PCR-based detection of the Mycobacterium tuberculosis complex in urine of HIV-infected and uninfected pulmonary and extrapulmonary tuberculosis patients in Burkina Faso

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INTRODUCTION

Burkina Faso registers a mean of 2000 new cases of tuberculosis (TB) per year (Burkina National TB Control Programme), of which smear-negative pulmonary TB (PTB) accounts for 12% (World Health Organization, 2004). In 2001, 7.2% of the population of Burkina Faso was estimated to be infected by human immunodeficiency virus (HIV) (National AIDS Control Programme, Burkina Faso, 2001). The last study available (1994) showed that the prevalence of HIV antibodies among Burkinae TB patients was 33.6% (Ledru et al., 1996). In other sub-Saharan African countries the rates of HIV infection among TB patients are even higher (44-5%) (Ngom et al., 1999) and smear-negative TB accounts for approximately 28% of the cases (World Health Organization, 2004).

The classical microbiological methods for diagnosing PTB and extrapulmonary tuberculosis (EPTB) lack both sensitiv-
ity and specificity (Chain, 1995). For example, the sensitivity of smear microscopy may be about 50% and culture on solid media requires up to 6 weeks to detect positive specimens (Small & Perkins, 2000; Daniel, 1990). However, only smear microscopy is routinely available in developing countries.

The absence of a positive smear in individuals still suspected of having TB is a diagnostic problem especially when there are no other tests available. Furthermore, acid-fast bacilli (AFB) smear-negative PTB is strongly associated with HIV infection (Elliott et al., 1993; Colebunders et al., 1989). In these patients, abnormal chest X-rays and other clinical symptoms are often absent, thus the diagnosis of active disease is done on scant clinical data. For this reason, treatment is frequently delayed. This delay in diagnosis and treatment contributes to the poor prognosis observed among HIV-infected smear-negative PTB patients (Hargreaves et al., 2001).

EPTB is difficult to detect without cytological and histological tests, which are frequently not available in Burkina Faso. The use of non-invasive simple molecular methods which could detect the presence of a few AFB of the *Mycobacterium tuberculosis* complex could be useful for diagnosing these cases.

Semi-automated commercial kits (Cobas Amplicor system from Roche Diagnostic Systems and Abbott LCx Probe System) have been used to diagnose PTB and EPTB. However, these diagnostic tests are expensive and their sensitivity is often similar or lower than that observed using the manual in-house PCR assay, in particular for smear-negative PTB (Yuen et al., 1997; Eing et al., 1998).

Sputa are not useful in diagnosing EPTB and are uncommonly produced by HIV-infected patients. Handling blood samples for diagnosing TB is hazardous and the detection of bacilli by PCR is not sensitive enough due to the presence of inhibitors (Folgueira et al., 1993, 1996). However, urine samples are easy to collect and the bacilli can be concentrated by centrifugation. Moreover, it has been reported that *M. tuberculosis* can be detected in urine samples by a one-tube nested PCR (OTN-PCR) using the IS6110 sequence as a target (Sechi et al., 1997; Aceti et al., 1999).

The aim of this study was to evaluate the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of the OTN-PCR analysis of urine in three different groups of HIV-infected and uninfected PTB patients: (1) microbiologically confirmed PTB, (2) smear- and culture-negative PTB and (3) EPTB.

**METHODS**

**Study population.** This study was conducted in four settings in Burkina Faso: the Tuberculosis Regional Centre and the pulmonology unit of the Sanou Sourou National Hospital, both in Bobo-Dioulasso (the second city of Burkina Faso), as well as the Tuberculosis National Centre and the Department of Pneumology of the Yalgado Ouedraogo National Hospital, both in Ouagadougou (the capital city). Consecutive adult patients were recruited from December 2000 to December 2001.

TB diagnosis was made using criteria established by the Burkina Faso National Control Program, following World Health Organization guidelines. In Burkina Faso, as the culture from sputum specimens is not done routinely, the patients are considered and notified by the physician as having smear-positive PTB if at least two out of three sputum specimens are positive by microscopy, and they are notified as having smear-negative PTB if two series of three sputum specimens are negative by microscopy but with at least one specimen subsequently found to be culture-positive, and a chest X-ray and clinical signs consistent with TB.

Diagnostic procedures and the start of anti-TB therapy of the patients were decided by the physician in charge of the patient regardless of the OTN-PCR test result. Patients already receiving anti-TB medication before the study started were excluded from the study. Urine collection for testing the OTN-PCR-based method was done before beginning the anti-TB treatment, at the same time that sputum specimens were collected.

In this study, since all sputum specimens were cultured, TB patients were finally classified on the bases of microbiological (microscopy and culture) and clinical findings into three groups: microbiological-positive PTB when smear and/or cultures were positives; microbiological-negative PTB when smear and cultures were negatives; and EPTB.

In the microbiological-positive PTB group, sensitivity and specificity were evaluated considering the culture as the gold standard; whereas for microbiological-negative PTB and EPTB, we considered both the culture and the national criteria for notifying TB cases. The ‘smear-negative PTB’ cases were diagnosed according to the criteria of the national program as follows (Dermot et al., 1997): (1) two series of three sputum specimens negative by microscopy, (2) chest X-ray with anomalies evocative of PTB and no response after treatment for 15 days with broad-spectrum antibiotics, (3) decision taken by the physician for treating with anti-TB therapy and (4) at least one specimen subsequently found to be culture-positive.

Diagnosis of EPTB was based on the epidemiological and clinical criteria such as a heavy endemic situation, contact status, extra-thoracic symptoms typical of TB (asthenia, weight loss of 10%, anorexia, prolonged fever resistant to broad-spectrum antibiotics, conjunctiva pale, evening sweat, amenorrhoea non gravid) and the presumptive anti-TB treatment. In addition, for each EPTB site, specific elements were considered for diagnosing each type of disease, e.g. chest X-ray excluding another obvious cause for miliary TB, serofibrinous pleural fluid with a characteristic cytology, excluding another obvious cause, for pleural TB, spine X-ray for Pott’s disease, localization and aspect of lymph nodes and presence of caseous material in biopsies for lymph node TB, painless tumefaction of scrotum for testicular TB, laparoscopy and the presence of serofibrinous ascitic fluid for tuberculous peritonitis and serofibrinous fluid for tuberculous pericarditis.

Control patients were individuals attending the clinics for respiratory diseases other than TB.

**Ethical approval.** The study was approved by the Ethical Committee of the Centre Muraz. A written informed consent was required from all patients before recruitment in the study.

**Sample collection and laboratory methods.** For each patient, three urine samples collected on three consecutive days as early morning urine (for the OTN-PCR test) and two sputum samples (for microscopy and culture) as well as 5 ml blood were collected for the purpose of this
study, in addition to the three sputum samples routinely required for TB diagnosis.

The laboratory staff performing the urine OTN-PCR were blinded to the clinical findings and to the other microbiological test results. Clinical, epidemiological and bacteriological data were collected by means of a standardized questionnaire.

**Microbiological analysis.** AFB staining was performed by the Ziehl–Neelsen method on two sputum specimens collected on consecutive days. Culture of mycobacteria was done on Löwenstein–Jensen slants after treating the samples by the Petroff method and the classical biochemical identification tests for the *M. tuberculosis* complex were used on the isolates grown.

**Serological test.** Antibodies to HIV type 1 (HIV-1), HIV-1 group O and HIV type 2 (HIV-2) were detected by a commercial enzyme immunoassay (Murex HIV-1.2.O from Abbott). In the case this test was reactive, discriminating between infections with HIV-1 and HIV-2 was done by means of two commercial monospecific tests, Wellozyme HIV Recombinant from Abbott for HIV-1 and Murex HIV-2 for HIV-2.

**OTN-PCR analysis**

**Urine processing.** The three urine specimens were pooled and centrifuged at 3000 g for 20 min. Supernatant was removed and the pellet was resuspended in 1 vol. 4% NaOH. After incubation for 15 min at 37 °C, 30 ml PBS was added and the samples were centrifuged at 3000 g for 20 min. The pellets were resuspended in 1 ml PBS, and aliquots of 500 µl were stored at −20 °C until analysis.

**Extraction of chromosomal DNA.** Aliquots of 500 µl of urine samples were heat-inactivated at 100 °C for 10 min. Debris was washed once with sterile distilled water and pelleted by centrifugation at 16 000 g for 5 min. The supernatant was flicked off and the pellet was resuspended in 50 µl 0.5% Tween 20 in sterile distilled water. Heating at 100 °C for 5 min followed by freezing at −80 °C for 5 min was performed twice followed by heating again at 100 °C for 5 min. The samples were centrifuged at 9000 g for 5 min and the supernatant was transferred to a clean microcentrifuge tube. One volume of chloroform was added and the sample was mixed by vortexing for 2 min. After centrifugation at 9000 g for 2 min, 10 µl of the aqueous supernatant was used for DNA amplification in a final volume of 30 µl.

**OTN-PCR.** The two sets of primers used for amplification were derived from the gene sequence encoding the insertion sequence IS6110, as follows. External primers were TB290 (5'-GGCGGGACAAAGCCGAATTTGCCGA-3') and TB856 (5'-CGAGGTTAGGGCTCGGTGTAAGGG-3'), and internal primers were TB431 (5'-TACTAGGACGACATCAACGC-3') and TB740c (5'-GGGGTGTGGCCGGGATCAAGGG-3'). The reaction mixture consisted of 20 mM Tris/HCl (pH 8.0), 50 mM KCl, 1.5 mM MgCl₂, 0.3 mM (each) dATP, dGTP, dCTP and dTTP, 0.01 M external primers, 0.1 M internal primers and 2 U Taq DNA polymerase (Invitrogen) in a total reaction volume of 30 µl. For the amplification reaction, the temperature cycling conditions consisted of: one cycle of 94 °C for 5 min; 40 cycles of 94 °C for 30 s, 64 °C for 40 s and 72 °C for 1 min; 25 cycles of 94 °C for 40 s, 54 °C for 40 s and 72 °C for 1 min; and a final cycle of 72 °C for 10 min. Positive and negative controls were included in each run, and all precautions to prevent cross-contamination were observed. Amplified product was visualized after electrophoresis in a 2% agarose gel in Tris-borate-EDTA buffer. Target bands of approximately 500 and 300 bp were visualized by staining with ethidium bromide.

**Statistical methods.** Data were collected on Epi Info version 6.04, and sensitivity, specificity, PPV and NPV with their 95% confidence intervals were calculated. The proportions were obtained by the Exact binomial method (CI 95%) and the chi square Pearson test was used to compare frequencies and proportions.

**RESULTS AND DISCUSSION**

**Cases**

Three hundred and thirty-one patients, including 145 HIV-infected, were diagnosed as TB patients according to the National criteria and were included in the study. Of these, 217 PTB cases (including 86 HIV-infected) were confirmed by microbiological tests; 210 were confirmed by microscopy and culture (including 82 HIV-infected) and seven were AFB smear-negative and culture-positive (including four HIV-infected). Some PTB patients had an additional infection site in addition to lung involvement, such as two of the 210 patients with microscopy- and culture-positive results (one miliary and one pleural) and two out of seven patients with AFB smear-negative and culture-positive results (two pleural). The group of microbiological-negative PTB accounted for 30 smear- and culture-negative patients (including 16 HIV-infected). Seven of these patients had an additional infection site in addition to lung involvement (four pleural, one miliary, one Pott’s disease and one tuberculous peritonitis).

Of the 247 PTB patients, 160 were male (mean age 36.8 years; range 15–75) and 87 were female (mean age 35.6 years; range 15–70).

Eighty-four patients presented with EPTB (including 43 HIV-infected). Of these patients, 58 were males (mean age 44–4 years; range 22–80) and 26 were females (mean age 32–5 years; range 15–60). The clinical presentations of this group were miliary TB for 18 patients (including eight HIV-infected), pleural TB for 38 patients (including 23 HIV-infected), urogenital TB for three patients (including three HIV-infected), Pott’s disease for seven (including one HIV-infected), lymph node TB for six patients (including four HIV-infected), tuberculous pericarditis for one patient (HIV-uninfected) and multifocal TB for eight patients (including three HIV-infected). Of these eight multifocal TB patients, three had miliary and pleural TB (one HIV-infected), one had miliary and pleural TB and tuberculous peritonitis (HIV-uninfected), one had pleural and lymph node TB (HIV-uninfected), two had miliary, pleural and lymph node TB (one HIV-infected) and one had pleural TB and tuberculous peritonitis (HIV-uninfected).

**Control cases**

Seventy-four patients were initially included as controls (including 31 HIV-infected). Nineteen had to be excluded from the analyses because they were lost to follow-up (*n* = 5), subsequently found to be TB patients (*n* = 12), living with a TB patient (*n* = 1) or died with a pleural effusion suggesting TB (*n* = 1). Finally, the remaining 55 controls (including 22 HIV-infected) were included in the
analyses. Among these patients 34 were males (mean age 45.6 years; range 21–80) and 21 were females (mean age 34.1 years; range 22–80). These patients did not meet the TB diagnostic criteria established by the Burkina Faso National Control Program, which follows World Health Organization guidelines.

**OTN-PCR analysis of urine**

Table 1 shows the PCR results in the different groups of patients. Fig. 1 shows an example of PCR amplification.

**M. tuberculosis** complex OTN-PCR was positive in urine samples from 88 of 217 (40.6 %) patients with microbiological-positive PTB, 20 of 30 (66.7 %) patients with microbiological-negative PTB and 48 of 84 (57.1 %) patients with EPTB. The detection of **M. tuberculosis** was higher in the microbiological-negative PTB and the EPTB groups than in the microbiological-positive PTB group (chi square test, \( P = 0.003 \)). The difference between the former two was not significant \( (P > 0.05) \), whereas the difference between the microbiological-positive PTB group and the microbiological-negative PTB or EPTB groups was significant (chi square test, \( P = 0.006 \) and \( P = 0.009 \), respectively).

In the group of patients with microbiological-positive PTB, the highest rate of detection was observed among AFB smear-negative culture-positive PTB patients with six out of seven urines positive by PCR (three HIV-positive and three HIV-negative).

Among patients with TB involving another site in addition to the lung we observed six out of seven urines positive by PCR in the group of microbiological-negative PTB and three out of four in the group of microbiological-positive PTB.

**M. tuberculosis** was significantly more frequently detected in HIV-infected versus uninfected patients with smear- and culture-positive PTB [43 of 82 (52.4 %) versus 39 of 128 (30.5 %); chi square test, \( P = 0.001 \)] and in patients with EPTB [29 of 43 (67.4 %) versus 19 of 41 (46.3 %); chi square test, \( P = 0.05 \)] but not in patients with an AFB smear-negative PTB with or without a positive culture [14 of 20 (70.0 %) versus 12 of 17 (70.6 %); chi square test, \( P > 0.05 \)].

Only one of 55 urine samples from controls had a positive signal by **M. tuberculosis** complex OTN-PCR that was considered as a false positive.

The sensitivity, specificity, PPV and NPV of the **M. tuberculosis** complex OTN-PCR results in urine samples from patients and controls are shown in Table 2.

**Table 1.** **M. tuberculosis** complex OTN-PCR results in urine samples from patients and controls

<table>
<thead>
<tr>
<th></th>
<th>OTN-PCR positive</th>
<th>Total</th>
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<tr>
<td></td>
<td>( n )</td>
<td>%</td>
</tr>
<tr>
<td><strong>Microbiological-positive PTB</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AFB smear-positive/HIV-positive</td>
<td>43</td>
<td>52.4</td>
</tr>
<tr>
<td>AFB smear-positive/HIV-negative</td>
<td>39</td>
<td>30.5</td>
</tr>
<tr>
<td>AFB smear-negative/HIV-positive</td>
<td>3</td>
<td>75.0</td>
</tr>
<tr>
<td>AFB smear-negative/HIV-negative</td>
<td>3</td>
<td>100.0</td>
</tr>
<tr>
<td>Total</td>
<td>88</td>
<td>40.6</td>
</tr>
<tr>
<td><strong>Microbiological-negative PTB</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HIV-positive</td>
<td>11</td>
<td>68.7</td>
</tr>
<tr>
<td>HIV-negative</td>
<td>9</td>
<td>64.3</td>
</tr>
<tr>
<td>Total</td>
<td>20</td>
<td>66.7</td>
</tr>
<tr>
<td><strong>EPTB</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HIV-positive</td>
<td>29</td>
<td>67.4</td>
</tr>
<tr>
<td>HIV-negative</td>
<td>19</td>
<td>46.3</td>
</tr>
<tr>
<td>Total</td>
<td>48</td>
<td>57.1</td>
</tr>
<tr>
<td><strong>Control patients</strong></td>
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<td></td>
</tr>
<tr>
<td>HIV-positive</td>
<td>1</td>
<td>4.5</td>
</tr>
<tr>
<td>HIV-negative</td>
<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td>Total</td>
<td>1</td>
<td>1.8</td>
</tr>
</tbody>
</table>

*Positive culture.
†Negative culture.
not in patients with microbiological-negative PTB [68.8% (95% CI 41.5–87.9) versus 64.3% (95% CI 35.6–86.0); chi square test, \( P < 0.05 \)]. The specificity and the PPV were high and the NPV relatively lower, irrespective of the HIV status.

As in most developing countries, laboratory diagnosis of TB in Burkina Faso remains problematic because of a lack of laboratory facilities. In sub-Saharan Africa, the HIV epidemic has contributed to the spread of \( M. \) \( tuberculosis \) disease frequently associated with an atypical clinical presentation (de Albuquerque et al., 2001). The high HIV seroprevalence accompanied by the often undiagnosed TB disease in these countries contribute to diagnostic and treatment delays. This finally results in further transmission of bacilli. The development of sensitive and rapid diagnostic tools to detect and confirm active TB is therefore desperately needed, especially among HIV-infected individuals in countries with a high burden of these two infectious diseases.

In our study, we evaluated the performance of a test based on an OTN-PCR amplification in urine using the IS6110 sequence of the \( M. \) \( tuberculosis \) complex as a target to be amplified.

In a previous evaluation of a similar technique, sensitivity was reported to be 28.6% (95% CI 17.9–41.3) in culture-confirmed PTB (Kafwabulula et al., 2002), a lower value than in our study; we found also a higher sensitivity in HIV-infected versus uninfected patients with either microbiological-positive PTB or EPTB. However, these two studies are not comparable in terms of patient selection and numbers, urine collection (in our study three void urine samples were collected on three consecutive days) and PCR protocols.

While evaluating the reproducibility of our \( M. \) \( tuberculosis \) complex OTN-PCR technique, we found that some samples of the same specimen gave inconsistent results (assays based on two separate decontaminated urine pellets; data not shown). This suggests that \( M. \) \( tuberculosis \) complex DNA is represented in low copy numbers and/or is unevenly distributed in urine samples, as already suggested by others (Kafwabulula et al., 2002; Jouveshomme et al., 1998).

The specificity of our OTN-PCR method is similar to that reported in other studies evaluating Sechi’s method and automated direct amplification tests (Yuen et al., 1997; Eing et al., 1998; Kafwabulula et al., 2002).

Diagnosis of TB in a resource-poor country is problematic. The cost and lack of suitably trained staff are the major problems. Although Centre Muraz in Bobo Dioulasso is a well-organized centre with professional personnel, implementation of a molecular technique in this setting must be considered carefully.

In the light of these results, \( M. \) \( tuberculosis \) complex OTN-PCR should not be recommended for routine detection of new TB patients in Burkina Faso. The technique may, however, be useful for confirming \( M. \) \( tuberculosis \) in patients suspected to be microbiological-negative PTB or EPTB when clinical and bacteriological diagnoses are not conclusive, especially in the presence of an HIV co-infection, considering that urine is a biological sample that can be obtained by a non-invasive technique.

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REFERENCES


