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Evaluation of PCR in Detection of *Mycobacterium tuberculosis* from Formalin-Fixed, Paraffin-Embedded Tissues: Comparison of Four Amplification Assays

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We compared the sensitivities and specificities of four nested PCR assays for the detection of *Mycobacterium tuberculosis* from formalin-fixed, paraffin-embedded tissues. Thirty-seven autopsy samples from human immunodeficiency virus-positive patients were analyzed: 15 were *M. tuberculosis* positive, 11 served as negative controls, and 11 were Ziehl-Neelsen positive without cultural confirmation of *M. tuberculosis*. Three genomic sequences (*mtp40*, 65-kDa antigen gene, and IS*6110*) with different molecular masses and numbers of repetitions within the *M. tuberculosis* genome were targeted. On the IS*6110* sequence, two fragments of different sizes (106 and 123 bp, respectively) were amplified with two separate pairs of primers. The highest sensitivity rates were obtained by amplifying the highly repetitive IS*6110* insertion sequence, and the different primers tested showed a sensitivity ranging from 80 to 87%. Amplification of the large 223-bp fragment of the *mtp40* sequence present in a single copy in the *M. tuberculosis* genome yielded a high rate of false-negative results, ranging from 66 to 80%. A poor sensitivity (from 47 to 60%) was also shown by PCR amplification of the 142-bp 65-kDa antigen gene. All the PCRs except that for the 65-kDa antigen gene showed a specificity of 100%. Moreover, different results were obtained with different dilutions of DNA, and DNA concentrations of 1 and 3 μg yielded the highest sensitivities depending upon which protocol was used. Application of the PCRs to the Ziehl-Neelsen-positive, culture-negative samples confirmed the sensitivities of the PCRs obtained with the control samples. In conclusion, PCR can successfully be used to detect *M. tuberculosis* from paraffin-embedded tissues and can be particularly useful in the validation of a diagnosis of tuberculosis in clinical settings in which the diagnosis is uncertain. However, the efficacy of PCR strictly depends on several amplification parameters such as DNA concentration, target DNA size, and the repetitiveness of the amplified sequence.

PCR for *Mycobacterium tuberculosis* has already proved to be a useful tool for the diagnosis of tubercular infection (9, 20). Several studies have shown that PCR performed with clinical specimens like sputum, fluid aspirates, and tissue homogenates allows a rapid diagnosis of tuberculosis, with a sensitivity comparable to that of a cultural examination but in a shorter amount of time (3 days for PCR versus the 2 to 6 weeks necessary for culture and identification) (2, 3, 6, 14, 18, 30).

For histopathologic investigations, human tissue samples are mostly stored as formalin-fixed, paraffin-embedded blocks. Widening of the applicability of amplification techniques to formalin-fixed, paraffin-embedded tissues could bring relevant improvements to the routine diagnosis of tubercular infections. This is particularly true when the microorganism fails to grow in culture as well as for those patients in whom an *M. tuberculosis* infection had not been clinically suspected and clinical samples had not been collected for culture. In both of these situations, the only available material may be a paraffin block, but the possibility of culturing diagnostic material is precluded by the tissue-preserving substances themselves. The limitations in the use of PCR in the diagnosis of tuberculosis in histopathological studies are the physical and chemical alterations of the DNA which affect the sensitivity and specificity of PCR. Several studies on the use of PCR assays have been reported, but none of these have thoroughly evaluated the most reliable protocol which could overcome the problems of amplification of *M. tuberculosis* DNA from archival material (10, 21–24, 28).

This study aimed to evaluate the usefulness of PCR in the detection of *M. tuberculosis* from formalin-fixed, paraffin-embedded tissues and to compare the effectiveness of four different PCR assays in order to determine and possibly standardize a PCR protocol suitable for performing a rapid *M. tuberculosis* diagnosis from formalin-fixed, paraffin-embedded tissues.

MATERIALS AND METHODS

Tissue samples. Thirty-seven formalin-fixed, paraffin-embedded samples were obtained from the Department of Pathology, "Luigi Sacco" Hospital, University of Milan. The tissue blocks ranged in age from 3 to 6 years. Paraffin blocks were collected from 37 different autopsy specimens from human immunodeficiency virus-positive patients (19 lymph node, 9 lung, 4 spleen, and 4 liver samples and 1 brain sample).

Of these 37 samples, a total of 26 were considered controls (15 positive controls and 11 negative controls). All 15 positive controls had to fulfill the following criteria: the patient from whom the sample was obtained had to have a clinical history of mycobacterial infection with microbiological confirmation by a positive smear for acid-fast bacilli (by Ziehl-Neelsen staining) and one specimen from the patient had to be culture positive for *M. tuberculosis*. Moreover, tuberculosis had to be the cause of death and a postmortem pathological pattern characterized by necrotizing Ziehl-Neelsen-positive lesions suggestive of *M. tuberculosis* infection had to be present. Of the 11 negative tissue controls, 6 had a clinicopathological pattern suggestive of a nontuberculous mycobacterial infection confirmed by a cultural examination positive for *Mycobacterium avium* complex and 5 had a clinicopathological pattern of cytomegalovirus infection, did not show any clinicopathological evidence of mycobacterial infection, and were all Ziehl-Neelsen negative and culture negative for *Mycobacterium* spp.

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The remaining 11 samples analyzed were Ziehl-Neelsen positive both while the patient was alive and postmortem, presented with a pathological suspicion of tuberculosis, but were culture negative for *M. tuberculosis*.

Sample processing for culture for acid-fast bacilli. Decontamination procedures were performed for all the nonaseptic samples by the standard protocol with *N*-acetyl-L-cysteine–4% NaOH; the samples were then concentrated by centrifugation at 3,000 × *g* for 15 min (17). After resuspension, two Lowenstein-Jensen slants for each specimen were inoculated at 37°C for an incubation time of 8 weeks and were examined weekly for growth. Bacterial colonies were identified as *M. tuberculosis* or other different species of mycobacteria by conventional identification methods (17).

DNA extraction. DNA was extracted from all 37 formalin-fixed, paraffin-embedded tissues. Three 20-μm-thick sections from each block were cut with a microtome (1500 Autocut; Reichter-Jung, Vienna, Austria). In order to prevent carryover of contaminating DNA, a fresh blade was used for each sample and the microtome overlay was covered with a piece of adhesive tape changed for every sample, and after processing each specimen it was subsequently cleaned with xylene and 100% ethanol. Due to the large number of samples, no more than 10 blocks were sectioned in the same batch.

As a negative extraction control, three serial 20-μm-thick sections were cut from formalin-fixed, paraffin-embedded tissue samples with a histopathological diagnosis other than mycobacterial infection and were processed in exactly the same manner as the test samples. Cut sections were collected in 1.5-ml microcentrifuge tubes and were melted at 65°C for 10 min. Paraffin was removed from the samples by adding 1 ml of xylene, vortexing the mixture, and incubating the mixture at room temperature for 30 min; this was followed by 5 min of centrifugation at 12,000 × *g*. The supernatant was then carefully removed and discarded. A further 1 ml of xylene was added to the pellet and the procedure was repeated. To facilitate pelletting and hydration of the samples, 1 ml of 100% ethanol was added. After vortexing, the samples were pelletted by centrifugation at 12,000 × *g* for 5 min and the supernatant was removed. The pellet was then air dried. The samples were resuspended in 300 μl of digestion buffer made up of 50 mM Tris-HCl (pH 7.5), 10 mM EDTA, 0.5% sodium dodecyl sulfate, 50 mM NaCl, and 300 μg of proteinase K per ml, and the mixture was incubated at 37°C under rocking conditions for at least 24 to 48 hours until most of the tissue was disintegrated. The proteinase K was inactivated by incubating the samples at 95°C for 10 min. DNA was extracted from the emulsified tissue samples by adding 300 μl of phenol, vortexing the mixture, and centrifuging the mixture at 12,000 × *g* for 3 min. The supernatant was then removed and transferred into a new tube to which 300 μl of phenol-chloroform (1:1) was added. After vortexing and centrifuging once again at 12,000 × *g* for 3 min, the supernatant was transferred into a new vial and 300 μl of chloroform-isoamyl alcohol (24:1) was added. After transferring the supernatant to a new tube, sodium acetate at a final concentration of 0.2 M and 99% ice-cold ethanol (500 μl) were added to precipitate the nucleic acids.

The samples were stored at −20°C for at least 1 h and were centrifuged at 12,000 × *g* for 20 min at 4°C. The supernatant was discarded, and the pellet was air dried and finally resuspended in 40 μl of distilled water.

DNA amplification by PCR. The amount of DNA extracted as described above was then determined with a spectrophotometer (Gene Quant II; Pharmacia Biotech, Uppsala, Sweden). Each amplification protocol was performed with 5, 3, and 1 μg of DNA.

PCR1. The first PCR (PCR1), a nested PCR which amplifies a region of the *mtp40* segment specific for *M. tuberculosis*, was performed (11).

The primers used in the first round (outer primers) corresponded to nucleotides 9 to 25 (primer PT1; 5′-CAACGCGCCGTCGGTGG-3′) and 385 to 401 (primer PT2; 5′-CCCCCCACGGCACCGC-3′) of the *mtp40* segment. These primers amplified a region of 396 bp (7, 15). The primers used in the second round of PCR1 (inner primers) corresponded to nucleotides 44 to 65 (primer PT3; 5′-CACCACGTTAGGGATGCACTGC-3′) and 244 to 265 (primer PT4; 5′-CTGATGGTCTCCGACACGTTCG-3′) and amplified a region of 223 bp (11).

The total reaction volume in each round was 50 μl and contained a mixture of the deoxynucleoside triphosphates (dNTPs) at concentrations of 200 μM each, 1.5 mM $MgCl_2$, 2.5 U of *Taq* DNA polymerase (Perkin-Elmer, Norwalk, Conn.), 5 μl of 10× *Taq* polymerase buffer (supplied with the enzyme), and 0.4 μM each primer used in that reaction. Ten microliters of DNA at different concentrations was added to the reaction mixture in the first round. Amplification reactions were conducted with an initial 3-min denaturation step at 94°C coupled to a repeating cycle of 1.5 min at 94°C, 1.5 min at 60°C, and 1.5 min at 72°C for 35 cycles, followed by a final extension at 72°C for 7 min. Five microliters of the first-round PCR mixture was transferred to 45 μl of a premixed solution containing the PCR reagents at the same concentrations listed above. The amplification procedure was repeated for 35 cycles with the same time and temperature parameters as described above, except that denaturation at 94°C for 4 min and extension at 72°C for 2 min were used.

PCR2. The second PCR assay (PCR2) used was a nested PCR which amplifies a small region of the gene encoding the 65-kDa antigen which is highly conserved in a variety of *Mycobacterium* species (29). Outer primers (primer MP1 [5′-AGGCGTTGGTTCGCGAGGG-3′] and primer MP2 [5′-TGATGACGCCCTCGTTGCC-3′]) amplified a 234-bp fragment corresponding to bases 538 to 771 of the 65-kDa antigen gene (21). Inner primers (primer MP3 [5′-CCAACCCGCTCGGTCTCAA-3′] and primer MP4 [5′-CCGATGGACTGGTCACCC-3′]) amplified a 142-bp product corresponding to bases 580 to 721 of the same gene (21). The total reaction volume in each round was 50 μl, containing 200 μM each dNTP, 1.5 mM $MgCl_2$, 2.5 U of *Taq* polymerase (Perkin Elmer), 5 μl of 10× *Taq* polymerase buffer (supplied with the enzyme), and 2.5 ng of each primer used in the reaction per μl. Thirty-one microliters of a DNA sample was added to the reaction mixture in the first amplification round. PCR was then conducted with an initial 4-min denaturation step at 94°C coupled to a repeating cycle of 1 min at 94°C, 2 min at 57°C, and 2 min at 72°C for 35 cycles; this was followed by a final extension at 72°C for 7 min. Five microliters of the first-round PCR product was transferred to the second-round PCR mixture. Amplification was repeated for 35 cycles by using the same temperature and time parameters described above, except that a 55°C annealing temperature was used.

PCR3. The third PCR protocol (PCR3) is a nested procedure which amplifies a region of IS*6110*, an insertion sequence usually represented in multiple copies within the *M. tuberculosis* complex genome. The outer primers, corresponding to nucleotides 216 to 236 (primer IS59; 5′-GCGCCAGGCGCAGGTCGATGC-3′) (32) and 858 to 877 (INS2; 5′-TTTGTCACCGACGCCTACGC-3′) (31), amplified a 662-bp fragment; the inner primers, corresponding to nucleotides 633 to 652 (primer INS1; 5′-CGTGAGGGCATCGAGGTGGC-3′) (31) and 718 to 738 (primer IS60; 5′-GCAGGACCACGATCGCTGATC-3′) (32), amplified a 106-bp fragment. The total reaction volume in each reaction was 50 μl and contained each dNTP at a concentration of 200 μM, 1.5 $MgCl_2$, 2.5 U of *Taq* DNA polymerase (Perkin-Elmer), 5 μl of 10× *Taq* polymerase buffer (supplied with the enzyme), and each primer (at 4 μM each) used in the reaction. Twenty microliters of a DNA sample was added to the reaction mixture in the first round. Both PCR rounds were conducted with an initial 3-min denaturation step at 94°C coupled to a repeating cycle of 1.5 min at 94°C, 105 s at 60°C, and 2.5 min at 72°C for 35 cycles; this was followed by a final extension at 72°C for 7 min. Five microliters of the first-round PCR product was transferred to the second-round PCR mixture for subsequent amplification.

PCR4. The fourth PCR procedure (PCR4) is a nested PCR based on the amplification of the repeated insertion sequence IS*6110*. The outer primers corresponded to nucleotides 695 to 724 (primer J; 5′-CGGGACCACCCGCGGCAAAGCCCGCAGGAC-3′) and 885 to 914 (primer K; 5′-CATCGTGGAAGCGACCCGCCAGCCCAGGAT-3′) of the IS*6110* sequence and amplified a 220-bp fragment (26). The inner primers (primer IS1 [5′-CCTGCGAGCGTAGGCGTCGG-3′] and primer IS2 [5′-CTCGTCCAGCGCCGCTTCGG-3′]) amplified a 123-bp fragment (8). The total reaction volume in the first PCR round was 50 μl, and the reaction mixture contained each dNTP at a concentration of 250 μM, 1 mM $MgCl_2$, 1.25 U of *Taq* DNA polymerase (Perkin-Elmer), 5 μl of 10× *Taq* polymerase buffer (supplied with the enzyme), and primers J and K at 0.1 μM each. The total reaction volume in the second round was 50 μl and contained *Taq* polymerase buffer (supplied with the enzyme), each dNTP at a concentration of 125 μM, 1.5 mM $MgCl_2$, 1.25 U of *Taq* polymerase (Perkin-Elmer), and primers IS1 and IS2 at 0.3 μM each. Twenty microliters of a DNA sample was added to the reaction mixture in the first round. Both PCR rounds were conducted with an initial 4-min denaturation step at 94°C coupled to a repeating cycle of 1.5 min at 94°C, 1.5 min at 63°C, and 1.5 min at 72°C for 20 (first round) and 40 (second round) cycles, followed by 7 min of final extension at 72°C. A total of 2.5 μl of the first-round PCR product was transferred to the second-round PCR mixture.

To better evaluate the sensitivities of PCR3 and PCR4, which were targeted to the IS*6110* fragment, the presence and copy number of IS*6110* were investigated by DNA fingerprinting of all the available *M. tuberculosis* culture-positive samples by restriction fragment length polymorphism (RFLP) analysis as described previously by van Embden et al. (33).

Detection of PCR products. For analysis of the amplified products of each PCR assay performed, 15 μl of the reaction solutions from the second round of amplification were resolved on 2% agarose gels containing 1 μg of ethidium bromide per ml, and the products were visualized by UV transillumination.

PCR control procedures. As a control for the integrity of template DNA, β-globin was amplified with the primers PC03 (5′-ACACAACTGTGTTCACTACC-3′) and PC04 (5′-GGTGAACGTGGATGAAGTTG-3′) as described previously (25).

In order to evaluate the reproducibilities of the experiments, all samples were subjected to the whole procedure, from DNA extraction to PCR amplification, three times.

In each amplification run with clinical specimens, multiple controls were included. As a control for the lysis reagents and procedure, a tube containing 10^3 CFU of the H37Rv strain of *M. tuberculosis* and a tube containing no organisms were processed along with each batch of clinical samples. A tube containing 100 pg of prepared *M. tuberculosis* DNA and a tube containing no DNA were included with each set of reactions as positive and negative amplification controls, respectively. An aliquot of water from the same bottle of distilled water used to resuspend the extracted sediments was processed and amplified with each run to rule out contamination during routine processing. A tube containing a set of primers but no template DNA was included with each set of reactions. In addition, steps were taken to minimize false-positive results. PCR product carryover was avoided by keeping the amplified products physically separated from the starting materials. Preparation of the reaction mixtures and setting up of the amplification procedures were performed in a "sterile area" room with no

circulating air and UV light. A circulation-free enclosure used for techniques requiring sterile conditions and outfitted with UV lighting was used while setting up all PCRs. Another room was dedicated to the processing and analysis of all products following amplification; a smaller room served as an anteroom for donning gowns, shoe and hair coverings, and gloves prior to entering the processing and analysis room. Other measures used to prevent cross-contamination included the use of a set of supplies (pipettes and tubes) dedicated only to PCR setup (one pipette was dedicated for nested PCR only); positive-displacement pipettes with disposable tips and plungers were also used. Laboratory techniques used to avoid cross-contamination between samples included frequent changing of gloves, minimal handling of samples, preparation of complete reaction mixtures before adding template DNA, and capping of all tubes immediately before proceeding to the next sample.

RESULTS

Amplification of the β-globin gene segment signifying intact DNA and PCR amplification was achieved for all the 37 autopsy samples.

All the experiments, from DNA extraction to PCR amplification, were performed three times, with concordant results obtained each time.

Data on the sensitivities and specificities of the four PCR assays for the detection of *M. tuberculosis* from formalin-fixed, paraffin-embedded tissues were collated for the 26 patient samples used as controls (15 positive controls and 11 negative controls). Each sample underwent amplification by the four different PCR protocols with each of the three DNA concentrations chosen to be tested (1, 3, and 5 μg).

Figure 1 shows the sensitivity and specificity data for the four assays with the different DNA concentrations tested.

Specificities of the PCRs in detecting *M. tuberculosis*. Except for PCR2, none of the PCRs was affected by false-positive results for each of the three concentrations tested. PCR2 showed false-positive results for 3 of 11 (27%) samples when 1 and 3 μg of DNA were used and false-positive results for 2 of 11 (18%) samples when 5 μg of DNA was used (average specificity of PCR2, 76%). It should be noted that all the false-positive results were for the subgroup of negative controls with a cultural diagnosis of *M. avium* complex. None of the five negative controls culture negative for *Mycobacterium* spp. were ever amplified by PCR2. The high rate of false-positive results obtained by PCR2 can be explained by the nature of the DNA fragment, itself the target of amplification by this protocol, which is shared by various members of the *Mycobacterium* genus.

Sensitivities of the PCRs in detecting *M. tuberculosis*. PCR1 had poor results in terms of sensitivity, with the following proportions of false-negative results: 10 of 15 (66%), 12 of 15 (80%), and 13 of 15 (87%) samples were false negative when DNA concentrations of 1, 3, and 5 μg, respectively, were tested. PCR2 yielded false-negative results for 6 of 15 (40%) samples when 1 μg of DNA was amplified, 4 of 15 (27%) samples when 3 μg of DNA was amplified, and 8 of 15 (53%) samples when 5 μg of DNA was amplified.

The best results in terms of sensitivity were obtained by amplifying the IS*6110* region of the *M. tuberculosis* complex genome. In particular, by PCR3 false-negative results were obtained for 4 of 15 (27%), 3 of 15 (20%), and 5 of 15 (33%) samples when 1, 3, and 5 μg of DNA, respectively, were used. PCR4 gave false-negative results for 2 of 15 (13%) samples when 1 and 3 μg of DNA were used and 5 of 15 (33%) samples when 5 μg of DNA was used.

The concentration of DNA used affected the outcome of the amplification protocols considerably, as shown in Fig. 1. The amplification of 5 μg of DNA seemed to be quite unsuccessful with each PCR protocol used. A concentration of 3 μg yielded the lowest percentage of false-negative results in PCR2 and PCR3. The use of 1 and 3 μg of DNA for amplification resulted in the lowest proportion of false-negative results by PCR4. These results demonstrate the strict interdependence of the DNA concentration and the PCR protocol itself in amplification performance with paraffin-embedded tissues.

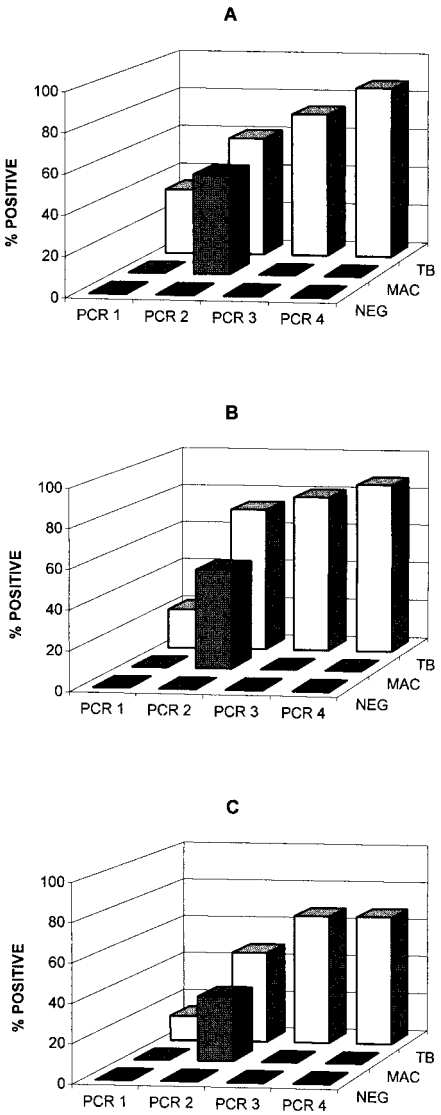

FIG. 1. Sensitivities and specificities of the four PCR assays for the detection of *M. tuberculosis* expressed in terms of the percentage of samples that were positive by each PCR. PCRs were performed with 1 (A), 3 (B) and 5 (C) μg of DNA. NEG, negative; MAC, *M. avium* complex; TB, *M. tuberculosis*.

Discordant trends in the sensitivities of the PCRs were observed according to the source of tissue analyzed. With respect to this issue, in PCR4 with 3 μg of DNA (the PCR protocol with the best performance in our study), unsatisfactory data were evidenced after amplification of DNA from liver, with 2 of 2 (100%) false-negative results. False-negative results were obtained for 1 of 4 (25%) lymph node samples. Conversely, no false-negative results were obtained by amplification of lung, spleen, and brain specimens.

DNA fingerprinting by RFLP analysis performed with all the 11 available *M. tuberculosis* cultures showed a genomic pattern characterized by the presence of multiple IS*6110* copies, indicating that the sensitivities of the IS*6110*-based amplification

TABLE 1. PCR results for histology- and Ziehl-Neelsen staining-positive samples negative by culture

No. of samples	PCR1 result	PCR2 result	PCR3 result	PCR4 result
4	−	−	+	+
2	−	−	−	+
3	+	+	+	+
1	−	+	−	−
1	−	+	+	−

assays were not biased because they lacked or contained insufficient amounts of genomic target.

By applying the four PCR assays to the 11 formalin-fixed, paraffin-embedded autopsy samples from patients with a clinical history of tuberculosis, a Ziehl-Neelsen-positive staining result, and a pathological pattern suggestive of mycobacterial infection but with a negative culture result we found various degrees of reactivity in each of the PCRs. Again, in the amplifications we used three different concentrations of DNA. Table 1 summarizes the results. In particular, it emerged that for 11 of 11 (100%) samples, at least one of the PCRs made it possible for us to detect mycobacterial DNA in samples which had shown no mycobacterial growth in culture. The overall higher proportion of positive results (without regard to the concentration used) was obtained with PCR3, by which 8 of 11 (73%) samples had positive results, and PCR4, by which 9 of 11 (82%) samples had positive results. By PCR1 3 of 11 (27%) samples had positive results, and by PCR2 5 of 11 (45%) samples had positive results. A positive amplification by each of the four PCR protocols with at least one of the DNA concentrations was obtained for only 3 of 11 (27%) samples. One sample yielded a positive result only when it was amplified with the primers homologous to sequences shared by a variety of *Mycobacterium* species (PCR2), suggesting an infection due to a mycobacterium other than *M. tuberculosis*.

DISCUSSION

Aside from the improvements to and the increasing use of PCR for the diagnosis of mycobacterial infections with fresh clinical specimens, there is still the need for the optimization of a sensitive and rapid PCR assay for the identification of mycobacteria from formalin-fixed, paraffin-embedded tissues. Major advantages would be gained by the assessment of an amplification technique for the detection of the *M. tuberculosis* genome in formalin-fixed, paraffin-embedded tissues. PCR is not restricted by the presence of viable organisms in the sample, thus rendering possible a retrospective diagnosis of tuberculosis from archival material with no cultural examination and whose storage in formalin severely hampers processing by usual bacteriological methods. Moreover, an amplification assay can give a result within 2 to 3 days, whereas culture of *M. tuberculosis* in common culture medium requires 2 to 6 weeks.

It has already been largely described in literature that the effectiveness of PCR with formalin-fixed, paraffin-embedded tissues is impaired by multiple interacting factors, including the type of fixative used (the best being 10% buffered formalin and the less desirable for amplification analysis being Carnoy's, Zenker's, and Bouin's fixatives) (12), the fixation time (12), the DNA extraction procedure, the length of the PCR target, the concentration of target DNA amplified, and the PCR protocol itself (1, 27). In our study we have evaluated the roles of some variables while keeping other variables stable. In particular, all of our samples were stored in 10% neutral buffered formalin for a relatively long exposure time of about 2 weeks because they were from autopsy material. The same procedure for the extraction of DNA from paraffin-embedded tissues was adopted, and the visualization of PCR products on agarose gels was performed under the same conditions. We have focused our attention on the influence of three variables on the effectiveness of each PCR assay: the concentration of the target DNA amplified, the molecular mass of the amplification product, and the repetitiveness of the fragment target of the amplification within the mycobacterial genome.

The question of the best DNA concentration to be used with formalin-fixed, paraffin-embedded samples is controversial. It is, in fact, well known that the relatively frequent failure of the amplification of DNA from formalin-fixed, paraffin-embedded material can be due to the presence of inhibitors whose nature (which is only partly known) seems to be endogenous as well as induced by formalin fixation and by all the other steps in tissue processing and deparaffinization. A way to remove such inhibitors could be to reduce the target DNA concentration (1, 5). On the other hand, reducing the amount of DNA to be amplified could possibly lead to a decrease in the sensitivity of the PCR, particularly in the presence of paucibacillary lesions. A low DNA concentration could also impair the specificity of the PCR for the easier formation of primer-dimer artifacts (5). To better evaluate the influence of such a parameter, each of the 26 control specimens was amplified by using 1, 3, and 5 μg of DNA. The highest number of false-negative results was obtained with DNA at a concentration of 5 μg, irrespective of the protocol used, probably as a consequence of the strong inhibition present. The amplification of 1 and 3 μg of DNA yielded the best results in terms of sensitivity, even if our data do not allow us to draw any definitive conclusion about the better concentration. The influence of the DNA concentration seems to be strictly linked to the amplification protocol itself.

The molecular mass of the amplification product plays an important role in the efficacy of the PCR protocol, mainly as a consequence of the high degree of degradation within the polynucleotide chain in formalin-fixed, paraffin-embedded tissues. It has already been shown that the longer the amplified fragment, the higher the likelihood of degradation and thus the lower the efficacy of the amplification itself (1, 5). This seems to be particularly true for the GC-rich *M. tuberculosis* genome due to the higher binding capacity of formalin to free amino groups present in the nucleotides mentioned above (16). Our results confirm this concept, and in fact, PCR3 and PCR4, whose final amplification products are 106 and 123 bp long, respectively, showed the best results in terms of sensitivity compared to those of PCR1 and PCR2, which amplify longer fragments (223 and 143 bp, respectively). This suggests the need to choose the correct primers, with those amplifying relatively shorter DNA sequences, which are thus less prone to fragmentation, being favored.

Moreover, the number of copies of the fragment that is the target of amplification present in the genome of the microorganism proved to be a controlling factor in the efficacy of the amplification. Again, the best sensitivities were shown by PCR3 and PCR4, which are based on the amplification of IS*6110*, a mobile genetic element usually present in multiple copies within the genomes of virtually all members of the *M. tuberculosis* complex (4, 32, 34, 35). On the contrary, the amplification of the species-specific *mtp40* region present in a single copy within the genome of *M. tuberculosis* (7, 19), showed an overall low sensitivity when applied to formalin-fixed tissues, which was opposite the result observed with fresh clinical specimens (11, 15). The sensitivity of PCR can thus be

possibly ameliorated by choosing as a target of the amplification DNA sequences likely to be present in multiple copies within the genome. In order to rule out the possibility of IS*6110*-based PCR detection failures due to isolates with few or even no IS*6110* elements all *M. tuberculosis* cultures available were typed by DNA fingerprinting by RFLP analysis (13, 33) to evaluate the presence and the number of IS*6110* copies within the genome. All the isolates presented a pattern with multiple IS*6110* bands.

Moreover, an additional parameter which may have an impact on the outcome of genomic amplification is the nature of the tissue tested. Choosing the more sensitive PCR assay (PCR4) with one of the better-performing DNA concentrations (3 μg), we observed variable results according to the tissue type, with DNA from the lung and the spleen samples being amplified in every case, liver samples not being amplified at all, and an amplification sensitivity of 75% being obtained with lymph node samples. Our data, even though they are partial considering the limited number of samples and the quite heterogeneous distribution of the different tissue types among the control samples that we had available, still seem to suggest that the amplification outcome may be influenced by the kind of tissue that is used.

Even though it did not prove to be more sensitive than cultural examination, PCR could still be of help to the clinician in all the settings where the tubercular etiology had not been suspected and material had not been collected for culture, as well as in the presence of a clinical history suggestive of tuberculosis, a positive Ziehl-Neelsen staining result, and a negative culture result. For the 11 Ziehl-Neelsen-positive, culture-negative autopsy samples, PCR allowed us to obtain a positive amplification for all specimens analyzed, thus suggesting that when making a diagnosis a positive PCR result should always be taken into consideration even when the culture result is negative. It must be stressed that only 3 of 11 samples were positive by each PCR assay with at least one of the DNA concentrations used, but the IS*6110*-based PCRs had sensitivities of 80%.

Moreover, PCR could possibly rapidly discriminate *M. tuberculosis* from nontuberculous mycobacteria. In regards to this last issue, of the four PCRs that we performed, PCR1 was highly species specific (7, 19), PCR3 and PCR4 amplify two different fragments of the insertion sequence IS*6110* specific for the *M. tuberculosis* complex (4), while the primers used in PCR2 amplify a small region of the gene encoding the 65-kDa heat shock protein (21). Due to the specificity of this region to the *Mycobacterium* genus, all the samples which were positive by PCR2 and negative by all the other assays could be presumptively considered to contain nontuberculous mycobacteria.

In order to better understand the 11 tissue samples smear positive and culture negative for acid-fast bacilli, the clinical records of the patients were reviewed. Four of the samples (36%) were from patients who had clinical and radiologic manifestations of tuberculosis or a suspect reactivation of a prior tubercular infection and who were under antitubercular treatment. This is in accord with what was previously stated in the literature as to the possibility of no cultural growth secondary to specific therapy (18) but with the persistence of PCR positivity even several weeks after the institution of treatment. Four samples belonged to patients with no apparent previous history of mycobacterial infection and who died within a few days after they were hospitalized.

Another explanation could be that broth media useful for recovering organisms from paucibacillary specimens were not used. In the years when the samples were collected, no media and/or incubation conditions able to detect fastidious mycobacteria such as *M. haemophilum* or *M. genavense* were in use.

The results of the present study indicate that the insertion sequence IS*6110* seems to be a good target for the amplification of DNA from formalin-fixed paraffin-embedded tissues. Even though in our experience the sensitivities shown by IS*6110*-based protocols do not appear to be optimal, they still reach maximum values of almost 90% and seem to be highly influenced by various interacting variables. On the other hand, these PCRs were shown to have good specificities, with no false-positive results, and seem to be useful in the differentiation of *M. tuberculosis* from other *Mycobacterium* spp.

Our study shows that a properly designed PCR assay can successfully be used to detect *M. tuberculosis* in formalin-fixed, paraffin-embedded tissues but highlight the necessity of paying particular attention to the choice of such parameters as target DNA size, DNA concentration, and target fragment repetitiveness within the mycobacterial genome. It is evident that further investigations need to be conducted in order to ameliorate and possibly standardize a protocol of DNA amplification from archival material. This could have a strong relapse as to the clinical application, rendering feasible a rapid and easy-to-perform retrospective diagnosis of *M. tuberculosis* infection, which would be particularly useful when there is a lack of growth on culture or when fresh material has not been collected for culture.

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