Universidade de Aveiro Departamento de Biologia
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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Microbiologia Molecular, realizada sob a orientação científica da Doutora Sónia Mendo, Professora Auxiliar do Departamento de Biologia da Universidade de Aveiro.

o júri

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resumo

Pseudomonas aeruginosa é um organismo patogénico, de Gram-negativo, frequentemente associado a infecções nosocomiais. O preocupante aumento de estirpes de *P. aeruginosa* resistentes a quinolonas constituíu a base para o presente trabalho. Assim, estudaram-se trinta e cinco isolados de *P. aeruginosa* resistentes a quinolonas, obtidos pelo serviço de patologia do Hospital Infante D. Pedro, Aveiro.

Numa primeira fase do trabalho procedeu-se à tipagem molecular dos isolados, os quais foram comparados por análise de restrição de DNA ribossomal amplificado (ARDRA), por BOX-PCR e por electroforese em campo pulsado (PFGE). Os resultados obtidos mostraram que os pacientes já se encontravam colonizados por mais do que um genótipo aquando da colheita, sugerindo uma elevada disseminação de *P. aeruginosa* fora do ambiente hospitalar. Por PFGE verificou-se também uma grande heterogeneidade entre os isolados. Assim, os resultados obtidos mostraram que a contaminação cruzada é uma importante via para aquisição de *P. aeruginosa.*

Os alvos das quinolonas são as topoisomerases do tipo II (DNA girase e topoisomerase IV). O aparecimento de mutações nos genes que codificam estas enzimas (*gyr*A, *gyr*B, *par*C and *par*E) foi averiguado. Os resultados revelaram a presença de algumas mutações novas em 16 dos isolados. Estas, foram identificadas na QRDR dos genes *gyr*B (codão 465), gene *par*C (codão 35) e gene *par*E (codões 431, 483, 487, 530, 538 e 544). Nenhuma das mutações foi anteriormente descrita em *P. aeruginosa*.

Dado o *background* genético dos isolados foi também estudada a expressão dos genes cujos produtos são intervenientes dos sistemas de efluxo. Por RT-PCR verificou-se que a estirpe Pa31 não expressava os genes *mex* B, *mex* F e *mex*Y. Este resultado conduziu a um estudo de crescimento, que mostrou tratar-se de um isolado com um crescimento mais lento do que os restantes o que possivelmente terá um impacto no “fitness” *in vivo* em meio de cultura sem antibiótico.

abstract

Pseudomonas aeruginosa is a Gram-negative pathogen associated with a number of nosocomial infections. The preoccupying increase of quinolone resistant *P. aeruginosa* was the basis for the present study. Thirty five quinolone resistant *P. aeruginosa* strains were obtained from the pathology service of Hospital Infante D. Pedro, Aveiro.

Firstly, the 35 isolates were typed using molecular methods. Those methods consisted in ribossomal restriction of amplified DNA (ARDRA), Box-PCR and Pulse Field Gel Electrophoresis (PFGE). The results obtained revealed that the patients were already colonized by more than one genotype on admission suggesting a rapid dissemination of *P. aeruginosa* outside the hospital environment. PFGE revealed a high degree of heterogeneity between isolates. The results obtained suggested that cross contamination is an important route for *P. aeruginosa* acquisition.

Type II topoisomerase (DNA gyrase and Topoisomerase IV) are the targets of quinolones. Mutations in the genes coding for these enzymes (*gyr*A, *gyr*B, *par*C and *par*E) were also studied. The results revealed the presence of mutations in 16 of the 35 strains. Some of the mutations found are new and have never been described before in *P. aeruginosa*. Those mutations were identified in the QRDR of *gyr*B gene, (codon 465) in the QRDR of *par*C gene (codon 35) and also in the *par*E gene (codon 431, 483, 487, 530, 538 and 544)

Given the genetic background of the isolates, expression of the genes coding for membrane proteins involved in the efflux systems was also analysed. RT PCR showed that strain Pa31 was down regulated to *mex* B, *mex* F and *mex*Y. A growth experiment was carried out on some strains showing QRDR mutations revealing that strain Pa31 shows a slower growth rate when compared to others. This is likely to have an impact on it's *in vivo* fitness in antibiotic free medium.

CONTENTS

INTRODUCTION

1 INTRODUCTION

1.1 *Pseudomonas aeruginosa* as a nosocomial agent

Pseudomonas aeruginosa is a Gram-negative, aerobic rod-shaped bacteria belonging to the Pseudomonadaceae family. The classification of pseudomonads is based on rRNA/DNA homology and common culture characteristics. In nature, the typical *Pseudomonas* bacterium might be found in biofilms, attached to some surface or substrate, or in a planktonic form, as a unicellular organism, actively swimming by means of its flagellum (Brooks *et al.*, 2001).

P. aeruginosa isolates may produce three colony types. Natural isolates from soil or water typically produce a small, rough colony. Clinical samples, in general, yield one or another of two smooth colony types. One type has a fried-egg appearance which is large, smooth, with flat edges and an elevated appearance. Another type, frequently obtained from respiratory and urinary tract secretions, has a mucoid appearance, which is attributed to the production of alginate slime. The smooth and mucoid colonies are presumed to play a role in colonization and virulence due to the presence of some virulence factors (Figure 1). *P. aeruginosa* strains produce two types of soluble pigments, the fluorescent pigment pyoverdin and the blue pigment pyocyanin. The latter is produced abundantly in media of low-iron content and has functions in iron metabolism in the bacterium. Pyocyanin (from "pyocyaneus") refers to "blue pus" which is a characteristic of suppurative infections caused by *P. aeruginosa* (Brooks *et al.*, 2001).

P. aeruginosa is an opportunistic pathogen, meaning that it exploits some break in the host defences to initiate an infection. It causes urinary tract infections, respiratory system infections, dermatitis, soft tissue infections, bacteremia, bone and joint infections, gastrointestinal infections and a variety of systemic infections, particularly in patients with severe burns, in cancer and AIDS patients who are immunosuppressed. The case fatality rate in these patients is 50 percent (*In*: http://www.bact.wisc.edu/Bact330/lecturepseudomonas).

Among nosocomial bacterial infections, those caused by *P. aeruginosa* are associated with highest mortality rate, and are difficult to eradicate from infected tissues or blood because those microrganisms are virulent and have a limited susceptibility to antimicrobials (Loureiro *et al.*, 2002). Nosocomial strains, in sharp contrast to community-acquired strains, exhibit high rates of resistance to antibiotics and are frequently multidrug resistant a fact probably related to the ease with which they can develop resistance in a hospital environment (Lomovskaya *et al.*, 2001).

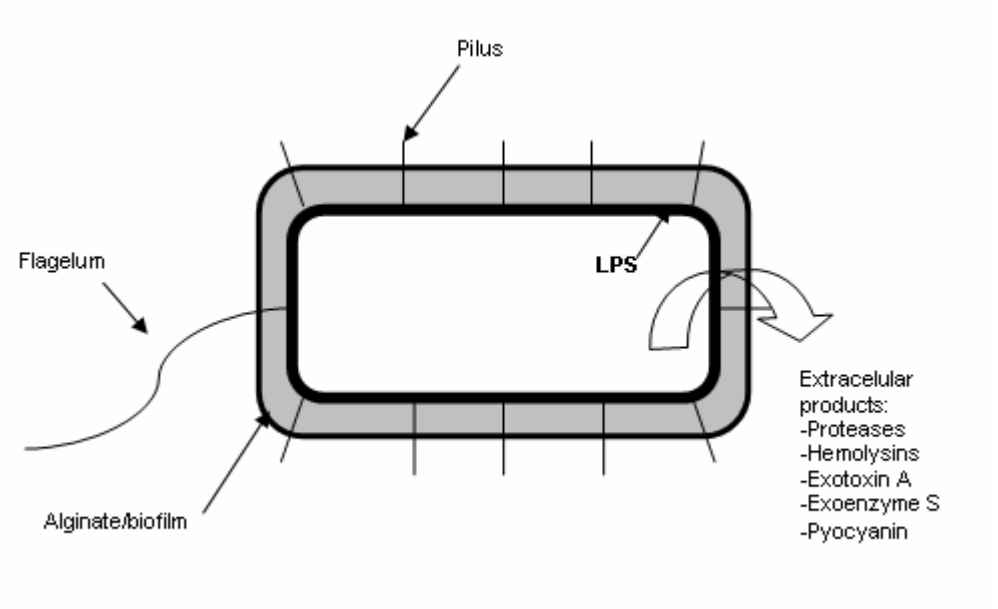

Figure 1- Virulence factors of *P. aeruginosa*: cell-associated (flagellum, pilus, alginate/biofilm, lipopolysaccharide [LPS]) and extracellular virulence factors (proteases, hemolysins, exotoxin A, exoenzyme S, pyocyanin).

P. aeruginosa shows significant degrees of intrinsic and acquired resistance to a wide variety of antimicrobial agents, including most *ß*-lactams, fluoroquinolones, tetracycline, chloramphenicol, erythromycin. That fact represents a serious clinical problem as *P. aeruginosa* is a major opportunistic pathogen and a leading cause of nosocomial infections (Tomofusa *et al.*, 2003).

1.2 Molecular epidemiology of bacterial strains

Several genotyping methods have been used for the molecular typing of bacterial strains with different purposes. These methods include: pulsed-field gel electrophoresis (PFGE) (Elaichouni *et al.*, 1994; Paraskaki *et al.*, 1996), ribotyping (Grothues *et al.*, 1988; Pfaller *et al.*, 1992), amplified ribosomal DNA restriction analysis (ARDRA) and a few polymerase chain reaction (PCR) based fingerprinting methods (Pfaller *et al.*, 1992; Speijer *et al.*, 1999). Each of these methods permits a certain level of phylogenetic classification, from the genus, species, subspecies, to strain specific level (Figure 2).

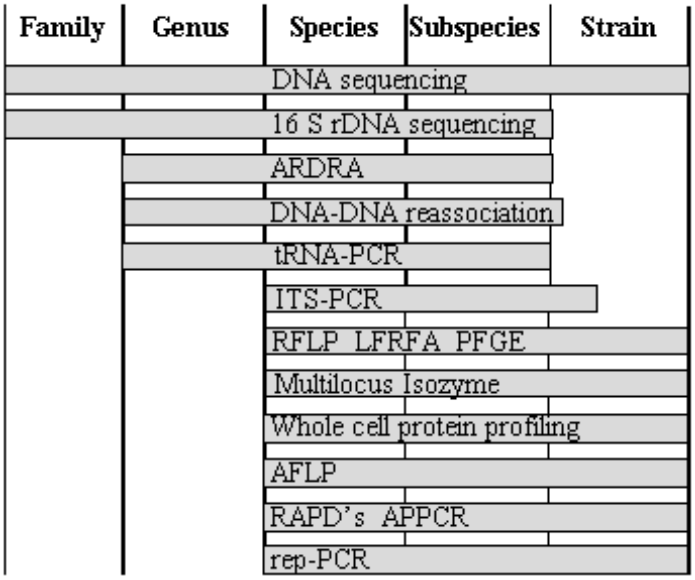

Figure 2- Relative resolution of various fingerprinting and DNA techniques (Rademaker *et al.*, 1997).

Molecular typing is performed to determine whether different isolates give the same or different results for one or more tests. Epidemiologically related isolates share the same DNA profile or fingerprint, whereas epidemiologically unrelated isolates have distinctly different patterns. If isolates from different patient/person share the same fingerprint, they probably originated from the same clone and were transmitted between patient/person by a common source or mechanism.

All these methods use electric fields to separate DNA fragments or proteins according to size on gels under the influence of strong electric field. This type of procedures separates shorter DNA fragments. Pulsed field gel electrophoresis (PFGE) is useful for separation of larger DNA molecules. At present, PFGE is considered to have both the reproducibility and resolving power of a standard technique for the epidemiological typing of bacterial isolates. DNA fingerprinting by PFGE of genomic DNA, after digestion with appropriate restriction endonucleases that cleave infrequently, has emerged as a technique that is broadly applied in molecular epidemiology studies being considered the gold standard technique with which all other molecular typing techniques are compared.

Some molecular typing methods rely on the use of the action of a temperature-resistant DNA polymerase, Taq polymerase, which catalysis growth from DNA primers. With the use of PCR, a single copy gene can be amplified out of a genomic sample, provided primers corresponding to known sequences of the gene can be synthesized. Because of the exponential amplification, PCR is very sensitive and can detect target sequences that are in extremely low copy number in a sample. PCR is generally useful in DNA diagnostics, in other words, in checking for the presence of a gene or the mutational state of a specific gene or, preparatively, in amplifying a defined segment.

The comparison of rRNA sequences constitutes a powerful tool for deducing phylogenetic and evolutionary relationships among bacteria, archaebacteria, and eucaryotic organisms (Weisburg *et al.*, 1991). Amplified ribosomal DNA restriction analysis (ARDRA) uses the 16S rRNA gene for this purpose. This molecular technique has been successfully used for bacterial community analysis in a great variety of environments (Giraffa *et al.*, 1998; Lagacé *et al.*, 2004). ARDRA is aimed at species but not strain differentiation, as it relies on the conserved nature of rDNA. ARDRA aims at overlooking intraspecific differences, which facilitates the recognition of species-specific patterns. This method has been shown to be of great help in the elucidation of the ecology and taxonomy of species of Acinetobacter and many other genera (Vaneechoutte *et al.*, 1993). Breafly, this technique implies a primary step were the 16S rDNA is amplified by PCR. Conserved regions proximal to the 5' and 3'

termini are found in all prokaryotic 16s rRNA sequences and are therefore used as primer sequences in the PCR to amplify almost the entire gene. In the second step, the experimentally obtained restriction patterns are normalized and combined by means of the pattern recognition (Heyndrickx *et al.*, 1996). The data thus obtained allow the rapid identification of related species and the construction of dendrograms.

Several examples of naturally occurring, highly conserved, repetitive DNA sequences, present in multiple copies in the genomes of most Gram-negative and several Gram-positive bacteria were described by Lupski and Weinstock, 1992. Three families of repetitive sequences have been identified, including the 35-40 bp repetitive extragenic palindromic (REP) sequence, the 124-127 bp enterobacterial repetitive intergenic consensus (ERIC) sequence, and the 154 bp BOX element (Versalovic *et al.*, 1994). These sequences appear to be located in distinct, intergenic positions around the genome. The repetitive elements may be present in both orientations, and oligonucleotide primers have been designed to prime DNA synthesis outward from the inverted repeats in REP and ERIC, and from the boxA subunit of BOX, in the PCR (Versalovic *et al.*, 1994). The use of these primer(s) and PCR, leads to the selective amplification of distinct genomic regions located between REP, ERIC or BOX elements. The corresponding protocols are referred to as REP-PCR, ERIC-PCR and BOX-PCR genomic fingerprinting respectively, and rep-PCR genomic fingerprinting collectively (Versalovic *et al.,*1991; Versalovic *et al.,*1994). The amplified fragments can be resolved in a gel matrix, yielding a profile referred to as a rep-PCR genomic fingerprint (Versalovic *et al.*, 1994) (Figure 3). REP-PCR, ERIC-PCR and BOX-PCR genomic fingerprints generated from bacterial isolates permit differentiation to the species, subspecies and strain level.

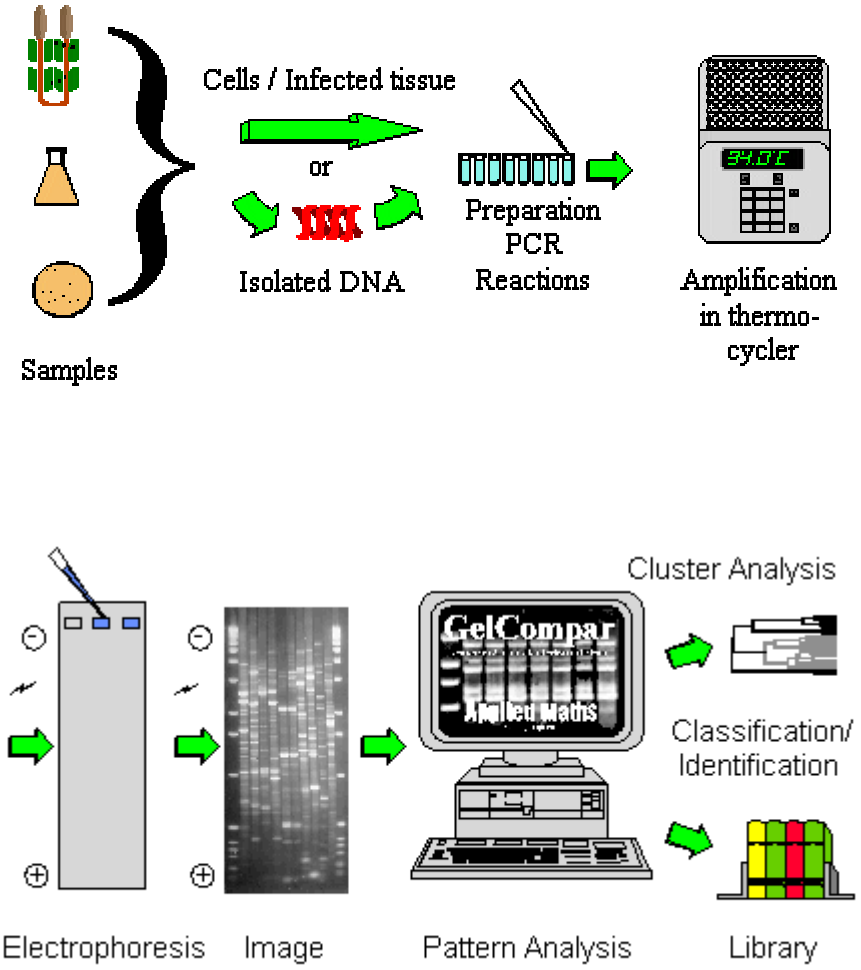

Figure 3- Basic procedure to achieve a phylogeny based on genomic fingerprints. (Rademaker *et al.*, 1997).

1.2.1 Molecular epidemiology of quinolone resistant *P. aeruginosa*

P. aeruginosa is frequently isolated from patients in which the strains are not necessarily epidemiologically or genetically related. Therefore, many studies have been directed at this microrganism. Genomic fingerprinting methods are now regarded as the most accurate methods for the typing of microrganisms for epidemiological purposes (Pfaller *et al.*, 1992). Typing of strains is important for eradication of

environmental sources as well as prevention of cross-infections and monitoring of antimicrobial therapy efficacy (Loureiro *et al.*, 2002).

In *P. aeruginosa* the discriminatory power of ribotyping when it is performed with the enzyme *Pvu* II has been claimed to be a valuable tool but slightly less than PFGE (Pfaller *et al.*, 1992; Brisse *et al.,* 2000).

RAPD, AFLP, RFLP were also used in several epidemiological studies (Speijer *et al*., 1999; Anthony *et al.,* 2002) but PFGE is currently considered the gold standard for bacterial typing and has been shown to be the method preferentially used for *P. aeruginosa* (Tassios *et al*., 1998).

1.3 Antibiotics

Drugs have been used for treatment of infectious diseases since the 17th century, however chemotherapy as a science began with Paul Ehrlich in the first decade of the 20th century. The current era of antimicrobial chemotherapy began in 1935, with the discovery of sulphonamides. In 1940, it was demonstrated the penicillin, which was discovered in 1929, could be an effective therapeutic substance. During the next 25 years, research on chemotherapeutic agents largely centered on substances of microbial origin called antibiotics (Brooks *et al*., 2001).

Antibiotics are substances produced by fungi and bacteria which, in small quantities, can inhibit the growth of microrganisms or even kill them. There are several classifications of the important antibiotics based on their chemical structure, their biological origin and their therapeutic use. Antibiotics of the same class have similar modes of action and spectra of activity; they generally share resistance and are similar in their toxicity. An ideal antimicrobial agent exhibits selective toxicity. Often selective toxicity is relative rather than absolute; this implies that a drug in a concentration tolerated by the host may damage an infecting microrganism. Selective toxicity may be a function of a specific receptor required for drug attachment, or it may depend on the organism but not on the host. A few antibiotics, which act selectively on certain pathogens, are called narrow-spectrum antibiotics. Broad-spectrum antibiotics are effective against a wide range of pathogens (Brooks *et al*., 2001).

The antibacterial activity of an antibiotic may be measured *in vitro* by determining the minimal inhibitory concentration (MIC). While some antibiotics merely inhibit the multiplication of bacteria and therefore called bacteriostactic, others actively kill microorganisms having thus a bactericidal activity. The most important bactericidal antibiotics are the penicillins, the cephalosporins and the aminoglycosides (Simon *et al.,* 1985).

The mechanisms of action of most antibiotics are well known, and are generally similar in drugs of the same group, giving rise to a pattern of complete cross-resistance. Antibiotics act by inhibiting bacterial cell wall synthesis, inhibiting the synthesis of cytoplasmic components, damaging the cytoplasmic membrane or disrupting nucleic acids synthesis. Knowledge of these mechanisms is important to understand the basis of the combined action of antibiotics, since a synergistic effect only occurs when the patterns in the combination have different sites of action (Simon *et al.,* 1985).

Sensitivity to antibiotics varies between different species of bacteria, between different strains of the same species and even within the same bacterial population (Simon *et al.,* 1985).

The antibiotic sensitivity pattern of some bacterial species, such as pneumococci, gonococci, meningococci and *Haemophilus influenzae*, is largely predictable; other species such as staphylococci, enterococci, *Escherichia coli*, *Klebsiella*, *P. aeruginosa*, *Proteus* and *Mycobacterium tuberculosis* show considerable variations (Simon *et al.,* 1985).

1.4 Quinolones

This is a group of synthetic antibacterial agents, analogs of nalidixic acid (Figure 4), effective in the treatment of selected community-acquired and nosocomial infections (Brooks *et al*., 2001).

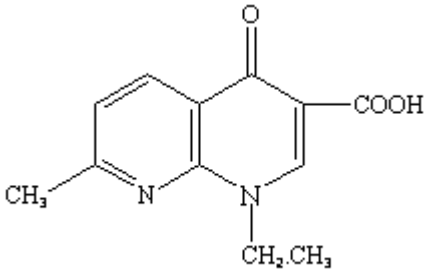

Figure 4- Acid Nalidixic molecule

Quinolones are bactericidal and exhibit concentration-dependent killing. The earlier quinolones did not achieve systemic antibacterial levels after oral intake and thus were useful only as urinary antiseptics (Mandigan *et al.,* 2003). Since the first quinolone, nalidixic acid, was developed the quinolones have undergone structural modifications, in particular the addition of fluorine at the position 6, to produce synthetic derivates, the fluoroquinolones. This change in the structure has seen their potency and pharmakokinetic profile greatly increased (Mandigan *et al.,* 2003).

Early quinolones, such as nalidixic acid, had poor systemic distribution and limited activity, and were primarily used for treatment of Gram-negative urinary tract infections. Nalidixic acid, was followed by a second generation of quinolone agents, the fluoroquinolones (e. g. ciprofloxacin, Figure 5, ofloxacin, norfloxacin, lomefloxacin, and enoxacin), that were more readily absorbed and displayed increased activity against Gram-negative bacteria. Newer fluoroquinolones (e.g., levofloxacin, sparfloxacin, trovafloxacin, and grepafloxacin) are broad-spectrum agents with enhanced activity against many Gram-negative and Gram-positive organisms.

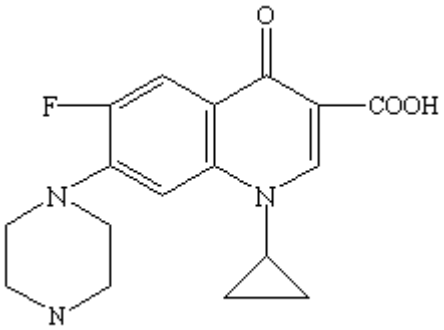

Figure 5- Structural modifications on the nalidixic acid molecule (addition of fluorine at position 6 and introduction of a piperzine and a cyclopropil) giving rise to the Ciprofloxacin molecule.

The quinolones are a potent group of drugs acting against DNA metabolism. Their targets are the essential bacterial enzymes DNA gyrase and topoisomerase IV (Higgins *et al.*, 2003). Overuse of these drugs, in certain situations, is selecting for quinolone resistant mutants and these may threaten the long term use of such compounds. Also, attempts to repair mechanisms by the bacterial cell may lead to the generation of resistant mutants. *In vitro* selection of resistance mutations has allowed the observation of how resistance is acquired and some of the modifications in newer fluoroquinolones have resulted in the shift of primary target from topoisomerase IV to gyrase in Gram-positive bacteria (Higgins *et al.,* 2003).

1.5 Mechanisms of action of Quinolones

Quinolones rapidly inhibit DNA synthesis by promoting cleavage of bacterial DNA in the DNA-enzyme complexes of DNA gyrase (encoded by genes *gyr*A and *gyr*B) and type IV topoisomerase (encoded by genes *par*C and *par*E), resulting in rapid bacterial death (Lampidis *et al.*, 2002). As a general rule, Gram-negative bacterial activity correlates with inhibition of DNA gyrase, and Gram-positive bacterial activity with the inhibition of DNA type IV topoisomerase (Hooper *et al.*, 1995), Figure 6.

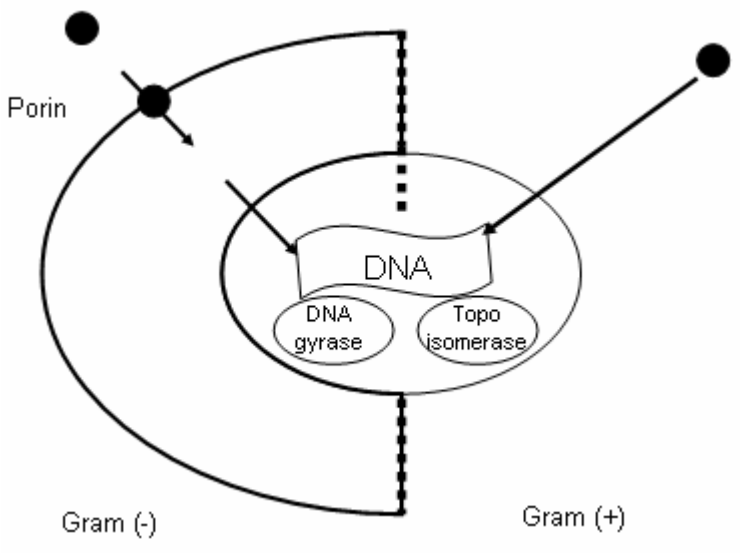

Figure 6- Mechanisms of action of quinoles in Gram-negative and Gram-positive bacteria. (Adapted from http://www.sbimc.org)

1.6 Mechanisms of Quinolones resistance

The causes for antimicrobial resistance are multifactorial. In the case of antibiotics, it has been well documented that resistance is mainly caused by continued over reliance on and imprudent use of these antibacterial agents (Herbert *et al.*, 2003). The consequence of continued exposure to antibacterials is an enrichment of bacteria that are intrinsically resistant to antimicrobials or have acquired resistance mechanisms to these substances. *P. aeruginosa*, *Burkholderia spp.* and *Stenotrophomonas maltophilia* are of clinical significance because of their innate multidrug resistance and their ability to acquire high-level multidrug resistance (Hooper *et al.*, 2001; Herbert *et al.*, 2003).

Bacterial resistance to quinolones may be acquired either by acquisition of foreign DNA or by modification of chromosomal DNA. Bacteria may possess structurally modified antibiotic target sites. Structural modifications result in a lower affinity of the target site for the antibiotic, so that antibiotic binding to the target is reduced or even prevented. Modified target sites may also cause new metabolic

pathways to be used, which no longer use the antibiotic as a substrate, so rendering it ineffectual(*In*:http://www.infectionacademy.org/downloads/AIM_quick_guide_to_antibiotic_resistance_Feb10a.pdf). A scheme of the mechanisms used by Gram-negative and Gram-positive bacteria to resist to quinolones is shown in the Figure 7.

Quinolones exert their toxicity on the bacterial cell by stabilizing the double-stranded break in DNA created by gyrase so that relegation becomes unfavourable. The ternary complex blocks transcription and, more importantly in terms of cell survival, DNA replication (Sifaoui *et al.*, 2003; Hooper *et al.*, 2001; Wentzell *et al.*, 2000). It is thought that blocking of DNA polymerase by the quinolone-topoisomerase complex triggers the release of broken DNA ends by an as yet undefined mechanism (Barnard *et al.*, 2001).

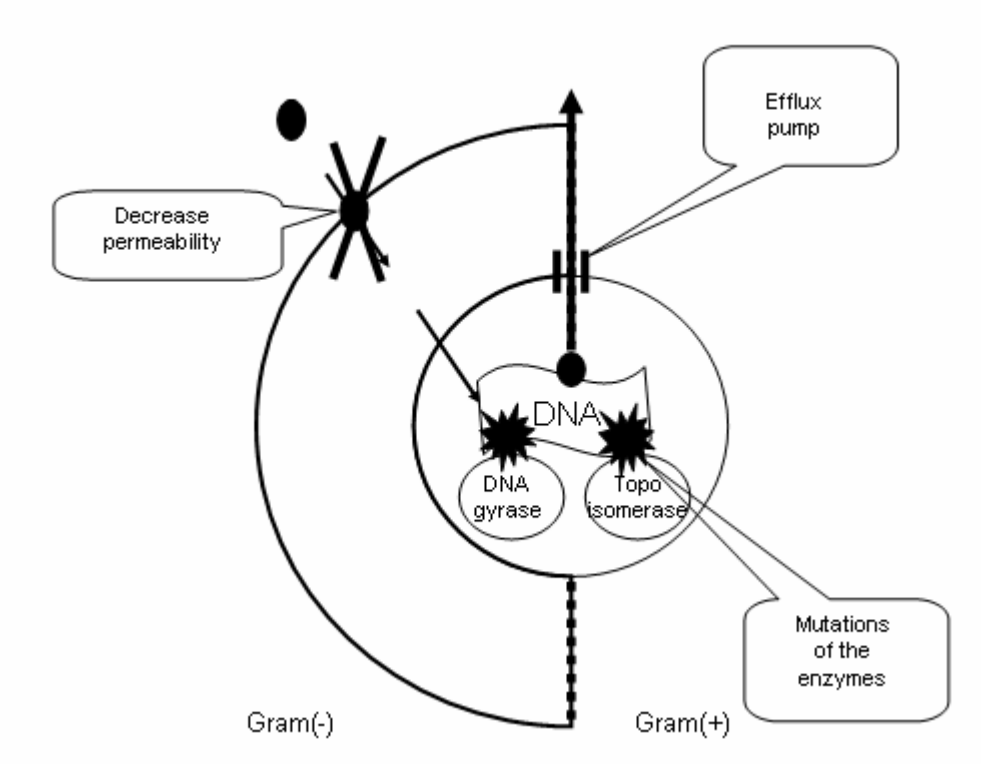

Figure 7- Mechanisms of Quinolones resistance in Gram-negative and Gram-positive bacteria. (Adapted from http://www.sbimc.org)

1.6.1 Reduced membrane permeation

The intrinsic resistance of Gram-negative bacteria has often been attributed entirely to the presence of the outer membrane barrier. This barrier does contribute to the resistance, as the narrow porin channels slow down the penetration of even small hydrophillic solutes, and the low fluidity of the lipopolysaccharide leaflet decreases the rate of transmembrane diffusion of lipophilic solutes. However, the outer membrane barrier cannot be the whole explanation, even in species such as *P. aeruginosa* which produces an outer membrane of exceptionally low permeability (Nakaido *et al.*, 1996). The molecular mechanism that produces intrinsic resistance in *P. aeruginosa* has remained an enigma. Clearly the low permeability of the outer membrane contributes to the resistance but other mechanisms are needed to explain the level of the intrinsic resistance (Li *et al.*, 1994).

Altered uptake of an antibiotic into a bacterial cell as a result of decreased permeability (entry into the organism) or increased efflux (pumping out of the organism) can be related to resistance. For example, the ineffectiveness of tetracycline against some Gram-negative pathogens is caused by inadequate permeability (Li *et al.*, 1994).

1.6.2 Efflux mechanisms

Although exclusion from the cell due to reduced outer membrane impermeability was thought to play a key role in the intrinsic resistance of *P. aeruginosa* and related bacteria to many antimicrobial compounds, this is now attributed to synergy between a low-permeable outer membrane and active efflux from the cell (Herbert *et al.*, 2003). In Gram-negative bacteria, as they possess two membranes separated by a periplasmic space, it is now generally assumed that resistance-nodulation-division (RND) efflux systems in these bacteria function as tripartite systems, wich enable the bacterium to extrude antibacterials from the cytoplasm all the way to the extracellular medium (Herbert *et al.*, 2003). Each tripartite pump contains a transporter located in the cytoplasmic membrane, an outer membrane channel, and a periplasmic linker protein, which is thought to bring the other two components into contact (Lomovskaya *et al.*, 2001) as shown in Figure 8.

So far, five major families of bacterial efflux systems have been identified. These include: i) the small multidrug resistance family (SMR); ii) the resistance-nodulation-division (RND) family, which is part of the larger RND permease superfamily; iii) the major facilitator superfamily (MFS); iv) the ATP-binding cassette family (ABC); and v) the multidrug and toxic compound extrusion family (MATE). In *P. aeruginosa* and related bacteria, the RND family is the best characterized and is of clinical significance (Li *et al.*, 1994; Herbert *et al.*, 2003). An analysis of the genome sequence of this bacterium reveals the existence of 12 potential RND efflux systems. At least six RND family drug efflux pumps are known to exist in cells of *P. aeruginosa*, MexAB–OprM (figure 8), MexCD–OprJ, MexEF–OprN, MexXY–OprM, MexJK–OprM and MexHI–OpmD (Tomofusa *et al.*, 2003).

Regulation of expression of membrane efflux transporters may contribute to quinolone susceptibility in both Gram-positive and Gram-negative bacteria (Hooper *et al.*, 1995).

With the exception of MexAB-OprM, which is constitutively expressed in wild-type cells cultivated under usual laboratory conditions, where it contributes to intrinsic resistance to quinolones and other antibiotics, the expression of most of these efflux systems is tightly regulated (Herbert *et al.*, 2003).

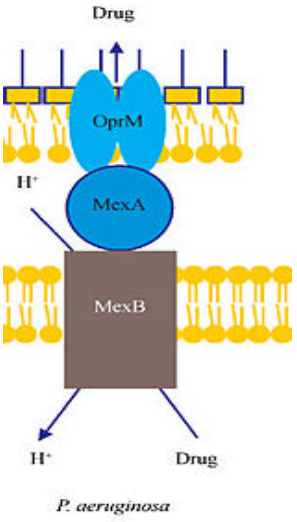

Figure 8- MexAB-OprM efflux pump of *P. aeruginosa*, which consists of I) an inner membrane (MexB); II) a trimeric channel forming (OprM) and III) a membrane-anchored periplasmic linker protein (MexA).

1.6.3 Target Mutations

Acquired resistance may result from spontaneous mutations, single or multiple changes in the bacterium's chromosomal DNA, occurring at a rate of about 1 in 10^6 to 1 in 10^9 per cell, in large bacterial populations (Hooper, 2001). Single point mutations in the N-terminal domains of subunit A of DNA gyrase or subunit C of the homologous topoisomerase IV, in the so-called quinolone resistance-determining-regions (QRDRs), have been found to be responsible for resistance to fluoroquinolones in *E. coli* and in a wide variety of pathogenic bacteria (Lampidis *et al.*, 2002). DNA gyrase is the primary target of Gram-negative organisms however, topoisomerase IV is the primary target of Gram-positive organisms. Within these enzymes there is a highly conserved region centered round the active site where resistance mutations occur. These mutations are almost always identical, irrespective of organism. In spite of the homology of this region, amino acid sequence analysis shows that there are defined differences between the Gram groups, particularly in topoisomerase IV, and it is speculated that herein lays the origin of target preference (Hooper, 2001).

Topoisomerase-based resistance to the fluoroquinolones occur step wise, with moderate levels of resistance arising from single mutations in the primary target of the drug (gyrase or topoisomerase IV, depending on the bacterial species). Once reduced sensitivity to the drug arises from the first mutation, higher levels of resistance may occur from additional mutations in both the primary and secondary enzyme targets (Zhao *et al.*, 1997).

Mutations in either the gyrase A gene (*gyr*A) or the gyrase B gene (*gyr*B) that affect quinolone susceptibility also affect drug binding, with resistance mutations causing decreased binding and hyper susceptibility mutations causing increased binding (Hooper, 1995).

1.6.3.1 Type II Topoisomerases

DNA gyrase and Topoisomerase IV are bacterial type II topoisomerases, which alter the topological state of the genetic material by passing an intact double helix through a transient double-stranded break that they generate in a separate DNA segment and facilitating chromosome segregation, during the replication process (Anderson *et al.*, 1998; Sifaoui *et al.*, 2003). Bacterial DNA exists in a supercoiled

form and the enzyme DNA gyrase, a topoisomerase, is responsible for introducing negative supercoils into the structure. Humans do possess DNA gyrase but it is structurally distinct from the bacterial enzyme and remains unaffected by the activity of quinolones. DNA gyrase plays critical roles in DNA replication, recombination, and transcription, as well as in the maintenance of genomic superhelical density. It is an essential enzyme that is responsible, in part, for the maintenance of DNA topology within the bacterial cell (Anderson *et al.*, 1998; Barnard *et al.*, 2001). Gyrase catalyzes the ATP-dependent introduction of negative supercoils into closed circular DNA, as well as ATP-independent relaxation of supercoiled DNA (Kampanis *et al*, 1998; Barnard *et al.*, 2001). It consists of two proteins, GyrA and GyrB, which form an A_2B_2 complex in the active enzyme. Gyrase introduces changes in the topology of closed circular DNA by cleaving the helix in both strands, forming a 4-base stagger, passing another segment of DNA through the break, and resealing the broken ends (Wentzell *et al.*, 2000; Barnard *et al.*, 2001), Figure 9a.

The second prokaryotic type II enzyme, topoisomerase IV, is comprised of the products of the *par*C and *par*E genes. Consistent with its critical role in chromosome segregation, topoisomerase IV displays a prejudice for catalyzing intermolecular DNA strand passage events (i.e. catenation/decatenation reactions) as opposed to intramolecular events (Anderson *et al.*, 1998), Figure 9b.

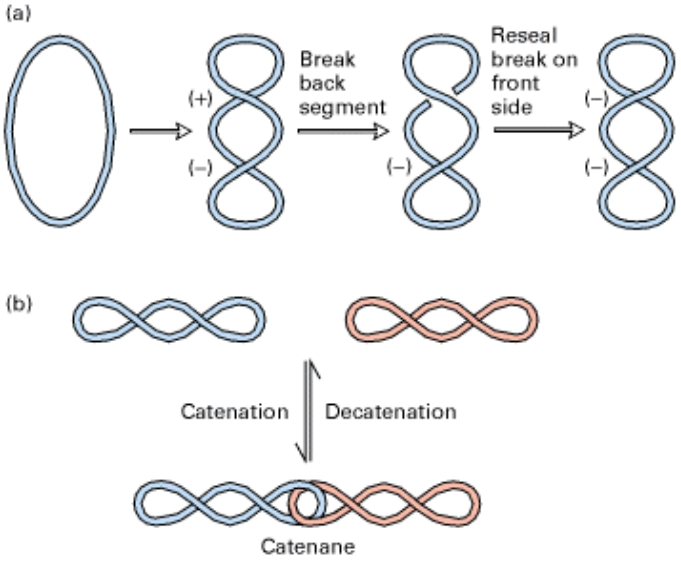

Figure 9- DNA gyrase (a) and Topoisomerase IV (b) action on the DNA molecule.

1.6.3.2 Detection of mutations

A few methods were successfully used to identify mutations in the so-called QRDR region. Those methods include i) a combination of DNA amplification by PCR followed by single-strand conformational polymorphism (SSCP) and ii) direct sequencing of amplified PCR product. SSCP analysis is based on the theory that denatured PCR fragments with different sequences migrate differently in a nondenaturating polyacrylamide gel because of their altered folded structures due to DNA sequence alteration(s) (Takenouchi *et al.,* 1999). Direct sequencing of PCR amplified genes to identify the changes in nucleotide sequences is an easy alternative to other methods when the number of strains to be analysed is considerably small (Weigel *et al.,* 1998).

Other methods have also been used to identify alterations in the genes associated with quinolone resistance, namely the *gyr*A and *gyr*B. These have included cloning of *gyr*A and *gyr*B from resistant strains and identification of the changes in nucleotide sequences as well as purification of GyrA and GyrB proteins to demonstrate drug resistance as a result of enzyme activity conferred by one subunit in a mixing experiment (Ouabdesselam *et al.*, 1995).

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AIMS

The continued and imprudent use of antimicrobials has tended to enrich for pathogens that are intrinsically antibiotic resistant or have acquired resistance mechanisms. Organisms such as *P. aeruginosa* are of increasingly clinical importance because of their innate resistance to multiple agents and their ability to develop high-level multidrug resistance (MDR). The organism's resistance can be attributed to different mechanisms namely i) alteration in target enzymes due to spontaneous mutations in the genes encoding the enzyme's subunits; ii) alteration in drug permeation, that requires the presence and enhanced expression of endogenous efflux system that actively pump the drug from the cytoplasm.

This study had three major objectives:

1) firstly, in order to assess the genetic variability within the *P. aeruginosa* population, selected quinolone resistant *P. aeruginosa* strains obtained in the pathology service of Hospital Infante D. Pedro, Aveiro, over a period of 6 months, will be typed using different molecular methods;

2) considering the fact that spontaneous mutations in key enzymes (type II topoisomerase) involved in DNA replication may contribute to increased resistance to quinolones, the detection of mutations in the QRDR region of *gyr*A, *gyr*B, *par*C and *par*E genes constitutes the second objective of the present work;

3) thirdly, expression of genes encoding for the membrane portion proteins involved in the efflux systems of the quinolone resistant clinical isolates will be investigated.

2 CHAPTER 1

Molecular genotyping as a tool for identifying dissemination of quinolone resistant *Pseudomonas aeruginosa* isolates

ABSTRACT

Thirty five quinolone resistant Pseudomonas aeruginosa strains were molecular genotyped using different DNA-based typing methods (PCR-ARDRA, BOX and Pulse field gel electrophoresis - PFGE).

The results obtained show the high discriminatory power of the methods used. Both methos revealed a highly heterogenous genetic background among the isolates. Also, the study showed a preoccupying clonal dessimination of quinolone resistant *P. aeruginosa* strains in the environment, suggesting that cross contamination and/or a common exogenous source is an important route of *P. aeruginosa* acquisition.

2.1 Introduction

P. aeruginosa is becoming one of the most important nosocomial opportunistic Gram-negative pathogens in humans. Under normal circumstances, colonization is harmless and infection only occurs when local or general defence mechanisms are reduced (Campa *et al.,* 2003). The intrinsic resistance of *P. aeruginosa* to a wide variety of antibiotics represents a major therapeutic challenge (Pfaller *et al.*,1992; Bergogne-Berezin *et al.*, 1996; Archibald *et al.*, 1997; Campa *et al.,* 2003) being the quinolones the class of antibiotics most effective for the treatment of *P. aeruginosa* infections in hospitals (OECD, 1997). *P. aeruginosa* is frequently isolated from patients whose strains are not necessarily epidemiologically or genetically related (Spencer *et al.*, 1996), therefore many studies have been directed at this microorganism.

Microbial strain delineation by DNA-based typing methods is a very powerful tool that contributes to our understanting of outbreaks, recurrent infections and the clonal dissemination of resistant bacterial strains (Pfaller *et al.*, 1992).

Genomic fingerprinting methods are now regarded as the most accurate methods for typing of microorganisms for epidemiological purposes (Spencer *et al.*, 1996, Versalovic *et al.*, 1994). Several methods have been used: i) pulsed-field gel

electrophoresis (PFGE) (Elaichouni *et al*., 1995; Speijer *et al*., 1999) which is considered worldwide the most powerful tool to perform hospital epidemiological studies of *P. aeruginosa* because of its high discriminatory power (Loureiro *et al.*, 2002); ii) ribotyping, that is used for the characterization of the restriction length polimorphism (RFLP) inside the rRNA operon and/or the genomic regions flanking the rRNA operons (Denamur *et al*., 1995; Spencer *et al.*, 1996); iii) amplified ribosomal DNA restriction analysis (ARDRA) that has been used as a suitable method for differentiation at the species level and also for isolate identification (Alves *et al*., 2002) and iv) other PCR based fingerprinting methods, which take advantage of the presence of interspersed repetitive sequences that are present in the genome of different bacterial species (Grothues *et al.*, 1986; Spencer *et al.*, 1996).The BOX element is one such sequence (Lupski *et al*.,1992; Rademark *et al*., 1997).

In the present study, clinical *P. aeruginosa* strains collected from external patients and patients admitted to a hospital from central Portugal, during a 6-month period in 2002, were studied. We aimed to investigate the clonal dissemination of quinolone resistant *P.aeruginosa* in an exogenous environment. Different genotyping methods were employed in order to assess the genetic heterogeneity and phylogenectic distances between the *P. aeruginosa* under study. The methods included PFGE, ARDRA and BOX- PCR.

2.2 Material and Methods

2.2.1 Strains

A total of thirty five clinical *P. aeruginosa* strains were isolated from the biological products from external patients or patients admitted to a hospital from central Portugal (Hospital Infante D. Pedro, Aveiro). The bacterial strains were identified using the Vitek System (Biomerieux). *P. aeruginosa* ATCC 15442 was used as reference strain. Bacterial strains were grown in Trypticase Soy Broth – TSB (DIFCO) and maintained at 4ºC on TSA plates.

2.2.2 DNA extraction

DNA was extracted from freshly grown overnight cultures in 5 mL of TSB (DIFCO) using a Genomic DNA Purification Kit (MBI Fermentas, Vilnius, Lithuania). Purified DNA was aliquoted in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and stored at -20 ºC until required. DNA purity and concentration were estimated spectrophotometrically.

2.2.3 PCR amplification of chromosomal DNA

Generally PCR reactions were performed in 50 µL reaction mixtures containing 1X PCR Buffer (PCR Buffer with $(NH_4)_2$ SO4), 3 mM $MgCl_2$, 5 % dimethylsulfoxide, 200 µM each nucleotide, 0.3 µM of each primer (MWG Biothec, Germany), 1 U Taq polymerase (MBI Fermentas) and 50-100 ng purified template DNA. Appropriate primers were used according to the amplifications to be performed.

Box-PCR analysis: single primer BOX A1R (5'-CTACGGCAAGGCGACGCTGA CG-3') was used to generate BOX-PCR profiles (Weisburg *et al.*, 1991). PCR was performed in a ThermoHybaid HBPXE 02 with an initial denaturation step at 95 ºC (7 min.) followed by 94 ºC (1 min.), 53 ºC (1 min.), 68 ºC (8 min.) for 35 cycles, and a final extension step at 65 ºC (16 min.). Amplified products were separated by electrophoresis at 80 V for 2 h on 1 % agarose gel with 1X TAE (Tris-acetate-EDTA) running buffer. A DNA ladder, GeneRuler 100-bp (MBI Fermentas) was used as DNA size standard. After staining with ethidium bromide, DNA was visualised under UV light. The gel was analysed both by visual inspection and by computer-aided methods.

The set of primers used for ARDRA analysis were as follows: fD1 (5'-AGAGTTT GATCCTGGCTCAG-3') and rD1 (5'-AAGGAGGTGATCCAGCC-3'). Those primers were used to amplify nearly full-length 16S rRNA genes (20). PCR was performed with initial denaturation step at 94 ºC (9 min.) followed by 94 ºC (30 s), 56 ºC (30 s) and 72 ºC (90 s) for 35 cycles, and a final extension step at 72 ºC (10 min). The amplicons were digested with the *Taq*I, *Mbo*I, *Alu*I (MBI Fermentas), as recommended by the manufacturer. After digestion ARDRA fragments were separated by electrophoresis at 80 V for 2 h on 2 % agarose gel with 1X TAE (Tris-acetate-EDTA) running buffer. A DNA ladder, GeneRuler 100-bp (MBI Fermentas) was used as DNA size standard. After staining with ethidium bromide DNA was visualised under

UV light. The gel was analyzed both by visual inspection and by computer-aided methods.

2.2.4 Typing with PFGE

Bacteria were grown to the late-exponential phase, embedded in agarose plugs and lysed with detergents and proteinase K. DNA plugs were digested at 37 ºC overnight (12 to 16 h) with 10 U of *Spe*I restriction enzyme (MBI Fermentas). Electrophoresis was performed in a CHEF-DRII apparatus (Bio-Rad, Richmond, Calif.) with the following conditions: 6 V/cm for 20 h at 12 ºC on 1% agarose with 0.5 Tris-borate-EDTA (TBE) with switch time intervals that ranged from 2 to 60 s. Gels were stained with ethidium bromide, photographed and visually examined.

According to Tenover *et al.*, (1995) isolates were considered non-identical if PFGE banding patterns differed by more than six fragments (bands). Isolates were considered related (subtypes) if only two to six bands differences between PFGE profiles were observed. Isolates with the same PFGE profile were considered indistinguishable.

2.2.5 Computer assisted analysis

Gels were photographed using an Imaging Densitometer (Bio-Rad) and recorded as TIFF images. The banding patterns were analysed with the software Quantity One (Bio-Rad) and converted to a two dimentional binary matrix (1-presence of band; 0-absence of band). Similarity matrices were calculated with the Dice coefficient (Priest *et al.*, 1993). Cluster analysis of similarity matrices was performed by the unweighted pair group method using arithmetic averages (UPGMA). The cophenetic correlation coefficient (r) was calculated to assess the goodness of the clustering method. Computer-assisted analysis was performed with the NTSYSpc2 program for Windows (Rohlf *et al.*, 1993).

2.3 Results

2.3.1 Analysis of 16S rDNA RFLPs

Nearly full-length 16S rDNA of the 35 quinolone resistant clinical *P.aeruginosa* was amplified by PCR using the universal primers fD1 and rD1. PCR products were digested with the restriction enzymes *Taq*I, *Mbo*I, *Alu*I. The choice of the restriction enzymes was made based on the number of restriction sites present on 16S nucleotide sequences deposited in the GenBank database belonging to *P. aeruginosa*. The RFLP pattern obtained with *Taq*I is shown in Figure 1. Identical profiles were obtained for all the strains being studied, confirming the all the strains belong to the same species.

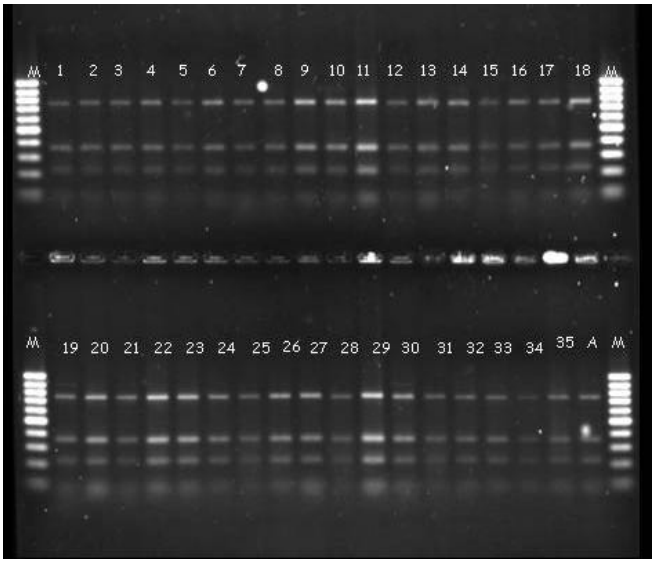

Figure 1- ARDRA patterns of digests of amplified 16S rDNA after restriction with *Taq*I separated in a 2 % agarose gel. M: GeneRuler 100-bp DNA Ladder; 1-35: clinical *P. aeruginosa;* A: *P. aeruginosa* ATCC 15442.

2.3.2 BOX analysis

Typing with BOX yielded a complex genomic fingerprinting as can be seen in Figure 2. The dendrogram obtained, Figure 3, reflects the overall profiles, revealing an extremely high number of clusters. Strains Pa30, Pa31, Pa34 and Pa35 are placed in a different cluster despite the fact that they were isolated from the same biological product of the same patient on the day of addmitance to the hospital. The same is true for strains Pa1 and Pa3 and also for strains Pa10, Pa11 and Pa12. This fact suggests that the patients were colonized on admission with more than one genotype meaning that a high

dissemination of *P. aeruginosa* outside the hospital environment is observed. Interestingly is the fact that strains Pa32 and Pa33 show and identical fingerprinting and are therefore placed in the same cluster and considered clones. These two strains were isolated from different patients that were admitted in the same day and that were staying at different wards while in the hospital. Again, this fact may suggest that cross acquisition and/or a common exogenous source is an important route of *P. aeruginosa* acquisition. However, the majority of the patients were colonized with *P. aeruginosa* isolates with unique genotypes as revealed by the complex dendrogram obtained suggesting a considerable high heterogeneity and genomic diversity between the clinical isolates.

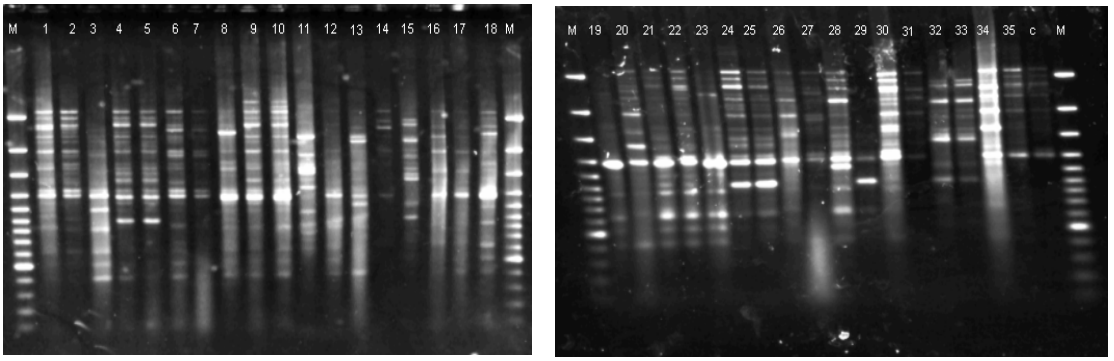

Figure 2- BOX- PCR fingerprints generated with the primer BOXA1R. M: GeneRuler 100-bp DNA Ladder Plus; 1-35: Clinical *P. aeruginosa*; C: *P. aeruginosa* ATCC 15442.

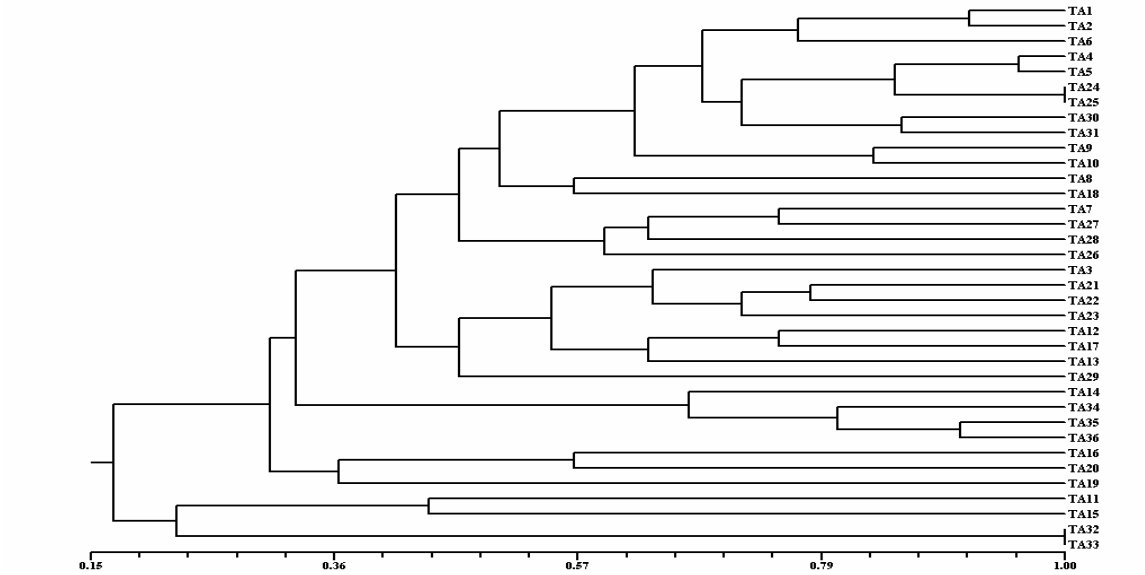

Figure 3- Dice/UPGMA cluster analysis of BOX fingerprints. Similarity is indicated as a percentage. *r* = 0, 78642

2.3.3 PFGE

DNA fingerprinting by PFGE of genomic DNA after digestion with appropriate restriction endonuclease has been considered to be the gold standard technique with which all other molecular typing techniques are compared. It has a higher discriminatory power when compared to other molecular typing techniques, and is broadly applicable to most nosocomial pathogens. Nevertheless, DNA degradation is a problem and can occur in the gel, preventing typing of some strains. This intrinsic degradation was also observed by us in the DNA of some isolates (Pa8, Pa18, Pa 19 and Pa 26). Several gels were run to overcome this problem, but systematically one or more samples became degraded during the long run of the gel.

Figure 4a and 4b shows the genomic fingerprinting obtained by PFGE for a few *P. aeruginosa* quinolone resistant isolates; the resulting dendrogram is shown in Figure 5. When the different fingerprints were compared on the dendrogram, seven main groups emerged where some of the isolates are more or less related to each other; these groups consisted of strains with pattern similarity equal or greater than 48%. Most of the isolates branched off at a point where the percentage of similarity between them is around 75 %. A different group was formed by strain Pa3; this strain shows a similarity of less than 35 % with the rest of the strains. Pa3 and Pa1 were isolated from the same patient.

By applying the criteria of Tenover et al., (1995), based on the numbers of band differences between strains, the results obtained were consistent with those previously obtained. A careful analysis of the dendrogram shown in Figure 5 suggests that a high degree of heterogeneity is present among the isolates as it was previously demonstrated with the BOX profile analysis.

Strains Pa32, Pa33, Pa34 and Pa35 are clones and therefore belong to the same cluster despite the fact that they were isolated from different patients that were admitted in the same day and that were staying at different wards while in the hospital. Still, strains Pa30, Pa31, Pa34 and Pa35 were isolated from the same patient but they have a different genomic profile and belong to different clusters. Strains Pa4 and Pa5 are also clones and were isolated from the same patient. Interestingly, strains Pa6 and Pa7 are also clones however they were isolated from different patients, one of them was admitted to the hospital and the other attended as an occasional external

patient. Once again, these results suggest that cross contamination and/or a common exogenous source is an important route of *P. aeruginosa* acquisition.

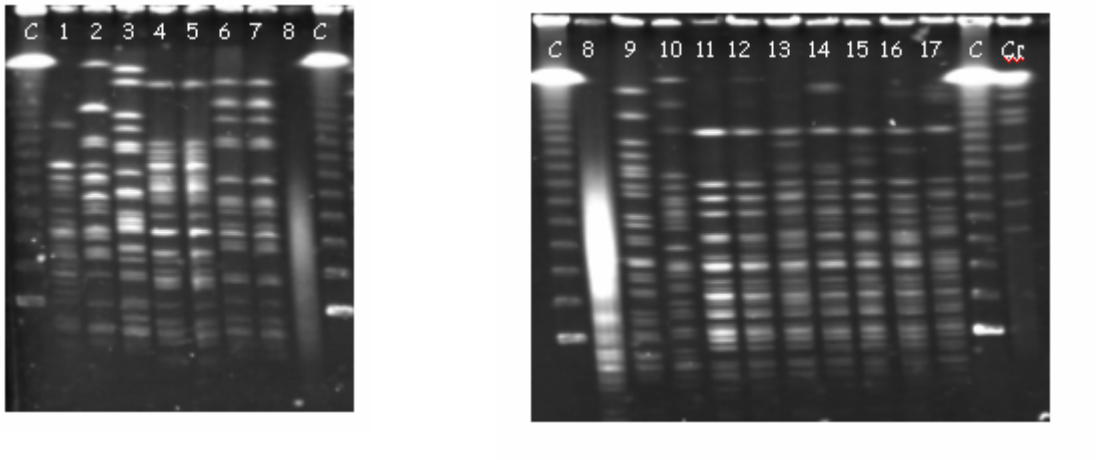

Figure 4a- PFGE profiles of *Spe*I-digested chromosomal DNA of clinical *P. aeruginosa*. C: DNA size standards lamda ladder (BioRad); Cr: DNA size markers yeast Chromossomal (BioRad); 1-17: resistant clinical *P. aeruginosa*.

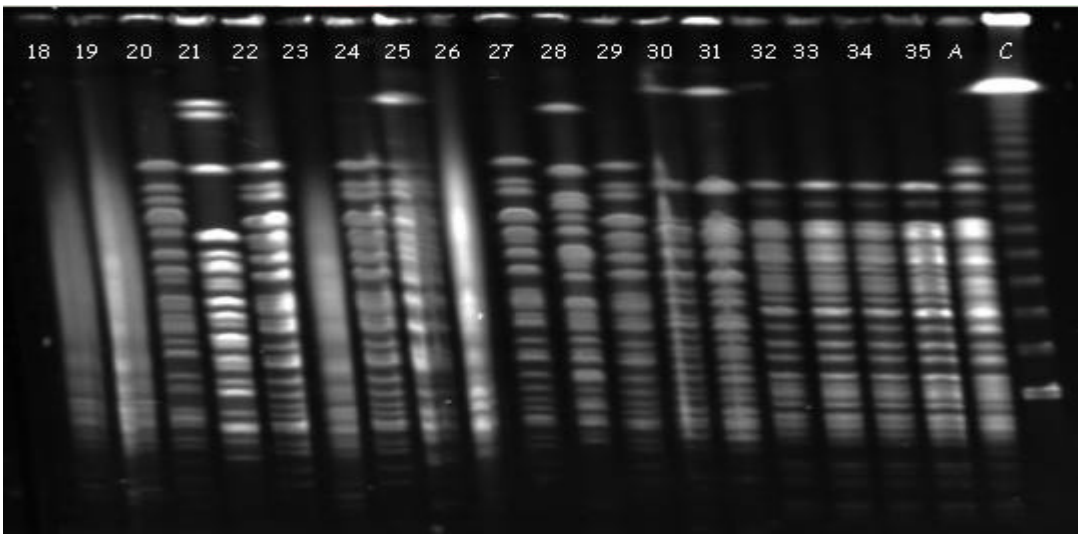

Figure 4b- PFGE profiles of *Spe*I-digested chromosomal DNA of clinical *P. aeruginosa.* C: DNA size standards lamda ladder (BioRad); Cr: DNA size markers yeast Chromossomal (BioRad); 18-35: resistant clinical *P. aeruginosa*; A- *P. aeruginosa* 15442 used as control.

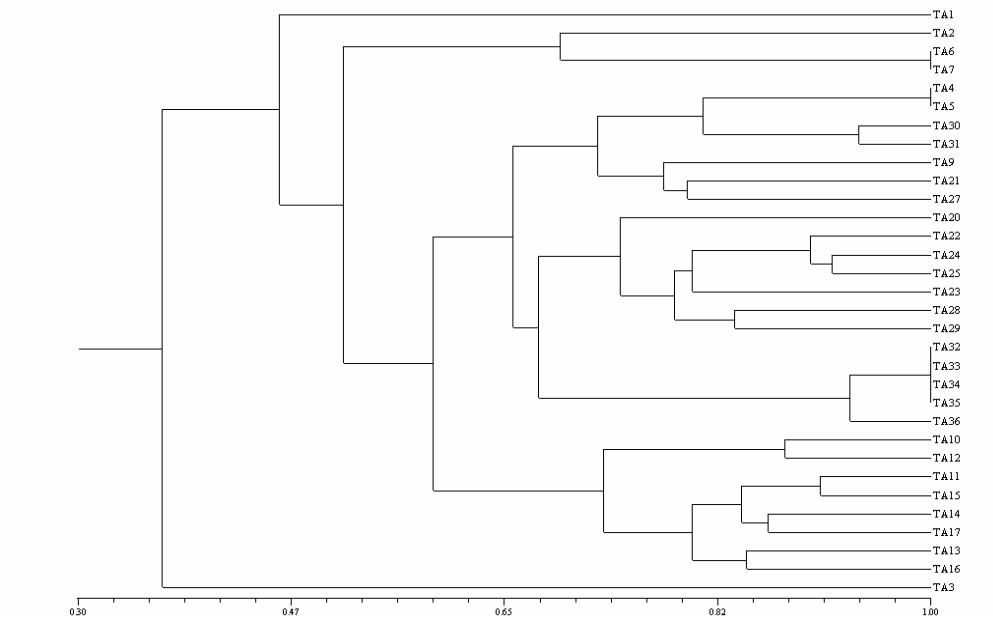

Figure 5- Dice/UPGMA cluster analysis of PFGE genomic fingerprints. Similarity is indicated as a percentage. *r* = 0,83285

2.3.4 Cluster analysis of combined BOX-PCR and PFGE

Cluster analysis was performed on the combination of BOX-PCR and PFGE genomic fingerprints. The resulting dendrogram is shown in Figure 6.The dendrogram reflects the differences observed among the isolates. Isolate Pa3 separates from all the rest of the isolates. The results show that the combined analysis of the BOX and PFGE genomic fingerprints is consistent with that previously obtained and clearly shows the enormous genomic heterogeneity amongst the *P. aeruginosa* isolates. Overall, similarity between the isolates is higher than 50 %, exception made for strain Pa3 that shows a lower similarity (35 %) when compared with the rest of the isolates.

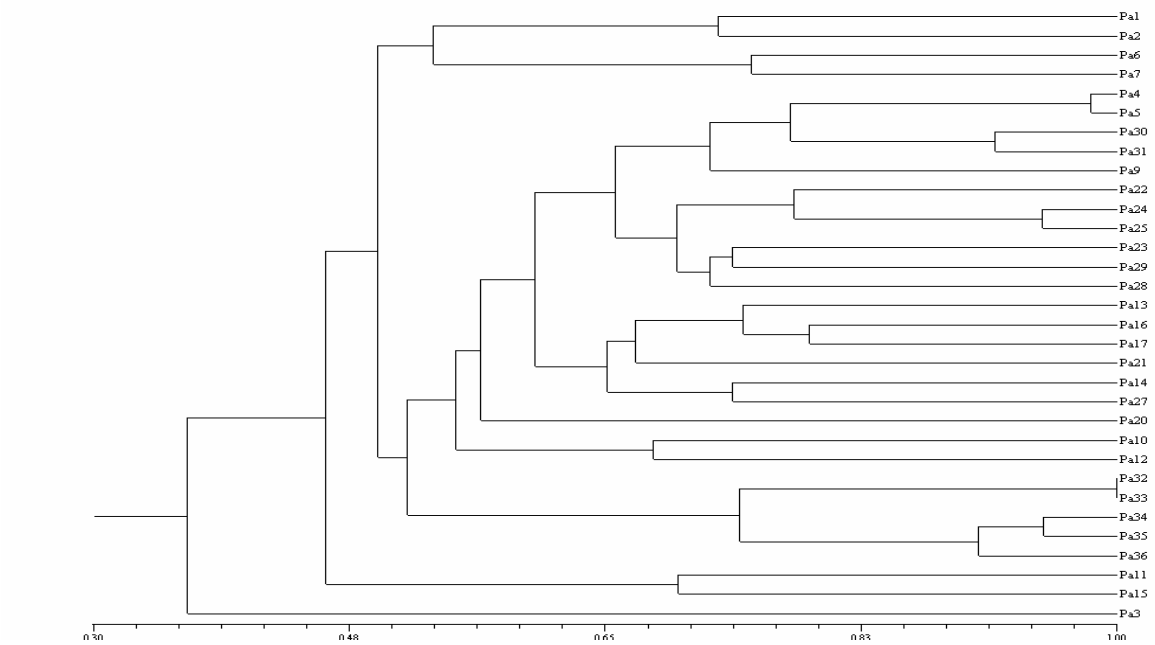

Figure 6- Dice/UPGMA cluster analysis of combined BOX and PFGE genomic fingerprints. Similarity is indicated as a percentage. *r* = 0,77789

2.4 Discussion

Numerous techniques are available to differentiate between bacterial isolates. Several different DNA–based typing methods fulfil these criteria. Among the most widely used are the chromosomal profiling using either PFGE or PCR based methods (Pfaller, 1999). Molecular typing is performed to determine whether different isolates give the same or different results for one or more tests. Epidemiologically related isolates share the same DNA profile or fingerprint, whereas unrelated isolates have distinctly different patterns (Pfaller, 2001). If isolates from a different patient/person share the same fingerprint they most probably originate from the same clone and were transmited from one person to the other or were acquired by a common contamination source.

In the present study three techniques for molecular typing of thirty five quinolone resistant clinical *P. aeruginosa* were used to averiguate the dissemination of *P. aeruginosa* outside the hospital environment.

Although PFGE is a very tedious and time consuming technique, in the present study, it proved to be very useful with a high discriminatory power when compared to BOX-PCR analysis. Still, DNA fingerprinting methods proved to be powerful tools to understand clonal dissemination of bacteria.

The computer assisted analysis allowed the separation of the isolates with more or less similar genotypes. Nevertheless, a high genomic heterogeneity was observed among the isolates.

The molecular techniques used proved their usefulness for tracking cross-contamination by the pathogenic quinolone resistant *P. aeruginosa*, outside the hospital environment, though it is almost impossible to establish the contamination source. All patients were colonized before admission. This suggests that cross-contamination is an important route of *P. aeruginosa* acquisition. Most of the patients were colonized with multiple strains with unique genotypes, nevertheless it was possible to identify a few clones colonizing patients with no relation to each other and that were not even hospitalised, or if so, not in the same ward and whose stays in the hospital did not occur at the same time. These results are suggestive of high clone dissemination in the environment.

Effective control programs are based on knowledge of the organisms and how they are spread but they can only be applied in confined environments (Anthony *et al.*, 2002). The major concern behind these results is the widespread of quinolone resistant isolates in the population. This fact would certainly represent a challenge for the treatment of *P. aeruginosa* infections, as quinolones are the class of antibiotics most effectively used in hospitals.

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3 CHAPTER 2

Novel mutations in the QRDR of *gyr*B and *par*E genes associated with quinolone resistance in *Pseudomonas aeruginosa*

ABSTRACT

Thirty-five quinolone-resistant clinical isolates of *Pseudomonas aeruginosa* were examined for the presence of mutations in the quinolone-resistance determining regions (QRDR) of the *gyr*A, *gyr*B, *par*C, *par*E genes. SSCP analysis was aplied to detect mutations in the *gyr*A and *gyr*B, but proved to be inconclusive. Nucleotide sequence of amplified fragments were determined and compared to the corresponding of *P. aeruginosa* PAO1 deposited in gene databases. In two strains (Pa4 and Pa24) a novel mutation in *gyr*B gene, codon 465 (CAG-Gly to CGG-Arg) was identified. In the *par*C gene a new mutation occurred in codon 35 leading to an amino acid substitution (Asp? Glu) in two of the strains (Pa6 and Pa31). Five novel mutations were identified in the *par*E gene in codons 431 (Leu? Val), 483 (Glu? Gln), 487 (Ala? Pro), 530 (Ala? Pro), 538 (Gly? Val) and 544 (Gln? His). Two of those mutations occurred inside the highly conserved motif EGDSA. Silent mutations were also found in the four genes.

A relation between the presence of mutations and MIC values is variable and can not be assumed as a straight forward relation due to the different and various genetic backgrounds of the isolates, e.g. the contribution of efflux pumps.

3.1 Introduction

The intrinsic resistance of *P. aeruginosa* to a wide variety of antibiotics still represents a major therapeutic challenge. Fluoroquinolones have potent antimicrobial activity against Gram-positive and Gram-negative bacteria and are often used in therapy of various bacterial infections including those caused by *P. aeruginosa* (Nakano *et al.,* 1997; Hooper, 1998; Takenouchi *et al.*, 1999; Akasaka *et al.*, 2001).

The targets of quinolones are considered to be the type II topoisomerases (DNA gyrase and Topoisomerase IV) which are the enzymes controlling the topological state of DNA during replication and transcription (Akasaka *et al.*, 2001). Gyrase appears to be the primary target for quinolones in Gram-negative bacteria

since mutations in *gyr*A genes are sufficient to render these organisms quinolone resistant (Marisol Goni- Urriza *et al.,* 2002). DNA gyrase is composed of two A and B subunits, which are encoded by the *gyrA* and *gyrB* genes, respectively. Topoisomerase IV is homologous to DNA gyrase and is composed by two subunits, ParC and ParE, which are encoded by the *parC* and *parE* genes, respectively (Takenouchi *et al.*, 1999; Akasaka *et al.*, 2001).

Virtually all mutations for quinolone resistance have been mapped in the quinolone-resistance-determining-region (QRDR) region of *gyr*A (Friedman *et al.*, 2001).

The emergence of fluoroquinolone resistance in *P. aeruginosa* since their introduction into clinical use and the development of resistant mutants during drug therapy have been reported (Takenouchi *et al.*, 1999). The mechanisms of quinolone resistance include the modification of DNA gyrase and topoisomerase IV, decreased permeability of cell wall, and multidrug efflux systems (Akasaka *et al.*, 2001). It has been reported that alterations in DNA gyrase or topoisomerase IV caused by mutations in the so-called ”quinolone-resistance-determining-region” QRDR region appear to play a major role in fluoroquinolone resistance in clinical isolates of *P. aeruginosa* (Cambau *et al.*,1995; Nakano *et al.,* 1997; Takenouchi *et al.*, 1999; Jalal *et al.*, 2000). The genetic basis of resistance to quinolones in Gram-negative bacteria has been defined mainly in *Escherichia coli*. Substitution of the highly conserved residue Ser-83 in GyrA is the most common alteration in both *in vivo* and *in vitro* resistant isolates. The mutation altering residue Asp-87 is usually found to augment the Ser-83 mutation for further increased resistance (Ruiz, 2003). Replacement of the residues Ser-80 and Glu-84 in *Par*C is commonly associated with the *gyr*A mutations resulting in high fluoroquinolone resistance (Heisig *et al.*, 1994; Nakano *et al.,* 1997). Mutations in *gyr* B and *par* E are not so common, however, the ones found in *gyr* B fragment in the codons 468 and 470 have been previously described in *E.coli* (Akasaka *et al.*, 2001).

Several methods have been used to identify mutations in the gyrase and topoisomerase genes associated with quinolone resistance. Some studies have applied SSCP analysis to the detection of *gyrA* mutations in several bacterial species (Ouabdesselam *et al.*, 1995; Takenouchi *et al.*, 1999).

The aim of the present study was to characterize the mutations occurring in the (QRDR) of the *gyr*A, *gyr*B, *parC* and *parE* genes of the 35 clinical ciprofloxacin and

pefloxacin resistant *P. aeruginosa* strains. For *gyr* A and *gyr* B genes, DNA was amplified by PCR followed by single-strand conformation polymorphism analysis (SSCP). Strains were grouped according to their SSCP profile and the DNA was sequenced to reveal any nucleotide change(s) which may be implicated in quinolone resistance. *parC* and *parE* genes were amplified and the DNA product was directly sequenced and analysed for nucleotide changes.

3.2 Material and Methods

3.2.1 Bacterial strains

The clinical isolates of *P. aeruginosa* were obtained in a hospital from central Portugal (Hospital Infante D. Pedro, Aveiro). The bacterial strains were identified using the Vitek System (Biomerieux). As reference strain, *P. aeruginosa* ATCC 15442 was used. Sensitive *P. aeruginosa* hospital isolates (S1, S2 and S3) were also used as control strains. Bacterial strains were grown in Trypticase Soy Broth -TSB (DIFCO) and stored at 4 ºC on TSA plates.

3.2.2 Determination of MICs.

The antimicrobial susceptibilities were evaluated according to NCCLS instructions (NCCLS**,** 2000) to the following antimicrobial agents: Meropenem, ciprofloxacin, pefloxacin and nalidixic acid. The MIC was determined as the lowest antimicrobial concentration that prevented visible growth of bacteria (Silbert *et al*, 2001). *P. aeruginosa* ATCC 15442 was used as control strain. The results were interpreted according to the National Committee for Clinical Laboratory Standards criteria (NCCLS**,** 2000).

3.2.3 DNA extraction

DNA was extracted from freshly grown overnight cultures in 5 mL of TSB (DIFCO) using the Genomic DNA Purification Kit (MBI Fermentas, Vilnius, Lithuania). Purified DNA was aliquoted in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and stored at -20 ºC until required. DNA purity and concentration were estimated

spectrophotometrically. Alternatively, for rapid PCR analysis chromosomal DNA present in crude cell lysates was prepared as described by Maguire *et al.*, (2001).

3.2.4 PCR amplification and DNA sequencing

A set of primers, GyrA_FW 5'- AGTCCTATCTCGACTACGCGAT-3' (nucleotides [nt] 320 to 341) and GyrA_RV 5' – AGTCGACGGTTTCCTTTTCCAG –3' (nt 676 to 697) were used to amplify a 209 bp fragment, from positions 421 to 630 (GenBank accession number L29417), containing the putative QRDR *gyr*A of *P. aeruginosa.* For amplification of the *gyrB* gene of *P. aeruginosa* (GenBank accession number AB00581) two primers, GyrB_FW 5'- GCGCGTGAGATGACCCGCCGT -3' (nt 1162 to 1182) and GyrB_RV 5' – CTGGCGGTAGAAGAAGGTCAG-3' (nt 1531 to 1551) were used. Those amplified from positions 1213 to 1455. Amplification of the *parC* gene (GenBank accession number AB003428) was achieved using the following set of primers: 5'-CGAGCAGGCCTATCTGAACTAT-3' (nt 214 to 235) and 5'-GAAGGACTTGGGATCGTCCGGA-39 (nt 496 to 517) that amplified the fragment from positions 350 to 481. For the amplification of the *parE* gene (GenBank accession number AB003429) the following primers were used: 5'-CGGCGTTCGTCTCGGGCGTGGTGAAGGA-3' (nt 1223 to 1250) and 59-TCGAGGGCGTAGTAGATGTCCTTGCCGA-3' (nt 1787 to 1814) that amplified the fragment from position 1378 to 1620.

The fragments were amplified in a Perkin-Elmer 2400 Thermal Cycler, in 50 µL volumes containing 0.3 µM of each primer (MWG Biothec, Germany), 200 µM each nucleotide, 1X reaction buffer with 10x $(NH_4)_2$ SO_4, 3 mM $MgCl_2$, 5 % dimethylsulfoxide, 1 U native *Taq* polymerase (MBI Fermentas) and 50-100 ng purified template DNA. An initial 3 min period of denaturation at 94 ºC was followed by 35 cycles of denaturation (30 s at 94 ºC), annealing (30 s at 55 ºC) and extension (1 min at 65 ºC or 72 ºC) and then a final cycle of 65 ºC for 16 min (Akasaka *et al.*, 2001). Amplification products were visualised after agarose gel electrophoresis and ethidium bromide staining to confirm the sizes of the gene fragments. PCR products were purified with MicroSpin^{TM}S-300 HR columns (Amersham Pharmacia Biotech Inc).

3.2.5 DNA sequencing and analysis

The DNA sequences of the PCR-amplified *gyr*A, gyrB, *par*C and *par*E fragments were determined using the Big Dye Terminator Cycle Sequencing Reaction kit, PE (Applied Biosystems) using an Abi Prism 310 Genetic Analyzer sequencer (Applied Biosystems). The DNA sequence of each fragment was determined for both strands of PCR products from two independent reactions. The Biological Sequence Alignment Editor, BioEdit version 7.0.0 was used for DNA and amino acid sequence alignments. Sequences obtained were compared to others deposited in the EMBL Genebank nucleotide sequence databases.

3.2.6 SSCP analysis

The procedure was essentially as previously described (Orita *et al.,* 1989). The single stranded PCR products were separated in a 12 % w/v acrylamide-bisacrylamide (20 %: 19:1) gel in TBE buffer (90 mmol / 1 Tris-borate, 2 mmol / 1 EDTA, pH 8.0) for 17 h at 80 V at room temperature. After electrophoresis, the gel was stained with Plus One TM silver staining kit (Pharmacia Biotech).

3.3 Results

3.3.1 MIC determination

MIC values obtained for the two fluoroquinolones tested and also to meropenem are shown in Table 1. MIC determinations were also evaluated for 3 sensitive strains (hospital isolates) and *P. aeruginosa* ATCC 15442. Overall, most of strains showed high-level of resistance to nalidixic acid with a MIC value for the quinolone resistant strains generally higher than (?256 ?g/ml).

However, a number of strains (Pa1, Pa24, Pa29 and Pa33) showed high-level resistance to ciprofloxacin and also to other groups of antibiotics namely beta-lactamics, macrolides and aminoglycosides (data not shown). These strains showed a Thr-83-Ile alteration in the *gyr*A gene.

Strain Pa5 was particularly resistant to other antimicrobial compounds tested (data not shown) and it prossesses mutations in the *gyr*A, *par*C and *par*E genes. No mutations were detected in the *gyr*B gene for this strain. These results emphasize that

the mutations detected may play an important role in fluoroquinolone resistance but other mechanism may also be involved and are currently being studied.

Table 1. Susceptibilities of fluoroquinolones resistant clinical isolates of *P. aeruginosa*.

Strain	Nalidixic Acid MIC (µg/ml)	Ciprofloxacin MIC (µg/ml)	Meropenem MIC (µg/ml)
PAO1	256	0,5	0,12
Pa1	4096	8	2
Pa3	256	0,25	0,5
Pa 4	4096	4	8
Pa5	4096	32	8
Pa6	4096	1	2
Pa9	128	0,12	0,5
Pa24	4096	4	1
Pa29	4096	16	0,25
Pa30	128	0,06	4
Pa31	1024	2	8
Pa33	4096	128	1
Pa34	256	2	2
Pa35	256	2	1
S1	64	0,06	0,12
S2	128	0,25	4
S3	128	0,25	0,06

S: sensitive clinical *P. aeruginosa*

Pa: Quinolone resistant clinical isolates

3.3.2 SSCP analysis of amplified *gyrA* and *gyrB* QRDR

The SSCP analysis of the PCR products was only applied to *gyr A* and *gyrB* genes. Although it proved to be a rather useful technique the resulting patterns obtained were sometimes difficult to interpret. Nevertheless, the isolates displayed different mobility patterns (data not shown). Migration patterns differed between resistant strains revealing the presence of mutations in the amplified fragment. Strains

were grouped according to the different patterns revealed. A total of ten different groups to *gyr* A fragment and twenty different groups to *gyr* B fragment were obtained. Based on these grouping two strains from each group were sequenced in order to detect the nucleotide replacements that could assign amino acid changes and that therefore would correspond to a mutation. Although SSCP proved to be a rapid analysis tool, when compared to DNA sequencing results it proved of little use in the present study as the different banding patterns obtained were also assigned to silent mutations that would not imply an amino acid change.

3.3.3 Genetic analysis of *gyrA*, *gyr*B, *par*C and *parE* QRDR region

The fragments corresponding to the genes under study, were amplified, sequenced and analysed for mutations leading to amino acid changes that could be associated with fluoroquinolone resistance. The mutations found in *gyrA*, *gyrB*, *parC* and *parE* QRDR of the 35 clinical ciprofloxacin and pefloxacin- resistant *P. aeruginosa* strains are described in Table 2. Mutations were examined individually for each particular gene.

***Gyr*A mutations**. The nucleotide sequences corresponding to the 209 bp fragment corresponding to the *gyrA* genes extended from nucleotides 421 to 630 and included the QRDR region. Comparison of the deduced amino acid sequences of the QRDRs of the 35 clinical isolates with those of the wild-type sensitive *P. aeruginosa* ATCC15442 and *P.aeruginosa* PAO1 revealed that only one amino acid substitution was observed at the position corresponding to codon 83 (ACC- Thr? ATC-Ile) in strains Pa1, Pa4 and Pa5. These mutations have been already described by Mouneimné *et al.* (1999). Comparison between the sequence of the *gyrA* fragment obtained in our isolates and that of *P. aeruginosa* PAO1 revealed the presence of some silent mutations in codon 113 (GTA to GTC, 2 strains), 128 (GCA to GCG, 2 strains).

***Gyr*B mutations.** Primers used to amplify the *gyrB* gene included the putative QRDR region. The fragment of 242 bp extended from nucleotides 1213 to 1455. The nucleotide sequence of the *gyrB* fragment from the wild-type sensitive *P. aeruginosa* ATCC15442 and *P.aeruginosa* PAO1 were identical.

Four of the ciprofloxacin and pefloxacin resistant *P. aeruginosa* strains (strains Pa30, 31, 33 and 35) harbored a *gyrB* mutation. This mutation occurred in codon 464, leading to the amino acid substitution of a serine (TCC) for a tyrosine (TAC). Two strains (Pa4 and Pa24) had a CAG to CGG mutation in codon 465, leading the substitution of a glutamine for an arginine. No other mutations leading to amino acid changes were found in all the remainder strains. However when compared to the sequence of the *gyrB* fragment obtained from *P. aeruginosa* PAO1, some silent mutations were found in codon 406 (AAA to AAG, 3 strains), 415 (CCG to CCA, 2 strains), 415 (CCG to CCC, 1 strain), 426 (GAC to GAT, 1 strain), 454 (GAA to GAG, 5 strains), 456 (GCG to GCA, 4 strains).

***par*C mutations.** Alignment of the nucleotide sequence corresponding to the 303 bp fragment of the *parC* gene of the wild-type sensitive *P. aeruginosa* ATCC15442, *P. aeruginosa* PAO1 and that of the ciprofloxacin and pefloxacin resistant clinical *P. aeruginosa* strains revealed that four strains (Pa5, Pa24, Pa29 and Pa33) harbored mutations in *par*C. These four strains exhibited a mutation (Ser-87 to Leu) that is located in the codon corresponding to Ser-80 in *E. coli par*C. Those mutations were already described by Cambau *et al.* (1995).

A novel mutation occurred in codon 35 leading to an amino acid substitution of an aspartic acid for a glutamic acid (Asp? Glu) in two of the strains (Pa6 and Pa31). Silent mutations were found in the *par*C QRDR at codons 46 (AAA to AAG, 2 strains), 60 (GGG to GGT, 1 strain), 87 (TCG to TCT, 1 strain), 97 (GCG to GCA, 1 strain), and 115 (GTC to GTG, 1 strain).

***par*E mutations.** In the nucleotide sequence of the *par* E gene a few amino acid substitutions were found. Those changes occurred in codons 431 (Leu? Val), 483 (Glu? Gln), 487 (Ala? Pro), 530 (Ala? Pro), 538 (Gly? Val) and 544 (Gln? His). However, we found that two of these replacements (Glu-483-Gln and Ala-487-Pro) have not been described so far and both occurred in the same strain (Pa5). This replacement occurred inside the highly conserved motif EGDSA that is present in the type II topoisomerase B subunit (Akasaka *et al*, 2001). Furthermore, silent mutations were found at codons 428 (GAA to GAG, 1 strain), 432 (CAA to CAG, 1 strain), 434 (GCG to GCA, 4 strains), 440 (AAC to AAT, 3 strains), 470 (GCC to GCT, 1 strain), 491 (GCG to GCT, 1 strain), 498 (GAA to GAG, 1 strain), 511 (AAC to AAT, 2 strains),

517 (GGC to GGT, 1 strain), 531 (GTG to GTA, 4 strains), 538 (GGT to GGC, 6 strains), 540 (AGT to AGC, 8 strains), 543 (GCC to GCT, 4 strains), 544 (CAG to CAT, 1 strain), 560 (GGG to GGC, 5 strains), 564 (GCC to GCT, 1 strain), 573 (CGC to CGT, 5 strains).

Table 2. Alterations in *gyr A, gyrB, parC and parE* QRDR region of the quinolone resistant and sensitive clinical *P. aeruginosa*.

	***Gyr*A**		**GyrB**		***Par*C**		***Par*E**					
Strain												
PAO1	Thr 83	Asp 87	Ser 464	Gln 465	Asp 35	Ser 87	Leu 431	Glu 483	Ala 487	Ala 530	Gly 538	Gln 544
Pa1	Ile[c)]											
Pa3							Val[a)]					
Pa4	Ile[c)]			Arg[a)]								
Pa5	Ile[C)]					Leu[c)]		Gln[a)]	Pro[a)]			
Pa6					Glu[a)]							
Pa9											Val[a)]	
Pa11												His[a)]
Pa17										Pro[a)]		
Pa24	Ile[c)]			Arg[a)]		Leu[c)]						
Pa29	Ile[c)]					Leu[c)]						
Pa30			Tyr[b)]									
Pa31			Tyr[b)]		Glu[a)]							
Pa33			Tyr[b)]			Leu[c)]						
Pa34			Tyr[b)]									
Pa35			Tyr[b)]									
S1												
S2												
S3												

a) New mutations identified in the present work

b) New mutation according to Cambau *et al.,* 1998; already described according Sato *et al*., 2001.

c) Previously discribed mutations

3.4 Discussion

The association of DNA gyrase and topoisomerase mutations with fluoroquinolone resistance has been established for *P. aeruginosa* by several studies (Weigel *et al.*, 1998; Takenouchi *et al.*, 1999; Akasaka *et al.*, 1999).

SSCP is one of the methods that have been used for detecting mutations in several genes. Some authors (Ouabdesselam *et al.*, 1995) suggested that SSCP analysis could be a useful screening method for identifying common *gyrA* mutations in clinical isolates of *E. coli*. This same technique was also used with success in the determination of mutations in genes of other bacterial species (Piddock *et al.,* 1998; Wang *et al*, 1998). Neverthelss, its application has been limited for the detection of the *gyrA* mutations (Ouabdesselam *et al*, 1995). We employed this technique to detect DNA mutation in DNA *gyrA and gyrB* genes. However, the SSCP patterns observed were not consistent with the nucleotide sequencing results obtained and proved to be inconclusive. Thus, because the number of strains being studied was not very high, DNA sequencing was applied to the detection of the mutations which may be implicated in quinolone resistance of the 35 ciprofloxacin and pefloxacin resistant clinical *P. aeruginosa*. We sequenced the amplified fragments containing the QRDR region corresponding to the *gyrA*, *gyrB, parC* and *parE* genes. DNA sequencing identified some mutations that lead to amino acid substitution in 16 out of the 35 strains.

In *E. coli*, a single point mutation in *gyr*A results in decreased susceptibility to fluoroquinolones, and high-level resistance is associated with double amino acid substitutions in the GyrA protein (Weigel *et al.*, 1998; Takenouchi *et al.*, 1999). Genetic studies revealed that the *gyrA* mutation is responsible for quinolone resistance and that the GyrA alteration leads to the resistance of DNA gyrase to inhibition by quinolones.

GyrA mutants containing a Thr-83-Ile alteration showed higher levels of resistance to fluoroquinolones than mutants containing a different single point mutation (Takenouchi *et al*, 1999). In this study the same mutations were detected and seem to be responsible for high-level resistance. In a number of strains the referred mutation is not present nevertheless, the strains also show resistance to fluoroquinolones suggesting that some of the other mutations detected could also play an important role, however, other resistance mechanisms could also be involved. In

seven of the strains mutations were detected in the *gyr*B gene. Those mutations correspond to amino acid substitution in codons 464 (strains Pa30-Pa35) and 465 (strains Pa4 and Pa24). One, we found a new mutation in codon 464 (serine TCC? tyrosine-TAC) that was already described in other species but not in *P. aeruginosa*. However, this mutation is described in *P. aeruginosa* at a different position corresponding to codon 468. This fact suggests that the selected QRDR region is subjected to slight changes that are later reflected in the amino acid replacements that are observed. At codon 465 a novel mutation *(*glycine-CAG? arginine–CGG*)* was detected that was not described before in *P. aeruginosa.*

Based on biochemical, genetic and epidemiological studies, DNA gyrase is known to be the primary target enzyme for fluoroquinolone, and topoisomerase IV is known to be the secondary target in *P. aeruginosa* (Akasaka *et al.*, 2001). Thus the alteration in ParC occurred after GyrA alteration and is associated with the development of higher fluoroquinolone resistance. In the present study this was not observed for the majority of the strains and, for example, strain Pa33 shows increased resistance to fluoroquinolones but no mutation was detected in the *gyr*A gene, however, strain Pa5 shows increased resistance to the fluoroquinolones tested and it presents mutations in the *gyr*A, *parC* and *parE genes*.

A new substitution was found in the QRDR of the *parC* gene in codon 35 that correspond to a change of an aspartic acid for a glutamic acid in two of the strains (Pa6 and Pa31). No mutations were found at position of codons 80, 83 or 84 where mutations are common in the homologous subunit, in *E.coli*. Since the A subunit of gyrase and the ParC subunit of the topoisomerase IV are highly homologous (Cambau *et al.*,1995) it has been suggested by Mouneimné *et al.,* (1999) that a serine at position 83 (or at a position corresponding to) in both DNA gyrase and topoisomerase IV is important in determining quinolone resistance. Mutations were identified in codon 87 where a serine was substituted by a leucine in four of the strains (Pa5, Pa24, Pa29 and Pa33). This mutation was already described in other *P. aeruginosa* (Akasaka *et al.*, 2001).

We also identified five new mutation in the *parE* gene among the isolates under study. Those mutations were found in codons 431 (leucine-CTG? valine-GTG), 483 (glutamic acid-GAG? glutamine-CAG), 487 (alanine-GCC? proline-CCC), 530 (alanine-GCG? proline-CCG), 538 (glicine-GGT? valine-GTT) and 544 (glutamine–

CAG? histidine-CAC). Two of those mutations occurred inside the highly conserved motif EGDSA, which is present in the type II topoisomerase B subunits. The mutations detected in the present study are novel and were not described so far in *P. aeruginosa,* however, a different mutation (Asp-419 to Asn), has been described in this motif of *P. aeruginosa* by other authors (Akasaka *et al.*, 2001). An alteration in this motif has been implicated in a fluoroquinolone-resistant *E.coli, par E*. Also, similar replacements have been described for quinolone-resistant strains of *Streptococcus pneumoniae par* E (Asp-435 to Asn) and *Staphylococcus aureus* GrlB (Par E) (Akasaka *et al.*, 2001). These results confirm that mutations in type Ⅱ topoisomerase subunit B are not as rare as they used to be.

Sensitive *P. aeruginosa* hospital isolates that were used as control, did not show any mutations in the QRDR region of any of the genes studied, *gyr A, parC* or *gyr* B and par*E*.

The relation between presence of mutations and MIC value is variable and can not be viewed as a straight forward assumption as clinical isolates have different and various genetic backgrounds. Multiple efflux pump systems have been described in *P. aeruginosa*. Overexpression of these pumps has been described for clinical fluoroquinolone resistant isolates (Akasaka *et al*, 2001). The levels of expression of these efflux pumps are now under study in the clinical isolates of *P. aeruginosa* and may help to clarify the results obtained in the present study.

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4 CHAPTER 3

Involvement of Efflux pump systems in quinolone resistance of clinical *Pseudomonas aeruginosa*

ABSTRACT

At least six broad-spectrum efflux pumps (Mex) are involved in high intrinsic antibiotic resistance as well as in acquired multidrug resistance in *Pseudomonas aeruginosa*. The genes encoding these pumps are arranged as operons. Three genes, *mex*B, *mex*F and *mex*Y, encoding the transporter involved in the export of the substrate across the inner membrane, were analysed for their expression. RT-PCR analysis revealed that all the strains tested were expressing the *mex*B and *mex*F and *mex*Y genes with the exception of strain Pa31 that showed lower or no expression at all of these genes.

A growth experiment was carried out with the same strains used for RT-PCR analysis, revealing that strain Pa31 shows a slower growth rate when compared with the other strains. This result suggested that strain Pa31 is in disadvantage to other strains due to the extra load that has to be put when this strain is grown in antibiotic free medium. In all the other strains antibiotic resistance does not seem affect bacterial fitness when the strains are grown in antibiotic free medium.

4.1 Introduction

P. aeruginosa is an opportunistic human pathogen characterized by an innate resistance to multiple agents and also by the ability to develop high-level multidrug resistance (MDR). (Pai *et al*., 2001; Li *et al*., 2003; Dean *et al.,* 2003). Recent studies showed that the major cause for resistance is the existence of multidrug efflux pumps (Poole, 2001). All known organisms have multidrug resistance pumps that can extrude chemically unrelated antimicrobials from the cell (Brooun *et al*., 2001), thus, providing resistance to this compounds (Schweizer *et al*., 1998; Poole, 2001; Li *et al*., 2003). The combination of tripartite multidrug efflux systems and outer membrane impermeability plays a major role in intrinsic antibiotic resistance in clinically relevant pathogens such as *P. aeruginosa* (Jo *et al*., 2003).

One important resistance mechanism is efflux mediated by the so-called resistance nodulation division (RND) family of efflux pumps (Dean *et al*., 2003).

At least six RND family drug efflux pumps are known to exist in cells of *P. aeruginosa*, MexAB-OprM, MexCD-OprJ, MexEF-OprN, MexXY-OprM, MexJK-OprM

and MexHI-OpmD (Li *et al*., 2003). RND pumps consist of an inner membrane transporter (MexB, MexD, Mex F and MexY), an outer membrane channel-forming component (OprM, OprJ and OprN) and a membrane fusion protein (MexA, MexC, MexE and MexX) (Nikaido, *et al*., 1998; Dean *et al*., 2003). The three proteins are believed to form a channel across the inner and the outer membranes. RND pumps show broad specificity, and their tripartite architecture allows extrusion of compounds directly from the cytoplasm to the external environment (Dean *et al*., 2003).

Because of its wide substrate specificity and its constitutive expression in wild type *P. aeruginosa* cells, the efflux pump system encoded by the *mex*A-*mex*B-oprM operon contributes significantly to the elevated resistance that this opportunistic pathogen naturally displays to a variety of antibiotics (Miller, 1994), as result of apparent synergism with the atypically impermeable outer membrane, which limits influx of antimicrobial agents (Brooun *et al*., 2001; Dean *et al*., 2003). Among these Mex pumps, expression of MexA-MexB-OprM is constitutive and is further enhanced in the presence of antimicrobial agents in the growth medium (Li *et al*., 2003). Also, it confers intrinsic resistance to a number of classes of antibiotics including certain ß-lactams, fluoroquinolones, tetracycline, chloramphenicol, erythromycin, macrolides. (Maseda *et al*., 2000; Brooun *et al*., 2001; Jo *et al*., 2003). Given the wide range of chemical structures recognized by the RND family pumps, mutations in efflux pump regulatory genes selected by exposure to a single antibiotic lead to MDR strains (Dean *et al*., 2003). Overexpression of MexA-MexB-OprM have been observed in *P. aeruginosa* clinical strains. (Dean *et al*., 2003). We investigated the expression of a few components of the inner membrane transporter (MexB, Mex F and MexY) in order to evaluate whether efflux mechanisms are up regulated and therefore responsible for the high resistance levels observed in a few of the strains under study.

Resistance to antibiotics frequently reduces the fitness of bacteria in the absence of antibiotics; this is referred to as “cost” resistance (Spratt, 1996). Chromosomal drug resistance-conferring mutations are commonly assumed to carry a fitness cost (Spratt, 1996). This is supported by the observation that some drug resistance mutations selected in vitro involve a significant decrease in bacterial fitness (Schweizer *et al*., 1998). In order to investigate whether quinolone resistance represented a cost for the bacterial fitness, a few strains were selected for this particular study.

4.2 Material and methods

4.2.1 Bacterial strains

Quinolone resistant clinical *P. aeruginosa* isolates were obtained in a hospital from central Portugal (Hospital Infante D. Pedro, Aveiro). The bacterial strains were identified using the Vitek System (Biomerieux). As reference strain, *P. aeruginosa* ATCC 15442 was used. Sensitive *P. aeruginosa* hospital isolates (S1, S2 and S3) were also used as control strains. Bacterial strains were grown in Trypticase Soy Broth -TSB (DIFCO) and maintained at 4 ºC on TSA plates. Antibiotic resistance pattern was investigated by MIC determination to several antimicrobial agents.

4.2.2 Drug susceptibility

The antimicrobial susceptibilities were evaluated according to NCCLS instructions. The MIC was determined as the lowest antimicrobial concentration that prevented visible growth of bacteria (Silbert *et al.*, 2001). *P. aeruginosa* ATCC 15442 and sensitive *P.aeruginosa* were used as control strains. After 24 h incubation at 37 ºC the results were registered and interpreted according to the National Committee for Clinical Laboratory Standards NCCLS criteria (NCCLS, 2000).

4.2.3 RNA isolation and reverse transcription-PCR (RT-PCR)

All the manipulations of RNA were carried out in RNAse free areas.

Cells of *P. aeruginosa* (2 mL cultures of strains S1, S2, S3, Pa24, Pa30 and Pa31) were harvested at the exponential phase of growth and treated with, RNAse free DNAse (Promega, USA, 1 U of enzyme/µg of RNA for 30 min at 37 ºC). Total cellular RNA was isolated from cells using the Qiagen RNeasy Mini Kit (Qiagen Inc., USA). Purified RNA was quantified spectrophotometrically and stored at –20 ºC until required. A sample containing 1 ?g of DNase- treated RNA was used as template for RT-PCR using the Qiagen One Step RT-PCR Kit (Qiagen) according to the manufacturer's reccomendations.

Primer pairs used for RT-PCR are listed in Table 1

Table 1 - Primers used to amplify *mex* B, *mex*F and *mex*Y.

Primers	Accession number
*mex*B - F-5'- CGCTCCGCAGCAAGGCGTGAC - 3 *mex*B - R- 5'- GACCGGCAGAGTGAGGATCG -3'	AF092566
mex F -.F- 5'- CTTCCAGTTGTCGATCAACAC - 3' *mex* F - R- 5'- CACCGAGAAGCGGTCCTTC- 3'	X99514
mex Y - F- 5'- GACTTCATGATCATGGTG ATG- 3' *mex* Y - R- 5'- CCTTGGCGAACGTCGCCAGC - 3'.	AB015853

PCR reaction (20 µL total volume) contained 12 µL of water, 5 µl of buffer, 1 µL of dNTP, 2,5 µL primer Fw, 2,5 µL primer Rv , 1µL of enzyme and were amplified in a PCT-100TM Programmable Thermal Controller (MJ Research, Inc.) with the following PCR programme: 50 ºC for 30 min; 95 ºC for 15 min; a total of 20 amplification cycles were performed consisting of a denaturation step at 94 ºC for 1 min, annealing 57 ºC for 1 min and extension 72 ºC for 1 min; and a final extension 72 ºC for 10 min 5µL of the RT PCR products were separated in a 2 % agarose gel in 1x TBE and visualised after ethidium bromide staining under UV light.

4.2.4 Determination of bacterial fitness

The cost of overall resistance shown by some isolates was determined by comparing the growth profile observed in drug resistant strains to that of the drug sensitive strains. Pre cultures of drug resistant and drug sensitive strains were used to inoculate antibiotic-free TSB medium (DIFCO). 1 mL of this culture with an optical density A_{600nm} of 1 was inoculated in 100 mL of fresh TSB medium without antibiotic. Every 20 min a volume 1 mL was withdrawn from the culture and optical density A_{600nm} was determined. Fitness was estimated based on the growth response exhibited by the selected group of strains individually. No competition experiment was carried out.

4.3 Results and Discussion

4.3.1 Drug susceptibility

MIC values were determined, for a number of antibiotics, to all the quinolone resistant *P. aeruginosa* strains, to three sensitive *P. aeruginosa* strains (S1, S2 and S3-hospital isolates) and to the control strain *P.aeruginosa* ATCC 15442. Results are shown in Table 2.

Overall, most of strains showed high-level resistance to nalidixic acid, with a MIC value for the quinolone resistant strains generally higher than (? 256 ?g/ml) and also to a number of other antibiotics. A few strains (Pa1, 24, 29 and 33) showed high-level resistance to ciprofloxacin and also to other groups of antibiotics tested, namely beta-lactamics, macrolides and aminoglycosides. Strain Pa5 was particularly resistant to ciprofloxacin (32 mg/L) and also to the other antimicrobial compounds tested.

4.3.2 RT-PCR

We investigated the expression of *mex*B, *mex*F and *mex*Y in some quinolone resistant *P.aeruginosa* strains. *mex*B it is known to be constitutively expressed in wild type *P. aeruginosa*, and was therefore the first to be analysed. Only a few strains were selected for this particular study based on the quinolone susceptibility pattern exhibited and also on the presence of mutations in the Topoisomerase II genes, in particular, in the *gyr*A and *gyr*B genes. In a first approach only nine strains were selected (Pa1, Pa3, Pa5, Pa 24; Pa30, Pa31 and the sensitive *P.aeruginosa* strains S1, S2 and S3).

RT-PCR analysis revealed that all the strains tested were expressing the *mex*B and *mex*F genes with the exception of strain Pa31 that showed lower or no expression at all of the referred genes (Figures 1, 2 and 3). For expression of *mex*Y gene only strains Pa24, Pa30, Pa31, S1, S2 and S3 were analysed because Pa30 and Pa31 showed exactly the same mutation in the *gyr*B gene, in codon 464 where a serine was replaced by a tyrosine. Strain Pa24 had a different mutation in the *gyr*B (codon 465, where a glutamine was replaced by an arginine) and it also possessed a mutation in the *gyr*A gene. The results revealed that this gene was not being expressed in strain Pa31, but it is constitutively expressed in the other strains.

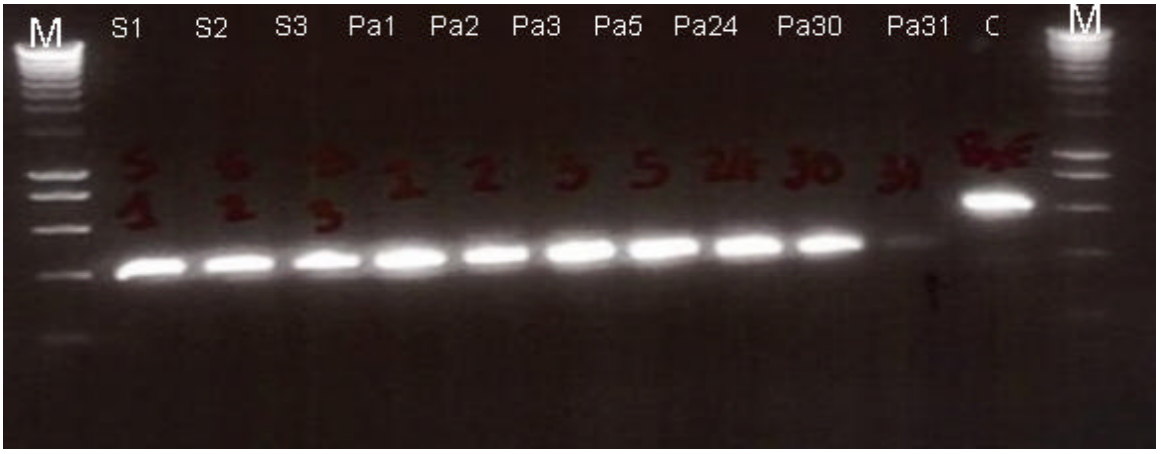

Figure 1- RT-PCR analysis. RT- PCR products indicating the expression of the *mex*B gene. M: Molecular weight marker (Hyper Ladder I); S1-S3: Sensitive clinical *P. aeruginosa*; Pa1-Pa3, Pa5, Pa24, Pa30 and Pa31: resistant clinical *P. aeruginosa*; C: *par*E gene (positive control).

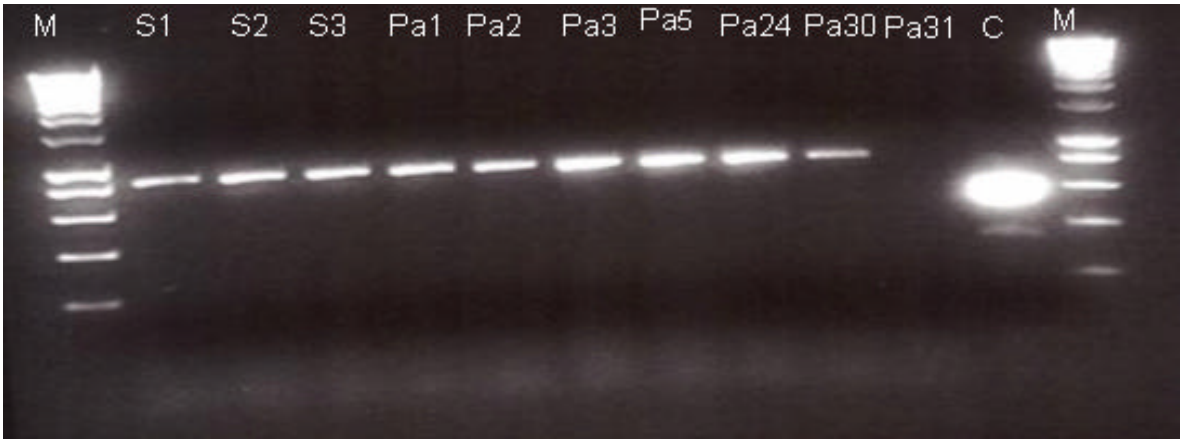

Figure 2- RT-PCR analysis. RT- PCR products indicating the expression of the *mex*F gene. M: Molecular weight marker (HyperLadder I); S1-S3: Sensitive clinical *P. aeruginosa*, Pa24, Pa30 and Pa31: resistant clinical *P. aeruginosa*; C: *par*E gene (positive control).

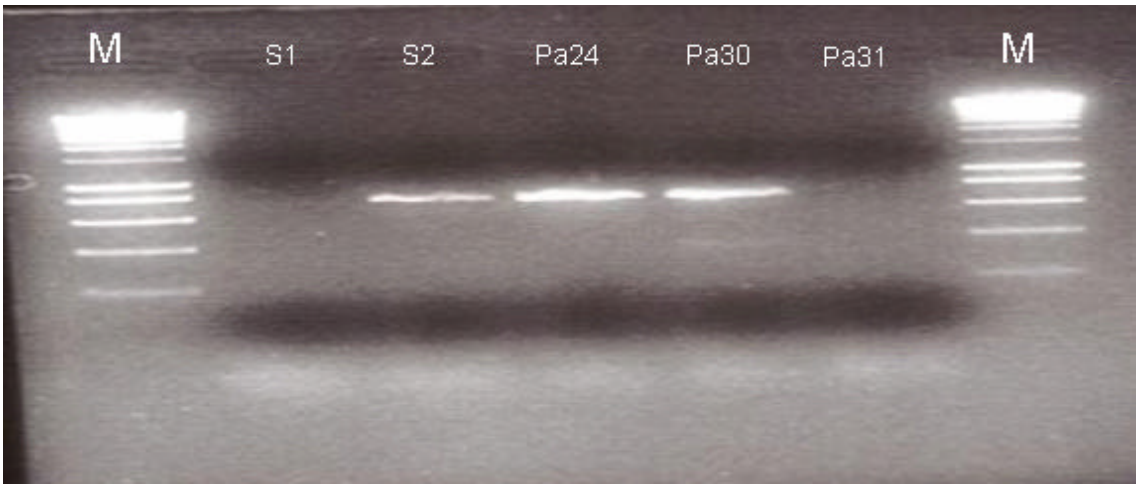

Figure 3- RT-PCR analysis. RT- PCR products indicating the expression of the *mex*Y gene. M: Molecular weight marker (HyperLadder I); S1 and S2: sensitive clinical *P. aeruginosa*, Pa24, Pa30 and Pa31: resistant clinical *P. aeruginosa.*

4.3.3 Bacterial growth experiments

The results previously obtained in RT- PCR experiment suggested that strain Pa31 was not expressing any of the analysed genes that are involved in transport of substrate across the membrane, dispite the fact that this same strain showed resistance to a number of antibiotics. Therefore growth experiments were carried out for the sensitive *P. aeruginosa* strains S1, S2 and S3 and also for the clinical quinolone resistant *P. aeruginosa* strains Pa24, Pa 30 and Pa31. Growth profiles in culture medium, without antibiotic, are shown in Figure 4. Analysis of this figure shows that strain Pa31, has a slower growth rate when compared to the other strains being studied. This result indicates that this particular strain did not show adaptation to the culturing conditions revealing a slower doubling time. All the remainder strains, in no competition conditions without drug, show a fitness advantage that is translated in shorter doubling times. One possible explanation for the decreased fitness of strain Pa31 was most probably the extra metabolic load of expressing the novel phenotypes and possibly the accumulation of novel proteins in the cell. Growth experiments in presence of the drug are the next step to follow in order to compare the behaviour of these strains.

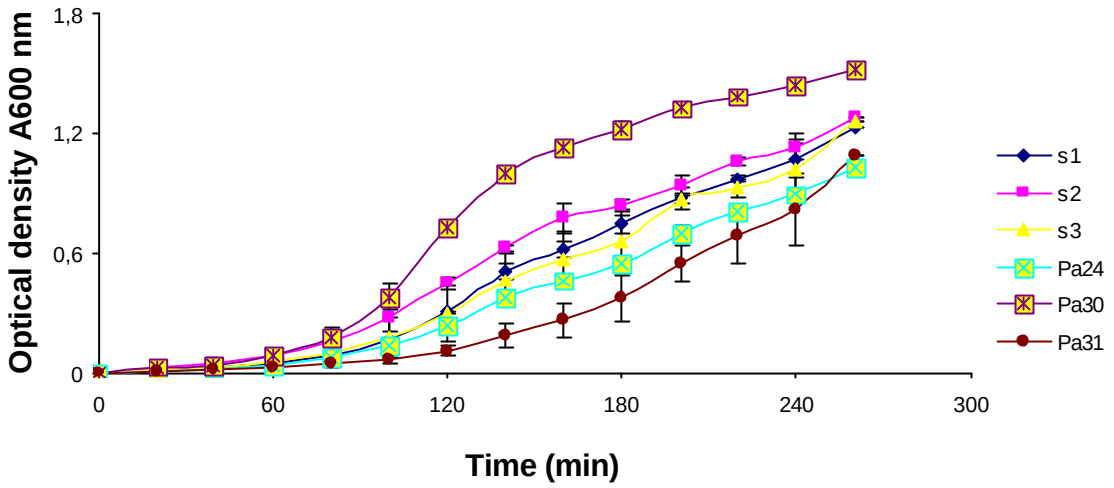

Figure 4- Growth curves of sensitive clinical *P. aeruginosa* (S1-S3) and clinical quinolone resistant *P. aeruginosa* (Pa24, 30 and 31) in antibiotic free medium (TSB) at 37 ºC. Three replicas of each growth curve were performed.

4.4 Discussion

It's well known that the expression of MexA-MexB-OprM in standard culture media is constitutive and contributes to the intrinsic resistance to a number of antimicrobials including fluoroquinolones among others (Poole *et al.,* 2001). Therefore analysis of the expression of this pump constituted the first target in our study. Study of the expression of other efflux systems was also attempted, namely MexEF-OprN, MexXY-OprM by RT-PCR. All of the selected strains were expressing the genes referred with the exception of strain Pa31 where all the operons were silent or expression was very weak. It is generally believed that of the four well characterized *P. aeruginosa* efflux pumps, only MexAB-OprM is expressed in wild type strains; the other pumps remain inactive until mutations have been acquired in repressors of these systems (Kievit *et al*., 2001). The presence of such mutations can also be suggested to exist in Pa31 strain. Considering the fact that all the strains used in the present study also showed at least a mutation in the QRDR region of each of the

following genes, *gyr*A, *gyr*B, *par*C and *par*E, it can be suggested that other mechanisms may also be responsible for the resistance pattern exhibited by the Pa31 strain.

Drug susceptibility tests indicated that all the *P. aeruginosa* strains show different resistance levels to a number of antibiotics. Nevertheless expression of the *mex*B, *mex*F and *mex*Y genes was determined in cells growing in antibiotic free medium, therefore under this circumstances it is not expected to observe overexpression of the referred genes, as the antibiotic does not need to be extruded from the cell. Therefore, the results obtained so far do not indicate that efflux systems are the only mechanism responsible for quinolone resistance in the *P. aeruginosa* clinical strains. Further studies are needed to clarify these results.

RT- PCR results and also those of drug susceptibility conducted to a bacterial fitness study were growth of strains in antibiotic free medium was evaluated. A study of this nature will reveal the capacity of adaptation of the bacterial strains to different environmental conditions. Pa31 showed a higher resistance level to different antibiotics when compared to that of strain Pa30, and also showed slower growth in standard antibiotic free medium. These results are consistent because we would expect that Pa31 would grow slower than Pa30 as it is well known that resistance to antibiotics frequently reduces the fitness of bacteria in the absence of antibiotic (Sander *et al*., 2002), as in the presence of a drug, a resistant genotype is expected to be at an advantage compared to less resistant phenotypes while in the absence of a drug, however, resistant genotypes may be at a disadvantage compared to their sensitive counterparts (Cowen *et al*., 2001). Nevertheless it would be expected that all quinolone resistant strains would exhibit approximate growth behaviour despite the fact of the different level of resistance observed.

Further studies into the regulation of the expression of these efflux systems will be required to evaluate their roles in determining quinolone resistance in the different clinical *P.aeruginosa* strains. Together with the mutations on the QRDR results, already obtained, we would expect to better understand the resistance mechanisms and the pattern exhibited by the *P.aeruginosa* strains being studied.

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5 DISCUSSION

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P. aeruginosa is a well-known opportunistic pathogen that has been repeatedly associated with sporadic or clustered cases of infection generally confined to single hospitalization units. Nevertheless, propagation of clones with high epidemic potency, proceeds through complex routes that may involve, for example, the hands of health care personnel, environmental reservoirs, medical equipment, or reagents (Hocquet *et al.*, 2003). Most of these isolates appear to be multidrug resistant, a property which may account for their maintenance in clinical environments under high antibiotic pressure. *P. aeruginosa* is innately resistant to many antibiotics therefore treatment options are limited to a few drugs such as ureidopenicillins, the carbapenems, ceftazidime and ciprofloxacin (Higgins *et al.*, 2003).

We investigated the dissemination of quinolone resistant *P. aeruginosa* isolated from patients admitted to a Hospital in central Portugal. Only the isolates collected on the day of admittance/external appointment were considered for the study.

Molecular genotyping methods based on PCR and also PFGE proved to be valuable tools for this type of study. A few clones were identified among *P. aeruginosa* population but, overall, most of the isolates are genetically different as revealed by the dendrograms obtained from BOX-PCR and PFGE analysis. It is suggested that a common and/or exogenous source of contamination is responsible for cross-acquisition of quinolone resistant *P. aeruginosa.*

Observed resistance to quinolones in a few clinical *P. aeruginosa* isolates can be attributed to the mutation in codon 83 of *gyr*A, were a threonine is replaced by a isoleucine. However new mutations were also found in the *gyr*B, *par*C and *par*E genes of the clinical isolates of *P.aeruginosa* being studied. Those mutations also seem to account for the increasing level resistance observed as most of the isolates that did not have a *gyr*A mutation showed a high level resistance to the fluoroquinolones.

Other resistance mechanisms can also contribute to the high level resistance to fluoroquinolones observed as, for instance, active efflux systems. RT-PCR on the membrane portion of the efflux pump (*mex*B, *mex*F and *mex*Y) revealed that all the strains selected for this particular experiment are expressing, apparently constitutively, the genes encoding the transporter involved in the export of the substrate across the inner membrane. Exception is made for strain Pa31 that is not expressing none of the referred genes. Those results conducted to a study of bacterial growth in antibiotic free medium that revealed that strain Pa31 shows a slow growth when compared to the rest of the isolates and this could have an overall impact in bacterial fitness.

Further studies are in progress to help elucidate the main mechanisms involved in quinolone resistance by the clinical bacterial isolates used in the present study.

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