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Detection of point mutations associated with antibiotic resistance in *Pseudomonas aeruginosa*

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Abstract

Excessive use of broad-spectrum antibiotics in hospitals has led to the emergence of highly resistant strains of *Pseudomonas aeruginosa*. To reduce the selection pressure for resistance, it is important to determine the antibiotic susceptibility pattern of bacteria so that hospital patients can be treated with more narrow-spectrum and target-specific antibiotics. This study describes the development of a technique for detecting point muations in the fluoroquinolone resistance-determining region of the *gyrA* and *parC* genes as well as the efflux regulatory genes *mexR*, *mexZ* and *mexOZ* that are associated with fluoroquinolone and aminoglycoside resistance. The assay is based on a short DNA sequencing method using multiplex-fast polymerase chain reaction (PCR) and Pyrosequencing™ for amplification and sequencing of the selected genes. Fifty-nine clinical isolates of *P. aeruginosa* were examined for mutations in the abovementioned genes. Mutations related to antibiotic resistance were detected in codons 83 and 87 of *gyrA* and codon 126 of the *mexR* regulatory gene. Results of this study suggest Pyrosequencing™ as a substitute for traditional methods as it provides a rapid and reliable technique for determinating the antibiotic resistance pattern of a given bacterial strain in <1 h.

Keywords

Pyrosequencing; *Pseudomonas aeruginosa*; Antibiotic resistance

1. Introduction

The emergence of drug-resistant bacteria occurs frequently in the Intensive Care Unit (ICU) involving both Gram-negative and Gram-positive organisms. This is a problem for critical care physicians because there are now several pathogens that can only be effectively treated with a

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limited number of antimicrobial agents, e.g. meticillin-resistant *Staphylococcus aureus* (MRSA), *Enterococcus faecium* and Gram-negative bacteria producing extended-spectrum β-lactamases [1].

Multidrug-resistant (MDR) bacterial infections are associated with increased mortality, length of hospital stay and cost of care [2]. For example, in a study from a large tertiary-care teaching hospital in Boston, MA, the median length of stay and hospital charges were significantly greater for patients with MRSA compared with those with meticillin-sensitive *S. aureus*.

Successful treatment of patients admitted to the ICU with nosocomial or community-acquired infections depends on adequate initial antibiotic use. A common strategy is to begin with broad-spectrum antibiotic therapy and later to de-escalate antibiotic therapy based upon culture and sensitivity data. Initial broad-spectrum therapy is necessary until culture data are available to guide focused antibiotic administration. However, broad-spectrum antibiotics are a leading cause of the emergence of drug-resistant bacteria [3].

Several strategies have been investigated as a means of reducing the emergence of MDR bacteria in the ICU. One such strategy is to employ only narrow-spectrum antibiotics directed at pathogens identified using rapid bedside detection devices. This strategy requires technology capable of detecting pathogens within minutes of sample collection, pathogen identification and analysis of antibiotic resistance patterns, and detection of organisms that may reside in the intracellular compartment.

One of the more common nosocomial pathogens is *Pseudomonas aeruginosa* [4]. Excessive use of broad-spectrum antibiotics has led to the emergence of highly resistant strains of *P. aeruginosa* that are a major threat to patients in the ICU. Adequate treatment of *P. aeruginosa* infections with modern antibiotics is difficult due to the intrinsic ability of the bacterium to adapt rapidly to new environments and acquire resistance to common therapies [5,6].

Fluoroquinolones and aminoglycosides are two important classes of antibiotics used in the treatment of *Pseudomonas* infections. Fluoroquinolones are members of the quinolone family that act as bactericidal agents by inhibiting bacterial DNA gyrase and topoisomerase IV, thereby inhibiting DNA transcription and replication. DNA gyrase is typically the target in Gram-negative organisms, whereas topoisomerase IV is the target in Gram-positive organisms. Aminoglycosides are a separate class of antibiotics that bring about their bactericidal action by binding to the bacterial 30S ribosomal subunit, inhibiting the translocation of peptidyl-tRNA from the A-site to the P-site, causing misreading of the mRNA and thus rendering the bacterium unable to synthesise proteins vital to its growth. *Pseudomonas* readily develops resistance to these agents, consequently reducing their utility. The main mechanisms of resistance are mutations in the genes that encode DNA gyrase (*gyrA*) and topoisomerase IV (*parC*). Other mechanisms include mutations in the regulatory genes of the multidrug efflux pumps, *mexAB-oprM* and *mexXY-oprM* [7]. The MexAB-OprM efflux system contributes to the natural resistance of bacteria to a wide range of antibiotics including fluoroquinolones, β-lactams and β-lactamase inhibitors, whereas MexXY-OprM contributes to aminoglycoside resistance. High expression of MexAB-OprM and MexXY-OprM may confer high levels of resistance to clinical strains as a result of mutations occurring mainly in their regulatory genes *mexR* and *mexZ* [8,9]. Another region related to aminoglycoside resistance is *mexOZ*, which is an intergenic region between the *mexZ* and *mexX* genes of *P. aeruginosa* [9].

In this study, we attempted to design a DNA-based technique for rapid determination of the antibiotic resistance pattern of *P. aeruginosa* compared with traditional methods such as disk diffusion or agar dilution. Multiplex polymerase chain reaction (PCR) and Sanger sequencing were used to find key mutations in the *gyrA*, *mexR*, *parC*, *mexZ* and *mexOZ* genes of *P.*

aeruginosa isolates exhibiting resistance to fluoroquinolones and aminoglycosides, and Pyrosequencing™ was used as the ultimate sequencing method to detect specific point mutations in these genes.

2. Materials and Methods

2.1. Clinical isolates of Pseudomonas aeruginosa

Fifty-nine previously identified clinical isolates of *P. aeruginosa* were obtained from the Microbiology Laboratory at Stanford Hospital (Stanford, CA) to evaluate mutations involved in antibiotic resistance. These isolates were previously tested by the Kirby–Bauer method for their susceptibility to fluoroquinolones and aminoglycosides. Samples were accordingly assigned to one of three groups, i.e. resistant, intermediate or susceptible to either fluoroquinolones or aminoglycosides. Fluoroquinolones and aminoglycosides used in the antibiotic susceptibility tests were ciprofloxacin/levofloxacin and tobramycin, respectively. Of the 59 clinical isolates, 12 were resistant, 6 were intermediate and 41 were susceptible to ciprofloxacin, levofloxacin or both (Table 1), and 43 were susceptible, 4 were intermediate and 12 were resistant to tobramycin.

2.2. DNA extraction and multiplex PCR

DNA was extracted from the 59 clinical isolates of *P. aeruginosa* using the Qiagen Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions and was used as the DNA template for multiplex/multiplex-fast PCR. Primers gyrA-1 (5'-GTGTGCTTTATGCCATGAG-3') and gyrA-2 (5'-GGTTTCCTTTTCCAGGTC-3') were used to amplify 287 bp of the fluoroquinolone resistance-determining region of the *gyrA* gene. Primers parC-1 (5'-CATCGTCTACGCCATGAG-3') and parC-2 (5'-AGCAGCACCTCGGAATAG-3') were used to amplify 267 bp of the fluoroquinolone resistance-determining region of *parC*. For the *mexR* regulatory gene, mexR-1 (5'-CTGGATCAACCACATTTACA -3') and mexR-2 (5'-CTTCGAAAAGAATGTTCTTAAA-3') primers were used to amplify the whole 503-bp region of the gene. Primers for amplification of *gyrA*, *parC* and *mexR* were designed with Primer 3 software (http://fokker.wi.mit.edu/primer3/input.htm) using known sequences available in GenBank with accession numbers L29417, AB003428 and U23763, respectively. The regulatory genes *mexZ* and *mexOZ* were amplified using primers from published data [9]. All primers were synthesised by Integrated DNA Technologies (Coralville, IA).

PCR amplification was performed in a 50 μL mixture containing 1× Thermo-Start™ Buffer (ABgene, Rockford, IL), 2.5 mM $MgCl_2$, 0.2 mM mix of deoxynucleotide triphosphates (Sigma-Aldrich, St Louis, MO), 10 pmol of each primer, 1 U of Thermo-Start DNA Polymerase (ABgene) and 150 ng of the DNA template. Amplification of the target regions was performed in 35 cycles consisting of initial heat activation at 95 °C for 15 min, denaturation at 95 °C for 45 s, annealing at 51 °C for 45 s and elongation at 71 °C for 1 min, with a final elongation at 71 °C for 7 min. The PCR products obtained from this step were used for Sanger sequencing.

2.3. Sanger sequencing

Dideoxy sequencing was performed using BigDye™ Terminator Chemistry v. 3.1 (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions, as described previously [10]. Forward and reverse primers for *gyrA*, *parC*, *mexR*, *mexZ* and *mexOZ* (Section 2.2) were used as sequencing primers using the ABI 3730 Bioanalyzer (Applied Biosystems).

2.4. Multiplex-fast PCR

Two fluoroquinolone-resistant isolates with mutations both on *gyrA* and *mexR* were chosen for multiplex-fast PCR. The primers used for amplification of *gyrA* and *mexR* with the Veriti™

96-Well Fast Thermal Cycler (Applied Biosystems) were identical to those used in traditional PCR (see Section 2.2). Forward *gyrA* and reverse *mexR* primers were biotin-labelled for single-strand separation. Amplification of the selected regions was performed in a 20 μL mixture with 2.5 mM $MgCl_2$, 0.2 mM mix of deoxynucleotide triphosphates (Sigma Aldrich), 10 pmol of each primer, 1 U of AmpliTaq Gold (Applied Biosystems) and 150 ng of the DNA template. Multiplex-fast PCR was performed in 25 cycles as follows: initial heat activation at 95 °C for 10 min, denaturation at 95 °C for 1 s, annealing at 46 °C for 15 s, elongation at 72 °C for 15 s and final elongation at 72 °C for 30 s. PCR products obtained from this step were used for Pyrosequencing™.

2.5. Sample preparation for Pyrosequencing™

Sample preparation for Pyrosequencing™ was performed according to the manufacturer's instructions and as described previously [11]. Single-stranded DNA amplicons were prepared semi-automatically using a Vacuum Prep Tool and Vacuum Prep Worktable (Biotage, Uppsala, Sweden). A 10 μL aliquot of biotinylated PCR products was immobilised onto 3 μL of streptavidin-coated Sepharose™ High Performance Beads (Amersham Biosciences, Piscataway, NJ) by incubating at 42 °C and agitation at 1400 rpm for at least 15 min in an Eppendorf Thermomixer R (Eppendorf AG, Hamburg, Germany). Double-stranded DNA immobilised on Sepharose beads was washed with 70% ethanol and denatured with 0.2 M NaOH. Unbound single-stranded DNA was washed with 0.1 M TE buffer [0.1 M Tris-HCl (pH 7.6) containing 1 mM ethylene diamine tetra-acetic acid (EDTA)]. All the steps were performed according to the manufacturer's instructions for the Vacuum Prep Station. The beads carrying single-stranded DNA amplicons were suspended in 12 μL of annealing buffer [20 mM Tris-acetate (pH 7.6), 2 mM Mg-acetate] containing 0.3 pmol of sequencing primers. The single-stranded DNA was annealed to the sequencing primer at 92 °C for 2 min followed by incubation for 5 min at room temperature.

2.6. Pyrosequencing™

Pyrosequencing™ (Biotage) was performed according to the manufacturer's instructions as described previously [11]. Single-stranded PCR products were sequenced using a PSQ™ HS 96A System (Biotage). Sequencing was performed according to the manufacturer's instructions in a total volume of 12 μL using PSQ™ 96 Gold Kit (Biotage).

2.7. Cloning

PCR amplicons of *gyrA* were cloned using the TOPO TA Cloning® Kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Cloned bacterial cells were cultured on LB medium (containing 10 g Bacto tryptone, 5 g Bacto yeast extract and 10 g NaCl in 1 L of ddH_2O) and incubated at 37 °C. Isolated single colonies from overnight cultures were suspended in 10 μL of water and incubated at 95 °C for 10 min. Fragments harbouring the *gyrA* regions were then amplified using 10 pmol of forward biotinylated *gyrA* primer, 10 pmol of reverse vector primer and 10 μL of the cell lysate containing the DNA template. Amplification of the cloned fragments was performed using the same conditions as in Section 2.2.

3. Results

Fifty-nine clinical isolates of *P. aeruginosa* were examined for the occurrence of mutations related to antibiotic resistance. Fragments of *gyrA*, *parC*, *mexR*, *mexZ* and *mexOZ* genes were amplified using multiplex PCR and the efficacy of the amplification was determined by gel electrophoresis. All fragments amplified adequately (data not shown). The PCR samples were then analysed for detection of point mutations in the fluoroquinolone resistance-determining regions of *gyrA* and *parC* as well as the efflux pump regulatory genes *mexR*, *mexZ* and

mexOZ using the Sanger sequencing method. To identify point mutations, sequences from clinical isolates were compared with that of wild-type *P. aeruginosa* PAO1. Results from the molecular analysis were compared with the antibiotic susceptibility profile of bacterial isolates to assess the correlation between mutations and resistance (Table 1).

The results showed that among the 12 fluoroquinolone-resistant isolates, 4 had a single mutation in *gyrA*, 4 had mutations both in *gyrA* and *mexR* and 4 had no mutations in the sequence areas examined (Table 1). Mutations in *mexZ* and *mexOZ* genes were highly variable within isolates, making it difficult to correlate a specific mutation with aminoglycoside resistance. Therefore, *mexZ and mexOZ* genes were not further evaluated using Pyrosequencing™.

Common mutations in fluoroquinolone-resistant strains occurred in codons 83 and 87 of the *gyrA* gene (Fig. 1). The nucleic acid alterations that occurred in these codons changed the amino acid profile from Thr to Ile and Asp to Asn (or Asp to Tyr), respectively, consistent with previous reports [7,12,13]. No mutations were found in *parC*. A novel mutation related to fluoroquinolone resistance in codon 126 of the *mexR* regulatory gene, changing amino acid Val to Glu, was detected (Fig. 2; Table 2).

To confirm the mutations related to fluoroquinolone resistance by Pyrosequencing™, the PCR products amplified from all isolates were sequenced again using a pre-programmed nucleotide dispensation, sequencing a 20-bp region starting 1 base upstream of the mutation site detected by the Sanger sequencing method. This pre-programmed sequencing was much more rapid and took only 20 min. Nucleotide patterns of each isolate were compared with those of the wild-type *P. aeruginosa* PAO1 for parts of the *gyrA* and *mexR* genes encoding amino acids 83, 87 and 126, respectively. Absent or added sequence signal peaks were designated as mutations (Fig 1 and Fig 2). All mutations detected by Sanger sequencing were confirmed by the Pyrosequencing™ method.

One isolate showed two peaks in a single nucleotide position that was expected to be an absent peak or a peak representing either one of the existing nucleotides (A or C). Because bacteria are haploid (one set of each gene), we suspected that this sample was a mixed sample rather than a single isolate. Therefore, the sample was cloned using a TA Cloning Kit. The cloned fragments were amplified and further analysed by Pyrosequencing™. Pyrosequencing™ was performed using a five-cycle ACGT nucleotide order dispensation. From the Pyrosequencing™ results, one-half of the colonies showed no mutations and were considered as wild-type and one-half showed a mutation. This confirmed that the sample had been a mixture, i.e. was contaminated by a wild-type or a mutant of *P. aeruginosa*. Fig. 3 shows the sequencing results before and after isolation of the sample by cloning.

For amplification using multiplex-fast PCR, two fluoroquinolone-resistant isolates with mutations both on *gyrA* and *mexR* were chosen and sequenced by Pyrosequencing™. The results from the signal peaks were identical to the signal peaks obtained by sequencing the amplicons from the multiplex PCR (data not shown). Multiplex-fast PCR together with Pyrosequencing™ took <1 h for detection of *gyrA* and *mexR* mutations.

4. Discussion

Fluoroquinolone and aminoglycoside resistance can lead to treatment failure in *P. aeruginosa* infections [7,8]. Known mutations responsible for resistance are found in the genes expressing DNA gyrase and topoisomerase IV as well as mutations in genes that regulate the expression of efflux pumps and mainly interfere with binding of these antibiotics to the target sites of the DNA gyrase and topoisomerase IV or lead to hyperextrusion of the drug by the bacterial efflux pumps. Because traditional microbiological culturing is time consuming,

empirical treatment is often started 18–24 h prior to definitive identification of the pathogen and 48 h prior to knowledge of its susceptibility profile. To eradicate drug-resistant strains of *P. aeruginosa*, it is crucial to design a molecular technique to identify resistance rapidly so that these infections may be treated appropriately.

Rapid and reliable methods are needed for the detection of resistant organisms, most of which can be identified through a limited number of mutations. Multiplex-fast PCR together with Pyrosequencing™ provides the advantage of lower sample volumes, significantly reducing the cost of performing sequencing reactions. Multiplex-fast PCR and DNA sequencing by Pyrosequencing™ using a pre-programmed sequencing approach as outlined here takes <1 h compared with the regular amplification and sequencing methods that can take up to 4–5 h.

Notably, all the mutations detected by the Sanger sequencing method were confirmed by the more efficient Pyrosequencing™ method. For *gyrA*, the main mutations found to be related to fluoroquinolone resistance were on codons 83 and 87 as previously reported [7,12–14]. We discovered a mutation in codon 126 of the *mexR* regulatory gene changing amino acid Val to Glu that correlated with fluoroquinolone resistance. Almost all the clinical isolates in this study that had a single mutation on *mexR* were susceptible and did not show any drug resistance (Table 2), indicating that a *mexR* mutation alone may not change the susceptibility of the bacterium but causes resistance when it co-occurs with a mutation on the *gyrA* gene. The mutations found on *gyrA* and *mexR* were also found in susceptible isolates, and not all resistant isolates had mutations on these genes (Table 1). Our results show that detection of *gyrA* and *mexR* mutations does not always imply resistance to fluoroquinolones, but that acquiring these mutations increases the likelihood of resistance. These discrepancies could suggest the existence of other additional molecular mechanisms for fluoroquinolone resistance.

Pyrosequencing™ was also able to distinguish a mixed sample from other clinical isolates. The pyrogram in Fig. 3a shows the presence of both A and C nucleotides at a single spot location in part of the *gyrA* gene. This could only be explained by assuming that this sample was mixed since bacteria are haploid. Pyrosequencing™ results after cloning the *gyrA* fragment confirmed this hypothesis.

In conclusion, point mutations in clinical isolates associated with antibiotic resistance are rapidly and reliably detected by DNA sequencing using Pyrosequencing™ and multiplex-fast PCR. With this novel approach, clinical isolates could be analysed quickly at lower cost. The panel of mutations screened can be readily expanded to cover other known resistance determinants in *P. aeruginosa* and to detect mutations involved in a variety of antibiotic resistance scenarios. Rapid and simple detection of resistance determinants at the genetic level could guide the choice of more appropriate antibiotics and enable effective employment of narrow-spectrum antibiotics. Ultimately, more accurate diagnosis and treatment could lower the incidence of resistance and improve outcomes for patients with severe bacterial infections.

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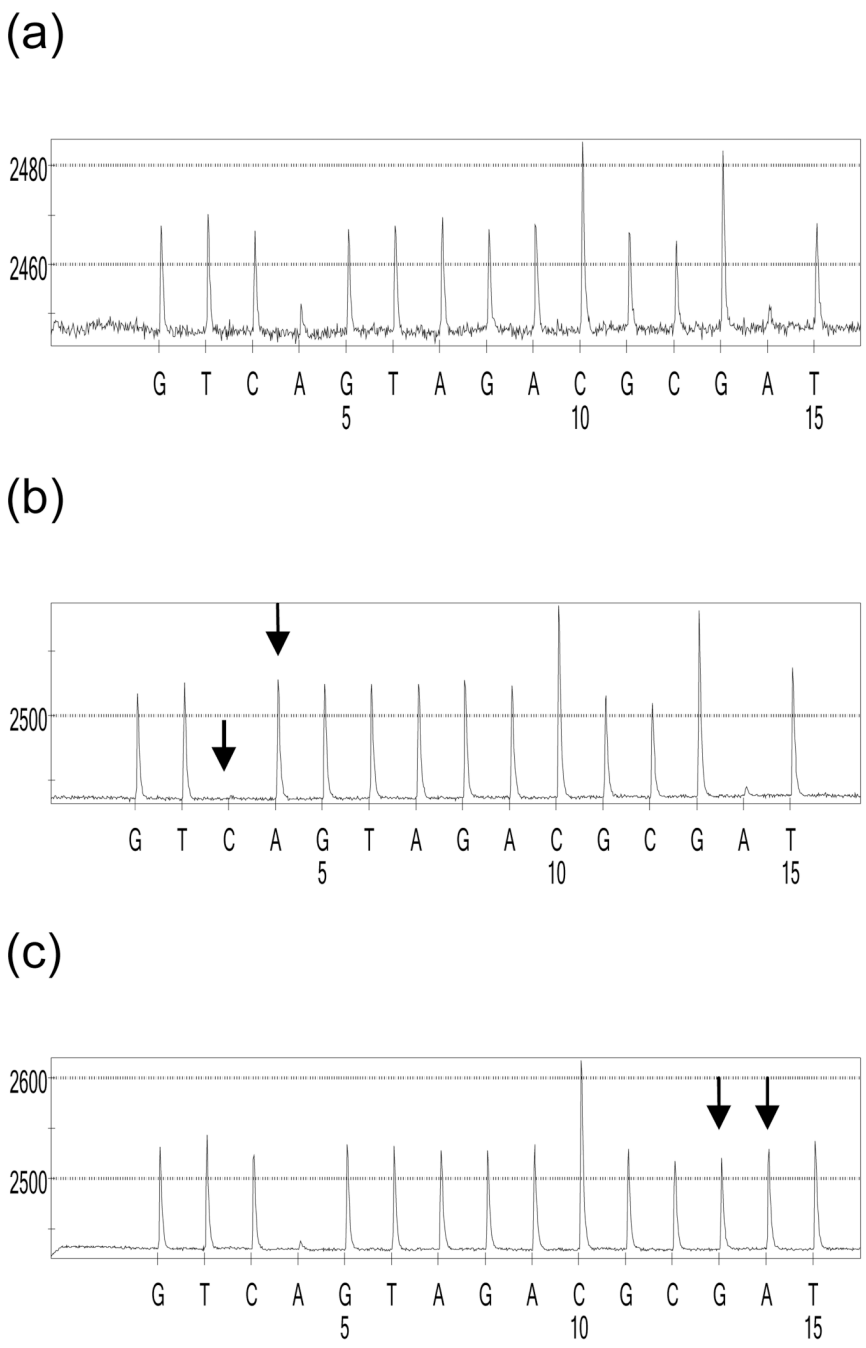

Fig. 1.

Pyrograms of the 20-bp sequence (amino acids 83–87) of the *gyrA* gene of *Pseudomonas aeruginosa* obtained by the pre-programmed DNA sequencing method: (a) wild-type sequence of *gyrA* with no alterations; (b) nucleotide C→A alteration in codon 83; and (c) nucleotide G→A alteration in codon 87. Arrows show the location of point mutations in the signal peaks.

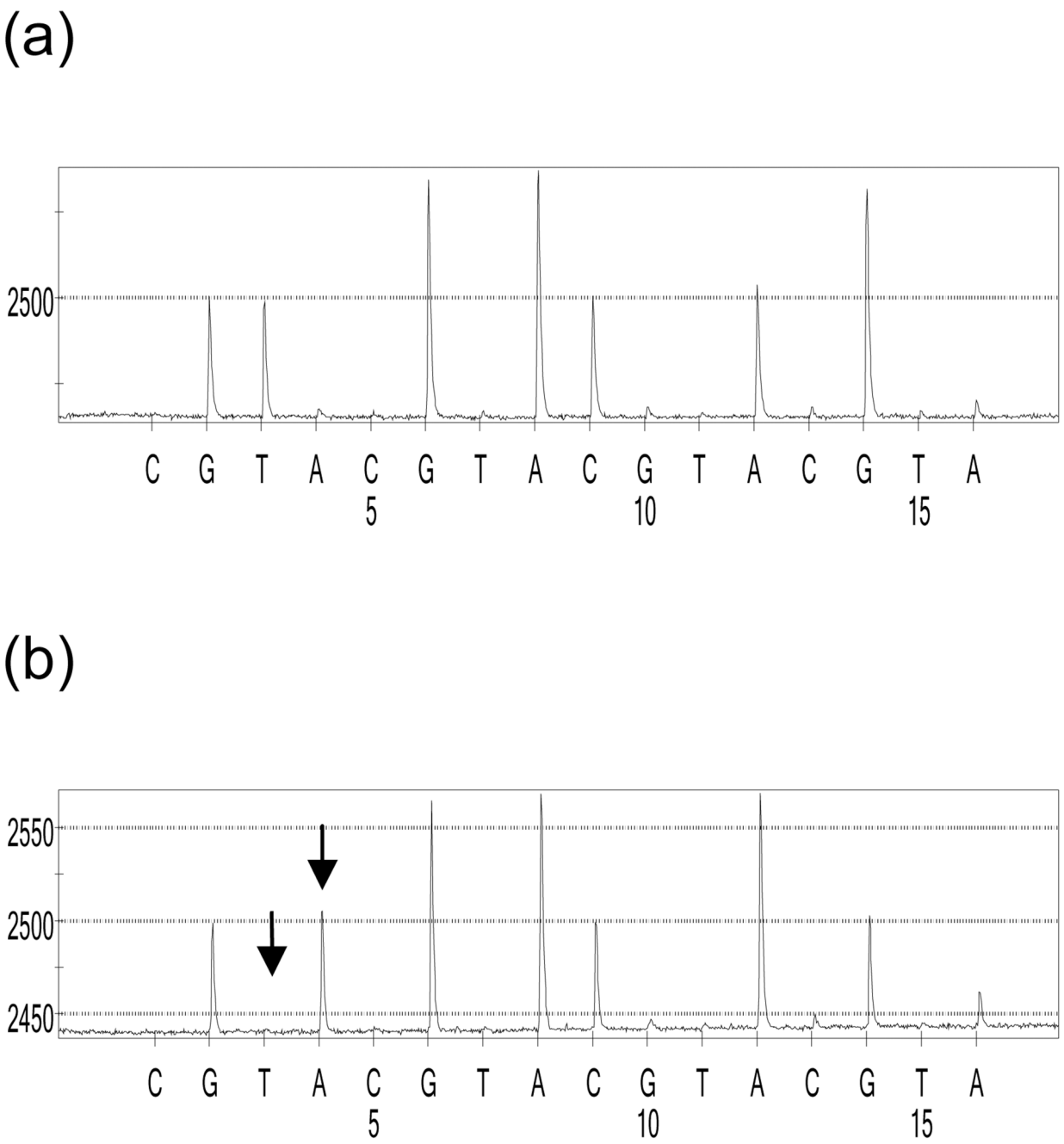

Fig. 2.
Pyrograms of the 20-bp sequence of the *mexR* regulatory gene of *Pseudomonas aeruginosa* (amino acids 126–128) obtained by the pre-programmed DNA sequencing method: (a) wild-type sequence of *mexR* with no alterations; and (b) nucleotide T→A alteration in codon 126. Arrows show the location of point mutations in the signal peaks.

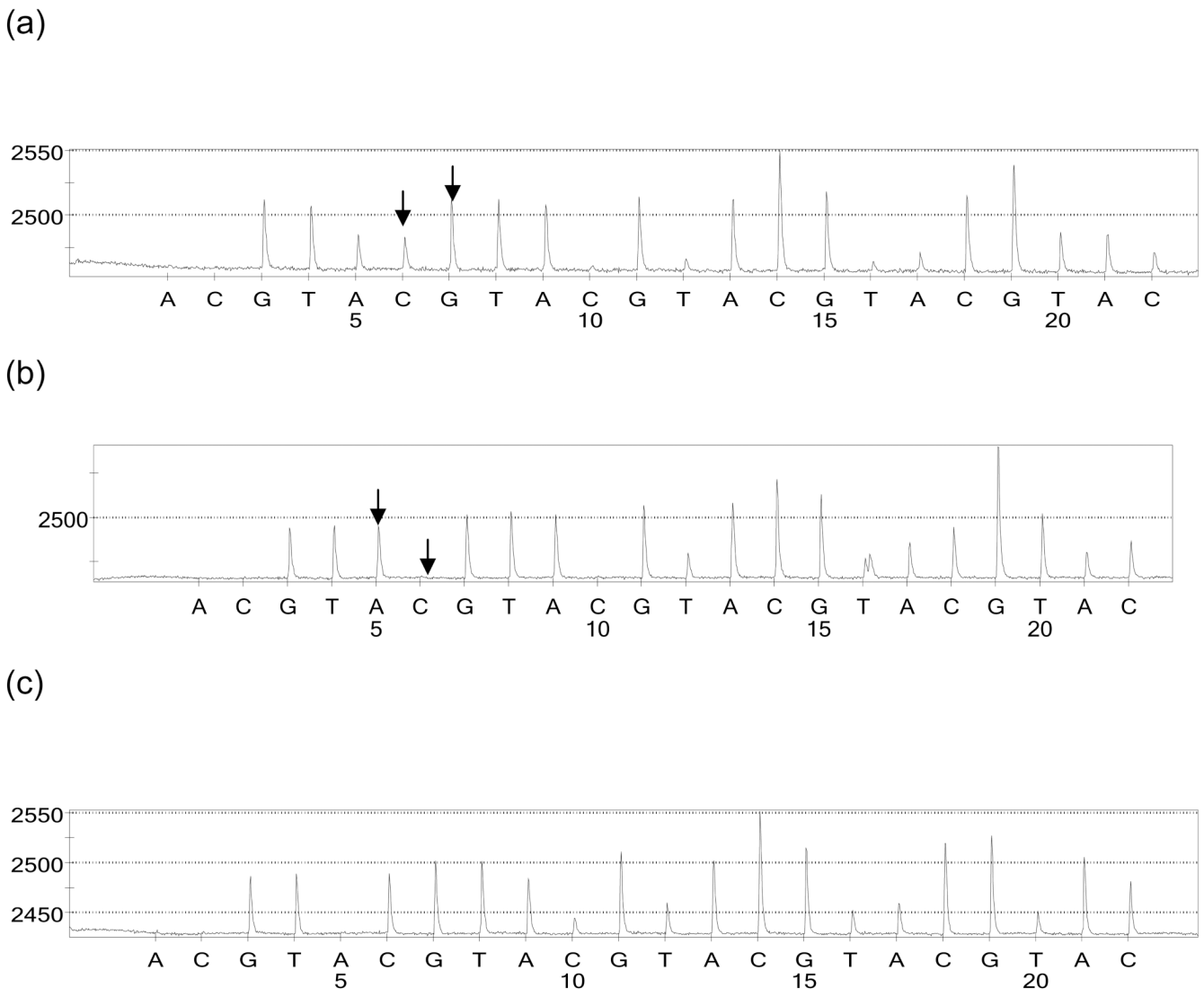

Fig. 3.

Pyrograms of the DNA sequence of *gyrA* for an isolate of *Pseudomonas aeruginosa*. (a) Sequencing data before cloning and isolation. The two arrows indicate the presence of both A and C nucleotides in the sequence. The *gyrA* gene was cloned into a plasmid vector, amplified by polymerase chain reaction (PCR) and sequenced by Pyrosequencing™ using a five-cycle ACGT dispensation. (b,c) Pyrograms after isolation of the mixed DNA sample; ca. 50% of the cloned vectors harboured DNA from wild-type (c) and 50% harboured DNA from a mutant of *P. aeruginosa* (b) (C→A alteration).

Table 1

Correlation between fluoroquinolone (ciprofloxacin/levofloxacin) susceptibility of 59 *Pseudomonas aeruginosa* clinical isolates and mutations in *gyrA* and *mexR* genes

Fluoroquinolone susceptibility (no. of isolates)	**Mutations in *gyrA* only**	**Mutations in *mexR* only**	**Mutations in both *gyrA* and *mexR***	**No mutations in *gyrA* or *mexR***
Resistant (12)	4	0	4	4
Intermediate (6)	1	1	1	3
Susceptible (41)	1	12	1	27

Table 2

Mutations in *gyrA*, *mexR*, *parC*, *mexZ* and *mexOZ* genes leading to amino acid alterations

Mutations in *gyrA* (codon)	**Mutations in *mexR* (codon)**	**Mutations in *parC***	**Mutations in *mexZ* and *mexOZ***
Asp→Asn (87)	Val→Glu (126)	None	Highly variable
Thr→Ile (83)			
Asp→Tyr (87)			