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Rapid Detection of Smear-Negative *Mycobacterium tuberculosis* by PCR and Sequencing for Rifampin Resistance with DNA Extracted Directly from Slides

MADHUMITA PATNAIK,* KARSTEN LIEGMANN, AND JAMES B. PETER

Research Department, Specialty Laboratories, Inc., Santa Monica, California 90404

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Conventional methods for identification of *Mycobacterium tuberculosis* from culture can take 6 weeks. To facilitate the rapid detection of *M. tuberculosis* and to assess the risks of drug resistance, we developed a technique of eluting DNA directly from sputum slides and performing PCR for the detection of *M. tuberculosis* DNA, followed by sequencing the *rpoB* gene to detect rifampin resistance. This entire process requires only 48 h. Forty-seven sputum specimens submitted for microscopy for detection of acid-fast bacilli (AFB) and for mycobacterial culture and susceptibility testing were assessed after elution from the slides and extraction. *M. tuberculosis*-specific DNA was amplified in a nested PCR with previously described primers (primers rpo95-rpo293 and rpo105-rpo273), followed by analysis on a 4% agarose gel for a 168-bp product. Automated sequencing was performed, and the sequences were aligned against a database for detection of anomalies in the *rpoB* gene (codons 511 to 533) which indicate rifampin resistance. Of the 47 sputum specimens tested, 51% (24 of 47) were culture positive (time to positive culture, 2 to 6 weeks). Smears for AFB were positive for 58% (14 of 24) of the specimens and were negative for 42% (10 of 24) of the specimens. All 24 culture-positive sputum specimens (14 microscopy-positive and 10 microscopy-negative sputum specimens) were positive by PCR with eluates from the smears. Forty-nine percent (23 of 47) of the sputum specimens were negative for *M. tuberculosis* by smear, culture, and PCR. Of the isolates from the culture-positive samples, five were rifampin resistant by sequencing; all five were also rifampin resistant by in vitro susceptibility testing. Of these rifampin-resistant *M. tuberculosis* isolates, two were microscopy negative for AFB. Patients who are negative for AFB and culture positive for *M. tuberculosis* can now be identified within a day, allowing institution of therapy and reducing isolation time and medical costs.

In the last decade, tuberculosis has reemerged as one of the leading causes of death, killing nearly 3 million people annually. With an estimated 8.8 million new cases every year and the continued proliferation of drug resistance, the public health implications are immense (5, 14). Conventional methods for positive identification of *Mycobacterium tuberculosis* from culture can take up to 6 weeks, and it has been reported that tuberculosis can be transmitted from 17% of patients who are smear negative, culture positive for *M. tuberculosis* (1). To facilitate the rapid detection of *M. tuberculosis* we have developed a technique of eluting DNA directly from a sputum slide and performing a nested PCR amplification. Additionally, this PCR product (a section of the *rpoB* gene) may be sequenced to determine missense mutations, insertions, or deletions which correlate at >96% with rifampin resistance (13). This entire process requires only 48 h.

MATERIALS AND METHODS

Forty-seven sputum specimens were obtained from the Microbiology Department at Specialty Laboratories for which smear results for acid-fast bacilli (AFB) and culture results had been determined. The sputa were spread onto slides, dried on a 75°C hot plate, and stored at room temperature for a period of at least a month to assess the acceptability of room temperature transport and storage and the stability of DNA on slides. The slides were placed into 50-ml centrifuge tubes, and a proprietary solution was used to elute the DNA. The DNA was then processed by proteinase K digestion, followed by extraction with phenol-chloroform. The primers used were as follows: rpo95 (5′-CCA CCC AGG ACG TGG AGG CGA TCA CAC-3′) and rpo293 (5′-AGT GCG ACG GGT GCA CGT CGC GGA CCT-3′) for the primary PCR and rpo105 (5′-CGT GGA GGC GAT CAC ACC GCA GAC GT-3′) and rpo273 (5′-GAC CTC CAG CCC GGC ACG CTC ACG T-3′) for the secondary PCR. A nested PCR was performed with the following conditions: 94°C for 1 min, 72°C for 1 min (primary PCR) and 74°C for 1 min (secondary PCR), and 72°C for 1 min for a total of 35 cycles.

Amplified products were separated by agarose gel electrophoresis on a 4% agarose gel, visualized with ethidium bromide, and photographed. The remainder of the PCR product for those samples positive for *M. tuberculosis* were then purified and sequenced. Sequencing reactions were performed with the Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin-Elmer), and automated sequencing used a 4% acrylamide gel on the ABI 377 DNA Sequencer (Applied Biosystems). The generated sequences were aligned against the sequences in a database for detection of mutations, insertions, and deletions in the *rpoB* gene (codons 511 to 533) to indicate rifampin resistance (9, 15).

RESULTS

Of the 47 sputum specimens tested, 51% (24 of 47) were culture positive (time to positive culture, 2 to 6 weeks; median time, 14 days). Forty-nine percent (23 of 47) of the sputum specimens were negative for *M. tuberculosis* by smears for AFB and culture.

All 24 culture-positive sputum specimens (14 positive for AFB and 10 negative for AFB) were positive by PCR for *M. tuberculosis* DNA on eluates from the smears, and correspondingly, all 23 culture-negative sputum specimens (negative for AFB) were negative by PCR with eluates from the smear. Nested PCR shows a clinical sensitivity of 100%, whereas detection of AFB by microscopy has a sensitivity of only 58%.

* Corresponding author. Mailing address: Research Department, Specialty Laboratories, Inc. Santa Monica, CA 90404. Phone: (310) 828-6543, ext. 2207. Fax: (310) 828-5173. E-mail: mpatnaik@specialtylabs.com.

TABLE 1. Results for rifampin resistance testing for 24 culture-positive samples

Sample category	No. of samples with the following result by testing for AFB:		
	Positive	Negative	Total
Culture-positive sputa	14	10	24
Rifampin resistant by *rpoB* gene sequencing	3	2	5
Rifampin resistant by in vitro susceptibility testing	3	2	5

Rifampin resistance in culture-positive sputa. Of the isolates in the culture-positive samples, five were rifampin resistant as determined by *rpoB* gene sequencing; all five were also rifampin resistant as determined by in vitro susceptibility testing. Of these five isolates, two were negative for AFB by microscopy. (Table 1).

DISCUSSION

Slide microscopy for the detection of AFB is a convenient, rapid, and economical test used for determination of *M. tuberculosis* infection. However, recent studies have shown that up to half of the new cases of tuberculosis are smear negative (2). This leads to delays in the institution of therapy, reduced rates of patient isolation, and increased medical costs.

The elution of nucleic acids directly from slides identical to those used for microscopy for AFB permits transport and storage of the samples to be assayed at room temperature (as opposed to the transport and storage of sputum at freezing temperatures). Thus, it allows many of the same aspects of convenience and economy found in microscopy for AFB. However, conventional nucleic acid amplification assays for direct detection of *M. tuberculosis* in smear-negative, culture-positive samples show low sensitivity (less than 60%) and less than optimal specificity (2, 3, 4, 10, 11, 12, 16, 17). The application of improved amplification techniques (in this case, nested PCR) allows greatly enhanced detection of smear-negative sputum specimens (8). Furthermore, rifampin resistance is detected using the amplification product as a template for sequencing for mutations conferring resistance, which is of practical interest as rifampin resistance represents a surrogate marker for multiple-drug resistant (MDR) *M. tuberculosis* (6, 14). The rapid detection of tuberculosis and rifampin resistance can substantially reduce the cost of inappropriate isolation of patients with risk factors for MDR *M. tuberculosis* (6).

Dye et al. (7) have published an extensive study on the global burden of tuberculosis and have shown that 80% of all incident tuberculosis cases are found in 22 countries, with more than half in five Southeast Asian countries. The case detection rates may also be low due to the extra burden of smear-negative disease, even in sophisticated testing facilities. These errors in case detection may be reduced by using the method described above on slides for detection of AFB for the earlier detection of smear-negative cases. The method may also be used as an aid for determination of appropriate treatment strategies for MDR *M. tuberculosis* prior to confirmation by culture.

These results demonstrate the feasibility of using nested PCR and sequencing of nucleic acids eluted directly from slides to detect the presence of *M. tuberculosis* and to determine resistance to rifampin in a manner far more rapid than culture determination and with greater sensitivity than microscopy for AFB.

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