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Use of PCR in routine diagnosis of treated and untreated pulmonary tuberculosis

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Abstract

***Aims*—To assess the routine use of a polymerase chain reaction (PCR) assay for the direct detection of *Mycobacterium tuberculosis* in expectorated sputum specimens.**

***Methods*—A pair of primers (20-mer) were designed to amplify the 38 kilodalton protein of *M tuberculosis*. The specificity of the assay was evaluated in 31 *M tuberculosis* strains, 15 atypical mycobacterium species, and several commensal bacteria of the upper respiratory tract. The assay was subsequently applied to 519 sputum specimens from 85 inpatients of a chest hospital in Hong Kong.**

***Results*—An amplified product of 239 base pairs was found in all *M tuberculosis* strains, standard strains of *M bovis*, and *M africanum* but not in the other bacterial strains tested. For the 51 patients with pulmonary radiographic lesions, the diagnosis of pulmonary tuberculosis was subsequently confirmed by both culture and PCR in 41 of them. Five patients who were treated before admission were positive by PCR alone. All but one patient in the control group (patients with acute exacerbation of chronic obstructive airway diseases) or those with atypical mycobacterial diseases were PCR negative. The PCR remained positive after four weeks of anti-tuberculosis treatment in 29 patients, 16 of whom had become culture negative.**

***Conclusion*—This PCR assay is a useful technique for the diagnosis of untreated and recently treated cases of pulmonary tuberculosis.**

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Pulmonary tuberculosis remains an important cause of morbidity and mortality (annual notification of 6283 cases and 410 deaths in 1991) in our locality despite a high standard of living.[1] For a specific diagnosis of this disease, the confirmed technique is the standard culture which takes weeks before a positive isolate can be identified and reported. Thus even in the absence of a positive Ziehl-Neelsen smear, the initiation of anti-tuberculosis treatment is often based on the presence of suggestive radiological lesions. Because early treatment can be effective in rendering the patients non-infectious, a rapid, specific, and sensitive diagnostic method would be useful by avoiding the use of more invasive diagnostic technique such as biopsy and the side effects of unnecessary empirical antimicrobial treatment.

The polymerase chain reaction (PCR) has been used to amplify Mycobacteria specific target DNA sequences.[2,3] Previous reports have emphasised the general applicability of PCR as a diagnostic tool by comparing the results of culture and PCR assay on individual specimens.[4,5] Though a prospective correlation of clinical diagnosis, conventional mycobacterial culture, and PCR assay was conducted by Brisson-Noel,[6] there was little information regarding the use of this technique in the follow up study of specific groups of patients. In this study PCR was incorporated with the conventional microbiological procedure in the initial diagnosis and systematic follow up of a group of patients with pulmonary diseases admitted to a chest hospital in Hong Kong.

Methods

Four hundred and twenty five serial expectorated sputum samples from 51 patients with the following clinical features were included in this study: (1) a history of productive cough for more than 4 weeks; (2) chest radiographic lesions suspicious of pulmonary tuberculosis (patchy or nodular infiltrate in upper lobes or superior segment of lower lobes, bilateral upper lobe infiltrates with patchy soft shadows with or without cavitation, pneumonic change with a hilar adenopathic shadow); and (3) absence of malignancy by negative sputum cytology in indicated cases. Two or more expectorated sputum samples were collected in γ-irradiated disposable plastic containers before and 4 weeks after anti-tuberculosis treatment with isoniazid, pyrazinamide, rifampicin and/or ethambutol, streptomycin. Six patients were already receiving treatment with anti-tuberculosis agents for less than 3 weeks when they were admitted. As controls, 94 specimens from 34 patients with acute exacerbation of chronic obstructive airway diseases were tested. These patients only received standard antibacterial drugs for their chest infection with *Streptococcus pneumoniae, Haemophilus influenzae, Branhamella catarrhalis* and unknown pathogens in some cases.

All samples were stored at −20°C if not

immediately processed. They were processed and assayed under code by investigators unaware of the clinical information. The samples were initially processed as routine sputum specimens as described.[7] Briefly, a direct smear was prepared for auramine fluorescent stain and if positive, Ziehl-Neelsen stain confirmation was performed. The specimens were then decontaminated and digested with an equal volume of Sputolysin (Oxoid) and 3% sodium hydroxide for 30 minutes at room temperature.

The mixture was neutralised with 0·048M NaH_2PO_4 (pH6·8) buffer and then centrifuged at 5000 rpm for 30 minutes. The sediment was resuspended in 400 μl of TE (50 mM TRIS-5 mM EDTA, pH 8·0) buffer. Each of two Lowenstein-Jensen agar slants was inoculated with 100 μl of suspensions and incubated at 37°C. The other half was transferred to an Eppendorf tube for DNA extraction.

DNA was extracted from the frozen aliquots of concentrated clinical samples, as described, with some modifications.[8] After digestion with lysozyme (1 mg/ml), the specimens was lysed by heating at 95°C with an alkaline sodium dodecyl sulphate solution, followed by neutralisation with TRIS-HCl buffer. DNA was extracted once with phenol-chloroform, precipitated with ethanol and resuspended in 100 μl sterile distilled water for PCR amplification.

Primer and probe sequences were selected from the region of *M tuberculosis* genome encoding the 38 kilodalton protein.[5 8] No clinically important homology was found between the primer sequences with the other known genomic sequences by computer search of the information in GenBank. The sequences of the primers were 5'-TGACGTTGGC GGAGACCGGT-3' and 5'-GCTGAGCGGA GATGGCTAGC-3'. The probe was located between the two primers and its sequence was 5'-GCT-GTTCAAC CTGTGGGGTC CGGC-CTTTC-3'.[5]

The primers and probes were synthesised on an Applied Biosystem 380B DNA synthesiser. They were successfully tested by PCR with 30 different clinical isolates of *M tuberculosis* and three standard strains of *M tuberculosis* complex. No false positive result occurred with 35 different standard strains of bacteria outside the *M tuberculosis* complex. These PCR negative strains included *M xenopi*, *M malmoense*, *M szulgi*, *M chelonae*, *M gordonae*, *M flavescens*, *M nonchromogenicum*, *M kansasii*, *M simiae*, *M scrofulaceum*, *M avium*, *M fortuitum*, *Corynebacterium* and *Nocardia* species. The primers were shown to be specific for *M tuberculosis*, *M bovis*, and *M africanum*. No target band or hybridisation with probe was found on PCR when other bacterial strains and human leucocytes were tested.

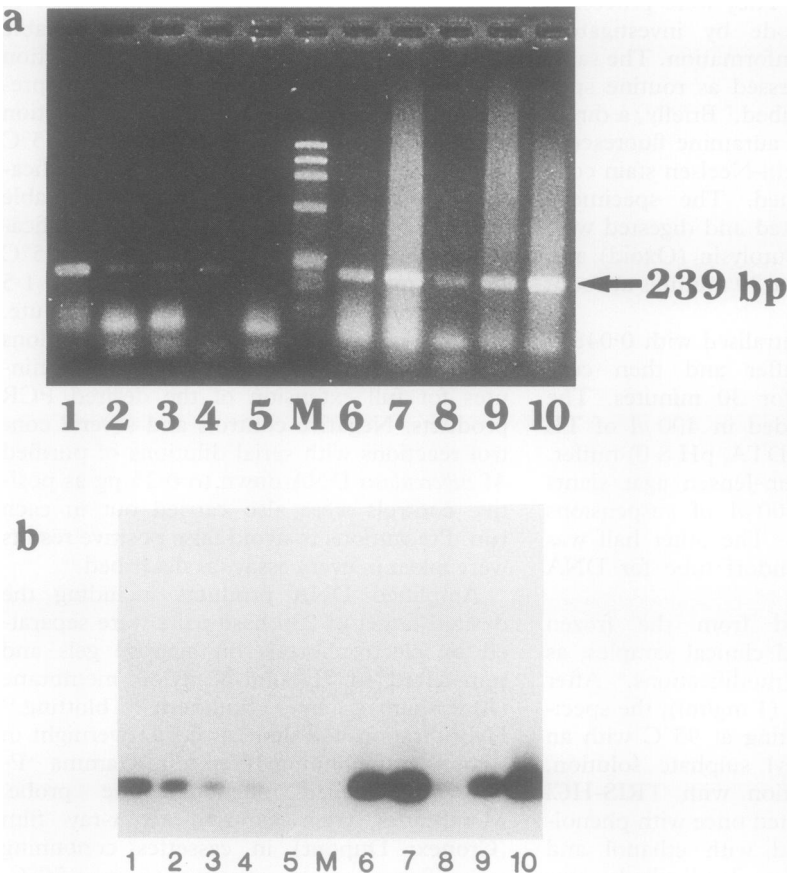
Each amplification reaction mixture (in a final volume of 100 μl) contained 10 mM TRIS-HCl (pH 8·3), 50 mM KCl, 1·8mM $MgCl_2$, 0·15 mM each deoxynucleotide triphosphate (Pharmacia), 75 pmol each of the two primers and 2 units of thermostable Taq DNA polymerase (Cetus). Each reaction was overlaid with mineral oil (75 μl) to prevent evaporation. The DNA in each reaction was initially denatured by incubating at 95°C for 5 minutes before 35 cycles of amplification were performed with a programmable thermal cycler (Coy, USA). Each amplification cycle consisted of denaturation at 95°C for 1·5 minutes, annealing at 59°C for 1·5 minutes and extension at 72°C for 1 minute. At the end of the 35 cycles, the reactions were maintained at 72°C for another 5 minutes for full extension of the desired PCR products. Negative controls and several control reactions with serial dilutions of purified *M tuberculosis* DNA down to 0·25 pg as positive controls were also carried out in each run. Precautions to avoid false positive results were taken in every assay, as described.[10]

Amplified DNA products including the desired target of 239 base pairs were separated by electrophoresis on agarose gels and transferred to Hybond-N nylon membrane (Amersham) by Southern blotting.[11] Hybridisation was done at 60°C overnight in a solution containing 10^6cpm/ml Gamma ^{32}P-5' end labelled oligonucleotide probe. Membranes were exposed to *x*-ray film (Cronex; Dupont) in cassettes containing intensifying screen overnight at −70°C. Typical PCR findings were shown in the figure. The results on the ethidium bromide stained agrose gel were recorded and subsequently confirmed by probing.

Results

A total of 519 specimens were processed from 51 patients with chest radiographic lesions suggestive of tuberculosis and 34 patients with acute exacerbation of chronic obstructive airways disease. All 53 smear positive specimens with subsequent positive culture for *M tuberculosis* were positive on PCR (table 1). However, five out of the 63 smear negative culture positive specimens were missed by the PCR assay (sensitivity 92%). Twenty specimens from four patients with radiographically suggestive lesions and one control yielded atypical mycobacteria on culture (4 *M avium intracellulare*, 1 *M chelonae*). None of their specimens was positive on PCR. With respect to the 383 culture negative specimens, PCR was positive in 57 specimens which were collected from both groups of patients (those initially culture positive for *M tuberculosis* and those receiving empirical treatment before admission) already receiving treatment with anti-tuberculosis agents.

When the status of individual patients was evaluated by the findings on multiple specimens, all 41 patients with positive cultures for *M tuberculosis* on admission were PCR positive (table 2). Though no documentation by culture was possible for the six patients who were already receiving treatment on admis-



Typical findings of the PCR assay on clinical specimens.
Lane 1–4: positive control reactions with serial dilutions of purified M tuberculosis DNA down to 0·25 pg.
Lane 5: negative control reaction.
Lane M: molecular size marker, RF-DNA of phage X-174, digested with Hae III.
Lanes 6, 7: persistent smear positive culture-positive specimens for M tuberculosis from a patient 4 weeks after and before treatment.
Lane 8: smear negative, culture positive specimen.
Lanes 9, 10: persistent smear positive specimens turning from culture positive to culture negative from a patient 4 weeks after and before treatment.

sion, five were PCR positive (of which two were smear positive). The PCR negative patient was subsequently found to have carcinoma of the lung. Among the other 34 negative controls one patient with chronic obstructive airways disease and silicosis was found to be PCR positive on three of his specimens. His pulmonary symptoms did not improve with the standard antibacterial treatment and the culture of his three specimens later turned out to be positive for *M tuberculosis*.

As for the effect of antituberculosis treatment on the conversion of findings on smear, culture, and PCR, 70% of the cases were still positive by PCR after 4 weeks of treatment (table 2) while only 32% of these patients remained culture positive. Although antituberculosis treatment was continued on the basis of the radiographic findings for the six patients who were already receiving treatment on admission, three of them remained PCR positive after another 4 weeks of treatment.

The turnround time required for reporting of confirmed positive cultures ranged from 26 days to 54 days (mean 34 days) and that for a confirmed PCR assay including hybridisation was 3 to 8 days even when the PCR was performed twice a week. Of the total of 168 PCR positive assays, 14 (8·3%) specimens were negative for the target band by direct staining of the agarose gel but only positive after DNA probing.

Discussion

To define the role of PCR assay in the management of patients with suspected pulmonary tuberculosis, we incorporated the PCR assay into our routine laboratory procedures using sputum specimens sent from patients who were admitted and followed up in a chest hospital for various pulmonary conditions. A set of primers from within the genomic sequences encoding the 38 kilodalton protein was designed for the PCR assay and found to be specific for the *M tuberculosis* complex both on standard strains and clinical isolates.

When the results of culture and PCR assay of individual specimens were correlated, false negative PCR results (8%) were observed among the smear negative, culture positive

Table 1 Comparison of PCR assay with smear and culture for detection of Mycobacterium tuberculosis in clinical specimens

Culture	*Positive for M tuberculosis*		*Positive for Atypical Mycobacteria*		*Negative*		
Smear	*Positive*	*Negative*	*Positive*	*Negative*	*Positive*	*Negative*	*Subtotal*
PCR assay							
Positive	53	58	0	0	8*	49*	168
Negative	0	5	3	17	0	326	351
Subtotal	53	63	3	17	8	375	519

*Specimens were taken from patients receiving antituberculosis treatment.

Table 2 Effect of four weeks of standard antituberculosis treatment on smear, culture, PCR status of patients

	On admission No (%)	*4 weeks after treatment No (%)*
Patients with radiographic changes suspicious of tuberculosis		
(1) Culture documented tuberculosis n = 41		
smear positive	28 (68)*	16 (39)
culture positive	41 (100)	13 (32)†
PCR positive	41 (100)*	29 (70)†
(2) Treated cases on admission n = 6‡		
smear positive	2	0
culture positive	0	0
PCR positive	5	3
(3) Cases with atypical mycobacteria n = 4		
smear positive	2	0
culture positive	4	3
PCR positive	0	0
Patients with acute exacerbation of chronic obstructive airways disease (controls) n = 34		
smear positive	0	—
culture positive	2§	—
PCR positive	1	—

*Significant difference ($p < 0·05$) by Fisher's exact test (2 tailed)
†Significant difference ($p < 0·005$) by Fisher's exact test (2 tailed)
‡One PCR negative patient was subsequently found to have carcinoma of the lung
§One patient had *M tuberculosis* (PCR positive) and the other had *M avium intracellulare* infection (PCR negative)

cases and might be related to imperfect extraction procedures, non-homogeneous distribution of the mycobacteria in the sputum samples or the presence of inhibitors.[3 11] No culture positive cases were missed if multiple specimens were sent for each patient in our series. PCR assay is more sensitive than the smear for diagnosis of tuberculous infection before treatment. It is also more sensitive than the culture after antimicrobial treatment has been started.

As for some patients who had started treatment before referral for various reasons, PCR assay was positive in five of our six patients with radiological lesions suggestive of tuberculosis despite up to 2 weeks of treatment. The only PCR negative case was subsequently found to have carcinoma of the lung. Three patients remained PCR positive after another 4 weeks of treatment during which all had turned smear negative. Thus PCR assay could be an important adjunct in the diagnosis of these treated cases.

No false positive results were found among patients with culture positive for atypical mycobacteria and the controls. Thus the PCR assay reliably distinguished cases with positive smear or culture due to atypical mycobacteria from the cases with tuberculosis. As for the 57 PCR positive specimens which were culture negative, all were taken from patients already receiving antituberculosis treatment. They should not be considered as false positive because the drug treatment could suppress the growth of mycobacteria despite continued shedding of these organisms into the sputum. Culture has conventionally been regarded as the gold standard for the evaluation of new diagnostic techniques, the data from our prospective study showed that the ability of PCR assay to detect non-viable or non-culturable mycobacterium after treatment is an additional advantage over this conventional method. The clinical importance of persistently positive PCR assays despite antituberculosis treatment in some cases still awaits further follow up study.

An extensive prospective study was reported by Brisson Noel *et al*, who compared the clinical features, mycobacterial culture, and the PCR assay results of 514 specimens from 318 patients with or without suspected tuberculosis. The nature of patients' specimens used in this study was diverse and these included gastric aspirate (21%), sputum (18%), buffy coat (14·5%), and others (46·5%). Though multiple specimens were obtained from some patients, no longitudinal follow up study was done and results of mycobacterial culture were not available for analysis in 13·2% of these specimens. In contrast to the findings of the present study, a longitudinal follow up study of 425 specimens from 51 patients with pulmonary diseases was carried out with clearly defined clinical criteria of enrolment and a complete set of conventional culture data correlating with PCR. A control group of 34 patients yielding 94 sputum samples was chosen to guard the assay. The results of the conventional culture, smear, and PCR assay before and 4 weeks after antituberculosis treatment were analysed.

With respect to the methodology, the two PCR assays used by Brisson-Noel *et al* were based on the amplification of the 65 kilodalton gene or the insertion sequence 6110. An overall sensitivity of 97·4% was achieved which was similar to that of the present study. There were six false negative, three false positive, and four misidentified strains. Southern hybridisation using ^{32}P-labelled or horseradish peroxidase labelled probes were used to confirm the identity of the amplicon. The correlation of findings on the gel and the Southern hybridisation was not given. Detection of PCR inhibitors was achieved by using PUC 91 plasmid target DNA and TEMA/TEMB primers in control experiments. Inhibitors were found in 16% of the buffy coat samples, 6·5% of the gastric aspirates and 3·2% of the sputum samples. Pretreatment with guanidium thiocyanate was proposed to reduce false negative results. In the present study the PCR assay was based on the amplification of the 38 kilodalton gene which is specific for *M tuberculosis* complex. No false positive results were found in specimens positive for atypical mycobacteria. The target band present on the agarose gel correlated with the Southern hybridisation in 91·7% of the positive assays. The remaining gel negative probe positive assays (8·3%) could be related to the low amount of target DNA present in the sample or presence of inhibitors. The use of ^{32}P-labelled probes was a setback to the routine application of this technique in clinical laboratories. We have already tested the use of digoxigenin-11-ddUTP (Boehringer Mannheim) labelled probes and detection by alkaline phosphatase catalysed chemiluminescent substrate AMPPD (3-(2'-spiroadamantane)-4-methoxy-4-(3"-phosphoryloxy)-phenyl-1,2-dioxetane) in 52 specimens which suggested similar sensitivity to ^{32}P-labelled probe (unpublished data). However, the recurrent cost of using these non-radioactive agents was higher. Another alternative to this problem is to use nested PCR, as the primer sequences used in this study were in between the primer sequences (MT-1 and MT-2) used by Sjobring *et al*. Though internal controls to detect inhibitors were not used in the present study, control PCR with primers for specific amplification of the DR-β gene in the HLA-class II region would be incorporated in further follow up studies.[12]

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Notes