Thorax 1992;47:690–694

Comparison of polymerase chain reaction amplification of two mycobacterial DNA sequences, IS6110 and the 65kDa antigen gene, in the diagnosis of tuberculosis

D A Walker, I K Taylor, D M Mitchell, R J Shaw

Abstract

Background **Knowledge of the sequences of mycobacterial genes and the availability of DNA amplification techniques have raised the possibility that identification of mycobacterial DNA may offer a rapid and specific diagnostic test for tuberculosis. The correlation between the presence of *Mycobacterium tuberculosis* DNA and clinical tuberculosis, however, is not known. This study compared the results of polymerase chain reaction amplification of two *M tuberculosis* DNA sequences, IS6110 and the gene encoding the 65kDa heat shock protein (65kDa Ag), from sputum, bronchoscopy washings, and bronchoalveolar lavage fluid and related these findings to the presence of active and past tuberculosis.**

Methods **Highly specific primers were used for amplification of IS6110 and 65kDa Ag DNA. Analysis was performed on one or more samples from 87 patients.**

Results **IS6110 DNA was identified in samples from all six patients with active tuberculosis, from 15 to 18 patients with past tuberculosis, from five of nine contacts of patients with tuberculosis, and from nine of 54 patients with lung disease unrelated to tuberculosis. The 65kDa Ag DNA was identified in samples from all patients with active and past tuberculosis, from contacts of patients with tuberculosis, and from 14 of 42 patients with non-tuberculous lung diseases.**

Conclusion **These data suggest that the presence of IS6110 DNA correlates more closely with a tuberculosis related diagnosis than that of 65kDa Ag DNA and that both DNAs are found in most subjects with past tuberculosis or contacts of patients with tuberculosis. This may limit the clinical usefulness of these tests.**

(*Thorax* 1992;47:690–694)

Department of Respiratory Medicine, St Mary's Hospital Medical School, Norfolk Place, London W2 1PG
D A Walker
I K Taylor
D M Mitchell
R J Shaw

Correspondence to:
Dr R J Shaw (reprints will not be available)

Accepted 19 March 1992

Tuberculosis continues to be a major cause of morbidity and mortality throughout the world, and its incidence is now increasing in developed countries.[1] It is difficult to confirm the diagnosis of tuberculosis as the organism grows slowly. Culture for up to eight weeks may be required to isolate the organism and further time is needed to type the mycobacteria. Bactec provides sensitive detection of radioactive carbon (^{14}C) released from the metabolised fatty acids in the medium and accelerates the time to diagnosis, but it may still take one to two weeks.

Amplification of specific mycobacterial DNA by polymerase chain reaction (PCR) offers the possibility of a rapid, sensitive, and specific test for tuberculosis. This technique raises interesting possibilities, including the ability to analyse a range of samples including cerebrospinal fluid and blood to diagnose tuberculosis, the study of subtle genomic differences in mycobacteria to explain the epidemiology of tuberculosis, and rapid identification of mycobacteria in immunocompromised patients.

Two of the many mycobacterial DNA sequences are candidates for a diagnostic test. These are the gene encoding the 65kDa heat shock protein (65kDa Ag)[2] and the sequence of IS6110, a mycobacterium specific DNA sequence present as repeats in the genome.[3–7] Studies using probes for the 65kDa Ag gene and probes combined with DNA amplification have succeeded in identifying mycobacteria obtained after routine culture,[8–12] and from Bactec culture.[13,14] Combined amplification and probing has also been used successfully in preliminary studies on clinical samples from patients with tuberculosis.[15,16]

The relation between the bacteriological identification of *Mycobacterium tuberculosis* and clinical disease is well recognised. Detection of specific DNA takes no account of organisms' viability or pathogenicity. There is no information on the relation between the identification of mycobacterial DNA in clinical samples by the new sensitive techniques of PCR and of probing and the presence of clinical disease. In this study, the results of DNA amplification by PCR of the two mycobacterial DNA sequences IS6110 and the 65kDa Ag gene in sputum and airway secretions are compared in relation to the clinical status of the patient.

Methods

PATIENTS AND CLINICAL SAMPLES

Sputum samples and samples from the bronchoscopy trap and bronchoalveolar lavage were obtained from 87 patients attending the chest clinic. The diagnosis of tuberculosis was made on the basis of clinical, radiological, and mycobacterial culture results. Six patients with bacteriologically confirmed active tuberculosis,

18 with past tuberculosis, 9 contacts of patients with tuberculosis, and 54 patients with other respiratory diseases were recruited. Pure laboratory strains of *M tuberculosis* were obtained as positive controls (gift of Dr I Brown, Bacteriology Department, St Mary's Hospital Medical School).

DNA PREPARATION

Sodium hydroxide was added to each sample to a final concentration of 0·05M NaOH. The sample was boiled for 10 minutes and the DNA was extracted twice in phenol/chloroform/isoamyl alcohol (25:24:1) and once in chloroform/isoamyl alcohol, followed by precipitation in 70% ethanol and resuspension in water.

DNA AMPLIFICATION

DNA amplification[17] used primers IS1 and IS2 to recognised sequences in the IS6110 gene,[4] and primers TB1 and TB2 to the 65kDa Ag gene[18] (table 1). In brief, the 50 μl reaction mixture contained 10 mmol/l tris-hydrochloric acid (pH 8·3) (Sigma, Poole, UK), 50 mmol/l potassium chloride (BDH, Poole, UK), 1·5 mmol/l magnesium chloride (Sigma), 10 mg/l gelatin (BDH), 200 μmol/l of each deoxyribonucleotide (Pharmacia, Uppsala, Sweden), 1 unit TAQ polymerase (Perkin Elmer Cetus, Emeryville, Ca, USA), 50 pmol of oligonucleotides TB1 and TB2 or IS1 and IS2 (synthesised on a Cyclone (Milligen, Watford, UK)), and 5 μl of clinical sample DNA containing 0·2–1·0 μg total DNA. Light mineral oil (25 μl) (Perkin Elmer Cetus) was layered over each mixture. The PCR conditions for IS6110 DNA amplification were an initial denaturation step of 94°C for five minutes, after which TAQ polymerase was added and 35 cycles of 94°C for two minutes, 68°C for two minutes and 72°C for two minutes were utilised, with an extension of five seconds added per cycle to the 72°C phase. For 65kDa Ag DNA identification, an initial denaturation step of 96°C for seven minutes was followed by 35 cycles of amplification under conditions of 94°C for one minute, 60°C for two minutes, 72°C for two minutes, with a final extension step at 72°C for 10 minutes.

DNA IDENTIFICATION

Amplification products were electrophoresed in a 2% agarose gel containing ethidium bromide. The specific fragments (123 base pairs for IS6110, 383 for 65kDa Ag) were initially identified by visualisation under ultraviolet light. DNA was transferred to Hybond N membrane (Amersham Pharmaceuticals, Amersham, Bucks, UK) and hybridised overnight at 55°C with the ^{32}P labelled oligonucleotide IS3 or TB4 (table 1).[15 18] The oligonucleotide had been previously end labelled by kinase (BRL, Gaithersburg, MD, USA) with ^{32}P labelled dATP (ICN, High Wycombe, UK). After hybridisation, the membrane was washed under increasingly stringent conditions with a final wash in 0·03 M NaCl, 6mM Na citrate and 0·1% sodium dodecyl sulphate (Sigma) at 55°C. The presence of mycobacterial DNA was confirmed by autoradiography with exposures of 2–24 hours.

Table 1 5'-3' Sequence of oligodeoxynucleotides used as primers and probes

TB1—5'GAGATCGAGCTGGAGGATCC-3'
TB2—5'-AGCTGCAGCCCAAAGGTGTT-3'
TB4—5'-CGAAATCGCTGCGGTGGCCG-3'
IS1 —5'-CCTGCGAGCGTAGGCGTCGG-3'
IS2 —5'-CTCGTCCAGCGCCGCTTCGG-3'
IS3 —5'-CATAGGTGAGGTCTGCTACC-3'

All experiments included positive control samples where mycobacterial DNA was included and negative control samples where sample DNA was omitted.

Results

IDENTIFICATION OF IS6110 AND 65KDA AG DNA IN SPUTUM FROM PATIENTS WITH TUBERCULOSIS

In initial studies, IS6110 DNA was detected in sputum from patients with tuberculosis but not from patients with other lung diseases by both ethidium bromide staining and subsequent probing with ^{32}P labelled IS3. Similarly, amplification of 65kDa Ag DNA and subsequent probing with ^{32}P labelled TB4 oligonucleotide identified the 65kDa Ag DNA in sputum from a patient with tuberculosis, while sputum from a patient with small cell lung cancer contained no 65kDa Ag DNA.

REQUIREMENT FOR ^{32}P PROBING

To determine whether ethidium bromide staining and ^{32}P probing were required to identify IS6110, the results in 55 patients were compared. Fourteen cases were positive by both indicators and 41 negative by both systems. No cases were positive on one detection system only, so ethidium bromide staining was used alone to identify IS6110 DNA in subsequent clinical samples. When ethidium bromide staining and ^{32}P probing were compared for the 65kDa Ag DNA, 11 cases were identified by both ethidium bromide and ^{32}P TB4 and eight by ^{32}P TB4 alone. ^{32}P probing was used to confirm the presence of 65kDa Ag DNA in subsequent clinical samples.

REPRODUCIBILITY

Reproducibility of IS6110 and 65kDa Ag gene detection was assessed when DNA from 46 samples (26 containing IS6110 DNA and 34 containing 65kDa Ag DNA) was subjected to amplification for IS6110 and 65kDa Ag DNA on a second occasion. IS6110 DNA was identified by ethidium bromide staining in 25 of 26 repeat assays (96%). 65kDa Ag DNA was identified by ethidium bromide staining in 28 of 34 repeat assays (82%), and after ^{32}P probing in 39 of 46 (85%) of assays.

COMPARISON OF IS6110 AND 65KDA AG DNA IDENTIFICATION IN RESPIRATORY SAMPLES FROM PATIENTS WITH LUNG DISEASE

Ninety respiratory samples—from sputum, bronchoscopy trap, and bronchoalveolar lavage—were collected from 87 patients. Several samples were obtained from seven

patients, and three patients gave more than one type of sample. IS6110 DNA was found in samples from all six patients with active pulmonary tuberculosis (seven of eight samples). It was also found in 15 of 18 patients with past tuberculosis (16 of 20 samples), five of nine tuberculosis contacts (five of nine samples), and five of 54 patients with non-tuberculous lung disease (six of 56 samples) (figure, table 2). Thus IS6110 DNA detection was a sensitive test for tuberculosis, but was not specific for active disease.

Detection of the 65kDa Ag DNA was also very sensitive, in that it was found in samples from four of four patients with active tuberculosis (four of five samples), four of four patients with past tuberculosis (five of eight samples) and four of four contacts (four of six samples). It was also present in samples from 14 of 42 patients with non-tuberculous lung disease (15 of 43 samples), suggesting lack of specificity.

The findings were similar when the results from each sample were compared (table 2). Both IS6110 and 65kDa Ag DNA were found in over three quarters of the samples from patients with active tuberculosis, and in over half the samples from patients with past tuberculosis or from contacts of cases of tuberculosis. IS6110 DNA was identified in six of 56 samples from patients with a lung disease other than tuberculosis, and 65kDa Ag DNA was identified in 15 of 43 samples from these patients. There was no difference in the yield of positive results when sputum, bronchoscopy trap, and bronchoalveolar lavage samples were compared.

Discussion

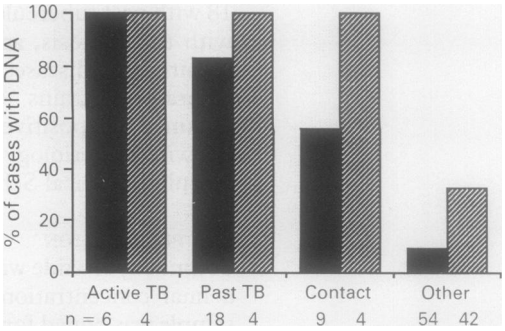
Many different mycobacterial genes have been proposed as targets for the detection of *M tuberculosis* in clinical samples.[4 5 19–24] The optimal target DNA has yet to be defined and the relation between positive identification of *M tuberculosis* DNA and clinical disease is unknown. We have compared the detection of two mycobacterial DNA sequences as a diagnostic test for tuberculosis and correlated the presence of these DNAs with clinically active or past exposure to tuberculosis.

The study makes two observations. Firstly, IS6110 DNA detection had some advantages over 65kDa Ag detection in the diagnosis of tuberculosis. IS6110 DNA was detected in a smaller proportion of patients with diseases unrelated to tuberculosis than 65kDa Ag DNA. IS6110 was also technically easier to detect than 65kDa Ag. The second observation was the high detection rate of both IS6110 and 65kDa Ag DNA in samples from patients with past tuberculosis and contacts of cases of tuberculosis.

In this study, both IS6110 DNA and 65kDa Ag DNA were identified in most samples from patients with tuberculosis, past tuberculosis and contacts. We had anticipated that IS6110 DNA detection would have been greater because unlike the 65kDa Ag DNA it is present as multiple repeats in the *M tuberculosis* genome.[3] For any mycobacterium there are thus several IS6110 sequences but only one 65kDa Ag DNA sequence available for amplification.

The ability to identify an organism through its DNA depends on the specificity of a particular sequence of DNA for a given organism and the specificity of the selected primers for a given DNA sequence. The primers we used were highly specific for each DNA sequence and were selected because they had been used by others.[4 18] Their specificity was confirmed by a search in Genbank (Clinical Research Centre, Harrow, UK, 1991). Both pairs of primers (IS1/IS2 and TB1/TB2) had 100% homology with IS6110 and 65kDa Ag DNA. Individual members of each pair of primers have 70–90% homology with a few DNA sequences from genes of organisms other than *M tuberculosis*. None of the genes from these organisms, however, had sequences with homology to the second member of the pair of primers. Thus, even if these other organisms had been present in the sample, their DNA sequence would not have been amplified as this amplification occurs only when both primers bind to the target sequence. Finally, the specificity was confirmed when the amplified products had the predicted size, indicating that these products contained the anticipated number of nucleic acid bases.

The presence of 65kDa Ag DNA in samples from up to a third of patients with a diagnosis unrelated to tuberculosis suggests that PCR amplification of 65kDa Ag DNA may have limited clinical use. Because the less sensitive detection system for 65kDa Ag yielded positive



Percentage of cases classified as active tuberculosis, past tuberculosis, tuberculosis contact, or other diagnosis in whom IS6110 DNA (black) or 65kDa Ag DNA (hatched) was found in respiratory samples (total number of cases in each group as indicated).

Table 2 Number of samples containing IS6110 or 65kDa Ag DNA divided by the total number of samples from sputum, bronchoscopy trap (Trap), and bronchoalveolar lavage fluid (BAL) and listed according to the clinical classification (in nearly all cases, each sample corresponds to a different patient, as only seven patients gave more than one sample, and only three gave samples in more than one category)

	Active tuberculosis	*Past tuberculosis*	*Contact*	*Other diagnosis*
IS6110:				
Sputum	2/2	9/13	4/5	2/15
Trap	4/5	6/6	1/2	3/24
BAL	1/1	1/1	0/2	1/17
Total	7/8	16/20	5/9	6/56
65kDa Ag:				
Sputum	2/3	3/5	4/5	5/11
Trap	2/2	1/2	0/2	6/17
Bal	0/0	1/1	0/0	4/15
Total	4/5	5/8	4/6	15/43

results and the more sensitive detection system for IS6110 DNA negative results in samples from most of these patients the DNA containing 65kDa Ag probably did not originate from *M tuberculosis*. The DNA sequence of the 65kDa Ag shares homology with the DNA sequence of *M leprae*,[2] the common antigen of pseudomonas, and the groEL protein of *Escherichia coli*.[25-27] The possibility that the PCR used in this study was amplifying DNA from other, non-tuberculous, mycobacteria in the lungs was reduced by probing with TB4, which bound with high affinity to *M tuberculosis* DNA only. The 65kDa Ag gene in *M tuberculosis* is thought to be identical in terms of amino acid sequence with the same gene in *M africanum* and *M bovis* BCG.[28] Similarly, IS6110 DNA is also found in *M bovis* and *M simiae* but not in other mycobacterial species.[4] We cannot exclude the possibility that the positive findings with IS6110 or 65kDa Ag DNA were the result of infection by other members of this small family of *M tuberculosis* like mycobacteria.

The other technical advantage of using IS6110 detection to diagnose tuberculosis was that it was more rapid. Results were documented by ethidium bromide staining of amplified products on a gel without the need to confirm the nature of the band by Southern blotting and hybridisation with the ^{32}P labelled IS3 oligodeoxynucleotide probe. As sequences similar to 65kDa Ag DNA are present in other mycobacteria, probing with ^{32}P labelled TB4 was required to confirm that the amplified product of the 65kDa Ag was derived from *M tuberculosis*. This additional step also increased the sensitivity of the test. Amplified 65kDa Ag DNA from eight patients was not clearly visualised by ethidium bromide staining but was identified following ^{32}P probing with the TB4 oligonucleotide.

The detection of both IS6110 and 65kDa Ag DNA in samples from patients with past tuberculosis and contacts of patients with tuberculosis was unexpected and poses a difficulty if these tests are to be clinically useful. Technical factors are unlikely to account for this observation. Although contamination of reagents by target DNA is a common problem with PCR,[29 30] this was excluded in our experiments as the negative controls were consistently negative. The laboratory equipment was cleaned regularly and checked by the "wipe test."[31] Initial concern that contamination of the bronchoscope might result in false positive results was unfounded as the incidence of false positive results in samples obtained at bronchoscopy was similar to that in sputum (table 2). We did not perform all the analyses in duplicate or triplicate, and this would be prohibitively expensive for a test aimed at routine clinical practice. The reproducibility was better for detection of IS6110 DNA than for 65kDa Ag DNA in that identical results were obtained in 96% of duplicate assays for IS6110 DNA compared with 85% for 65kDa Ag DNA.

An alternative explanation for the positive results in samples from patients with past tuberculosis and from contacts might have been that, unlike bacteriological culture, PCR, with or without probing, can detect non-viable organisms. It would be expected to detect mycobacteria in samples from patients being treated for tuberculosis some months after culture had become negative, as suggested by Brisson-Noel *et al.*[15] Our data suggest that *M tuberculosis* DNA from organisms that are not viable or rendered non-pathogenic was present in respiratory samples from many patients for long periods after completion of tuberculosis treatment, and in samples from contacts of patients with tuberculosis.

It is questionable whether PCR amplification in its present form is suitable as a routine clinical test. The technique involves three basic steps, DNA extraction, PCR amplification, and visualisation of the amplified product. DNA extraction uses toxic organic chemicals and there is a risk of contamination of the sample with DNA from other samples or amplified product from a previous reaction. PCR requires expensive reagents and manual dexterity in handling very small volumes without causing contamination. Visualisation of the product involves running a gel and either staining the gel with ethidium bromide or transferring the DNA to a membrane and probing this with a radiolabelled probe.

Comparison of IS6110 and 65kDa Ag DNA detection on technical grounds and in terms of their correlation with clinical disease suggests that detection of IS6110 DNA in respiratory samples may prove more useful in the clinical setting. A negative result with this technique seemed to identify those patients in whom mycobacteria would not subsequently be detected. Further refinements are required to discriminate patients with tuberculosis from those with previously treated tuberculosis or contacts of patients with tuberculosis. The technique provided only qualitative data on the presence or absence of specific DNA. With the development of PCR methods to quantitate the number of molecules of a particular DNA in a sample, it may be possible to discriminate further between different patient groups.

This sensitive technique may also be used to investigate the relation between infection of the host by *M tuberculosis* and the development of clinical disease. The identification of *M tuberculosis* DNA in samples from patients with treated tuberculosis and from contacts suggests that host immune responses may contain the organisms in a non-viable or non-pathogenic dormant state for long periods. This is in keeping with clinical experience, in that reactivation of past tuberculosis is a well recognised consequence of iatrogenic or acquired immunodeficiency.

The authors would like to thank Dr Howard Beswick for his assistance in the synthesis of the oligonucleotides and the use of Genbank. This work was supported by grants from the Chest, Heart and Stroke Association and the Locally Organised Research Committee. It was presented in part at the American Thoracic Society meeting, Anaheim, May 1991.

1 Murray JF. The white plague: down and out, or up and coming? *Am Rev Respir Dis* 1989;**140**:1788–95.
2 Shinnick TM. The 65-kilodalton antigen of *Mycobacterium tuberculosis*. *J Bacteriol* 1987;**169**:1080–8.

3 Eisenach KD, Crawford JT, Bates JH. Repetitive DNA sequences as probes for *Mycobacterium tuberculosis*. *J Clin Microbiol* 1988;**26**:2240–5.
4 Eisenach KD, Cave MD, Bates JH, Crawford JT. Polymerase chain reaction amplification of a repetitive DNA sequence specific for *Mycobacterium tuberculosis*. *J Infect Dis* 1990;**161**:977–81.
5 Patel RJ, Freis WU, Piessens WF, Wirth DF. Sequence amplification by polymerase chain reaction of a cloned DNA fragment for identification of *Mycobacterium tuberculosis*. *J Clin Microbiol* 1990;**28**:513–8.
6 Thierry D, Cave MD, Eisenach KD, Crawford JT, Bates JH, Gicquel B, Guesdon JL. IS6110, an IS-like element of *Mycobacterium tuberculosis* complex. *Nucleic Acids Res* 1990;**18**:188–9.
7 Thierry D, Brisson-Noel A, Vincent-Levy-Frebault V, Nguyen S, Guesdon J-L, Gicquel B. Characterization of a *Mycobacterium tuberculosis* insertion sequence, IS6110, and its application in diagnosis. *J Clin Microbiol* 1990; **28**:2668–73.
8 Gonzales R, Hanna BA. Evaluation of Gen-Probe DNA hybridization systems for the identification of *Mycobacterium tuberculosis* and *Mycobacterium avium-intracellulare*. *Diagn Microbiol Infect Dis* 1987;**8**:69–77.
9 Roberts MC, McMillan C, Coyle MB. Whole chromosome DNA probes for rapid identification of *Mycobacterium tuberculosis* and *Mycobacterium avium* complex. *J Clin Microbiol* 1987;**25**:1239–43.
10 Drake TA, Hindler AJA, Berlin OG, Bruckner DA. Rapid identification of *Mycobacterium avium* complex in culture using DNA probes. *J Clin Microbiol* 1987;**25**:1442–5.
11 Musial CE, Tice LS, Stockman L, Roberts GD. Identification of mycobacteria from culture by using the Gen-Probe rapid diagnostic system for *Mycobacterium avium* complex and *Mycobacterium tuberculosis* complex. *J Clin Microbiol* 1988;**26**:2120–3.
12 Sherman I, Harrington N, Rothrock A, George H. Use of a cutoff range in identifying Mycobacteria by the Gen-Probe rapid diagnostic system. *J Clin Microbiol* 1989;**27**:241–4.
13 Kiehn TE, Edwards FF. Rapid identification using a specific DNA probe of *Mycobacterium avium* complex from patients with acquired immunodeficiency syndrome. *J Clin Microbiol* 1987;**25**:1551–2.
14 Ellner PD, Kiehn TE, Cammarata R, Hosmer M. Rapid detection and identification of pathogenic mycobacteria by combining radiometric and nucleic acid probe methods. *J Clin Microbiol* 1988;**26**:1349–52.
15 Brisson-Noel A, Gicquel B, Lecossier D, Levy-Frebault V, Nassif X, Hance AJ. Rapid diagnosis of tuberculosis by amplification of mycobacterial DNA in clinical samples. *Lancet* 1989;ii:1069–71.
16 Shanker P, Manjunath N, Mohan KK, Prasad K, Behari Shrinwas M, Ahuja GK. Rapid diagnosis of tuberculosis meningitis by polymerase chain reaction. *Lancet* 1991; **337**:5–7.
17 Saiki RJ, Gelfand DH, Stoffel S, Scharf SJ, Higuchi R, Horn GT, *et al*. Primer-directed amplification of DNA with a thermostable DNA polymerase. *Science* 1988;**239**:487–91.
18 Hance AJ, Grandchamp PB, Levy-Frebault V, Lecossier D, Rauzier J, Bocart D, *et al*. Detection and identification of mycobacteria by amplification of mycobacterial DNA. *Mol Microbiol* 1989;**3**:843–9.
19 De Wit D, Steyn L, Shoemaker S, Sogin M. Direct detection of *Mycobacterium tuberculosis* in clinical specimens by DNA amplification. *J Clin Microbiol* 1990;**28**:2437–41.
20 Fries JW, Patel RJ, Piessens WF, Wirth DF. Genus- and species specific DNA probes to identify mycobacteria using the polymerase chain reaction. *Mol Cell Probes* 1990;**4**:87–105.
21 Hermans PW, Schuitema AR, Van-Soolingen D, Verstynen CP, Bik EM, Thole JE, *et al*. Specific detection of mycobacteria tuberculosis complex strains by polymerase chain reaction. *J Clin Microbiol* 1990;**28**:1204–13.
22 Hermans PW, van Soolingen S, Dale JW, Schuitema ARJ, McAdam R A, Catty D, *et al*. Insertion element IS986 from *Mycobacterium tuberculosis*: a useful tool for diagnosis and epidemiology of tuberculosis. *J Clin Microbiol* 1990;**28**:2051–8.
23 Kaneko K, Onodera O, Miyatake T, Tsuji S. Rapid diagnosis of tuberculous meningitis by polymerase chain reaction (PCR). *Neurology* 1990;**40**:1617–8.
24 Pao CC, Yen JB, Maa JS, Fiss EH, Chang CH. Detection and identification of *Mycobacterium tuberculosis* by DNA amplification. *J Clin Microbiol* 1990;**28**:1877–80.
25 Young D, Garbe T, Lathigra R, Abou-Zeid C, Zhang Y. Characterization of prominant protein antigens from mycobacteria. *Bull Int Union Tuberc Lung Dis* 1991;**66**: 47–51.
26 Young DB, Kaufmann SHE, Hermans PWM, Thole JER. Mycobacterial protein antigens: a compilation. *Mol Microbiol* 1992;**6**:133–45.
27 Shinnick TM, Vodkin MH, Williams JC. The *Mycobacterium tuberculosis* 65-kilodalton antigen is a heat shock protein which corresponds to common antigen and to the *Escherichia coli* groEL protein. *Infect Immun* 1988;**56**: 446–51.
28 Lu MC, Lien MH, Becker RE, Heine HC, Buggs AM, Lipovsek D, *et al*. Genes for immunodominant protein antigens are highly homologous in *Mycobacterium tuberculosis*, *Mycobacterium africanum*, and the vaccine strain *Mycobacterium bovis BCG*. *Infec Immun* 1987;**55**:2378–82.
29 Kwok S, Higuchi R. Avoiding false positives with PCR. *Nature* 1989;**339**:237–8.
30 Porter-Jordan K, Garret CT. Source of contamination in polymerase chain reaction assay. *Lancet* 1990;**335**:1220.
31 Cone RW, Hobson AC, Huang M-L W, Fairfax MR. Polymerase chain reaction decontamination: the wipe test. *Lancet* 1990;ii:686–7.