Direct detection of *Mycobacterium tuberculosis* in respiratory samples from patients in Scandinavia by polymerase chain reaction

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Objective: To investigate the use of DNA amplification by the polymerase chain reaction (PCR) for the detection of *Mycobacterium tuberculosis* directly in human respiratory specimens.

Methods: The PCR assay employed was the Amplicor *M. tuberculosis* Test (Roche Diagnostics, Switzerland), which uses the 16S rDNA as the target template. Nine hundred and sixty samples from 741 patients in two clinical microbiology laboratories in Norway and Sweden were processed by routine culture analysis and PCR.

Results: Of the 56 specimens containing cultivatable *M. tuberculosis*, 49 (87.5%) were detected by PCR. Among the 904 culture-negative specimens, 897 samples were negative also by PCR and seven (0.8%) were positive by PCR. In comparison with culture, the sensitivity, specificity, and positive and negative predictive values of PCR were 91.7%, 99.6%, 94.2% and 99.4% for laboratory 1 and 80.0%, 98.7%, 76.2% and 99.0% for laboratory 2, respectively. For both laboratories combined the values were 87.5%, 99.2%, 87.5% and 99.2%.

Conclusions: These results indicate that multiple (two or three) respiratory samples from each patient should be tested, to allow sufficient accuracy in detecting *M. tuberculosis* in the specimens. Still, the labor-intensive format of this test necessitates strong clinical indications and patient prioritization to provide a service feasible within the current limits of routine laboratories.

Key words: Tuberculosis, polymerase chain reaction (PCR), 16S rDNA, rapid diagnostics

INTRODUCTION

There is clearly a demand for more rapid and reliable laboratory methods for the diagnosis of *Mycobacterium tuberculosis* infections for public health and therapeutic reasons [1]. The introduction of the radiometric BACTEC system represents a major improvement in the cultivation of mycobacteria by providing rapid

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detection and a high recovery rate of mycobacterial cultures [2,3]. Species-specific nucleic acid probes have significantly improved the opportunity for rapid confirmation of culture results for several mycobacterial species [4]. Still, days to weeks may be required for sufficient growth for identification. The use of the polymerase chain reaction (PCR) for species identification of mycobacteria, particularly *M. tuberculosis*, from early BACTEC cultures has been favorably explored [5,6].

Several groups have previously validated PCR assays for the identification of *M. tuberculosis* directly in clinical specimens [7–11]. Several nucleic acid targets have rendered sufficient sensitivity and representative species-specific differentiation, such as the 16S rRNA gene [12,13], IS elements [14–16], and the genes

encoding the 32-kDa and 65-kDa proteins [7,17]. Nucleic acid amplification techniques other than PCR, such as transcription-mediated amplification [14,18] and, more recently, strand displacement [19] and Q-beta-replicase probe amplification assays [20], are also being widely used.

We evaluated the Amplicor PCR assay (Roche, Switzerland) for detection of M. tuberculosis directly in respiratory specimens. The target DNA to be amplified was the mycobacterial 16S rDNA signature region [13,21]. The aim of our study was to establish whether this test was useful for direct detection of M. tuberculosis in respiratory samples in Scandinavian laboratories, one in Norway and one in Sweden.

MATERIALS AND METHODS

Material

All routine clinical specimens submitted for cultivation of mycobacteria were processed by standard procedures. The specimens in laboratory 1 were decontaminated by the N-acetyl-L-cysteine/NaOH method [22]. Two volumes of NALC/NaOH solution (2% NaOH, 1.45% sodium-citrate, 0.5% N-acetyl-L-cysteine) were mixed well with the specimen and allowed to digest for 15 to 30 min at room temperature. Ten volumes of 10 mM phosphate buffer (pH 6.8) were added for dilution, before centrifugation at 3000g for 15 min. In laboratory 2 the samples were decontaminated by the sodium lauryl sulfate method [23] and centrifuged at 3500g for 30 min. Sediments were resuspended in 3 to 5 ml of phosphate-buffered saline. Smears were prepared, stained according to Ziehl-Nielsen or with auramine, and examined for acid-fast bacilli (AFB) by microscopy. Five hundred and eighty-two sputum samples and 378 bronchioalveolar lavage (BAL) samples from 741 patients were processed. Five hundred and forty-four samples were investigated by laboratory 1 and 416 by laboratory 2.

Culture protocol

BACTEC Middlebrook 12B vials (Becton Dickinson Diagnostic Instruments, Sparks, Md, USA) [2,3] were inoculated with 0.5 mL of each specimen. The 12B vials were monitored by using the BACTEC 460 radiometric reader (Becton Dickinson Diagnostic Instruments) on a regular basis for 6 weeks. Once a 12B vial attained a growth index (GI) of $>=100$, the presence of AFB was confirmed by Ziehl-Nilsen staining. Laboratory 2 inoculated each sample on Lowenstein-Jenssen (LJ) medium. The PCR assay was incorporated into the laboratory routine without any change in practices, and specimens were processed 5 to 6 days/week.

Culture identification

Hybridization assays were performed directly on lysed AFB using commercially available nucleic acid probes for the M. tuberculosis complex and M. avium-intracellulare (MAC) (Accuprobe, Gen-Probe, San Diego, CA, USA) [4]. Mycobacterial species other than the M. tuberculosis complex and MAC were identified by conventional procedures.

PCR analysis

Sample preparation

One hundred microliters of the decontaminated sputum or BAL was added to 500 μL of Tris-HCl with 1% Triton X-100 and 0.05% sodium azide, mixed and centrifuged at 12,500g for 10 min. The supernate was carefully removed, and 100 μL of the lysis solution containing 1% Triton X-100, 0.4% sodium hydroxide and 0.05% sodium azide was added. The pellet was dissolved by vortex mixing and incubated at 60 °C for 45 min. After centrifugation, 100 μL Tris-HCl with 0.05% sodium azide was added. One positive control containing M. tuberculosis DNA and three negative buffer controls were included in each experiment for reference purposes.

PCR amplification reaction

Genus-specific primers KY18 and KY75 derived to correspond to a highly conserved region of the 16S rRNA gene (rDNA) of mycobacteria were used to amplify a 584-base-pair fragment (bases 15 to 598 of the M. tuberculosis 16S rDNA sequence, accession no. 52917 in Genbank). Fifty microliters of each sample were added to 50 μL of PCR reaction mixture (Amplicor, Roche, Basel, Switzerland) containing 10 mM Tris-HCl, 50 mM KCl, 2 mM MgCl₂, 200 μM concentrations of each deoxynucleotide triphosphate (dATP, dCTP, dGTP and dUTP), 0.001% (w/v) gelatin, uracil-N-glycosylase (UNG), biotinylated primers, and 0.5 U of Ampli-Taq polymerase (Perkin-Elmer Cetus, Norwalk, CT, USA). Each sample was first heated at 50 °C for 2 min, and then amplified in two cycles of 20 s at 98 °C, 20 s at 62 °C and 45 s at 72 °C, and then in 35 cycles of 20 s at 94 °C, 20 s at 62 °C and 45 s at 72 °C in a thermal cycler (Perkin Elmer Cetus TA9600). dUTP, instead of dTTP, was used as a substrate for UNG in order to prevent carryover of the amplified DNA [24]. Finally, samples were heated at 72 °C for 5 min until further processing to complete the initiated DNA polymerase activity and not allow UNG, which could have survived the extensive heating, to have an effect on dUTP-containing PCR products.

Hybridization reaction

After the PCR amplification process, the amplified products were chemically denatured and added to a microwell plate containing a bound, *M. tuberculosis*-specific oligonucleotide probe, KY172T3. This probe was selected from the hypervariable region of the 16S rRNA gene [3,21]. The biotin-labeled PCR products were then hybridized to the probe and thus 'captured'.

Detection reaction

After washing to remove unbound material, an avidin–horseradish peroxidase (Av-HRP) conjugate was added to the plate. After washing to remove the unbound conjugate, the bound Av-HRP was reacted with peroxide (H_2O_2) and tetramethylbenzidine (TMB) to form a color complex. The reaction was stopped by the addition of weak acid. The optical density at 450 nm was measured in an automated microwell plate reader and the results were compared to the cut-off value of 0.350. A clinical specimen with an A_{450} reading equal to or greater than 0.35 is positive, and a specimen with a reading less than 0.35 is negative for the presence of *M. tuberculosis* DNA.

Chemical prevention of PCR product contamination

The recommended procedures to prevent false-positive reactions as a result of target or amplified product contamination were followed.

RESULTS

Detection of mycobacteria by culture

In total, 88 specimens were positive by culture (9.2%) (Table 1). Of these, 56 (5.8%) were *M. tuberculosis* isolates from 33 patients. Moreover, 15 isolates of MAC, 12 *M. malmoense*, two *M. chelonae*, and one *M. xenopi* were detected.

Table 1 Comparison of results obtained by culture and PCR in detection of *M. tuberculosis* in respiratory specimens in Scandinavia. The numbers for laboratory 1 and 2 are given on the upper line, and the total numbers for both laboratories are given in bold type

	Culture Mtb+	Culture Mtb− Culture MOTT−	Culture Mtb− Culture MOTT+	Total
PCR+	33+16	2ª+5	0+0	35+21
	49	**7**	**0**	**56**
PCR−	3ᵇ+4	487+378	19+13	509+395
	7	**865**	**32**	**904**
Total	**56**	**872**	**32**	**960**

ªOther samples from these two patients were *M. tuberculosis* culture positive.
ᵇRetesting by PCR (AmpliCor) gave a positive result in one of these samples; PCR confirmation testing by Roche in Basel (blind testing) gave positive results in the remaining two samples.

Detection of *M. tuberculosis* by PCR

The same 960 respiratory specimens were tested for presence of *M. tuberculosis* by the nucleic acid amplification method (PCR). In total, 49 of the 56 specimens which yielded *M. tuberculosis* by culture were positive for *M. tuberculosis* DNA by PCR (Table 1). PCR was positive for an additional seven specimens from four patients, which were negative by culture. Based on the findings in Table 1, the overall sensitivity, specificity, and positive predictive and negative predictive values of this particular PCR test in comparison with culture were 87.5%, 99.2%, 87.5% and 99.2%, respectively (Table 2). The values of the PCR test were 91.7%, 99.6%, 94.2% and 99.4% for laboratory 1 and 80.0%, 98.7%, 76.2% and 99.0% for laboratory 2, respectively (Table 2).

No positive result for the *M. tuberculosis* complex was obtained by the PCR system for the specimens which were positive for atypical mycobacteria or other bacterial species grown in the BACTEC 12B or LJ media. Four patients whose sputum specimens were culture negative, but *M. tuberculosis* PCR-positive, had other PCR-positive samples, a past history of tuberculosis and/or clinical response to recent antituberculosis chemotherapy.

DISCUSSION

In comparison with culture, the sensitivity of PCR was 91.7% for laboratory 1 and 80.0% for laboratory 2, respectively (Tables 1 and 2). In comparison with previously published studies for direct detection of *M. tuberculosis* by PCR, other groups have found that the sensitivity of their PCR assays when compared with culture ranged from 82% to 94% [7,14–17]. Seven samples in five patients were *M. tuberculosis* culture negative and PCR positive and can as such be strictly regarded as false positive. But when other factors are taken into account for evaluating the patient as '*M. tuberculosis* positive' by having other samples positive by culture and/or PCR or other factors indicating tuberculosis, most of this specificity problem is resolved.

Table 2 The sensitivity, specificity, and positive predictive value and negative predictive value of the PCR test as compared to culture, based on the results in Table 1

Laboratory	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)
Laboratory 1	91.7	99.6	94.2	99.4
Laboratory 2	80.0	98.7	76.2	99.0
Laboratories 1 and 2	87.5	99.2	87.5	99.2

Different decontamination procedures may account for the major part of the discrepant sensitivity in the two laboratories. Laboratory 1 used the NALC decontamination procedure, while laboratory 2 used the sodium dodecylsulfate (SDS) method. The NALC procedure is clearly the decontamination method recommended by the Amplicor manufacturing company and for PCR and other amplification techniques [14,15,18]. The higher sensitivity of PCR in comparison with culture for laboratory 1 could also be due to less optimized culture techniques than in laboratory 2, laboratory 1 using only BACTEC detection and not including solid media [3]. Possibly, recently documented batches of BACTEC vials with reduced performance in cultivating both *M. tuberculosis* and MOTT might be involved [25]. Other factors include the time elapsed between sampling and processing, sample handling, and the technical quality of the sample preparation, lysis and PCR set-up. The samples examined by PCR by laboratory 2 were transported after decontamination and sample preparation and frozen at -70 °C before PCR analysis.

The sample lysis and pretreatment procedure with Triton X-100 and optimized buffering clearly facilitates direct detection of *M. tuberculosis* by PCR. The UNG enzyme inactivates up to 10^9 copies of uracil-containing *M. tuberculosis* amplified DNA [24]. This reduced the likelihood of false-positive results arising due to contamination with pre-existing PCR products. Furthermore, the inclusion of the hybridization event ensured that only *M. tuberculosis*-specific PCR products were detected, increasing the overall specificity of the test. These actions, in addition to careful laboratory precautions in sample processing and work habits, have now minimized the occurrence of false-positive PCR results.

Factors lowering the sensitivity of PCR are interfering substances present in clinical specimens [7,11,15] and inadequate amounts of the microbial DNA to be detected. An uneven distribution of bacteria or DNA, even after lysis of the material, as may particularly apply to mucous material in sputum, may cause an arbitrary sampling effect. In our hands, the PCR assay worked just as well directly on respiratory specimens which were not subjected to decontamination (unpublished results). The elimination of factors inhibitory for PCR in clinical specimens remains a challenge in the use and acceptance of all amplification assays in the diagnostic setting. Certainly, the inclusion of a positive amplification control test is useful to assess the inhibiting factors which may be present in clinical material.

Despite promising results of numerous published reports, the routine use of PCR to detect *M. tuberculosis*

directly in clinical specimens has been hampered for a variety of reasons, such as contamination, expense, and lack of sensivity and/or specificity [7,9–11,13,15,17]. In addition, the routine use of PCR in the clinical laboratory sets limitations because of the complex procedures required for amplification, such as cumbersome sample preparation and detection methods.

Still, using PCR in the identification of *M. tuberculosis* directly in clinical samples offers unique improvements in this diagnostic field. This PCR assay offers a sensitive and specific test for *M. tuberculosis* performed within 5 to 6 h. More automation and lower assay expenses are required. For the future, this and other amplification techniques can facilitate the direct detection of the infecting agent and its antibiotic susceptibility pattern [26], as well as epidemiological mapping [27]. Potentially, all of these goals can be achieved in one single multiplex assay.

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