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Detecting mutations related to antibiotic resistance in Pseudomonas aeruginosa

Neda Nemat-Gorgani
San Jose State University

DETECTING MUTATIONS RELATED TO ANTIBIOTIC RESISTANCE IN *PSEUDOMONAS AERUGINOSA*

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Neda Nemat-Gorgani

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by
Neda Nemat-Gorgani

APPROVED FOR THE DEPARTMENT OF BIOLOGICAL SCIENCES

Dr. John Boothby, Department of Biological Sciences — Date 8/31/09

Dr. Cleber Ouverney, Department of Biological Sciences — Date 8/31/09

Dr. Nader Pourmand, Biomolecular Engineering, University of California at Santa Cruz — Date 9/2/09

APPROVED FOR THE UNIVERSITY

Associate Dean Office of Graduate Studies and Research — Date 8/31/09

ABSTRACT

DETECTING MUTATIONS RELATED TO ANTIBIOTIC RESISTANCE IN *PSEUDOMONAS AERUGINOSA*

by Neda Nemat-Gorgani

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 above-mentioned 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 determining the antibiotic-resistance pattern of a given bacterial strain in < 1 h.

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PREFACE

This thesis is organized in three chapters. Chapter I presents a general introduction on *Pseudomonas aeruginosa* and its resistance to antibiotics and the aim of the research study. Chapter II describes the mechanisms of antibiotic resistance in *P. aeruginosa*. Chapter III includes the details of the study, prepared as a manuscript entitled "Detection of point mutations associated with antibiotic resistance in *Pseudomonas aeruginosa*" (submitted to the *Journal of Microbiological Methods*).

The last part of the thesis is composed of appendixes, which provides additional details about the data collected.

CHAPTER I

INTRODUCTION

Pseudomonas aeruginosa is a notorious opportunistic pathogen and is isolated mostly from patients with urinary tract infections, wound infections, and severe burns. *P. aeruginosa* infection is also known to be a serious problem in patients hospitalized with cystic fibrosis, cancer, and burns. Half of these infections are fatal. *P. aeruginosa* has been considered a nosocomial pathogenic bacterium in a number of studies. What makes *P. aeruginosa* infections problematic is the bacterium's resistance to antibiotics and disinfectants (Lambert, 2002). Antibiotic and disinfectant resistance have been attributed to 1) intrinsic resistance to a wide variety of antimicrobial agents due to low membrane permeability, 2) genetic capacity to express a wide range of resistance mechanisms, 3) acquisition of resistance to antibiotics through chromosomal mutations, and 4) acquisition of resistance genes from other organisms via plasmids, bacteriophages, and/or transposons (Lambert, 2002).

P. aeruginosa is an extremely adaptive organism. It can grow on a wide range of substrates and quickly responds to environmental alterations (Lambert, 2002). It has a large genome (6.26 Mbp encoding 5,567 genes) compared to other common human opportunistic pathogens such as *Escherichia coli* K12 (4.64 Mbp), *Haemophilus influenzae* (1.83 Mbp encoding 1,714 genes), and *Staphylococcus aureus* N315 (2.81 Mbp encoding 2,594 genes) (Lambert, 2002). *P. aeruginosa* consequently possesses substantial additional genetic capacity compared to other bacteria. This capacity may confer its

extremely adaptive nature, including the capacity to develop resistance when antibiotics are employed extensively (Lambert, 2002).

Mechanisms of resistance to antibiotics in *P. aeruginosa* are either based on non-mutational intrinsic resistance or mutational acquired resistance. Fluoroquinolones and aminoglycosides are two important classes of antibiotics used in the treatment of *Pseudomonas* infections. *Pseudomonas* readily develops resistance to these agents, reducing the antibiotic effectiveness.

In this study, we developed a DNA-based technique to determine the antibiotic-resistance pattern of *P. aeruginosa* in a shorter period of time compared with traditional methods such as disk diffusion or agar dilution. We used multiplex polymerase chain reaction (PCR) and Sanger sequencing to find key mutations in the *gyrA*, *mexR, parC, mexZ,* and *mexOZ* genes of *P. aeruginosa* isolates exhibiting resistance to fluoroquinolones and aminoglycosides. Furthermore, Pyrosequencing™ was used as the ultimate method to detect specific point mutations in the above-mentioned genes.

CHAPTER II

MECHANISMS OF ANTIBIOTIC RESISTANCE IN *PSEUDOMONAS AERUGINOSA*

This section reviews the resistance of *P. aeruginosa* to antibiotics and antimicrobial agents based on non-mutational intrinsic resistance and mutational acquired resistance.

1. Non-mutational intrinsic resistance

P. aeruginosa has natural resistance to many antimicrobial agents. This is primarily due to active efflux systems that are present in all wild-type strains, its membrane permeability properties, and plasmid and chromosomal β-lactamase genes (Aires et al., 1999, Giwercman et al., 1990, Lambert, 2002, Livermore, 2002).

The most well-known efflux system that leads to intrinsic antibiotic resistance is the MexAB-oprM system. Efflux pumps are active transporters that are localized in the cytoplasmic membrane of all bacterial strains and reduce the antibiotic levels at the site of activity in the cell. Antibiotics are the most clinically important substrates of efflux systems. The MexAB-oprM efflux system is expressed constitutively in wild-type strains of *P. aeruginosa* and confers resistance to a broad range of drugs including quinolones, chloramphenicol, β-lactam, β-lactam inhibitors, trimetoprime, sulfamethoxasole, tetracycline, and novobiocin (Aires et al., 1999).

2. Mutational acquired resistance

P. aeruginosa acquires resistance to antibiotics through chromosomal mutations. These mutations include 1) mutations in the regulatory genes for the drug efflux pump systems, 2) mutations in the target genes encoding DNA gyrase (*gyrA*) and topoisomerase IV (*parC*), 3) mutations causing cell wall impermeability due to loss of OprD, a porin that forms narrow transmembrane channels, 4) mutations causing alterations in penicillin-binding proteins, and 5) impulsive mutations in the regulatory genes for the bacteria's β-lactamase resulting in derepression of β-lactamase.

2.1. Derepression of β-lactamase

Chromosomally encoded β-lactamase is increasingly proven as a cause of resistance to novel β-lactam antibiotics (Giwercman et al., 1990). All strains of *P. aeruginosa* express a chromosomal AmpC β-lactamase. In the absence of β-lactam antibiotics, this enzyme is expressed at a very low basal level but can increase to much higher levels in the presence of the antibiotic. However, this stimulation is a transient response to β-lactam antibiotics. Strains displaying constant AmpC derepression produce higher amounts of β-lactamase, possibly due to the over expression resulting from spontaneous mutations in regulatory genes (Livermore, 2002). Antibiotics such as imipenem, benzylpenicillin, and cefoxilin are strong inducers, whereas newer β-lactam antibiotics such as piperacillin are weak inducers under *in vitro* conditions (Giwercman et al., 1990).

Three genes related to peptidoglycan recycling are implicated in *ampC* stimulation. These genes include *ampD* (encodes a cytosolic *N*-acetyl-anhydromuramyl-*L*-alanine amidase and specifically hydrolyzes 1,6-anhydromuropeptide), *ampR* (encodes a transcriptional regulator of the LysR family), and *ampG* (functions as a permease for 1,6-anhydromuropeptide, the signal molecule for stimulation of *ampC* expression, and encodes a transmembrane protein) (Langaee et al., 2000). Inactivation of AmpD results in cytoplasmic buildup of 1,6-anhydromuropeptide and finally constitutive over expression of AmpC (Langaee et al., 2000). In *P. aeruginosa*, three phenotypes of distorted AmpC expression have been related to β-lactam resistance, and two out of three have been related to mutations in the *ampD* gene (Langaee et al., 2000). AmpR and AmpD regulators also control the inducible expression of the *ampC* gene (Langaee et al., 2000).

Plasmid-encoded β-lactamases active against cephalosporins and penicillins may provide a mechanism for β-lactam resistance (Giwercman et al., 1990, Lambert, 2002). For newer stable β-lactam antibiotics, the rapid appearance of β-lactam resistance during drug therapy and frequent therapeutic failures related to the expansion of multiple resistances to β-lactam antibiotics are now common (Giwercman et al., 1990).

2.2. Multidrug efflux pumps

The combination of numerous resistance genes was first thought to be the exclusive cause of multiple antibiotic resistances in bacteria, each encoding resistance to

a separate drug. More recently, resistant phenotypes have been found to be due to the activity of drug efflux pumps (Nikaido, 1998). Several of these efflux pumps reveal broad ranges of specificity covering nearly all chemotherapeutic agents, inhibitors, detergents, and antibiotics (the exemption possibly being highly hydrophilic complexes). These efflux pumps work efficiently through their synergetic interaction with the outer membrane barrier in some Gram-negative bacteria, including *P. aeruginosa* (Nikaido, 1998).

Three protein components make up the multidrug efflux system: an outer membrane porin, an energy dependent pump positioned in the cytoplasmic membrane, and a linker protein which couples these two membrane components. This tripartite arrangement removes toxic molecules such as antibiotics that find their way into the cytoplasmic membrane, the periplasm, or the cytoplasm (Lambert, 2002).

In *P. aeruginosa* four different efflux systems have been observed: MexAB-oprM, MexXY-oprM, MexCD-oprJ, and MexEF-oprN. The pump proteins MexB and MexY are positioned in the cytoplasmic membrane, whereas MexA and MexX which are so called "membrane fusion" proteins are anchored in the inner membrane, but extend to the periplasm. Two of these pumps, MexXY-oprM and MexAB-oprM supply the intrinsic resistance of wild-type strains to antibiotics. MexXY-oprM is responsible for extrusion of aminoglycosides, whereas MexAB-oprM extrudes β-lactams, quinolones, and a large range of disinfectants (Lambert, 2002). The genes for these systems exist in all strains of *P. aeruginosa,* but are not expressed in high amounts (Lambert, 2002).

MexAB-oprM may lead to elevated antibiotic resistance in clinical strains when it is expressed in high levels as a result of mutations that take place in the *mexR* regulatory gene of the bacteria (Aires et al., 1999). The two other efflux pump systems, MexCD-oprJ and MexEF-oprN are not constitutively formed in the wild-type strains of *P. aeruginosa.* These efflux pumps are homologous to MexAB-oprM, and are more substrate-restricted than MexAB-oprM. They are capable of accommodating complexes as structurally diverse as trimethoprime, chloramphenicol, and quinolones. *NfxB* and *mexT (nfxC)* are regulatory genes for MexCD-oprJ and MexEF-oprN, respectively. Mutations that take place in these genes are also associated with higher levels of antibiotic resistance (Aires et al., 1999).

MexY, homologous to AcrD in *Escherichia coli,* is related to a membrane fusion protein MexX and serves as a drug proton anti-porter. Elevated levels of expression of MexXY efflux system has been observed in few aminoglycoside resistant types of *P. aeruginosa,* and has been illustrated as a major source of aminoglycoside resistance in isolates collected from the lungs of CF patients (Islam et al., 2004). It has been suggested that MexXY efflux pump system shares the OprM channel with MexAB, since it does not have a gene for an outer membrane protein that should be located downstream of *mexY.* The expression of MexXY is under negative regulation by another protein called MexZ. This protein is encoded by a gene that is located 263 bp upstream of *mexX* and is transcribed divergently. MexZ is thought to have a DNA-binding domain at its *N*-terminal that contains a helix-turn-helix motif. In wild-type *P. aeruginosa* isolates deletion of MexXY increases their susceptibility to antibiotics such as erythromycin,

tetracycline, and gentamycin. MexXY-oprM is also inducible by gentamycin and tetracyclin antibiotics and has the ability to pump out fluoroquinolones, macrolides, and carbapenems (Islam et al., 2004).

2.3. Mutational impermeability

In *P. aeruginosa* the outer membrane limits the rate of penetration of small hydrophilic molecules and at the same time excludes larger molecules. The outer membrane also serves as an important barrier to the penetration of antibiotics. β-lactams and quinolones are small hydrophilic molecules which can only pass the outer membrane through the aqueous channels supplied by porin proteins. Several different porins are formed in *P. aeruginosa*. OprF is one of the major porins that is present in all strains of *P. aeruginosa*. Although mutant strains lacking the OprF porin have been documented, lack of this porin has not been reported to have a significant effect on antibiotic resistance of the organism to any drugs, most probably because such strains have limited capability to take up hydrophilic compounds (Lambert, 2002).

Resistance to imipenem and reduced susceptibility to meropenem is reported to be associated with loss of OprD porin. OprD is a porin which shapes narrow transmembrane channels and is only utilized by carbapenems but not other β-lactams. OprD is co-regulated with Mex-EF-oprN. Therefore, the *nfxC* (*mexT*) mutants that are selected by antibiotics such as fluoroquinolones (but not carbapenems) have up-regulated MexEF-oprN and lower OprD levels. These mutants subsequently have abridged

susceptibility to meropenem and resistance to both imipenem and fluoroquinolones (Livermore, 2002).

2.4. Alterations in penicillin-binding proteins

In *P. aeruginosa* alterations in penicillin-binding proteins are also related to β-lactam resistance. The efflux pump system adds only weakly to β-lactam resistance (caused by PBP mutations) most likely because the increased antibiotic buildup predicted in the deletion copies is still inadequate to overcome the lowered affinity of the PBP for β-lactams (Masuda et al., 2000).

2.5. DNA gyrase and topoisomerase IV mutations

The DNA gyrase is a holoenzyme which is a tetramer consisting of A and B subunits. These subunits are products of *gyrA* and *gyrB,* respectively (Kureishi et al., 1994). The DNA gyrase enzyme is a type II DNA topoisomerase responsible for introducing negative superhelical coils into covalently attached DNA in a process that is ATP-dependent (Kureishi et al., 1994). It also functions in DNA decatenation, replication, and transcription regulation of some promoters that are supercoil sensitive (Kureishi et al., 1994). The A subunits are in charge of DNA reunion and breakage, and the B subunits are dedicated to ATP hydrolysis (Kureishi et al., 1994). DNA gyrase has been proven to be a suitable target for antibiotics (Kureishi et al., 1994). Quinolones such as ciprofloxacin and nalidixic acid inhibit the action of the A subunits while

antibiotics such as coumermycin A1 and novobiocin are known to inhibit the action of B subunits (Kureishi et al., 1994). The glycocinnamoylspermidine agent cinodine and microcin (an antibiotic peptide) have also been reported to inhibit the function of the DNA gyrase enzyme (Kureishi et al., 1994).

In most countries fluoroquinolones are the only accessible antibiotics for oral treatment of infections caused by *P. aeruginosa* (Jalal et al., 2000). However, it easily becomes resistant to these antibiotics, rigorously constraining their effectiveness. Mutations in the target genes that encode DNA gyrase (*gyrA*) and topoisomerase IV (*parC*) are the main mechanisms of resistance to fluoroquinolones (Jalal et al., 2000). Mutational studies have shown that *parC* mutations in codon 80 (Ser→ Leu), and codon 84 (Glu→Lys) have been associated with higher fluoroquinolone resistance (Nakano et al., 1997). *GyrA* mutations in codon 83 (Thr→Ile) and codon 87 (Asp→Asn or Asp→Tyr) are associated with elevated fluoroquinolone resistance (Yonezawa et al., 1995). Multiple mutations in both *parC* and *gyrA* genes in codons 80, 83, 84, and 87 are linked to much more elevated resistance in *P. aeruginosa* (Mouneimne et al., 1999, Nakano et al., 1997.)

A recent study on fluoroquinolone resistant *P. aeruginosa* (MIC>32) clinical isolates have shown that *gyrA* mutations with an additional mutation in the *mexR* regulatory gene leads to accelerated resistance. This signifies that antibiotic resistance associated with DNA gyrase in general corresponds to efflux and *gyrA* mutations (Jalal et al., 2000, Nakajima et al., 2002).

2.6. P. aeruginosa biofilms and resistance

Creation of an alginate barrier is another resistance mechanism in *P. aeroginosa*. *P. aeruginosa* grows in microcolonies enclosed in an alginate barrier composed of various polysaccharides (Lambert, 2002). This kind of growth typifies biofilm (Lambert, 2002). Notable resistance to antibiotics is common to all biofilms (Lambert, 2002). Although documented for many years, the complete biological events in bioflim formation and antibiotic resistance are not well understood. Physical segregation of the antibiotic and high bacterial concentration are factors that participate in the resistance of bacteria in biofilm (Lambert, 2002). In general, reactions to physiological alterations may arise in cells in the biofilm in which defensive mechanisms are provoked and key metabolic pathways are shut down. It is apparent that cells in the biofilm can alter their properties according to surrounding cells by sensing their presence (quorum sensing) (Lambert, 2002). The biofilm consists of a heterogeneous population of cells, including slow and fast growing cells. Some cells are resistant through production of efflux pumps and inactivating enzymes, whereas others are not (Lambert, 2002). Therefore, the general resistance relies on the interaction of the entire population, and therapies need to be aimed against a community that is multicellular (Lambert, 2002).

2.7. Propositions for therapy

Efflux and restricted permeability are essential properties of the organism and are crucial in developing resistant phenotypes to antibiotics as diverse as quinolones, β-

lactams, and aminoglycosides. In other words, innate resistance results from the action of efflux systems and restricted permeability of the cell wall, and may be enhanced by elevated expression of special efflux pump systems (Lambert, 2002).

In CF patients, the incidence of more specific mechanisms which engage alteration and inactivation in target molecules reflects the selective pressure due to reliance of these patients to these drugs. For example, expression of chromosomal β-lactamase can be increased by spontaneous mutations in the chromosomal genes. These mutants are chosen under the stress of antibiotic treatment especially when monotherapy is used. Increased recognition of the role of efflux pumps in causing natural resistance to antibiotics has resulted in the search for efflux pump inhibitors which could serve as therapeutic adjuncts. Correspondingly, understanding of the multifaceted interaction in biofilm populations may ultimately lead to developing novel therapeutic approaches. However, *P. aeruginosa* has always adapted to antibiotic therapy. The threatening size of its genome and the present lack of information of the role of many of its genes will continue to be an important issue in developing novel therapies (Lambert, 2002).

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CHAPTER III

DETECTION OF POINT MUTATIONS ASSOCIATED WITH ANTIBIOTIC RESISTANCE IN *PSEUDOMONAS AERUGINOSA*

Neda Gorgani[a,b,1], Scott Ahlbrand[c,1], Andrew Patterson[c] and Nader Pourmand[a,d]*

[a] *Stanford Genome Technology Center, Stanford University, Palo Alto, CA 94304, USA*
[b] *Department of Biological Sciences, San Jose State University, San Jose, CA 95192, USA*
[c] *Stanford School of Medicine, Stanford University, Palo Alto, CA 94305, USA*
[d] *Biomolecular Engineering, University of California at Santa Cruz, Santa Cruz, CA 95064, USA*

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* Corresponding author. Tel.: +1 831 502 7315; fax: +1 831 459 4829.
E-mail address: pourmand@soe.ucsc.edu (N. Pourmand).

[1] These authors have equal contribution to this work.

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 above-mentioned 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 determining the antibiotic-resistance pattern of a given bacterial strain in < 1 h.

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 limited number of antimicrobial agents, e.g. methicillin-resistant *Staphylococcus aureus* (MRSA), *Enterococcus faecium*, and Gram-negative bacteria producing extended-spectrum β-lactamases (Kollef and Micek, 2005).

Multidrug-resistant (MDR) bacterial infections are associated with increased mortality, length of hospital stay, and cost of care (Geissler et al., 2003). 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 methicillin-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 (Nseir et al., 2005).

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* (Sherertz and Sarubbi, 1983). 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 (Lambert, 2002, Livermore, 2002).

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 the peptidyl-tRNA from the A-site to the P-site, causing misreading of the mRNA and thus rendering the bacterium unable to synthesize 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* (Jalal et al., 2000). 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* (Aires et al., 1999, Islam et al., 2004). Another region related to aminoglycoside resistance is *mexOZ,* which is an intergenic region between the *mexZ* and *mexX* genes of *P. aeruginosa* (Islam et al., 2004).

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 P. 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-Baur 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 (Islam et al., 2004). All primers were synthesized by Integrated DNA Technologies (Coralville, IA).

PCR amplification was performed in a 50 µl mixture containing 1X Thermo-Start™ Buffer (ABgene, Rockford, IL), 2.5 mM $MgCl_2$, 0.2 mM mix of deoxynucleotide triphosphates (Sigma-Aldrich, St Louis, MO), 10 pmole of each primer, 1U 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 (Gharizadeh et al., 2006). 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 the 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 pmole of each primer, 1U 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 (Gharizadeh et al., 2005).

Single-stranded DNA amplicons were prepared semi-automatically, using a Vacuum Prep Tool and Vacuum Prep Worktable (Biotage, Uppsala, Sweeden). A 10 µl aliquot of biotinylated PCR products was immobilized onto 3 µl 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 Eppendorf Thermomixer R (Eppendorf AG, Hamburg, Germany). Double-stranded DNA immobilized 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 pmole 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 (Gharizadeh et al., 2005). Single-stranded PCR products were sequenced using a PSQ™ HS 96A System (Biotage). Sequencing was preformed 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 harboring 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 analyzed 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).

Table 1

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

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

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 (Jalal et al., 2000, Nakano et al., 1997, Yonezawa et al., 1995). No mutations were found in *parC*. A novel mutation related to

fluoroquinolone resistance occurred in codon 126 of the *mexR* regulatory gene, changing amino acid Val to Glu was detected (Fig. 2; Table 2).

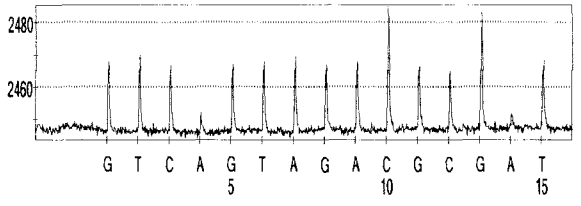
Table 2

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

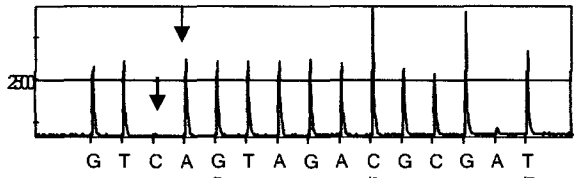
Fluoroquinolone susceptibility (no. of isolates)	Mutations in *gyrA* only	Mutations in *mexR* only	Mutations in both *gyrA* and *mexR*	No mutations in *gyrA* or *mexR*
Resistant (12)	4	0	4	4
Intermediate (6)	1	1	1	3
Suscpetible (41)	1	12	1	27

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* PA01 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 (Figs. 1 and 2). All mutations detected by Sanger sequencing were confirmed by the Pyrosequencing™ method.

(a)



(b)



(c)

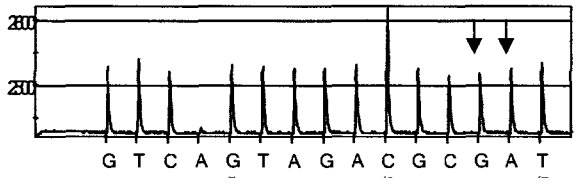
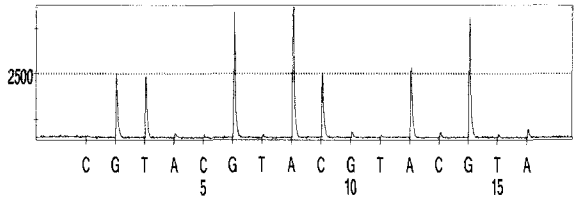


Fig. 1. Pyrograms of the 20-bp sequence (amino acids 83-87) of the *gyrA* gene of *P. aeruginosa* obtained by the pre-programmed DNA-sequencing method:
(a) the wild-type sequence of *gyrA* with no alterations; (b) nucleotide C → A alteration in codon 83; and (c) nucleotide G → A alteration in codon 87. Arrows show the location of point mutations in the signal peaks.

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 analyzed 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.

(a)



(b)

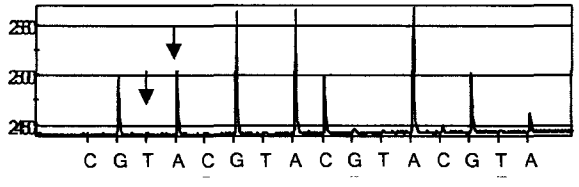


Fig. 2. Pyrograms of the 20-bp sequence of the *mexR* regulatory gene of *P. aeruginosa* (amino acids 126-128) obtained by the pre-programmed DNA-sequencing method: (a) the wild-type sequence of *mexR* with no alterations; and (b) nucleotide T → A alteration in codon 126. Arrows show the location of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

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.

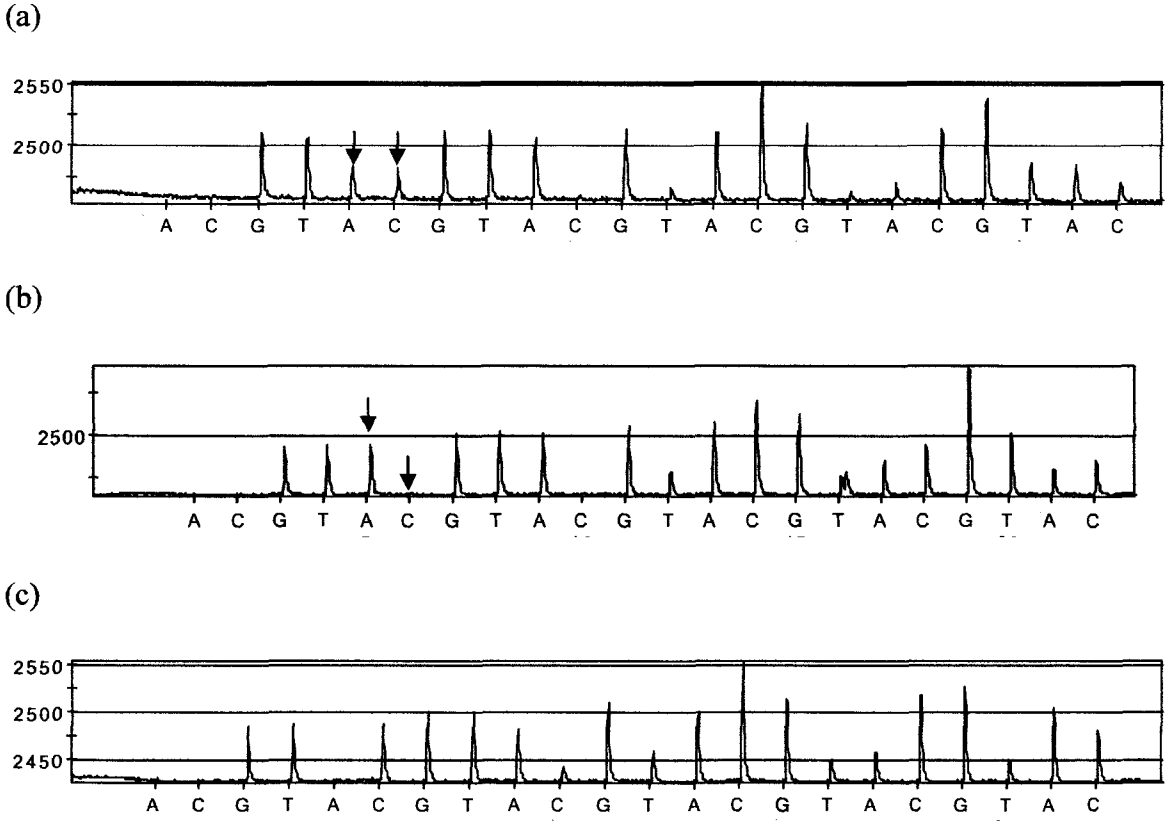


Fig. 3. Pyrograms of the DNA sequence of *gyrA* for an isolate of *P. aeruginosa.* (a) Sequencing data before cloning and isolation. The two arrows indicate the presence of both A and C nucleotides in the sequence. The *gyrA* gene was cloned into a plasmid

vector, amplified by polymerase chain reaction (PCR) and sequenced by Pyrosequencing™ using a five- cycle ACGT dispensation. (b and c) pyrograms after isolation of the mixed DNA sample; ca. 50% of the cloned vectors harbored DNA from wild-type (c) and 50 % harbored DNA from a mutant of *P. aeruginosa* (b) (C → A alteration).

4. Discussion

Fluoroquinolone and aminoglycoside resistance can lead to treatment failure in *P. aeruginosa* infections (Aires et al., 1999, Jalal et al., 2000). Known mutations responsible for resistance are found in the genes expressing DNA gyrase, and topoisomerase IV and in genes that regulate the expression of efflux pumps. These mutations 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 requiring 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 to other amplification and sequencing methods that take up to 5 hours.

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 (Jalal et al., 2000, Jalal and Wretlind, 1998, Nakano et al., 1997, Yonezawa et al., 1995). We discovered a mutation in codon 126 of the *mexR* regulatory gene, changing amino acid Val to Glu, which correlated with fluoroquinolone resistance. Almost all the clinical isolates in this study that had a single mutation in *mexR* were susceptible and did not show any drug resistance, 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 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 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 analyzed 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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GENERAL CONCLUSIONS

Opportunistic *P. aeruginosa* infection is a major problem in medical care. The growing threat from resistant strains calls for development of accurate diagnostic methods and effective treatment strategies. Recent studies have focused on molecular mechanisms of antibiotic resistance and genetic parameters involved in development, acquisition, and spread of resistance genes. Reports have demonstrated several molecular mechanisms of antibiotic resistance. Understanding the distribution and combinations of genetic resistance mechanisms will contribute to identify potential targets for new antibiotics, and developing better diagnostic tools and treatment strategies to meet the challenges of *P. aeruginosa* infection.

In this study, a new molecular approach using DNA-sequencing methods (Pyrosequencing™ and multiplex-fast PCR), clinical isolates of *P. aeruginosa* were analyzed quickly for point mutations leading to resistance to current antibiotics. The method described in this study, can be readily expanded to cover a variety of known resistance determinants in *P. aeruginosa*. This technique could also be used as a general approach for mutation detection in other microbial resistance studies. Rapid and simple determination of resistance determinants at the genetic level could guide the choice of more appropriate antibiotics, and enable the development of narrower spectrum antibiotics. Ultimately, more accurate diagnosis and treatment could lower the incidence of resistance, and improve outcomes for patients with severe bacterial infections.

In this study, we designed a molecular technique for rapid, easy, and cost-effective diagnosis of antibiotic resistance in clinical isolates of *P. aeruginosa* that can have application in epidemiological studies, and developing and implementing strategies for infectious diseases. Further investigation on larger numbers of isolates collected from different medical centers will be required. These isolates should include resistant as well as susceptible strains with their relevant susceptibility profiles which could not be validated in this preliminary study. Epidemiological data indicating the patient's status and the type of infectious diseases not included in this study will be helpful in interpreting the results.

APPENDIXES

APPENDIX A. SUMMARY OF DNA-SEQUENCING RESULTS

Table 1A. *GryA*, *parC*, *mexR* mutations, and fluoroquinolone susceptibility of *P. aeruginosa* clinical isolates.

Sample ID	*gyrA* Mutations	*parC* Mutations	*mexR* Mutations	Fluoroquinolone Susceptibility
M583111	none	none	none	susceptible
M586966	none	none	(126) Val→Glu	susceptible
M587161	none	none	(126) Val→Glu	susceptible
M586030	none	none	none	susceptible
W607182	none	none	none	susceptible
M586970	none	none	none	susceptible
M581880	none	none	none	susceptible
M586981	none	none	(79) Asn→Ser (126) Val→Glu	susceptible
F598875	none	none	none	susceptible
T613503-1	none	none	none	susceptible
T613503-2	none	none	none	susceptible
T613898-2	none	none	(132) Val→Ala	susceptible
T614089-2	none	none	(126) Val→Glu	susceptible
F597588-1	none	none	none	susceptible
F597588-3	none	none	none	susceptible
F596760-3	none	none	(126) Val→Glu	susceptible
F596760-2	none	none	none	susceptible
T614204-2	none	none	none	susceptible
T614052-1	none	none	none	susceptible
T612031-1	none	none	none	susceptible
T612031-2	none	none	none	susceptible
T613324-3	none	none	(126) Val→Glu	susceptible
T613324-2	none	none	none	susceptible
T614084-1	none	none	none	susceptible
T614084-2	none	none	none	intermediate
H605658-1	(87) Asp→Asn	none	none	susceptible
M595294-2	none	none	(126) Val→Glu	susceptible

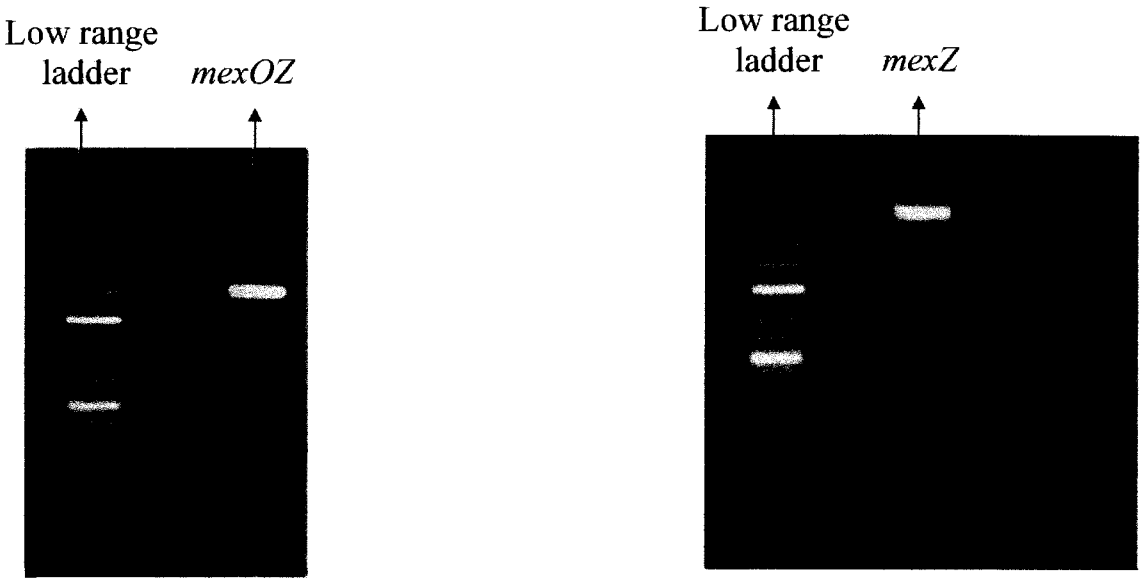
W618073	(87) Asp→Tyr	none	(132) Val→Ala (126) Val→Glu	susceptible
F615319	none	none	none	susceptible
F618171	none	none	none	susceptible
X360656	none	none	(126) Val→Glu	susceptible
M628794	none	none	none	susceptible
H640	none	none	none	susceptible
W643857	none	none	(126) Val→Glu	susceptible
F644797	none	none	(126) Val→Glu	intermidiate (levofloxacin)
H638643-3	(87) Asp→Tyr	none	none	intermidiate (levofloxacin)
F35726-2	none	none	(126) Val→Glu	susceptible
H638643-2	(87) Asp→Tyr	none	none	resistant
H638636	none	none	none	intermidiate (levofloxacin)
F624989	none	none	(126) Val→Glu	susceptible
M674446-2	none	none	none	resistant
M674446-1	none	none	none	resistant
H682001-5	(83) Thr→Ile	none	(126) Val→Glu	intermediate
F676239-6	none	none	none	intermediate
F676239-5	none	none	none	susceptible
M665266-1	(83) Thr→Ile	none	(126) Val→Glu	resistant
F660729-2	none	none	none	susceptible
H663398	(83) Thr→Ile	none	none	resistant
T682138-4	(83) Thr→Ile	none	(126) Val→Glu	resistant
M665266-2	(83) Thr→Ile	none	(126) Val→Glu	resistant
F660729-1	none	none	none	susceptible

Table 1B. *MexZ and mexOZ* mutations, and tobramycin susceptibility of *P. aeruginosa* clinical isolates.

Sample ID	*mexZ* Mutations	*mexOZ* Mutations	Tobramycin Susceptibility
M583111	no amplification	295, 459 bps	susceptible
M586966	(138) Leu→Arg (186) Asn→ser	295, 459 bps	susceptible
M587161	no amplification	244bp	susceptible
M586030	none	244 bp	susceptible
W607182	none	Insertion (frame shift)	susceptible
M586970	none	244, 358 bps	susceptible
M581880	none	244 bp	susceptible
M586981	no amplification	199, 229, 268, 415	susceptible
F598875	none	244bp	susceptible
T613503-1	Insertion (frame shift)	244 bp	susceptible
T613503-2	(95) Glu→Stop	244 bp	susceptible
T613898-2	none	244, 302 bps	
T614089-2	(138) Leu→Arg (186) Asn→ser (58) Val→Ala	295, 459 bps	susceptible
F597588-1	Deletion (frame shift)	244 bp	susceptible
F597588-3	Deletion (frame shift)	244 bp	susceptible
F596760-3	none	244, 274 bps	susceptible
F596760-2	none	244, 274 bps	susceptible
T614204-2	Deletion (frame shift)	none	susceptible
T614052-1	(131) Lys→Arg	244 bp	susceptible
T612031-1	(131) Lys→Stop	none	susceptible
T612031-2	(131) Lys→Stop	none	susceptible
T613324-3	Deletion (frame shift)	244 bp	susceptible
T613324-2	none	244 bp	susceptible
T614084-1	Deletion (frame shift)	244 bp	susceptible
T614084-2	(191) Met→Arg	244 bp	intermediate
H605658-1	(163) Leu→Pro	244 bp	susceptible
M595294-2	none	244 bp	susceptible
M647759	(46) Gly→Cys	244, 201 bps	resistant
W670409-2	Deletion (frame shift)	244 bp	susceptible

W670409-3	Deletion (frame shift)	244 bp	susceptible
F660729	Deletion (frame shift)	no amplification	resistant
H663398-3	none	244 bp	resisant
T660514	none	244 bp	resistant
F624989	Deletion (frame shift)	295, 459 bps	susceptible
H649427	none	244, 324 bps	intermediate
W618073	no amplification	244 bp	susceptible
F615319	none	244 bp	susceptible
F618171	none	none	susceptible
X360656	(138) Leu→Arg (186) Asn→Ser	295, 459 bps	susceptible
M628794	none	244, 453 bps	susceptible
H640927	Deletion and insertions (frame shift)	none	susceptible
W643857	Deletion (frame shift)	244, 358 bps	susceptible
F644797	none	244 bp	intermediate
H638643-3	no alignment	244, 277 bps	susceptible
F35726-2	none	244, 489 bps	susceptible
H638643-2	(144) Ala→Val	244 bp	susceptible
H638636	none	244 bp	susceptible
F624989	Deletion (frame shift)	295, 459 bps	susceptible
M674446-2	none	244 bp	resistant
M674446-1	none	244 bp	resistant
H682001-5	(138) Leu→Arg (186) Asn→Ser	295, 335, 459 bps	resistant
F676239-6	(62) Met→Lys	244, 489 bps	resistant
F676239-5	(62) Met→Lys	244, 489 bps	resistant
M665266-1	(138) Leu→Arg	295, 459 bps	intermediate
F660729-2	(209) Asp→Gly	no amplification	susceptible
H663398	none	244 bp	resistant
T682138-4	no alignment	295, 459 bps	resisant
M665266-2	no alignment	295, 459 bps	resistant
F660729-1	none	no ampliication	susceptible

APPENDIX B. AGAROSE GEL ELECTROPHORESIS RESULTS



P. aeruginosa *mexOZ* **(382bp)** and *mexZ* **(594bp)** PCR product

Figure 1A and 1B. Agarose gel electrophoresis (1.5%) results of *P. aeruginosa* PCR products using *mexOZ* and *mexZ* primers.

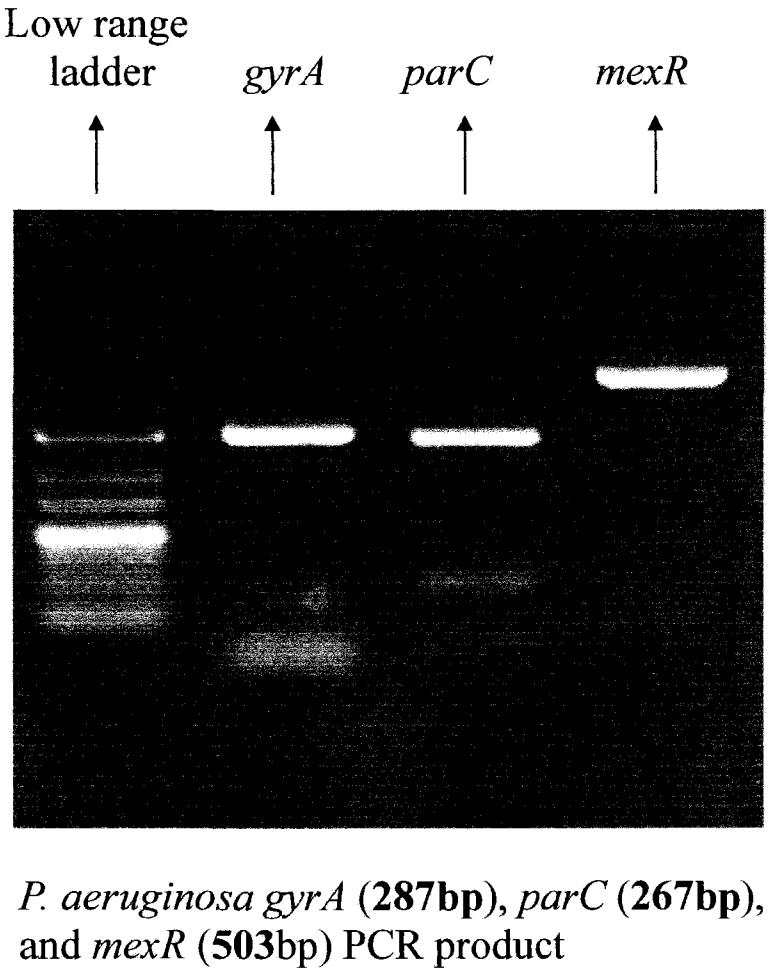


Figure 2. Agarose gel electrophoresis (1.5%) result of *P. aeruginosa* PCR products using *gyrA, parC,* and *mexR* primers.