Diagnosis of tuberculosis by DNA amplification in clinical practice evaluation

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Various polymerase chain reaction (PCR) assays have been devised for the rapid identification of mycobacteria in clinical specimens. To assess the value of such assays in routine laboratory work the results obtained by PCR were compared with those obtained by standard microbiological methods for 514 specimens collected for investigation of mycobacterial infection. Specimens were tested for the presence of *Mycobacterium tuberculosis* complex and atypical mycobacteria in two assays, one based on amplification of the 65 kDa gene and the other on the IS*6110* insertion sequence. For the 489 samples that did not contain inhibitors of the amplification reaction PCR findings correlated well with bacteriological and/or clinical data in 476 (97·4%). 6 PCR results turned out to be false negatives, 3 to be false positives and 4 to be mis-identification of strains. Pre-treatment of samples with guanidium thiocyanate reduced the proportion of false-negative results and of samples that contained inhibitors. This study confirms the potential of DNA amplification for early diagnosis of mycobacterial infections.

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Introduction

Isolation of mycobacteria from clinical samples takes several weeks for culture on solid medium, and 10–20 days by the 'Bactec' system (Becton Dickinson, Townson, MD, USA), which is based on the measurement of carbon dioxide released by bacteria during growth in liquid medium. We and others have reported that amplification of mycobacterial DNA by use of the polymerase chain reaction (PCR) technique permits rapid detection of mycobacteria directly in clinical samples. The various PCR assays used for identifying mycobacterial DNA include (i) amplification of the genes encoding mycobacterial antigens such as the 65 kDa protein,[1,2] MPB64 protein,[3,4] or antigen b;[5] (ii) amplification of repetitive sequences;[6-9] and (iii) amplification of ribosomal RNA.[10] To examine the feasibility of applying this technique to the routine diagnosis of mycobacterial infections in a clinical laboratory, we have done a large-scale survey with assays based on the amplification of the gene *groEL* coding for the 65 kDa antigen[1,11] or on amplification of the insertion sequence IS*6110*,[6,12] followed by hybridisation with specific DNA probes.

Materials and methods

Clinical specimens

Specimens were obtained from 318 patients with or without suspected tuberculosis who were inpatients at the Hôpital Pasteur, Hôpital Desgenettes (Lyon, France), or Hôpital Sainte-Anne (Toulon, France). Two or more samples were obtained from 97 patients.

Sample treatment

All specimens (buffy coat in the case of blood) were treated using sodium hydroxide lysis and phenol extraction as previously described.[1] Samples containing PCR inhibitors or giving false-negative results were further treated as follows before being re-tested with the IS*6110* assay: 50 to 100 µl of sample were lysed in guanidium thiocyanate and DNA was purified on silica particles as described by Boom et al.[13]

DNA amplification

Amplification by polymerase chain reaction[14] was done as already described,[6] with 10 µl of DNA extracted from the clinical sample. Thermal cycles were 1 min at 94°C, 1 min at 60°C, and 1 min at 72°C for 40 cycles. Controls with purified *M tuberculosis* DNA and without DNA were included in each experiment.

Labelling of probes

Oligonucleotide probes labelled at the 5′ end with horse-radish peroxidase (HRP) were provided by Sanofi Elf Bio-Recherches, Labège, France. Alternatively, oligonucleotides were radiolabelled at the 5′ by use of polynucleotide kinase (Boehringer Mannheim, Germany) and (γ^{32}P)-ATP (specific activity 3000 Ci/mmol, Amersham). DNA fragments were labelled by nick-translation with (α^{32}P)-dATP and (α-^{32}P)-dCTP (specific activity 3000 Ci/mmol, Amersham).

Hybridisation experiments

DNA was separated by electrophoresis on agarose gels and transferred to 'Hybond N' nylon filters (Amersham, Great Britain) by Southern blotting.[15] Hybridisation was done at 42°C for 3 h with 25 ng/ml HRP-labelled probe or with 2×10^5 cpm/ml ^{32}P-labelled probe. HRP was detected by chemiluminescence by use of enhanced chemiluminescence detection reagents (Amersham) according to manufacturers' recommendations, and membranes were exposed to radiographic films for 30 min. ^{32}P-labelled probes were detected by autoradiography for 18 h at −80°C with an intensifying screen.

Amplification assays

Each clinical specimen was amplified with three sets of primers. Primers TB1/TB2[1,11] and ISTB2/ISTB7[6] were specific for the *groEL* and IS*6110* sequences, respectively. As previously described,[6] pUC19 plasmid and TEMA/TEMB primers were used in control experiments to detect the presence of amplification inhibitors. All amplified products were electrophoresed on agarose

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TABLE I—NATURE OF SAMPLES TESTED

Samples	No of samples*	Samples	No of samples*
Gastric	107 (6·5%)	Bone marrow	6 (17%)
Sputum	93 (3·2%)	Serum	4
Bronchial washing	32	Biopsy†	20
Bronchial aspirate	50	Ascitic fluid	5
Pleural fluid	21	Pus	2
Urine	43 (4·6%)	Cystostomy	3
Cerebrospinal fluid	27	Miscellaneous	26
Blood	75 (16%)		

*Numbers in parentheses refer to % with inhibitors.
†Biopsy (eg, lymph node, lung, liver).

TABLE II—COMPARISON OF PCR WITH BACTERIOLOGICAL ANALYSIS

PCR results	Culture positive		Culture negative	Not cultured	Total
	TB complex	MOTT			
Positive (TB complex)	120	3	54	16	193
Positive (MOTT)	1	2	30	1	34
Negative	5	1	217	39	262
Presence of inhibitors	4	5	4	12	25
Total	130	11	305	68	514

MOTT mycobacteria other than tuberculosis

gels and hybridised with specific probes. For the TB1/TB2 assay, amplification products were detected either with an oligonucleotide probe specific for *M tuberculosis* (TB) complex or with a fragment probe allowing the detection of atypical mycobacteria. The use of ^{32}P- or HRP-labelled probes led to identical results. Samples were considered as positive for TB when repeatedly positive (ie, in two or three independent experiments) with either the IS*6110* and/or the *groEL* assays.

Results

Comparison of amplification results with bacteriological data

514 specimens (table I) were tested by PCR. Of these 446 were also sent for culture on Loewenstein-Jensen or Coletsos solid medium (table II). For the remaining 68 samples, bacteriological data were unavailable. 25 specimens contained inhibitors of the amplification reaction (9 were culture positive, 4 were culture negative, and 12 were not tested by culture). Of the 433 samples that were tested by culture and that did not contain inhibitors, 339 gave the same result whether tested by DNA amplification or culture. These specimens were either positive for TB complex (119 *M tuberculosis* and 1 *M africanum*), positive for atypical mycobacteria (*M xenopi* and *M terrae*), or negative (217 samples, including 30 samples from patients without any indication of tuberculous infection). Discrepancies were found in 94 specimens—84 were culture negative and PCR positive, 6 were culture positive and PCR negative, and 4 were culture positive but differently identified by PCR. Of the 56 samples not microbiologically tested and inhibitor-free, 17 were PCR positive and 39 PCR negative.

When samples containing inhibitors or otherwise giving false-negative results had been treated with guanidium thiocyanate/silica, positive results were obtained for the 6 samples that were culture-positive and PCR-negative with the sodium hydroxide/phenol protocol. Pretreatment with guanidium thiocyanate/silica also removed inhibitors of amplification (data not shown).

TABLE III—CORRELATION OF PCR FINDINGS WITH CLINICAL DATA

Certainty of diagnosis	Number of patients (n=68)	Number of samples (n=101)
Definite (isolation of mycobacteria in an other specimen)	26	36
Strong presumption		
Typical chest X-ray ± other samples PCR positive	20	26
Meningeal syndrome ± other samples PCR positive	3	3
Improvement under antituberculous treatment	3	4
Suspicion of tuberculosis		
Deterioration of general state of health ± other samples PCR positive	5	14
AIDS or HIV seropositivity	8	8
Other		
Bacteriological contamination	3	3
Lung cancer, stomach cancer, pneumothorax	3	3

TABLE IV—COMPARISON OF MICROBIOLOGICAL AND PCR METHODS AS DIAGNOSTIC TOOLS IN DETECTION OF MYCOBACTERIAL INFECTIONS

Microbiological diagnosis	PCR diagnosis	Number of patients (percentage) (n=318)
Negative	Negative	156 *(49%)*
Positive	Positive	108 *(34%)*
Negative	Positive	39 *(12·3%)*
Positive	Negative	2 *(0·6%)*
Negative	False positive	3 *(0·9%)*
..	Inhibitors	10 *(3·2%)*

Comparison of DNA amplification results with clinical criteria

101 specimens were positive by PCR analysis but culture negative (84 specimens) or not tested by culture (17 specimens). PCR-positive findings were examined in relation to clinical criteria for mycobacterial infections (table III). 36 of these samples came from 26 patients with bacteriological confirmation of *M tuberculosis* infection in other specimens. For another 26 samples (20 patients), tuberculosis was a very strong possibility because of the findings on chest X-ray, presence of meningeal syndrome with characteristic changes in the cerebrospinal fluid (lymphocytosis and hypoglycorrachia), or clinical improvement with antituberculous treatment. In some patients, several different samples were PCR positive, which strengthened the evidence for tuberculous infection. In other patients, various symptoms of mycobacterial infection were seen. For example, blood samples containing *M tuberculosis* complex (6 cases) or atypical mycobacteria (2 cases) as determined by PCR were obtained from 8 HIV seropositive individuals or AIDS patients in deteriorating state of health. Three positive PCR results were due to laboratory contamination by *M tuberculosis*. Three false-positive PCR results came from patients with cancers or pneumothorax.

Discussion

Our investigation of how well PCR works in the laboratory diagnosis of tuberculosis shows that the DNA amplification technique is more sensitive than bacteriological culture. Of the 489 specimens that were free of amplification inhibitors, 476 (97·3%) gave results that accorded well with bacteriological (339 samples) and/or with clinical data (36 positive samples from patients with diagnosed tuberculosis, 62 positive samples from patients with clinical features of tuberculosis, and 39 negative samples). Of specimens from patients with a definite diagnosis of tuberculosis, 80% were positive by DNA

amplification and only 67% by culture. In addition, 61 specimens from patients with strong evidence of tuberculosis were positive by DNA amplification only. Similar observations were reported by Pao et al, who examined 284 specimens with a PCR assay based on the *groEL* gene.[2]

A few discrepancies were observed. The false-negative results could have resulted from: (i) the presence of inhibitors not detected by the control amplification; (ii) non-homogeneous distribution of bacteria in the specimen so that the fraction tested does not contain mycobacteria; or (iii) low numbers of bacilli in the specimen (one false-negative sample resulted in growth of only four colonies), which decreases the probability of presence of mycobacteria in the fraction analysed by PCR. Thus to minimise the possibility of obtaining false-negative results, the principles that apply to culture for mycobacteria should also be followed for DNA amplification—ie, testing several specimens for each patient; choosing the most appropriate and good quality (eg, presence of human cells in sputum) specimens; and concentrating specimens before analysis (by centrifugation or classical decontamination). Two forms of misidentification occurred—3 samples were positive for TB complex but resulted in growth of atypical mycobacteria (perhaps because these strains masked the presence of *M tuberculosis*), whereas another sample hybridised only with the *Mycobacterium* probe but yielded *M tuberculosis* on culture (but the reason for this is not clear). False-positives were detected in patients having cancers or pneumothorax in the absence of classical clinical and biological indications of tuberculosis. However, only single test samples were obtained from these patients.

Inhibitors of the amplification reaction were detected in 5% samples, mostly blood, sputum, gastric fluid, and urines. They were not identified but could result from residual traces of haemoglobin, sodium dodecyl sulphate, or phenol, which are known to be potent inhibitors of the *Taq* polymerase. They might also be patient-associated since samples containing inhibitors were repeatedly obtained from two patients. An assessment of the ability of guanidium thiocyanate/silica to remove inhibitors will be published elsewhere. For some samples results with one amplification assay differed from that obtained with the other. Better sensitivity of the IS*6110* assay or the presence of inhibitors specific for one or the other system might be the explanation. The lesson is that two amplification assays should be done in parallel.

12% of mycobacteria-positive patients were identified by DNA amplification but not by microbiological methods (table IV). This high percentage may be attributed to the fact that our study includes a high proportion of positive patients (34%, table IV), which does not reflect the actual situation in most clinical laboratories. Among these PCR-positive/culture-negative patients, 67% of these patients were infected with TB complex, 33% with atypical mycobacteria (33%), and 20% also had HIV infection. Unless all the patients with atypical mycobacteria had clinical signs suggestive of a mycobacterial infection, identification of the atypical mycobacterial sequences that were amplified will permit us to determine whether these results have a real clinical significance or result from current mycobacterial contaminants such as *M gordonae*.

In conclusion, nucleic acid amplification and specific probe hybridisation is a reliable method for the early diagnosis of mycobacterial infections. This method would be especially useful in the diagnosis of tuberculosis meningitis, tuberculous pleurisy, and TB in HIV patients since infection is usually disseminated in these cases.

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From The Lancet

Hat reform

In [a recent] issue we took occasion to notice a proposal that a hat of particular make should be used as a distinguishing mark of medical practitioners. It now appears that the desire for peculiarity has not been limited to this country. As if by a side wind of suggestion, medical opinion in Berlin has, it is said, arrived at nearly the same point. There is a difference, however, and a material one. This time it is not the doctor, but his coachman, who is about to undergo alteration. In future a white hat will be for him like the fireman's helmet—a sign of urgency before which ordinary traffic will be expected to give way. The idea, it must be allowed, gives promise of some practical advantage, and many busy practitioners would be only too well pleased if by so simple a device they could scatter the obstructions which in town practice render the daily round a protracted and a devious task. By the sick whom they visit it should be hailed as an earnest of speedy relief. If, therefore, any suitable distinction commends itself to professional taste, no serious objection, we presume, could be urged against it. There would still remain the uncertainty whether the charioteers of other interests would be willing to admit the claim of medicine to precedence on the road. On the whole, therefore, we should prefer, before advising the adoption of any scheme of hat reform, to watch the progress of the new departure in the German capital.

(April 18, 1891)