Comparison of Amplicor, In-House PCR, and Conventional Culture for Detection of *Mycobacterium tuberculosis* in Clinical Samples

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Five hundred four clinical specimens (337 sputum and 167 bronchial samples) from 340 patients were tested for the presence of *M. tuberculosis* complex by the Amplicor *M. tuberculosis* test and by an in-house PCR. The results were compared with those obtained by conventional culture and by direct microscopy. Thirty specimens (from 14 patients) were positive by in-house PCR, 25 (from 13 patients) were positive by the Amplicor *M. tuberculosis* test, and 24 (from 10 patients) were positive by culture. Cultures from 16 specimens were contaminated with other bacteria. Strong inhibition of in-house PCR was found with three samples. After discordancy analyses, with clinical data as supportive evidence for tuberculosis, 27 true-positive and 458 true-negative samples were defined. On the basis of these figures, the sensitivities of the Amplicor *M. tuberculosis* test, in-house PCR, and microscopy were 70.4, 92.6, 88.9, and 52.4%, respectively. The specificities of all four tests were higher than 98%. The good performance of the in-house PCR for detection of *M. tuberculosis* makes it a very useful additional tool in *M. tuberculosis* diagnostics. In contrast, the Amplicor test needs to be improved. Twenty-three of the Amplicor-negative samples were further tested for inhibition of the Amplicor system by retesting the DNA extracts after the addition of the 16S rRNA gene. In 15 of these samples, 5 true positives and 10 true negatives, inhibition of the Amplicor test was demonstrated. This might explain the lack of sensitivity of the Amplicor test. If the inhibition problem can be solved, the Amplicor *M. tuberculosis* test, which is already rapid, very user-friendly, and reasonably priced, may certainly become very useful in microbiological laboratories.

For the detection of *Mycobacterium tuberculosis*, microscopic examination of acid-fast stained smears and culture are still the methods of choice in most microbiological diagnostic laboratories. However, none of these methods are really satisfactory. Direct staining for acid-fast bacilli (AFB) takes less than an hour but lacks sensitivity (7, 7a). Moreover, a positive result with this test does not discriminate between the *Mycobacterium* species. Culturing of *M. tuberculosis* is sensitive and specific but may take 6 to 10 weeks. The recently developed nucleic acid amplification methods may provide us with very sensitive, specific, and rapid tests for the detection of *M. tuberculosis*, thus combining the advantages of both of the classical methods. Several reports on the successful use of the PCR for the detection of *M. tuberculosis* complex in clinical specimens have been published (1, 3, 4, 8, 12, 15, 18). However, recent studies clearly showed that PCR can only reliably be used for the detection of *M. tuberculosis* complex when both sensitivity and specificity are carefully monitored (7, 16).

Commercial nucleic acid amplification kits might be attractive alternatives for the relatively cumbersome in-house PCRs, assuming that these kits supply all the necessary specificity and sensitivity controls. In the Amplified *M. tuberculosis* Direct Test (Gen-Probe), the target for amplification is *M. tuberculosis* rRNA. This commercially available test has been reported to be a reasonably reliable tool in the diagnosis of tuberculosis (10, 13, 17). The Amplicor *M. tuberculosis* test (ROCHE) is a PCR kit used for amplifying part of the 16S rRNA gene. The test is marketed in Europe for diagnostic use with sputum and bronchial alveolar lavage specimens.

The aim of this study was to compare the commercial Amplicor *M. tuberculosis* kit and our in-house PCR with the conventional culture and direct staining methods.

**MATERIALS AND METHODS**

**Clinical specimens.** A total of 504 clinical specimens (337 sputum samples, 135 bronchial washes, and 33 bronchial secretions) from 340 patients suspected of having tuberculosis were selected from samples which were sent to our routine laboratory by hospital clinics. The only selection criterion was that the volume of the specimen should be large enough to perform culture, in-house PCR, and the Amplicor *M. tuberculosis* test. For culture and PCR, the samples were liquefied and decontaminated by standard procedures with 1 volume of 0.005% N-acetyl i-cysteine in 0.5 M NaOH-0.05 M sodium citrate (9). Aliquots for the Amplicor test and for the in-house PCR were taken immediately after the N-acetyl i-cysteine incubation step.

**Microscopy and culture.** Microscopy for AFB was performed on (most of) the original nondecontaminated samples by Ziehl-Neelsen staining (7a). For culture, at least 2 ml of each decontaminated sample was diluted immediately with phosphate buffer, concentrated by centrifugation for 15 to 20 min at 4,000 × g, resuspended, and inoculated in triplicate in Löwenstein-Jensen medium (12a).

**Amplicor *M. tuberculosis* test.** The Amplicor *M. tuberculosis* kit consists of three different subkits: the Specimen Preparation kit, the Amplification kit, and the *Mycobacterium tuberculosis* Detection kit. The complete Amplicor *M. tuberculosis* test was performed according to the manufacturer’s instructions, starting with the addition of 500 μl of Sputum Wash Solution to 100 μl of the decontaminated sample and by centrifugation at 12,500 × g for 10 min. After this initial step, the cell pellets were stored at −20°C until batchwise performance of the actual tests in a later stage (in consultation with the manufacturer). The cell pellets were resuspended in 100 μl of Sputum Lysis Reagent, incubated at 60°C for 45 min, and then neutralized by the addition of 100 μl of Sputum Neutralization Reagent. Portions (50 μl) of the pretreated patient samples and one positive and three negative controls were processed in a GeneAmp PCR system 9600 thermocycler (37 cycles; duration, 1.5 h). The cycle parameters were 20 s at 98°C (only cycles 1 and 2) or 94°C (cycles 3 to 37), 20 s at 62°C, and 45 s at 72°C. After amplification the samples were denatured by 100 μl of Denaturing Solution for 10 min at room temperature. Hybridization was performed by incubation of 25 μl of the denatured product and 100 μl of Hybridization Buffer for 1.5 h at 37°C in probe-coated microwells. Then the wells were washed five times with Working Wash Solution and incubated for 15 min at 37°C with 100 μl of
TABLE 1. Comparison of the Amplicor M. tuberculosis test, in-house PCR, and culture for detection of M. tuberculosis in 504 clinical specimens

<table>
<thead>
<tr>
<th>PCR</th>
<th>Culture</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pos.</td>
</tr>
<tr>
<td>Amplicor pos.—in-house PCR pos.</td>
<td>18</td>
</tr>
<tr>
<td>Amplicor pos.—in-house PCR neg.</td>
<td>0</td>
</tr>
<tr>
<td>Amplicor neg.—in-house PCR pos.</td>
<td>4</td>
</tr>
<tr>
<td>Amplicor neg.—in-house PCR neg.</td>
<td>2</td>
</tr>
<tr>
<td>Amplicor neg.—in-house PCR inh.</td>
<td>0</td>
</tr>
</tbody>
</table>

a pos., positive; neg., negative; inh., inhibition of in-house PCR by 1/10 dilution of DNA extract; contam., no culture result because of contamination with other bacteria.

b Four of these samples exhibited low optical densities just above the cutoff value in the Amplicor test (all were negative in the retest).

c Fivefold (3 samples) dilution of the DNA extracts.

d The 16 samples with contaminated cultures and 3 samples with persistent inhibition of the in-house PCR are not included in this table.

RESULTS

The comparison of the results of the Amplicor test with those of the in-house PCR and culture for 504 clinical specimens is shown in Table 1. Thirty clinical specimens (from 14 patients) were positive by in-house PCR, 25 (from 13 patients) were positive by the Amplicor M. tuberculosis test, and 24 (from 10 patients) were positive by culture. No culture results could be obtained for 16 specimens because of contamination with other bacteria. Inhibition of in-house PCR was found with 18 samples which were all negative by culture and by the Amplicor M. tuberculosis test. In only three of these cases, inhibition persisted at a dilution of 1/10. In the other 15 cases, the inhibition could be bypassed by a twofold (12 samples) or fivefold (3 samples) dilution of the DNA extracts. The 16 samples with contaminated cultures and the 3 samples with persistent inhibition of the in-house PCR were not further evaluated. The overall results of culture, in-house PCR, and the Amplicor test were concordant for 447 (92.3%) of the remaining 485 samples. In contrast, whereas 38 samples were positive by at least one of the three tests, only 18 (47%) of them were positive by all three tests. The Amplicor test clearly failed to detect M. tuberculosis in 6 of the 24 culture-positive samples. Only two of these samples were also negative by the in-house PCR. When the mycobacteria obtained by culture from these two culture-positive–in-house-PCR-negative samples were also tested by in-house PCR, the results were positive, indicating that the initial in-house-PCR-negative results were not caused by sequence variation. In both cases, the corresponding Ziehl-Neelsen preparations were negative, which suggests a limited bacterial load. For the other 14 discordant, culture-negative samples, we further investigated the clinical data. Only three of them, all positive by in-house PCR, were from (two) patients with clinical symptoms of tuberculosis, which suggests that M. tuberculosis in these samples had been missed by culture. One of these three samples was also positive by the Amplicor test.

Direct staining for AFB on 438 of the 485 above-evaluated specimens was positive in 12 cases (from five patients). From one of these AFB-positive samples, mycobacteria other than M. tuberculosis were grown. This sample was negative by the other three tests. The other 11 AFB-positive samples were positive by both culture and in-house PCR. Only nine of them were positive by the Amplicor test.

For the further evaluation, two groups of samples were considered to be M. tuberculosis true-positive samples: firstly, samples which were culture positive and, secondly, samples which were culture negative but positive by at least one PCR method (either in-house PCR or the Amplicor test) and were from patients with either clinical symptoms of tuberculosis or with other culture-positive clinical specimens in the same period. On this basis, we defined 27 true-positive samples (from 11 patients) and 458 true-negative samples, resulting in an M. tuberculosis prevalence of 5.6% in the samples studied. Microscopy for AFB was performed on only 21 true-positive and 417 true-negative samples, corresponding to an M. tuberculosis prevalence of 4.8% in these samples. The numbers of true-positive and true-negative samples identified by each test system are shown in Table 2. The in-house PCR missed 2 of the...
TABLE 3. Overall performances of the Amplicor test, in-house PCR, culture, and direct microscopy for the detection of M. tuberculosis

<table>
<thead>
<tr>
<th>Method</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>Predictive value (%) Positive</th>
<th>Predictive value (%) Negative</th>
<th>Accuracy (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amplicor test</td>
<td>70.4</td>
<td>98.7</td>
<td>76.0</td>
<td>98.3</td>
<td>98.1</td>
</tr>
<tr>
<td>In-house PCR</td>
<td>92.6</td>
<td>98.9</td>
<td>83.3</td>
<td>99.6</td>
<td>98.6</td>
</tr>
<tr>
<td>Culture</td>
<td>88.9</td>
<td>100</td>
<td>100</td>
<td>99.3</td>
<td>99.4</td>
</tr>
<tr>
<td>Microscopy</td>
<td>52.4</td>
<td>99.8</td>
<td>91.7</td>
<td>97.7</td>
<td>97.5</td>
</tr>
</tbody>
</table>

* Values were calculated for 485 samples for the PCR assays and for culture. For microscopy, 438 samples were included. For further details, see the text and Table 2.

72 true-positive samples, whereas 3 were missed by culture. In contrast, no less than 8 (of 27) and 10 (of 21) true-positive samples were missed by the Amplicor test and microscopy, respectively. None of the test systems detected infection in all of the infected patients: the Amplicor test, the in-house PCR, and culture recognized infection in 7, 9, and 10 of the 11 infected patients, respectively. With direct microscopy, infection in no more than 4 patients was detected (eight infected patients were tested).

Further characteristics of the different test systems are listed in Table 3. All four tests showed very good specificities (>98%) and accuracies (>97%). However, the sensitivities were less satisfactory. Whereas the low level of sensitivity (52.4%) of microscopy might have been expected (8), the level of sensitivity of the Amplicor test (70.4%) was very disappointing and much lower than the levels of sensitivity of culture (88.9%) and in-house PCR (92.6%).

**DISCUSSION**

When the performance of a new test system is evaluated, the performance of the standard method is a critical parameter. For the detection of M. tuberculosis, culture has always been considered to be the “gold standard.” The specificity of culture is usually considered to be 100%. The sensitivity, however, though quite satisfactory, is often reported to be <90% (10, 15), as was also found in the present study. For culture of M. tuberculosis, we used conventional Löwenstein-Jensen medium only, as is often done when sputum samples and bronchial washes are studied (1, 15, 21). However, for an optimal recovery of M. tuberculosis, the additional use of broth-based culture systems is currently recommended (14). The recent introduction of broth media in our laboratory has not markedly improved the recovery of M. tuberculosis from sputum samples and bronchial washes so far. Therefore, we think that our Löwenstein-Jensen medium produced reasonably acceptable culture results with the samples studied. Irrespective of the validity of this judgment, any culture system will undoubtedly miss some true-positive samples. For that reason, the calculated sensitivities and specificities of the various tests (Table 3) were based not only on the performance of the culture method but in addition on the clinical and historical laboratory data.

Besides favorable sensitivities and specificities, diagnostic laboratories especially need acceptable predictive values of test results. Table 3 shows that the negative predictive values of all of our tests were very high (>97%). The positive predictive values were very good for culture (100%, by definition) and microscopy (91.7%) but were lower for the in-house-PCR (83.3%) and the Amplicor test (76.0%). Therefore, interpretation of positive results obtained with our in-house PCR or the Amplicor test in low-prevalence situations should always include consideration of clinical and other laboratory findings.

In situations with a higher prevalence of M. tuberculosis, the positive predictive values of both PCR systems would improve considerably. It can be calculated that with an M. tuberculosis prevalence of 10%, the positive predictive values of our in-house PCR and the Amplicor test would be 90.3 and 85.7%, respectively, which is in the same range as the values published for the Gen-Probe system at comparable prevalences (13, 17).

The good overall performance of the in-house PCR for detection of M. tuberculosis makes it a very useful additional tool in M. tuberculosis diagnostics, as was also reported earlier by other investigators (1, 4, 12, 15, 18). In contrast, the performance, and especially the sensitivity (70.4%), of the Amplicor test is not good enough yet. A similar low level of sensitivity (66.7%) of the Amplicor M. tuberculosis test was recently also reported elsewhere (5).

Two possible causes of the disappointing low level of sensitivity of the Amplicor test were briefly investigated: the possibility of a low intrinsic level of sensitivity of the Amplicor test system itself and inhibition of the amplification step by “clinical” DNA extracts. The sensitivities of the different test systems were recalculated for two subgroups of the true-positive samples separately: the AFB-positive samples, which are considered to contain relatively large amounts of M. tuberculosis bacteria, and the AFB-negative samples. The sensitivities of the Amplicor test, the in-house PCR, and the cultures were 81.8, 100, and 100%, respectively, in the AFB-positive group (n = 11) and 60, 90, and 70%, respectively, in the AFB-negative group (n = 10). Clearly, the Amplicor test, and also to a lesser extent the culture, was much more affected by changing from large to small numbers of bacilli than the in-house PCR. This suggests that the Amplicor test, especially, had a relatively low intrinsic level of sensitivity. One of the reasons for this may be the relatively small sample volume used. Whereas our in-house amplification reaction was performed with DNA originating from 50 µl of sputum, only 12.5 µl was used in the Amplicor test.

One could imagine that the Amplicor test would be particularly susceptible to inhibition, since the sample pretreatment procedure contains only one simple wash step. Therefore, inhibition of the Amplicor test was investigated by retesting the DNA extracts from 23 Amplicor-negative samples after the addition of M. tuberculosis DNA. As is shown in Table 4, this inhibition control was selectively performed on five of the true-positive samples missed by the Amplicor test, but not by the in-house PCR, and on all 18 negative samples showing inhibition of the in-house PCR test. No less than 15 of these 23...
selected samples showed inhibition of the Amplicor test. This included all five Amplicor false-negative samples tested, which suggests that inhibition was the major cause of the observed lag in the sensitivity of the Amplicor test. Naturally, the adverse effects of inhibition would be most noticeable in low positive, AFB-negative, samples. Although the overall results obtained with the 18 negative samples suggest some correlation between the degree of inhibition of the in-house PCR and inhibition of the Amplicor test, the inhibition patterns observed with the five true-positive samples were clearly discordant (Table 4). This indicates that the chemical substances inhibiting both systems are not completely identical.

The usefulness of the Amplicor test might be greatly improved when alternative test protocols aimed at a more effective avoidance of inhibition are developed. For instance, it might be better to remove the aliquots for the Amplicor tests from the final concentrated bacterial suspensions, which are also used for culture, instead of just after the decontamination step. By doing so, the aliquots might contain lower concentrations of inhibitory substances and certainly larger numbers of bacteria. However, it remains to be seen whether such an alternative approach is really better: other investigators, using the final bacterial suspensions—in fact the manufacturer’s instructions on this point were somewhat inconsistent—also reported a low level of sensitivity for the Amplicor test (5). Our results clearly show that inhibition controls should be included in the Amplicor M. tuberculosis test system. The most obvious way to check for inhibition would be to add a small amount of M. tuberculosis DNA to duplicate samples, as we did in our in-house PCR or, preferably, to coamplify modified sequences in one single test tube (11, 15). In addition, it would be useful to advise the users of the test to testinetuberculosis samples at low dilutions.

The attractiveness of new diagnostic tests will not only be determined by the characteristics discussed above but will also largely be influenced by the price of the test and the ease of performance, compared with those for alternative in-house tests. The Amplicor system is rapid and relatively easy to perform, certainly when compared with an in-house PCR. Still, in-house PCR is often cheaper, although this will depend on the number of samples tested per week and also on the local prices of reagents, kits, and labor. Moreover, in-house PCR protocols can often be simplified by introducing homemade enzyme-linked immunosorbent assay systems for the hybridization and detection of amplified sequences (21). This development will reduce the prices of the in-house tests considerably. The conventional tests for detection of M. tuberculosis, culture and microscopy, are undoubtedly much cheaper than the PCR systems or related systems. However, none of these conventional methods combine a reasonable sensitivity with a reasonable test time, as the PCR systems and related amplification systems do. Since the Amplicor system is rapid, very user-friendly, and reasonably priced (in Europe), resolution of the inhibition and sensitivity problems might make the Amplicor M. tuberculosis test a real gain for M. tuberculosis diagnostics.

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REFERENCES