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Urine as an adjunct specimen for the diagnosis of active pulmonary tuberculosis

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Summary
Background: The diagnosis of pulmonary tuberculosis (PTB) is conventionally established by examination of three Ziehl—Neelsen stained smears; however, negative results do not preclude active TB. Since tubercle bacilli or their nucleic acids are also expected to be excreted through the kidneys, we assessed spot urine as a supplementary specimen for diagnosing PTB.
Methods: A total of 164 respiratory specimens (147 sputum, 15 bronchoalveolar lavage, and two gastric lavage) from 81 suspected PTB cases were prospectively collected and processed. A total of 112 non-TB controls were also included in the study. For three consecutive days, morning urine specimens were collected from all patients and controls, and were processed for culture by BACTEC™ MGIT 960 (mycobacteria growth indicator tube) and Lowenstein—Jensen methods and for PCR by amplifying a 441-bp fragment of the *hsp65* gene (*Mycobacterium* genus-specific) and a 786-bp fragment of the *cfp32* gene (TB complex-specific).
Results: Of the 81 patients suspected of having PTB, 46 (56.8%) were sputum culture-positive. Of these, 12 (26.1%) were also urine culture-positive for *Mycobacterium tuberculosis*. Of the 35 sputum culture-negative cases, three (8.6%) were urine culture-positive. The TB complex specific PCR (*cfp32*) was positive in 52.2% (24/46) of the bacteriologically-confirmed and 28.6% (10/35) of the bacteriologically-negative PTB patients. In none of the control subjects were urine culture or PCR found to be positive for *M. tuberculosis*.
Conclusions: Specific PCR and culture examination of spot urine samples from suspected PTB patients significantly improved the detection rate of PTB and should be encouraged in resource-limited settings and where multiple pulmonary specimens are not feasible.

Introduction

Tuberculosis (TB) is a major public health problem of global importance and it is the second leading cause of death worldwide, killing nearly 1.6 million people in 2005.[1] Several

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studies have identified the clinical manifestations and symptomatology of sputum smear-positive individuals.[2–4] The most common method for the diagnosis of pulmonary tuberculosis (PTB) is microscopic examination of Ziehl–Neelsen stained smears, which has a variable detection rate of 20–70%.[5] However, approximately 20–50% of patients with PTB are smear-negative, and 10% of these patients remain culture-negative, even on three consecutive days.[5] This phenomenon is more common in patients infected with multidrug-resistant *Mycobacterium tuberculosis* (MDR-TB).[6] Several studies have also shown that most of the smear- and culture-negative patients will develop bacteriologically-positive disease in the course of time.[2,5,7] In sputum-scarce PTB cases, bronchoalveolar lavage (BAL) fluid is a preferred clinical specimen in adults and gastric lavage in young children, but these specimens can be obtained only in a tertiary healthcare setting and have very low detection rates.[5,8]

Colby postulated that tubercle bacilli could be excreted through the kidneys and that the organisms could be demonstrated in the urine of active TB patients who have no symptoms pertaining to the urinary tract.[9] This hypothesis was confirmed by studies carried out in HIV-negative[10–12] and HIV-positive cases.[13,14] These studies showed that urine could be used as an adjunct specimen due to the convenience and non-invasive nature of collection. However, in smear- and culture-positive cases it may not be necessary to include other samples, and such studies may be a matter for the records only. A study by Torrea et al.[15] confirmed the utility of urine specimens for diagnosing PTB by nested PCR with a sensitivity of 64.3% in culture-negative PTB cases and 46.3% in extra-pulmonary tuberculosis (EPTB) cases. Though India harbors the majority of TB cases, no such study has yet been undertaken in this country. Therefore we carried out this study in order to evaluate the utility of urine as an adjunct or alternative specimen for diagnosing smear- and/or culture-negative PTB, using culture and PCR methods.

Materials and methods

Patients and specimens

This prospective hospital-based study was conducted from July 2005 to June 2006 at the Department of Laboratory Medicine, All India Institute of Medical Sciences (AIIMS), New Delhi, India. AIIMS is a tertiary care medical center. During the study period, the clinical data of 215 patients with suspected PTB, referred from various clinics of this institute and other hospitals in and around Delhi for mycobacterial culture, were investigated. Only 81 of these fulfilled the inclusion criteria and were recruited into this study.[16,17] A total of 112 healthy volunteers, including 15 laboratory staff, with no present or past history of TB were also included in the study as control subjects. Cases of suspected genitourinary TB with symptoms of burning micturition, unexplained sterile pyuria, or hematuria, and/or cases ultrasonographically suggestive of pyelonephritis were excluded from the study. Also only anti-tuberculosis treatment (ATT)-naïve patients were included in the study. The results of routine laboratory investigations such as erythrocyte sedimentation rate (ESR), total leukocyte count, and liver enzyme values were recorded for both the suspected TB and control subjects.

To establish the presence of PTB, at least two sputa (spot and early morning) were collected from each patient with a productive cough. BAL or gastric lavage specimens were taken from sputum-scarce patients. The sputum specimens were collected and brought to the laboratory by the patients themselves. The sputa received from the suspected PTB patients were first decontaminated by NALC-NaOH (modified Petroff's method) as previously described.[18]

An early morning or spot urine sample (approximately 500 ml) was also collected simultaneously from the patients and controls in a sterile wide-mouthed container for three consecutive days; the first two samples were stored in a refrigerator and all three samples were then pooled and processed on the third day. The pooled urine specimens from each patient were centrifuged at 3000 $\times$ *g* for 20 min. The resulting pellet was decontaminated with an equal amount of 4% NaOH. After incubation for 15 minutes, the suspension was neutralized with phosphate-buffered saline (PBS; pH 6.8) and again centrifuged at 10 000 rpm for 20 min.

The pellets of decontaminated respiratory and urine specimens were resuspended in PBS; smears were made for Ziehl–Neelsen staining and 0.1 ml was inoculated on Lowenstein–Jensen (L–J) slants while 0.5 ml was inoculated in BACTEC™ MGIT 960 tubes (mycobacteria growth indicator tube; BD Diagnostics, Sparks, MD, USA) for culture isolation. The remaining aliquots of the suspended pellets were stored at −80 °C and processed further for DNA isolation and analysis by PCR. Sputum and urine samples were processed separately to avoid possible cross-contamination.

The L–J slants were incubated at 37 °C for 6 weeks.[18] The inoculated MGIT 960 tubes were loaded into the BACTEC™ MGIT 960 system, and the growth was continuously monitored in fluorescence units, which flash positive after reaching a cut-off growth set by the manufacturer. Ziehl–Neelsen staining for acid-fast bacilli (AFB) was used to confirm positive BACTEC™ MGIT 960 tubes and any growth on L–J medium; Gram staining was also carried out to check for contamination. The specimens having AFB with contaminants were reprocessed as per the protocol above, while the others were discarded. Cultures were considered negative for mycobacterial culture only after 42 days, as per the manufacturer's guidelines. The *Mycobacterium* species isolated from the clinical specimens were identified by phenotypic and biochemical tests, including heat stable catalase, nitrate, niacin, and arylsulfatase tests.[18]

DNA isolation and analysis by PCR

The DNA from an aliquot of the decontaminated specimens was isolated as described previously by Ausubel et al.[19] In brief, the decontaminated pellets were lysed with lysozyme and proteinase K–SDS, and DNA extracted by the phenol chloroform method followed by precipitation with 70% ice-cold ethanol. The resulting DNA pellets were solubilized in Tris–EDTA buffer and used for the PCR. *Mycobacterium* genus-specific PCR was done by amplifying a 441-bp fragment of the *hsp65* gene[20] and TB complex-specific PCR by amplifying a 786-bp fragment of the *cfp32* gene.[21] The PCR master mix was prepared by combining 500 mM KCl, 100 mM Tris HCl