Denaturing High-Performance Liquid Chromatography Detection of Ribosomal Mutations Conferring Macrolide Resistance in Gram-Positive Cocci

Annie Canu,^{1,2} Ahmed Abbas,³ Brigitte Malbruny,^{2,4} François Sichel,³ and Roland Leclercq^{2,4*}

UFR des Sciences Pharmaceutiques, Groupe Régional d'Etudes sur le Cancer, Université de Caen/Basse-Normandie,¹
Laboratoire de Cancérologie Expérimentale, Centre François Baclesse,³ Service de Microbiologie, Hôpital Côte
de Nacre, UFR de Médecine,⁴ and EA2128 Immunopathologie et infection,² Caen, France

Received 11 September 2003/Returned for modification 25 September 2003/Accepted 3 October 2003

Mutations in genes coding for L4 (*rplD*) or L22 (*rplV*) ribosomal proteins or in 23S rRNA (*rrl* gene) are reported as a cause of macrolide resistance in streptococci and staphylococci. This study was aimed at evaluating a denaturing high-performance liquid chromatography (DHPLC) technique as a rapid mutation screening method. Portions of these genes were amplified by PCR from total DNA of 48 strains of *Streptococcus pneumoniae* (*n* = 22), *Staphylococcus aureus* (*n* = 16), *Streptococcus pyogenes* (*n* = 6), *Streptococcus oralis* (*n* = 2), and group G streptococcus (*n* = 2). Thirty-seven of these strains were resistant to macrolides and harbored one or several mutations in one or two of the target genes, and 11 were susceptible. PCR products were analyzed by DHPLC. All mutations were detected, except a point mutation in a pneumococcal *rplD* gene. The method detected one mutated *rrl* copy out of six in *S. aureus*. This automated method is promising for screening of mutations involved in macrolide resistance in gram-positive cocci.

Mutations affecting sites involved directly (23S rRNA) or indirectly (ribosomal proteins L22 and L4) in ribosomal binding of macrolides have been reported in recent years as an increasing cause of resistance to these antimicrobial agents (1, 19, 23, 25). The importance of 23S rRNA mutation was first recognized for organisms with only one or two copies of the *rrl* genes encoding this structure, such as *Mycobacterium avium* and *Helicobacter* (31). Since then, target mutations have been found in a variety of gram-positive cocci with several rRNA operons, including *Staphylococcus aureus* and *Streptococcus pneumoniae* (16, 23, 25, 31). Most of these mutations are localized in domains V and II of 23S rRNA. In particular, mutations at positions 2058 and 2059 in domain V (*Escherichia coli* numbering) are reported for a great number of bacterial species (31). Other mutations, at positions 2611, 2610, and 2062 (domain V) and at position 752 (domain II), have also been described (31). Mutations in the *rplD* and *rplV* genes encoding ribosomal proteins L4 and L22, respectively, have also been detected in clinical isolates of pneumococci that are resistant to macrolides in various countries (19, 21, 23, 30), as have *rplD* mutations in clinical isolates of *Streptococcus pyogenes*, group G, and oral streptococci (1, 17; A. Canu, B. Malbruny, M. Slaoui, M. Coquemont, X. Haristoy, A. Lozniewski, and R. Leclercq, Abstr. 41st Intersci. Conf. Antimicrob. Agents Chemother., abstr. C1-1815, 2001). In staphylococci, mutations in 23S rRNA and ribosomal proteins have been pointed out as the major mechanism of resistance to macrolides in strains isolated in patients with cystic fibrosis (25, 26). Characterization of mutations in strains resistant to macrolides involves intensive labor and time-consuming procedures, since

it requires sequencing of several ribosomal targets of the macrolides, including the *rplD*, *rplV*, and *rrl* genes. These techniques are unsuitable for rapid analysis of macrolide resistance mechanisms in strain series. This is the reason why rapid methods of screening, such as single-strand conformational polymorphism, have been proposed for the detection of ribosomal mutations (1, 2, 17, 25). When a mutation is suspected with use of this technique, results should be confirmed by sequencing the relevant gene region.

A technique recently developed in human genetic studies, denaturing high-performance liquid chromatography (DHPLC), allows the rapid automated detection of single base substitutions as well as small insertions or deletions, employing a combination of temperature-dependent denaturation of DNA and ion pair chromatography (5, 6, 12, 22). DHPLC has been used in its early stages for bacterial identification, typing, and molecular epidemiology and has been recently developed for the analysis of resistance to quinolones in laboratory strains of *S. aureus* and *Yersinia pestis* and clinical isolates of *Salmonella* (3, 4, 7, 8, 9, 28).

We applied this technique to a collection of 48 strains of streptococci and staphylococci, 37 of which harbored previously characterized mutations representative of the different types found in gram-positive strains (1, 2, 17, 18, 25, 31; Canu et al., 41st ICAAC).

MATERIALS AND METHODS

Bacterial strains. Forty-eight laboratory strains and clinical isolates belonging to five species, *S. aureus* (*n* = 16), *S. pneumoniae* (*n* = 22), *S. pyogenes* (*n* = 6), *Streptococcus oralis* (*n* = 2), and group G streptococcus (*n* = 2), were analyzed in this study (1, 2, 17, 18, 25; Canu et al., 41st ICAAC). Thirty-seven of these strains harbored one or several mutations in one or two genes which have been identified by sequencing. Mutations included point mutations, deletions, and insertions and are listed in Tables 1 and 2. Eleven susceptible strains were used as controls.

Portions of the *rplD*, *rplV*, and *rrl* genes encoding the entire ribosomal proteins

* Corresponding author. Mailing address: Roland Leclercq, CHU de Caen, Service de Microbiologie, Avenue Côte de Nacre, 14033 Caen cedex, France. Phone: (33) 2 31 06 45 72. Fax: (33) 2 31 06 45 73. E-mail: leclercq-r@chu-caen.fr.

TABLE 1. Mutations in the *rplD* and *rplV* genes encoding L4 and L22 ribosomal proteins, respectively, in gram-positive cocci analyzed by DHPLC

Species	No. of mutated strains	Mutations ^a		Reference
		Type of L4 mutation ^b	Type of L22 mutation ^b	
<i>S. aureus</i>	2	None	¹⁰⁰ SAINKRT (i)	18
	1	None	⁹⁷ KTIHITI (i)	18
	1	None	⁸³ GPTLK (i)	18
	1	None	⁷⁸ GP (d)	18
	1	None	¹⁰⁰ SAINKRT (i) + ⁹⁷ KR (d)	18
<i>S. pneumoniae</i>	1	G71R	G95D	2
	2	None	G95D	2
	1	None	P99Q	2
	1	None	A93E	2
	1	None	A93E, P91S, G83E	2
<i>S. oralis</i>	1	⁷⁰ RREKGTG (i)	None	Canu et al., 41st ICAAC
<i>S. pyogenes</i>	1	⁶⁹ KG (i)	None	17
	1	⁷² RA (i)	None	1
	1	⁶³ WR (d)	None	1
	1	⁶⁸ TG (d)	None	1
Group G streptococcus	1	⁷¹ EGTGR (i)	None	Canu et al., 41st ICAAC

^a (i), insertion; (d), deletion.^b *E. coli* numbering; for insertions and deletions, the number of amino acid after which the insertion or the deletion occurred is shown.

L4 and L22 and domains II and V of 23S rRNA, respectively, were amplified from total DNA of each strain as described previously (2, 17, 18, 25) (Table 3). For *rml* genes, several copies are present in bacteria (four in *S. pneumoniae*, five or six in *S. aureus*, and six in *S. pyogenes*). For *S. pneumoniae* and *S. pyogenes* each individual copy was amplified as described previously and analyzed (2, 25, 30). Overall, 80 PCR products were obtained for DHPLC analysis.

DHPLC. DHPLC screening for DNA mutation was undertaken on the Helix Varian system (Varian, les Ullis, France). Injection was performed by an autosampler, model 430. The column was a 510 Pro-star model; the solvent delivery module was a Pro-star 210 model; and the UV detector was a 310 Pro-star model.

The hydrophobic stationary phase is composed of a polystyrene-divinyl benzene copolymer coated with a positively charged ion-pairing agent, triethyl ammonium acetate (TEAA), which mediates the binding of DNA. Removal of the DNA from TEAA-coated beads is dependent upon the mobile organic phase in the form of a linear acetonitrile gradient. In this method, wild-type and mutant

crude PCR products are mixed in an approximately equimolar ratio, heated to denature each strand, and then allowed to cool slowly (reannealing) until they form a mixed population of homoduplexes plus heteroduplexes containing the mismatched bases. Under conditions of partial denaturation temperature, the heteroduplexes elute from the column earlier than the homoduplexes because of their reduced melting temperature (22).

Five microliters of crude PCR products from each mutant and wild-type DNA were mixed. Each type of PCR product of wild-type sample was analyzed alone as a reference. All samples were heated at 94°C and cooled slowly to 55°C to form heteroduplexes before being loaded on the column. Each sample was analyzed at two or three temperatures as described below.

The gradient was created by mixing buffers A (100 mM TEAA plus 0.1 mM EDTA [pH 7]) and B (100 mM TEAA plus 0.1 mM EDTA plus 25% [vol/vol] acetonitrile [pH 7]). Buffer B gradient increased from 45 to 50% in 30 s and from 50 to 68% in 5 min 30 s and finally remained constant for 1 min. The constant flow rate was 0.45 ml/min. This gradient allows separation of fragments based on presence, number, and/or size of heteroduplexes.

RESULTS

Determination of DHPLC conditions. DHPLC data analysis is based on a qualitative comparison of peak number and/or shape of a single peak between the sample and reference chromatograms and therefore is optimal if all heteroduplexes patterns are clearly distinguishable from wild-type homoduplexes (reference patterns) (14, 22, 29). The resolution of homo- and heteroduplexes depends mostly on the temperature of partial denaturation. So, the temperature at which the samples are run is the critical parameter of the procedure, and previous studies have specified the necessity of analyzing the samples at different temperatures to optimize the mutation detection conditions (24, 27). We determined the optimal temperature in two steps. First, the predicted temperature (T_p) (temperature prediction software) required to separate homo- and heteroduplexes was given by the DHPLCMelt program (<http://insertion.stanford.edu/melt.html>),

TABLE 2. Mutations in *rml* gene (23S rRNA) in gram-positive cocci analyzed by DHPLC

Species	Mutation	No. of wild-type copies/no. of mutated copies	No. of strains	Reference
<i>S. aureus</i>	A2058T	1A/4T	1	25
	A2058G	1A/4G	3	25
	A2058G	2A/4G	1	25
	A2059G	2A/3G	1	25
<i>S. pneumoniae</i>	A752 (deletion)	0/4	1	2
	A2058T	1A/3T	2	2
	A2058G	1A/3G	1	2
	A2059G	0A/4G	1	2
	C2610T	2C/2T	1	2
	C2611T	2C/2T	2	2
	C2611T	0C/4T	2	2
	C2611A	1C/3A	1	2
<i>S. pyogenes</i>	C2611T	0C/6T	1	17

TABLE 3. PCR products tested and column temperature conditions proposed by the DHPLCMelt program (<http://insertion.stanford.edu/melt.html>) and optimized by melting curve analysis

Gene (rRNA or ribosomal protein) or position	Species	Primer sequences (5' to 3' as synthesized)	PCR product size (bp)	T_p^a (°C)	O_pT^b (°C)
<i>rfl</i> (domain II) 578–850 ^c	<i>S. pneumoniae</i>	CGGCGATTACGATATGATGCC TCTAATGTCGACGCTAGCC	273	61	60–61
<i>rfl</i> (domain V) 1990–2134 ^c	<i>S. pneumoniae</i>	CTGTCTCAACGAGAGACTC CTTAGACTCCTACCTATCC	144	60	60–61
1990–2404 ^c	<i>S. pneumoniae</i>	CTGTCTCAACGAGAGACTC GGAACCACCGGATCACTAAG	416	61	60–61
	<i>S. aureus</i>	Same set as for <i>S. pneumoniae</i>	416	61	61–62
2331–2769 ^c	<i>S. pneumoniae</i>	GTATAAGGGAGCTTGACTG GGTTTCACACTTA GATG	439	62	62
	<i>S. pyogenes</i>	Same set as for <i>S. pneumoniae</i>	439	62	62
<i>rplD</i> (L4) + 148 + 567 ^d	<i>S. pneumoniae</i>	GCTGTAAAAACCGCTCTG GCTATTTGCGATGTCAAG	420	61	60–63
–93 + 385 ^d	<i>S. pneumoniae</i>	AAAGGTAACGTACCAGGTGC GAGCGTCTACAGCTACG	478	55–60	55–64
	<i>S. oralis</i>	Same set as for <i>S. pneumoniae</i>	478	58	58
–51 + 400 ^d	<i>S. pyogenes</i>	CAAGTCAGCAGTTAAAGCTGC GGTGCTGCAAATGAAAGGCC	451	55–60	58
	Gr G streptococcus ^e	Same set as for <i>S. pyogenes</i>	451	55–60	58
<i>rplV</i> (L22) –41 + 396 ^d	<i>S. pneumoniae</i>	GCAGACGACAAGAAAACACG GCCGACACGCATACCAATTG	437	59	59–60
–68 + 388 ^d	<i>S. aureus</i>	CAAAGGACACGTTGCAGACGACAAGAAA ATTTTTTGACCCACAAGTATTCCTCCTT	459	58	58–59

^a T_p , temperature recommended (DHPLCMelt program).^b O_pT , optimal temperature (melting curve).^c *E. coli* numbering.^d Base relative to ATG.^e Group G streptococcus.

which predicts the temperature on the basis of the PCR fragment composition. Then, other temperatures were tested as follows. Wild-type samples were run at a series of 10 column temperatures on the range of 5 degrees over and 5 degrees under the T_p as described previously (27). For each type of mutation, the chosen temperature was that at which the retention time decreased significantly, corresponding to the 75% DNA denaturation point (11). In practice, each sample was analyzed at the T_p and at one or two consecutive temperatures defined by the melting curve to achieve greater sensitivity (Table 3). In order to assess the reproducibility of the elution profiles, the *rfl* gene fragment containing the adenine residue 2058 from the wild-type *S. aureus* sample was tested at the same temperature conditions in four independent assays. All the obtained chromatograms were similar, confirming the excellent reproducibility of the method (data not shown). However, to control the column stability, we included systematically in each run a negative control from a wild-type strain and a positive control from a strain with a known mutation.

***rplV* mutations.** For the 22 *S. pneumoniae* PCR products analyzed, the five strains containing a single point mutation were more easily detected at the optimal temperature of 60°C than at the T_p (59°C), as shown in Fig. 1A. All samples containing the same single base substitution had identical profiles. The only strain which harbored a triple mutation had a specific profile that was clearly detected at the T_p (59°C) and at 60°C. The six *S. aureus* mutants which contained an insertion or/and deletion were detected at both tested temperatures 58 and 59°C without association of a specific profile with an insertion or deletion (Fig. 1B).

***rplD* mutations.** All insertions and deletions in the *rplD* genes from *S. oralis*, group G streptococcus, and *S. pyogenes* were detected at the same temperature, 58°C (Table 3). All the chromatograms profiles from mutated strains presented in Fig. 2 showed two distinct peaks without any possibility of differentiating insertions from deletions.

Only one *S. pneumoniae* point mutation (G211A) could not

FIG. 1. DHPLC analysis of PCR products with mutations located in the *rplV* gene. The temperature tested for each set of experiments is noted on the left. The entire *rplV* genes of the *S. pneumoniae* (A) and *S. aureus* (B) strains were amplified and analyzed by DHPLC as described in Materials and Methods. The profiles of mutants were compared to that of *S. pneumoniae* 3 (WT) at the predicted temperature, 59°C, and at the optimal temperature, 60°C. For *S. aureus*, the profiles of mutants were compared to that of *S. aureus* 740 (WT).

FIG. 2. DHPLC analysis of PCR products with mutations located in the *rplD* gene. The temperature tested for each set of experiments is noted on the left. A portion of the *rplD* gene of *S. pyogenes* (A), *S. oralis* (B), and group G streptococcus (C) strains was amplified and analyzed by DHPLC as described in Materials and Methods. The profiles of mutants were compared to that of the wild-type (WT) strains (*S. pyogenes* 11V1, *S. oralis* CIP 103216, and group G streptococcus UCN39).

FIG. 3. DHPLC analysis of PCR products with mutations in the *rrl* gene. The temperature tested for each set of experiments is noted on the left. Portions of the *rrl* genes were amplified and analyzed by DHPLC as described in Materials and Methods. For domain II (A), the profile of the *S. pneumoniae* mutant was compared to that of the wild type, *S. pneumoniae* 2 (WT). For domain V for the mutants at positions 2610 to 2611, the profiles of *S. pneumoniae* mutants were compared to that of the wild type, *S. pneumoniae* 2 (B); the profiles of the *S. pneumoniae* (C) and *S. aureus* (D) mutants at positions 2058 to 2059 were compared to that of the wild types, *S. pneumoniae* 2 and *S. aureus* 740, respectively. (E) Sensitivity of the DHPLC. PCR product containing the 2058 adenine residue from *S. aureus* ATCC 29213 was mixed with PCR product containing the mutated 2058 guanine residue from *S. aureus* UCN13 (25) in a ratio ranging from 0/6 to 6/6.

be differentiated from the wild type, although we tested two PCR products, of 478 and 420 bp, in the range of temperatures from 55 to 64°C for the first and 60 to 63°C for the second (Table 3). The elution peaks corresponding to the wild and mutated strains had the same elution retention time and were perfectly superimposable.

***rrl* mutations.** In the only strain of *S. pneumoniae* with a point deletion in domain II, the mutation was detected at T_p (61°C). Figure 3A shows a subtle change of shape without any shift in retention time when the wild-type and mutant chromatograms are overlaid. The patterns of chromatograms for all the other strains, devoid of mutation, were similar to that of the wild type. All T, G, or C domain V mutations at positions 2058 to 2059 in *S. aureus* and *S. pneumoniae* were detected at the T_p , 61°C. However, the wild-type profiles had a better resolution at the optimal temperature of 60°C. These results are presented Fig. 3C and D. Interestingly, for the totality of the mutants, two clearly separated elution peaks were obtained even for strains with 50% mutated copies.

All the mutations affecting the bases 2610 or 2611 were detected at the T_p of 62°C for *S. pyogenes* and *S. pneumoniae* PCR products and presented similar profiles. Figure 3B shows an example for an *S. pneumoniae* strain with a C2611T point mutation, where a single peak with a very small modification of shape and a modest shift in retention time could be observed (retention time peaks between 5.84 and 5.86 min for the wild types and between 5.76 and 5.77 min for the mutated strains).

To test if the DHPLC technique was sensitive enough to detect a single mutated copy in an *rrl* multicopy gene, we used a PCR product amplified from a single copy containing the mutated adenine residue 2058 from *S. aureus* as published previously (25). Since this microorganism possesses up to six copies of the *rrl* gene, the mutated PCR product (A2058G) was mixed with the wild-type PCR product from *S. aureus* ATCC 29213 in a range of ratios from 0/6 to 6/6 and analyzed to mimic the presence of one to six mutated copies in the sample. Figure 3E shows that one mutated copy was clearly detectable, showing that the method was highly sensitive for detection of heteroduplexes with only one mutated copy. It should be noted that the wild-type copies sample (6A/0G) and the sample that contained six mutated copies (0A/6G) presented similar profiles and that the DHPLC technique cannot be used to quantify the number of mutated copies.

Influence of fragment size. Previously reported experiments have suggested that for DHPLC analysis, the size of the PCR fragment should ideally be in the range of 150 to 700 bp (12, 15, 32). We achieved maximal sensitivity with fragments in the range of 270 to 480 bp. A 144-bp fragment that we previously used in single-strand conformational polymorphism analysis for the detection of A2058 and A2059 mutations in *S. pneumoniae* was not suitable for DHPLC (2). By contrast, amplification of a larger fragment of 416 bp in size allowed detection of point mutations at this position, confirming that the size of the amplified product plays an important role in DHPLC sensitivity.

Another advantage to use of short-length fragments comprised, on average, of between 150 and 500 bp is that the probability of misreading caused by the polymerase is low (13). In our study, we did not observe any false-positive result using a standard, non-proofreading, DNA polymerase.

DISCUSSION

We successfully applied a DHPLC technique to the detection of a variety of mutations affecting several genes implicated in macrolide resistance. In our standardization conditions, 97.2% (36 of 37) of mutations and all the wild-type controls were correctly detected, which is similar to results obtained in other studies on the human genome (10, 22). No false-positive results were noted.

As mentioned above, the choice of column temperature is critical to increasing the efficiency of mutation detection. The standardization with the DHPLC Melt program may underestimate the melting temperature, and some authors have proposed an arbitrary increase of predicted temperature by 2°C to detect mutations which could be missed at T_p (10, 24, 32). To determine the analysis temperature with the highest accuracy, we combined analyses at T_p and at the temperature deduced from melting curves (27). In our study, analysis at T_p resolved the mutations in most cases. In particular, all the types of point mutations affecting the *rrl* gene were detected. However, we obtained a sharper profile resolution for the *S. pneumoniae* mutations at positions 2058 to 2059 at the optimal temperature, 60°C (Fig. 3C). Similarly, although all insertions affecting the *rplV* gene were clearly detected at T_p , the point mutations in *S. pneumoniae* were more readily distinguished at a higher optimal temperature (Fig. 1A). These results confirmed the need to test the samples at several temperatures.

After analysis of about 1,500 to 2,000 samples, the column had to be changed, and minor differences in the chromatographic profiles might be observed for the same sample even under the same experimental conditions (24). To control the column stability, we introduced systematically before each assay a set of control samples corresponding to wild and identified mutated strains.

Despite this preliminary delicate standardization of the melting temperature, which is crucial for heteroduplex detection, automation of the method offers considerable advantages over classical techniques. The analysis is rapid (6 min per sample) and easy to perform because the procedure is fully automated after the introduction into the autosampler of the small-volume sample (5 μ l) of crude PCR products in the range from 270 to 480 bp. We obtained a sufficient sensitivity, since we have demonstrated that, for *S. aureus*, one mutated *rrl* copy mixed with five wild-type copies was detected. This result agrees with the report of the detection of a mutation in the human Y-chromosome DYS271 locus present at only 2% of the concentration of the wild-type DNA (14). In another report, detection was also successful with one variant in a pool of 20 alleles (32).

We used the same sets of primers for the detection of *rrl* gene mutations in three bacterial species. The primers were chosen as "universal" within highly conserved bacterial DNA sequences. Therefore, the technique could be applied to a large number of bacterial species. Since conserved regions can be identified throughout the entire *rrl* gene, it should be possible to apply the DHPLC method to the analysis of a variety of gram-positive microorganisms. Ten pairs of primers already designed to amplify overlapping fragments of the entire *rrl* genes of *S. aureus*, *S. pyogenes*, and *S. pneumoniae* should be tested (1).

It is not clear why the point mutation in the *S. pneumoniae* *rplD* gene was missed. Other detection failures were also reported, but the mutations not detected at the T_p were all found at the optimal temperature, except for one case, in which C→G and G→C transversions could not be discriminated successfully (5, 10, 22).

Since screening of mutations requires comparison of elution patterns between susceptible and resistant strains, the choice of the susceptible control is crucial. In an ideal situation, the wild-type and mutated sequences should differ by only the mutation responsible for resistance. This is the case for many types of mutational resistance, such as resistances to macrolide, quinolones, and rifampin, where mutations are located within highly conserved regions (4, 20). The susceptible control can thus be easily chosen after analysis of the bacterial genomes entirely sequenced. In the case where the targeted sequences are variable, the choice of the control strain might be difficult. We have found that the *rplV* genes of *S. pneumoniae* R6 (NC_003098.1), *S. pneumoniae* TIGR4 (NC_003028.1, <http://www.tigr.org>), and *S. pneumoniae* 670 (TIGR_189423) differed by only a C or a T at position 216. In both cases, the corresponding codon encoded an asparagine. Either nucleotide was also identified in five clinical strains susceptible to macrolides (2). Therefore, in the analysis of clinical strains, migration patterns suggestive of a mutation could be observed related to substitutions of nucleotide 216 but not to mutations involved in resistance. To overcome this drawback, the two alleles should be used as controls.

We conclude that the DHPLC method under optimized conditions is highly accurate, rapid, and efficient in detecting mutations and may demonstrate utility in screening of mutations in epidemiological studies of antibiotic resistance.

REFERENCES

- Bingen, E., R. Leclercq, F. Fitoussi, N. Brahimi, B. Malbrun, D. Deforche, and R. Cohen. 2002. Emergence of group A streptococcus strains with different mechanisms of macrolide resistance. *Antimicrob. Agents Chemother.* **46**:1199–1203.
- Canu, A., B. Malbrun, M. Coquemont, T. A. Davies, P. C. Appelbaum, and R. Leclercq. 2002. Diversity of ribosomal mutations conferring resistance to macrolides, clindamycin, streptogramin, and telithromycin in *Streptococcus pneumoniae*. *Antimicrob. Agents Chemother.* **46**:125–131.
- Cooksey, R. C., G. P. Morlock, B. P. Holloway, J. Limor, and M. Hepburn. 2002. Temperature-mediated heteroduplex analysis performed by using denaturing high-performance liquid chromatography to identify sequence polymorphisms in *Mycobacterium tuberculosis* complex organisms. *J. Clin. Microbiol.* **40**:1610–1616.
- Eaves, D. J., E. Liebana, M. J. Woodward, and L. J. Piddock. 2002. Detection of *gyrA* mutations in quinolone-resistant *Salmonella enterica* by denaturing high-performance liquid chromatography. *J. Clin. Microbiol.* **40**:4121–4125.
- Ellis, L. A., C. F. Taylor, and G. R. Taylor. 2000. A comparison of fluorescent SSCP and denaturing HPLC for high throughput mutation scanning. *Hum. Mutat.* **15**:556–564.
- Gross, E., M. Kiechle, and N. Arnold. 2001. Mutation analysis of p53 in ovarian tumors by DHPLC. *J. Biochem. Biophys. Methods* **47**:73–81.
- Hannachi-M'Zali, F., J. E. Ambler, C. F. Taylor, and P. M. Hawkey. 2002. Examination of single and multiple mutations involved in resistance to quinolones in *Staphylococcus aureus* by a combination of PCR and denaturing high-performance liquid chromatography (DHPLC). *J. Antimicrob. Chemother.* **50**:649–655.
- Hurtle, W., D. Shoemaker, E. Henchal, and D. Norwood. 2002. Denaturing HPLC for identifying bacteria. *BioTechniques* **33**:386–388, 390–391.
- Hurtle, W., L. Lindner, W. Fan, D. Shoemaker, E. Henchal, and D. Norwood. 2003. Detection and identification of ciprofloxacin-resistant *Yersinia pestis* by denaturing high-performance liquid chromatography. *J. Clin. Microbiol.* **47**:3273–3283.
- Jones, A. C., J. Austin, N. Hansen, B. Hoogendoorn, P. J. Oefner, J. P. Cheadle, and M. C. O'Donovan. 1999. Optimal temperature selection for mutation detection by denaturing HPLC and comparison to single-stranded conformation polymorphism and heteroduplex analysis. *Clin. Chem.* **45**:1133–1140.
- Keller, G., A. Hartmann, J. Mueller, and H. Hoffer. 2001. Denaturing high pressure liquid chromatography (DHPLC) for the analysis of p53 mutations. *Lab. Invest.* **81**:1735–1737.
- Kota, R., M. Wolf, W. Michalek, and A. Graner. 2001. Application of denaturing high-performance liquid chromatography for mapping of single nucleotide polymorphisms in barley (*Hordeum vulgare* L.). *Genome* **44**:523–528.
- Kristensen, V. N., D. Kelefotis, T. Kristensen, and A. L. Borresen-Dale. 2001. High-throughput methods for detection of genetic variation. *BioTechniques* **30**:318–322, 324, 326.
- Kuklin, A., K. Munson, D. Gjerde, R. Haefele, and P. Taylor. 1997–1998. Detection of single-nucleotide polymorphisms with the WAVE DNA fragment analysis system. *Genet. Test.* **1**:201–206.
- Larsen, L. A., M. Christiansen, J. Vuust, and P. S. Andersen. 2001. Recent developments in high-throughput mutation screening. *Pharmacogenomics* **2**:387–399.
- Leclercq, R., and P. Courvalin. 2002. Resistance to macrolides and related antibiotics in *Streptococcus pneumoniae*. *Antimicrob. Agents Chemother.* **46**:2727–2734.
- Malbrun, B., K. Nagai, M. Coquemont, B. Bozdogan, A. T. Andrasevic, H. Hupkova, R. Leclercq, and P. C. Appelbaum. 2002. Resistance to macrolides in clinical isolates of *Streptococcus pyogenes* due to ribosomal mutations. *J. Antimicrob. Chemother.* **49**:935–939.
- Malbrun, B., A. Canu, B. Bozdogan, B. Fantin, V. Zarrouk, S. Dutka-Malen, C. Feger, and R. Leclercq. 2002. Resistance to quinupristin-dalfopristin due to mutation of L22 ribosomal protein in *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* **46**:2200–2207.
- Musher, D. M., M. E. Dowell, V. D. Shortridge, R. K. Flamm, J. H. Jorgensen, P. Le Magueres, and K. L. Krause. 2002. Emergence of macrolide resistance during treatment of pneumococcal pneumonia. *N. Engl. J. Med.* **346**:630–631.
- Musser, J. M. 1995. Antimicrobial agent resistance in mycobacteria: molecular genetic insights. *Clin. Microbiol. Rev.* **8**:496–514.
- Nagai, K., P. C. Appelbaum, T. A. Davies, L. M. Kelly, D. B. Hoellman, A. T. Andrasevic, L. Drukalska, W. Hryniewicz, M. R. Jacobs, J. Kolman, J. Miculeviciene, M. Pana, L. Setchanova, M. K. Thege, H. Hupkova, J. Trupl, and P. Urbaskova. 2002. Susceptibilities to telithromycin and six other agents and prevalence of macrolide resistance due to L4 ribosomal protein mutation among 992 pneumococci from 10 central and Eastern European countries. *Antimicrob. Agents Chemother.* **46**:371–377.
- Oefner, P. J. 2000. Allelic discrimination by denaturing high-performance liquid chromatography. *J. Chromatogr. B. Biomed. Sci. Appl.* **739**:345–355.
- Pihlajamäki, M., J. Kataja, H. Seppälä, J. Elliot, M. Leinonen, P. Huovinen, and J. Jalava. 2002. Ribosomal mutations in *Streptococcus pneumoniae* clinical isolates. *Antimicrob. Agents Chemother.* **46**:654–658.
- Premstaller, A., and P. J. Oefner. 2003. Denaturing high-performance liquid chromatography. *Methods Mol. Biol.* **212**:15–35.
- Prunier, A. L., B. Malbrun, D. Tande, B. Picard, and R. Leclercq. 2002. Clinical isolates of *Staphylococcus aureus* with ribosomal mutations conferring resistance to macrolides. *Antimicrob. Agents Chemother.* **46**:3054–3056.
- Prunier, A. L., B. Malbrun, M. Laurans, J. Brouard, J. F. Duhamel, and R. Leclercq. 2003. High rate of macrolides resistance in *Staphylococcus aureus* strains from patients with cystic fibrosis reveals high proportions of hypermutable strains. *J. Infect. Dis.* **187**:1709–1716.
- Rudolph, J. G., S. White, C. Sokolsky, D. Bozak, C. Mazzanti, R. H. Lipsky, and D. Goldman. 2002. Determination of melting temperature for variant detection using DHPLC: a comparison between an empirical approach and DNA melting prediction software. *Genet. Test.* **6**:169–176.
- Shlush, L. I., D. M. Behar, A. Zelazny, N. Keller, J. R. Lupski, A. L. Beaudet, and D. Bercovich. 2002. Molecular epidemiological analysis of the changing nature of a meningococcal outbreak following a vaccination campaign. *J. Clin. Microbiol.* **40**:3565–3571.
- Spiegelman, J. I., M. N. Mindrinos, and P. J. Oefner. 2000. High-accuracy DNA sequence variation screening by DHPLC. *BioTechniques* **29**:1084–1092.
- Tait-Kamradt, A., T. Davies, M. Cronan, M. R. Jacobs, P. C. Appelbaum, and J. Sutcliffe. 2000. Mutations in 23S rRNA and ribosomal protein L4 account for resistance in pneumococcal strains selected in vitro by macrolide passage. *Antimicrob. Agents Chemother.* **44**:2118–2125.
- Vester, B., and S. Douthwaite. 2001. Macrolide resistance conferred by base substitutions in 23S rRNA. *Antimicrob. Agents Chemother.* **45**:1–12.
- Xiao, W., and P. J. Oefner. 2001. Denaturing high-performance liquid chromatography: a review. *Hum. Mutat.* **17**:439–474.