Comparison of PCR with the Routine Procedure for Diagnosis of Tuberculosis in a Population with High Prevalences of Tuberculosis and Human Immunodeficiency Virus

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Direct smear examination with Ziehl-Neelsen (ZN) staining for the diagnosis of tuberculosis (TB) as employed in most low-income countries is cheap and easy to use, but its low sensitivity is a major drawback. The low specificity of chest X-rays, used for the diagnosis of smear-negative TB, risks high levels of overdiagnosis. Major advances in molecular techniques, which rapidly identify mycobacterial DNA in sputa, may overcome these obstacles. In this study, the AMPLICOR PCR system was used to diagnose pulmonary TB in a developing country with high prevalences of both TB and human immunodeficiency virus (HIV). The sensitivity and specificity of this technique were compared to those of the usual diagnostic techniques. Sputum specimens were collected from 1,396 TB suspects attending the Rhodes Chest Clinic, Nairobi, Kenya. The specimens were analyzed for the presence of Mycobacterium tuberculosis by PCR; culture on Löwenstein-Jensen medium was used as the “gold standard.” All culture-positive samples were genotyped to identify the mycobacterial species. The sensitivity and specificity of PCR were 93 and 84%, respectively. HIV status did not affect the sensitivity of PCR. A total of 99.7% of the true smear-positive and 82.1% of the true smear-negative TB patients were correctly identified by PCR. PCR detected M. tuberculosis in 11.7% of the culture-negative suspects, 60% of which had one or two PCR-positive sputum specimens. Of the 490 positive cultures, 486 were identified as M. tuberculosis. The high sensitivity of Amplicor PCR merits usage in a clinical setting with high TB and HIV burdens. Thus, PCR can be considered as an alternative to ZN staining in combination with chest X-ray for diagnosis of TB; however, cost-effectiveness studies and operational studies are required to support an evidence-based decision of introducing PCR for TB control in high-burden environments.

Tuberculosis (TB) is one of the most serious infectious diseases and a considerable public health problem due to its high risk of person-to-person transmission, morbidity, and mortality. Both the human immunodeficiency virus (HIV) epidemic and social deterioration have contributed to the overall increase in the Mycobacterium tuberculosis infection rate, especially in developing countries, where resources are scarce (13). In Nairobi the case detection rate increased from 78 per 100,000 in 1991 to 581 per 100,000 in 2001, with a total number of 12,963 cases.

Early diagnosis followed by adequate treatment is essential to prevent both morbidity and mortality. Although the conventional technique of direct smear examination with Ziehl-Neelsen staining (ZN) is cheap and easy to perform, its low sensitivity is a major drawback. Depending on the number of specimens examined, ZN detects 30 to 60% of the culture-positive “TB suspects” (7). Furthermore, it requires sputum samples collected on consecutive days, making the procedure slow and making patient compliance with the diagnostic process difficult.

New techniques are very much needed (7), and molecular amplification assays such as PCR have been shown to be promising alternatives even for developing countries (2). PCR has the potential to be a cost-effective alternative, provided the diagnosis can be determined with one sputum examination (8). If diagnosis can be established faster, and the diagnostic process becomes less cumbersome for the patient, PCR may reduce delay both in diagnosis and in the start of treatment.

Depending on the “gold standard” and other methodological factors, studies show PCR sensitivities ranging from 77% to more than 95% and PCR specificities of >95% for smear-positive specimens (4, 9, 10, 12). However, sensitivities for smear-negative TB patients have been reported to be below 90% (9). Most PCR studies have been performed in industrial countries (4, 9, 10, 12) where the TB and HIV burdens are low.

To investigate the performance and feasibility of PCR in an environment of TB endemicity and high prevalences of HIV and AIDS, a study was conducted in Nairobi, Kenya, comparing PCR to conventional routine diagnostic methods within a program setup. In this study, the Roche Amplicor Mycobacteria PCR test for the direct detection of M. tuberculosis was used on sputum specimens from TB suspects attending a chest clinic in Nairobi. Its performance was compared with those of the basic routine diagnostic procedures according to the national
guidelines (6), including clinical findings, ZN, and chest X-rays (CXR), on smear-negative suspects. Löwenstein-Jensen (LJ) culture results were used as the gold standard.

MATERIALS AND METHODS

Study population. Between March 2000 and March 2001, TB suspects (15 to 65 years old), defined by a cough of 3 weeks’ duration and/or hemoptysis, were enrolled from the Rhodes Chest Clinic, a large diagnostic center in Nairobi. The selection of the suspects entering the diagnostic process strictly followed routine diagnostic procedures as described previously (11). Suspects were counseled to deliver three specimens. The first specimen was collected when the patient attended the clinic for the first time. A container was given to the patient for collecting an early morning sample at home the next day, and a third was collected again at the clinic when the patient brought back the early morning specimen. The suspects were also requested to give a blood sample for HIV testing on a voluntary basis. Those who were not willing to have an HIV test remained eligible for study inclusion. The Kenya Medical Research Institute (KEMRI) Ethical Committee approved this study, and written informed consent was obtained from every participating individual.

Acid-fast staining. A direct smear was made from each sputum specimen, stained by the ZN method, and read at the Rhodes Chest Clinic, after which the specimen was sent to KEMRI for culture and PCR. A suspect was diagnosed as a smear-positive TB patient when at least one of the three smears was positive by ZN.

Culture and identification. For culture and PCR techniques, sputum samples were first decontaminated and concentrated by centrifugation. From each sputum specimen decontaminated with 2% N-acetyl-L-cysteine NaOH, 0.25 ml was inoculated in duplicate onto slants of LJ medium (Becton Dickinson Microbiological Systems, Cockeysville, Md.) (5). The inoculated LJ medium slants were incubated at 37°C and examined for growth twice weekly for the first 2 weeks and once weekly thereafter up to 8 weeks, after which a definitive result was obtained. Cultures that showed no growth after 8 weeks were scored as “negative.” A patient was defined as a “TB-positive case” if one of the three sputum specimens had a positive culture and as a “non-TB case” if none of the three sputum specimens showed growth.

The identity of the mycobacterium was determined on one positive culture from each culture-positive TB case by hybridization to genus-specific probe (Accu probe; GenProbe). When results were inconclusive, DNA sequencing on appropriate ribosomal DNA sequences was performed (1).

CXR. CXR were taken for those suspects whose sputum smear ZN results were negative. A suspect was diagnosed as a smear-negative TB case when three sputum smears were negative for ZN and a CXR result was consistent with TB (11).

PCR. The Amplicor test system (Roche Diagnostic Systems, Basel, Switzerland), which utilizes biotinylated genus-specific primers to amplify a 584-bp ribosomal DNA sequence specific to the M. tuberculosis complex, was used. Testing was performed according to the manufacturer’s instructions. Specimens with a final A₅₅₀ of 0.35 in the enzyme amplification detection assay were interpreted as positive for M. tuberculosis. One positive and three negative amplification controls were included in all runs. The results of the controls fulfilled the criteria set by the manufacturer.

A coamplified internal control sequence allowed the monitoring of inhibition of the PCR in each specimen. When inhibition was found, samples were diluted and restested. In 0.5% of all samples tested, no PCR result could be obtained due to inhibition. A suspect was labeled PCR positive when at least one out of three specimens tested positive and PCR negative when all three specimens tested negative.

HIV. Antibodies to HIV were detected by the Virinostika HIV Uni-Form II plus 0 assay from Organon Teknika (Bostel, The Netherlands) according to the manufacturer’s instructions.

Data analysis. The laboratory staff was “blinded” to the clinical data. Patient data were recorded on routine forms and a special register. These data were entered into a computer database and analyzed by appropriate statistical software (Epi Info and SPSS).

RESULTS

Study population. In total, 1,398 individuals were enrolled. For 867 (62%) suspects, culture, ZN, and PCR results were available from all three specimens, in addition to CXR results for establishing the diagnosis of smear-negative TB. Of these 867 suspects, 490 (57%) were culture positive and 377 (43%) were culture negative. Two hundred seventeen individuals gave consent for HIV testing; 77 (35%) were HIV positive, and 140 (65%) were HIV negative.

Routine diagnostic procedure. The routine diagnostic procedure yielded 302 smear-positive and 278 smear-negative TB patients (Table 1). Taking the culture result into account, 2.3% of the smear-positive and 44.2% of the smear-negative individuals were falsely diagnosed (overdiagnosis), mainly due to the low specificity of CXR. Forty (13.9%) of the 287 suspects labeled as having no TB were culture positive (underdiagnosis).

PCR performance. PCR yielded 514 positive suspects, 60 of whom (11.7%) were culture negative (Table 2). Of these 60 suspects, 36 (60%) had two or three PCR-positive specimens; only 1 patient had a positive result by ZN smear microscopy.

Of the 353 PCR-negative suspects, 36 (10.2%) were culture positive. Two of these positive cultures were identified as Mycobacterium mucogenicum, and the other 34 were identified as M. tuberculosis, by molecular probing. Of the 454 culture- and PCR-positive samples, 452 were identified as M. tuberculosis, 1 was identified as a mixture of mycobacterial species, and 1 was identified as another mycobacterial species (OMS-33) (1).

Comparison of routine diagnostic procedures and PCR. Table 3 shows the sensitivities, specificities, and likelihood ratios of ZN alone, ZN in combination with CXR, and PCR. PCR had a sensitivity of 93%; its specificity of 84% was lower than that of ZN alone (98%) but higher than that of ZN in combination with CXR (66%). PCR correctly identified 294 (99.7%) of the 295 true smear-positive (positive by both culture and ZN) and 160 (82.1%) of the 195 true smear-negative (positive by culture and negative by ZN) TB cases.

| TABLE 1. Comparison of routine diagnosis (ZN followed by CXR) with culture as the gold standard |
|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|
| Result by routine diagnosis*                  | No. (%) with the indicated result by routine diagnosis | No. (%) with the following result by culture: |
| Smear-positive TB                              | 302 (34.8)                                      | 295 (97.7)                                    | 7 (2.3)                                           |
| Smear-negative TB                              | 278 (32.1)                                      | 155 (55.8)                                    | 123 (44.2)                                       |
| No TB                                         | 287 (33.1)                                      | 40 (13.9)                                     | 247 (86.1)                                       |
| Total                                         | 867 (100)                                       | 490 (56.5)                                    | 377 (43.5)                                       |

* Diagnosis made by a clinician based on routine diagnostic procedures (6).

<p>| TABLE 2. Comparison of PCR on three specimens with culture as the gold standard |
|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|</p>
<table>
<thead>
<tr>
<th>PCR result</th>
<th>No. (%) with the indicated PCR result</th>
<th>No. (%) with the following result by culture:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>514 (59.3)</td>
<td>454 (88.39)</td>
</tr>
<tr>
<td>Negative</td>
<td>353 (40.7)</td>
<td>36 (10.2)</td>
</tr>
<tr>
<td>Total</td>
<td>867 (100)</td>
<td>490 (56.5)</td>
</tr>
</tbody>
</table>

* PCR results were labeled positive when at least one out of three sputum specimens was positive and negative when all three specimens were negative.
Test performance stratified by sputum sample. ZN microscopy used for the first specimen detected 157 (32%) of the 490 culture-positive suspects; the second morning specimen added another 113 (23%), and the third specimen added 26 (5%). The overall sensitivities were 32% (95% CI, 28 to 36%), 47% (95% CI, 42 to 51%), and 31% (95% CI, 27 to 35%), respectively, for the first, second, and third sputum specimens.

Similarly, PCR detected 421 (86%) of the 490 culture-positive suspects upon testing of the first sputum specimen; the second specimen added another 27 (6%) PCR-positive results, and the third sample added another 6 (1%). The sensitivity of PCR on the first sputum sample was 86% (95% CI, 84 to 88%) and the specificity was 90% (95% CI, 88 to 92%). The positive and negative likelihood ratios were 8.52 and 0.16, respectively. The sensitivities for the second and third specimens were 85% (95% CI, 84 to 88%) and 82% (95% CI, 79 to 86%), respectively.

Effect of HIV on the performance of PCR and ZN. The sensitivity of PCR for HIV-positive TB patients (n = 36) was 89%, compared to 95% for HIV-negative TB patients (n = 79) (P = 0.43 by the chi-square test with Yates’ correction). The sensitivity of ZN was significantly lower for HIV-positive patients (44%) than for HIV-negative patients (63%) (P = 0.05 by the chi-square test).

DISCUSSION

Current global TB control efforts are based on diagnosis of cases followed by adequate treatment. It is important that diagnosis be established early and efficiently in order to prevent continued transmission and misdiagnosis. In environments with high prevalences of TB and HIV, better tests and more-efficient diagnostic processes are needed.

The sensitivity of PCR reported here compares well to that reported from studies performed in industrialized countries (4, 10, 12). One study conducted in Lusaka reported a sensitivity of 55% (3). However, that study used a low-cost “in-house” one-tube nested PCR and a gold standard incorporating both microbiological and clinical data. This demonstrates the need for standardization of reagents and methodology, and particularly for standardization of the gold standard.

In our study population, where both TB and HIV prevalences are high, PCR performed better than the current routine diagnostic process of ZN smear microscopy on three sputum samples followed by CXR. PCR detected a similar proportion of culture-positive TB patients (93 versus 92%) but had a higher specificity (84 versus 66%), resulting in less over-diagnosis. When PCR results of the first sputum sample only were considered, the sensitivity was reduced to only 86% while the specificity increased to 90%.

The high sensitivity of PCR on three samples (93%) and even on the first sample (86%) opens the way to a simplified diagnostic screening process in which both ZN and CXR can be avoided. Moreover, unlike that of ZN, the sensitivity of PCR is not influenced by the HIV status of the patient. While the sensitivity of ZN is dependent on the type and quality of the specimen—with the second, early morning specimen yield-
ing the highest sensitivity—the sensitivity of PCR is independent of the time of sputum collection, as shown in this study.

The specificity of the PCR as calculated in this study (84% on three sputum examinations and 90% on the first specimen only) is lower than that of ZN microscopy (98%) and lower than that reported in many other studies where automated culture systems were used as the gold standard (4, 10, 12). We used positive culture on LJ medium as the gold standard for the presence of TB. More sensitive methods exist, including the use of automated culture systems and incorporation of the clinical diagnosis and treatment outcome. It may well be that the PCR is more sensitive than the LJ culture method used here, effectively resulting in an underestimation of the specificity of the PCR when culture only is used as the gold standard. This possibility is supported by the fact that the majority of PCR-positive but culture-negative suspects (60%) had two or more positive PCR results. Cross-contamination was not a problem, since all negative controls remained negative throughout the study. However, the design of this study did not allow clinical follow-up of PCR-positive but culture-negative patients. Identification of the positive cultures revealed that PCR gave a false-positive result for one of the four mycobacterial strains identified as other than \textit{M. tuberculosis}. The causative organism of pulmonary tuberculosis in this population seemed to be primarily \textit{M. tuberculosis}.

The performance of a test expressed as sensitivity and specificity is independent of the prevalence of the disease. The chance, however, that a positive or negative test result reflects a positive or negative TB status does depend on the prevalence of TB among the suspects. The prevalence of TB in the study population was relatively high (57%).

The question is how the performance of PCR can be compared to that of the routine diagnostic process in environments with different pretest probabilities for TB. As shown in Fig. 1, ZN on its own is the best test for confirming a diagnosis of TB, but a negative result does not rule out TB. However, the posttest probability of confirming TB is higher with PCR than with the routine diagnostic procedure (ZN followed by CXR); the difference is highest in environments with medium to high pretest probabilities of TB among suspects, which is the case in most sub-Saharan cities. PCR performs as well as the routine diagnostic process at ruling out TB.

Apart from the test performance, there are other factors to consider that determine the choice to introduce a certain test. An advantage of PCR is that it can be performed on a single specimen and yields a result within a day. This makes the diagnostic process shorter and more patient-friendly, which may reduce dropout levels and altogether contribute to reduced transmission.

However, there are also disadvantages. We found that various issues had to be addressed: (i) training of personnel, (ii) organization of appropriate working space with a constant supply of water and a power backup, (iii) maintenance of equipment, and (iv) timely procurement of the PCR kits, which have a short shelf life and need cooling. From our experience, maintenance of the equipment and provision of continuous supplies were the main problems. Finally, the costs of PCR are considerable.

PCR has great potential, but its evaluation in low-income countries has been limited. This study showed that PCR can be considered as an alternative to ZN in combination with CXR for the diagnosis of TB. However, cost-effectiveness studies and operational studies are required to support an evidence-based decision to introduce PCR for TB control in high-burden environments.

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