower than that of ^{32}P-labeled probes, and some nonspecific background coloring exhibited with nonhomologous DNAs could have interfered with reading of the results. A less expensive and somewhat simpler approach to nonradioactive labeling of DNA probes is that developed by Orgenics Co. Our tests with this system, which is based on DNA sulfonation, yielded results at least as sensitive as those obtained by biotinylation, while showing increased specificity as expressed by lower background staining with nonhomologous DNAs.

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LITERATURE CITED

1. **Bak, A. L., F. T. Black, C. Christiansen, and E. A. Freundt.** 1969. Genome size of mycoplasmal DNA. Nature (London) **224**:1209–1210.
2. **Brunner, H., B. Prescott, H. Greenberg, W. D. James, R. L. Horswood, and R. M. Chanock.** 1977. Unexpectedly high frequency of antibody to *Mycoplasma pneumoniae* in human sera as measured by sensitive techniques. J. Infect. Dis. **135**:524–530.
3. **Clyde, W. A., Jr., and P.-C. Hu.** 1986. Antigenic determinants of the attachment protein of *Mycoplasma pneumoniae* shared by other pathogenic *Mycoplasma* species. Infect. Immun. **51**:690–692.
4. **Kenny, G. E., and F. D. Cartwright.** 1984. Immunoblotting for determination of the antigenic specificities of antibodies to the Mycoplasmatales. Isr. J. Med. Sci. **20**:908–911.
5. **Langer, P. R., A. A. Waldrop, and D. C. Ward.** 1981. Enzymatic synthesis of biotin-labeled polynucleotides: novel nucleic acid affinity probes. Proc. Natl. Acad. Sci. USA **78**:6633–6637.
6. **Lind, K., B. Ø. Lindhardt, H. J. Schütten, J. Blom, and C. Christiansen.** 1984. Serological cross-reactions between *Mycoplasma genitalium* and *Mycoplasma pneumoniae*. J. Clin. Microbiol. **20**:1036–1043.
7. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
8. **Marmur, J.** 1961. A procedure for the isolation of deoxyribonucleic acid from microorganisms. J. Mol. Biol. **3**:208–218.
9. **Razin, S., and S. Rottem.** 1976. Techniques for the manipulation of mycoplasma membranes, p. 3–26. *In* H. Maddy (ed.), Biochemical analysis of membranes. Chapman & Hall, Ltd., London.
10. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
11. **Taylor-Robinson, D., P. M. Furr, and N. F. Hanna.** 1985. Microbiological and serological study of nongonococcal urethritis with special reference to *Mycoplasma genitalium*. Genitourin. Med. **61**:319–324.
12. **Tully, J. G., D. L. Rose, R. F. Whitcomb, and R. P. Wenzel.** 1979. Enhanced isolation of *Mycoplasma pneumoniae* from throat washings with a newly modified culture medium. J. Infect. Dis. **139**:478–482.
13. **Verdlov, E. D., G. S. Monastyrskaya, L. I. Guskova, T. L. Levithan, V. I. Scheichenko, and E. I. Budowsky.** 1974. Modification of cytidine residues with a bisulfite-o-methylhydroxylamine mixture. Biochim. Biophys. Acta **340**:153–165.
14. **Viera, J., and J. Messing.** 1982. The pUC plasmids, and M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene **19**:259–268.
15. **Yogev, D., and S. Razin.** 1986. Common deoxyribonucleic acid sequences in *Mycoplasma genitalium* and *Mycoplasma pneumoniae* genomes. Int. J. Syst. Bacteriol. **36**:426–430.