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RAPID DETECTION OF MYCOBACTERIA IN PATIENTS WITH HIV INFECTION

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RUBINA J. PATEL

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Division of Microbiology
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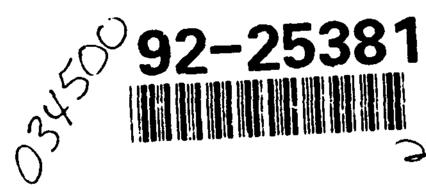

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Polymerase chain reaction (PCR) and DNA probes were used to detect and identify mycobacteria (potentially) present in patient specimens processed by the Mycobacteriology laboratory at Walter Reed Army Medical Center (WRAMC). Reagents for the assays were aliquoted into PCR tubes and stored at -70°C. When required, the tubes were thawed on ice. *Taq* polymerase, primers, and 10 ul of the specimen (also thawed on ice) were added. Positive and negative controls were included with each set of reactions. After 30 to 35 cycles of amplification in a Perkin-Elmer Thermal DNA Cycler (denaturation at 95°C for one minute, annealing at 55°C for two minutes, and extension at 72°C for three minutes), one-tenth of each reaction was run on an agarose gel, stained with ethidium bromide, and transferred by vacuum to a nylon membrane for hybridization with digoxigenin-labelled (non-radioctive) probe(s). Results were recorded on X-ray film by chemiluminescence. Of 76 patient samples obtained in 1989, 7 (9%) were positive by standard methods alone, whereas 13 (22%) were positive by PCR and DNA probes. The non-radioactive PCR/probe results were obtained in four days whereas standard methods took four to six weeks.

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X In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

Rubina Patel   30 June 1992

Rubina J. Patel, Ph.D.   Date

Principal Investigator

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INTRODUCTION

Nature of the Problem

Mycobacterium avium and M. intracellulare (also known as the M. avium complex or MAC) are the etiologic agents of the most common systemic bacterial infections in patients with the Acquired Immunodeficiency Syndrome (AIDS) (8, 13, 20, 30). Tuberculosis also remains a major health problem in urban areas in the U.S. as well as in developing countries. Although treatment for M. avium/M. intracellulare infections is usually disappointing (20, 30), effective chemotherapy is available for tuberculosis; however, efficient patient management and control of transmission (9) are compromised by inadequacies in current laboratory techniques resulting in a delay of six to eight weeks before a definitive diagnosis, i.e., demonstration and speciation of acid-fast bacilli (AFB) can be accomplished. The recent outbreak of drug-resistant tuberculosis makes the need for rapid identification even more acute.

Although relatively small numbers of bacilli may be detected by microscopy (32), mycobacterial species cannot be differentiated by morphology alone. Specimens containing acid-fast microorganisms must be cultured for differentiation by criteria such as growth rate, colony morphology, biochemical and drug susceptibility tests (35). Since mycobacteria, especially M. tuberculosis, are rather difficult to culture and have a generation time of 15 to 20 hours (36), biochemical identification can take up to eight weeks. Specific and rapid detection of M. tuberculosis and/or M. avium, directly from body fluids or tissue biopsies, would eliminate the lengthy and labor-intensive methods of standard mycobacteriology and allow implementation of appropriate treatment almost immediately.

Background of Previous Work

In recent years, several groups have proposed methods to shorten the time necessary for specific identification. These methods include a very rich medium (BACTEC), microscopy (1, 21) antigen and/or antibody assays (13, 27, 28, 34), differentiation at the DNA level using restriction fragment length polymorphism (RFLP's) (4, 5, 13, 14), hybridization with DNA probes, and amplification of target DNA (2, 3, 6, 7, 12, 13-15, 22, 25, 26, 29, 31, 33, 37, 38, 39). Although these approaches have shown promise, their routine application has been hampered by one or more of the following limitations: (i) radioactive detection requiring special safety and waste handling procedures, (ii) low specificity and/or sensitivity of the assay, (iii) necessity for culturing the organisms, and (iv) elaborate methods of extracting DNA/RNA. Patel and Fries (10, 11, 23, 24) described methods to circumvent these problems. Combining the polymerase chain reaction (PCR) and DNA probes, it was possible to detect and correctly identify mycobacteria from frozen stock cultures, log-phase liquid cultures, and patient specimens cultured on solid medium.

Purpose of the Present Work

The goals of this work were: (i) to verify the specificity and the sensitivity of the assay developed by Patel and Fries, and (ii) apply it to patient specimens, especially those from HIV positive individuals, for rapid identification of mycobacteria.

Methods of Approach

Small aliquots of processed patient specimens that had been used to inoculate various media were obtained from the Mycobacteriology laboratory at WRAMC. Taq polymerase, the appropriate primers, and 10 ul of the patient sample were added to core reagents aliquoted into PCR tubes. Amplification of mycobacterial DNA was carried out in a Perkin-Elmer Thermal DNA Cycler. One-tenth of each reaction was run on an agarose gel, stained with ethidium bromide, and transferred to a nylon membrane by vacuum. After hybridization with the digoxigenin-labelled probe, results were recorded on X-ray film by chemiluminescence.

BODY

Experimental Methods

Patient specimens. Small aliquots of "sediments" which had been used for smears and cultures at the Mycobacteriology Laboratory, Walter Reed Army Medical Center were obtained and stored at -70°C. and then processed with the genus-, M. avium- and M. tuberculosis- specific primers and probes.

Primers and probes. Primers detailed in Patel (23) and Fries (10) were synthesized in-house on an Applied Biosystems DNA synthesizer Model 380B. From published sequences of recombinant mycobacterial DNA, 30- and 50-base oligonucleotides were synthesized for use as probes.

Amplification of target DNA using polymerase chain reaction (PCR). Fifty ml of reaction mix (50 mM KCl, 10 mM Tris-HCl, pH 8.4, 1.5 mM $MgCl_2$, 0.01% gelatin, and 0.2 mM dATP, dCTP, dGTP, dTTP) were mixed thoroughly and pipetted into PCR tubes (Perkin-Elmer Cetus, Norwalk, CT) in 88-ul aliquots. The tubes were stored at -70°C until required. To the mixes thawed on ice were added 3.5 U Taq polymerase, primers to 1 mM, and 10 ul of the sediment. Negative controls consisted of tubes wherein one of the critical reagents was missing. C1 contained no template DNA, C2 no primers, and C3 no Taq polymerase. Positive controls C4 and C5 had 100 ng of purified DNA from the type strains of M. tuberculosis (ATCC 27294) or M. avium (ATCC 25291), respectively. The tubes were loaded on a Perkin-Elmer Cetus DNA Thermal Cycler for 25 to 35 cycles of denaturation at 94°C for 1 minute, annealing at 55°C for 2 minutes, and synthesis at 72°C for 3 minutes. Tubes were stored at 4°C, and as much mineral oil as possible was removed.

Analysis of amplification reactions. One-tenth of the reaction volume was run on 1.8% agarose gels in 1X Tris-Borate-EDTA (TBE) buffer (0.089 M Tris, 0.089 M boric acid, 0.002 M EDTA, pH 8.0) for 2.5 h at 100 V. After staining with 0.5 ug/ml ethidium bromide, PCR products were photographed with Polaroid 667 under ultraviolet light.

Southern transfer and hybridization. Initially DNA was transferred from agarose gels by capillary action overnight (24). In later experiments a Bio-Rad Model 785 vacuum blotter was used with a transfer time of 90 minutes (18). Oligonucleotide, synthesized probes were 3' end-labeled with digoxigenin-11-dUTP (Boehringer Mannheim Biochemicals) according to the manufacturer's protocols. HindIII- and HaeIII-digested lambda and phiX174 RF DNA, respectively, were labeled with digoxigenin-11-dUTP by random-priming. Hybridization was carried out as described by Laxer et al. (18).

Tissue samples. Eight blocks of paraffin-embedded tissue suspected of mycobacterial infection were submitted for analysis by PCR and DNA probes. Sources of the material were: mycobacterial synovitis of the wrist (two blocks), an AIDS patient with dual infection (two blocks), an armadillo and a dog.

Tissue processing. Each paraffin block was processed as described by Laxer et al. (17) with the following modifications. From each sample, six 6 uM-thick sections were cut on a microtome; two sections were placed in each of three 1.5 ul microcentrifuge tubes. Sections were deparaffinized in two changes of 500 ul xylene at room temperature and centrifuged at 12,000 x g for 5 minutes. The xylene was discarded and the sections were rinsed in absolute ethanol for one minute. Sections were rehydrated in 95% and 70% ethanol for one minute each, followed by centrifugation at 12,000 x g after each rinse. All supernate was removed, the samples were dried under vacuum, then resuspended in 240 ul of digestion buffer, 50 mM Tris-HCl, pH 8.0, 1 mM CaCl, 30 ul Proteinase K (20mg/ml) and incubated at 55°C for 2 hours. Following incubation, the sample was boiled for 10 mins to denature the Proteinase K and then cooled on ice. Ten ul of the sample were added to PCR tubes for amplification with the genus-, M. avium- and M. tuberculosis-specific primers as described above.

Results

Analysis of laboratory specimens. For any given set of reactions, no results were acceptable unless the negative control lanes were clear in both the gel and lumigram and the positive control lanes showed the characteristic 321-bp band with the genus-specific primers, 95-bp band with M. avium primers, and 400-bp with M. tuberculosis primers. Background on lumigrams (digoxigenin-labelled probes) had to be comparable with that of autoradiograms (radioactive probes).

Bands seen in the gel other than the target fragment were the result of non-specific amplification. They did not hybridize with the probes although two or three distinct bands are not unusual with the M. tuberculosis probe since it was derived from a repeated sequence. In sample lanes, even with the occurrence of non-specific amplification, either there was the characteristic band or the lane was clear. When positive signals were seen in any of the negative control lanes (usually the result of contamination with PCR product) all reagents were discarded and the reactions were repeated. Whereas results of culture previously were obtained after six to eight weeks, results of PCR/probe assays now were available within four days.

Results of the 85 patient specimens identified by microscopy, culture, and DNA probes are presented in Appendix 1. All specimens that were positive by microscopy were positive by culture; all specimens that were positive by culture were positive by PCR/probes. Three samples positive by microscopy and culture for M. gordonae (a common environmental contaminant) were negative by PCR/probes. Chi-square analysis (Figure 1) showed that 10 more samples were positive by PCR/probes than by culture alone -- an increase of 12%.

Tissue samples. Despite occurrence of non-specific amplification in some of the samples, no hybridization was seen if mycobacterial DNA was not present. Three of the samples (from the AIDS patient and the dog) were positive for mycobacterial disease, possibly M. avium. Results with the M. tuberculosis probe are unclear at this time.

PCR & Probes	Culture +	Culture −	
+	4	13	17
−	3	65	68
	7	78	85

FIG. 1. Comparison of mycobacterial identification by culture or PCR and DNA probes, n = 85.

CONCLUSIONS

Implications of Current Research

Thus far identification by PCR and DNA probes of mycobacteria in specimens from the Mycobacteriology Laboratory is similar, if not better than, microscopy and culture; however, many more samples have to be processed before any unequivocal conclusions may be made.

Regarding the three samples positive by microscopy and culture for M. gordonae (a common environmental contaminant) either there was contamination during preparation of the smears and inocula, or our assay did not have the requisite level of detection.

Results with the tissue samples are encourgaging but clearly the protocol has to be modified for optimal detection, especially in the case of small numbers of organisms.

By using both recombinant DNA technology and non-radioctive techniques, results are available within four days, an improvement over the six to eight weeks required by standard methodology.

In summary:

(1) It is possible to identify mycobacteria directly from patient specimens without having to culture the organisms.
(2) Samples may be stored for at least two years, but freeze-thawing is not recommended.
(3) No special safety measures for personnel or radioactive waste are required.
(4) Under optimal conditions, unequivocal results are available within four days.
(4) This approach may limit possible contamination with environmental mycobacteria.
(5) The procedure does not require prior training in molecular biology; however, a concentrated effort and attention to detail is essential.

Recommendations

The following modifications in current conditions are necessary to obtain optimal results:

(1) Two work areas, pre- and post-PCR, strongly recommended by Perkin Elmer-Cetus (patent holder and consultants for PCR technology) should be made and adhered to rather strictly to minimize or eliminate contamination with genomic DNA or PCR product.
(2) All equipment used for PCR should be serviced regularly by the authorized agent(s).
(2) Overall detection can be maximized by varying the concentration of reagents and polymerase, and modifying the annealing temperature.
(3) Improve detection of organisms in paraffin-embedded tissue by amending protocol for maximum exposure of mycobacterial DNA.

Since many *M. avium* or *M. intracellulare* infections are detected by blood culture, an assay reducing heme to <1% without excessive dilution of the sample would be extremely useful.

The lowest level of detection using digoxigenin-labelled probes should be determined by spiking patient specimens with known numbers of mycobacteria. The sensitivity should then be compared with that of radiolabelled (^{32}P or ^{35}S) probes.

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APPENDIX 1

SPECIMENS FROM MYCOBACTERIOLOGY LABORATORY
1989

WRAMC No.	Source	Smear	Culture	Genus	*M. av*	*M. tb*
1684	sputum	neg	neg	pos	neg	
1685	sputum	neg	neg	pos	neg	
1686	B A L*	neg	neg	pos	neg	
1687	urine	neg	neg	neg	neg	
1690	ascites fluid	neg	neg	pos	neg	
1693	biopsy	neg	neg	pos	neg	
1694	B A L	pos	*M. fortuitum*	pos	neg	
1695	B A L	pos	*M. fortuitum*	pos	neg	
1698	sputum	neg	neg	pos	neg	
1699	lung swab	pos	*M. tuberculosis*	pos	neg	
1700	biopsy	neg	neg	pos	neg	
1701	abscess	neg	neg	neg	neg	
1702	B A L	neg	neg	pos	neg	

*B A L = bronchial alveolar lavage

Appendix 1

WRAMC No.	Source	Smear	Culture	Genus	M. av	M. tb
1703	R max sinus	pos	M. tuberculosis M. gordonae	pos	neg	
1704	L max sinus	pos	M. gordonae conf. by Gen-Probe	neg	neg	
1709	urine	neg	neg	pos	neg	
1710	sputum (5 July)	neg	neg	pos	neg	
1711	sputum (6 July)	neg	neg	pos	neg	
1712	B A L	neg	neg	neg	neg	
1722	B A L	neg	neg	neg	neg	
1723	B A L	neg	neg	neg	neg	
1724	bronchial brush	neg	neg	neg	neg	
1725	sputum	neg	neg	neg	neg	
1729	foot	neg	neg	neg	neg	
1730	stool	neg	neg	neg	neg	
1734	urine	neg	neg	neg	neg	
1735	urine	neg	neg	neg	neg	
1736	B A L	neg	neg	neg	neg	
1737	sputum	neg	neg	neg	neg	

Appendix 1

WRAMC No.	Source	Smear	Culture	Genus	M. av	M. tb
1738	B A L	neg	neg	neg	neg	
1739	stool	neg	neg	neg	neg	
1743	urine	neg	neg	neg	neg	
1744	urine	neg	neg	neg	neg	
1746	sputum	neg	neg	neg	neg	
1749	sputum	neg	neg	neg	neg	
1750	sputum	pos	M A I* M. avium by Gen-P**	neg	neg	
1751	urine	neg	neg	neg	neg	
1752	skin lesion	neg	neg	neg	neg	
1753	L finger tissue	neg	neg	neg	neg	
1757	urine	neg	neg	neg	neg	
1758	sputum	neg	neg	neg	neg	
1759	finger tisuue	neg	neg	neg	neg	
1761	sputum	neg	neg	neg	neg	
1763	sputum	neg	neg	neg	neg	
1764	sputum	neg	neg	neg	neg	

Appendix 1

WRAMC No.	Source	Smear	Culture	Genus	*M. av*	*M. tb*
1765	sputum	neg	neg	neg	neg	
1766	sputum	neg	neg	neg	neg	
1767	urine	neg	neg	neg	neg	
1768	urine	neg	neg	neg	neg	
1769	urine	neg	neg	neg	neg	
1770	urine	neg	neg	neg	neg	
1773	pleural fluid	neg	neg	neg	neg	
1776	C S F*	neg	neg	neg	neg	
1781	sputum	neg	neg	neg	neg	
1782	sputum	neg	neg	neg	neg	
1783	sputum	neg	neg	neg	neg	
1784	sputum	neg	neg	neg	neg	
1785	stool	neg	neg	neg	neg	
1786	bone tissue	neg	neg	neg	neg	
1787	C S F	neg	neg	pos	pos	

*C S F = cerebrospinal fluid

Appendix 1

WRAMC No.	Source	Smear	Culture	Genus	M. av	M. tb
1789	sputum	neg	neg	neg	neg	
1803	paratracheal mass	neg	neg	neg	neg	
1804	B A L	neg	neg	neg	neg	
1805	sputum	neg	neg	neg	neg	
1806	sputum	neg	neg	neg	neg	
1807	sputum	neg	neg	neg	neg	
1808	sputum	neg	neg	neg	neg	
1809	sputum (10 July)	neg	neg	neg	neg	
1810	sputum (11 July)	neg	neg	neg	neg	
1811	sputum (12 July)	neg	neg	neg	neg	
1813	urine	neg	neg	neg	neg	
1814	peritoneal fluid	neg	neg	neg	neg	
1815	C S F	neg	neg	neg	neg	
1817	C S F	neg	neg	pos	pos	
1821	sputum	neg	neg	neg	neg	
1822	sputum	neg	neg	neg	neg	

Appendix 1

WRAMC No.	Source	Smear	Culture	Genus	*M. av*	*M. tb*
1823	sputum	neg	neg	neg	neg	
1824	sputum	neg	neg	neg	neg	
1825	sputum	neg	neg	neg	neg	
1832	urine	neg	neg	neg	neg	
1833	sputum	neg	neg	neg	neg	
1834	sputum	pos	*M. gordonae*	neg	neg	
1835	sputum	neg	neg	neg	neg	
1836	tissue	neg	neg	neg	neg	
1837	sputum	neg	neg	neg	neg	