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DETECTION OF FLUOROQUINOLONE RESISTANCE AND EFFLUX PUMPS ACTIVITY BY FLOW CYTOMETRY

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DETECTION OF FLUOROQUINOLONE RESISTANCE AND EFFLUX PUMPS ACTIVITY BY FLOW CYTOMETRY

DETECÇÃO DA RESISTÊNCIA E ACTIVIDADE DAS BOMBAS DE EFLUXO ÀS FLUOROQUINOLONAS POR CITOMETRIA DE FLUXO

Thesis presented to *Escola Superior de Biotecnologia* of the *Universidade Católica Portuguesa* to fulfill the requirements of Master of Science degree in Applied Microbiology

by

Rita Ferreira Nogueira

Place: Faculdade de Medicina da Universidade do Porto – Serviço de Microbiologia (Porto, Portugal)

Supervision: Cidália Pina-Vaz, PhD (Faculdade de Medicina da Universidade do Porto, Porto, Portugal)

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ABSTRACT

Fluoroquinolones are bactericidal drugs which have been widely used due to their great activity and wide spectrum namely against gram negative bacteria like *Enterobacteriacea*. Multidrug resistance is a rising health concern worldwide and increased AcrAB-TolC efflux pump expression has been documented in association with resistance to fluoroquinolones. The classic susceptibility methods are based on growth in the presence of antimicrobial drugs which takes at least 24 to 48 hours and empiric therapy usually is used to overcome this delayed answer. A rapid assay was created to determine the susceptibility of gram negative bacteria to ciprofloxacin and levofloxacin; facing a resistant phenotype, another protocol was developed to detect the presence of efflux pump over-expression. These are rapid and accurate protocols based on flow cytometry that demonstrated great advantages for clinical Microbiology. Sixty two resistant and susceptible clinical isolates of *Enterobacteriaceae* were tested for ciprofloxacin and fifty three were tested for levofloxacin, previously evaluated by Vitek2®. Genetically modified *E. coli* K12 with AcrAB-TolC efflux system inactivated, over-expressed and wild-type were used as efflux controls. For susceptibility profile, the cells were incubated with antimicrobial breakpoints according to CLSI, then fixed with ethanol 70% (v/v) and stained with SYBR-Green I, a nucleic acid probe. CFU assays were performed before flow cytometric analysis. For efflux activity study thirty resistant strains were tested. Bacteria were diluted in PBS supplemented with glucose and subinhibitory concentrations of ciprofloxacin and stained with Ethidium Bromide. In parallel the same strains were incubated with chlorpromazine, a pump inhibitor, and the protocol repeated. Flow cytometric analysis was performed in FL1 (520 nm) and FL3 (600 nm) for susceptibility phenotype and efflux determination, respectively. In the susceptibility test, susceptible strains showed a decrease in the fluorescence intensity compared to the control; conversely, resistant strains maintained approximate values, even after incubation with high concentrations of the drugs. Correlation between conventional CFU assay and flow cytometry was successfully achieved. In the efflux assay, a comparison between the fluorescence intensity with and without chlorpromazine was done. When it decreased there was AcrAB-TolC over-expression, if the values maintained there wasn't. Flow cytometry demonstrated to be an excellent approach to evaluate the resistance to fluoroquinolones and the responsibility of efflux pumps on such resistance.

RESUMO

As fluoroquinolonas são fármacos bactericidas amplamente utilizadas devido à sua elevada actividade e largo espectro nomeadamente contra bactérias gram negativo como é o caso das *Enterobactereaceae*. A resistência a antibacterianos é um tema preocupante a nível mundial. Os métodos de susceptibilidade clássicos baseiam-se no crescimento bacteriano na presença de antibacterianos, cujos resultados demoram pelo menos 24 a 48 horas a obter, sendo muitas vezes necessário recorrer a terapia empírica. Foi criado um teste rápido para determinar a susceptibilidade de bactérias gram negativo à ciprofloxacina e levofloxacina; no caso de fenótipos resistentes foi desenvolvido um outro protocolo para detectar a presença de sobre-expressão de bombas de efluxo, um mecanismo de defesa bacteriano bem documentado. Tratam-se de testes rápidos e precisos que utilizam a citometria de fluxo e demonstraram grandes vantagens para a Microbiologia clínica. Sessenta e dois isolados clínicos de *Enterobactereaceae* foram testados para a ciprofloxacina e cinquenta e três para a levofloxacina, previamente avaliados pelo sistema Vitek2®. *E. coli* K12 geneticamente modificadas com o sistema de efluxo AcrAB-TolC inactivado, sobre-expresso e *wild-type* foram utilizadas como controlos de efluxo. Para o perfil de susceptibilidade, as células foram incubadas com os *breakpoints* fornecidos pelo CLSI, em seguida fixadas com etanol 70% (v/v) e marcadas com SYBR-Green I, uma sonda de ácidos nucleicos. A contagem de UFC foi realizada antes da análise citométrica. Para o estudo do efluxo, trinta isolados foram estudados onde as células são diluídas em PBS suplementado com glucose e concentrações subinibitórias de ciprofloxacina e marcadas com brometo de etídio. Em paralelo, as mesmas estirpes são incubadas com clorpromazina, um inibidor de efluxo, e o protocolo repetido. A análise citométrica foi realizada em FL1 (520 nm) e FL3 (600 nm) para a avaliação de susceptibilidade e a determinação do efluxo, respectivamente. As estirpes susceptíveis incubadas com os antibacterianos demonstraram uma diminuição da intensidade de fluorescência comparando com as não tratadas. As estirpes resistentes mantiveram valores aproximados, mesmo após incubação com altas concentrações de fármaco. Verificou-se correlação entre a contagem de UFC e a citometria de fluxo. Para o ensaio de efluxo, comparou-se a intensidade de fluorescência com e sem clorpromazina. Nos casos de sobre-expressão da bomba de efluxo AcrAB-TolC a intensidade de fluorescência diminuiu. A citometria de fluxo demonstrou ser uma excelente ferramenta para avaliar a resistência às fluoroquinolonas e a responsabilidade do efluxo nessa mesma resistência.

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% – Percentage

µg – Microgram

µl – Microliter

^{o}C – Celsius degree

β – Beta

ml – Milliliter

OD_{600nm} – Optical density at 600 nanometers

pH – Hydrogen potential

(v/v) – Concentration volume per volume

rpm – Rotations per minute

< – Minor

LIST OF ABBREVIATIONS

AST – Antibiotic Susceptibility Testing

ATCC – American Type Culture Collection

ATP – Adenosine triphosphate

CFU – Colony Forming Units

Cipro – Ciprofloxacin

CLSI – Clinical Laboratory Standard Institute

CPZ – Chlorpromazine

$DiBAC_4(3)$ – Bis-(1,3-dibutylbarbituric acid)

DNA – Deoxyribonucleic acid

dsDNA – Double-stranded DNA

ESBL – Extended-Spectrum β-Lactamase

EtBr – Ethidium Bromide

EUCAST – European Comittee on Antimicrobial Susceptibility Testing

FC – Flow Cytometry

FI – Fluorescence Intensitity

FQ – Fluoroquinolone

FSC – Forward-scatter

LB – Luria Bertani broth

Levo – Levofloxacin

MH – Müeller-Hinton broth

MIC – Minimal Inhibitory Concentration

PBS – Phosphate buffered saline

PMQR – Plasmid-Mediated Quinolone-Resistance

QRDR – Quinolone Resistance Determining Region

RNA – Ribonucleic acid

ROC – Receiver Operating Characteristic

SGI – SYBR-Green I

SPSS – Statistical Package for the Social Sciences

SSC – Side-scatter

ssDNA – Single-stranded DNA

USA – United States of America

1. INTRODUCTION

1.1. General considerations

Nalidixic acid was the first quinolone introduced in the 1960's. In the late 1980's many fluoroquinolones (FQs) were commercialized: ciprofloxacin (Cipro) in 1987 and ten years later levofloxacin (Levo), after a period of a certain idling in fluoroquinolone research and development. The chemical structure of quinolones is composed of a bicyclic aromatic core. FQs are synthetic quinolone derivatives with an addition of a fluorine atom in the 6 position that enhances antimicrobial activity, alters pharmacokinetic properties, improves potency, providing enormous advantages over quinolones. The addition of different substituents to the different positions of the quinolone nucleus accounts for the variability in antimicrobial spectrum of activity. Their mechanism of action is unique among available antibiotics (Andriole, 2005; Dalhoff, 2012).

Since FQs were introduced in clinical practice, they allowed physicians to prescribe them orally and parenterally as broad spectrum drugs in a large range of infections such as urinary tract infections, gastrointestinal infections, respiratory tract infections, chronic osteomyelitis and sexually transmitted diseases (Dalhoff, 2012; Greenfield, 1993).

The earliest FQs were predominantly active against gram negative agents, especially *Enterobacteriaceae*. The newer ones, including Cipro, had a wider spectrum of activity, including gram positive and some atypical bacteria. The more recent FQs, such as Levo, have an enhanced activity against gram positive cocci, but are also active against gram negative microorganisms (Ball, 2000; Gootz & Brighty, 1996).

During the last several years FQs were used in large scale, especially in outpatients. Different rates of FQ resistance have been reported worldwide in both nosocomial and community-acquired infections. Several factors contribute for these differences: the patients, local epidemiological factors, antibiotic policies, inadequate usage, lack of information and their use in animals are some of the factors (Acar & Goldstein, 1997; Dalhoff, 2012). More worrisome are recent reports of an overall increase in resistance to FQs amongst bacteria causing community-acquired infections, such as *E. coli*. Surveillance data from the late 1990's demonstrated that FQ resistance had to be associated with particular bacterial species on one hand, and patient populations on the other hand (Acar & Goldstein, 1997). Not much has changed since then and resistance increased to alarming high rates.

The continued increase in FQ resistance affects patient management and demands a change in some current guidelines (Dalhoff, 2012).

Another major concern is the association of FQ resistance and Extended-Spectrum β-Lactamase (ESBL)-production in *Enterobacteriaceae*. One to two thirds of *Enterobacteriaceae* producing ESBL's were FQ resistant too, thus limiting the FQ use in the treatment of community, as well as healthcare acquired infections, in all those geographic areas in which FQ resistance rates and/or ESBL-production is high (Dalhoff, 2012).

Successful clones of resistant bacteria are often spread horizontally either due to poor hygiene, transfer of patients from one ward to another or from a hospital to a nursing home, as well as interregional migration and international population mobility. Meaning, humans are mobile vectors of drug resistance (MacPherson et al., 2009). Both, exposure of bacterial pathogens to antibacterials and environmental factors have a role in the emergence and spread of resistance. Furthermore, inappropriate antibiotic policies, poor compliance, suboptimal dosing, diagnostic and laboratory error, ineffective infection control, counterfeit or altered drugs contribute to the selection of resistance (Dalhoff, 2012).

This should be taken into account when empiric treatment is prescribed (Barenfanger, Drake, & Kacich, 1999; Dalhoff, 2012).

1.2.Mechanism of action of fluoroquinolones

DNA gyrase is a type II topoisomerase that negatively supercoils deoxyribonucleic acid (DNA) in the presence of adenosine tryphosphate (ATP). It also catenates and decatenates circular double-stranded DNA (dsDNA) molecules, resolves knots and relaxes negatively supercoiled DNA in the absence of ATP. Thereby, this enzyme is indispensable for most vital processes involving dsDNA such as transcription, replication and recombination making it an appropriate target for antibacterials. DNA gyrase cleaves the double-strand, passes another duplex through it and reseals the molecule. The biochemical characterization of gyrase from *E. coli* shows that the enzyme is composed of two subunits GyrA (97-kDa protein) and GyrB (90-kDa protein) (Jacoby, 2005). The C-terminal one-third of GyrA is responsible for wrapping DNA around itself in a positive superhelical way. The N-terminal two-thirds has the cleavage-religation activity. Regarding the GyrB subunit, it's N-terminal half is responsible for hydrolysing ATP and the C-terminal half is involved in the binding between DNA and the GyrA subunit (Hooper, 1998). Topoisomerase IV can also remove positive and negative supercoils and is even better at decatenation than gyrase. It is also

composed of two subunits, ParC and ParE. Both enzymes work together in the replication, transcription, recombination and repair of DNA. A few bacteria are able to function with only DNA gyrase, but most bacteria have both enzymes. In gram negative microorganisms, gyrase is more susceptible to quinolones than topoisomerase IV, and on the other hand, in gram positive bacteria topoisomerase IV is the prime target, thus gyrase is less susceptible to inhibition (Jacoby, 2005).

A range of inhibitors have been found that interfere with the enzymatic reactions of DNA gyrase leading to its inactivation, and quinolones are one of them.

When gyrase is performing DNA supercoiling the GyrA subunit covalently binds to the double-stranded helix, in an ATP-dependent process, where both DNA strands are cleaved at certain 4 base pair staggered sites. At this location described, DNA is present as single strands, forming a bubble-shaped "quinolone binding pocket". Two quinolone molecules assemble to form a dimer structure inside the gyrase induced binding pocket. This way, the progress of the supercoiling procedure that includes rearrangement of the DNA segments, reattachment and resealing of the cuts, is locked up. Permanent gaps in the DNA strands induce synthesis of repair enzymes (exonucleases), initiating an uncoordinated repair process. This results in the breakdown of the DNA molecule leading to irreversible damage and, consequently, to cell death (Cabral et al., 1997; Heddle & Maxwell, 2002; Lupala, Gomez-Gutierrez, & Perez, 2012; Mason, Power, Talsania, Phillips, & Gant, 1995). FQs are the only class of antimicrobial agents in clinical use that are direct inhibitors of bacterial DNA synthesis (Dalhoff, 2012).

1.3.Mechanisms of resistance

There are three main mechanisms of resistance to quinolones which are: mutations of target-enzymes, mutations that reduce drug accumulation in the bacterial cell and transference of plasmids that protect the cells from the effects of the antimicrobial (Fabrega, Madurga, Giralt, & Vila, 2009).

Target-enzyme resistance mechanisms – This is the main mechanism of resistance (Strahilevitz, Jacoby, Hooper, & Robicsek, 2009). As stated before, the prime targets of quinolone action are the indispensable enzymes DNA gyrase in gram negative bacteria and topoisomerase IV in gram positive bacteria. In *E. coli* and *Klebsiella*, mutations in the *gyr*A gene, that are chromosome enconded, result in resistance to FQs. Such is due to amino acid substitutions in *gyr*A, in a region called Quinolone Resistance Determining Region (QRDR),

that is located in the DNA-binding surface of the enzyme, and has "hot spots" that differ according to bacterial species (Cabral et al., 1997; Mazzariol, Zuliani, Cornaglia, Rossolini, & Fontana, 2002). The most accepted reason for the diminishing in susceptibility is that mutations reduce drug affinity to gyrase (Barnard & Maxwell, 2001).

Once a first-step mutation has occurred, additional mutations in *gyr*A, *gyr*B or *par*C (in topoisomerase IV) can be possible augmenting resistance, despite, by themselves, they wouldn't be enough to confer resistance in a wild-type GyrA, once the level of susceptibility is determined by the most susceptible target, which is GyrA. In other words, multiple mutations are generally required to confer clinically important resistance, since wild-type strains are highly susceptible (Jacoby, 2005; Strahilevitz et al., 2009). Furthermore, reduced target expression has been described as another mechanism leading to low level quinolone resistance (Dalhoff, 2012).

Efflux resistance mechanisms – Quinolones must cross the cell wall and the cytoplasmic membrane in order to reach their targets. Particularly in gram negative bacteria, they must penetrate an additional outer membrane. These microorganisms can regulate the permeability of the membrane, by altering expression of outer membrane porins that create channels for passive diffusion. Furthermore, bacteria in general have energy-dependent nonspecific efflux systems. Some of them are constitutively expressed, others are induced by mutations or regulated by global control systems. The AcrAB-TolC efflux system is present in most *Enterobacteriaceae*, belongs to the resistance-nodulation-division family of transporters that only exists in gram negative bacteria and allows the extrusion of substrates from the cytoplasm and/or periplasm (Paixao et al., 2009; Yang, Clayton, & Zechiedrich, 2003). This transporter consists of three parts: TolC, an outer membrane protein, AcrB, an inner membrane protein and AcrA, a membrane fusion protein that facilitates the connection between AcrB and TolC as depicted in Figure 1.3.1 (Bratu, Landman, George, Salvani, & Quale, 2009; Yang et al., 2003; Zgurskaya & Nikaido, 1999). The AcrAB-TolC system plays a major role in quinolone efflux in *E. coli* and *K. pneumoniae* and has multiple controls (Fenosa et al., 2009; Jacoby, 2005; Padilla et al., 2010).

Since these efflux pumps have a non specific character, they can be activated in response to a variety of other compounds such as non-quinolone antimicrobials, antiseptics, detergents, sodium salicylate, amongst others. This illustrates the complexity of fluoroquinolone resistance mechanisms since there can be coselection of resistance by chemically unrelated classes of antibacterials and antiseptics (Dalhoff, 2012; Jacoby, 2005).

A study done with *K. pneumoniae* and Cipro showed that an efficient efflux pump system could eliminate about 90% of the cell-associated Cipro. The resistant isolates, with a Minimal Inhibitory Concentration (MIC) over 1 µg/ml, had medium to high levels of efflux activity and the ones with MICs over 16 µg/ml had high to very high levels of efflux activity. The most resistant strains also had associated target site mutations. This group also verified that high levels of efflux pump activity appeared to be the main or the only mechanism of resistance to FQs in some isolates with no target alterations, which leads to the conclusion that efflux plays a major role in resistance in *K. pneumoniae* strains (Aathithan & French, 2011).

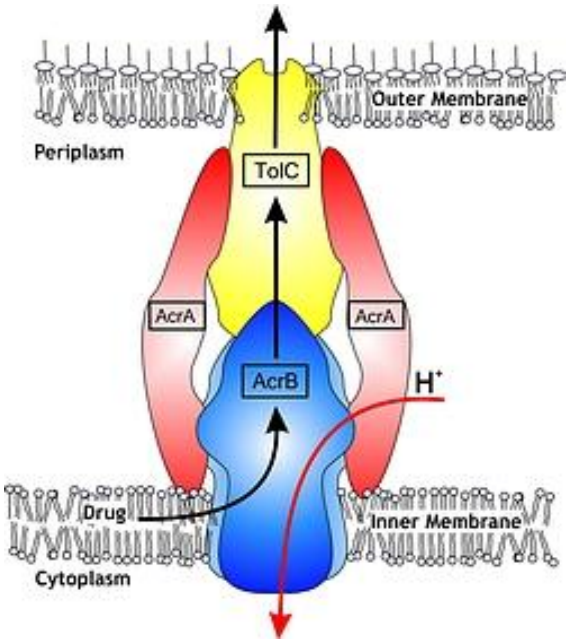

Figure 1.3.1 – Schematic drawing of tripartite RND multidrug efflux system AcrAB–TolC of the gram negative bacteria *Escherichia coli*. The activated AcrAB transporter triggers opening of the periplasmic tip of TolC to allow diffusion of substrates across the outer membrane. *Reprinted from* (Pos, 2009).

In *E. coli*, there is evidence that AcrAB-TolC over-expression is important for quinolone resistance, but for actual clinical resistance has to be added to mutations in target enzymes and/or is associated with over-expression of other efflux pumps from other transporter families (Yang et al., 2003). In gram negative organisms in general, resistance due to efflux pump over-expression does not account for high level resistance by itself, but helps increase the MICs (Hernandez, Sanchez, & Martinez, 2011).

Plasmid-mediated resistance – Until recent times, the mechanisms of resistance to quinolones in *Enterobacteriaceae* were believed to be only two chromosome encoded: alterations in the target enzymes and over-expression of naturally-occurring efflux.

However, emergence of Plasmid-Mediated Quinolone-Resistance (PMQR), which consists of horizontally transferable elements, was first reported in 1998 in a *K. pneumoniae* isolate. Three PMQR mechanisms are known: Qnr proteins, aminoglycoside acetyltrasferase AAC(6')-lb-cr and efflux pump QepA. Five types of Qnr proteins have been reported and they bind and protect DNA gyrase and type IV topoisomerase from quinolone action acting as part of a Stress-Response mechanism (Dalhoff, 2012; Guillard et al., 2011; Ruiz, Pons, & Gomes, 2012). They are widespread in *Enterobacteriaceae* (Da Re et al., 2009) and usually plasmids carrying *qnr* genes also encode ESBL's, which could be one of the reasons for the high frequency of resistance to quinolones observed in ESBL-producing bacteria. The expression of these genes is upregulated by the presence of Cipro (Briales et al., 2012; Dalhoff, 2012; Jacoby, 2005). The AAC(6')-lb-cr acetylates several FQs and has two amino acid substitutions compared to the wild type. The more recently reported QepA are plasmid-encoded efflux pumps that extrude hydrophilic FQs, such as Cipro. So far, Qnr and AAC(6')-lb-cr have been reported worldwide even from unrelated enterobacterial species. PMQR confers a low level resistance and is usually associated with other mechanisms of resistance to confer clinical non-susceptibility (Guillard et al., 2011; Jacoby, 2005).

1.4.Detection of susceptibility profile *in vitro*

It is a wide-held premise that an ideal susceptibility test is based on cellular growth and viability. Routine susceptibility tests have made possible the quantitative and qualitative assessment of drug susceptibility.

The development and standardization of susceptibility tests to antimicrobials *in vitro* is crucial in predicting and ensuring the success of therapeutic outcome. Despite their importance, these tests are not always required by the physician, who then applies an empirical based therapy. The results of the *in vitro* susceptibility tests are affected by several factors such as *inoculum* concentration, composition and pH of the culture media, temperature, incubation time and the physical and chemical properties of the antimicrobial.

Such complexity has led to the development of reference susceptibility protocols, standardized and provided by the Clinical and Laboratory Standards Institute (CLSI). These protocols are based on microdilution and allow the determination of MICs with good reproducibility (CLSI, 2013). Later, the European Comittee on Antimicrobial Suseptibility Testing (EUCAST) has also developed standardized protocols for bacterial susceptibility. Although the CLSI and EUCAST protocols are equivalent, there are some differences, such

as incubation times, *inoculum* concentrations and breakpoints, which can compromise the comparison of results from different laboratories (Hombach, Bloemberg, & Bottger, 2012; Rodriguez-Martinez et al., 2011).

Meanwhile, there are other alternative tests to evaluate susceptibility to FQs *in vitro*, such as the agar diffusion test, the commercial method Etest® (AB BIODISK, Solna, Sweden) and Vitek2® (bioMérieux, Marcy l'Étoile, France). The agar diffusion method uses discs impregnated with the antimicrobials, allows a qualitative measurement and it's relatively inexpensive and simple to perform. Etest® is based upon a concentration gradient of the antimicrobial and is a quantitative test as it can provide MIC values. On the other hand, it is an expensive test and sometimes has a weak correlation between the drugs and the reference method. Vitek2® is also a quantitative colorimetric method, simple to perform and reproducible.

All of these current techniques provide results only after a period of 24 to 48 hours, since they are based upon microbial growth. They do not consider heterogeneity of the bacterial population or mechanisms of resistance (Alvarez-Barrientos, Arroyo, Canton, Nombela, & Sanchez-Perez, 2000).

1.5.Flow cytometry

Flow cytometry (FC) is a very useful technique with important applications in Biology and Medicine. In the field of Microbiology, this technique has proven its potential namely in the study of susceptibility phenotypes both in bacteria and yeasts (Faria-Ramos et al., 2013; Pina-Vaz & Rodrigues, 2010; Wickens, Pinney, Mason, & Gant, 2000). FC allows to count, analyze and classify cells in a suspension. Simultaneously, this technique performs a multiparametric analysis of the physical and chemical characteristics of each cell, using optical and/or electronic detection devices (Bergquist, Hardiman, Ferrari, & Winsley, 2009).

There are two types of flow cytometers, depending on the illumination source: laser light source or an arc lamp light source. Arc lamp flow cytometers are more adequate for Microbiology applications due to their increased versatility and better light scattering resolution making them ideal for the accurate study of microorganisms (Alvarez-Barrientos et al., 2000).

Summarily, the FC process is based on the circulation of large number of particles (about 50 000) on a hydrodynamically-focused stream (constant velocity), one by one, through an excitation source and the measuring of the magnitude of the impulse

generated by the deviation of that light, using the appropriate filters and detectors (Figure 1.5.1).When the cell is intercepted by the light beam, the transmitted light is composed of scattered and fluorescent light (if fluorescence probe is added) that is received by lenses or mirrors that transfer that light to a detector - photomultiplier tube. The number of detectors varies according to the machine and each photomultiplier collects light at different wavelengths. The scattered light collected in the same direction as the incident light (forward-scatter) is related to cell size, as the scattered light collected at a 90º degree angle from the light source (side-scatter) gives information about cell complexity. Size and complexity are considered intrinsic parameters since they can be obtained without fluorescent staining. Later the photomultiplier amplifies and converts the signal received that is later electronically evaluated and computerized. Such analysis allows quantifying cells with identical characteristics (size and complexity) by creating a histogram of the analyzed population; additionally it is also possible to deduce its heterogeneity (Alvarez-Barrientos et al., 2000; Bergquist et al., 2009).

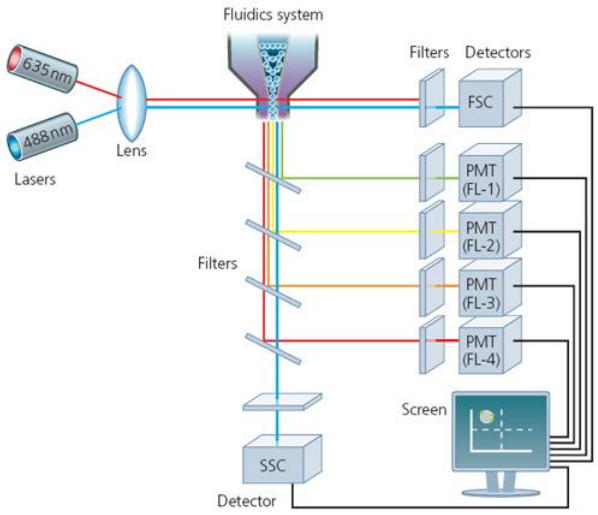

Figure 1.5.1 – Schematic overview of a typical flow cytometer setup. Scheme of optic systems of a flow cytometer with 6 parameters detected: cell size by forward-scatter (FSC), complexity by side-scatter (SSC) and dye fluorescence by 4 different photomultipliers tubes (PMT). *Reprinted from* (Bio-Rad, 2013).

Certain cells may be metabolically active and able to replicate, while others present metabolic action with intact membranes but are not able to replicate. The ability to distinguish different cellular stages is crucial in the assessment of survival of pathogenic microorganisms (Czechowska, Johnson, & van der Meer, 2008; Wickens et al., 2000). FC proved to be a

useful tool on the redefinition of the concept of bacterial viability, broadening the horizons in classical Microbiology.

Using FC and fluorescent labels it is possible to measure biological parameters such as the nucleic acid content, breathing rate, intracellular enzymatic activity, integrity of cytoplasmic membrane or cell wall, evaluate different viability stages, amongst others, analysing many cells in a few minutes (Czechowska et al., 2008).

Some cytometers, called cell sorters, are able to physically separate cells, based on their characteristics that are previously defined (Davey, 2002).

In addition to being an automated technique, it has the additional advantage to provide results in real time that are of the out most importance in clinical Microbiology. Several studies have presented FC as a fast, accurate and reproducible technique in the evaluation of the susceptibility to antimicrobials (Alvarez-Barrientos et al., 2000; Faria-Ramos et al., 2012; Faria-Ramos et al., 2013; Pina-Vaz, Costa-de-Oliveira, & Rodrigues, 2005; Silva, Lourenco, Queiroz, & Domingues, 2011).

2. AIMS OF THIS WORK

The aim of this study was to develop a fast and simple *in vitro* susceptibility test for *Enterobacteriaceae* to FQs based upon FC; additionally it was pretended to understand the role of efflux pumps on such resistance, also using FC.

3. MATERIALS AND METHODS

3.1.Bacterial strains

Sixty two strains of *E. coli* and *K. pneumoniae* were tested for Cipro and fifty three strains for Levo with different susceptibility phenotypes, according to Vitek2® System panel Antibiotic Susceptibility Test (AST)-192 card.

Two reference strains from the American Type Culture Collection (ATCC) (*Escherichia coli* ATCC 25922 and *Klebsiella pneumoniae* ATCC 700603) were used, as recommended by the CLSI for susceptibility evaluation (CLSI, 2013).

For the efflux study, the control strains were *E. coli* K-12 AG100 wild-type (*arg*E*3 thi-l rps*L*xylmtl Δ (gal-uvr*B) supE44) that contains the AcrAB-TolC efflux system functional, *E. coli* AG100A (Δ*acr*AB::Tn*903*Kan^r) that has the AcrAB-TolC efflux system inactivated due to the insertion of the transposon Tn*903* in the *acr*AB operon and *E. coli* $AG100_{TET}$ which is an AG100 progeny with an induced resistance to tetracycline, that over-expresses the *acr*AB efflux pump amongst other efflux systems. These strains were gratefully provided by Miguel Viveiros (Universidade Nova de Lisboa, Lisboa, Portugal).

All the bacterial isolates were frozen at -80 ºC and prior to every experiment they were subcultured twice in Müeller-Hinton (MH) broth to assure purity and the efflux control strains were also subcultured in MH supplemented with the respective selective antibiotic.

3.2.Antimicrobial drugs and reagents

Cipro and Levo were purchased from Sigma-Aldrich (St. Louis, Missouri, United States of America (USA)) as powder and stock solutions (1mg/ml) were diluted in a 0.1 M HCl solution for solubility. MH in powder was acquired from Liofilchem (Abruzzo, Italy) used to prepare agar and broth medium.

SYBR-Green I (SGI) was also purchased from Sigma-Aldrich and stock solution was prepared in a Tris-EDTA buffer for stability at a concentration of 1/1000 (v/v) (commercial concentration not available).

Phosphate Buffered Solution (PBS), chlorpromazine (CPZ), kanamycin, tetracycline and ethidium bromide (EtBr) were also acquired from Sigma-Aldrich. Kanamycin stock solution was prepared with distilled water and filtered with 0.22 µm syringe and tetracycline stock solution was prepared in methanol.

All working solutions were prepared with distilled water. Luria Bertani (LB) used to prepare broth and glucose were also purchased from Liofilchem.

3.3.Susceptibility evaluation using flow cytometry

3.3.1. Growth and antibiotic exposure

Prior to every experiment, bacteria were plated in a MH Petri dish overnight at 37ºC. One bacterial colony was transferred to filtered MH and was grown using shaking bottles at 37ºC and 180 rpm until early exponential phase was reached (OD_{600nm}=0.1).

The culture was then diluted in new filtered MH until an OD_{600nm}=0.06, corresponding approximately to $5x10^6$ cells/ml (Walberg, Gaustad, & Steen, 1997). Aliquots of 1 ml were transferred into shaking bottles with 10 ml of new MH supplemented with the antibiotics added at concentrations corresponding to the breakpoints (1, 2 and 4 µg/ml for Cipro and 2, 4 and 8 µg/ml for Levo) stipulated by the CLSI, following incubation for 1 hour at 37ºC and 180 rpm. This protocol was also tested in cultures directly from the plate (stationary phase).

3.3.2. Bacterial fixation and staining

After incubation with the antibiotics, 1ml aliquots were pelleted (10 minutes, 10000 rpm at room temperature) and were immediately fixed with 70% ethanol for 10 minutes, followed by 5 minutes of centrifugation (10000 rpm at room temperature). Finally, the samples are loaded with 1ml of SGI working solution 1/100000 (v/v), for 1 hour at 37ºC in the dark. 1 ml aliquots were taken for flow cytometric analysis.

3.3.3. Flow Cytometric analysis

After staining, bacterial cells were analyzed in a BD FACSCalibur™ (BD Biosciences, Sidney, Australia) flow cytometer with an argon arc lamp as light source. SGI is excited at 290, 380 and 497 nm, and its emission is at 520 nm, detected in FL1 channel (green fluorescence). Data was collected for 20000 events/sample.

For each strain, a suspension of non-stained and non-treated cells was also analyzed with the purpose to determine the cell´s autofluorescence and define the acquisition settings. Then, non-treated and treated cells stained with SGI were analysed and compared. The data generated by FC was analyzed by Cell Quest Pro software (version 4.0.2, BD Biosciences). The percentage of treated cells was quantified based upon gated population and the area under the obtained curve, which is automatically normalized by the cytometer software.

3.3.4. Cytometric susceptibility phenotype and Cipro/Levo Index

In order to assess the effect of such drugs on the isolates, an Index was calculated for each drug, Cipro Index for ciprofloxacin and Levo Index for levofloxacin, as the ratio between the percentage of treated cells (exposed to the breakpoints of each drug) *versus* the percentage of non-treated cells (absence of drug treatment).

Based on the results of the Index obtained for each bacterial strain, a cut-off value was set with the objective of classify them as susceptible or resistant.

3.3.5. Viability studies

The number of viable cells in the treated and non-treated cellular suspensions was determined by the count of the number of Colony Forming Units (CFU)/ml in MH agar plates. For every sample 1:10 serial dilutions were performed until 10^{-3}, followed by 100 µl inoculation of the plates for each suspension. The counting was performed after 24 hours incubation at 37ºC. Every assay was executed in triplicate.

3.3.6. Statistical Analysis

To study the agreement between FC and the classic method resulting in categorical variables (Susceptible *versus* Resistant), two complementary parameters were calculated: agreement proportion and the *kappa* value.

To evaluate the accuracy of FC as a suitable diagnostic method for detecting the susceptibility phenotype of the tested bacteria different parameters were calculated: area under Receiver Operating Characteristic (ROC) curve, sensitivity, specificity, positive and negative predictive values and overall accuracy.

All the parameters were calculated using the classical method Vitek2® as the reference method with a 95% confidence interval (P<0.001).

Statistical analysis was performed with Statistical Package for the Social Sciences (SPSS) software version 21.0.

3.4.Efflux detection by flow cytometry

3.4.1. Efflux control strains

The efflux control strains were grown in 10 ml of aerated LB using shaking bottles at 37ºC and 220 rpm until *mid-log* phase (OD_{600nm}=0.6). One ml aliquots were collected and centrifuged for 3 minutes at 13000 rpm and the supernatant discarded. The pellet was washed

twice with PBS (13000 rpm for 3 minutes), the supernatant discarded and diluted in PBS supplemented with 0.4% (v/v) of glucose.

EtBr working solution was added at a final concentration of 1 μg/ml followed by incubation at 37ºC for 60 minutes in the dark.

3.4.2. Efflux assessment in clinical strains

The clinical strains were grown in 10 ml of aerated LB using shaking bottles at 37ºC and 220 rpm until *mid-log* phase (OD_{600nm}=0.6). 1 ml aliquots were recultured in the same previous conditions in 10 ml of fresh LB supplemented with Cipro: 4 μg/ml for resistant strains and ½ MIC for susceptible strains (CLSI, 2013).

After adjusting the OD_{600nm}=0.3 for each sample with LB, 1 ml aliquots were collected and centrifuged for 3 minutes at 13000 rpm and the supernatant discarded. The pellet was washed twice with PBS (13000 rpm for 3 minutes), the supernatant discarded and diluted in PBS supplemented with 0.4% (v/v) of glucose.

EtBr working solution was added at a final concentration of 1 μg/ml. In parallel, to the same strains, 1 μg/ml of EtBr was added simultaneously with CPZ (efflux inhibitor) at a final concentration of 20 μg/ml, followed by incubation at 37ºC for 60 minutes in the dark.

3.4.3. Flow cytometric analysis

After staining, 0.5 ml aliquots were collected for fluorescence measurements in both control and clinical strains. The samples were analyzed in a BD FACSCalibur™. EtBr is excited at 530 nm and its emission is at 600 nm, detected in FL3 channel (red fluorescence).

4. RESULTS

4.1.Optimization of FC protocol for susceptibility evaluation

When a flow cytometric susceptibility protocol is being designed from scratch it is necessary to optimize some parameters such as *inoculum*, treatment and fluorochrome concentrations, as well as incubation times. There were few papers regarding susceptibility by FC that provided leads for the optimal bacterial concentration and there was also no published work at the time using SGI as a susceptibility probe for *Enterobacteriaceae*. Therefore, four different concentrations of SGI were tested in order to assess the optimal fluorochrome concentration. Figure 4.1.1 presents the histogram overlay of non-treated cells incubated with several fluorochrome concentrations, where A (blue) was the sample with the lowest concentration of SGI and D (purple) was the most concentrated. Samples B (red) and C (black) show the best results since peak D had too much FI and A did not have enough discriminating FI. SGI 1/100000 (v/v) (B) was considered the optimal concentration of fluorochrome since it was the lowest concentration with the best results.

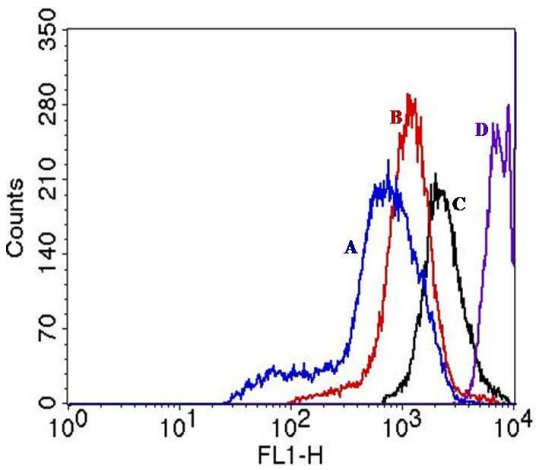

Figure 4.1.1 – Histogram of the susceptible strain *E. coli U54218* analysed in FL1 (520 nm) after staining for 1 hour with different concentrations of SGI with no antimicrobial treatment. A – 1/1000000 (v/v) SGI; B – 1/100000 (v/v) SGI; C – 1/10000 (v/v) SGI; D – 1/1000 (v/v) SGI.

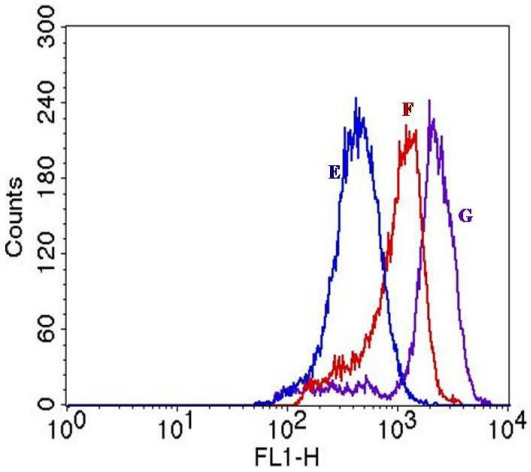

Figure 4.1.2 – Histogram of the susceptible strain *E. coli U40354* analysed in FL1 (520 nm) after treatment with Cipro for 30 minutes and 1 hour and staining for 1 hour with 1/100000 (v/v) SGI. G – no Cipro treatment; F – 1 µg/ml Cipro for 30 minutes; E – 1 µg/ml Cipro for 1 hour.

Regarding optimal incubation time with antimicrobial to observe differences between non-treated and treated cells it was experimented 30 minutes and 1 hour of Cipro incubation followed by 1 hour staining with SGI. In Figure 4.1.2, the blue peak E (1 hour incubation with Cipro) demonstrated better discrimination from the control peak G (purple) then F peak, although with 30 minutes incubation it was already possible to observe difference between peaks G and F. Since Cipro and Levo have similar mechanisms of action, the same protocol conditions were considered for both drugs.

4.2.Susceptibility evaluation by means of FC

The typical results obtained for FI distribution are depicted in Figure 4.2.1. The cells intrinsic autofluorescence was always detected in the first decade of the logarithmic scale (not represented), not affecting the assessment of nucleic acid content by SGI since intrinsic cell autofluorescence intensity was very low.

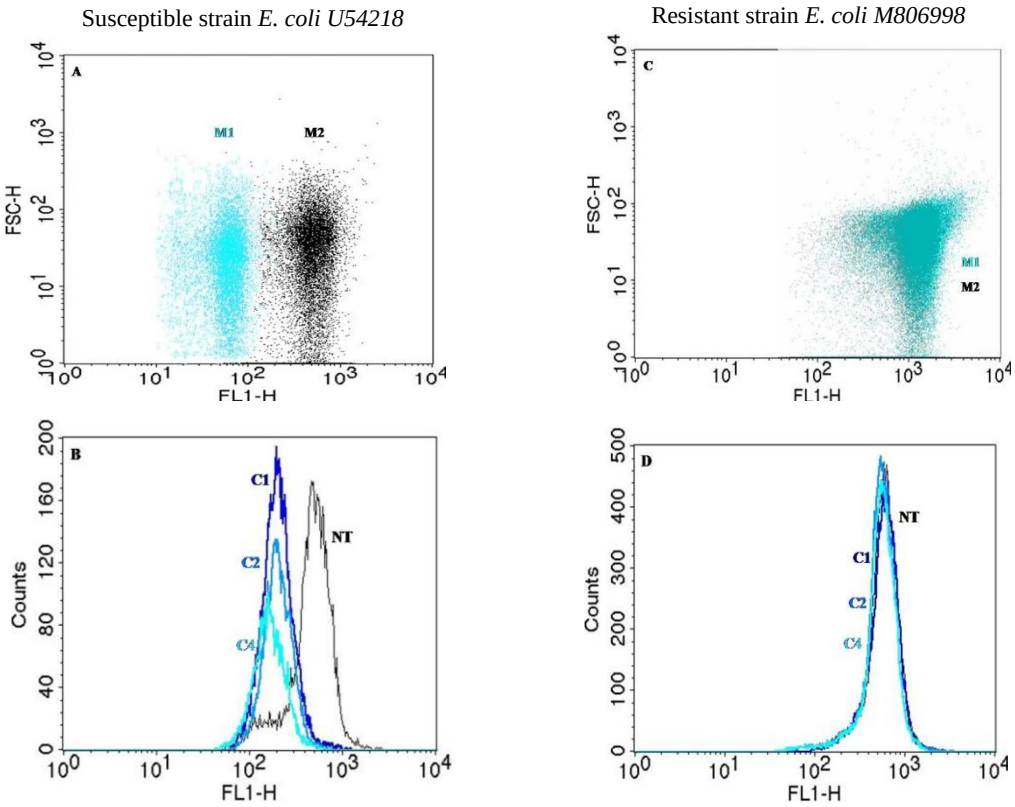

Figure 4.2.1 – Flow Cytometric analysis of two clinical strains treated for 1 hour with Cipro and stained with SGI. *E. coli U54218* represents the susceptible strain (A, B) and *E. coli M806998* is a resistant example (C,D). A and C: scattergram overlay of cell size (FSC-H) *versus* fluorescence (FL1-H at 520 nm) where are represented two distinct cell populations. M1 (blue) represents the subpopulation treated with 4 µg/ml Cipro and M2 (black) corresponds to the non-treated subpopulation. **B and D:** histogram overlay representing the different cell populations. NT (black) represents the non-treated cells. C1 stands for the cells treated with Cipro 1 µg/ml, C2 to the cells treated with Cipro 2 µg/ml and C4 to the cells treated with Cipro 4 µg/ml.

In Figure 4.2.1A, the Cipro susceptible strain presented two different subpopulations. M2 corresponded to the non-treated cells, displaying a higher FI than M1. On the other hand, M1 represented the susceptible subpopulation treated with Cipro 4 µg/ml for 1 hour displaying a lower FI. In terms of cell size there were no significant alterations once M1 and M2 subpopulations were approximately in the same decade of FSC-H (ordinate axis).

The resistant strain (Figure 4.2.1C) was exposed to the same conditions, but contrarily to the susceptible strain, it showed the two subpopulations M1 and M2 superimposed, which translates in little alterations in cell size and FI. Analysing the histogram of the susceptible strain (Figure 4.2.1B), there were four distinct fluorescence peaks where NT corresponded to the non-treated subpopulation, C1 C2 and C4 peaks corresponded to the FI after treatment with CLSI breakpoints 1 µg/ml, 2 µg/ml and 4 µg/ml respectively.

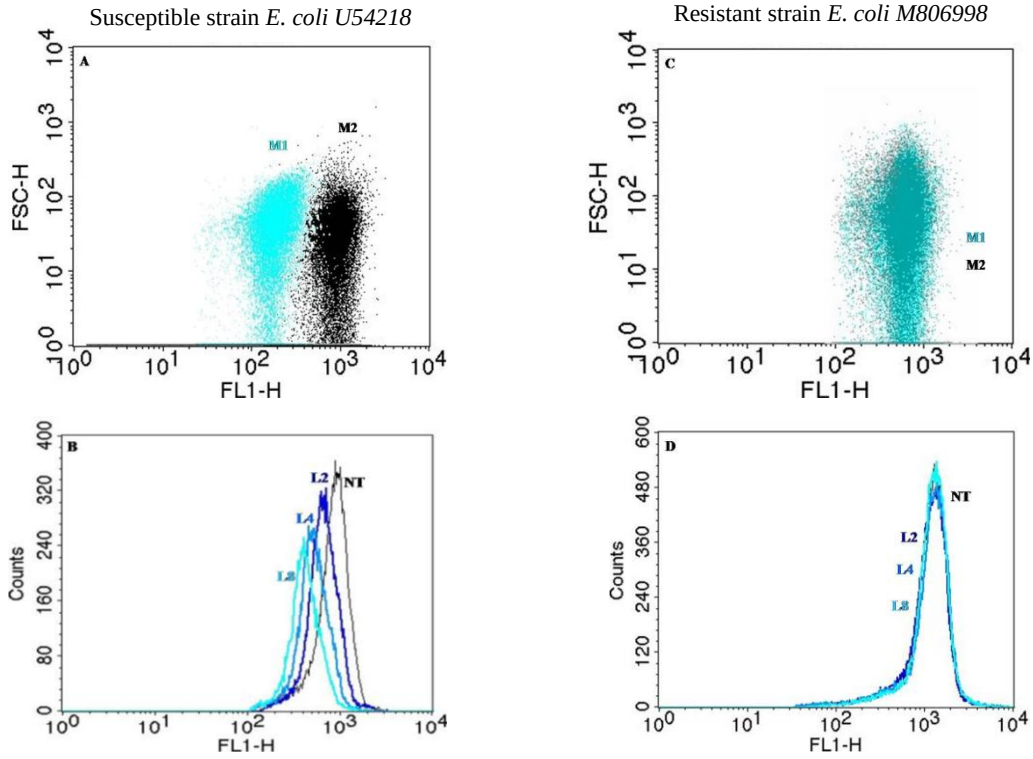

Figure 4.2.2 – Flow Cytometric analysis of two clinical strains treated for 1 hour with Levo and stained with SGI. *E. coli U54218* represents the susceptible strains (A, B) and *E. coli M806998* is a resistant example (C,D). A and C: scattergram overlay of cell size (FSC-H) *versus* fluorescence (FL1-H at 520 nm) where are represented two distinct cell populations. M1 (blue) represents the subpopulation treated with 8 µg/ml Levo and M2 (black) corresponds to the non-treated subpopulation. **B and D:** histogram overlay representing the different cell populations. NT (black) represents the non-treated cells. L2 stands for the cells treated with Levo 2 µg/ml, L4 to the cells treated with Levo 4 µg/ml and L8 to the cells treated with Levo 8 µg/ml.

As in Figure 4.2.1B, NT peak showed higher FI corresponding to the M2 subpopulation. C1, C2 and C4 had lower FI in a dose-dependent manner, translating into a displacement of the peaks to the left in the histogram, where C4 corresponded to M1 subpopulation. The histogram of the resistant strain (Figure 4.2.1D) showed an overlay of the NT, C1, C2 and C4 peaks in the same logarithmic decade corresponding to high FI just like M1 and M2 subpopulations. Since the susceptibility protocol for Levo is the same as for Cipro, similar results were expected. Resemblant to Figure 4.2.1, the susceptible strain dotplot also presented two different subpopulations M1 with low FI and M2 with high FI, as well as the resistant strain showed M1 and M2 overlaid in the high FI decade (Figure 4.2.2A and 4.2.2C). In addition, the susceptible strain histogram (Figure 4.2.2B) also exhibited four distinct peaks where the NT (control) presented the highest FI and the L2, L4 and L8 peaks treated with the CLSI breakpoints 2 µg/ml, 4 µg/ml and 8 µg/ml respectively, showed lower FI in a dose-dependent fashion. Finally, the histogram displayed in Figure 4.2.2D also presented, as expected, a superimposition of the four peaks NT, L2, L4 and L8 indicating that, besides treatment with Levo, the strain maintained a similar FI profile.

The action of Cipro and Levo was tested not only in late *log* phase (broth culture) but also in stationary phase (plate culture), without satisfactory results.

The results of the susceptibility evaluation of the clinical strains by means of FC, using SGI are presented in Table 4.2.3 and Table 4.2.4. The susceptibility phenotype of the strains used in this assay (n = 62 for Cipro and n=53 for Levo) was previously determined by Vitek2® using the AST-192 card. The Cipro and Levo Index were calculated for each breakpoint as the ratio between the percentage of treated cells in M2 population *versus* the percentage of non-treated cells also in M2 population (see Figures 4.2.1A and 4.2.2A). Since treatment with the lowest concentration of each antibiotic demonstrated visible alterations in nucleic acid content, enough to discriminate susceptible from resistant bacteria, the Cipro and Levo Index used for the classification was the correspondent to the first breakpoint concentration.

The phenotype classification by FC was determined using the cut-offs from Table 4.3.1. If Cipro and Levo Index are equal or inferior to the cut-off the strain is considered susceptible and if Cipro and Levo Index are superior to the cut-off the strain is resistant.

Based on this principle and regarding Cipro-treated bacteria (n=62), phenotype classification using FC agreed with the classic method in all cases except in three

Table 4.2.3 – Characterization of the tested isolates according to the susceptibility phenotype and FC analysis after Cipro treatment with the CLSI breakpoints and SGI staining.

Strain ID	SP Vitek2®	Cipro Index 1 µg/ml	Cipro Index 2 µg/ml	Cipro Index 4 µg/ml	SP FC
K. pneumoniae U96072	R	1.003	1.014	0.961	R
E. coli M804966	R	1.004	1.003	0.950	R
E. coli M826482	R	1.004	0.998	1.001	R
E. coli U23674	R	0.994	0.984	0.966	R
E. coli U42274	R	1.030	0.988	0.964	R
E. coli U50189	R	1.009	1.002	0.999	R
K. pneumoniae M805349	R	0.870	0.920	0.909	R
K. pneumoniae U54098	R	1.011	1.027	0.977	R
E. coli M950444	R	1.063	1.054	0.933	R
E. coli M949814	R	0.961	0.966	0.974	R
E. coli M950520	R	0.969	0.958	0.977	R
E. coli M950214	R	0.978	1.040	1.017	R
K. pneumoniae M959357	R	0.975	0.913	0.848	R
K. pneumoniae M959517	R	1.030	0.992	0.909	R
K. pneumoniae M962998	R	1.002	1.016	1.036	R
E. coli M964188	R	1.019	1.018	0.990	R
K. pneumoniae M964900	R	1.025	0.992	0.999	R
E. coli M965063	R	0.984	1.014	0.993	R
E. coli M966234	R	0.985	0.989	1.003	R
E. coli U721289	R	1.000	1.037	1.022	R
E. coli U720234	R	1.026	1.037	1.012	R
E. coli U559684	R	0.964	0.975	0.909	R
K. pneumoniae M973171	R	0.876	0.844	0.889	R
E. coli M933974	R	1.010	0.995	0.977	R
E. coli M933838	R	1.020	1.023	1.021	R
E. coli M977747	R	0.947	1.023	1.001	R
E. coli U659159	R	1,001	0.995	1.003	R
K. pneumoniae M960363	R	0.869	0.868	0.882	R
K. pneumoniae U96073	R	1.082	1.140	1.053	R
E. coli M806998	R	1.023	1.040	1.116	R
K. pneumoniae M117014	R	0.922	0.843	0.870	R
E. coli U54218	S	0.521	0.330	0.240	S
E. coli U54220	S	0.697	0.655	0.374	S
E. coli U36850	S	0.130	0.209	0.249	S
E. coli M804190	S	0.593	0.317	0.448	S
E. coli U40350	S	0.634	0.243	0.253	S
E. coli M826482	S	0.252	0.511	0.408	S
E. coli M824163	S	0.347	0.546	0.087	S
E. coli M950471	S	0.857	0.841	0.829	S
E. coli M950556	S	0.781	0.766	0.680	S
K. pneumoniae M959335	S	0.662	0.760	0.856	S
E. coli M964022	S	0.737	0.540	0.527	S
K. pneumoniae M964159	S	0.786	0.718	0.820	S
K. pneumoniae U16125	S	1.010	1.020	0.997	R
E. coli M973472	S	0.753	0.716	0.609	S
E. coli M804959	S	0.768	0.688	0.667	S
E. coli U15662	S	0.722	0.670	0.609	S
E. coli U38786	S	0.781	0.571	0.674	S
E. coli U46883	S	0.869	0.808	0.725	R
E. coli U45787	S	0.691	0.535	0.530	S
K. pneumoniae M806673	S	0.673	0.608	0.561	S
E. coli U20358	S	0.846	0.721	0.633	S
E. coli M934084	S	0.687	0.533	0.506	S
E. coli U40354	S	0.654	0.341	0.360	S
E. coli M824162	S	0.654	0.385	0.436	S
E. coli M937535	S	0.928	0.713	0.243	R
K. pneumoniae M934352	S	0.385	0.270	0.235	S
E. coli U655836	S	0.698	0.506	0.389	S
E. coli U655837	S	0.751	0.478	0.381	S
K. pneumoniae M973472	S	0.521	0.369	0.351	S
E. coli M934091	S	0.675	0.585	0.572	S
E. coli M936862	S	0.600	0.531	0.504	S

Strain ID – Strain identification; SP Vitek2®– Susceptibility profile determined using Vitek2® automated system; SP FC – Susceptibility profile determined using FC based on the first breakpoint; S – Susceptible; R – Resistant.

Table 4.2.4 – Characterization of the tested isolates according to the susceptibility phenotype and FC analysis after Levo treatment with the CLSI breakpoints and SGI staining.

Strain ID	SP Vitek2®	Levo Index 2 µg/ml	Levo Index 4 µg/ml	Levo Index 8 µg/ml	SP FC
E. coli M804966	R	1.004	1.001	1.003	R
E. coli U42274	R	0.968	0.954	0.821	R
E. coli U50189	R	1.011	0.985	0.794	R
K. pneumoniae U54098	R	0.813	0.884	0.895	S
E. coli M950444	R	1.035	1.005	1.035	R
E. coli M949814	R	0.994	0.854	0.896	R
E. coli M950520	R	0.885	0.969	0.960	S
E. coli M950214	R	1.049	1.023	1.003	R
K. pneumoniae M959357	R	0.959	0.890	0.832	R
K. pneumoniae M959517	R	1.017	0.986	0.952	R
K. pneumoniae M962998	R	1.040	1.029	1.021	R
E. coli M964188	R	1.014	0.986	0.986	R
K. pneumoniae M964900	R	1.005	1.006	1.000	R
E. coli M965063	R	0.998	0.994	0.986	R
E. coli M966234	R	0.992	0.992	0.980	R
E. coli U721289	R	1.018	0.988	0.933	R
E. coli U720234	R	0.968	1.025	0.929	R
E. coli U559684	R	0.969	0.855	0.826	R
K. pneumoniae M973171	R	0.883	0.838	0.818	R
E. coli M933974	R	1.003	0.903	0.791	R
E. coli M933838	R	1.006	1.015	1.016	R
E. coli M977747	R	1.010	1.011	0.930	R
E. coli U659159	R	1.000	0.981	0.816	R
K. pneumoniae M960363	R	1.019	0.992	0.830	R
K. pneumoniae U96072	R	1.106	1.176	1.111	R
E. coli M806998	R	1.106	1.014	0.998	R
K. pneumoniae M117014	R	0.862	0.819	0.872	R
E. coli U54218	S	0.775	0.455	0.569	S
E. coli M804190	S	0.849	0.729	0.456	S
E. coli M950471	S	0.670	0.708	m	S
E. coli M950556	S	0.750	0.626	0.768	S
K. pneumoniae M959335	S	0.604	0.886	0.871	S
E. coli M964022	S	0.659	0.609	0.572	S
K. pneumoniae M964159	S	0.790	0.544	0.553	S
K. pneumoniae U16125	S	1.014	0.988	0.977	R
E. coli M973472	S	0.647	0.571	0.571	S
E. coli M804959	S	0.667	0.611	0.607	S
E. coli U15662	S	0.659	0.666	0.600	S
E. coli U38786	S	0.724	0.768	0.708	S
E. coli U46883	S	0.821	0.764	0.667	S
E. coli U45787	S	0.787	0.653	0.559	S
K. pneumoniae M806673	S	0.780	0.698	0.638	S
E. coli U20358	S	0.789	0.690	0.652	S
E. coli M934084	S	0.673	0.552	0.444	S
E. coli U40354	S	0.681	0.528	0.323	S
E. coli M824162	S	0.703	0.581	0.440	S
E. coli M937535	S	0.912	0.728	0.622	R
K. pneumoniae M934352	S	0.434	0.429	0.352	S
E. coli U655836	S	0.696	0.342	0.274	S
E. coli U655837	S	0.616	0.414	0.337	S
K. pneumoniae M973472	S	0.489	0.458	0.428	S
E. coli M934091	S	0.566	0.571	0.535	S
E. coli M936862	S	0.529	0.444	0.372	S

Strain ID – Strain identification; SP Vitek2®– Susceptibility profile determined using Vitek2® automated system; SP FC – Susceptibility profile determined using FC based on the first breakpoint; S – Susceptible; R – Resistant; m – missing value.

isolates *(K. pneumoniae U16125, E. coli U46883* and *E. coli M937535*) that were misevaluated as resistant(Table 4.2.3). In susceptible isolates the Cipro Index may vary between strains, but in most cases diminishes within the same strain in a dose-dependent manner as the drug concentration increases. In the resistant isolates Cipro Index also varies amongst strains but with values close to one for all three breakpoints.

Regarding Levo treatment, the clinical strains analyzed (n=53) displayed a similar behavior as portrayed in Table 4.2.4. The resistant strains also presented Index values close to one and the susceptible strains also had lower Index values as the breakpoint concentration increased, in a dose-dependent fashion. FC method agreed in all cases with the classic routine method except in three isolates (*K. pneumoniae U54098, K. pneumoniae U16125* and *E. coli M937535*) as one false negative and two false positives, correspondingly.

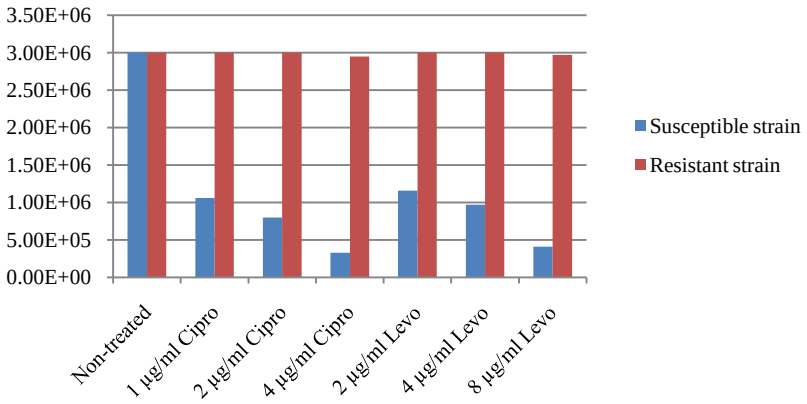

Figure 4.2.5 – Number of CFU/ml of a representative example of a Cipro and Levo susceptible strain (*E. coli U54218*) and a Cipro and Levo resistant strain (*E. coli M806998*) after 60 minutes exposure to Cipro (1, 2, 4 µg/ml) and Levo (2,4, 8 µg/ml).

The results obtained by FC are supported by the viability assay as demonstrated in Figure 4.2.5. A dose-dependent decrease in the CFU/ml is observed for the representative susceptible strain for both drugs and the values are very proximate for the representative resistant strain, after 1 hour incubation with the drugs.

4.3. Agreement between classic and FC methods

To evaluate the agreement between the routine susceptibility approach and the FC method the agreement proportion and *kappa* value were calculated, as well as the accuracy measures of sensitivity, specificity, positive and negative predictive values and overall accuracy. All the parameters were calculated with a 95% confidence interval ($P<0.001$).

Table 4.3.1 summarizes the statistical analysis performed. Both agreement and accuracy measures presented values close to one demonstrating excellent results for the validation of FC as a susceptibility determining tool. The cut-offs were calculated, for each breakpoint of both drugs using the ROC curve model, and the values obtained allowed to discriminate weather a strain was susceptible or resistant comparing these values with the Cipro and Levo Index.

Table 4.3.1 – Statistical parameters of agreement and accuracy to validate FC as a suitable methology as well as the cut-offs to determine the susceptibility phenotype.

		Agreement measures		Accuracy measures					Cut-off
		Agreement Proportion	*kappa* Value	Sensitivity	Specificity	Positive Predictive Value	Negative Predictive Value	Overall Accuracy	
1 BP	Cipro	0.9500	0.9300	1.0000	0.9032	0.9118	1.0000	0.9516	0.8630
	Levo	0.9400	0.9200	0.9630	0.9231	0.9286	0.9600	0.9434	0.8555
2BP	Cipro	0.9800	0.9700	1.0000	0.9677	0.9688	1.0000	0.9839	0.8420
	Levo	0.9600	0.9500	1.0000	0.9231	0.9310	1.0000	0.9623	0.7935
3BP	Cipro	0.9700	0.9600	1.0000	0.9355	0.9394	1.0000	0.9677	0.8385
	Levo	0.9600	0.9500	1.0000	0.9231	0.9310	1.0000	0.9623	0.7795

1 BP – First breakpoint (1 µg/ml for Cipro and 2 µg/ml for Levo); 2 BP – Second breakpoint (2 µg/ml for Cipro and 4 µg/ml for Levo); 3 BP – Third breakpoint (4 µg/ml for Cipro and 8 µg/ml for Levo).

4.4.Efflux detection by FC

Efflux is a major contributor for clinical resistance to FQs in *Enterobacteriaceae*, particularly the efflux pump AcrAB-TolC. In order to assess if there was probable over-expression of this pump as a resistance mechanism in any of the studied strains, control cells were analysed. Figure 4.4.1 presents the three FI peaks corresponding to the three control strains used. IE clearly corresponded to the population with inactivated AcrAB-TolC system since it has the highest FI resulting from the retention of EtBr within the cell. OE (blue) represents the control population with over-expressing AcrAB-TolC efflux pump once it had FI values very low due to the extrusion of the fluorochrome. Finally, WT (black) refers to the wild-type control that showed inferior FI comparing with IE, but still much higher FI values than OE.

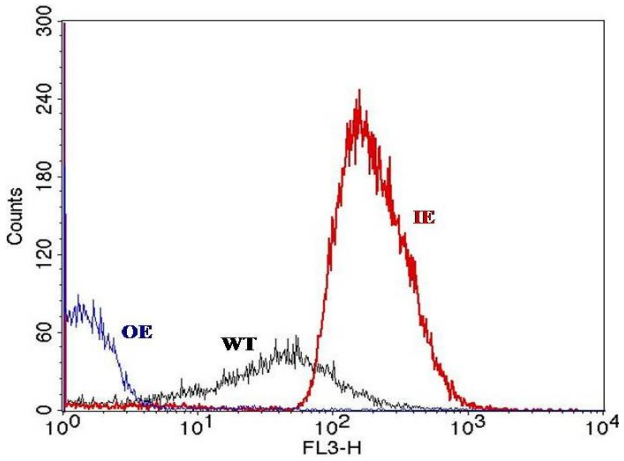

Figure 4.4.1 – EtBr efflux by the *E. coli* AG100 control strains analysed in FL3 (600 nm) in a 0.4% (v/v) glucose medium. OE – *E. coli* $AG100_{TET}$ with over-expressing AcrAB-TolC efflux; WT – *E. coli* K-12 AG100 wild-type; IE – *E. coli* AG100A with inactivated AcrAB-TolC efflux.

After studying the well characterized controls, clinical strains were analysed. Figure 4.4.2 is a typical example of a resistant strain with low efflux rate. EB (red) peak corresponds to the population exposed to Cipro (stress) and later stained with EtBr. The EB+CPZ (green) is the control peak exposed to the same conditions, except there was AcrAB-TolC complex inhibition by CPZ action, translating into maximum EtBr accumulation within the cell.

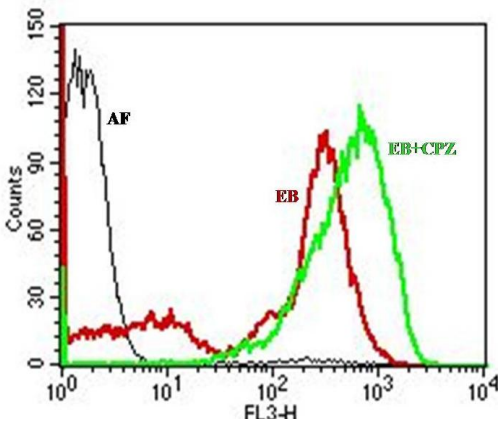

Figure 4.4.2 – Histogram of the resistant strain *E. coli M826482* analysed in FL3 (600 nm), after 1 hour efflux stimulation with Cipro followed by 1 hour EtBr staining. AF - Autofluorescence; EB – stained with 1 µg/ml EtBr; EB+CPZ – treated with 20 µg/ml of CPZ and simultaneously stained with 1 µg/ml EtBr.

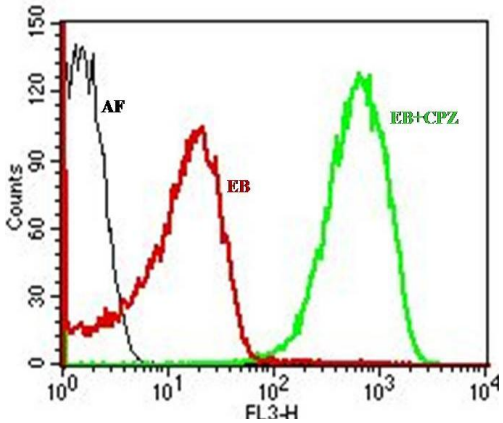

Figure 4.4.3 – Histogram of the resistant strain *E. coli U23674* analysed in FL3 (600 nm), after 1 hour efflux stimulation with Cipro followed by 1 hour EtBr staining. AF - Autofluorescence; EB – stained with 1 µg/ml EtBr; EB+CPZ – treated with 20 µg/ml of CPZ and simultaneously stained with 1 µg/ml EtBr.

Comparing the two, there is closely an overlay of the peaks in the same decade of FI as the IE peak in Figure 4.4.1, leading to the idea that this particular strain must not have efflux as a major clinical resistance contributor. Moreover it is important to observe the presence of a second small peak of the EB population in the first decade of fluorescence, meaning that a small percentage of the cells extruded EtBr.

Table 4.4.4 – Efflux evaluation of the clinical strains (n=30) using FC after 1 hour staining with EtBr.

		EtBr		EtBr+CPZ	
Strain ID	SP Vitek2®	P1 % Gated	P2 % Gated	P1 % Gated	P2 % Gated
E. coli U40354	S	15.38	66.27		99.12
E. coli U54218	S	64.26	30.45		97.71
E. coli M950471	S	77.31	16.72		85.51
E. coli M950556	S	27.85	65.07		89.95
E. coli U42274	R	83.97			98.33
E. coli M806998	R	70.52	14.79		97.38
E. coli M950444	R	86.87			86.23
E. coli M950520	R	35.39	50.79		87.15
E. coli M94814	R	79.49	8.390		91.89
E. coli M950214	R	85.16			95.53
K. pneumoniae M959357	R	82.25		21.29	75.00
K. pneumoniae M959517	R	84.08			82.29
E. coli M959232	R	55.89	37.67	30.74	65.01
K. pneumoniae M962998	R	49.18	31.49		82.04
E. coli M964188	R	83.79	10.51		76.71
K. pneumoniae M964900	R	33.30	61.54		90.22
E. coli M965063	R	89.78			87.70
E. coli M966234	R	73.18	10.30		87.82
E. coli U721289	R	67.09	15.60		93.72
E. coli U720234	R	73.99	11.64		89.90
K. pneumoniae M973171	R	85.33			96.73
K. pneumoniae U54098	R	82.44			89.99
E. coli U50189	R	85.23			96.79
E. coli M804966	R	90.07			98.75
E. coli M826482	R	34.01	65.17		98.33
E. coli U50194	R	68.34	29.62		95.05
E. coli U559684B	R	83.45			97.90
E. coli M933974	R	87.84			85.28
E. coli M933838	R	85.50			95.68
E. coli U23674	R	90.75			97.29

EtBr – Samples treated only with EtBr; EtBr+CPZ – Samples treated simultaneously with EtBr and CPZ; Strain ID – Strain identification; SP Vitek2®– Susceptibility profile determined using Vitek2® automated system; P1 % Gated – percentage of gated population in Peak 1; P2 % Gated – percentage of gated population in Peak 2; S – Susceptible; R – Resistant.

On the other hand, in Figure 4.4.3 a different resistant strain depicted the two peaks clearly apart: EB+CPZ peak is situated in the same decade the IE peak (see Figure 4.4.1) and EB peak showed much lower FI, meaning that this strain extruded practically all of the EtBr that entered the cell. Thereby, it is very probable that in this particular *inoculum* efflux over-expression must be a major contributor for clinical resistance.

The results obtained in all clinical strains analysed (n=30) are presented in Table 4.4.4. In both EtBr and EtBr+CPZ columns, corresponding to the EB and EB+CPZ population respectively (Figures 4.4.2 and 4.4.3), in certain bacteria, two distinct peaks were obtained: P1 (Peak 1) and P2 (Peak 2). P1 corresponds to a subpopulation with low FI (EtBr extrusion from the cell), usually portrayed in first decade of fluorescence (almost autofluoresce) and P2 is the subpopulation with high FI values (EtBr remained inside the cell). The strains analysed may present only P1, P2 or both simultaneously, meaning there were two different populations in that sample. One clear example is clinical strain *E. coli U50194* where 29.62% of the population was in P2 and 68.34% of the population was in P1 – most of the cell population extruded the EtBr.

Within the resistant strains analysed (n=26) there were three different efflux profiles obtained: those that had a higher percentage of cells that extruded EtBr from the cell (higher P1 % gated values), the ones who kept most of the EtBr (higher P2 % gated values) and finally those that had approximate values in P1 and P2. In the susceptible strains (n=4) only two efflux profiles were present: higher percentage values in P1 or P2.

The EtBr+CPZ column corresponds to the control population with efflux inhibited by CPZ. As expected, the strains presented FI only in P2 with values close to 100% of the population, since the EtBr was arrested inside the cell. Only two strains did not follow this pattern (*K. pneumoniae M959357* and *E. coli M959232*) but still with higher percentage values in P2.

5. DISCUSSION

The problematic increase of resistance to FQs demands an update in susceptibility detection and treatment. The current routine methods available are time-consuming procedures that take too long to provide results and a rapid diagnostic is important to prescribe the right antibiotherapy. In response to this problem, a novel susceptibility assay based on FC was developed. This new assay allows an accurate and rapid assessment of the susceptibility phenotype and provides a multiparameter cell-by-cell analysis. The susceptibility profile of all of the clinical isolates used in this assay was previously determined by Vitek2®, used as a comparative method.

Cipro and Levo were chosen as FQ representatives since they are some of the most prescribed drugs of the Quinolone family and because Cipro is more active against gram negative bacteria and Levo more active towards gram positives. *E. coli* and *K. pneumoniae* were used in this assay since they are the most prevalent microorganisms, particularly in minor infections treated with FQ.

When selecting a fluorescent probe for a susceptibility assay, the drug's mechanism of action is the main parameter to take into account. In this particular case, since FQs cause the fragmentation of the DNA molecule it would be wise to choose a fluorochrome that binds to nucleic acids, specially to dsDNA in order to detect alterations in DNA conformation due to molecule breakdown (Cabral et al., 1997; Heddle & Maxwell, 2002; Lupala et al., 2012; Mason et al., 1995).

Although there are several DNA-binding probes available, SGI seemed to be a suitable candidate (Barbesti et al., 2000; Gregori, Denis, Seorbati, & Citterio, 2003). This fluorochrome has a wide spectrum of applications in molecular biology, fluorescence imaging and also in FC due to its excellent properties. These include favourable photophysical properties, temperature stability, selectivity for dsDNA, even in the presence of ribonucleic acid (RNA) and single-stranded DNA (ssDNA), and high sensitivity. This probe is a membrane-permeant dye that binds to nucleic acids mainly by external binding (minor groove binding) (Zipper, Brunner, Bernhagen, & Vitzthum, 2004). Its commercial concentration is not available.

When a susceptibility assay by means of FC is developed, it is necessary to optimize the protocol's parameters such as incubation times, bacteria and dye concentration. After choosing SGI as a suitable probe, several different concentrations were tested in order to determine the most appropriate. In a fixed *inoculum* around $5x10^6$ cells/ml often a high

concentration of fluorochrome can cause unspecific staining, hence the importance to do a titration with different concentrations of dye in order to achieve optimal signalling/results (Gregori et al., 2003). The bacterial concentration used was based on the work developed by Faria-Ramos et al, later published in 2013 (Faria-Ramos et al., 2013).

Experiments with other probes were conducted, such as with Bis-(1,3-dibutylbarbituric acid) ($DiBAC_4(3)$) that enters cells with depolarized membranes and Propidium Iodide that binds to nucleic acids after cell-membrane compromise. No satisfactory results were obtained after 1 hour treatment with FQs (data not shown). Probably it would be required more incubation time with the antimicrobials since the primary cell lesion is DNA fragmentation (FQ mechanism of action). Also, in 2000 Mortimer et al. suggested to rethink the use of nucleic acid binding dyes that are non-membrane-permeant in antimicrobial susceptibility testing, such as Propidium Iodide, TO-PRO-1 and SYTOX green, since the drug's mechanism of action influences fluorochrome uptake by damaged cells (Mortimer, Mason, & Gant, 2000).

As depicted in Figure 4.1.1, peak B, that corresponds to 1/100000 (v/v), demonstrated to be the best SGI concentration. Peak C also showed a suitable result but cost-wise, it is better to use the lowest concentration possible. Peak D had FI values too high (off-scale) and in peak A seemed to have occurred nonspecific binding resulting from the low concentration of SGI: dye concentration is not high enough and probably starts binding to other cellular components, explaining the small peak that was forming on the left side of peak A.

Drug incubation time was also tested with Cipro for 30 minutes and 1 hour, based on the review work of Walberg et al in 1997 and Álvares-Barrientos et al in 2000, to keep the protocol as brief as possible (Figure 4.1.2) (Alvarez-Barrientos et al., 2000; Walberg et al., 1997). The results were satisfactory for both incubation times, although peak E (1 hour) displayed even better discrimination power from the non-treated cells in peak G then F, probably because in 1 hour there is more DNA damage then in 30 minutes. Since both drugs have similar mechanisms of action, the same conditions were also applied for Levo treatment.

In this assay, FI is a direct measure of intact DNA content and the distribution of light scattering translates alterations in cell's complexity and size. For susceptible strains exposed to breakpoints of Cipro and Levo according to CLSI (CLSI, 2013), the FI values decrease compared to the NT controls (Figure 4.2.1B and 4.2.2B). Since SGI is believed to bind to the minor groove of the helical structure, fragmentation of DNA causes a loss in FI since there are limited biding sites for the dye due to the presence of intermediate sized fragments (Mortimer et al., 2000; Walberg et al., 1997). This descent in DNA content is dose-dependent

and results were observed after just 1 hour incubation with the drugs; this study aims for detection of primary cell lesions in order to be rapid, so cell disintegration wasn't observed.

Although SGI is cell permeable, 70% (v/v) ethanol fixation after antimicrobial treatment is very important to arrest cell cycle and replication, especially in resistant strains where there might be continued replication without cell division, still maintaining cell conformation and DNA content (Mortimer et al., 2000).

After close observation, in Figure 4.2.1B the Cipro treatment peaks (C1, C2 and C4) are more separated from the control NT peak than in Figure 4.2.2B (L2, L4 and L8) after Levo treatment. Since Levo is more active against gram positive microorganisms, targeting preferentially topoisomerase IV than gyrase, the Levo MIC is consequently higher than Cipro MIC, explaining the shorter distance between the NT and treatment peaks in Figure 4.2.2B (still in a dose-dependent manner). In fact, this phenomenon may lead to the conclusion that the shorter the distance between the NT and treatment peaks, the higher will be the MIC to that antimicrobial.

Concerning light scattering, in Figures 4.2.1A and 4.2.2A, the cells are in about the same decade of FSC-H in the dotplot, which denotes no significant changes in cell size. Wickens et al stated that only 3 hours of drug incubation could allow to observe light-scattering alterations such as cell filamentation (Wickens et al., 2000).

The abscissa axis, that concerns FI (FL1-520 nm), M1 (treated) subpopulation has lower FI than M2 (non-treated), confirming the results obtained in the histograms. In addition, it is also verified the occurrence of the same phenomenon described above: the Levo dotplot also displayed a smaller distance between M1 and M2 subpopulations than the Cipro-treated, due to being a gram negative organism and consequently having a higher MIC to Levo.

On the other hand, in resistant isolates, the FI revealed approximate values, both in controls and treated cells, meaning the DNA molecules synthesized were in their normal conformation, without fragmentation, presenting the binding-sites for SGI to fluoresce (Mortimer et al., 2000). Such is observed by the superimposition of the NT and treated peaks in the histograms (Figures 4.2.1D and 4.2.2D) as well as M1 and M2 subpopulations in the dotplots (Figures 4.2.1C and 4.2.2D).

This susceptibility protocol was also tested in late *log* phase (broth culture) and in stationary phase (plate culture) yet with no satisfactory results probably due to the drug's mechanism of action. Since FQs act in DNA synthesis, which happens during bacterial growth, if cells are in stationary phase with no growth or even in late *log* phase, where nutrient availability is decreasing rapidly as well as bacterial growth, there are not optimal

conditions for DNA replication. Such is backed by the research of Lebaron et al. in 1998, where they concluded that SYTOX green, another nucleic acid dye, wasn´t suitable to assess the viability of starved cultures due to degradation or alteration of fluorochrome binding-sites (Lebaron, Catala, & Parthuisot, 1998).

In order to assess the effect of the studied drugs on the clinical isolates, an Index was calculated for each drug, Cipro Index and Levo Index, as the ratio between the percentage of treated cells gated in M2 (population with non-damaged DNA) *versus* the percentage of non-treated cells also gated in M2 population (See Figures 4.2.1A and 4.2.2A). The Index values vary among isolates.

The cut-offs to determine whether a strain is susceptible or resistant to each antibiotic were calculated using the ROC curve which is a graphical plot that illustrates the performance of a binary classifier system (Susceptible vs. Resistant) (Zou, O'Malley, & Mauri, 2007). In more detail, they were calculated by the evaluation of the curve´s coordinates that presented simultaneously the highest sensitivity value as well as the best sensitivity/specificity equilibrium. Due to the limited sample size, it was observed a data extrapolation for the curve construction and therefore it was analysed if the values around the proposed coordinate could present more accurate values and selected the ones where such occurred.

In practice, the technician will compare the Cipro or Levo Index, obtained after the FC protocol has been applied, with the cut-off calculated in the statistical analysis. If the Cipro or Levo Index are equal or inferior to the respective cut-off the bacterial strain is considered susceptible, on the other hand, if the Index is superior to the respective cut-off the strain is considered resistant.

Once it was possible to observe significant differences between the control population and treated population in terms of nucleic acid content, already with the lowest drug concentration applied, the cut-offs used to determine the susceptibility phenotype were the ones calculated for the first breakpoint. Therefore, the classification was made comparing Cipro Index with 0.8630 and Levo Index with 0.8555 (see Table 4.3.1).

Tables 4.2.3 and 4.2.4 display the susceptibility phenotype previously determined by Vitek2® and the classification obtained with FC after comparing the Cipro/Levo Index with the cut-offs of the first breakpoint (1BP).

Particularly in Table 4.2.3 after Cipro treatment, there was agreement between both methods in the majority of the cases except in three isolates wrongly classified by FC as resistant (false positives).

After Levo treatment, as depicted in Table 4.2.4, there was agreement as well between FC and Vitek2® phenotype, except in three strains mistakenly classified by FC. Curiously, two of the isolates were the same wrongly classified by FC after Cipro treatment.

The strains that showed no agreement between FC and Vitek2® were tested with an alternative susceptibility method to confirm their phenotype to both drugs, given by the reference method.

To confirm the action of the tested antibiotics, a viability assay was performed for each isolate after incubation with every drug breakpoint during the susceptibility protocol. Representative susceptible and resistant isolates are depicted in Figure 4.2.5 to example the typical results obtained. As expected, the susceptible strain portrayed a dose-dependent decrease in the CFU/ml for both drugs, although the Levo-treated cells showed slightly superior values due to the usual higher MICs to this drug. Also as expected, the representative resistant strain exhibited very approximate values for all drug concentrations, thus corroborating the results obtained by FC.

The evaluation of agreement between FC and the classic method, resulting in categorical variables, is better characterized using the complementary measures of agreement proportion and *kappa* value (Petrie & Sabin, 2009). The agreement proportion analyses the proportion of cases where both techniques agree relative to the total amount of case studies. This measure varies between 0 and 1, where 0 represents total disagreement and 1 total agreement (Petrie & Sabin, 2009). The results obtained (Table 4.3.1) indicate an excellent agreement, with values close to 1 ranging from 0.9500 to 0.9800 for Cipro and 0.9400 to 0.9600 for Levo.

The *kappa* value corresponds to the agreement proportion with correction of chance and varies between -1 and 1, where -1 corresponds to total disagreement, 1 is total agreement and 0 corresponds to the agreement expected if the classifications where made by chance (Petrie & Sabin, 2009). Landis and Koch suggest that a *kappa* value under 0.40 is poor, between 0.40 and 0.75 is intermediary/good and over 0.75 is excellent (this classification is merely orientative since prevalence can affect the *kappa* value) (Gordis, 2011). According to these authors, the classification obtained for both antibiotics to the three breakpoints is excellent ranging from 0.9300 and 0.9700 for Cipro and 0.9200 and 0.9500 for Levo.

The accuracy of a diagnostic test translates into its ability to distinguish resistant (with disease) from susceptible (no disease) subjects (Gordis, 2011). To study the accuracy of FC as a diagnostic method in detecting the susceptibility phenotype different parameters were calculated such as sensitivity, specificity, positive and negative predictive values, overall accuracy and area under ROC curve (Kramer & Mausner, 2007; Zou et al., 2007).

According to Table 4.3.1, the results show that FC is highly sensitive, with values equal to 1 in all cases except in the first breakpoint for Levo (0.9630), and specific since the values obtained are close to 1. The positive predictive values are also very high, for example, 0.9118 for Cipro and 0.9286 for Levo (first breakpoint). On the other hand, the negative predictive value also has excellent results being 1.0000 for all cases, except for the Levo first breakpoint (0.9600) which is also very good, meaning that true positives and true negatives are adequately diagnosed (Kramer & Mausner, 2007). Regarding the overall accuracy, there were also obtained values close to 1, where the lowest value is 0.9434 and the highest is 0.9839. This means, for example for Levo (first breakpoint), that 94.34 % of the strains analysed will have the same outcome (Kramer & Mausner, 2007).

Both the agreement and accuracy parameters were calculated using a 95% confidence interval and a margin of error of 5%.

The estimated costs of reagents and consumables per sample used in this new assay are fairly low, without considering the FC equipment and technician, even though nowadays such equipment is often found in most hematology and immunology laboratories.

Based on existing research, there are important clinical and financial benefits in developing rapid bacterial identification and susceptibility tests, such as a decrease in turnaround time, mortality, morbidity and patient-stay as well as a reduction in hospital total and variable costs. A prompter susceptibility test will allow physicians to have access to crucial information earlier and enable them to prescribe the appropriate antimicrobial therapy or to change to a more suitable antibiotic sooner and subsequently avoiding an empirical prescription (Barenfanger et al., 1999; Doern, Vautour, Gaudet, & Levy, 1994).

Besides the accuracy, simplicity and cost-effectiveness of this method, it also reduces the waiting time of the results. Despite the necessary incubation time to reach early exponential phase (an average of 1,5 hours), by using this susceptibility protocol results can be obtained in 2,5 hours, much faster than the bacterial growth based routine methods that usually give results in 24 to 48 hours (Alvarez-Barrientos et al., 2000), bringing a very important advantage.

Since the resistance rate of *Enterobacteriaceae* is increasing dramatically throughout the years and the availability of new antibiotics is diminishing, it seems pertinent to find which are the resistance mechanisms and address them in order to overcome this limitation (Paixao et al., 2009; Piddock, 2012). If such would be possible, the joint administration of antibiotics associated with a resistance mechanism inhibitor the bacterial MICs could potentially be

lowered with no need to develop that many new drugs to bridge this resistance problematic (Marquez, 2005; Piddock, 2012).

Taking this into account, and also that efflux is a major contributor for clinical FQ resistance in *Enterobacteriaceae*, a FC protocol was developed based on the research of Paixão et al in 2009 in order to indirectly detect over-expression of the efflux pump AcrAB-TolC (main FQ efflux system in *E. coli* and *K. pneumoniae*), *in vivo* and in real-time, that translates into different degrees of efflux amongst the studied strains. Since this protocol evaluates overall efflux and is not specific for one particular efflux system, mutant control strains for AcrAB-TolC were previously analysed (Paixao et al., 2009).

In short, cells are incubated with the fluorochrome EtBr (nucleic acid binding) in such a low concentration that it does not bind to nucleic acids. In fact, it has low FI in aqueous solution (cell exterior) and high FI when weakly-binded to celular components in the periplasm. This binding has to be weak in order to allow it's extrusion from within the cell and use it as an efflux probe. EtBr uptake is through passive diffusion and its extrusion by active transport (Paixao et al., 2009), hence the use of glucose in the protocol as energy source for the efflux.

Cells are also incubated with CPZ, an inhibitor of proton-motive force-dependent efflux pumps, such as AcrAB-TolC (Paixao et al., 2009). For efflux to occur, the hydrolysis of ATP is necessary and contributes for the activation of the AcrAB-TolC system. It is believed that CPZ is an inhibitor of calcium binding to proteins, such as ATPase, thus affecting the efflux of EtBr (Marquez, 2005; Martins et al., 2011).

Figure 4.4.1 exhibits the histograms of the control strains: IE peak (inactivated efflux) shows the maximum FI from EtBr retention, OE (over-expressing efflux) has practically autofluorescence levels of FI from EtBr extrusion and WT (wild-type) has intermediate levels of FI amongst the first and the second decade in FL3 (600nm).

Comparing the two given examples, that portray the typical efflux profile of the clinical resistant strains analysed with the controls, there is resistant isolate *E. coli M826482* (Figure 4.4.2) where the EB and EB+CPZ peaks are almost totally overlaid in the same FI decade as the IE control. This analysis means that this resistant isolate retains most of the EtBr within the cell, therefore this strain must have other mechanisms than efflux over-expression contributing for clinical resistance.

By contrast, *E. coli U23674* presented in Figure 4.4.3, reveals the EB and EB+CPZ peaks totally apart, where the EB+CPZ peak is also in the same decade as the IE population and EB peak has much lower FI (dislocation to left of the histogram). This means the given isolate

actively extruded most of the EtBr taken by the cell, leading to the conclusion that this strain probably has AcrAB-TolC over-expression as a major contributor for clinical resistance.

The efflux profile of all the clinical isolates analysed (n=30) is displayed in Table 4.4.4, where P1 corresponds to the peak with low FI (extruded EtBr) and P2 corresponds to high FI (no EtBr efflux). The EtBr+CPZ column represents the control population with inhibited efflux by CPZ treatment, thus the high percentage of cells in P2. Within the resistant isolates (n=26) only two have not shown almost total efflux inhibition (*K. pneumoniae M959357* and *K. pneumoniae M959232*) probably due to human error, deduced by the presence of cell percentage in P1, and consequently a lower percentage in P2.

Most of the resistant strains in the EtBr column showed higher gated cell percentage in P1, leading to the conclusion that these isolates most likely have efflux over-expression, with a histogram similar to Figure 4.4.2 (red peaks). On the other hand, there were three resistant isolates that revealed low EtBr efflux rate (*E. coli M950520*, *K. pneumoniae M964900* and *E. coli M826482*) by having higher cell percentage in P2. For example, strain *E. coli M826482* had 65.17% gated population that arrested EtBr and 34.01% extruded it – mechanisms other than efflux over-expression must be responsible for clinical resistance.

There is also a third efflux profile verified in the resistant strains: those that present approximate gated cell percentage in P1 and P2. Both *E. coli M959232* and *K. pneumoniae M962998* display approximate percentages in P1 and P2, so probably they don't possess efflux as a clinical resistance contributor.

In sum, there were three different efflux profiles verified in the resistant clinical isolates: those with high efflux rates, where AcrAB-TolC seems to be over-expressed as a resistance mechanism, those with low efflux rates and lastly those who possess intermediate efflux.

Four susceptible clinical isolates were also analysed using this protocol. *E. coli U40354* and *E. coli M950556* demonstrated low efflux rates by having a high cell percentage in P2, which is expected in a susceptible strain. Contrarily to what would be expected, the other two susceptible strains *E. coli U54218* and *E. coli M950471* presented higher cell percentage in P1, so most EtBr was extruded from the cell, demonstrating the presence of efflux over-expression even in susceptible strains.

A possible strategy to surpass bacterial resistance would be the use of inhibitors of the resistance mechanisms that would allow to potentiate the activity of existing antimicrobials (Marquez, 2005; Piddock, 2012).

Based on this principle, aiming to try to diminish the clinical resistance of the tested strains in the efflux assay, a microdilution protocol was developed, based on the CLSI

protocol for Cipro susceptibility determination (CLSI, 2013), where the isolates were incubated with sub-inhibitory concentrations of CPZ. In some isolates, it was possible to slightly lower the MIC, but not significantly enough. A reversion of the phenotype would be impossible since there are other mechanisms that confer resistance, namely, mutations in target enzyme.

This protocol allows real-time monitoring of EtBr efflux and the information obtained can be of great importance in understanding resistance phenotypes and also in finding new ways to help overcome this problem by targeting resistance mechanisms.

6. CONCLUSION

The problematic increase of resistance to FQs demands an update in susceptibility detection and treatment. The current routine methods available are time-consuming procedures that take too long to provide results and a rapid diagnostic is important to prescribe the right antibiotherapy. In response to this problem, a novel susceptibility assay using FC was developed based on the principles of the routine methods. This new assay developed allows an accurate and rapid assessment of the susceptibility phenotype. It also provides a multiparameter cell-by-cell analysis and presented an excellent agreement with Vitek2®, used as a comparative method.

Additionally, another rapid FC protocol was developed in order to obtain the bacterial efflux profile in order to understand the role of efflux pump over-expression as a contributor for clinical resistance. It also has the advantage to provide a real-time analysis and it can be used as a tool to study and develop efflux inhibitors to overcome this mechanism.

In conclusion, the susceptibility protocol described is a promising method that proved to be an excellent alternative to the routine tests as well as the efflux assay that demonstrated to be a great tool to study and overcome bacterial resistance through efflux.

7. FUTURE WORK

The results presented in this thesis may provide the way for future research.

This work belongs to an international patent Ref. PPI 45744/12 - "KIT AND METHOD OF DETECTING THE RESISTANT MICROORGANISMS TO A THERAPEUTIC AGENT" - UNIVERSIDADE DO PORTO - Cidália Irene Azevedo (Pina Vaz et al.). Therefore, more susceptibility protocols must be developed for different bacterial strains and antimicrobial agents. There were some experiments performed with FQs and *Pseudomonas aeruginosa* and also with cocci using FC that showed similar results for both susceptible and resistant isolates (number of tested isolates not statistically significant), meaning that this protocol might also be suitable for these bacteria.

Once this is a susceptibility test, this technique should be allied to a rapid isolate identification method such as the MALDI-TOF technology; these two methods are potential complementary techniques to be used in the clinical routine setting (Harris, Winney, Ashhurst-Smith, O'Brien, & Graves, 2012).

Albeit being rapid and simple, in order to optimize the cost-benefit ratio, further automation is necessary. Flow cytometers need to be adapted to robotics for automatic sample handling and there's the need to develop software to analyse the results. This kind of software is already available for Hematology, so an adaptation for Clinical Microbiology shouldn't be difficult. The major step should be to create the need for the companies to invest and develop this kind of technology.

Regarding the efflux study, although the results are strongly backed-up by the control strains, a genomic approach is required to corroborate these results. Also, further work should be done in finding new approaches to block efflux, for example, the study and development of efflux pump inhibitors that can be administered in association with the antimicrobial to potentiate its activity.

Therefore, future studies that address these questions should have a positive impact on the diagnostic and treatment of *Enterobacteriaceae* infections as well as the understanding of the contribution of efflux as a resistance mechanism to FQ.

8. REFERENCES

Aathithan, S., & French, G. L. (2011). Prevalence and role of efflux pump activity in ciprofloxacin resistance in clinical isolates of Klebsiella pneumoniae. *Eur J Clin Microbiol Infect Dis,* **30:** 745-752.

Acar, J. F., & Goldstein, F. W. (1997). Trends in bacterial resistance to fluoroquinolones. *Clin Infect Dis,***24:** 67-73.

Alvarez-Barrientos, A., Arroyo, J., Canton, R., Nombela, C., & Sanchez-Perez, M. (2000). Applications of flow cytometry to clinical microbiology. *Clin Microbiol Rev,* **13**: 167-195.

Andriole, V. T. (2005). The quinolones: past, present, and future. *Clin Infect Dis,* **41:** 113-119.

Ball, P. (2000). Quinolone generations: natural history or natural selection? *J Antimicrob Chemother,* **46:** 17-24.

Barbesti, S., Citterio, S., Labra, M., Baroni, M. D., Neri, M. G., & Sgorbati, S. (2000). Two and three-color fluorescence flow cytometric analysis of immunoidentified viable bacteria. *Cytometry,* **40**: 214-218.

Barenfanger, J., Drake, C., & Kacich, G. (1999). Clinical and financial benefits of rapid bacterial identification and antimicrobial susceptibility testing. *J Clin Microbiol,* **37**: 1415-1418.

Barnard, F. M., & Maxwell, A. (2001). Interaction between DNA gyrase and quinolones: effects of alanine mutations at GyrA subunit residues Ser(83) and Asp(87). *Antimicrob Agents Chemother,* **45**: 1994-2000.

Bergquist, P. L., Hardiman, E. M., Ferrari, B. C., & Winsley, T. (2009). Applications of flow cytometry in environmental microbiology and biotechnology. *Extremophiles,* **13**: 389-401.

Bio-Rad, L. (Producer). (2013). Introduction to Flow Cytometry.

Bratu, S., Landman, D., George, A., Salvani, J., & Quale, J. (2009). Correlation of the expression of acrB and the regulatory genes marA, soxS and ramA with antimicrobial resistance in clinical isolates of Klebsiella pneumoniae endemic to New York City. *J Antimicrob Chemother,* **64**: 278-283.

Briales, A., Rodriguez-Martinez, J. M., Velasco, C., de Alba, P. D., Rodriguez-Bano, J., Martinez-Martinez, L., & Pascual, A. (2012). Prevalence of plasmid-mediated quinolone resistance determinants qnr and aac(6')-Ib-cr in Escherichia coli and

Klebsiella pneumoniae producing extended-spectrum beta-lactamases in Spain. *Int J Antimicrob Agents,* **39**: 431-434.

Cabral, J. H., Jackson, A. P., Smith, C. V., Shikotra, N., Maxwell, A., & Liddington, R. C. (1997). Crystal structure of the breakage-reunion domain of DNA gyrase. *Nature,* **388**: 903-906.

CLSI. (2013). *Performance Standards for Antimicrobial Susceptibility Testing; Twenty-third Informational Supplement*. CLSI document M100-S23. Clinical and Laboratory Standards Institute, Wayne, PA.

Czechowska, K., Johnson, D. R., & van der Meer, J. R. (2008). Use of flow cytometric methods for single-cell analysis in environmental microbiology. *Curr Opin Microbiol,* **11**: 205-212.

Da Re, S., Garnier, F., Guerin, E., Campoy, S., Denis, F., & Ploy, M. C. (2009). The SOS response promotes qnrB quinolone-resistance determinant expression. *EMBO Rep,* **10**: 929-933.

Dalhoff, A. (2012). Global fluoroquinolone resistance epidemiology and implictions for clinical use. *Interdiscip Perspect Infect Dis*, **2012**: 1-37.

Davey, H. M. (2002). Flow cytometric techniques for the detection of microorganisms. *Methods Cell Sci, **24***: 91-97.

Doern, G. V., Vautour, R., Gaudet, M., & Levy, B. (1994). Clinical impact of rapid in vitro susceptibility testing and bacterial identification. *J Clin Microbiol,* **32**: 1757-1762.

Fabrega, A., Madurga, S., Giralt, E., & Vila, J. (2009). Mechanism of action of and resistance to quinolones. *Microb Biotechnol,* **2**: 40-61.

Faria-Ramos, I., Costa-de-Oliveira, S., Barbosa, J., Cardoso, A., Santos-Antunes, J., Rodrigues, A. G., & Pina-Vaz, C. (2012). Detection of Legionella pneumophila on clinical samples and susceptibility assessment by flow cytometry. *Eur J Clin Microbiol Infect Dis,* **31**: 3351-3357.

Faria-Ramos, I., Espinar, M. J., Rocha, R., Santos-Antunes, J., Rodrigues, A. G., Canton, R., & Pina-Vaz, C. (2013). A novel flow cytometric assay for rapid detection of extended-spectrum beta-lactamases. *Clin Microbiol Infect,* **19**: 8-15.

Fenosa, A., Fuste, E., Ruiz, L., Veiga-Crespo, P., Vinuesa, T., Guallar, V., Vinas, M. (2009). Role of TolC in Klebsiella oxytoca resistance to antibiotics. *J Antimicrob Chemother,* **63**: 668-674.

Gootz, T. D., & Brighty, K. E. (1996). Fluoroquinolone antibacterials: SAR mechanism of action, resistance, and clinical aspects. *Med Res Rev,* **16**: 433-486.

Gordis, L. (2011). *Epidemiologia* (4th edition ed.).

Greenfield, R. A. (1993). Symposium on antimicrobial therapy. VII. The fluoroquinolones. *J Okla State Med Assoc,* **86**: 166-174.

Gregori, G., Denis, M., Seorbati, S., & Citterio, S. (2003). Resolution of viable and membrane-compromised free bacteria in aquatic environments by flow cytometry. *Curr Protoc Cytom,* **11**: 11-15.

Guillard, T., Moret, H., Brasme, L., Carlier, A., Vernet-Garnier, V., Cambau, E., & de Champs, C. (2011). Rapid detection of qnr and qepA plasmid-mediated quinolone resistance genes using real-time PCR. *Diagn Microbiol Infect Dis,* **70**: 253-259.

Harris, P., Winney, I., Ashhurst-Smith, C., O'Brien, M., & Graves, S. (2012). Comparison of Vitek MS (MALDI-TOF) to standard routine identification methods: an advance but no panacea. *Pathology,* **44**: 583-585.

Heddle, J., & Maxwell, A. (2002). Quinolone-binding pocket of DNA gyrase: role of GyrB. *Antimicrob Agents Chemother,* **46**: 1805-1815.

Hernandez, A., Sanchez, M. B., & Martinez, J. L. (2011). Quinolone resistance: much more than predicted. *Front Microbiol,* **2**: 1-6.

Hombach, M., Bloemberg, G. V., & Bottger, E. C. (2012). Effects of clinical breakpoint changes in CLSI guidelines 2010/2011 and EUCAST guidelines 2011 on antibiotic susceptibility test reporting of Gram-negative bacilli. *J Antimicrob Chemother,* **67**: 622-632.

Hooper, D. C. (1998). Bacterial topoisomerases, anti-topoisomerases, and anti-topoisomerase resistance. *Clin Infect Dis,* **27***:* 54-63.

Jacoby, G. A. (2005). Mechanisms of resistance to quinolones. *Clin Infect Dis,* **41***:* 120-126.

Kramer, S., & Mausner, J. S. (2007). *Introdução à Epidemiologia* (4th edition ed.).

Lebaron, P., Catala, P., & Parthuisot, N. (1998). Effectiveness of SYTOX Green stain for bacterial viability assessment. *Appl Environ Microbiol,* **64**: 2697-2700.

Lupala, C. S., Gomez-Gutierrez, P., & Perez, J. J. (2012). Molecular Determinants of the Bacterial Resistance to Fluoroquinolones: A Computational Study. *Curr Comput Aided Drug Des,* **9**: 281-288.

MacPherson, D. W., Gushulak, B. D., Baine, W. B., Bala, S., Gubbins, P. O., Holtom, P., & Segarra-Newnham, M. (2009). Population mobility, globalization, and antimicrobial drug resistance. *Emerg Infect Dis,* **15**: 1727-1732.

Marquez, B. (2005). Bacterial efflux systems and efflux pumps inhibitors. *Biochimie,* **87**: 1137-1147.

Martins, A., Machado, L., Costa, S., Cerca, P., Spengler, G., Viveiros, M., & Amaral, L. (2011). Role of calcium in the efflux system of Escherichia coli. *Int J Antimicrob Agents,* **37**: 410-414.

Mason, D. J., Power, E. G., Talsania, H., Phillips, I., & Gant, V. A. (1995). Antibacterial action of ciprofloxacin. *Antimicrob Agents Chemother,* **39**: 2752-2758.

Mazzariol, A., Zuliani, J., Cornaglia, G., Rossolini, G. M., & Fontana, R. (2002). AcrAB Efflux System: Expression and Contribution to Fluoroquinolone Resistance in Klebsiella spp. *Antimicrob Agents Chemother,* **46**: 3984-3986.

Mortimer, F. C., Mason, D. J., & Gant, V. A. (2000). Flow cytometric monitoring of antibiotic-induced injury in Escherichia coli using cell-impermeant fluorescent probes. *Antimicrob Agents Chemother,* **44**: 676-681.

Padilla, E., Llobet, E., Domenech-Sanchez, A., Martinez-Martinez, L., Bengoechea, J. A., & Alberti, S. (2010). Klebsiella pneumoniae AcrAB efflux pump contributes to antimicrobial resistance and virulence. *Antimicrob Agents Chemother,* **54**: 177-183.

Paixao, L., Rodrigues, L., Couto, I., Martins, M., Fernandes, P., de Carvalho, C., Viveiros, M. (2009). Fluorometric determination of ethidium bromide efflux kinetics in Escherichia coli. *J Biol Eng,* **3**: 1-13.

Petrie, A., & Sabin, C. (2009). *Medical Statistics at a Glance* (3rd edition ed.).

Piddock, L. J. (2012). The crisis of no new antibiotics--what is the way forward? *Lancet Infect Dis,* **12**: 249-253.

Pina-Vaz, C., Costa-de-Oliveira, S., & Rodrigues, A. G. (2005). Safe susceptibility testing of Mycobacterium tuberculosis by flow cytometry with the fluorescent nucleic acid stain SYTO 16. *J Med Microbiol,* **54**: 77-81.

Pina-Vaz, C., & Rodrigues, A. G. (2010). Evaluation of antifungal susceptibility using flow cytometry. *Methods Mol Biol,* **638**: 281-289.

Pos, K. M. (2009). Trinity revealed: Stoichiometric complex assembly of a bacterial multidrug efflux pump. *Proc Natl Acad Sci U S A,* **106**: 6893-6894.

Rodriguez-Martinez, J. M., Briales, A., Velasco, C., Diaz de Alba, P., Martinez-Martinez, L., & Pascual, A. (2011). Discrepancies in fluoroquinolone clinical categories between the European Committee on Antimicrobial Susceptibility Testing (EUCAST) and CLSI for Escherichia coli harbouring qnr genes and mutations in gyrA and parC. *J Antimicrob Chemother,* **66**: 1405-1407.

Ruiz, J., Pons, M. J., & Gomes, C. (2012). Transferable mechanisms of quinolone resistance. *Int J Antimicrob Agents,* **40**: 196-203.

Silva, F., Lourenco, O., Queiroz, J. A., & Domingues, F. C. (2011). Bacteriostatic versus bactericidal activity of ciprofloxacin in Escherichia coli assessed by flow cytometry using a novel far-red dye. *J Antibiot (Tokyo)*, **64**: 321-325

Strahilevitz, J., Jacoby, G. A., Hooper, D. C., & Robicsek, A. (2009). Plasmid-mediated quinolone resistance: a multifaceted threat. *Clin Microbiol Rev*, **22**: 664-689.

Walberg, M., Gaustad, P., & Steen, H. B. (1997). Rapid assessment of ceftazidime, ciprofloxacin, and gentamicin susceptibility in exponentially-growing E. coli cells by means of flow cytometry. *Cytometry*, **27**: 169-178.

Wickens, H. J., Pinney, R. J., Mason, D. J., & Gant, V. A. (2000). Flow cytometric investigation of filamentation, membrane patency, and membrane potential in Escherichia coli following ciprofloxacin exposure. *Antimicrob Agents Chemother*, **44**: 682-687.

Yang, S., Clayton, S. R., & Zechiedrich, E. L. (2003). Relative contributions of the AcrAB, MdfA and NorE efflux pumps to quinolone resistance in Escherichia coli. *J Antimicrob Chemother*, **51**: 545-556.

Zgurskaya, H. I., & Nikaido, H. (1999). Bypassing the periplasm: reconstitution of the AcrAB multidrug efflux pump of Escherichia coli. *Proc Natl Acad Sci U S A*, **96**: 7190-7195.

Zipper, H., Brunner, H., Bernhagen, J., & Vitzthum, F. (2004). Investigations on DNA intercalation and surface binding by SYBR Green I, its structure determination and methodological implications. *Nucleic Acids Res*, **32**: 1-25.

Zou, K. H., O'Malley, A. J., & Mauri, L. (2007). Receiver-operating characteristic analysis for evaluating diagnostic tests and predictive models. *Circulation*, **115**: 654-657.