Rapid molecular detection of extrapulmonary tuberculosis by automated GeneXpert® MTB/RIF system

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Short title: Evaluation of Xpert® MTB/RIF for extrapulmonary TB screening
Abstract

In total, 521 non-respiratory specimens (91 urine, 30 gastric aspirate, 245 tissue, 113 pleural fluid, 19 cerebrospinal fluid (CSF), and 23 stool specimens) submitted to the German National Reference Laboratory for Mycobacteria (NRL) from May 2009 to August 2010 were comparatively investigated with the new molecular based GeneXpert® MTB/RIF system and conventional liquid and solid culture methods. 20 (3.8%) of the 521 specimens gave no interpretable result. Whereas the sensitivity of Xpert assay with tissue specimens was 69.0 % (20 out of 29 culture positive cases detected), 100% sensitivity was found in the urine and stool specimens. The combined sensitivity and specificity of the Xpert assay was calculated to 77.3% and 98.2%, respectively.

Introduction

With an estimated 9 million new cases and 2 million deaths every year, tuberculosis (TB) remains a leading public health problem worldwide (6). In the majority of cases the disease affects the lungs, but there is also a not negligible amount of cases of about 15 % with extra-pulmonary involvement in low-incidence countries. There are even higher rates in high-incidence countries. HIV-coinfected TB often develops extrapulmonary and may progress rapidly unless diagnosed and treated appropriately (8).

Extrapulmonary infections with *Mycobacterium tuberculosis* Complex (MTBC) remain a diagnosis that is often difficult to establish, since the number of bacteria is often lower as in pulmonary specimens. Furthermore, extrapulmonary material often requires invasive procedures, and it is not easy to obtain additional samples. In recent
times, attention has been devoted to new nucleic acid amplification diagnostic technologies owing to their rapidity, sensitivity and specificity.

One of the latest systems, the GeneXpert® MTB/RIF assay, was evaluated only recently in a large study on pulmonary specimens. The Xpert assay uses hemi-nested real-time PCR to amplify an MTB-specific sequence of the \textit{rpoB} gene. To determine rifampicin (RMP) resistance the \textit{rpoB} gene is probed with molecular beacons within the rifampicin-resistance determining region (7). The assay can be carried out nearly fully automated, including bacterial lysis, nucleic acid extraction, amplification and amplicon detection. The test runs on the GeneXpert platform (Cepheid, Sunnyvale, CA) using a disposable plastic cartridge with all required reagents (16).

It could be shown that the Xpert assay detected pulmonary TB with a high sensitivity of over 97% of all TB patients, including over 90% of smear-negative patients (2).

The purpose of this study was to test the efficiency and reliability of the Xpert system for the detection of TB bacteria in extrapulmonary specimens, and to compare it to conventional culture methods.

Materials and Methods

Specimens. In the study all non-respiratory specimens that were submitted to the German National Reference Laboratory for Mycobacteria (NRL) from May 2009 to August 2010 were included. The specimens originated from patients with suspect of TB or NTM infection based on clinical criteria or to rule out these infections. They are used consecutively and not selected by special criteria. In total, 521 specimens
were tested which comprised of 91 urine, 30 gastric aspirate, 245 tissue, 113 pleural fluid, 19 cerebrospinal fluid (CSF), and 23 stool specimens.

**Culture medium inoculation, incubation, and test duration.** All specimens were processed by the standard N-acetyl-L-Cysteine and sodium hydroxide (NALC/NaOH) method, with the final concentration of 1% for NaOH (according to the DIN-guidelines, [6]). After the centrifugation step the sediment was resuspended in 1.0-1.5 ml of sterile phosphate-buffer (pH 6.8). This suspension was used for inoculation of culture medium. Different culture media were inoculated. (i) **MGIT 960.** MGIT tubes were inoculated with 0.5 ml of the processed specimen. The tubes were incubated in the MGIT 960 instrument at 37°C. For tissue samples a MGIT tube was inoculated with 0.5 ml specimen and incubated at 31°C. From the positive tube, a smear was prepared for examination of AFB, and further differentiation of mycobacteria with molecular methods was performed. (ii) **Solid media.** For each specimen one LJ and one Stonebrink slant (Becton Dickinson Diagnostic Systems Sparks, MD) was inoculated with 0.1 ml suspension and incubated at 37°C (6). From all tissue samples one LJ, Stonebrink and 7H10 agar slants were additionally inoculated and incubated at 31°C. Bacterial colonies were investigated by AFB smear and further by molecular methods. For the purpose of data analysis, the different media were regarded as a single culture-medium system.

**AFB smears.** After the processing of specimens, smears were prepared from all samples other than urine and were examined at the NRC for the presence of acid-fast bacteria (AFB). All smears were stained by the Kinyoun method and examined with a light microscope (5).
Drug susceptibility testing (DST). DST for RMP was performed with the BACTEC MGIT 960 method (MGIT 960; Becton Dickinson Diagnostic Systems Sparks, MD) with the standard critical concentration of 1 µg/ml RMP.

Identification of mycobacteria and analysis of discrepant by DNA sequencing.

For the identification of MTBC organisms and the differentiation of NTM from positive cultures two commercially available DNA strip assays were used, the GenoType MTBC and CM/AS assay (Hain Lifescience GmbH, Nehren, Germany) (17, 18). The assays were performed according to the instructions of the manufacturer. In all discordant cases the 81 bp rpoB hot spot region of culture isolates was analyzed by PCR and DNA sequencing with primers TR8 and TR9 to identify the presence or absence of rpoB core mutations (20). Direct sequencing of the PCR products was carried out with an ABI Prism 3100 capillary sequencer (Applied Biosystems, CA, USA) and the ABI Prism BigDye Terminator kit v.1.1. according to the manufacturer’s instructions. The NCBI-available Blast2 Sequences computer program was used for DNA sequence comparisons (http://www.ncbi.nlm.nih.gov/BLAST/).

Xpert procedure. The Xpert assay was performed as recently described (11). Sample reagent was added in 3:1 ratio to decontaminated specimens ≥ 0.5 ml volume. The closed tube was manually agitated twice during a 15 min. incubation period at room temperature before 2 ml of the inactivated sample reagent:sample mixture was transferred to the Xpert test cartridge. Cartridges were inserted into the GeneXpert device and the automatically generated results were red after 90 min.
Results

In this study 521 specimens were included, all sent to the National Reference Center for Mycobacteria between 2009 and 2010. Among 245 tissue samples, the majority was lymph nodes, but also included skin, kidney, spleen, liver, bone, and lesions. Overall, 62 (11.9%) of the 521 specimens tested were positive for mycobacteria by culture. Out of these 62 positive cultures, 6 (9.7%) also were smear positive for acid-fast bacilli. Thirty MTBC and 17 NTMs could be isolated from the tissue specimens, 8, 5 and 2 MTBC strains from gastric fluid, urine, and stool, respectively.

Most of the MTBC strains originated from single patients (n=38). Only one patient was represented by 3 urine specimens (all of them culture positive and Xpert positive), one patient with 2 stool specimens (both of them culture positive and Xpert positive), and one with 2 skin tissues (both of them culture positive, but only one Xpert positive, the other negative).

The NTMs comprised of 11 *M. marinum*, 3 *M. avium*, 1 *M. kansasii*, 1 member of the *M. abscessus* group and 1 *M. chelonae*. Not any NTMs were recovered from the other specimen types and the highest rates of MTBC strains were isolated from gastric aspirates (8 of 30 [26.7%]).

Comparison of the Xpert assay with the culture method results for the detection of MTBC.

In total, with the Xpert assay 501 (96.2%) of the 521 specimens gave an interpretable result and 20 (3.8%) were excluded from further analysis (Table 1). The Xpert indeterminate results are indicated in the GeneXpert software as “invalid”, “error” or “no result”. With respect to the specimen type the highest rates of indeterminate
results were found with the stool specimens (3 out of 23 [13.0%]). Contaminated cultures were excluded for the Xpert assay sensitivity and specificity calculation. Overall the combined sensitivity and specificity was calculated to 79.5% and 98.2%, respectively. The sensitivities differed markedly between the specimen types. Whereas the Xpert assay detected only 20 out of 29 culture positive cases among the tissue specimens, all of them were found in the urine and stool specimens. Furthermore, some Xpert results showed a MTB positive result but the culture was contaminated (one gastric fluid, 2 stools), which could indicate a higher sensitivity among these specimens. This indication may be supported by the isolation of TB strains from these patients from alternative materials. The specificities varied to a lesser extend, at which stool specimens showed the lowest specificity (91.7%). None of the isolated nontuberculous mycobacteria were found to be positive in the Xpert assay.

Xpert® MTB/RIF assay for RMP resistance detection

From 29 MTB isolates RMP resistance was tested by conventional drug susceptibility testing. All strains were found to be susceptible to RMP. Of positive Xpert tests, 3 of 29 (10.3%) had an indeterminate RMP resistance result. For the remaining 26 samples, 25 were detected as susceptible and one as resistant. For this case, the phenotypic drug susceptibility testing was repeated first. Furthermore, sequencing showed a \textit{rpoB} mutation in codon 533 resulting in a lysine to proline exchange, which is known to be debatable of being RMP resistance-associated (12, 15, 19).
Discussion

Conventional laboratory techniques like direct microscopy for the diagnosis of tuberculosis are far from being sensitive. Moreover, cultures are time consuming, require biosafety measures and need trained laboratory personnel.

Molecular techniques have heavily changed the field of tuberculosis diagnosis and have been proven to yield rapid results while being highly sensitive (2). Numerous PCR assays employing a number of different \textit{M. tuberculosis} targets have recently been described (9, 14). The new Xpert assay tested in our study targets the rifampicin resistance associated \textit{rpoB} gene region as hemi-nested PCR by 3 specific primers and combines the sensitive detection of tuberculosis DNA and determination of RMP resistance. Furthermore, the hand on time is short due to automation of bacterial lysis, DNA extraction, real-time PCR amplification, and amplicon detection in a single system. A recent study showed the high sensitivity of over 97\% and specificity of the Xpert assay for pulmonary specimens (2). Numerous studies have assessed the yield of PCR techniques for the diagnosis of extrapulmonary tuberculosis (3, 10, 13).

Nevertheless, this is the first study to verify the usefulness of the Xpert assay applied to the rapid diagnosis of extrapulmonary tuberculosis. Overall, the sensitivity and specificity of the Xpert assay was very high, correctly identifying 77.3\% of all culture positive isolates. The high sensitivity is not self-evident, since only small amounts of DNA are expected in any extrapulmonary clinical samples. However, sensitivity in tissue specimens (69.0\%) was lower compared to the other specimen types. To improve the sensitivity a preincubation step with proteinaseK could be useful to enhance the capacity of the provided lysis buffer.
A potential advantage of the Xpert assay (as also for other nucleic acid based techniques) is the low liability for secondary bacteria from specimens with a high contamination rate such as stool or urines. In case of 2 stool specimens with contaminated cultures (5 were contaminated in total) tuberculosis DNA was detected, however also the indeterminate rate was higher compared to all other specimens (3 out of 23 [13.0%]).

In some cases the Xpert result was positive, but the culture remained negative. Of the 7 patients with discrepant results, two patients had a pulmonary TB, proven by several cultures from different specimens. Two patients suffered from a TB (culture confirmed) one year and two years before, and were presumably still or again under treatment at the time of sampling. For the remaining 3 patients no clinical data were available to give an indication for the resolution of the discrepancies.

Furthermore, a contamination might be the reason leading to a false positive Xpert PCR, although the Xpert real-time technology is less prone for contamination due to the closed reaction chamber. Furthermore, measures to avoid contamination with bacterial DNA are the extensive cleaning of the surfaces where the specimens are processed.

One limitation of this study is the small sample size of the different specimen types. Definitive interpretation of the results in each category of specimens should be done with great care. Furthermore only few MTB positive strains and no rifampicin resistant strain were isolated in order to assess sensitivity and specificity of RMP resistance. Nevertheless, from our result one can conclude that the Xpert assay can be applied on extrapulmonary specimens with a high sensitivity and specificity, which,
coupled with its speed and simplicity, make this technique a very useful tool for the
diagnosis of extrapulmonary tuberculosis.
Acknowledgement

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Table 1 Comparison of the Xpert assay with the culture method results for the detection of MTBC

<table>
<thead>
<tr>
<th>Specimen type (total number=521)</th>
<th>Xpert result</th>
<th>Culture result</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>indeterminate</td>
<td>Neg.</td>
</tr>
<tr>
<td>Tissue (245)</td>
<td>6 (2.4%)</td>
<td>216</td>
</tr>
<tr>
<td>CSF (19)</td>
<td>0</td>
<td>19</td>
</tr>
<tr>
<td>Gastric fluid (30)</td>
<td>0</td>
<td>22</td>
</tr>
<tr>
<td>Pleural fluid (113)</td>
<td>7 (6.2%)</td>
<td>103</td>
</tr>
<tr>
<td>stool (23)</td>
<td>3 (13.0%)</td>
<td>15</td>
</tr>
<tr>
<td>Urine (91)</td>
<td>4 (4.4%)</td>
<td>81</td>
</tr>
</tbody>
</table>

CSF: cerebrospinal fluid
Table 2 Sensitivity and specificity of the Xpert assay with the culture method as reference standard

<table>
<thead>
<tr>
<th>Specimen type</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tissue</td>
<td>69.0%</td>
<td>98.4%</td>
</tr>
<tr>
<td>CSF</td>
<td>not calculable</td>
<td>100.0%</td>
</tr>
<tr>
<td>Gastric fluid</td>
<td>87.5%</td>
<td>100.0%</td>
</tr>
<tr>
<td>Pleural fluid</td>
<td>not calculable</td>
<td>98.1%</td>
</tr>
<tr>
<td>stool</td>
<td>100.0%</td>
<td>91.7%</td>
</tr>
<tr>
<td>Urine</td>
<td>100.0%</td>
<td>98.6%</td>
</tr>
<tr>
<td>total</td>
<td>77.3%</td>
<td>98.2%</td>
</tr>
</tbody>
</table>

CSF: cerebrospinal fluid