Use of Microelectronic Array Technology for Rapid Identification of Clinically Relevant Mycobacteria

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We developed a new method based on the Nanochip microelectronic array technology for identification of various clinically relevant mycobacterial species. PCR-amplified rRNA genes obtained from 270 positive Mycobacteria Growth Indicator Tube cultures were successfully tested by hybridizing them with species-selective probes, and the results agreed with those of conventional identification methods. The system is rapid and accurate and opens new perspectives in clinical diagnostics.

Mycobacteria are slow-growing microorganisms causing a variety of infections that involve lung, skin, soft tissue, skeletal system, and lymph nodes in immunocompromised and immunocompetent patients (5). Among them, Mycobacterium tuberculosis is the most common pathogen, causing 1.7 million deaths a year worldwide according to 2003 World Health Organization estimates (http://www.who.int/mediacentre/factsheets/fs104/en/print.html). Rapid and accurate identification of these microorganisms at the species level is important to establish a prompt and appropriate therapy, considering that antibiotic treatment may vary according to the species encountered (2). Molecular assays that offer an alternative approach to time-consuming culture-based methods have recently been developed to improve identification of mycobacteria (14).

In this study, we developed a new method, based on microelectronic chip array technology, to rapidly identify a variety of clinically relevant mycobacterial species by use of the Nanochip Molecular Biology Workstation (Nanogen, Inc., San Diego, CA). The instrument uses a proprietary semiconductor microchip, which incorporates a 10-by-10 array of microelectrodes coated with a permeation layer containing streptavidin, for the rapid transport and concentration of negatively charged nucleic acid molecules through the selective application of a positive electronic bias to selected test sites. The nucleic acid may then be immobilized by direct attachment to the permeation layer or by hybridization to a previously addressed nucleic acid. This technique has been used for single-nucleotide polymorphism assays using fluorescently labeled reporter probes and biotinylated amplified DNA samples (4).

A 205-bp highly conserved region of the Mycobacterium 16S rRNA gene was amplified from genomic DNA samples using the pMyc14 (5′-biotinylated) and pMyc7 primers and PCR conditions as previously described (7). A small amount of biotinylated PCR product was electronically deposited and anchored by streptavidin linkage on the microchip surface, denatured in situ, and hybridized with 13 dye-labeled oligonucleotide probes, seven of which were selective for M. tuberculosis complex, M. avium, M. xenopi, M. gordonae, M. terrae, M. tripex, and M. malmoense, respectively. The other six probes were each selective for two or multiple mycobacterial species, due to the high similarity between their PCR-amplified 16S rRNA gene fragments (Table 1).

Almost of these species are very closely related: M. intracellular versus M. chimaera (17), M. chelonae versus M. abscessus and M. massilense (1); and M. kansasii versus M. gaudi (8), or belong to the same taxonomic group: M. fortuitum versus M. porcinum, M. neworleansense, and M. boenickei (12) (Table 1). Probes were designed by the use of Vector NTI Advance 9.0 software (Invitrogen, Inc., Milan, Italy), taking into account their secondary structure, C+G content, length, and melting temperature. In addition, a general probe, specific for the genus Mycobacterium, was included as a positive control. The specificity of these probes was assessed by hybridization with DNA from bacterial species belonging to the genera Corynebacterium, Nocardia, and Rhodococcus, which are closely related to the genus Mycobacterium. Probes were coupled on the basis of their melting temperature and synthesized so that the oligonucleotides of each pair contained either a 5′-Cy3 or 5′-Cy5 fluorophore (Table 1). The probe pairs were used in consecutive hybridization runs.

Amplicon addressing (the process by which each amplicon was electronically placed in the microchip) was performed following the Nanogen User’s Guide instructions. Briefly, a 96-well microtiter plate was filled as follows: one well contained 60 μl of 0.3 M NaOH for sample passive (not electronic) pre-treatment; another well contained 60 μl of 50 mM histidine (30 μl of 100 mM histidine plus 30 μl of high-performance liquid chromatography-grade water) as the background control; the other wells each contained one sample to be processed (15 μl of PCR product, 15 μl of high-performance liquid chromatography-grade water, and 30 μl of 100 mM histidine); the last well contained 60 μl of 0.1 M NaOH for sample passive denaturation. Then, the microtiter plate and Nanochip cartridge were put into the...
Hybridization solutions were prepared by mixing in ice 2 µl of high-salt buffer (50 mM sodium phosphate, pH 7.0), and the cartridge was then cooled to 4°C and left at this temperature for 3 min. Heating at 56°C was introduced to avoid the formation of secondary structures. After hybridization, three washing steps were performed with low-salt buffer (50 mM sodium phosphate, pH 7.4), and the cartridge was then cooled to 4°C and scanned using a two-laser system. Data were analyzed, and samples with a signal-to-noise ratio of >5 were registered as positive. To perform a new hybridization step with a different probe pair, bound probes were removed with NaOH (0.3 M). A single cartridge could be stripped and rehybridized at least 10 times.

To set up the assay, to evaluate probes efficacy and to discriminate hybridization thermal stringency, we performed preliminary experiments using mycobacterial reference strains M. tuberculosis ATCC 27294 and ATCC 35801; M. avium ATCC 25291; M. chelonae ATCC 35752; M. fortuitum ATCC 6841; M. intracellularare ATCC 13950; M. kansasii ATCC 12478; M. marinum ATCC 927; M. xenopi ATCC 19250; M. smegmatis ATCC 607; and M. terrae ATCC 15755 and clinical isolates of M. gordonae, M. triplex, and M. malmoense. Specific fluorescence signals were obtained for most of the probes tested in a series of independent experiments. Probes that failed to give reproducible hybridization results were redesigned by choosing another target sequence within the 16S rRNA gene region (Table 1).

We tested PCR products obtained from 270 primary liquid cultures of clinical specimens from patients with suspected tuberculosis or other mycobacterial infections that were detected as positive by the BACTEC Mycobacteria Growth Indicator Tube 960 system (Becton Dickinson, Sparks, MD). Briefly, a 1-ml aliquot from each Mycobacteria Growth Indicator Tube culture was incubated at 100°C to inactivate mycobacteria. Genomic DNA was then extracted as previously described (11). After amplification, the PCR products were purified and desalted by QIAquick PCR purification kit (QIAGEN, Hilden, Germany), according to the manufacturer’s instructions with the following modifications: in step 5, two washes with buffer PE plus 1 wash with 0.75 ml of 70% ethanol were performed; in step 8, elution was performed using 30 µl of high-salt buffer.

<table>
<thead>
<tr>
<th>Probe</th>
<th>Target species</th>
<th>Sequence (5’–3’)</th>
<th>Probe pair Tm (°C)</th>
<th>Probe pair designation</th>
</tr>
</thead>
<tbody>
<tr>
<td>pGeneral3</td>
<td>Mycobacterium spp.</td>
<td>Cy5-CGATTGACCGCT</td>
<td>38.5</td>
<td>Pair 1</td>
</tr>
<tr>
<td>pTub2</td>
<td>M. tuberculosis complex</td>
<td>Cy3-AGCCTGATTC</td>
<td>37.4</td>
<td>Pair 2</td>
</tr>
<tr>
<td>pAvi1</td>
<td>M. avium</td>
<td>Cy5-ACATGGGCGCTA</td>
<td>39.4</td>
<td>Pair 3</td>
</tr>
<tr>
<td>pXen1</td>
<td>M. xenopi</td>
<td>Cy3-ACCATGAAATGG</td>
<td>39.4</td>
<td>Pair 3</td>
</tr>
<tr>
<td>pInt2</td>
<td>M. intracellularare, M. chimaeraa</td>
<td>Cy5-ACATGGGCGCTA</td>
<td>37.7</td>
<td>Pair 4</td>
</tr>
<tr>
<td>pChe13</td>
<td>M. chelonae, M. abscessus, M. massiliensec</td>
<td>Cy3-GGCATCAGTG</td>
<td>38.4</td>
<td>Pair 5</td>
</tr>
<tr>
<td>pTripl2</td>
<td>M. triplex</td>
<td>Cy5-GGACATCAGTC</td>
<td>40.1</td>
<td>Pair 6</td>
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<tr>
<td>pMar2</td>
<td>M. marinum, M. ulcerans, M. lipifidii, M. pseudoshottsiid</td>
<td>Cy5-GATCCGGTGG</td>
<td>37.5</td>
<td>Pair 7</td>
</tr>
<tr>
<td>pTer1</td>
<td>M. terrae</td>
<td>Cy3-ACAGAACATGCAT</td>
<td>37.5</td>
<td>Pair 7</td>
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<tr>
<td>pFor3</td>
<td>M. fortuitum, M. fortuitum third biovariant complexd</td>
<td>Cy5-ATCCGGTGG</td>
<td>38.4</td>
<td>Pair 5</td>
</tr>
<tr>
<td>pKan3</td>
<td>M. kansasii, M. gastroi</td>
<td>Cy3-GGCATCAGTG</td>
<td>37.5</td>
<td>Pair 7</td>
</tr>
<tr>
<td>pSme2</td>
<td>M. smegmatis, M. flavescens</td>
<td>Cy3-GGCATCAGTG</td>
<td>37.5</td>
<td>Pair 7</td>
</tr>
<tr>
<td>pMal2</td>
<td>M. malmoense</td>
<td>Cy3-GGCATCAGTG</td>
<td>37.5</td>
<td>Pair 7</td>
</tr>
</tbody>
</table>

* Included in the M. avium complex, M. chimaera has recently been proposed as a new species (17).

b Having identical 16S rRNA gene hypervariable region sequences, like M. abscessus, M. massiliena can be discriminated from M. chelonae by biochemical tests (1, 8) or by PCR restriction analysis of the hsp65 Telenti fragment (16).

c These species are pathogenic for animals (9, 10).

d Among the species included in this group, only 16S rRNA gene hypervariable region sequences of M. abscessus, M. xenopi, and M. massiliena can be discriminated from M. chelonae by biochemical tests (1, 8) or by PCR restriction analysis of the hsp65 Telenti fragment (16).
FIG. 1. Examples of Nanochip hybridization results for three sequence-selective fluorescently labeled probe pairs against the 13 mycobacterial species tested. Graphs show fluorescence signal values obtained in each of the three experiments. Histidine was used as a negative control.
not shown). Among the remaining 44 PCR products, 12 hybridized with probe pInt2, 11 with probe pChel3, 9 with probe pFor3, 6 with probe pKan3, 5 with probe pSme2, and 1 with probe pMar2; correspondingly, the culture isolates were identified as *M. intracellulare* (12 isolates), *M. chelonae* (11 isolates), *M. fortuitum* (9 isolates), *M. kansasi* (6 isolates), *M. smegmatis* (5 isolates), and *M. marinum* (1 isolate), using biochemical and molecular tests (8, 16). Figure 1 shows the results of hybridization obtained with PCR products from 13 different mycobacterial species using three probe pairs. As expected, strong signals were observed only between the PCR products and their selective oligonucleotide probes.

Microelectronic chips or arrays are innovative methods for DNA hybridization analysis of point mutations, single-nucleotide polymorphism, short tandem repeats, and gene expression. Electronic molecule addressing and hybridization are carried out on these devices, where the electric field and the use of low-conductance buffers (histidine, etc.) greatly accelerate the hybridization reactions on the selected test sites (4, 15). The feasibility of these devices, initially developed for genotyping purposes (6, 13, 20), makes them adaptable for other clinically important applications (3, 19).

In this work, we applied the Nanogen Nanochip Molecular Biology Workstation to the field of bacterial diagnostics for the first time, focusing on the species identification of mycobacteria. We adapted this technology to our purpose by creating a hybridization assay in which multiple species-selective probes, matching a highly polymorphic region of the *Mycobacterium* 16S rRNA gene, were used in consecutive reactions on a single platform. This gene has been used in several molecular methods (11, 14), and, recently, in a microarray system developed by Troesch et al. (18), for the species identification of mycobacteria. Like the last method, the microelectronic chip has the potential answer to many diagnostic questions associated with the genus *Mycobacterium*, since it is possible to expand the spectrum of the mycobacterial species by testing additional probes on the same chip. In our assay, we included probes directed against either most clinically relevant mycobacterial species (i.e., *M. tuberculosis* complex and *M. avium*) or *Mycobacterium* species rarely causing human disease, such as *M. triplex*. The choice of these probes reflected the epidemiology of *Mycobacterium* infections in our hospital, but obviously, the panel of probes can be modified according to the patient population to which diagnostic investigation is referred.

In conclusion, the Nanochip system offers a great deal of promise for rapid identification, especially if used in combination with PCR detection methods. A similar approach has been ideated by Westin et al. (20), in which multiplex strand displacement amplification was combined with microelectronic chip array to detect the factor V Leiden mutation. Our results confirm the great flexibility of the Nanochip system and open new perspectives for its large-scale use in clinical diagnostics. In spite of the remarkable qualities of this system, such as accuracy, ability to select a minimal signal-to-noise ratio, need for only a small amount of DNA, reduced turnaround time, and easy performance, the Achilles' heel remains the attention in designing probes of interest to avoid additional synthesis costs, although this is of little importance compared to a 100-site array cost (approximately 500 Euros). As the system is capable of testing up to 100 samples simultaneously, we feel that the Nanochip system presented here is particularly suitable for routine use in mycobacteriology laboratories that process a wide number of clinical specimens.

REFERENCES


