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Rapid identification of *Mycobacterium tuberculosis* complex on urine samples by Gen-Probe amplification test

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Abstract The aim of the study was to evaluate the applicability to urine samples of the Amplified *Mycobacterium tuberculosis* Direct Detection Test (AMTD), which is currently used to identify this organism in respiratory specimens within a few hours. The study was performed on 95 patients, comprising 35 subjects with a high index of suspicion for active tuberculosis of the urinary tract and 60 subjects with evidence of nonmycobacterial disease. One urine specimen from each subject was examined by microscopy, culture and AMTD. AMTD was positive in 38 specimens and negative in 57. Assuming culture as the reference standard, the sensitivity, specificity, positive predictive value and negative predictive value of AMTD were 100%, 91.93%, 86.84% and 100%, respectively. Reassessing the discrepancies between AMTD and culture by review of patients' charts, the sensitivity, specificity, positive predictive value and negative predictive value of AMTD were 100%, 93.44%, 89.47% and 100%. The results of the study as well as the characteristics of AMTD encourage its use for the rapid recognition of urinary tract tuberculosis, although its findings should be interpreted cautiously when the clinical picture is not consistent with an active tuberculosis.

Key words *Mycobacterium tuberculosis* · Amplification test · Urine specimens

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Introduction

The Amplified *Mycobacterium tuberculosis* Direct Detection Test (AMTD; Gen-Probe, San Diego, Calif.) is a method for the rapid recognition of the causative agent of tuberculosis. Based on rRNA amplification, it is currently used to identify the organisms directly in specimens from the respiratory tract within a few hours. The purpose of our study was to evaluate the applicability of AMTD to urine samples.

Materials and methods

The study was performed on 95 samples of early morning urine from as many subjects (age range 20–80 years; mean age 57 years). These subjects, selected from patients admitted to our hospital between January 1994 and July 1996, were evaluated on the basis of the clinical data and the findings of chest radiography, urography and ultrasound examination. The clinical data included the response to the intradermal test with 5 U of PPD (0.1 ml) (Biocine, Siena, Italy), in which an induration of at least 10 mm after 48 h was considered a positive response.

The study population was divided into two groups. The first included 35 subjects (age 20–74 years; mean age 52 years) with a high index of suspicion for active tuberculosis of the urinary tract. The second group included 60 subjects (age 31–80 years; mean age 60 years) with evidence of nonmycobacterial disease, represented by benign prostatic hyperplasia in 38 cases, renal lithiasis in 15, bladder lithiasis in 4, and renal cysts in 3. No subject in the second group had a history of previous active tuberculosis, in any location. The PPD skin test was positive in 33 subjects of the first group and 45 of the second.

Specimens were stored at −20°C prior to processing. They were then decontaminated using the *N*-acetyl-L-cysteine (NALC)– 3% NaOH protocol [12], concentrated by centrifugation at 3000 *g* for 20 min, and resuspended in 1.5 ml phosphate buffer (pH 6.8).

Microscopy

Direct smears prepared from specimens were stained by the Kinyoun method and examined using a Leitz Diaplan light microscope.

Culture

Aliquots (0.3 ml) from the processed sediment were inoculated into Loewenstein-Jensen medium, and incubated at 37°C for 10 weeks

in a humidified atmosphere. Cultures were checked twice a week during the first 2 weeks and then weekly. The identification of organisms was based on growth rates, colonial morphology, pigmentation, photochromogenicity and biochemical tests (niacin, nitrate reduction, aryl sulfatase, 68° catalase, tellurite reduction, Tween hydrolysis and urease).

AMTD

Fifty milliliters of decontaminated sample was added to 200 ml of dilution buffer in a lysing tube and sonicated for 15 min. Then 25 ml of reconstituted amplification reagent was placed in a reaction tube and covered with 200 ml of oil. Fifty milliliters of lysate was transferred to the amplification tube, incubated at 95°C for 15 min, and then cooled at 42°C for 5 min. An enzyme reagent mix (25 ml) was added, and the mixture incubated at 42°C for 2 h. To terminate the amplification, 20 ml of termination reagent was added to each tube, and the mixtures kept at 42°C for 10 min. Further steps were the addition of labeled probe (100 ml), incubation at 60°C for 15 min, addition of selection reagent (300 ml), and reincubation at 60°C for 10 min. Finally, the light signal emitted by the labeled probe was measured with a PAL luminometer. Results were expressed as relative light units (RLU), setting the cut-off value at 30 000 RLU. Values of ≥30 000 RLU were taken as positive; values of < 30 000 RLU were taken as negative.

Results

Microscopy was positive in 27 specimens and negative in 68. Culture was positive in 33 specimens and negative in 62 (all isolates were identified as *M. tuberculosis hominis*). AMTD was positive in 38 specimens and negative in 57 (the 5 AMTD-positive/culture-negative specimens were confirmed by repeat testing). RLU mean values were 804 888 (SD 375 293, SE 60 880) for positives and 8621 (SD 4649, SE 615) for negatives. The results of the examinations in the two groups of patients are shown in Table 1.

Taking culture as the reference standard, the results of AMTD were true positives in 33 cases, false positives in 5, false negative in none and true negatives in 57, giving a sensitivity, specificity, positive predictive value and negative predictive value of 100%, 91.93%, 86.84% and 100%, respectively (Table 2).

The five AMTD-positive/culture-negative specimens were re-evaluated on the basis of the patients' charts. One of these specimens, collected from a patient of the first group who had undergone nephrectomy, was correctly identified by AMTD, since the identification was sustained by both clinical-radiological evidence of active tuberculosis and a histopathological picture of renal granulomatous-caseating lesions. The remaining AMTD-positive/culture-negative specimens (each collected from subjects of the second group with a positive reaction to the PPD intradermal test) proved to be false positives. In conclusion, the adjusted results of AMTD were 34 true positives, 4 false positives, no false negative and 57 true negatives. After correction of the discrepancies, the sensitivity, specificity, positive predictive value and negative predictive value of AMTD were 100%, 93.44%, 89.47%, and 100%, respectively; the sensitivity, specificity, positive predictive value and negative predictive value of culture were 97.05%, 100%, 100%, and 98.38%; and the sensitivity, specificity, positive predictive value and negative predictive value of microscopy were 79.41%, 100%, 100%, and 89.70% (Table 3).

Table 1 Results of the examinations in the two groups of patients (*AMTD* Amplified *Myocobacterium tuberculosis* Direct Detection Test)

Examination	First group (35 subjects)		Second group (60 subjects)	
	Positive	Negative	Positive	Negative
Microscopy	27	8	0	60
Culture	33	2	0	60
AMTD	34	1	4	56

Table 2 Performance of microscopy and AMTD assuming culture as the reference standard

Microscopy	
True positives: 27	Sensitivity: 81.81%
False positives: 0	Specificity: 100%
False negatives: 6	Positive predictive value: 100%
True negatives: 62	Negative predictive value: 91.17%
AMTD	
True positives: 33	Sensitivity: 100%
False positives: 5	Specificity: 91.93%
False negatives: 0	Positive predictive value: 86.84%
True negatives: 57	Negative predictive value: 100%

Table 3 Performance of microscopy, culture and AMTD after the resolution of discrepancies

Microscopy	
True positives: 27	Sensitivity: 79.41%
False positives: 0	Specificity: 100%
False negatives: 7	Positive predictive value: 100%
True negatives: 61	Negative predictive value: 89.70%
Culture	
True positives: 33	Sensitivity: 97.05%
False positives: 0	Specificity: 100%
False negatives: 1	Positive predictive value: 100%
True negatives: 61	Negative predictive value: 98.38%
AMTD	
True positives: 34	Sensitivity: 100%
False positives: 4	Specificity: 93.44%
False negatives: 0	Positive predictive value: 89.47%
True negatives: 57	Negative predictive value: 100%

Discussion

In spite of the availability of effective antimycobacterial drugs, the incidence of tuberculosis has recently increased in industrialized countries, mainly as a consequence of migration and the HIV epidemic. This has emphasized the limits of conventional procedures for the detection of mycobacteria, which are represented by direct microscopy (with Ziehl-Neelsen, Kinyoun or auramine staining) and by culture examination. Micros-

copy is characterized by a high detection limit [0.5–1×10^4 colony forming units (CFU) per ml] and provides a presumptive diagnosis, as it can not differentiate *Mycobacterium tuberculosis* complex from nontuberculous mycobacteria. Culture examination, characterized by a lower detection limit (10–30 CFU/ml) and by sensitivities of 70–90% and specificities of 95–100%, permits the identification of mycobacterial species on the basis of phenotypical and biochemical parameters. Conventionally used as a reference standard, it is nevertheless a time-consuming procedure, requiring no less than 3–4 weeks.

The exigency of anticipating the identification of organisms led to the elaboration of alternative techniques, represented by both culture-dependent and culture-independent methods. The former, developed since the early 1980s, include Bactec, High-Performance Liquid Chromatography (HPLC), Mycobacteria Growth Indicator Tube (MGIT) and the AccuProbe System, which permit detection of mycobacteria on solid or liquid media within 10–15 days. The latter, developed between the late 1980s and early 1990s, are represented by the polymerase chain reaction (PCR) and other amplification procedures, such as AMTD, ligase chain reaction (LCR), QB replicase amplification (QBRA) and strand displacement amplification (SDA). These techniques, which have an extremely low detection limit (few to one mycobacterial cell/ml), allow the identification of organisms directly on specimens within 24 h or less.

Amplification procedures are widely used in studies on fluids from the respiratory tract, obtaining sensitivities and specificities comparable to those of culture. Far fewer data are available on the applicability of such techniques to urine specimens.

In the wake of previous limited experiences [4, 11], Van Vollenhoven et al. [19] tested 92 urine specimens collected from 83 patients with suspected urinary tract tuberculosis with both PCR and Bactec: though 2 urine specimens were positive by both PCR and Bactec and 90 were negative by both methods, the evaluation of the results was vitiated by the presence of inhibitors of PCR in 10% of specimens.

Our study employed AMTD, a transcriptase-mediated amplification system. By this method a *M. tuberculosis* complex-specific rRNA sequence is replicated via DNA intermediates (both the target and the major amplification product are single-stranded). The amplicons, hybridized with a chemiluminescent probe, and detected by a so-called hybridization protection assay (HPA), are measured luminometrically as RLUs. Thirty thousand RLUs are taken as a reliable cut-off value. AMTD is highly automated (amplification and analysis occur in the same tube) and extremely rapid (its completion time is approximately 5 h). On account of its characteristics AMTD is suited to laboratories with high work flow [1–3, 6, 8, 9, 13, 15, 20].

To our knowledge, up to now AMTD has been used for urine examination only on 16 samples in a study by Vlaspolder et al. [20] and on five samples in a study by Fairfax [8]. Although the results of the tests with AMTD were totally consistent with culture in both studies, their small number does not allow a valid conclusion.

Additional data are provided by our study, which was based on a larger quantity of urine samples, collected from both subjects with suspected active tuberculosis of the urinary tract and subjects who were neither suspected of having the disease nor had a history of previous active tuberculosis.

Thirty-three specimens were AMTD-positive and culture-positive, whereas 57 were negative by both methods; results were discrepant for 5 specimens, which proved positive by AMTD and negative by culture (Table 1). The AMTD-positive and AMTD-negative results were clearly discriminated by RLU magnitudes. Taking culture as the reference standard, the sensitivity, specificity, positive predictive value and negative predictive value of AMTD were 100%, 91.93%, 86.84% and 100%, respectively (Table 2). Reassessing the discrepancies between AMTD and culture by review of patients' charts, one AMTD-positive/culture-negative specimen turned out to be a true positive, while the 4 remaining discrepancies were confirmed as AMTD false positives. After resolution of the discrepancies the sensitivity, specificity, positive predictive value and negative predictive value of AMTD were 100%, 93.44%, 89.47% and 100%, while the sensitivity, specificity, positive predictive value and negative predictive value of culture were 97.05%, 100%, 100% and 98.38% (Table 3).

The discrepancies between amplification procedures and culture, also recorded by a large number of studies on respiratory fluids, are a topical issue. When active tuberculosis is suspected on the basis of clinical data, the amplification test-positive/culture-negative results probably relate to the higher detection limit of culture and/or to the uncultivability of the organisms (owing to metabolic exigencies, disruption of the cell wall due to decontamination procedures, or overgrowth of non-mycobacterial bacilli) [1–3, 6, 8, 9, 13, 15–18, 20]. The evaluation of amplification test-positive/culture-negative results proves more difficult when no evidence of active tuberculosis exists. This phenomenon could be explained by the ability of amplification techniques to record subclinical active disease or even latent infection by dormant bacilli [3, 17, 18, 21]: yet the latter possibility, which would cast doubts on the clinical utility of amplification tests, seems unlikely in the light of recent longitudinal investigations on respiratory fluids with both PCR [10] and AMTD [14]. Authentic false positives with amplification techniques may be caused by contamination with products of previous tests or nucleic acids. This risk, which is notable for "home-grown" PCR procedures [4, 11, 19], is probably lower for AMTD since aerosol-producing manipulations are limited by the use of the single test tube; moreover single-stranded RNA is less likely than double-stranded DNA to survive in the laboratory environment [1–3, 6, 8, 9, 13, 15, 20]. False AMTD positives can also result from nonspecific background chemiluminescence due to

blood impurities in the samples [8, 17]. At any rate the blood interference can be minimized by different measures, which include sample pretreatment with a solution of 10% sodium dodecyl sulfate (SDS)-EDTA (50 mM) [7]; the use of specimen blanks, containing the sample from the subject but no probe, in order to ascertain the background chemiluminescence provided by the sample itself [7]; and the combination of short centrifugations to eliminate blood cells and centrifugation at 9600 *g* for 1 min to capture the organisms [5].

A variable rate of amplification test-negative/culture-positive results derives from inhibition of the *Taq* polymerase of PCR or of the RNA polymerase of AMTD. Inhibitors include exogenous substances as well as endogenous metabolites, contained in respiratory fluids, urine, blood and other specimens; a variety of procedures has been proposed to inactivate such inhibitors [1, 2, 6, 8, 9, 13, 15, 17, 19]. In particular, the NALC-NaOH protocol of decontamination, utilized in our study, is credited with reducing the inhibition of AMTD by approximately 80% [6]. Finally, false negative results with amplification tests may result from the missing of organisms, due to sampling variability and small sample volumes [1, 2, 6, 8, 9, 13, 15, 17, 19]; an augmentation sample size is planned for the coming generation of AMTD.

In conclusion, the satisfactory results from AMTD recorded by our study, as well as the manageability and great rapidity of this method, encourage its adoption for the early diagnosis of tuberculosis of the urinary tract. However, possible positive AMTD results due to tuberculous latent infection are debated, while validation of technical improvements is still required. Consequently, in accordance with the assessments available for pulmonary tuberculosis [1–3, 9, 13, 15], AMTD should supplement rather than substitute the conventional microbiological examinations and the clinical evaluation. In this way AMTD can represent a rapid and reliable diagnostic tool as long as clinical-radiological data are compatible with active tuberculosis, whereas its results should be interpreted cautiously in follow-up examinations or when subjects with a previous history of tuberculosis are examined.

The use of AMTD should in any case be restricted to regional laboratories, because of the technical expertise required by this examination and its cost (approximately $70–80 per sample).

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