Development and Evaluation of a Molecular Assay for Detection of Nontuberculous Mycobacteria by Use of the Cobas Amplicor Platform

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We have developed and evaluated a semiautomated assay for detection of nontuberculous mycobacteria (NTM) from clinical samples based on the Cobas Amplicor Mycobacterium tuberculosis test (Roche Diagnostics, Switzerland). A capture probe, specific for mycobacteria at the genus level, was linked to magnetic beads and used for the detection of amplification products obtained by the Cobas Amplicor M. tuberculosis assay. We demonstrate that the analytical sensitivity of the genus assay is similar to that of Cobas Amplicor M. tuberculosis detection. Four hundred sixteen clinical specimens were evaluated for the presence of NTM DNA. Sensitivities for smear-positive and smear-negative specimens were found to be 100% and 47.9%, respectively. Specificity was 97.7%, the positive predictive value 84.6%, and the negative predictive value 93.1%. The genus assay is easy to perform, produces reliable results, and was found to be a valuable diagnostic tool for rapid diagnosis of infections with NTM. The genus assay has the potential to detect NTM not routinely recovered by culture and to discover new mycobacterial species.

Nontuberculous mycobacteria (NTM) are frequently isolated from environmental sources, and about one-third of the species described have been associated with human disease (11, 16). Their pathogenic potential is variable, and infections preferentially occur in patients with underlying immunocompromising conditions, although immunocompetent individuals can also be affected. Clinical conditions caused by NTM include cutaneous ulcers, soft-tissue infections, lymphadenitis, joint infections, lung disease, and disseminated infections (11). Rapid laboratory diagnosis and differentiation from Mycobacterium tuberculosis are crucial, since treatment of NTM differs significantly from that of M. tuberculosis infections. Given the slow growth of most mycobacteria, fast and accurate molecular techniques are important for the diagnosis of suspected mycobacterial infections.

A variety of PCR-based test systems for direct detection of M. tuberculosis complex DNA from clinical specimens are commercially available and widely used, e.g., Cobas Amplicor M. tuberculosis (Roche Diagnostics, Switzerland) and AMTD2 (Gen-Probe) (15). In contrast, molecular genetic assays for direct detection of NTM are only infrequently implemented in routine diagnostics, although various in-house assays have been described (2, 4, 5, 8, 12, 21, 22). This is mainly due to the fact that existing assays are time-consuming and evaluation data are limited. Recently a commercial assay based on a manual line blot format was released for the direct detection of N. meningitidis from clinical samples (Hain Lifescience, Nehren, Germany) (7). However, numerous NTM well known to cause disease in humans are not included in this assay (e.g., M. marinum, M. abscessus, M. chelonae, M. xenopi, and M. ulcerans) (1, 6, 11, 14).

Here we present the development of an assay capable of detecting a large number of nontuberculous mycobacteria from clinical specimens using a semiautomated system well established for the detection of M. tuberculosis complex DNA.

MATERIALS AND METHODS

Decontamination of specimens, microscopy, and culture. Specimens were decontaminated using the N-acetyl-l-cysteine-sodium hydroxide method for respiratory samples or the sodium hydroxide method for samples from sterile sites, following standard protocols (10). Microscopy was performed using auramine-rhodamine fluorochrome staining; positive microscopy results were confirmed using Zielh-Neelsen staining (10). Standard media (Lowenstein-Jensen, 7H11 plates, and BBL MGIT [Becton, Dickinson and Company]) were used for cultural recovery, and cultures were incubated for 8 weeks at 37°C. For specimens from sterile sites, microscopy and inoculation of culture media were performed prior to decontamination. Mycobacteria grown in culture were identified by sequence analysis of the 16S rRNA gene as described previously (3, 12).

Clinical specimens. Over a period of 3 months, the genus assay was performed with all samples for which molecular detection of M. tuberculosis was requested. In addition, we retrospectively included patient samples in the study which were positive for NTM by culture to allow calculation of sensitivity. Retrospectively analyzed samples were decontaminated and stored at 4°C prior to DNA extraction.

Although the Cobas Amplicor M. tuberculosis test is approved only for respiratory specimens, it is also widely used for nonrespiratory specimens (13, 17, 20, 24). Consequently, we included respiratory as well as nonrespiratory specimens in our study.

DNA extraction. DNA was extracted from 0.5 ml of decontaminated specimen using the respiratory specimen preparation kit (Roche Diagnostics, Switzerland), following instructions for the Cobas Amplicor M. tuberculosis test (18). Detection of M. tuberculosis complex DNA. The Cobas Amplicor M. tuberculosis test was performed according to the manufacturer’s instructions (Roche Diagnostics, Switzerland). Briefly, 50 µl master mix containing AmpliTaq DNA polymerase, biotinylated primers KY18 and K75, and an internal control plasmid were transferred into the detection ring, and 50 µl of DNA extract was added. The denaturation reagent, conjugate reagent, substrate, internal control detec-
tion reagent, and *M. tuberculosis* detection reagent were applied to the Cobas Amplicor analyzer as described in the operator’s manual (18). Results were interpreted according to the manufacturer’s instructions. A run was considered valid if the optical density at 660 nm (OD$_{660}$) of the positive control was >2.0 and the OD$_{660}$ of the negative control was <0.25. A sample was interpreted as positive if the OD$_{660}$ was >0.35. A sample was interpreted as negative if the OD$_{660}$ was <0.15 and the OD$_{660}$ of the internal inhibition control was <0.35.

**Labeling of magnetic beads with *Mycobacterium* genus-specific capture probe.** To adapt the Cobas Amplicor *M. tuberculosis* platform for the detection of nontuberculous mycobacteria, we constructed a *Mycobacterium* genus-specific capture probe. Magnetic beads harboring carboxylic acid groups on their surfaces were coupled to a *Mycobacterium* genus-specific oligonucleotide probe with an amino modification. Magnetic beads (Dynabeads M-270 carboxylic acid; Invitrogen, Germany) were washed four times with 25 mM 2-(N-morpholino)ethanesulfonic acid (MES) (Sigma-Aldrich Chemie GmbH, Germany) (pH 5) and incubated with oligonucleotide 259 (12) carrying an amino modification at the 5′ end (NH$_2$-TTT CAC GAA CCA CGC GAC AA; Tib Molbiol, Germany) at a concentration of 2.1 nmol oligonucleotide per mg beads for at least 30 min at room temperature. Subsequently, N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide HCl (EDC) (Sigma-Aldrich Chemie GmbH, Germany), resolved in cold 100 mM MES (pH 5), was added to the beads at a concentration of 10 mg per mg beads and incubated overnight at 4°C with shaking at 900 rpm. Labeled beads were washed four times using 50 mM Tris-HCl (pH 7.4) and stored in 50 mM Tris-HCl-0.1% Tween 20-0.02% sodium azide (pH 7.4) at 4°C until usage. All buffers were subject to sterile filtration using a vacuum filtration system equipped with 0.22-μm membrane filters (Techno Plastic Product, Switzerland).

**Detection of *Mycobacterium* genus DNA using Cobas Amplicor platform.** For detection of *Mycobacterium* genus DNA, we prepared a *Mycobacterium* genus-specific detection reagent. Beads labeled with the genus-specific probe (4.5 mg) were washed four times with 50 mM Tris (pH 7.4), resuspended in 1.3 ml of 25 mM 2-(N-morpholino)ethanesulfonic acid (MES) (Sigma-Aldrich Chemie GmbH, Germany) (pH 5) and incubated with oligonucleotide 259 (12) carrying an amino modification at the 5′ end (NH$_2$-TTT CAC GAA CCA CGC GAC AA; Tib Molbiol, Germany) at a concentration of 2.1 nmol oligonucleotide per mg beads for at least 30 min at room temperature. Subsequently, N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide HCl (EDC) (Sigma-Aldrich Chemie GmbH, Germany), resolved in cold 100 mM MES (pH 5), was added to the beads at a concentration of 10 mg per mg beads and incubated overnight at 4°C with shaking at 900 rpm. Labeled beads were washed four times using 50 mM Tris-CHCl (pH 7.4) and stored in 50 mM Tris-CHCl-0.1% Tween 20-0.02% sodium azide (pH 7.4) at 4°C until usage. All buffers were subject to sterile filtration using a vacuum filtration system equipped with 0.22-μm membrane filters (Techno Plastic Product, Switzerland).

**Results**

**Establishment of direct detection assay for *Mycobacterium* genus DNA from clinical specimens.** We developed and validated an assay for direct detection of nontuberculous mycobacteria from clinical specimens using the Cobas Amplicor platform. Brieﬂy, the Cobas Amplicor *M. tuberculosis* assay includes the following steps: (i) isolation of DNA from clinical samples, (ii) amplification of a 584-bp fragment of the 16S rRNA gene with biotinylated pannycobacterial primers (2, 23), (iii) capture of the PCR product with an *M. tuberculosis*-specific probe linked to magnetic beads, and (iv) photometric detection of the captured PCR product. The reaction mix contains an internal control plasmid to control for inhibition of the PCR. In order to use the Cobas Amplicor platform for the detection of nontuberculous mycobacteria, we generated a *Mycobacterium* genus-specific capture probe to be used as the specific detection reagent. Samples positive in the genus assay were amplified with pannycobacterial primers, and resulting amplification products were subjected to nucleic acid sequence determination to identify the *Mycobacterium* species.

**Sensitivity of the genus assay compared to the Cobas Amplicor *M. tuberculosis* test.** The analytical sensitivity of the genus assay was determined using a dilution series of *M. tuberculosis* DNA (H37RV) and compared to that of the Cobas Amplicor *M. tuberculosis* assay. The detection limit of both assays was 8 to 80 fg DNA per PCR, which is equivalent to approximately 2 to 20 genome copies (molecular weight of the *M. tuberculosis* genome, 4 fg) (9) (Fig. 1). Clinical evaluation showed that 15 of 431 specimens tested positive for the presence of *M. tuberculosis* DNA using the Cobas Amplicor *M. tuberculosis* assay. All 15 samples also gave a positive result using the genus assay (data not shown), further indicating that the genus assay is as sensitive as the commercial Cobas Amplicor *M. tuberculosis* assay.

**Evaluation of the genus assay with clinical specimens.** To evaluate the performance of the genus assay on clinical samples, the assay was incorporated into the routine diagnostic workflow. Samples screened for the presence of *M. tuberculosis* DNA were subsequently tested for the presence of NTM DNA using the genus assay. This strategy enabled us to screen a large number of clinical samples for the presence of NTM DNA using a semiautomated platform.

In total, 467 specimens were tested for the presence of *M. tuberculosis* and *Mycobacterium* genus DNA. This included 342 respiratory and 125 nonrespiratory specimens (46 puncture specimens, including aspirates from the pleural and abdominal cavities, 42 biopsies, 13 CSFs, 15 urines, 4 gastric aspirates, 3 stool samples, and 2 bone marrow specimens).

Inhibition of amplification as determined by negative internal control signals was detected in 36 of 467 (7.8%) specimens.
In 15 (3.5%) of the remaining 431 samples, *M. tuberculosis* DNA was detected by both the genus assay and the Cobas Amplicor *M. tuberculosis* test (see above). These samples were excluded from the evaluation of the genus assay; thus, a total of 416 specimens were evaluated for the presence of NTM DNA.

In the genus assay, 52 of the 416 (12.5%) specimens tested positive, and the *Mycobacterium* species was further determined by sequence analysis. Samples were considered to be true positive in the genus assay if (i) the corresponding NTM was grown in culture, (ii) the corresponding NTM could be cultivated from other samples of the same patient, or (iii) the assay identified a noncultivable *Mycobacterium* species associated with a typical disease (e.g., *M. leprae*).

For 39 of 52 (75.0%) of the genus assay-positive samples species, identity was confirmed by culture (Table 1). Mycobacterial culture was negative for 13 samples positive in the genus assay. These discrepant samples were further analyzed to determine if NTM infection was present in the corresponding patients (Table 2). Three of the thirteen samples originated from patients with confirmed *M. kansasii* infections and were thus considered true positives. Two of the thirteen samples revealed the presence of *M. leprae* DNA; these samples were likewise considered true positives. For analysis of the remaining eight samples positive in the genus assay, see below.

For 64 specimens, culture revealed growth of nontuberculous mycobacteria (39 genus assay-positive samples and 25 genus assay-negative samples) (Table 1), resulting in a total number of 69 NTM-positive samples (Table 3). For smear-positive samples, the genus assay showed a sensitivity of 100% (21/21). For smear-negative samples, where the number of acid-fast bacilli can be as low as a single bacterium, the sensitivity was found to be 47.9% (23/48). Taking together smear-positive and -negative samples, the genus assay was able to detect 44/69 NTM-positive samples, resulting in an overall sensitivity of 63.8%. Species detected by the genus assay included *M. kansasii*, *M. abscessus*, *M. chelonae*, *M. avium*, *M. intracellulare*, *M. xenopi*, and *M. leprae*.

By culture, 347 samples were considered to be negative for

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**TABLE 1. Results of genus assay in comparison to those of culture and smear assays**

<table>
<thead>
<tr>
<th>Smear test result</th>
<th>Genus assay positive</th>
<th>Genus assay negative</th>
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<tbody>
<tr>
<td></td>
<td>Culture positive</td>
<td>Culture negative</td>
</tr>
<tr>
<td>Positive</td>
<td>17</td>
<td>4</td>
</tr>
<tr>
<td>Negative</td>
<td>22</td>
<td>9</td>
</tr>
<tr>
<td>Total</td>
<td>39</td>
<td>13</td>
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positive (3.935) M. kansasii, positive (0.928) no sequence, Corynebacterium positive (1.059) M. leprae, positive (1.173) M. kansasii, positive (1.149) M. smegmatis, positive (1.359) M. kansasii (gastro). For the remaining four samples, sequence determination of the amplification product did not result in a clear sequence (Table 2).

Taken together, the specificity of the genus assay was 97.7%, the positive predictive value (PPV) 84.6%, and the negative predictive value (NPV) 93.1%. Sensitivity, specificity, PPV, and NPV determined for the Mycobacterium genus assay compare well with those published for the Cobas Amplicor M. tuberculosis test (15).

### DISCUSSION

The possibility of genus-specific molecular assays in detection of mycobacterial infections has been recognized since the early 1990s (2). However, it has proven difficult, if not impossible, for routine clinical microbiological laboratories with little expertise in molecular diagnostics to implement homemade assays in their diagnostic workflow. To address this issue, we wished to develop a genus-specific molecular detection assay for mycobacteria in the setting of a widely available commercial instrumentation platform. Here we present the development and evaluation of a semiautomated assay for direct detection of nontuberculous mycobacteria from clinical samples. The Cobas Amplicor M. tuberculosis assay is well established in diagnostic laboratories. An assay based on this system has several advantages: (i) the basic test and platform are commercially available; (ii) a semiautomated detection system with little hands-on time allows high throughput, which is especially important in populations with a high proportion of negative samples; (iii) the PCR product obtained from the Cobas Amplicor M. tuberculosis test allows screening for both M. tuberculosis DNA and mycobacterial genus DNA; (iv) the positive control included in the Cobas Amplicor M. tuberculosis test can be used as a positive control for the genus assay (by using the same positive control, the performance of the genus assay can be directly compared to the Cobas Amplicor M. tuberculosis test for quality assurance); and (v) an internal control plasmid is included in the Cobas Amplicor M. tuberculosis test, which controls for inhibition of amplification.

To determine the analytical sensitivity of the genus assay, we tested a dilution series of M. tuberculosis DNA and demonstrated that there is no difference between the detection limit of the genus assay and that of the Cobas Amplicor M. tuberculosis assay (Fig. 1). The detection limit is in accordance with the published analytical sensitivity for purified DNA of five genome copies per PCR in 100% of the PCRs (18).

For evaluation of the genus assay in a routine diagnostic setting, 467 clinical samples were tested for the presence of mycobacterial DNA. These included 342 respiratory and 125 nonrespiratory specimens. The sensitivities of the genus assay were found to be 100%, 47.9%, and 63.8% for smear-positive, smear-negative samples, and overall sensitivity, respectively. These numbers are similar to the sensitivities published for the Cobas Amplicor M. tuberculosis test for extrapulmonary and respiratory specimens, ranging from 87.5 to 100% for smear-positive samples, from 17.2 to 70.8% for smear-negative samples, and from 27.3 to 85% for overall sensitivity, as reviewed by Piersimoni et al. (15). For respiratory speci-

| TABLE 3. Performance of genus assay with samples positive or negative for NTM |
|------------------------------|------------------|------------------|------------------|
| Smear test result | Genus assay positive | Genus assay negative | Genus assay positive | Genus assay negative |
| Total | 44 | 25 | 8 | 339 |

* A total of 69 samples were positive and 347 negative for NTM.
mens, the sensitivity for smear-positive and smear-negative samples and the overall sensitivity, as described in the manual of the manufacturer, are 96.4%, 72.8% and 86.6%, respectively (18). We conclude that the sensitivity of the genus assay is similar to that of the Cobas Amplicor M. tuberculosis test. The calculated overall sensitivity of the genus assay is relatively low in our study, since the number of smear-negative samples included in the study was more than twofold higher than that of smear-positive specimens tested.

A total of 52 samples were positive using the genus assay. Positive PCRs were confirmed by culture for 39 of the 52 samples. The remaining 13 samples were further analyzed to resolve the discrepancy (Table 2). For three (5.8%) genus assay-positive, culture-negative samples, sequence analysis of the PCR product revealed fast-growing mycobacteria of doubtful clinical significance. Given that fast-growing mycobacteria are found in various environmental sources, these results were considered most likely to represent contaminants. Since non-tuberculous mycobacteria are frequently encountered in the environment, minimizing the risk of specimen contamination remains challenging. This not only involves assay setup but also includes all preanalytical steps (sample collection, transport, processing, decontamination, and DNA extraction). In one sample, Corynebacterium sp. was detected by nucleic acid analysis of the PCR product. The 16S rRNA sequences used as primer target sites in the Cobas Amplicor M. tuberculosis test are highly conserved within the genus Mycobacterium, including the majority of clinically relevant mycobacteria, with the exception of mycobacteria of the M. simiae group (19, 23). However, these 16S rRNA sequences are not exclusively specific for mycobacteria. Cross-reactivity with closely related bacteria, such as Corynebacterium species, Nocardia species, and Rhodococcus species, has been described (23). Cross-reactivity with these bacteria had only a minor impact on the performance of the genus assay, since all positive results were subjected to sequencing. Corynebacterium sp. was identified in 1/52 (1.9%) of genus assay-positive samples, indicating that cross-reactivity with these genera was a rather rare event in our evaluation. For four (7.7%) genus assay-positive specimens, sequence determination of the PCR product did not result in a defined sequence. Most likely this is a result of unspecific amplification in the genus assay.

In five (9.6%) genus assay-positive, culture-negative samples, we identified mycobacteria well established to cause disease in humans. Three samples revealed M. kansasii. All three samples were from patients with confirmed M. kansasii infections. Negative cultures are most likely due to intrasample variation and impaired viability of the organisms under antibiotic therapy. Two samples revealed the presence of M. leprae DNA; we consider these five samples to be correct positives in the genus assay.

Our results do point to the potential of the genus assay to detect mycobacteria which cannot be recovered by culture under normal laboratory conditions. This includes noncultivable mycobacteria (e.g., M. leprae), mycobacteria with special growth requirements (e.g., M. ulcerans), very slow-growing mycobacteria that exceed routine standard incubation times, and mycobacteria that fail to be cultivated, e.g., due to antibiotic therapy or harsh sample decontamination.

The specificity of the genus assay was 97.7%, the PPV 84.6%, and the NPV 93.1%. Again, these values are comparable to the described data for the Cobas Amplicor M. tuberculosis test, ranging from 91.3 to 100%, 73.3 to 100%, and 80.8 to 99.2%, respectively (15). The overall specificity, PPV, and NPV for respiratory specimens published in the manufacturer’s manual is 99.7%, 96.3%, and 98.9%, respectively (18).

The eight false-positive samples in the genus assay were all smear negative and culture negative, and nucleic acid sequencing of the PCR product revealed sequences of fast-growing mycobacteria (n = 3) or Corynebacterium sp. (n = 1) or did not result in a defined sequence (n = 4) (Table 2). As a result of our evaluation, we report a positive genus assay result only if one of the following conditions is met: (i) the identified Mycobacterium species is clearly associated with disease; (ii) the sample is smear positive; or (iii) the same sequence has been obtained from other samples of the patient, which may include the laboratory request for additional analysis of samples in case only a single sample from a patient has been submitted to the laboratory. This strategy can increase the specificity and PPV to close to 100%, although it is difficult to determine true-positive and true-negative results when cultures remains negative and no additional reference method is available.

In conclusion, we have successfully developed a direct detection assay for NTM from clinical specimens based on the Cobas Amplicor M. tuberculosis platform. The genus assay and subsequent sequence analysis were able to identify the vast majority, 44/52 (84.6%), of NTM-positive samples correctly. The Mycobacterium species detected included M. kansasi, M. abscessus, M. chelonae, M. avium, M. intracellulare, M. xenopi, and M. leprae, which are all associated with human disease. The developed assay enables rapid and accurate diagnosis of infections with NTM, as well as recognition of unknown and noncultivable mycobacterial species.

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