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RAPID DETECTION OF *MYCOBACTERIUM TUBERCULOSIS* IN CLINICAL SPECIMENS BY POLYMERASE CHAIN REACTION

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ABSTRACT

We investigated the use of DNA amplification by polymerase chain reaction (PCR) for detection of *Mycobacterium tuberculosis* in 300 patients who were suspected of having pulmonary tuberculosis and compared the results with culture results which were performed in parallel with PCR. Two-thirds of each sample was processed for smear and culture by standard methods and one-third was prepared for DNA extraction, amplification and detection using *Mycobacterium tuberculosis* specific PCR primers. In this study 45 patients were positive for *M. tuberculosis* by PCR and probe hybridization (sensitivity and specificity 100%) whereas 42 patients (93%) exhibited growth of *M. tuberculosis*. Of 42 culture positive specimens 3 exhibited negative PCR results.

Smear positivity rate for PCR positive specimens was 73.2%. For analysis of discrepant results 3 variables such as the source of specimen, the concentration of bacteria in the original specimen and the presence of inhibitor were examined. It was found that only 3 sputum specimens (6.6%) gave discrepant results, which were found to contain inhibitor of amplification. It remains to be shown whether positive PCR results in smear and culture negative patients mean false positivity or an early laboratory finding which predicts a subsequent reactivation of a prior tuberculosis infection or whether asymptomatic patients may carry PCR amplifiable *Mycobacterium tuberculosis* DNA without any clinical relevance.

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INTRODUCTION

Tuberculosis is a disease of worldwide importance, particularly in third world countries. A recent report suggests that there are 20 million cases of active tuberculosis in the world and that around 5000 people die from tuberculosis every day.[6] In developed countries, increases are related not only to AIDS and homelessness, but also to the aging of the population.[3,14] Culture and subsequent identification of isolates is the traditional method of confirming the diagnosis of tuberculosis. However because the organism is slowly growing, laboratory diagnosis by conventional methods can take as long as 10 weeks.[4] Despite the acceptance of culture as the definitive tool for the diagnosis of tuberculosis, some microscopy positive specimens fail to yield *Mycobacteria* on culture.[4] This may be due to the harsh chemical treatment which is used to decontaminate specimens, contamination with other bacteria or to the presence of nonviable *Mycobacteria*. The most promising new approach to this problem

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is PCR.[15]

Several research groups have described different PCR systems and performed clinical studies based on PCR.[5,7, 10, 11, 18, 21, 27, 28, 29] The authors reported widely different results with respect to sensitivity and specificity of PCR. One reason for this may be methodological differences concerning sample preparation, another may be the design of amplification and detection procedures.[8] Different clinical parameters were used to evaluate PCR results in relation to the microbiological and clinical finding.

These methodological differences complicate the comparison of investigations and estimations of clinical value of PCR methods.[4] In the present study, we used a fast DNA preparation method as well as a non-radioactive highly specific and rapid amplicon detection method for comparison of PCR with conventional diagnostic methods for *Mycobacterium tuberculosis* using well characterized clinical specimens for patients who were assessed according to standard parameters for *M. tuberculosis*.[1]

MATERIAL AND METHODS

In a prospective study from September 2000 to August 2001, we investigated 300 patients who were suspected of having pulmonary *Mycobacterium tuberculosis* on the basis of history, pulmonary infiltrates on their chest X ray, tuberculin skin test, (0.1 to 100 TU) and computerized axial tomography scan. The clinical and paraclinical studies were done in Respiratory Disease and Microbiology Departments of Royal Victoria Hospital of Mcgill University of Montreal Canada. The type and percentage of specimens were as follows: 200 sputum (66.6%), 60 bronchial washings (20%) and 40 pleural fluid (13.3%). For patients whose sputum were used it was analysed 3 times. Clinical specimens were processed by standard mycobacterial laboratory method.[20, 23] Specimens were pretreated by 2.5% N- acetyl- L cysteine-NaOH and decontaminated by Kirchner solution [23] as appropriate (eg, sputum). Concentration of specimens was performed by centrifugation at 3000×g for 15 min. After resuspension of the sediment in 1.5mL of 0.67M phosphate buffer (pH:5.6), two-thirds of each sample was used for smear preparation (fluorochrome acid fast staining) and culture inoculation, and one-third was prepared for PCR test. Culture for mycobacterium was performed by inoculation of the processed material onto Lowenstein-Jenson and Middlebrook and onto one Bactec 12B Bottle and incubated for 6 weeks.

The quantity of growth observed was tabulated as follows: <1+ = growth in BACTEC bottle only or < 50 colonies on either slants, 1+ = 50 to 100 colonies, 2+ =100 to 200 colonies, 3+ = 200 to 500 colonies and 4+ = > 500 colonies. Identification of acid fast isolates was performed by standard procedures.[23]

Preparation of specimens for PCR test

Preparation of respiratory specimens for PCR was performed according to standard methods.[4, 24] We performed an alkaline DNA preparation method after pretreatment with 2.5% N- acetyl- L cysteine-NaOH solution by Cobas Amplicor MTB Kit (Roch Diagnostic System). The cell pellets were lysed with 100µL of 50mM NaOH for 15 minutes at 95°C under an oil overlay, then was neutralized with 1M Tris-HCl (8µl per 50µl of NaOH). Details of alkaline lysis are according to reference No.[24] In this research we tested three amplification systems. Two primer pairs produce MTB specific amplicons: a 158 (2.4 kb) DNA insert and a 240 bp amplification product.[4, 28] The third system amplifies a 383 bp fragment which evaluates the presence of MTB as well as mycobacteria other than MTB (MOTT) using a genus specific primer pair.[5] In a methodological pilot study we found no difficulty with respect to the sensitivity of detecting MTB DNA. For diagnostic PCR, 1µL of the prepared biological sample was used in a 50µL assay, 200µM of each of the deoxynucleoside triphosphates (d ATP, dCTP, dGTP and dTTP), 0.3µM of each single oligonucleotide primer and 1.25 IU of Taq polymerase (Perkin-Elmer Cetus, Norwalk, Conn.) were used. The mixture was subjected to 35 cycles of amplification and the temperature profile for denaturation and annealing was as follows: 50 seconds at 95°C, 40s at 60°C and 40s at 72°C. Mycobacterium internal control, a non-infectious synthetic DNA, containing identical primer binding sequences and a signal sequence distinct from that of the target DNA in Tris-HCl, served as an internal PCR amplification control (IC) for the detection of amplifiable DNA purified from each sample. For quality control each PCR run included one processed *M. tuberculosis* positive control and one processed *M. tuberculosis* negative control and the results were measured at the wavelength of 660nm. The possibility of PCR contamination was minimized by keeping the amplified products physically separated from starting materials. All pre- PCR handlings, such as aliquoting all reagents, packing tips and tubes, etc., were performed in one room. Setting up all PCRs was done in a second room, and another room located on a different floor was dedicated to the processing and analysis of amplification products. Equipment in these rooms (displacement pippetes, disposable tips, etc.) was used exclusively for PCR. For all reaction steps gloves were worn and changed frequently. Interpretation of results was as follows: For specimens with A660 < 0.35 and IC results >0.35 specimens were interpreted as negative for *M. tuberculosis,* the results of A660 <0.35 and IC <0.35 were interpreted as invalid and another ali-

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quot of the original specimen was processed. Specimens with A660 >0.35 were interpreted as positive for *M. tuberculosis* regardless of the IC results. About 40% of specimens in duplicate PCRs demonstrated a congruity of 100%. Because of the high level of comformity of PCR results about 60% of the samples were not tested further in duplicate. In this study a PCR was positive if MTB specific primer revealed positive results in the hybridization procedure. The specimens which were positive by culture but negative by PCR were evaluated as inhibitory. For evaluation of inhibition of PCR, all available specimens that were positive for growth of *M. tuberculosis* but negative by PCR, and a group of randomly selected AFB culture positive and negative reference test specimens were analyzed for inhibition of PCR. Several procedures could be used: (i) heat treatment at 95°C for 10 min prior to PCR, (ii) freeze-thawing twice in liquid nitrogen prior to PCR, (iii) pretreatment of the sample at 4°C, (iv) a 10-fold dilution with amplicor transport buffer or (v) a 10-fold dilution in amplicor transport buffer and subsequent heating of the sample at 95°C for 10 min. prior to PCR. In this study we performed a second PCR on the lysates specimens with an aliquot of a 10-fold dilution in Amplicor transport buffer.[34, 35, 25]

RESULTS

As shown in Table I, of 300 specimens under study 51 (17%) were smear positive, 78 (26.1%) were culture positive for acid fast bacilli, and 42 (14%) specimens exhibited growth of *M. tuberculosis*. 36 (46.1%) specimens were culture positive for mycobacteria other than *M. tuberculosis* (MOMT). Of 54 (27%) culture positive specimens 28 sputums (51.8%) were positive for *M. tuberculosis*. In this study of 300 specimens, 45 specimens (15%) were *M. tuberculosis* PCR positive (Table II).

As shown in Table III, of 45 *M. tuberculosis* PCR positive specimens 30 specimens (66.6%) were smear and culture positive, 3 specimens (6.6%) were smear positive, culture negative, 10 specimens (22.2%) were smear negative culture positive and 2 specimens (4.6%) were smear and culture negative for *M. tuberculosis*. Smear positivity rate for PCR positive specimens was 73.2%. Of 43 *M. tuberculosis* culture positive specimens, 3 specimens (sputum) were negative by PCR which were found to contain inhibitor of amplification. As sputum specimens are concerned, of 33 PCR positive sputums,

Table I. Species distribution and smear results for culture positive acid fast bacilli.

Species	Specimen sources			
	All		Sputum	
	Culture positive (%)	Smear positive (%)	Culture positive (%)	Smear positive (%)
M. tuberculosis	42 (14)	27 (52.4)	28 (51.8)	16 (44.4)
M. kansasii	13 (4.3)	9 (17.6)	9 (16.6)	7 (19.5)
M. avium-intracellulare	12 (4)	8 (15.6)	10 (18.5)	7 (19.5)
M. gordonae	7 (2.3)	4 (7.8)	3 (5.5)	4 (11.1)
M. fortuitum	4 (1.4)	3 (5.9)	4 (7.4)	2 (5.5)
Total	78 (26.1)	51 (17)	54 (27)	36 (16)

Table II. Detection of *M.tuberculosis* from 300 clinical specimens by culture and PCR.

PCR results	Culture results		
	Positive (%)	Negative (%)	Total (%)
Positive for *M. tuberculosis*	40 (13.3)	5 (1.7)	45 (15)
Negative for *M. tuberculosis*	2 (1)	253 (84)	255 (85)
Total	42 (14.3)	258 (100)	300 (100)

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Table III. Smear and culture results for *M. tuberculosis* PCR positive specimens.

Smear results	PCR positive specimens		
	Culture positive (%)	Culture negative (%)	Total (%)
Positive	30 (66.6)	3(6.6)	33 (73.2)
Negative	10 (22.2)	2 (4.6)	12 (26.8)
Total	40 (88.8)	5 (11.2)	45 (100)

Table IV. Comparison of growth intensity of *M. tuberculosis* using liquid and solid media with PCR results.

Growth intensity	No. of specimens with the indicated PCR results			
	PCR results for all specimens		PCR results for sputum	
	Positive (%)	Negative (%)	Positive (%)	Negative (%)
>1+	27 (60)	2 (66.6)	19 (57.5)	2 (66.6)
1 +	7 (15.5)	0 (0)	6 (18.1)	0 (0)
< +1	6 (13.3)	1 (33.4)	3 (9.9)	1(33.4)
No growth	5 (11.2)	0 (0)	5 (14.2)	0 (0)
Total	45 (100)	3 (100)	33 (100)	3 (100)

16 sputums (48.4%) were smear and culture positive for *M. tuberculosis* and 12 (36.3%) were culture positive, smear negative. For analysis of discrepant results (culture positive, PCR negative specimens), 3 interrelated variables were examined: The source of the specimen, the concentration of bacteria in the original specimen and the presence of inhibitor. In this study only sputum specimens yielded culture positive PCR negative results which contained inhibitor of amplification. The concentration of bacteria in the original specimen can be estimated by the density of growth. Table IV compares growth on original media with PCR results for specimens positive for *M. tuberculosis*. As Table IV indicates, 27 PCR positive specimens (60%) exhibited growth intensity of >1+ and 6 specimens (13.3%) exhibited growth intensity of < 1+ , 5 (11.1%) PCR positive specimens exhibited no growth in culture media of which 3 were smear positive. 2 of 3 culture positive PCR negative specimens which were sputum (Table IV) exhibited growth intensity of >1+ and one sputum exhibited growth intensity of <1+. All of these 3 sputums contained inhibitor. After retesting of 1/10 dilution aliquots of these specimens they exhibited positive PCR and positive probe hybridization results. For these patients bronchial wash and/or pleural fluid were tested by PCR and probe hybridization performed, which gave positive results too.

On examining the histories of these patients we could not find why their particular specimens (sputums) had inhibitors and why the other specimen taken from the same patients did not. All of these 3 patients had tuberculosis and were immunosuppressed (two HIV positive patients and one patient with myeloid metaplasia).

In this study , 2 of 5 PCR positive, culture negative specimens (40%) had negative smear (Table III), but only 10 of 46 (25%) PCR positive, culture positive specimens were smear negative. Of 5 patients with PCR positive but culture negative results, 2 patients had positive culture results 5 months after the first examination. One patient had had tuberculosis 20 years ago and had recently undergone renal transplantation. One patient was HIV seropositive and one patient who was a 70 years old man had a chronic pneumonia. In this study sensitivity and specificity of PCR was 100% but sensitivity of culture was 93.3%.

DISCUSSION

Although there have been numerous studies of the sensitivity and specificity of PCR for detecting *M. tuberculosis* in cultures or limited numbers of clinical specimens,[9] there have been few studies on its usefulness for large numbers of routine clinical specimens. The present study demonstrates that PCR is very helpful in diagnosis of tuberculosis because highly standardized protocol for DNA preparation, appropriate precautions for avoidance of contamination and a sensitive hybridization assay were used.

The PCR methodology reduces the time period for obtaining results from more than 3 weeks to one to two

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days in smear negative patients. The clinical sensitivity of the PCR found in our study was 100% which is in accordance with results of other groups which are between 74% to 100%.[5, 10, 11, 15, 17] Schluger et al.[32] assayed 65 inpatients with PCR. When correlated with cultures and clinical history, the sensitivity of PCR was 100%. However some studies reported a relatively low PCR sensitivity, e.g., Pierre et al.[21] and Soini et al.[30] found sensitivity for their PCR assays of 63% and 55.9% respectively. Such low sensitivity in PCR studies may be explained by suboptimal assay conditions. We compared PCR on clinical specimens with stain and culture methods and investigated the clinical significance of discrepant results. The sensitivity of concomitantly performed culture was 88.8%. In the evaluation of the PCR results, one should carefully analyze the stain and culture standards against which the test will be measured. Our rate of smear positivity of 73.3% for the specimens that exhibited positive PCR results for *M. tuberculosis* was higher than that reported by others.[12, 19] probably because of differences in technology (the fluorochrome stain after digestion and centrifugation, is more sensitive than the Ziehl-Neelsen stain).[9] However this rate is similar to that reported by Lipsky et al.[13] and Savic et al.[31] When culture is used as the standard technique in a comparison study, specimens containing nonculturable bacteria which may lead to a positive PCR are initially identified as false positive samples. In the absence of an ideal gold standard, it is not clear what proportion of the specimens with initial false positive results actually contains noncultivable bacteria. Jonas et al.[17] described 21 specimens which were PCR positive but culture negative. Forbes and Hicks[11] investigated two culture negative patients with positive PCR results, which is near to our results.

At present there is no adequate comparison to evaluate a new method for MTB diagnosis other than culture results or clinical assessment. Culture does not detect nonviable or nonculturable organisms and clinical assessment is not always reliable. Because of these results, the authors hypothesize that PCR assays not only can prove more sensitive than culture but can modify our present understanding of MTB infection. We detected MTB in samples from patients with a history of tuberculosis but no active disease. We know it is not always possible to determine the clinical relevance of a positive PCR result. The fact that PCR correctly detects MTB DNA in such situations is supported by observations about the presence of mycobacteria in patients with disease other than tuberculosis and the surveillance of PCR detectable MTB in non-active tuberculosis patients[27, 34] as well as the high percentage of MTB infected persons without relevant clinical signs.[2] In addition because of high sensitivity of PCR, it can detect tuberculosis in patients with severe bronchial disease. In this study of 3 patients who were missed by culture but detected by PCR, one had chronic pneumonia, one was HIV seropositive and one had renal transplantation.

One factor which influences PCR results (exhibits a false negative PCR) is the presence of inhibitor in specimens. In this study 3 specimens (sputums) were defined to have inhibitor of amplification reaction. We did not attempt to identify the nature of inhibitors but it has been suggested that substances such as heparin, hemoglobin, phenol and sodium dodecyl-sulfate may be potent inhibitors.[13, 16, 22]

At the end we can demonstrate that if a high grade of standardization (including DNA preparation procedures and contamination controls parallel to all PCR steps) is used, PCR is a fast and also a reliable method for the exclusion of tuberculosis and has a large impact on hospital costs. Nevertheless it remains to be shown whether positive PCR results in smear and culture negative patients means false positivity, an early laboratory finding which predicts a subsequent reactivation of a prior tuberculosis infection or whether asymptomatic patients may carry PCR amplification MTB without any clinical relevance.

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