Rapid diagnosis of tuberculosis through the detection of mycobacterial DNA in urine by nucleic acid amplification methods

Clare Green, Jim F Huggett, Elizabeth Talbot, Peter Mwaba, Klaus Reither, Alimuddin I Zumla

Tuberculosis kills over 1.7 million people worldwide every year and nearly 40% of patients with active tuberculosis remain undiagnosed because of the poor sensitivity of the current, century old diagnostic method: sputum microscopy. Sputum microscopy is not able to easily detect paediatric, extrapulmonary, or HIV-associated tuberculosis, which are now important causes of morbidity and mortality in developing countries. Newer diagnostic methods for tuberculosis remain less sensitive than sputum microscopy. Alternative strategies to diagnose tuberculosis by use of nucleic acid amplification methods to detect fragments of mycobacterial DNA in urine have been developed over the past decade with varying sensitivities and specificities. Methods using quantitative PCR on urine samples to detect transrenal mycobacterial DNA are under development. The detection of transrenal DNA makes it possible to assay the total body burden of mycobacterial infection in any age group and in extrapulmonary tuberculosis with urine samples, which can be collected non-invasively. This Review discusses the developments and application of nucleic acid amplification of mycobacterial transrenal DNA for improved tuberculosis diagnostics.

Introduction

Worldwide, tuberculosis is a health problem of enormous proportions. Every year about 9 million people develop tuberculosis and over 1.7 million die as a consequence. Sub-Saharan Africa has the greatest tuberculosis burden, with 363 people per 100 000 of the population with active disease, and 63% of the worldwide number of people with tuberculosis that are HIV positive. Case-detection rates remain low, despite widespread implementation of the WHO directly observed treatment, short-course strategy over the past decade (the case-detection rate in Africa is 46% compared with the 70% Millennium Development Goals global target). Although this low case-detection rate is largely because of unsatisfactory national tuberculosis programme activities, it is compounded by the fact that diagnostic algorithms in use at present in countries with a high tuberculosis burden are based on inaccurate tests that have been in clinical use for many decades. Bacteriological testing for tuberculosis in the majority of laboratories in disease-endemic countries is restricted to microscopic examination of the acid-fast stained sputum smear, a method that was introduced over a century ago. Even in resource-rich settings where a variety of diagnostics are more readily available, a delay in tuberculosis diagnosis increases mortality.

A major shortcoming of conventional microscopy is its relatively low sensitivity compared with culture. Furthermore, sputum microscopy is not sensitive enough for the detection of tuberculosis in children, who frequently cannot produce sputum, or in patients with HIV-associated or extrapulmonary tuberculosis. HIV coinfection tends to reduce caseating necrosis and lower the numbers of acid-fast bacilli in the airway. In some settings, HIV might also reduce the specificity of sputum microscopy by increasing the number of patients infected with non-tuberculous mycobacteria. Alternative diagnostic methods commonly used for non-pulmonary forms of tuberculosis include culture or nucleic acid amplification techniques on tissue biopsies, blood, urine, gastric aspirates, or stool. These examinations are done if tuberculosis is suspected. Autopsy studies have shown the presence of Mycobacterium tuberculosis DNA in various organs including the kidney in adults with a known pulmonary tuberculosis diagnosis. It is likely that extrapulmonary tuberculosis is under-reported because of its non-specific presentation and the difficulty of its diagnosis. A cross-sectional study of 525 naphophily patients in Spain found tuberculosis in 259 per 100 000 people, substantially higher than the national average of 35 per 100 000 people, of which 86% had extrapulmonary involvement.

The lack of accurate and rapid diagnostic testing for tuberculosis is an important impediment to worldwide tuberculosis control. There is a great need to develop alternative rapid-diagnostic methods—appropriate for use in both developing and developed countries—that are more sensitive and specific than sputum microscopy and better able to detect tuberculosis disease anywhere in the body.

Alternative methods to sputum microscopy

Nucleic acid amplification techniques have been shown to be useful for the rapid identification of Mycobacterium tuberculosis in respiratory samples (sputum, bronchoalveolar lavage, and oral washes). Many nucleic acid amplification methods are much more sensitive than sputum microscopy, and results can be available within several hours. The cost and complexity of existing nucleic acid amplification platforms has limited their application in resource-poor settings and in countries with a high
prevalence of tuberculosis, although newer developments are specifically addressing these problems.

Urine for transrenal DNA diagnosis of tuberculosis

Evidence is building that urine contains fragments of DNA that are derived from the cell-free nucleic acids in plasma and blood resulting from the breakdown of DNA released from dying human cells and microorganisms. Some of these fragments pass through the kidney and are excreted in urine as transrenal DNA (figure 1). Cell-free nucleic acids in plasma were first identified when male fetal sequences were detected in maternal plasma. Both the cell-free nucleic acids in plasma and transrenal DNA fragments are much smaller than genomic DNA, which is believed to be why they can pass through the kidney and enter the urine. At least 70% of cell-free nucleic acids in plasma are smaller than 300 bp, including fragments large enough to be of diagnostic value. The use of transrenal DNA for the accurate diagnosis of acute and chronic infectious diseases (particularly tuberculosis), and as a biomarker for disease activity, cure, and relapse, needs further definition and development.

Transrenal DNA in urine for medical monitoring of disease states

The presence of DNA in urine has long been associated with specific disease states and used as a disease-activity indicator, for example for nephrotoxicity, transplant rejection, ultraviolet exposure, and prostate cancer. The advantage of cell-free DNA in body fluids is the opportunity such samples provide to amplify DNA without the need to determine the site of infection (eg, cancer biomarkers) and without the need for invasive sampling (eg, fetal genetic testing). Cell-free nucleic acids in plasma are routinely used in clinical settings for fetal Rhesus blood group D determination in mothers negative for Rhesus blood group D. The use of cell-free nucleic acids in plasma for fetal diagnoses is based on the high concentration of fetal nucleic acid in the maternal plasma, which increases with gestation to a maximum 6-2% (range 2.3-11.4%) of total plasma DNA. However, the quantity of DNA in urine varies substantially, and, in mice, only 5% of cell-free nucleic acids in plasma are subsequently detectable as transrenal DNA.

Almost a decade ago, several investigators, including our group, did sentinel studies detecting M tuberculosis DNA in the urine of patients with pulmonary tuberculosis using nucleic acid amplification (see table 1 for summary). In this Review we discuss the hurdles and explore the possibilities of using transrenal DNA as a target for tuberculosis diagnosis with close reference to the seven published studies that have reported detecting M tuberculosis DNA in the urine of patients with non-genitourinary tuberculosis.

As outlined, urinary-based diagnosis of tuberculosis is extremely attractive, and, for this reason, transrenal DNA has been looked at in a more coordinated way for this infectious disease than for many other diseases. In 1999, the year before the transrenal DNA was described, Aceti and colleagues did a small study amplifying M tuberculosis DNA from the urine of Italian recruits infected with HIV and showed 100% sensitivity (n=13) and 98% specificity (n=66). Other subsequent studies of nucleic acid amplification techniques on the urine of patients with pulmonary tuberculosis showed substantial variation in test sensitivity (7-79% for pulmonary tuberculosis; table 1), and little homogeneity or standardisation of test methods. The variability of nucleic acid amplification methods, differences in the collection and storage of urine specimens, and poor understanding of the nature of transrenal DNA have contributed to the lack of consistency in the published performance of urine-based detection of M tuberculosis DNA (table 2). We have recently shown that conditions that render urinary DNA stable in one geographical location do not have the same effect in another. Additionally, incomplete information regarding methodologies and storage of the urine before extraction, as outlined in table 2, make interpretation of these data and replication of the findings practically impossible.
Since these studies were published, progress in the development of a more accurate and sensitive urine based nucleic acid amplification test for tuberculosis has been slow. More recently, improved technology and DNA amplification and quantification methods have been developed and refined, although these have not generally been applied to urine. With increasing knowledge of transrenal DNA, optimum strategies for diagnoses of the basis of transrenal DNA are being devised. A multidisciplinary group funded by the European Union is developing these strategies and our experience in doing these studies has shown us that every step involved needs to be standardised and controlled.

### Optimising *M tuberculosis* transrenal DNA detection

There are many variables to consider when urine is used as a clinical specimen. Urine can vary in its quantity, bacterial DNA content, specific gravity, and protein content, and this might influence the sensitivity and specificity of any diagnostic test. The stage and type of clinical presentation of tuberculosis at diagnostic centres in any country can vary substantially and could consist of, at any point in time: healthy people, sick people not aware of their symptoms, sick people with symptoms suggestive of pulmonary tuberculosis, sick people with symptoms suggestive of pulmonary tuberculosis but not tuberculosis, and people with extrapulmonary tuberculo-

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### Table 1: Summary of studies on urine-based detection of mycobacterial DNA by PCR amplification

<table>
<thead>
<tr>
<th>Location*</th>
<th>Before extraction†</th>
<th>Extraction method</th>
<th>Urine volume used per PCR reaction</th>
<th>PCR inhibition assessed?</th>
<th>Percentage sensitivity of detection (n)‡</th>
</tr>
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<tbody>
<tr>
<td>Sechi et al34</td>
<td>Italy</td>
<td>Yes</td>
<td>None</td>
<td>ND: volume of urine concentrated not stated</td>
<td>No</td>
</tr>
<tr>
<td>Aceti et al35</td>
<td>Italy</td>
<td>Yes</td>
<td>Yes; conditions ND</td>
<td>None</td>
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<tr>
<td>Kafwabuluula et al36</td>
<td>Zambia</td>
<td>Yes</td>
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<td>Rebollo et al38</td>
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<td>ND</td>
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<td>ND</td>
</tr>
<tr>
<td>Cannas et al39</td>
<td>Italy</td>
<td>Yes</td>
<td>Yes, 0.5 M EDTA, -80°C, for an unstated length of time</td>
<td>Guanidine isothiocyanate; sepharose resin and column</td>
<td>320 μL</td>
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<td>Gopinath and Singh40</td>
<td>India</td>
<td>Yes</td>
<td>Yes, up to 3 days at 4°C</td>
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The tabulated data present the most relevant comparisons between studies relating to transrenal DNA detection. For all studies, except the initial study by Sechi and colleagues34 who relied on empirical observations, the gold standard for pulmonary diagnosis was sputum smear or culture positives. Data have been divided to distinguish between different presentations where appropriate. D=disseminated. ND=not disclosed. *Location of participant recruitment. †Handling of urine sample before extraction. ‡Confirmed pulmonary tuberculosis patients only.

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### Table 2: Summary of storage, extraction, and detection methodology for mycobacterial DNA in urine by PCR amplification

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an additional confounding factor influencing detection sensitivity is the choice of extraction method. Transrenal DNA fragment detection was improved as a result of an extraction method using silica. Even for an advanced specialty such as the study of cell-free nucleic acids in plasma, extraction methods can have a substantial affect on detection sensitivity: a 2.3 mean factor improvement in fetal DNA yield can be achieved by using different extraction techniques. It has been shown that for optimum detection of transrenal DNA the purification method must equally purify small molecules. The affect on the fragment size of the physiological processes producing transrenal DNA is unclear, though for DNA that is filtered from the blood through the kidney, the upper size limit is likely to be determined by the glomerulae and is predicted to be about 70 kDa, hypothetically corresponding to about 100 bp. Many details of the mechanisms of transrenal DNA production remain to be clarified, and recent indications are that in transplant recipients, not all the transrenal DNA fragment is comprised of DNA that has physically passed through the kidney from the blood. Of the seven reported studies on the amplification of M tuberculosis DNA from urine, only one amplified targets whose size fell within the most abundant transrenal DNA fragments, therefore sub-optimum transrenal DNA detection is highly likely. Possibly making extraction more complex, the small size of transrenal DNA has made it necessary to amplify smaller-sized targets that have, as would logically be anticipated, increased sensitivity of detection. Ultrashort targets for the detection of fetal transrenal DNA in maternal urine allow for increased sensitivity: 100% for a 25 bp target compared with 25% for a 65 bp target \((n=10)\). Of the previous studies examining M tuberculosis DNA in urine, five of the seven have used a nested PCR approach to increase sensitivity by essentially performing two sets of reactions on the same target (table 1). The small size of transrenal DNA might make this approach impossible since the target is not long enough to design two sets of primers for amplification. However, sensitivity of the PCR assay can be improved by increasing the proportion of extracted DNA added to the reaction while ensuring that PCR inhibition is not occurring. These parameters have a substantial effect on detection, yet both increasing template DNA and assessing assay specific inhibition were not detailed in any or only one, respectively, of the publications analysing M tuberculosis DNA in urine to date (table 2).

Multicopy insertion sequences, common in bacterial genomes, have proven popular targets for amplification where the organism of interest is anticipated to be present in low numbers. Even for PCR-based approaches to M tuberculosis detection in sputum, nested amplification of a multicopy insertion sequence (IS6110) outperformed other strategies. Despite this, the use of IS6110 specifically as a diagnostic target is controversial. This insertion sequence has been estimated to be present in
the M tuberculosis genome at up to 25 copies, but it might also be present in far fewer (zero to five) copies. Low copy-number strains can be found in sub-Saharan Africa, precisely where HIV-associated tuberculosis is a serious problem. Regions of IS6110 share homology with DNA from many other mycobacteria, a characteristic that could enable the identification of non-tuberculous mycobacteria common in HIV coinfections alongside an M tuberculosis diagnosis if the conserved regions were used to amplify mycobacterium-specific transrenal DNA. This insertion sequence was the target for all of the six studies to date reporting M tuberculosis DNA detection in urine of patients with pulmonary infection (table 1).

Transrenal DNA for tuberculosis diagnosis: potential for the future?

Despite the large number of new tuberculosis diagnostic tests now commercially available (eg, interferon γ release assays, loop-mediated isothermal amplification, immunochromatographic rapid diagnostic tests, etc), none have yet been proven to be more sensitive than sputum microscopy. In high-burden settings where HIV is prevalent, validation of these new tests is yielding varying results, showing the need for careful evaluation of new diagnostics in this group of patients. Equally, despite a number of patents being filed for diagnostics based on transrenal DNA (US Patent 6,251,638; 6,287,820; and 6,492,144 for Xenomics Inc), no transrenal DNA diagnostic test is yet commercially available. The use of nested conventional PCR for detection of M tuberculosis transrenal DNA is a cumbersome process. Since much work to clarify the optimum storage, extraction, and molecular targets remains undone, real-time or quantitative PCR is an ideal platform for developing diagnostics based on transrenal DNA. This benefit is because the quantitative result enables assessment of the amount of transrenal DNA in relation to the patient’s disease state, response to therapy, and drug resistance. The closed nature of the system also reduces the possibility of contamination of samples with PCR product, although this cannot be ruled-out without the inclusion of appropriate negative controls during sample collection and extraction. Until such advances in visual detection of signal amplification and isothermal reactions become routine, quantitative PCR is the most suitable technology for transrenal DNA detection.

Transrenal DNA in diagnostic centres

Tuberculosis diagnosis by transrenal DNA need not be restricted to resource-rich settings, and could be applied to many resource-poor settings with high disease burden. What are required in the resource-poor setting are affordable, easy tests that are done on easily obtainable samples at the point of care. PCR-based diagnostics is a flexible platform (applicable to many diseases) that can be tailored to the microscopy-centre level. The real-time quantitative PCR machinery can be modified to address any transrenal DNA target with the development and optimisation of an appropriate assay. In addition to tuberculosis transrenal DNA detection, the benefit of using nucleic acid amplification techniques to study the transrenal DNA fraction is the chance to look at many diseases in one test or from one processed sample. Newer methods allowing simplified quantifiable multiplexing raise the possibility of a single urine test for tuberculosis, malaria, HIV, bacterial pneumonia, pneumocystis pneumonia, and many more.

Quantitative PCR is tied to a constant electricity supply and expensive reagents requiring consistent cold storage at temperatures of −20°C or below. The development of a solar-powered thermocycling platform and interface, and lyophilised reagents to reduce the need for a functioning cold chain, will help to spread this technology more widely. Additionally, as the specialty investigating newer non-nucleic acid amplification DNA detection methods grows, the possibilities of dipstick-type diagnostic methods that detect DNA from urine by merging the knowledge of the two specialties becomes ever closer. However, while such molecular methods offer tremendous potential, for new approaches to have any affect on tuberculosis they must be standardised if they are to be used by the national tuberculosis programme.

Transrenal DNA in specific groups of patients

The focused use of transrenal DNA for diagnosis could benefit certain neglected groups of patients with tuberculosis. The emergence of the dual HIV–tuberculosis pandemics has further complicated tuberculosis diagnosis in many high-burden settings. Over 31% of newly infected tuberculosis patients are HIV positive in continental Africa and the poor ability of smear microscopy is well-documented in HIV positive patients. Data from the studies assessing urine for tuberculosis diagnosis show that assay sensitivity was increased for patients with HIV compared with people that were HIV negative by at least 10%, and at most 41% irrespective of approach (table 1). An increase in detection sensitivity of DNA from urine in patients infected with HIV was also recorded for visceral leishmania. It is unclear whether the improved detection of transrenal DNA in patients that are HIV positive is because of HIV-associated nephropathy, reflects a reduced compartmentalisation ability in these patients, or is unrelated, perhaps indicating disease dissemination to the kidneys or genitourinary tract. Optimisation of existing extraction techniques and tailoring of a transrenal DNA assay for tuberculosis diagnosis for people that are HIV positive could enable rapid diagnosis of precisely the patient group for which the current diagnostic methods increasingly fail. An approach based on transrenal DNA could also improve diagnosis of tuberculosis in children, from whom sputum collection can be very difficult. The vulnerability of children to active tuberculosis increases
with HIV infection, and it is these children who are more likely to develop difficult to diagnose extrapulmonary signs of the disease.

Conclusions

Transrenal DNA provides a challenging new target for molecular tuberculosis diagnosis from an accessible and abundant sample. The reason an approach to tuberculosis diagnosis based on transrenal DNA is still to be developed is because of the variability in consistency and sensitivity of detection. Studies examining the storage and extraction of urine have shown the affect these upstream processes can have on DNA yield, and have emphasised the need for rigorous standardisation of methodology to improve the foundation for the implementation of optimally designed nucleic acid amplification techniques. A more coherent analysis of all the steps that contribute to the final result based on DNA amplification should enable researchers to identify its true worth for tuberculosis diagnosis in all groups of patients. However, with substantial additional research and development, we anticipate that transrenal DNA provides a potentially valuable target that could eventually produce a simple urine test that will rapidly diagnose tuberculosis and other infections at the point of care in peripheral clinics and be available worldwide.

Contributors

The idea to develop the review came from CG, JH, and AZ and was agreed upon by the other authors as part of the tuberculosis transrenal DNA research consortium. The initial draft was written by CG, JH, and AZ and contributions received from PM, KR, and ET. The final draft was reviewed and edited by all authors.

Conflicts of interest

We declare that we have no conflicts of interest.

References
