

Comparison of polymerase chain reaction for IS6110 and Amplicor in the diagnosis of tuberculosis

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Abstract

Background – Polymerase chain reaction (PCR) amplification of *Mycobacterium tuberculosis* DNA offers the potential of a sensitive and specific diagnostic test for tuberculosis. To evaluate this technique from the clinician's perspective, samples were collected from patients with chronic respiratory disease and the sensitivity and specificity of a newly introduced commercially available PCR kit (Amplicor) was compared with that of an established method to detect the target sequence IS6110.

Methods – Sputum or bronchial washings from patients with active tuberculosis, previously treated tuberculosis or other selected respiratory illnesses were analysed by both techniques and their sensitivity and specificity determined.

Results – Amplicor was more specific than IS6110 in the diagnosis of active infection (98% versus 79%). Both techniques were equally sensitive (92%).

Conclusion – These results suggest that analysis of respiratory samples by Amplicor PCR in inner city populations of patients has greater specificity for a diagnosis of active tuberculosis than PCR for IS6110, and thus Amplicor PCR may aid the clinician in making a diagnosis of active tuberculosis.

(Thorax 1996;51:320-322)

Keywords: tuberculosis, polymerase chain reaction, Amplicor.

ence of IS6110 DNA in respiratory samples from patients living in an area with a high prevalence of tuberculosis who do not themselves have active tuberculosis. There is also doubt about the interlaboratory reproducibility of IS6110 PCR methodology.⁴ A further problem is that most studies are laboratory based and samples are included irrespective of the clinical status of the patient. The Amplicor PCR system (Roche Molecular Systems, Branchburg, New Jersey, USA) for the detection of *M tuberculosis* DNA has recently been introduced. This uses an alternative target sequence of DNA which encodes the 16S ribosomal RNA (rRNA) gene and incorporates techniques to prevent contamination by previously amplified samples. The target sequence of DNA is present in one copy of the chromosome and has been used by others.⁵ The method avoids the need for a reverse transcription step required if the equivalent sequence of the rRNA is used.⁶

We have undertaken a study to determine whether the Amplicor system is better able to discriminate between patients with active tuberculosis requiring treatment and those with other chronic respiratory conditions including past tuberculosis than PCR for IS6110. To this end samples were examined which had been collected from specifically selected groups of patients with chronic respiratory disease who lived in an area with a high prevalence of tuberculosis.

Methods

Sputum or bronchial washings were collected from selected inpatients and outpatients attending St Mary's Respiratory Medicine Service. Over a three month period, a sputum specimen was obtained from all patients diagnosed as having pulmonary tuberculosis before or within 48 hours of commencing treatment. Similarly, sputum was obtained from patients previously treated for tuberculosis attending the outpatients who had been treated with a conventional rifampicin based regimen and a few currently on treatment. Patients with other selected chronic respiratory diseases including cancer, chronic obstructive airways disease, bronchiectasis, and sarcoidosis (table) were recruited and those with a productive cough provided a sputum sample. Patients in these groups without a productive cough who were undergoing diagnostic fiberoptic bronchoscopy were recruited and 10 ml of bronchial trap fluid was collected. All

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Received 16 March 1995
Returned to authors
5 June 1995
Revised version received
14 August 1995
Accepted for publication
3 November 1995

Tuberculosis is a major cause of morbidity and mortality worldwide. In developed countries the plateau in the fall in the number of notifications of tuberculosis, the increase in drug resistance, and the interaction with human immunodeficiency virus (HIV) infection have increased concern over tuberculosis control. To date the diagnosis of pulmonary tuberculosis remains dependent on staining and culture of sputum or other clinical specimens. Staining does not differentiate tuberculosis from other mycobacterial infections and culture is slow, with the results available many weeks later. Amplification of DNA sequences specific to *Mycobacterium tuberculosis* by polymerase chain reaction (PCR) offers the potential of a rapid and specific diagnostic test.¹

The most commonly used sequence is IS6110.² However, our group³ has found evid-

Comparison of Amplicor and PCR for IS6110 on respiratory samples from patients with lung disease

Diagnosis	No.	Sputum	Bronchial washings	Smear and culture +	Amplicor +	IS6110 +
Active tuberculosis	12	9	3	12	11	11
Tuberculosis on treatment	3	2	1	1	0	2
Past tuberculosis	12	9	3	0	0	2
Carcinoma	15	4	11	0	1	4
COPD	13	9	4	0	0	2
Bronchiectasis	11	11	0	0	0	5
Active sarcoidosis	3	1	2	0	0	0
Other	8	0	8	0	0	1
<i>M. kansasii</i>	1	1	0	1	0	0

Other = haemoptysis of unknown cause 4, cryptogenic fibrosing alveolitis 1, rheumatoid vasculitis 1, pneumonia 1, prominent hila 1.

patients gave informed consent and the study was approved by the local ethics committee.

The age, sex, and clinical diagnosis of the patients was recorded. The specimens were analysed by PCR by an individual blinded to the code. Aliquots from every specimen were stained and cultured for mycobacteria and submitted to PCR for IS6110 and the Amplicor system.

AMPLIFICATION USING THE AMPLICOR SYSTEM

The sputum samples were decontaminated with NaOH sodium citrate N-acetyl-L-cysteine and concentrated after decontamination.⁷ To 100 µl of decontaminated sputum or bronchial washings was added 500 µl of sputum wash solution. The samples were vortexed and centrifuged at 12 500g for 10 minutes; 100 µl of sputum lysis reagent was added to the pellet and vortexed. Positive and negative controls were prepared as follows. Briefly, to 100 µl of positive and negative controls (provided with the Amplicor kit) was added 400 µl of the sputum lysis reagent. 100 µl of controls and processed specimens were incubated at 60°C in dry heat block for 45 minutes. The tubes were pulse centrifuged for five seconds and 100 µl of sputum neutralisation reagent was added. All the reagents were provided with the Amplicor kit.

A 584 base pair sequence using genus specific primers located in a highly conserved region of 16S ribosomal RNA (rRNA) gene of mycobacteria was amplified. The primers were biotinylated. Master mix (Tris-HCl solution containing 20% glycerol, biotinylated primers, dATP, dCTP, dGTP, dUTP, Amplitaq and sodium azide) with AmpErase (Tris-HCl solution containing uracil N-glycosylase) was prepared by adding 100 µl of AmpErase to one tube of Master mix. 50 µl of Master mix with AmpErase was added to each PCR tube containing 50 µl of processed samples and positive or negative controls. Amplification was carried out using GeneAmp PCR system 9600 thermal cycler (Perkin Elmer, Warrington, Cheshire, UK). The conditions used for PCR were two minutes at 50°C followed by two cycles of 20 seconds at 98°C, 20 seconds at 62°C, and 45 seconds at 72°C and then 35 cycles of 20 seconds at 94°C, 20 seconds at 62°C, and 45 seconds at 72°C and five minutes at 72°C. The PCR amplified specimens and controls were denatured by adding 100 µl of denaturing so-

lution. The amplified products were detected in multiwell plates coated with *M. tuberculosis* specific probe for the hypervariable region of the 16S rRNA using the Avidin-HRP conjugate system.

AMPLIFICATION USING PCR FOR IS6110

Decontaminated sputum samples and bronchial washings (100 µl) were mixed with 400 µl of a lysis buffer containing 15% sucrose, 0.05 M Tris-HCl (pH 8.5) and 0.05M EDTA. DNA was purified according to the method of Ross *et al.*⁸ Two oligonucleotide primers within the IS6110 insertion sequence designated primers Pt8 (5-GTGCGGATGGTCGCAGAGAT-3) and Pt9 (5-CTCGATGCCCTCACGGTTC-3) were used for PCR⁹ to amplify a 541 bp PCR fragment. To prevent contamination primers were used to a region of IS6110 which has not previously been amplified in our laboratory. The PCR mixture contained 10 mM Tris-HCl (pH 8.3), 50 mM MgCl₂, 0.01% (wt/vol) gelatin, 0.2 mM of each of the deoxynucleoside triphosphates (dATP, dCTP, dGTP and dTTP), 0.2 mM of each of the primers Pt8 and Pt9, and 1 U of Taq Polymerase (Promega Ltd, UK). The conditions used for DNA amplification were as described previously by Walker *et al.*³ In all PCR series a positive control (DNA from H37Rv strain of *M. tuberculosis*) and negative control (sterile distilled water) were included. The PCR products were analysed by agarose gel electrophoresis on 2% agarose gel stained with ethidium bromide.

Results

Samples of sputum and bronchoscopic washings were collected from 78 patients and the results of smear/culture, Amplicor, or IS6110 PCR were compared (table). Samples from 12 patients were shown to contain *M. tuberculosis* by smear and later confirmed by culture. No additional isolates were identified by culture. Both Amplicor and IS6110 had equal sensitivity in that they identified *M. tuberculosis* DNA in 11 of 12 samples confirmed bacteriologically. The study included three patients on treatment for pulmonary tuberculosis. *M. tuberculosis* DNA was found by Amplicor in none of the samples from these patients on treatment, but from two patients using IS6110 PCR. When diagnoses other than active tuberculosis were considered, the Amplicor PCR was more specific in that it identified *M. tuberculosis* DNA in a sample from only one patient (who had lung cancer) out of the 63 who had no evidence of active tuberculosis and who were not receiving treatment for tuberculosis. By contrast, PCR for IS6110 DNA was less specific and gave positive results in samples from 14 patients who did not have active tuberculosis; these included two patients with past tuberculosis, four with lung cancer, two with chronic obstructive airways disease, five with bronchiectasis, and one with vasculitis. When the patients on therapy were excluded the sensitivity of the Amplicor test was 92% and the specificity 98%, whereas IS6110 detection had

a sensitivity of 92% and specificity of 79%. Of interest, one patient initially thought to have tuberculosis on sputum smear was later found to have *M. kansasii* and his samples were negative by both Amplicor and IS6110 PCR.

Discussion

The advent of PCR assays for DNA specific *M. tuberculosis* has raised hopes of a rapid and accurate diagnostic test for tuberculosis. The technique of PCR amplification when directed at any of a number of *M. tuberculosis*-specific DNA sequences is a sensitive method for identifying patients with smear positive *M. tuberculosis*. However, the clinician needs to know whether the technique discriminates between patients with active tuberculosis which requires treatment and those with diseases which may appear similar on clinical grounds. This question was addressed in the present study. Furthermore, since a new commercially available technique (Amplicor) has become available, this method was compared with the more established technique of PCR to detect IS6110 DNA. Recent large laboratory based studies have found Amplicor to have a high sensitivity and specificity, but these studies have not focused on patient groups which have posed diagnostic difficulty.¹⁰⁻¹³ The present study did not focus on the equally important group of smear negative patients suspected of having tuberculosis which is a subject of future work.

The results reported here suggest that both tests were sensitive. However, the Amplicor test proved more specific in that IS6110 DNA was detected in 14 of 66 patients who did not have clinical evidence of active tuberculosis. The IS6110 PCR methodology used in this study is similar or identical to that used by other authors. However, there is a well recognised interlaboratory variation in the results using PCR to detect the IS6110 sequence.⁴

One explanation for the greater number of positive results for IS6110 DNA may be that IS6110 offers multiple targets for amplification since it is present as multiple repeats in the *M. tuberculosis* genome. This may increase the likelihood of a positive response in the presence of small, possibly clinical insignificant, numbers of *M. tuberculosis* organisms. Thus, an occasional non-viable mycobacterium in a macrophage might register a positive result with PCR for IS6110, but not with Amplicor.

The Amplicor test is technically easier and more rapid than IS6110 detection. It has the advantage that contamination from past assays is prevented, but it is considerably more expensive than routine bacteriological examination. The cost per test is likely to be in the region of £50-100 but time from sample collection to results is potentially less than two days. The Amplicor system has been designed for sputum analysis and there is little experience with other biological fluids. Furthermore, it requires a specific Perkin Elmer thermal cycler.

As anticipated, the degree of diagnostic accuracy of both PCR techniques was similar in smear positive disease to direct examination and staining of sputum or bronchial washings. Unlike other laboratory based studies this study was designed specifically to compare the specificity of two techniques by comparing samples from patients with tuberculosis with samples from patients with selected diseases which might cause diagnostic confusion with tuberculosis. By chance all the patients with tuberculosis were smear positive and hence clinically did not present a management problem. This small study did not provide data on how the two techniques would compare in the more clinically challenging smear negative patients who are suspected of having pulmonary tuberculosis. The results did indicate that the Amplicor test had a greater specificity for patients with active tuberculosis than those with chronic respiratory disease. The high sensitivity and specificity of the Amplicor test are encouraging, although the high cost suggests that it will not be used for most clinical samples. Further studies are needed to determine the clinical indications for using the Amplicor test in routine clinical practice.

This study was supported by the British Lung Foundation and further assistance was provided by Roche Products Ltd.

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