Routine application of the polymerase chain reaction for detection of *Mycobacterium tuberculosis* in clinical samples

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Abstract

**Aim**—To investigate the use of the polymerase chain reaction (PCR) in the routine laboratory for the detection of *Mycobacterium tuberculosis* in clinical samples.

**Methods**—Samples were divided and processed separately for the detection of *M. tuberculosis* by microscopy, culture and PCR. PCR was performed with primers specific for the insertion element IS6110 and the product was analysed by agarose gel electrophoresis, Southern blotting or dot blotting and hybridisation with a digoxigenin labelled internal probe. Each sample was tested for inhibitors of Taq polymerase with the aid of an internal control. Multiple negative and positive controls were used to monitor each step of the procedure.

**Results**—The data from two laboratories, using the same operating procedures, were combined. Of 1957 specimens, 79 (4%) were culture and PCR positive, while 1839 (93-9%) were negative in both tests. Thirty specimens (1-5%) were PCR positive only and nine (0-5%) were culture positive but PCR negative.

**Conclusion**—Using culture and clinical history as the gold standard, sensitivity and specificity for PCR were 92-1% and 99-8%, respectively. With elaborate precautions, PCR is a suitable and reliable method for the detection of *M. tuberculosis* in clinical samples in a routine microbiology laboratory.

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Keywords: *Mycobacterium tuberculosis*, PCR, application, clinical samples.

After years of declining incidence, the number of registered patients with tuberculosis in The Netherlands has increased since 1987. Rapid diagnosis of infectious cases of tuberculosis is of major importance for public health services. Drawbacks of the conventional *Mycobacterium tuberculosis* detection techniques are that direct staining for acid fast bacilli lacks sensitivity and specificity and that culturing of mycobacteria is very time-consuming. Furthermore, microscopy and culture are difficult in cases of extrapulmonary infection, and it is for this reason that some laboratories still use inoculation of guinea pigs for samples which are difficult to obtain. With the polymerase chain reaction (PCR) it is possible to identify *M. tuberculosis* directly in clinical samples. Many papers describe the development and the use of DNA amplification methods for detection of *M. tuberculosis*. However, Noordhoek et al recently showed that reliable detection of *M. tuberculosis* using PCR can be difficult because of the occurrence of false positive results through contamination and lack of sensitivity, usually caused by inhibitors present in Taq polymerase by biological substances present in the sample.

In The Netherlands, the prevalence of tuberculosis infections is still very low, less than 4% of the samples submitted to a routine laboratory are culture positive for *M. tuberculosis*. Thus, if the few positive results are not to be missed, the sensitivity of a rapid and specific test needs to be close to 100%. To investigate the feasibility of routine PCR testing in a clinical laboratory, both conventional tests and PCR were carried out on a large number of clinical samples. Insertion sequence IS6110, which is specific for the *M. tuberculosis* complex group of bacteria, was used as the target for DNA amplification. The protocol was optimised to obtain maximal sensitivity and specificity.

**Methods**

**CLINICAL SPECIMENS**

The samples tested at the microbiology departments of the Leeuwarden or Enschede Public Health Laboratories were routine specimens obtained from patients suspected of having tuberculosis. In Leeuwarden some of the samples were preselected. During the first four months of the project, all sputum and bronchial washings received in the laboratory were included in the study. Thereafter, PCR on sputum and bronchial washings was carried out only when acid fast bacilli were found on microscopy or when there was strong clinical evidence of tuberculosis. On the day of receipt, taking appropriate precautions in a laminar flow hood, the specimens were divided into two portions, one for PCR and one for conventional detection methods. In the mycobacteria laboratory, direct smears were analysed for acid fast bacilli using microscopy after staining with auramine and/or Ziehl-Neelsen. Sputum, bronchial washing and pleural fluid samples were decontaminated using standard procedures and inoculated onto Löwenstein-Jensen medium. Specimens from other sites of the body were, after standard pretreatment, inoculated onto Löwenstein-Jensen and/or Middlebrook 7H11 medium.
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PRETREATMENT OF SAMPLES FOR PCR

PCR was performed according to the recommendations of Kwok,10 using three separate locations for the preparation of the PCR reaction mixture, sample and DNA preparation, and the analysis of PCR products, respectively. Positive displacement pipettes or filtertiks were used throughout and for most of the specimens dUTP-uracil-N-glycosylase (UDG) (Gibco-BRL) was used to avoid contamination with dUTP from previous reactions.8,11 Sputum, bronchial washing, pleural fluid, and pus samples were pretreated with 1 to 10 volumes of 0-5 M NaOH/0-05 M sodium citrate/0-5% N-acetyl-L-cysteine and centrifuged for 15 minutes at 15 000 x g in a microcentrifuge. Samples which did not need liquefaction, such as urine and cerebrospinal fluid, were concentrated by centrifugation at 15 000 x g in a microcentrifuge. The pellets were washed once with a solution containing 1% Triton X100, 20 mM Tris/HCl (pH 8·3) and 1 mM EDTA (TEX), and resuspended in 100 µl TEX. Tissue biopsy specimens were homogenised with razor blades and incubated for 12 to 16 hours at 55°C in 100 to 500 µl lysis buffer containing 1% sodium dodecyl sulphate (SDS), 100 µg/ml proteinase K in 10 mM Tris/HCl (pH 8·3). Proteinase K was inactivated by heating at 100°C for 15 minutes; 100 µl of the lysate was used for further analysis. DNA was extracted from all samples as described previously.12 Briefly, 1 ml guanidinium thiocyanate (GuSCN) containing L6 buffer and 25 µl silica particles were added to 100 µl of the pretreated sample suspension. After thorough mixing, the samples were incubated for 20 minutes at 80°C for complete lysis of mycobacteria, followed by 15 to 60 minutes mixing at room temperature to allow binding of the bacterial DNA to the silica particles. The particles were washed twice with L2 buffer, twice with 70% ethanol and once with acetone. DNA was released by addition of 65 to 100 µl distilled water and incubation for 30 to 60 minutes at 60°C. The number of samples analysed at any one time was variable, depending on the number of clinical samples sent to the laboratory. To monitor for cross-contamination during pretreatment and DNA extraction, one negative sample to every four or five clinical samples was included in each series. The efficiency of DNA extraction was monitored in each test by including two sputum samples to which 100 or 1000 Mycobacterium bovis BCG bacteria had been added.

POLYMERASE CHAIN REACTION

PCR was performed using primers INS1 and INS2 homologous for sequences in the insertion sequence IS6110, which is specific for M tuberculosis complex bacteria.13 The extracted DNA (10 µl) was added to 40 µl of PCR mixture. In Leeuwarden the samples were also tested at a 10-fold dilution. The PCR mixture was prepared as described previously.3 dUTP was used instead of dTTP and 0·2 to 0·02 units of UDG were added per 40 µl of PCR mixture.8 Two approaches were used to check each sample for inhibition of Taq polymerase. In Leeuwarden 50 fg DNA from recombinant Mycobacterium smegmatis strain 1008 was added to the PCR mixture. M smegmatis 1008 contains a modified IS6110 sequence.13 When this modified IS6110 sequence is amplified using primers INS1 and INS2, a 301 base pair fragment is obtained which is distinguishable from the 245 base pair PCR product from M tuberculosis on agarose gel electrophoresis. In Enschede 10 fg of M tuberculosis DNA was added to a duplicate of each sample.8

Performance of the amplification reaction was monitored by testing one sample without DNA and three samples containing 2·5, 25 and 250 fg M tuberculosis DNA. The PCR was performed in a thermocycler (Perkin Elmer 480 or Bio-med 60 for Leeuwarden and Enschede, respectively). In Leeuwarden the PCR cycle profile was optimised by using a "touch down" protocol to increase the specificity of the reaction.14 The reaction started with six minutes at 37°C for UDG incubation and six minutes at 94°C for UDG inactivation and DNA denaturation, followed by PCR cycles of one minute at 94°C for denaturation, one minute for primer annealing and one minute for primer extension at 72°C. The annealing temperature of the first 10 cycles was decreased by 1°C every second cycle from 70°C to 66°C, followed by 40 cycles with an annealing temperature of 65°C. In Enschede the PCR cycle profile was used as described previously.3 PCR products were kept at 72°C until analysis.

ANALYSIS OF PCR PRODUCTS

In Leeuwarden 10 µl of the amplification product was examined by ethidium bromide agarose gel electrophoresis and Southern blotting. In Enschede the products were analysed by dot blotting. In both laboratories the blots were hybridised with an internal 188 base pair probe which was labelled with digoxigenin during amplification of M tuberculosis DNA.3 Probe-target hybrids were detected using the DIG nucleic acid detection kit (Boehringer Mannheim) as recommended by the manufacturer. Results were considered valid only if all controls were correct. Samples with a negative PCR result were only reported as negative when there was no evidence of inhibition in the sample. Samples with a positive result for M tuberculosis were retested for confirmation.

Results

COMPARISON OF PROCEDURES OF THE TWO LABORATORIES

This study combines the results from two laboratories using similar protocols for detecting M tuberculosis by PCR. The sensitivity of the procedures in both laboratories was compared by testing serial dilutions of chromosomal M tuberculosis DNA and by exchanging sets of test sputum samples containing 100 and 1000 M bovis BCG bacteria. The two laboratories had the same level of detection.
INHIBITION OF TAQ POLYMERASE

All samples were tested for the presence of substances inhibiting Taq polymerase by using *M. smegmatis* 1008 DNA or by spiking duplicate samples with *M. tuberculosis* DNA. Inhibition was found in a few samples (figure, lanes 1 and 3). In many cases inhibition disappeared after the extracted DNA was diluted 10-fold (figure, lanes 2 and 4). However, dilution also decreased the detection limit of the test. This was shown in smear negative samples in which only a few colonies of mycobacteria were cultured. Undiluted samples were PCR positive, whereas the samples diluted 10-fold were negative (figure, lanes 9 and 10). The undiluted samples contained the minimal amount of target DNA to give a detectable PCR product. In Leewarden no PCR result could be obtained in nine (1.5%) of 605 samples because of persistent inhibition. In Enschede, where samples were not routinely tested at the 10-fold dilution, 61 (4.1%) of 1494 samples showed inhibition.

COMPARISON OF MICROSCOPIEC, CULTURE AND PCR RESULTS

Over 14 months, 2099 specimens from 1638 different patients were submitted for detection of *M. tuberculosis* by microscopy, PCR and culture. No culture results were obtained in 55 specimens because of contamination with other bacteria. In 17 samples mycobacteria other than *M. tuberculosis* were cultured. Persistent inhibition of Taq polymerase was found in 70 samples. These 142 samples were not evaluated further in this study.

The results of the *M. tuberculosis* PCR were compared with culture for *M. tuberculosis* in 1957 specimens (table 1). Of the samples, 4% were culture and PCR positive and 94% were negative in both tests. Thirty samples were PCR positive and culture negative. Of these 30 samples, 26 originated from patients with clinical symptoms of tuberculosis or from patients in whom a previous sample was culture positive. Most of these patients were receiving treatment with antituberculosis drugs. The positive PCR supported the clinical diagnosis of tuberculosis and it is unlikely that these positive PCR results were due to contamination of samples with *M. tuberculosis* or with amplicons. The remaining four samples came from three different patients. Retesting of extracted DNA from one patient's pleural fluid sample resulted in weak equivocal PCR products. New DNA extracts from the other two patients were also weakly positive on PCR. The clinical histories of the three patients did not show evidence of tuberculosis infection at investigation or one year after sampling. Although dUTP was used throughout, UDG was not used during the early phase of the study. These positive results occurred during this early period. Therefore, we have to regard these PCR results as probable false positives. Contamination might have occurred during pretreatment or DNA extraction.

We found nine PCR negative samples, although acid fast bacilli were observed in direct smears and *M. tuberculosis* was cultured. The *M. tuberculosis* strains cultured from these samples were all PCR positive, indicating that the *IS6110* fragment was present. The negative PCR results in these clinical specimens were probably because of unequal distribution of mycobacteria in the sample. In the smear preparations few acid fast bacilli were observed and only one or two of the three inoculated tubes containing Löwenstein-Jensen medium were positive.

To evaluate our PCR, we have used the definitions "true" positive and "true" negative. We regarded a test result as "true" positive when *M. tuberculosis* was cultured, when direct microscopy and PCR were positive but culture was negative and when direct microscopy and culture were negative but PCR was positive and other material from the patient was positive on culture or had been positive in the past. In table 2 the results of culture, microscopy and PCR are compared using the definitions as described above. These results show that our PCR test has a specificity of 99.8% and a sensitivity of 92.1%, whereas culture had a sensitivity of 77.2%. In total, the PCR test was positive in 34 (1.7%) "true" positive samples with a negative microscopy result.

Hybridisation of blots increased the sensitivity of the test (data not shown). In a few
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Table 2 Comparison of results obtained on culture, smear and PCR with true positives and true negatives

<table>
<thead>
<tr>
<th>PCR positive</th>
<th>Culture positive</th>
<th>Culture negative</th>
<th>PCR negative</th>
<th>Culture positive</th>
<th>Culture negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Smear positive</td>
<td>63</td>
<td>8</td>
<td>9</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Smear negative</td>
<td>16</td>
<td>18</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

cases a 245 base pair band was not visible on the gel and was only detected after hybridisation of the Southern blot. Hybridisation was also necessary to control the specificity of the reaction. We found that a *Nocardia farcinica* isolate from a sputum sample produced a 245 base pair fragment after PCR with the INS1-INS2 primer set which did not hybridise with the 188 base pair internal probe. This PCR product will be analysed further.

Discussion

PCR is a rapid, sensitive and specific DNA amplification technique for the detection of *M tuberculosis*. Most *M tuberculosis* complex strains contain multiple copies of the insertion sequence IS6110, which makes this element a widely used target for amplification. Recently, about seven *M tuberculosis* strains lacking IS6110 have been reported.15-17 However, these strains were isolated from patients in South East Asia and all European strains tested to date contain one or more IS6110 copies (D van Soolingen, personal communication). We consider the IS6110 element to be suitable for the detection of *M tuberculosis* infection in our region.

Three studies have described the feasibility of using a PCR test in the routine clinical laboratory where many samples, including a large proportion of negative specimens, need to be tested daily.18-20 Using PCR without prior DNA purification, Claridge *et al* reported a sensitivity of 86-1%, while 7-3% of the specimens contained Taq polymerase inhibitors.18 Forbes *et al* achieved a sensitivity of 87-2% without monitoring for inhibition.19 Nolte *et al* used internal controls during PCR and reported that Taq polymerase was inhibited in 10 to 17% of sputum samples when no DNA purification was performed.20 After phenol extraction the inhibiting substances were removed from all samples. Inhibition of Taq polymerase is a problem described by many investigators.21-24 We have chosen a PCR method with a DNA extraction procedure in which each individual sample is monitored for enzyme inhibition in order to achieve the highest possible sensitivity. In The Netherlands the number of culture positive specimens does not exceed 4%, so we considered sensitivity a priority. We found that, even after DNA extraction with GuSCN and silica particles, some samples contained inhibiting substances. Dilution of the sample led to some decrease in sensitivity although, for many samples, the benefit of diluting the inhibiting substances was larger. Alternatively, phenol extraction followed by ethanol precipitation will remove inhibitors from GuSCN/silica purified DNA. A nested PCR will also improve sensitivity.25-26 However, nested PCR is not recommended in a routine laboratory in view of the risk of contamination with amplicons.

Commercial tests based on nucleic acid amplification for the detection of *M tuberculosis* in samples from the respiratory tract are now available.27-30 However, the commercial tests are rather expensive and need further development. Experience obtained from in house DNA amplification tests shows that commercial test systems need to include controls which enable one to monitor individual specimens for the presence of enzyme inhibitors.31 We did not encounter false positive results caused by contamination with amplicons as dUTP-UDG was used in the reaction mixture and because the laboratory procedures described by Kwok were followed.8-10 However, carry-over of bacteria or DNA sometimes occurred from a sample with a high load of *M tuberculosis* bacteria. The use of tubes with screw caps for the extraction procedure solved this problem.

The percentage of positive samples in this study, 5%, is not representative of the specimens received by our laboratories because some of the samples were preselected. If all sputum and bronchial washing samples had been analysed, we would have expected the number of positive results to be not more than 3%, which is the mean prevalence for The Netherlands. All samples were investigated within a week of receipt, so the PCR results were available much earlier than the culture results and could be used for clinical management.

A PCR test result needs to be interpreted in its clinical context. A PCR positive result does not always conform to culture results—for example, the case of a 30 year old man with suspected early pulmonary tuberculosis. On admission, the patient's sputum sample was positive on microscopy and PCR, but was culture negative. Two weeks later all three tests were positive. After 76 days of treatment with antituberculosis drugs, sputum was negative on microscopy but was culture and PCR positive. On day 188, only the PCR was positive. The first specimen was regarded as positive because it was positive on both microscopy and PCR. The last specimen was interpreted as positive based on the clinical presentation of the consecutive specimens.

Although the routine use of PCR is expensive, we conclude that, when combined with microscopy, nucleic acid amplification is very useful for the rapid and specific detection of *M tuberculosis* DNA in respiratory tract samples. Furthermore, for investigation of pleural fluid and extrapulmonary samples such as tissue biopsy specimens and cerebrospinal
fluid, PCR has become a reliable and sensitive alternative to inoculation of guinea pigs (data not shown). Animal testing is no longer necessary for the diagnosis of tuberculosis. PCR can also be used to identify mycobacterial species rapidly. Discrimination between \textit{M. tuberculosis} complex bacteria and other mycobacterial species is essential for adequate treatment and isolation of the patient.

PCR is not a substitute for culture unless a wide spectrum of mycobacteriocal species can be detected. If such a test becomes available, a routine laboratory could use PCR to screen all incoming respiratory and extrapulmonary samples and perform culture only on the PCR positive samples in order to test antibiotic susceptibility. The cost of such an approach will be offset by the benefit to patients, in whom treatment is instituted only when the presence of mycobacteria has been confirmed, and the savings gained by restricting culture only to those samples containing mycobacteria.

At present, PCR is an important addition to the routine battery of laboratory tests for the rapid and definitive diagnosis of tuberculosis.

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