Rapid detection of specific gene mutations associated with isoniazid or rifampicin resistance in *Mycobacterium tuberculosis* clinical isolates using non-fluorescent low-density DNA microarrays

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**Background:** A new, fast ‘low cost and density’ DNA microarray (LCD array), designed for the detection of mutations that confer isoniazid or rifampicin resistance in *Mycobacterium tuberculosis* isolates, has been developed and was evaluated using 46 resistant clinical isolates from Barcelona.

**Methods:** LCD chips are pre-structured polymer supports using a non-fluorescent detection principle based on the precipitation of a clearly visible dark substrate. One LCD chip consists of eight identical microarrays, designed to detect mutations within the 90 bp *rpoB* region, codon 315 in the *katG* gene and the *mabA*-inhA regulatory region. A total of 22 strains with a *katG*315 mutation, 19 strains with alterations in the *mabA*-inhA regulatory region and 16 strains with mutations in the *rpoB* region, characterized previously, were studied.

**Results:** The identification of S315T and S315N mutations using the LCD was 100% concordant with the sequencing data. A strain with the S315R mutation, which is not tiled on the LCD array, was detected by the absence of hybridization using the wild-type probe. Of 19 strains with low-level isoniazid resistance related to the *mabA*-inhA regulatory region, 18 were identified correctly. The detection of mutations in the *rpoB* region was 93.8% concordant with the sequencing data. One *mabA*-inhA and *rpoB* mutated strain showed a cross-hybridization.

**Conclusions:** The LCD array protocol takes 45 min (15 min ‘hands-on’ time) after prior PCR amplification. Only minimal laboratory equipment is required. LCD arrays provide a rapid and economical method to characterize mutations in codon 315 of the *katG* gene, in the *mabA*-inhA regulatory region and in the *rpoB* gene.

**Keywords:** drug resistance, tuberculosis, *mabA*-inhA, *katG*, *rpoB*

**Introduction**

Tuberculosis is currently the second leading cause of adult mortality due to infectious diseases and is responsible for approximately two million deaths a year worldwide. The WHO estimates that one-third of the world’s population is infected with *Mycobacterium tuberculosis*.¹ Multidrug-resistant tuberculosis (MDR-TB) is an emerging public health problem in many regions of the world, particularly in developing nations.⁷ Accurate and rapid diagnosis is essential in the management of drugs and MDR-TB, not only to optimize treatment but also to prevent transmission. Mutations confined to a short 81 bp DNA region in the *rpoB* gene, encoding the β-subunit of the RNA polymerase, have been found in ~95% of rifampicin-resistant strains.³,⁴ Mutations in this region are an excellent marker for MDR-TB.⁴ However, the molecular mechanisms of isoniazid resistance are highly complex. They have been associated with a variety of mutations which affect one or several genes involved in mycolic acid biosynthesis or overexpressed as a response to the build-up or cellular toxicity of isoniazid.⁵,⁶ Several studies have revealed that mutations in the *katG* gene are responsible for 60–70% of isoniazid-resistant strains,⁷ with the most frequent mutation...
occurring at codon 315. Mutations in the \textit{mabA-inhA} regulatory region that exhibit both low-level isoniazid resistance and ethionamide resistance have been found in clinical isolates.\(^8,9\)

Less common, strains with mutations in the structural gene \textit{inhA} have been described.\(^6\) At least 18 alternative genes have been implicated in the mechanism of resistance to isoniazid.\(^6\)

Among these, there are \textit{ndh} (encoding NADH dehydrogenase),\(^5\) \textit{fadE23}, \textit{fadE24} (involved in fatty acid \(\beta\)-oxidation), \textit{Rv1592c}, \textit{Rv1772} (of unknown function but induced transcriptionally by isoniazid) and the \textit{iniBAC} region (\textit{Rv0340} genes, \textit{iniA}, \textit{iniB} and \textit{iniC}) induced by both isoniazid and ethambutol. Mutations in the \textit{oxyR-ahpC} region, a gene that encodes an alkyl hydroperoxide reductase (AhpC), do not appear to play a direct role in isoniazid resistance, although mutations in this region can be used as a marker of resistance when KatG activity is low or absent.\(^10\)

We recently presented a molecular analysis of isoniazid and rifampicin resistance mechanisms in \textit{M. tuberculosis} isolates recovered from Barcelona\(^11\) and determined that a rapid genotypic assay including the 315-TG kodon and \(-15\) nt of the \textit{mabA-inhA} regulatory region would cover 62\% of isoniazid-resistant strains in Barcelona. Moreover, targeting of the 81 bp region of \textit{rpoB} would detect all rifampicin-resistant isolates.

The aim of this study was to evaluate a new, fast and economical low-density DNA microarray (LCD array) for its ability to detect rifampicin and isoniazid resistance in \textit{M. tuberculosis} isolates. In recent years, several molecular techniques have been applied to detect mutations related to antituberculous drug resistance. These include amplification and restriction fragment length polymorphism,\(^12,13\) amplification and sequencing,\(^14\) PCR single-strand conformational polymorphism,\(^9\) EIA hybridization\(^15\) and real-time PCR using fluorescent labelled hybridization probes.\(^16\) DNA microarrays have been applied widely in fundamental research, human genetics, infectious disease diagnosis, genotyping, genetic expression monitoring, pharmacogenomics, environmental control and, more recently, in the identification and detection of mutations in genes responsible for drug resistance.\(^17\) High-density oligonucleotide arrays have been used for parallel species identification and the detection of mutations in genes responsible for drug resistance.\(^17\) High-density oligonucleotide arrays have been used for specific species identification and the detection of mutations that confer rifampicin resistance in mycobacteria,\(^18\) and more specifically for the detection of \textit{M. tuberculosis} strains resistant to rifampicin\(^19–22\) or isoniazid, kanamycin, streptomycin, pyrazinamide and ethambutol.\(^23,24\)

However, owing to high costs, complex protocols and the need for substantial additional laboratory equipment, DNA microarrays have not yet become part of routine molecular diagnostics. The working principle of the LCD arrays used in this study is comparable to the robust protocols used for membrane-based reverse hybridization techniques, such as reverse line blots\(^25\) and line probe assays.\(^26\)

We have compared the LCD array technology with the accuracy of conventional DNA sequencing to detect mutations associated with isoniazid and rifampicin resistance in \textit{M. tuberculosis} strains.

\section*{Materials and methods}

\textit{M. tuberculosis} strains

DNA from 46 resistant \textit{M. tuberculosis} isolates recovered from the Barcelona area over a 2 year period was available for this study. The resistance phenotypes and molecular mechanisms associated with the resistance to isoniazid and rifampicin have been characterized previously\(^17\) (Table 1), and the LCD array was evaluated as a blind assay. Strains with both high and low levels of isoniazid resistance were included. A total of 22 strains with MICs of isoniazid between 4 and 16 mg/L and a mutation at amino acid position 315 in the \textit{katG} gene were studied: 20 strains with the molecular change \textit{S}\textit{315T}, 1 with \textit{S}\textit{315N} and 1 with \textit{S}\textit{315R}. Also, 19 strains with MICs between 0.25 and 2 mg/L and a mutation at amino acid position 315 in the \textit{mabA-inhA} regulatory region were included: 16 with \textit{S}\textit{315T}–\textit{T}, 2 with \textit{S}\textit{315C}–\textit{C} and 1 with \textit{S}\textit{315H}–\textit{C}. A total of 16 \textit{M. tuberculosis} strains with mutations related to rifampicin resistance were analysed: 7 strains with the mutation \textit{S}\textit{531L}, 2 with \textit{H}\textit{526D}, 2 with \textit{H}\textit{526Y}, 2 with \textit{D}\textit{516V}, 1 with \textit{S}\textit{531W}, 1 with \textit{D}\textit{516F} and 1 with \textit{D}\textit{516Y}.

\section*{LCD array design}

The LCD array is a transparent, pre-structured polymer support containing eight identical microarrays in well-separated, individually addressable hybridization fields. The outer dimensions of 50 × 50 mm combined with the non-fluorescent detection principle, based on the formation of a clearly visible substrate precipitate, allow the use of a very economical transmission-light film scanner to generate greyscale images of \(\sim 10\) \(\mu\)m resolution for the data analysis. The capture probes are between 16 and 22 nt long, and carry an immobilization tag at their 5′ end. They are spotted as duplicates in a 9 × 9 pattern, with average spot diameters of 300 \(\mu\)m. Each array is designed to detect mutations within the 90 bp \textit{rpoB} region, codon 315 in the \textit{katG} gene and the \textit{mabA-inhA} regulatory region (Figure 1). The capture probes for the \textit{rpoB} gene cover a 90 bp region coding for amino acids 504–534. Six capture probes, representing the wild-type (wt) sequence and 12 capture probes for the most frequent mutations are immobilized. The capture probes for the \textit{katG} gene span the sequence region of amino acid position 315. One probe represents the wt (315S) and two probes harbour the sequence for the mutations \textit{S}\textit{315T} and \textit{S}\textit{315N}. The capture probes of the \textit{mabA-inhA} regulatory region have been designed for three point mutations, \textit{S}\textit{8T}–\textit{C}, \textit{S}\textit{15C}–\textit{T} and \textit{S}\textit{17G}–\textit{T}, and two probes represent the wt in the 5′ non-coding region. A control probe with an unrelated sequence motif (fungal origin) is immobilized in three angles of each field, working as a functional control for successful hybridization and staining steps, and enables the user or the software to position the analysis grid correctly. LCD arrays were manufactured by Chipron GmbH, Berlin, Germany.

\section*{Amplification, hybridization and reading}

The LCD array protocol for the analysis of \textit{M. tuberculosis} resistance is based on a PCR amplification of the three regions of interest (\textit{rpoB}, \textit{katG} and \textit{mabA-inhA}). A multiplexed single reaction, with simultaneous product biotinylation, was performed using \(\sim 200\) ng of DNA in a 25 \(\mu\)L reaction volume. The primers used were obtained from Chipron GmbH, Germany (Table 2). The amplification was carried out as follows: 2 min at 98°C; 45 cycles of 20 s at 96°C, 20 s at 62°C and 20 s at 72°C; and 1 min at 72°C. Subsequently, hybridization and staining procedures were performed according to the manufacturer’s instructions using the material supplied. In brief, 12.5 \(\mu\)L of the biotinylated PCR products was combined with 25 \(\mu\)L of the foramide-based hybridization buffer. These mixtures were applied to the individual fields of the LCD arrays and hybridized for 30 min at 36°C. Following a 2 min washing step in a ‘low-salt’ wash buffer, the chips were

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dried using brief centrifugation. The specifically bound, biotinylated PCR products were further incubated with a streptavidin–horseradish peroxidase conjugate for 5 min at room temperature. Following an additional 2 min wash and drying cycle, the specific hybridization events were visualized by incubation with an enzyme substrate for 2 min at room temperature, leading to the formation of a dark precipitate at positions of peroxidase conjugate binding. Although these signals are clearly visible to the human eye, high-resolution greyscale images (10 mm resolution) were taken using a transmission-light film scanning device (Chipron, Germany). These images allow the use of commercially available software for image analysis and data storage.

Two software packages (SlideReader 1.1, Chipron, Germany and GenePix Pro 5.0, Axon Instruments, Inc., USA) and the online analysis module (Chipron, Germany) available at www.chipron.com were used to analyse the hybridization signals. All three pieces of software revealed the same data interpretation, as shown in Table 1. The whole protocol takes <3 h and up to 48 samples can easily be analysed in parallel owing to its simplicity.

### Results

The results obtained using the LCD array for all regions evaluated are summarized in Table 1.

**Detection of M. tuberculosis katG 315 mutants**

All strains with the S315T mutation (n = 20) (Figure 2a, strain 2R) and the strain with the S315N mutation were identified correctly using the LCD array. The strain with an S315R mutation, which was not represented by a specific capture probe, was identified indirectly since no hybridization with the wt probe occurred (Figure 2c, strain 16R).

**Detection of M. tuberculosis mabA-inhA regulatory region mutants**

Of the 16 strains with mutation at the position –15C→T, 15 were identified correctly (Figure 2b, strains 3R and 47R).

### Table 1. Isoniazid and rifampicin phenotype and correlation between sequence analysis and microarray hybridization

<table>
<thead>
<tr>
<th>No. of strains&lt;sup&gt;b&lt;/sup&gt;</th>
<th>INH and RIF phenotype</th>
<th>Results of sequencing analysis&lt;sup&gt;11&lt;/sup&gt;</th>
<th>Result of LCD array&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>katG mabA-inhA rpoB</td>
<td>katG mabA-inhA rpoB</td>
</tr>
<tr>
<td></td>
<td></td>
<td>wt mut wt mut wt mut wt mut</td>
<td>wt mut wt mut wt mut</td>
</tr>
<tr>
<td>8R</td>
<td>H</td>
<td>S315N wt wt</td>
<td>NEG 315N POS NEG POS NEG</td>
</tr>
<tr>
<td>16R</td>
<td>H+R</td>
<td>S315T wt S531L</td>
<td>NEG 315T POS NEG POS (1–5) 531L</td>
</tr>
<tr>
<td>44R, 48R, 56R</td>
<td>H+R</td>
<td>S315T wt S531L</td>
<td>NEG 315T POS NEG POS (1–5) 531L</td>
</tr>
<tr>
<td>18R</td>
<td>H+R</td>
<td>S315T wt H526Y</td>
<td>NEG 315T POS NEG POS (1–4,6) 526Y</td>
</tr>
<tr>
<td>19R</td>
<td>H+R</td>
<td>S315T wt D516F</td>
<td>NEG 315T POS NEG POS (1,2,4–6) NEG</td>
</tr>
<tr>
<td>63R, 64R</td>
<td>H+R</td>
<td>S315T wt D516V</td>
<td>NEG 315T POS NEG POS (1,2,4–6) 516V</td>
</tr>
<tr>
<td>61R</td>
<td>H</td>
<td>wt –15C→T</td>
<td>POS NEG POS (22) –15T POS</td>
</tr>
<tr>
<td>47R</td>
<td>H+R</td>
<td>wt –15C→T H526Y</td>
<td>POS NEG POS (22) –15T POS</td>
</tr>
<tr>
<td>3R</td>
<td>H+R</td>
<td>wt –15C→T H526D</td>
<td>POS NEG POS (22) –15T POS</td>
</tr>
<tr>
<td>52R</td>
<td>H+R</td>
<td>wt –15C→T S531L</td>
<td>POS NEG POS (22) –15T POS</td>
</tr>
<tr>
<td>26R</td>
<td>H</td>
<td>wt –17G→T wt</td>
<td>POS NEG POS (22) –17T POS</td>
</tr>
<tr>
<td>34R</td>
<td>H</td>
<td>wt –8T→C wt</td>
<td>POS NEG POS (24) –8C POS</td>
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<tr>
<td>42R</td>
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<td>wt –8T→C wt</td>
<td>POS NEG POS (24) –8C POS</td>
</tr>
<tr>
<td>5R</td>
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<td>wt S516Y</td>
<td>POS NEG POS (1,2,4–6) POS</td>
</tr>
<tr>
<td>7R</td>
<td>H+R</td>
<td>wt S531W</td>
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<tr>
<td>17R</td>
<td>H+R</td>
<td>wt S531L</td>
<td>POS NEG POS (1–5) 526N, 526Y, 531L</td>
</tr>
<tr>
<td>28R</td>
<td>H+R</td>
<td>wt H526D</td>
<td>POS NEG POS (1–4,6) POS</td>
</tr>
<tr>
<td>39R</td>
<td>H+R</td>
<td>wt S531L</td>
<td>POS NEG POS (1–5) 531L</td>
</tr>
</tbody>
</table>

**INH (H), isoniazid; RIF (R), rifampicin; wt, wild-type; mut, mutation; POS, hybridization positive; NEG, hybridization negative.**

The inconclusive results are shown in bold.

<sup>a</sup>The numbers in parentheses correspond to the probe numbers given in Figure 1.

<sup>b</sup>Numeration of the strains according to Coll et al.11

<sup>c</sup>The mechanism of INH resistance was associated with mutations in other regions of the katG gene: 5R, deletion codon 234; 7R, W204 stop; 17R, G560A; 28R, A172T; and 39R, no detected mutations.
The other strain hybridized with the –15C→T mutated probe but also showed an additional signal with the mabA-inhA –15/-17 wt probes (Table 1). The two strains with mutation at position –8T→C and the strain with the mutation –17G→T were identified correctly (Figure 2a, strains 26R and 34R).

**Detection of M. tuberculosis rpoB mutants**

Of the 16 strains with rpoB mutations, 14 were identified correctly using the LCD array (Table 1). All showed a strong signal of hybridization with the mutated probe and absence of hybridization with the respective wt probe. One strain with the mutation D516F did not hybridize with the wt probe for amino acids 514–519 (Figure 2c, strain 19R), thereby indicating a mutation within that region. A specific capture probe for this mutation was not tiled on the array (Figure 1). One strain, which showed a mutation S531L on sequencing analysis, displayed hybridization with five of the six wt probes and several mutated probes (Table 1), and we were therefore unable to determine the presence of the mutation using the LCD array.
Results with the wt regions

A total of 24 strains with a wt katG315, 30 strains with a wt rpoB core region and 27 strains with a wt mabA-inhA regulatory region hybridized with the corresponding wt probes and did not show any signals with the mutation-specific probes (Table 1).

Results with the multidrug-resistant strains

Of the 46 strains analysed, 16 were multidrug resistant. In 10 strains, the mutations responsible for isoniazid resistance were well characterized using the LCD array. Strain 16R had a S315R mutation in the katG gene. This strain showed an absence of hybridization with the wt probe, but the mutation was not detected because the specific probe was not tiled on the array (Figure 2c). In five strains, the isoniazid resistance mechanism was not a mutation in the katG gene 315 codon or the mabA-inhA regulatory region (Table 1). These strains were not therefore detected by the LCD array as isoniazid resistant. The mutations responsible for the rifampicin resistance were well characterized in 14 strains. Strain 19R had a D516F mutation in the rpoB gene. It showed an absence of hybridization with the 514–519 wt probe, but the mutation was not detected because the specific probe was not tiled on the array (Figure 2c). Finally, the S531L rpoB mutation was not identified in the strain 17R as multiple hybridization signals were observed.
**Discussion**

Delayed diagnosis, inadequate treatment regimens and mortality characterize drug-resistant and MDR-TB. Steps should be taken to ensure that all patients are diagnosed and treated effectively to avoid creation and transmission of the resistant strains in the community. The retrieval of antibiotics for *M. tuberculosis* is severely delayed when drug susceptibility testing is culture based. Routine application of rapid molecular tests in the clinical management of drug-resistant tuberculosis is essential.

In recent years, some light has been shed on the molecular basis for the mechanism of action of antituberculosis agents and the way in which organisms become resistant. The screening of codon 315 of the *katG* gene and the *mabA-inhA* upstream region should be included in a rapid genotypic assay for the direct detection of isoniazid resistance. However, if an 81 bp region of *rpoB* were selectively targeted, more than 95% of rifampicin-resistant isolates would be detected rapidly. The detection of *M. tuberculosis* resistance by means of microarrays has generally been limited to rifampicin. To our knowledge, few studies have detected isoniazid resistance to *M. tuberculosis*.

**62.2% in New York, 32 59.2% in South Korea, 33 34.6% in Madrid, 32 32% in Barcelona,11 and in none of the Equatorial Guinea strains.34 Conversely, the frequency of *mabA-inhA* regulatory region mutations reported is 80.5% in Equatorial Guinea strains.34**

The detection of *M. tuberculosis* resistance by means of microarrays has generally been limited to rifampicin. To our knowledge, few studies have detected isoniazid resistance to *M. tuberculosis* by means of DNA microarrays.24,28

**The results for the core region of the *rpoB* gene obtained with the LCD array were 93.8% concordant with the sequencing analysis. In previously published studies, the ability to detect rifampicin resistance with DNA chips has ranged between 83.6 and 94.29%.19,20,22,28 It was not possible to identify the presence of the S531L specific mutation in one strain because, although it did not hybridize with the corresponding wt probe, it hybridized with several other mutated probes. A D516F mutated rifampicin-resistant strain, whose mutation was not tiled on the LCD array, was suspected because it did not hybridize with the wt probe covering amino acid positions 514–519.**

The array detected 97.5% of the isoniazid-resistant isolates and it characterized 95.1% of the mutations responsible for resistance. In a recent study, the ability to detect isoniazid resistance with oligonucleotide microarrays was 80%. The detection of a mutation in codon 315 in the *katG* gene was 95.4% concordant with the sequencing data. The LCD array did not incorporate a probe to detect the 315R mutation. Nevertheless, resistance to isoniazid in a strain with such a mutation could be inferred due to the fact that this strain did not hybridize with the wt probe. The LCD array identified 18 out of 19 (94.7%) strains with a mutation in the *mabA-inhA* regulatory region. One strain with a –15 mutated probe was detected using sequencing analysis hybridized with the –15 wt probe but also with the wt probes. The signal intensity was the same for both –15 mutated and –15–17 wt probes. In this strain, we were therefore unable to determine using the LCD array the presence or absence of the mutation.

There are geographical variations in the molecular mechanism responsible for isoniazid resistance. An S315T mutation in the *katG* gene has been detected in 91.7% of isoniazid-resistant strains in St Petersburg, 29 87% in Brazil,30 83.9% in Lithuania,31 62.2% in New York,32 59.2% in South Korea,33 34.6% in Madrid,32 32% in Barcelona,11 and in none of the Equatorial Guinea strains.34 Conversely, the frequency of *mabA-inhA* regulatory region mutations reported is 80.5% in Equatorial Guinea strains.34 32% in Barcelona,11 30.4% in Philippines,32 24% in KwaZulu Natal and South Korea,33,36 16.3% in Lithuania31 and 14.5% in Poland.37 The frequency and type of mutations in one country or geographical region are not therefore applicable generally. The design of the LCD array presented here should allow identification of 62% of isoniazid-resistant isolates and all isolates that have been tested positive for rifampicin resistance in Barcelona.11 However, it is useful to distinguish between the *katG* 315 mutated genotypes that confer high-level isoniazid resistance and the *mabA-inhA* mutated genotypes that confer low-level isoniazid resistance.

The LCD array was able to characterize the molecular mechanism of isoniazid and rifampicin resistance in 56.2% of the 16 multidrug-resistant strains. More precisely, the array detected 62.5% and 87.5% of the isoniazid and rifampicin resistance mechanisms, respectively. As commented on above, in all but one of the seven strains not detected as multiresistant, the molecular mechanism was not located in the targeted zones. Nevertheless, rifampicin resistance is usually associated with isoniazid resistance. Therefore, in our series, multiresistance could be suspected either because both molecular mechanisms were detected by the LCD array (nine strains) or because the rifampicin one was (six strains).

Finally, other advantages of this new LCD array technology compared with membrane-based reverse hybridization techniques25,26 are the short, time-saving protocol (45 min for hybridization, staining and analysis) and the opportunity for automated software-supported data analysis, data storage and report generation. Moreover, the LCD array offers the opportunity to work with an increased throughput (eight samples per chip, several chips in parallel) with a low production cost, as well as with the possibility of introducing new capture probes for additional gene regions or mutations of interest.

In summary, the LCD array provides an easy, rapid and convenient method to characterize mutations in codon 315 of the *katG* gene, at positions –8, –15 and –17 of the *mabA-inhA* regulatory region and in the 90 bp *rpoB* region. Incorporation of wt probes for these genomic regions has allowed us to detect even those point mutations that are not represented by specific capture probes.

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**Transparency declarations**

None to declare.

**References**


