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Real-time PCR for single-nucleotide polymorphism detection in the 16S rRNA gene as an indicator for extensive drug resistance in *Mycobacterium tuberculosis*

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Objectives: A real-time PCR screening system was established for rapid detection of single-nucleotide polymorphisms (SNPs) at positions 1401, 1402 and 1484 of the 16S rRNA gene of *Mycobacterium tuberculosis* leading to resistance to amikacin, kanamycin and capreomycin. Resistances to the respective drugs may indicate the presence of an extensively drug-resistant (XDR) strain of *M. tuberculosis*.

Methods: Fifty-seven *M. tuberculosis* isolates that tested phenotypically susceptible or resistant to amikacin, capreomycin or both were subjected to 1401-2/1484 real-time PCR to screen for SNPs in the respective *rrs* region.

Results: 1401-2 and 1484 wild-type and mutant *M. tuberculosis* strains displayed distinct melting peaks. Of the cross-resistant strains, 86.7% displayed A1401G SNPs, 76.9% of amikacin-resistant strains did not display *rrs* SNPs and one capreomycin-resistant strain showed a C1402T SNP.

Conclusions: Phenotypic drug susceptibility testing takes several weeks, but with the 1401-2/1484 real-time PCR a preliminary diagnosis can be made within a few hours. SNPs in the *rrs* region are not exclusively involved in the development of resistances to amikacin and capreomycin. However, 80.0% of XDR-tuberculosis samples tested were detected with the real-time PCR screening assay of the present study.

Keywords: XDR-TB, amikacin, capreomycin

Introduction

Multidrug-resistant *Mycobacterium tuberculosis* (MDR-TB), resistant to at least isoniazid and rifampicin, and extensively drug-resistant *M. tuberculosis* (XDR-TB), defined as MDR-TB with additional resistance to any of the fluoroquinolones and to amikacin, kanamycin or capreomycin [the injectable second-line drugs (SLDs)], are a major health problem worldwide. However, it is assumed that many XDR-TB cases are never diagnosed since testing for resistance to SLDs is not provided for in every laboratory.[1]

Kanamycin resistance of *M. tuberculosis* has been shown to be associated with single-nucleotide polymorphisms (SNPs) in the 16S rRNA gene (*rrs*) at positions 1401, 1402 and 1484;[2] moreover, high-level amikacin/kanamycin cross-resistance is mostly linked to SNP 1401 in *rrs*.[3,4] Capreomycin resistance was shown to be predominantly associated with point mutations in the *tlyA* gene,[5] while *rrs* SNP 1401 was observed in capreomycin/kanamycin cross-resistant strains.[5] Overall, capreomycin/amikacin, capreomycin/kanamycin and kanamycin/amikacin cross-resistances were primarily linked to SNP 1401, 1402 and 1484 in *rrs*,[6,7] mutations that seem to be indicators of the presence of XDR-TB. Phenotypic drug susceptibility testing requires approximately 2–4 weeks; the aim of this study was therefore to develop a screening method for rapid and accurate detection of resistance to injectable SLDs in patients.

Materials and methods

Microorganisms

A total of 57 *M. tuberculosis* isolates (MDR-TB, $n=28$; and XDR-TB, $n=5$), preferentially phenotypically amikacin and/or capreomycin resistant ($n=30$), were provided by the Austrian National Reference Laboratory for Tuberculosis.

Additionally, DNA of one artificial clone carrying SNP G1484T in the *rrs* region—kindly provided by S. Niemann and D. Hillemann, National Reference Center for Mycobacteria (Borstel, Germany)—was included in the current study.

Primers and probes were tested for specificity by subjecting DNA of frequently found bacteria (*Mycobacterium avium*, *Mycobacterium intracellulare*, *Mycobacterium kansasii*, *Mycobacterium fortuitum*, *Mycobacterium xenopi*, *Mycobacterium gordonae*, *Staphylococcus aureus*, *Staphylococcus epidermis*, *Enterococcus faecalis*, *Listeria monocytogenes*, *Pseudomonas aeruginosa*, *Klebsiella oxytoca*, *Escherichia coli*, *Citrobacter koseri*, *Legionella pneumophila* and *Bordetella pertussis*) to real-time PCR.

Sample preparation

DNA was extracted from bacterial cultures by dispersing a loop full of cells into 500 μL of physiological saline, heating to 95°C for 30 min and finally centrifuging for 5 min at 12 500 **g**. Patient samples [$n=10$, positive by PCR and culture for *M. tuberculosis* complex (MTC)] were processed using *N*-acetyl-L-cysteine/sodium hydroxide solution (in-house preparation) and a Cobas Amplicor Sample Preparation Kit (Roche Diagnostics) according to the manufacturer's recommendations.

Amplification of rrs and melting curve analysis

All primers and probes were designed using the *M. tuberculosis rrs* gene (gene ID 2700429) as the template. Real-time PCR and melting curve analysis were performed using a LightCycler® 2.0 instrument, software version 4.1 (Roche Diagnostics). For both 1401-2 and 1484 real-time PCR, reaction volumes of 20 μL were applied containing 5 mM $MgCl_2$, 10 pmol of primer rrs_for (5′-CTG CGG TGA ATA CGT TCC-3′) and 2.5 pmol of primer rrs_rev (5′-CTC GCC CAC TAC AGA CAA G-3′), 8 pmol of probe [either 1401-2 (1401-2_anc_la 5′-AGG CCA CTG GCT TCG GGT GTT ACC-Fl, 1401-2_sen_la LC640-CTT TCA TGA CGC GAC GGG C-Ph) or 1484 (1484_anc_laneu 5′-CCA GCC GCA CCT TCC GGT AC-Fl, 1484_sen_laneu LC640-CTA CCT TGT TAC GAC TTA GTC CCA ATC G-Ph)], 2 μL of Mastermix (LightCycler® FastStart DNA Master HybProbe Kit, Roche Diagnostics), 2.3 μL of PCR grade water and 2 μL of target DNA as a target. Real-time PCR conditions were 95°C for 10 min and 50 cycles of 95°C for 5 s, 54°C for 5 s (acquisition mode single) and 72°C for 10 s; after amplification a melting curve was obtained by one cycle of 95°C for 10 s, 40°C for 10 s and 80°C for 0 s (acquisition mode continuous, 0.1°C/s), followed by a cooling step (50°C for 1 min).

For detection limit testing of primers and probes, one DNA extract was selected for preparation of a dilution series (from 1×10^{-1} to 1×10^{-6}, measured using a Picodrop® UV/Vis Spectrophotometer; Picodrop Limited) and subsequent real-time PCR.

DNA sequencing

All PCR products were sequenced after purification using a MinElute PCR Purification Kit (Qiagen) or exonuclease I and shrimp alkaline phosphatase (Fermentas), following the manufacturers' instructions. Sequencing reactions were performed following the manufacturer's instructions using a BigDye Terminator v1.1 Kit (Applied Biosystems) with primer rrs_for or rrs_rev. Samples were purified using Centri-Sep™ columns (Princeton Separations Inc.) and sequenced on a 3130 Genetic Analyzer (Applied Biosystems).

Results

SNPs

Of 57 samples tested by 1401-2/1484 real-time PCR, 16 displayed SNP A1401G, 1 displayed SNP C1402T and none displayed SNP G1484T; 40 samples did not show any alteration in the respective *rrs* region (Table 1).

As previously determined by BACTEC MGIT 960 (BD), 15 samples displayed amikacin/capreomycin cross-resistance, 13 samples were resistant to amikacin only, 2 samples were resistant to capreomycin only and 27 samples were susceptible to both drugs.

Of 15 samples showing phenotypic amikacin/capreomycin cross-resistance, 13 (86.7%) displayed SNP A1401G in the *rrs* hotspot region; 1 of the 13 samples showed genotypic heteroresistance, displaying both wild-type and A1401G melting peaks. Of 13 samples resistant to amikacin, 3 (23%) showed SNP A1401G in the tested 250 bp *rrs* section, 1 of them being heteroresistant with wild-type and A1401G melting peaks. One of two samples (50.0%) phenotypically resistant to capreomycin showed SNP C1402T. Four of 5 XDR-TB samples (80.0%) showed SNP A1401G ($n=3$) or SNP C1402T ($n=1$), in comparison with 13 of 28 MDR-TB samples (46.4%) displaying SNP A1401G only (Table 1).

Both susceptible and resistant samples displayed distinct melting curves, the former with median melting peaks at 57.4°C (1401-2 wild-type) and 64.9°C (1484 wild-type) (ranges of 55.7–57.6°C and 64.6–65.1°C) and the latter with median melting peaks at 67.3°C (A1401G; range 67.1–67.7°C), 52.0°C (C1402T) and 68.2°C (G1484T) (Figure 1). Melting peak variation within one parameter was determined using 10 samples chosen for each allele (1401-2 wild-type, A1401G and 1484 wild-type).

Real-time PCR detection limit

The DNA concentration of the sample used for the dilution series was 25.35 ng/μL. At each dilution step, 2 μL of DNA was subjected to 1401-2/1484 real-time PCR. The sample could be detected with both real-time PCRs to a dilution of 5×10^{-5}, which is equivalent to a detection limit of approximately 0.1 pg.

Real-time PCR specificity

DNA samples of *S. aureus*, *S. epidermis*, *E. faecalis*, *L. monocytogenes*, *P. aeruginosa*, *K. oxytoca*, *E. coli*, *C. koseri*, *L. pneumophila* and *B. pertussis* yielded negative results when tested with 1401-2/1484 real-time PCR. Non-tuberculous mycobacteria

Table 1. Number of strains and their *rrs* region mutations (*rrs* SNP) in relation to their respective phenotypic antibiotic resistance pattern (numbers of isolates designated MDR and XDR are given in round and square brackets, respectively)

rrs SNP	AMK^R and CAP^R	AMK^R	CAP^R	AMK^S and CAP^S
No mutation	2, (2)	10, (9)+[1]	1, (1)	27, (3)
A1401G	13[a], (10)+[3]	3[a], (3)		
C1402T			1, [1]	
Total	15	13	2	27

AMK, amikacin; CAP, capreomycin; R, resistant; S, susceptible.
[a]One sample with heteroresistance displaying a wild-type and an A1401G melting peak.

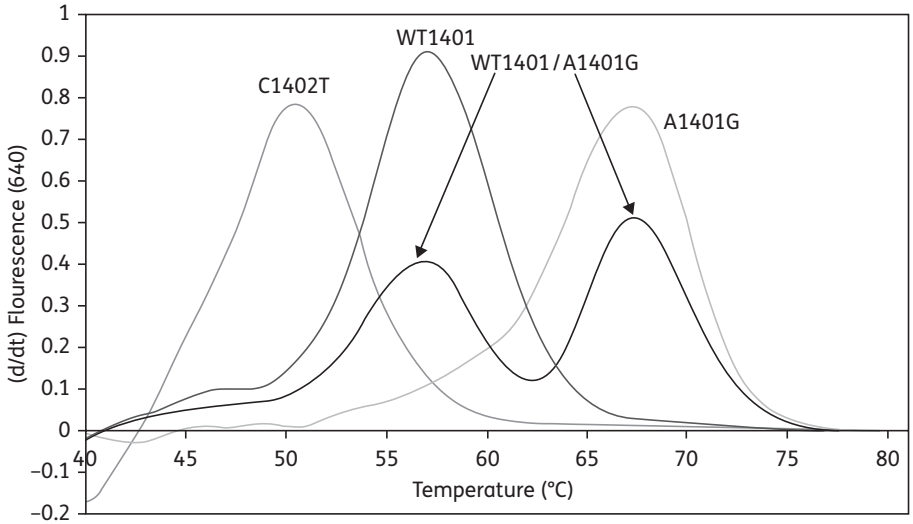

Figure 1. Melting peaks for 1401-2. Melting peak for 1401-2 wild-type (WT) at 57.4°C (median), melting peak for A1401G at 67.3°C (median) and melting peak for C1402T at 52.0°C.

(NTM) samples showed weak positive results with 1401-2/1484 real-time PCR.

Sequencing of the 16S rRNA gene

Sample sequencing of the *rrs* region confirmed SNP A1401G for 16 samples tested, SNP C1402T for 1 sample tested and SNP G1484T for 1 sample tested. As detected by 1401-2/1484 real-time PCR, all other samples displayed a wild-type sequence. Two samples showing genotypic heteroresistance in 1401-2 real-time PCR were confirmed by a mixed peak for both nucleotides A and G at position 1401.

Discussion

Fast detection of *M. tuberculosis* drug resistance is a major issue for successful treatment of tuberculosis. We established a real-time PCR for simultaneous detection of SNPs at nucleotide positions 1401, 1402 and 1484 within *rrs* of *M. tuberculosis* associated with amikacin, kanamycin and capreomycin resistance.[2,3,6–8]

Differences between wild-type and mutant melting points were significant—52.0°C for mutation C1402T, 57.4°C for the 1401-2 wild-type and 67.3°C for mutation A1401G. Interestingly, the 1401-2 real-time PCR showed varying melting points for 1401-2 wild-type—with a difference between lowest and highest melting points of 1.9°C, which can be explained by concentration variations of samples; the lower the DNA concentration [measured by crossing point (Cp) values], the lower the melting point (data not shown). This phenomenon was not observed when applying wild-type DNA to 1484 real-time PCR. In contrast, 10 samples tested carrying mutation A1401G had a melting point range of only 0.6°C, presumably due to a perfectly matching sensor probe.

Two samples with heteroresistance were detected with 1401-2/1484 real-time PCR and confirmed by sequencing. To our knowledge, this is the first record of heteroresistance in *M. tuberculosis* associated with amikacin and capreomycin resistance.

In contrast to other methods[6,7,9] and GenoType® MTBDR*sl* (Hain Lifescience), the 1401-2/1484 real-time PCR used in this study is much faster and easier to apply.

Interestingly, our results show that four of five XDR-TB samples tested (80.0%) could be detected with this real-time PCR. Similar results were found by Feuerriegel *et al.*[6] and Evans and Segal,[7] corroborating the findings of this study. Therefore, we think 1401-2/1484 real-time PCR can be used for mutation detection in the respective *rrs* region, indicating the possibility of an XDR-TB that has to be confirmed by additional fluoroquinolone resistance testing.

Like GenoType® MTBDR*sl*,[10] 1401-2/1484 real-time PCR can be applied to MTC PCR-positive patient samples only, due to weak positive signals generated by NTM. However, as determined within the current study, the detection limit of GenoType® MTBDR*sl* is about one power of 10 lower (1.25 pg) than the detection limit of 1401-2/1484 real-time PCR (0.1 pg). Thus, the real-time PCR of the present study is faster to perform and less expensive than any commercial assay.

In our opinion, 1401-2/1484 real-time PCR works as a rapid indicator of the presence of XDR-TB. Further studies are required to prove its value, together with *rpoB* genotypic screening, as a fast and reliable tool for the diagnosis of suspected XDR-TB.

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Transparency declarations

None to declare.

References

1 WHO. *Multidrug and Extensively Drug-Resistant TB (M/XDR-TB): 2010 Global Report on Surveillance and Response*. Geneva: WHO, 2010.

2 Suzuki Y, Katsukawa C, Tamaru A *et al*. Detection of kanamycin-resistant *Mycobacterium tuberculosis* by identifying mutations in the 16S rRNA gene. *J Clin Microbiol* 1998; **36**: 1220–5.

3 Alangaden GJ, Kreiswirth BN, Aouad A *et al*. Mechanism of resistance to amikacin and kanamycin in *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother* 1998; **42**: 1295–7.

4 Maus CE, Plikaytis BB, Shinnick TM. Molecular analysis of cross-resistance to capreomycin, kanamycin, amikacin, and viomycin in *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother* 2005; **49**: 3192–7.

5 Maus CE, Plikaytis BB, Shinnick TM. Mutation of *tlyA* confers capreomycin resistance in *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother* 2005; **49**: 571–7.

6 Feuerriegel S, Cox HS, Zarkua N *et al*. Sequence analyses of just four genes to detect extensively drug-resistant *Mycobacterium tuberculosis* strains in multidrug-resistant tuberculosis patients undergoing treatment. *Antimicrob Agents Chemother* 2009; **53**: 3353–6.

7 Evans J, Segal H. Novel multiplex allele-specific PCR assays for the detection of resistance to second-line drugs in *Mycobacterium tuberculosis*. *J Antimicrob Chemother* 2010; **65**: 897–900.

8 Krüüner A, Jureen P, Levina K *et al*. Discordant resistance to kanamycin and amikacin in drug-resistant *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother* 2003; **47**: 2971–3.

9 Perdigão J, Macedo R, Malaquias A *et al*. Genetic analysis of extensively drug-resistant *Mycobacterium tuberculosis* strains in Lisbon, Portugal. *J Antimicrob Chemother* 2010; **65**: 224–7.

10 Hillemann D, Rüsch-Gerdes S, Richter E. Feasibility of the GenoType MTBDR*sl* assay for fluoroquinolone, amikacin-capreomycin, and ethambutol resistance testing of *Mycobacterium tuberculosis* strains and clinical specimens. *J Clin Microbiol* 2009; **47**: 1767–72.