Rapid Molecular Detection of Extrapulmonary Tuberculosis by the Automated GeneXpert MTB/RIF System[▽]

Doris Hillemann,[1]* Sabine Rüsch-Gerdes,[1] Catharina Boehme,[2] and Elvira Richter[1]

*Nationales Referenzzentrum für Mykobakterien, Forschungszentrum Borstel, Borstel, Germany,[1] and
Foundation for Innovative New Diagnostics (FIND), Geneva, Switzerland[2]*

In total, 521 nonrespiratory 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 (Xpert) assay system and conventional liquid and solid culture methods. Twenty (3.8%) of the 521 specimens gave no interpretable result. Whereas the sensitivity of the Xpert assay with tissue specimens was 69.0% (20 of 29 culture-positive cases detected), 100% sensitivity was found with the urine and stool specimens. The combined sensitivity and specificity of the Xpert assay were calculated to be 77.3% and 98.2%, respectively.

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 are also not negligible numbers of cases (about 15%) with extrapulmonary involvement in low-incidence countries. There are even higher rates in high-incidence countries. HIV-coinfected TB patients often develop extrapulmonary involvement and may progress rapidly unless the infection is diagnosed and they are treated appropriately (8).

Extrapulmonary infection with members of the *Mycobacterium tuberculosis* complex (MTBC) remains a diagnosis that is often difficult to establish, since the number of bacteria in extrapulmonary specimens is often lower than the number in pulmonary specimens. Furthermore, collection of 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 (Xpert) assay, was evaluated only recently in a large study with pulmonary specimens. The Xpert assay uses heminested real-time PCR to amplify an *M. tuberculosis*-specific sequence of the *rpoB* gene. To determine rifampin (RMP) resistance, the rifampin resistance-determining region of the *rpoB* gene is probed with molecular beacons (7). The assay can be carried out in a nearly fully automated manner, including bacterial lysis, nucleic acid extraction and 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 in all TB patients, including over 90% of smear-negative patients, with a high sensitivity of over 97% (2).

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

MATERIALS AND METHODS

Specimens. All nonrespiratory specimens that were submitted to the German National Reference Laboratory for Mycobacteria (NRL) from May 2009 to August 2010 were included in the study. The specimens originated from patients with suspected *M. tuberculosis* or nontuberculous mycobacterial (NTM) infection on the basis of clinical criteria or to rule out these infections. Consecutive specimens were used, and specimens were not selected by the use of special criteria. In total, 521 specimens were tested. These comprised 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 a final NaOH concentration of 1% (according to the Deutsches Institut für Normung guidelines [4]). After the centrifugation step, the sediment was resuspended in 1.0 to 1.5 ml of sterile phosphate buffer (pH 6.8).This suspension was used for inoculation of culture media. Different culture media were used.

(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 further MGIT tube was inoculated with 0.5 ml specimen and incubated at 31°C. For tubes identified as positive, a smear of a sample from the tube was prepared for examination for acid-fast bacilli (AFB), and further differentiation of mycobacteria was performed with molecular methods.

(ii) Solid media. For each specimen, one Löwenstein-Jensen (LJ) slant and one Stonebrink slant (our own production and Becton Dickinson Diagnostic Systems, Sparks, MD) were inoculated with 0.1 ml suspension and incubated at 37°C (4). For all tissue samples, one slant each of LJ, Stonebrink, and 7H10 agar was additionally inoculated and incubated at 31°C. Bacterial colonies were investigated by AFB smear and were further investigated by molecular methods. For the purpose of data analysis, each of the different media was regarded as a single culture medium system.

AFB smears. After processing of the specimens, smears were prepared from all samples other than urine and were examined at the German National Reference Laboratory for Mycobacteria (NRC) for the presence of AFB. All smears were stained by the Kinyoun method and examined with a light microscope (5).

DST. Drug susceptibility testing (DST) for RMP was performed with the Bactec MGIT 960 method (MGIT 960; Becton Dickinson Diagnostic Systems) with the standard critical concentration of 1 μg/ml RMP.

* Corresponding author. Mailing address: Nationales Referenzzentrum für Mykobakterien, Forschungszentrum Borstel, Parkallee 18, 23845 Borstel, Germany. Phone: 49 4537188761. Fax: 49 4537188311. E-mail: dhillemann@fz-borstel.de.

TABLE 1. Comparison of Xpert assay with culture method results for detection of MTBC

Specimen type[a]	No. (%) of specimens						
	Xpert assay			Culture			
	Indeterminate	Negative	Positive	Negative	Positive for M. tuberculosis	Positive for NTM	Contaminated
Tissue (245)	6 (2.4)			3	1	2	
		216		189	9	15	3
			23	3	20		
CSF (19)	0						
		19		19			
			0			0	
Gastric fluid (30)	0						
		22		19	1		2
			8		7		1
Pleural fluid (113)	7 (6.2)			6			1
		103		103			
			3	2			1
Stool (23)	3 (13.0)			2			1
		15		11			4
			5	1	2		2
Urine (91)	4 (4.4)			4			
		81		70			11
			6	1	5		

[a] A total of 521 specimens were tested. The numbers in parentheses are the number of each specimen type.

Identification of mycobacteria and analysis of discrepant results by DNA sequencing. For the identification of MTBC organisms and the differentiation of MTBC and NTM from positive cultures, two commercially available DNA strip assays were used, the GenoType MTBC and CM/AS assays (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, Foster City, CA) and an ABI Prism BigDye Terminator cycle sequencing kit (version 1.1), according to the manufacturer's instructions. The Blast2 Sequences computer program, available from NCBI, 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 a 3:1 ratio to ≥0.5 ml of decontaminated specimen. 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 read after 90 min.

RESULTS

This study included 521 specimens, all of which were sent to the National Reference Center for Mycobacteria between May 2009 and August 2010. Among the 245 tissue samples, the majority were lymph node specimens, but they also included skin, kidney, spleen, liver, bone, and lesion specimens.

Overall, 62 (11.9%) of the 521 specimens tested were positive for mycobacteria by culture. Out of these 62 positive cultures, 6 (9.7%) were also smear positive for acid-fast bacilli. Thirty MTBC and 17 NTM strains could be isolated from the tissue specimens, and 8, 5, and 2 MTBC strains were isolated from gastric fluid, urine, and stool specimens, respectively.

Most of the MTBC strains originated from single patients (n = 38). However, three strains were recovered from urine specimens from one patient (all of them were culture positive and Xpert assay positive), two stool specimens from one pa-

tient were positive for MTBC (both of them were culture positive and Xpert assay positive), and two skin tissue specimens from one patient were positive for MTBC (both of them were culture positive, but one was Xpert assay positive and the other was Xpert assay negative).

The NTMs comprised 11 M. marinum isolates, 3 M. avium isolates, 1 M. kansasii isolate, 1 member of the M. abscessus group, and 1 M. chelonae isolate. No NTMs were recovered from the other specimen types, and the highest rates of MTBC strain detection were for gastric aspirates (8 of 30 [26.7%]).

Comparison of Xpert assay with culture method results for detection of MTBC. In total, 501 (96.2%) of the 521 specimens gave an interpretable result with the Xpert assay and 20 (3.8%) were excluded from further analysis (Table 1). The Xpert assay indeterminate results are indicated "invalid," "error," or "no result" by the GeneXpert software. 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 were calcu-

TABLE 2. Sensitivity and specificity of Xpert assay with culture method as reference standard

Specimen type	Sensitivity (%)	Specificity (%)
Tissue	69.0	98.4
CSF	Not calculable	100.0
Gastric fluid	87.5	100.0
Pleural fluid	Not calculable	98.1
Stool	100.0	91.7
Urine	100.0	98.6
Total	77.3	98.2

lated to be 77.3% and 98.2%, respectively (Table 2). The sensitivities between the specimen types differed markedly. Whereas the Xpert assay detected only 20 out of 29 culture-positive cases among the tissue specimens, the Xpert assay detected all culture-positive cases among the urine and stool specimens. Furthermore, some Xpert assay results showed an *M. tuberculosis*-positive result but the culture was contaminated (one gastric fluid and two stool specimens), which could indicate higher sensitivity with these specimens. This possibility may be supported by the isolation of TB strains from alternative materials from these patients. The specificities varied to a lesser extent, with stool specimens showing 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. Twenty-nine *M. tuberculosis* isolates were tested for RMP resistance by conventional drug susceptibility testing. All strains were found to be susceptible to RMP. Of the isolates positive by Xpert tests, 3 of 29 (10.3%) had an indeterminate RMP resistance result. For the remaining 26 samples, 25 were found to be susceptible and 1 was found to be resistant. For this case, phenotypic drug susceptibility testing was repeated first. Furthermore, sequencing showed an *rpoB* mutation in codon 533, resulting in a lysine-to-proline exchange, whose association with RMP resistance is debated (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 substantially 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 *M. tuberculosis* targets have recently been described (9, 14). The new Xpert assay tested in our study targets the rifampin resistance-associated *rpoB* gene region by heminested PCR with three specific primers and combines the sensitive detection of *M. tuberculosis* DNA and determination of RMP resistance. Furthermore, the hands-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 application of the Xpert assay to the rapid diagnosis of extrapulmonary tuberculosis. Overall, the sensitivity and specificity of the Xpert assay were very high, with the assay correctly identifying 77.3% of all culture-positive specimens. The high sensitivity is not self-evident, since only small amounts of DNA are expected in any extrapulmonary clinical sample. However, sensitivity of detection in tissue specimens (69.0%) was lower than that in the other specimen types. To improve the sensitivity, a preincubation step with proteinase K to enhance the capacity of the provided lysis buffer would be useful.

A potential advantage of the Xpert assay (as well as for other nucleic acid-based techniques) is the low probability of detection of secondary bacteria from specimens with a high contamination rate, such as stool and urine specimens. *M. tuberculosis* DNA was detected in 2 stool specimens with contaminated cultures (5 were contaminated in total); however, the indeterminate rate for stool specimens was also higher than that for all other specimens (3 out of 23 [13.0%]).

In some cases, the Xpert assay result was positive but the culture remained negative. Of the seven patients with discrepant results, two patients had pulmonary TB, proven by several cultures of different specimens. Two patients had had TB (culture confirmed) 1 year and 2 years before and were presumably still or again under treatment at the time of sampling. For the remaining three patients, no clinical data were available to give an indication for the resolution of the discrepancies.

Furthermore, contamination might be the reason for a false-positive Xpert PCR assay result, although the Xpert real-time technology is less prone to contamination due to the closed reaction chamber. Furthermore, the surfaces where the specimens are processed were extensively cleaned to avoid contamination with bacterial DNA.

One limitation of this study is the small sample size for each of the different specimen types. Definitive interpretation of the results for each category of specimens should be done with great care. Furthermore, only a few *M. tuberculosis* strains and no rifampin-resistant strain were isolated in order to assess the sensitivity and specificity of RMP resistance detection. Nevertheless, from our results, one can conclude that the Xpert assay can be applied to 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.

REFERENCES

1. Reference deleted.
2. **Boehme, C. C., et al.** 2010. Rapid molecular detection of tuberculosis and rifampin resistance. N. Engl. J. Med. **363:**1005–1015.
3. **Cheng, V. C., et al.** 2004. Clinical evaluation of the polymerase chain reaction for the rapid diagnosis of tuberculosis. J. Clin. Pathol. **57:**281–285.
4. **Deutsches Institut für Normung.** 1986. Medical microbiology. Diagnosis of tuberculosis. Part 3. Detection of mycobacteria by culture methods. DIN 58943-3. Beuth Verlag, Berlin, Germany.
5. **Deutsches Institut für Normung.** 1995. Medizinische Mikrobiologie. Tuberkulosediagnostik. Teil 32. Mikroskopische Methoden zum Nachweis von Mykobakterien. DIN 58943-32. Beuth Verlag, Berlin, Germany.
6. **Dye, C.** 2006. Global epidemiology of tuberculosis. Lancet **367:**938–940.
7. **El-Hajj, H. H., S. A. Marras, S. Tyagi, F. R. Kramer, and D. Alland.** 2001. Detection of rifampin resistance in *Mycobacterium tuberculosis* in a single tube with molecular beacons. J. Clin. Microbiol. **39:**4131–4137.
8. **Ellner, J. J.** 2009. The emergence of extensively drug-resistant tuberculosis: a global health crisis requiring new interventions. Part II. Scientific advances that may provide solutions. Clin. Transl. Sci. **2:**80–84.
9. **Greco, S., M. Rulli, E. Girardi, C. Piersimoni, and C. Saltini.** 2009. Diagnostic accuracy of in-house PCR for pulmonary tuberculosis in smear-positive patients: meta-analysis and metaregression. J. Clin. Microbiol. **47:**569–576.
10. **Hasaneen, N. A., M. E. Zaki, H. M. Shalaby, and A. S. El-Morsi.** 2003. Polymerase chain reaction of pleural biopsy is a rapid and sensitive method for the diagnosis of tuberculous pleural effusion. Chest **124:**2105–2111.
11. **Helb, D., et al.** 2010. Rapid detection of Mycobacterium tuberculosis and rifampin resistance by use of on-demand, near-patient technology. J. Clin. Microbiol. **48:**229–237.

12. **Hillemann, D., S. Rüsch-Gerdes, and E. Richter.** 2007. Evaluation of the GenoType MTBDRplus assay for rifampin and isoniazid susceptibility testing of *Mycobacterium tuberculosis* strains and clinical specimens. J. Clin. Microbiol. **45:**2635–2640.

13. **Hofman, V., et al.** 2003. Value of PCR amplification from formalin-fixed paraffin-embedded tissues in the diagnosis of *Mycobacterium tuberculosis* infection. Ann. Pathol. **23:**206–215.

14. **Ling, D. I., L. L. Flores, L. W. Riley, and M. Pai.** 2008. Commercial nucleic-acid amplification tests for diagnosis of pulmonary tuberculosis in respiratory specimens: meta-analysis and meta-regression. PLoS One **3:**e1536.

15. **Ma, X., et al.** 2006. *rpoB* gene mutations and molecular characterization of rifampin-resistant *Mycobacterium tuberculosis* isolates from Shandong Province, China. J. Clin. Microbiol. **44:**3409–3412.

16. **Raja, S., et al.** 2005. Technology for automated, rapid, and quantitative PCR or reverse transcription-PCR clinical testing. Clin. Chem. **51:**882–890.

17. **Richter, E., M. Weizenegger, S. Rüsch-Gerdes, and S. Niemann.** 2003. Evaluation of genotype MTBC assay for differentiation of clinical Mycobacterium tuberculosis complex isolates. J. Clin. Microbiol. **41:**2672–2675.

18. **Richter, E., S. Rüsch-Gerdes, and D. Hillemann.** 2006. Evaluation of the GenoType Mycobacterium assay for identification of mycobacterial species from cultures. J. Clin. Microbiol. **44:**1769–1775.

19. **Taniguchi, H., et al.** 1996. Rifampicin resistance and mutation of the rpoB gene in *Mycobacterium tuberculosis*. FEMS Microbiol. Lett. **144:**103–108.

20. **Telenti, A., et al.** 1997. Genotypic assessment of isoniazid and rifampin resistance in Mycobacterium tuberculosis: a blind study at reference laboratory level. J. Clin. Microbiol. **35:**719–723.