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Evaluation of Molecular-Beacon, TaqMan, and Fluorescence Resonance Energy Transfer Probes for Detection of Antibiotic Resistance-Conferring Single Nucleotide Polymorphisms in Mixed *Mycobacterium tuberculosis* DNA Extracts

Hasan Yesilkaya,[1] Francesca Meacci,[2] Stefan Niemann,[3] Doris Hillemann,[3] Sabine Rüsch-Gerdes,[3] LONG DRUG Study Group,† Michael R. Barer,[1] Peter W. Andrew,[1]* and Marco R. Oggioni[2]

Department of Infection, Immunity and Inflammation, University of Leicester, Leicester LE1 9HN, United Kingdom[1]; Laboratorio di Microbiologia Molecolare e Biotecnologia, Dipartimento di Biologia Molecolare, Universit à di Siena Policlinico Le Scotte (lotto 5, piano 1), 53100 Siena, Italy[2]; and National Reference Center for Mycobacteria, Forschungszentrum, Borstel, Germany[3]

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The ability of fluorescence resonance energy transfer, molecular-beacon, and TaqMan probes to detect single nucleotide polymorphism (SNP) in the presence of a wild-type allele was evaluated using drug resistance-conferring SNPs in mixed *Mycobacterium tuberculosis* DNA. It was found that both the absolute quantity and the ratio of alleles determine the detection sensitivity of the probe systems.

Several studies have shown that single nucleotide polymorphisms (SNPs) in *Mycobacterium tuberculosis* confer resistance to rifampin and isoniazid (5, 15). Ninety-five percent of SNPs giving resistance to rifampin localized in a 100-bp region of *rpoB* (5, 14). Mutation at codon 315 of *katG* has been reported in a very high percentage of isoniazid-resistant isolates (1, 7, 12). Even in drug-free environments, rifampin resistance occurs at an estimated rate of 1 in 10^8 bacilli, while isoniazid resistance arises in approximately 1 in 10^6 bacilli (10). Consequently, mixed populations of resistant and susceptible organisms may occur even when the apparent phenotype of the culture is susceptible (14). Under selective pressure, while the susceptible bacilli are gradually eliminated, the drug-resistant bacilli multiply and become the dominant entity in the population.

Culture-based tests for drug susceptibility in *M. tuberculosis* may be insensitive in detection of mixed populations, and they are time-consuming (3). Furthermore, processing of clinical specimens may have a negative impact on the viability of the few resistant organisms that may be present (18). Hence, a sensitive and swift molecular method for detecting low numbers of resistant bacilli in the backdrop of drug-sensitive ones is desirable, as an inappropriate course of therapy and delayed follow-up of susceptibility tests in heteroresistance cases could permit additional resistance to develop (13).

* Corresponding author. Mailing address: Department of Infection, Immunity and Inflammation, University of Leicester, Leicester LE1 9HN, United Kingdom. Phone: (44) 116 2522941. Fax: (44) 116 2525030. E-mail: pwa@le.ac.uk.

† The LONG DRUG study group is composed of Marco R. Oggioni and Francesca Meacci, C Trappetti Università di Siena; Francesco Checchi and Maryline Bonnet, Epicentre Paris; Graziella Orefici, Manuela Pardini, and Lanfranco Fattorini, Istituto Superiore di Sanità Roma; Peter Andrew, Mike Barer, and Hasan Yesilkaya, University of Leicester; Heinz Rinder, University of München; Sabine Rüsch-Gerdes and Stefan Niemann, Research Centre Borstel; Germano Orrù, Università di Cagliari; Francis Varaine, Médecins Sans Frontières Paris; and Thierry Jarosz, 3Es Paris.

Our aim was to investigate whether or not resistance-conferring SNPs could be identified in a rapid and efficient fashion in the presence of sensitive alleles. Real-time detection chemistries have been reported as specific and sensitive assays of SNPs. However, while many studies tested their utility in homogenous cultures or DNA solutions (2, 4, 9, 16), data on their sensitivity and specificity in detecting a single allele difference in heterogeneous DNA samples are sparse. Hence, we evaluated the usefulness of molecular-beacon, TaqMan, and fluorescence resonance energy transfer (FRET) probes in detecting resistance-conferring SNPs in the presence of the wild-type allele and vice versa.

A pair of 6-carboxyfluorescein (FAM)-labeled molecular beacons, L531mt+ and S531wt+ (Table 1), synthesized by TIB MOLBIOL (Berlin, Germany), targeted either the rifampin resistance (TTG) or wild-type (TCG) allele at codon 531 of *rpoB*. The 20-μl reaction mixture contained 20 μM molecular beacon, 2 mM Mg^{2+}, 20 μM concentrations each of the MARF and MARR primers (Table 1), and 10 μl 2× HotStarTaq DNA polymerase kit reagents (QIAGEN, West Sussex, United Kingdom). As a source of template, threefold dilutions of 3.4 ng/μl wild-type *M. tuberculosis* H37Rv chromosomal DNA and of 2.13 ng/μl of chromosomal DNA of a clinical strain, designated 53-7, that was resistant to all first-line but susceptible to all second-line antimycobacterial drugs and with an SNP at codon 531 of *rpoB* (TCG to TTG), mixed in various ratios, were used (Table 2). The isolate was obtained from a patient who had been treated with isoniazid, rifampin, streptomycin, ethionamide, and pyrazinamide. A Stratagene MX4000 cycler was used for the reactions, and the following were the settings for PCR: 15 min at 95°C enzyme activation followed by 40 cycles of 95°C for 30 s, 60°C for 1 min, and 72°C for 30 s. The experiment with each probe was repeated at least four times, and the cycle number at which the signal was above the threshold fluorescence (C_T) was determined.

The TaqMan minor groove binder (MGB) probes (Table 1) were purchased from ABI (Weiterstadt, Germany) and tar-

TABLE 1. Primers and probes used in this study

Probe or primer	Oligonucleotide[a]	Position in H37Rv genome
L531mt+	6-FAM-<u>CCTGG</u>AGCCGACTGTT<u>T</u>GGCGCTGT<u>CCAGG</u>-DABCYL	761149–761161
S531wt+	6-FAM-<u>CGACC</u>GACTGTCGGCGCT<u>GGTCG</u>-DABCYL	761149–761161
MARF	GGCCGGTGGTCGCCGCG	761050–761066
MARR	ACGTGACAGACCGCCGGGC	761165–761183
TaqMan MGB mt+	FAM-CGATGCCGCTGGT-TAMRA	2155160–2155172
TaqMan MGB wt+	JOE-CGATGCCGGTGGT-TAMRA	2155160–2155172
katG-F	TGCTCCGCTGGAGCAGAT	2155225–2155242
katG-R	TCGAGGAAACTGTTGTCCCATT	2155111–2155132
OG76	CCCCGAACCCGAGGCT	2155242–2155257
OG77	AACGGGTCCGGGATGGT	2155009–2155025
OG78	LCred705-CCTCGATGCCGCTGGTGAT	2155157–2155175
OG79	FI-TTTCGTCGGGGTGTTCGTCCATAC	2155131–2155154
OG84	AGTTCTTCGGCACCA	761074–761088
OG85	CACATCCGGCCGTAG	761225–761239
OG86	LCred640-ACTGTCGGCGCTGGG	761150–761164
OG87	FI-GGTTGACCCACAAGCGCC	761131–761148

[a] The sequences not homologous to the target in the molecular beacons are underlined. TAMRA, 6-carboxytetramethylrhodamine; DABCYL, 4-dimethylaminophenylazobenzoic acid; FI, fluorescein.

geted either the isoniazid resistance allele (ACC) or the wild-type allele (AGC) of codon 315 of *katG* (7). The 20-μl reaction mixture contained 10 μM probes, 20 μM concentrations each of *katG*-F and *katG*-R primers (Table 1), and 1× commercial ready-to-use PCR reagents (Roche, United Kingdom). As a source of template, threefold dilutions of 3.4 ng/μl wild-type *M. tuberculosis* H37Rv chromosomal DNA and of 2.13 ng/μl of chromosomal DNA of clinical strain 53-7, which carries an SNP at codon 315 of *katG* (ACC to AGC), mixed in various ratios, were used (Table 3). By mixing the two probes in equimolar ratios, the assay became a multiplex with two fluorophores producing emission maxima at separate wavelengths (FAM at 520 nm for the mutant and JOE [dichlorodimethoxyfluorescein] at 555 nm for the wild-type allele), allowing simultaneous detection of wild-type and mutant alleles in the same reaction. Real-time PCR analyses were performed in a Corbett Research Rotor Gene 2000, and the parameters for cycling were 94°C for 10 min followed by 40 cycles of 92°C for 15 s and 60°C for 1 min.

FRET probes (Table 1) targeted either a 19-bp-long region in *katG* encompassing codon 315 with the OG78 probe and OG79 anchor or targeted a 15-bp mutation hot spot in the *rpoB* gene with the OG86 probe and OG87 anchor. The probes were designed on the wild-type sequence; thus, the presence of the mutated allele was distinguished by the difference in the melting temperatures of amplified products. The real-time PCR was done essentially as described previously (11), using amplification products of the OG76 and OG77 primers for *katG* and the OG84 and OG85 primers for *rpoB* targets (Table 1). The 20-μl reaction mixture contained 10 pmol of primers, 2 pmol of FRET probes, 4 mM $MgCl_2$, 1× master hybridization probes reaction mixture (Roche Diagnostics, United Kingdom), and the dilutions of 3.4 ng/μl chromosomal wild-type DNA and 2.13 ng/μl mutated DNA, with SNPs in either

TABLE 2. Performance of molecular-beacon probes for the detection of a specific allele of *rpoB* in heterogeneous DNA solution

Beacon probe and mutant DNA concn (ng/μl)	C_T value with wild-type DNA at a concn (ng/μl) of:							
	3.4	1.13	0.38	0.126	0.042	0.014	0.0047	0
Wild type specific								
2.13	24.5	27.33						
0.71	24.6	26.46	28.01	29.5				
0.24	24.25	26.45	27.65	30.05	32.1			
0.079	25.03	26.3	28.2	29.81	32.4	33.5		
0.026	25.15	26.19	28.05	29.71	32.9	33.76	36.68	
0.0087	24.49	26.9	29.26	29.72	31.46	33.33	36.51	
0.0029	25.67	27.15	28.41	29.98	32.33	32.7	36.18	
0	25.28	27.28	29.03	30.1	32.3	33.94	36.79	
Mutant specific								
2.13	27.34	26.23	26.98	27.3	27.01	26.54	27.24	27.07
0.71	30.26	30.21	30.21	29.89	29.61	29.82	29.95	29.89
0.24		31.41	31.28	31.33	31.37	31.19	30.91	31.55
0.079				32.86	32.62	33.25	33.01	32.4
0.026				34.08	34.46	34.93	34.91	34.77
0.0087								36.45
0.0029								38.20
0								

TABLE 3. Performance of TaqMan probes for the detection of a specific allele of *katG* in heterogeneous DNA solution

TaqMan probe and mutant DNA concn (ng/μl)	C_T value with wild-type DNA at a concn (ng/μl) of:							
	3.4	1.13	0.38	0.126	0.042	0.014	0.0047	0
Wild type specific								
2.13	23.93	27.27	27.29					
0.71	23.67	26.96	28.64	36.71				
0.24	24.65	25.39	28.08	32.94	37.84			
0.079	23.66	26.04	28.36	32.37	36.87	37.71		
0.026	22.53	25.96	27.36	32.11	36.06	38.22		
0.0087	24.69	27.02	27.97	31.8	36.29	37.71	38.64	
0.0029	25.1	27.54	29.44	32.11	35.66	37.15	38.96	
0	22.03	27.25	27.87	32	35.99	37.92	38.8	
Mutant specific								
2.13	27.09	27.66	28.85	27.53	27	27.72	26.91	28.39
0.71	29.5	29.74	29.12	31.37	30.09	29.65	28.64	30.24
0.24	31	31	29.6	31.45	35.21	35.07	33.66	35.03
0.079		32	33	38.03	36.99	36.54	36.09	36.7
0.026					38.45	38.5	38.89	37.59
0.0087						39.84	39.28	38.14
0.0029								39.63
0								

the *rpoB* gene or the *katG* gene as described above. The PCR cycling parameters consisted of an initial denaturation step at 95°C for 30 s, followed by 40 cycles of repeated denaturation (0 s at 95°C), annealing (10 s at 57°C), and polymerization (10 s at 72°C). The temperature transition rate was 20°C/s in all segments. In the final cycle, the melting curve was determined in the samples by initial heating to 95°C, cooling to 45°C, and subsequent controlled heating to 95°C with a temperature transition rate of 0.1°C/s.

Molecular-beacon and TaqMan probes. The results with molecular-beacon and TaqMan probes showed that when only a single mutant or wild-type allele was present, the C_T value decreased with an increasing amount of initial template quantity, as expected. An amplification signal could be obtained with the lowest template quantity that was tested, which was approximately 3 to 5 pg for both alleles, with either probe system (Tables 2 and 3). When both alleles were present in the reaction, the detection ability was determined first by the amount of each allele and second by the ratio of alleles, irrespective of the probe system (Tables 2 and 3). Regardless of whether a second allele was present, detection of both mutant and wild-type alleles was achieved consistently with both methodologies when the reaction mixtures contained between 0.126 and 3.4 ng DNA for the wild-type allele and 0.079 and 2.13 ng DNA for the mutant allele (Tables 2 and 3). In 16 reactions with each method, DNA ratios ranged from 1 to 43, and in 14 reactions both alleles could be detected, provided the ratios of the alleles did not exceed 1:5.6 (Table 4). The only exception to this was the detection of a 1:14 ratio with the TaqMan probe (Tables 2 and 4).

With both systems, in mixtures containing low quantities of templates (0.0047 to 0.042 ng of the wild-type allele and 0.0029 to 0.026 ng of the mutant allele), both alleles could be detected provided the ratio of alleles did not exceed 1:4.8 (Tables 2, 3, and 4). In all other cases, only the higher-quantity allele could be detected (Tables 2, 3, and 4). This shows that the ratio of the alleles adversely affected the detection ability of both probe systems.

The presence of a second allele did not influence the specificity of probe systems. This was evident from the fact that similar C_T values were obtained for mixed and homogenous reactions containing the same quantity of target DNA. For example, while the C_T value of reactions containing 3.4 ng wild-type allele and up to 2.13 ng mutant allele ranged from

TABLE 4. Summary of detection of specific SNPs in heterogeneous DNA samples by TaqMan and molecular-beacon probes

Quantity of mutant DNA (ng/μl)	Detection of SNPs with wild-type DNA at a concn (ng/μl) of[a]:							
	3.4	1.13	0.38	0.126	0.042	0.014	0.0047	0
2.13	**MIX**	**MIX**	**MIX**[2]	MT	MT	MT	MT	MT
0.71	**MIX**	**MIX**	**MIX**	**MIX**	MT	MT	MT	MT
0.24	**MIX**[1]	**MIX**	**MIX**	**MIX**	**MIX**	MT	MT	MT
0.079	WT	**MIX**[1]	**MIX**	**MIX**	**MIX**	**MIX**	MT	MT
0.026	WT	WT	WT	**MIX**[3]	**MIX**	**MIX**	**MIX**[3]	MT
0.0087	WT	WT	WT	WT	WT	**MIX**[1]	**MIX**	MT
0.0029	WT	WT	WT	WT	WT	WT	WT	MT
0	WT	WT	WT	WT	WT	WT	WT	NEG

[a] The reactions where both alleles could be detected by both TaqMan and molecular-beacon probes are in boldface. WT, wild-type allele; MT, mutant allele; MIX, mixed mutant and wild-type alleles; NEG, no amplification. The superscript numerals indicate the following: 1, the molecular-beacon probe detected only the WT allele; 2, the molecular-beacon probe detected only the MT allele; and 3, the molecular-beacon probe detected both alleles.

25.6 to 24.5, the C_T value of reactions containing only 3.4 ng wild-type DNA was 25.2 (Table 2). The differences in observed C_T values were not statistically significant ($P > 0.05$).

FRET probes. When the chromosomal DNA mixtures were tested with FRET probes, no amplification signal could be detected whatever the quantity or ratio, even though in reactions containing solely wild-type or mutant allele successful amplification was obtained with as little as 3 to 5 pg for both alleles. This could be due to interference by high-molecular-weight nonspecific chromosomal DNA. Indeed, it was only after a preamplification step that the detection of either allele alone could be achieved to a sensitivity similar to that of the molecular-beacon and TaqMan probes.

The diagnostic value of SNP analysis in the presence of a wild-type DNA is good not only for speedy detection of drug-resistant *M. tuberculosis* but also in nonmicrobiological situations, for example, the diagnosis of SNPs associated with biologically significant phenotypic traits, such as cancer and mitochondrial diseases (6, 17). Although several methods are available for SNP detection in mixed DNA samples, for example, bioluminometric assay coupled to modified primer extension reaction and DNA microarray technology (8), the requirement for multiple procedures and the costs limit their routine use. In this study, we demonstrated the use of fluorogenic allele-specific detection probes that allow PCR amplification and allele detection in a single procedure. It was shown that molecular-beacon and TaqMan probes can specifically detect individual alleles in a mixture, but this was governed by the ratio of the alleles as well as the absolute quantity of each allele. We found that FRET probes can also reach the detection level of molecular beacons and TaqMan probe systems but only after target enrichment by primary PCR, suggesting that it may be a less useful technique here.

The implication of our results is that these techniques can be used for detection of heteroresistance earlier than is possible with culture. However, the requirement to design individual probes for each SNP increases the costs. Consequently, beacon and TaqMan probes may be best suited for detecting commonly encountered SNPs, such as those in codon 315 of *katG* or codon 531 of the *rpoB* gene. On the other hand, although FRET probes required two rounds of PCR amplification, they are ideal for initial screening of multiple SNPs and they are more cost efficient for high-throughput screening. Preliminary data on field application of these three approaches indicate a detection limit near that of phenotypic drug susceptibility testing and hence their usefulness as molecular tools.

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REFERENCES

1. **Bakonyte, D., A. Baranauskaite, J. Cicenaite, A. Sosnovskaja, and P. Stakenas.** 2003. Molecular characterization of isoniazid-resistant *Mycobacterium tuberculosis* clinical isolates in Lithuania. Antimicrob. Agents Chemother. **47:**2009–2011.
2. **Bellete, B., P. Flori, J. Hafid, H. Raberin, and R. Tran Manh Sung.** 2003. Influence of the quantity of nonspecific DNA and repeated freezing and thawing of samples on the quantification of DNA by the Light Cycler. J. Microbiol. Methods **55:**213–221.
3. **Canetti, G., W. Fox, A. Khomenko, H. T. Mahler, N. K. Menon, D. A. Mitchison, N. Rist, and N. A. Smeley.** 1969. Advances in techniques of testing mycobacterial drug sensitivity, and the use of sensitivity tests in tuberculosis control programmes. Bull. W. H. O. **41:**21–43.
4. **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.
5. **Garcia de Viedma, D.** 2003. Rapid detection of resistance in *Mycobacterium tuberculosis*: a review discussing molecular approaches. Clin. Microbiol. Infect. **9:**349–359.
6. **Gibson, N. J.** 2006. The use of real-time PCR methods in DNA sequence variation analysis. Clin. Chim. Acta **363:**32–47.
7. **Hillemann, D., T. Kubica, R. Agzamova, B. Venera, S. Rüsch-Gerdes, and S. Niemann.** 2005. Rifampicin and isoniazid resistance mutations in *Mycobacterium tuberculosis* strains isolated from patients in Kazakhstan. Int. J. Tuber. Lung Dis. **9:**1161–1167.
8. **Kwok, P. Y., and X. Chen.** 2003. Detection of single nucleotide polymorphisms. Curr. Issues Mol. Biol. **5:**43–60.
9. **Lin, S. Y., W. Probert, M. Lo, and E. Desmond.** 2004. Rapid detection of isoniazid and rifampin resistance mutations in *Mycobacterium tuberculosis* complex from cultures or smear-positive sputa by use of molecular beacons. J. Clin. Microbiol. **42:**4204–4208.
10. **Loddenkemper, R., D. Sagebiel, and A. Brendel.** 2002. Strategies against multidrug-resistant tuberculosis. Eur. Respir. J. Suppl. **36:**66s–77s.
11. **Meacci, F., G. Orru, E. Iona, F. Giannoni, C. Piersimoni, G. Pozzi, L. Fattorini, and M. R. Oggioni.** 2005. Drug resistance evolution of a *Mycobacterium tuberculosis* strain from a noncompliant patient. J. Clin. Microbiol. **43:**3114–3120.
12. **Mokrousov, I., O. Narvskaya, T. Otten, E. Limeschenko, L. Steklova, and B. Vyshnevskiy.** 2001. High prevalence of KatG Ser315Thr substitution among isoniazid-resistant *Mycobacterium tuberculosis* clinical isolates from northwestern Russia, 1996 to 2001. Antimicrob. Agents Chemother. **46:**1417–1424.
13. **Niemann, S., E. Richter, S. Rusch-Gerdes, M. Schlaak, and U. Greinert.** 2000. Double infection with a resistant and a multidrug-resistant strain of *Mycobacterium tuberculosis*. Emerg. Infect. Dis. **6:**548–551.
14. **Rattan, A., A. Kalia, and N. Ahmad.** 1998. Multidrug-resistant *Mycobacterium tuberculosis*: molecular perspectives. Emerg. Infect. Dis. **4:**195–209.
15. **Slayden, R. A., and C. E. Barry III.** 2000. The genetics and biochemistry of isoniazid resistance in *Mycobacterium tuberculosis*. Microbes Infect. **2:**659–669.
16. **Varma-Basil, M., H. El-Hajj, R. Colangeli, M. H. Hazbón, S. Kumar, M. Bose, M. Bobadilla-del-Valle, L. G. García, A. Hernández, F. R. Kramer, J. S. Osornio, A. Ponce-de-León, and D. Alland.** 2004. Rapid detection of rifampin resistance in *Mycobacterium tuberculosis* isolates from India and Mexico by a molecular beacon assay. J. Clin. Microbiol. **42:**5512–5516.
17. **Vignal, A., D. Milan, M. SanCristobal, and A. Eggen.** 2002. A review on SNP and other types of molecular markers and their use in animal genetics. Genet. Sel. Evol. **34:**275–305.
18. **Yesilkaya, H., M. R. Barer, and P. W. Andrew.** 2004. Antibiotic resistance may affect alkali decontamination of specimens containing mycobacteria. Diagn. Microbiol. Infect. Dis. **50:**153–155.