Universidade de Aveiro Departamento de Biologia

2009

Cátia Raquel Talhas Santos

Pesquisa de integrões classe 1 em isolados clínicos de Gram-negativo

Screening of class 1 integrons in clinical isolates of Gram-negative bacteria

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

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palavras-chave

Integrões de class 1, disseminação de genes de resistência aos antibióticos, bactérias de Gram-negativo, ambiente hospitalar.

resumo

Actualmente, é cada vez mais frequente a associação de bactérias oportunistas e comensais resistentes a antibióticos com infecções nosocomiais. Este problema clínico tornou-se preocupante e deve-se ao uso indiscriminado de antibióticos. Perante esta pressão selectiva, as bactérias desenvolvem diferentes mecanismos de resistência a estes compostos. A presença de estruturas capazes de transportar genes de resistência, designadas por integrões, que contribuem para a disseminação destes genes bem como a sua associação com o perfil de resistência de bactérias constitui o objectivo do presente trabalho.

Assim, foram recolhidas amostras de superfícies das instalações sanitárias, do serviço de Medicina II, do Hospital Infante D. Pedro, Aveiro. Após o isolamento das bactérias em meio selectivo para Gram-negativas (MacKonkey), todos os isolados foram sujeitos a tipagem molecular por BOX-PCR. O perfil de bandas obtido após electroforese foi analisado com o programa GelCompar II software (Applied Maths, Kortrijk, Belgium), permitindo distinguir diferentes grupos clonais. De cada grupo clonal foi seleccionado um isolado para os estudos posteriores, resultando num total de 45 isolados distintos.

A pesquisa de integrões classe 1 iniciou-se por um "screening" para o gene da integrase. Nos 25 isolados positivos para este gene, foi amplificada e caracterizada a respectiva região variável. A sequência nucleotídica dos amplicões foi comparada com outras depositadas na base de dados.

Os resultados mostraram a presença de integrões em diferentes espécies (*Pseudomonas putida*, *Klebsiella pneumoniae*, *Escherichia coli*, *Pseudomonas mendocina*, *Proteus mirabillis* e *Morganella morganii*). As regiões variáveis apresentavam diferentes tamanhos e diferentes arranjos de genes. Em geral, predominam gene *cassettes* que conferem resistência aos aminoglicosídeos, trimetoprime e metalo-β-lactamases. A localização destas estruturas no genoma bacteriano foi efectuada por "southern blot"; o DNA genómico foi digerido com a enzima S1, e sujeito a hibridação com sondas para os genes 16S e da integrase revelando que a maioria dos integrões estão localizados em plasmídeos.

Como conclusão geral, verifica-se a prevalência de isolados contendo determinantes genéticos de resistência em superfícies inanimadas do ambiente hospitalar (53.33%), os quais podem constituir um potencial risco para os pacientes, uma vez que se trata de bactérias oportunistas. O facto de estes genes de resistência estarem associados a elementos genéticos móveis, nomeadamente transposões e muitas vezes plasmídeos, facilita a sua disseminação no ambiente hospitalar, principalmente por transferência horizontal de genes.

Keywords

Class 1 integrons, multidrug-resistance, dissemination of resistance genes, Gram-negtive bacteria, hospital environment.

abstract

Currently, it is becoming frequent the association of antibiotic resistant opportunistic and commensal bacteria with nosocomial infections. This is a clinical problem of concern and is based on the indiscriminate use of antibiotics. Given the selective pressure within the hospital environment, bacteria develop different resistance mechanisms to these compounds. The presence of structures, referred as integrons, that carry and disseminate these resistance genes among bacteria and their association with the bacteria resistance profile constitutes the aim of the present study.

To this end we collected samples from surfaces of sanitary facilities, of the Medicine II service of the Hospital Infante D. Pedro, Aveiro. After bacteria isolation on a selective medium (MacKonkey) for Gram negatives, all the isolates were molecular typed by BOX-PCR. After electrophoresis, the banding pattern was analysed with the GelCompar II software (Applied Maths, Kortrijk, Belgium), which allowed for the selection of different clonal groups. One isolate was selected from each group for further studies.

Forty five isolates were selected for the screening of class 1 integrons. In the twenty-five positive isolates respective variable region was amplified and characterized. Amplicons nucleotide sequences were compared with others deposited in databases.

The results revealed the presence of integrons in different species (*Pseudomonas putida*, *Klebsiella pneumoniae*, *Escherichia coli*, *Pseudomonas mendocina*, *Proteus mirabillis* e *Morganella morganii*). Different lenghts of variable regions and different genes arrays were found. Generally gene cassettes conferring resistance to aminoglycosides trimethoprim and metallo-β-lactamases were predominante. Southern hybridization of S1 digested genomic DNA with 16 rDNA and integrase genes labeled probes revealed that the majority of the integrons are located in plasmids.

To conclude, is important to refer that there is a prevalence of opportunistic bacteria possessing integrons in inanimate surfaces within the hospital environment, which can constitute risk to the debilitated patients. Moreover, these structures are associated with mobile genetic elements, mainly transposons and many times plasmids, which facilitates the dissemination of these antibiotic resistance genes in the hospital environment, mainly by horizontal gene transfer.

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LIST OF ABBREVIATIONS

3'-CS: Conserved segment 3'

5'-CS: Conserved segment 5'

59-be: 59 base element

aac: Aminoglycoside acetyltransferase

aad: Aminoglycoside adenyltransferase

arr: Rifampin ADP-ribosyl transferase

***attI*:** Recombination site associated to 5'-CS

***attC*:** Recombination site associated to gene cassette

Bp: Bases of pairs

Cat: Cloramphenicol acetyltransferase

CR: Common region

CS: Conserved segment

CTX-M: Cephalosporinase

Dhfr: Dihydrofolate reductase

Dhps: Dihydropteroate synthase

DNA: Deoxiribonucleic acid

ERIC: Enterobacterial repetitive intergenic consensus

ESBL: Extended-spectrum β-lactamases

Gyr: Topoisomerase II type (gyrase)

HGT: Horizontal gene transfer

IMP: Imipenemase

IntI: Integrase enzyme

IS: Insertion sequence

ISCR: Insertion sequence common region

Kb: Kilobases

MBL: Metallo-β-lactamases

MDR: Multi-drug resistance

MGE: Mobile genetic element

MIC: Minimal Inhibitory Concentration

NCCLS: National Committee for Clinical Laboratory Standards

ORF: Open reading frame

OXA: Oxacillinase

P2: Second promoter

Par: Topoisomerase IV type

PBP's: Penicillin Binding Proteins

***Pc* or P_{ant}:** Promoter present in 5'-CS

PCR: Polymerase chain reaction

PFGE: Pulsed-field gel electrophoresis

***qacEΔ*:** Resistance gene to quaternary ammonium compounds

Qnr: Quinolone resistance gene

REP: Repetitive extragenic palindromic

RC: Rolling-circle

RI: Resistance integrons

RNA: Ribonucleic acid

SHV: Penicillinase

SI: Super-integrons

Sul: Dihydropteroate synthase

TEM: Penicillinase

Tn: Transposon

***tni* module:** Tranposition genes

tnpA: putative transposase

VGT: Vertical gene transfer

VIM: Verona imipenemase

VR: Variable region

I. Introduction

1. ANTIBIOTICS

Simple microorganisms such as bacteria, appeared on our planet about 3.5 billion years ago, and had to adapt to the inhospitable conditions of that time, in order to survive. Several studies have concluded that these microorganisms have a remarkable ability to adapt and proliferate in any environment, no matter how harsh it is (Bennett 2008).

In the 30´s, Alexander Fleming discovered a new solution that came to end with the pain and death caused by many diseases. This was based on the natural and spontaneous production of an antibacterial compound – penicillin, produced by a fungus, *Penicillium* spp*.,* marking the beginning of the antibiotic era and antimicrobial chemotherapy (Ruiz 2003; Abeylath and Turos 2008). It started to be used in 1940 to treat infections caused by *Staphylococcus aureus* (Smith and Romesberg 2007; Yamaguchi 2009).

The euphoria triggered by the discovery continued over the next two decades, where new classes of antibiotics were discovered and introduced as shown in Figure 1. Most of these antibiotic compounds were of natural origin, since they were produced by microorganisms, such as bacteria and fungi, but nowadays a big part of the compounds are of synthetic origin (Levy and Marshall 2004).

It was really a breakthrough in the field of medicine (Clewell 2005), since these compounds were only toxic to the bacterial cell, because the target of the antibiotic is absent in the host cell (B. Henriques Normark 2002), and some experts believed that infectious diseases would be conquered (JM Conly 2005). But in reality that did not happened and the initial optimism was lost (Smith and Romesberg 2007; Yamaguchi 2009), being necessary to discover new solutions for this problem.

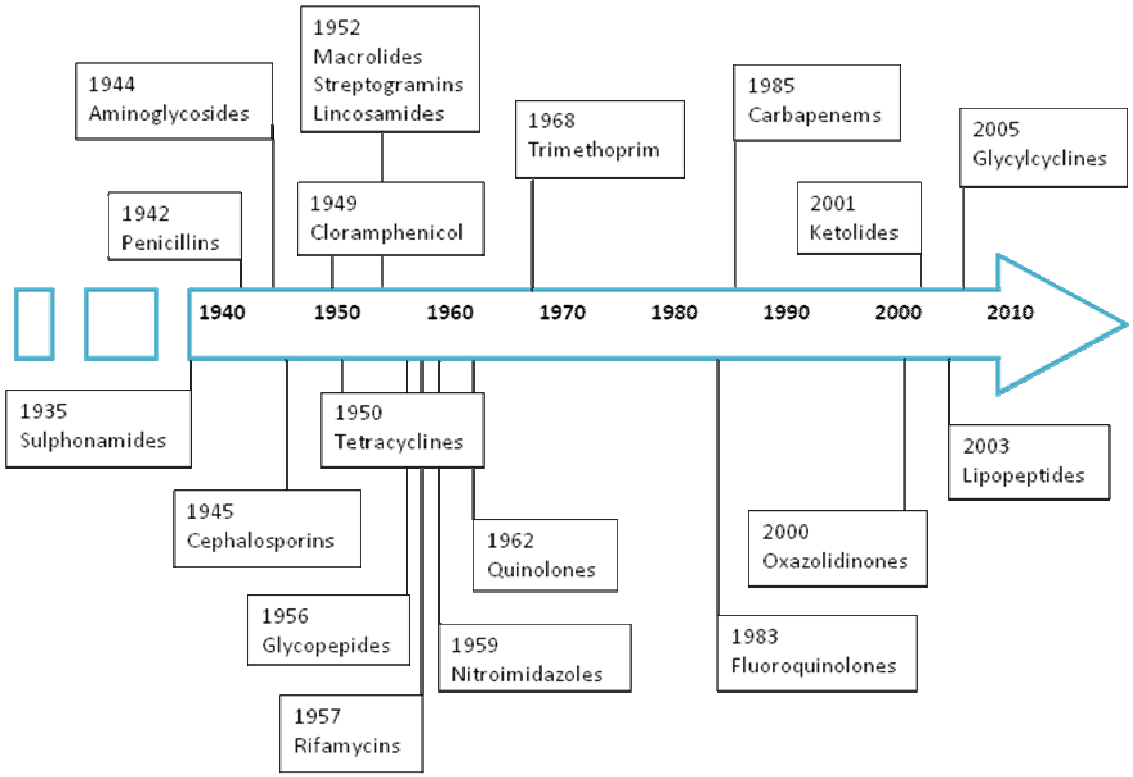


Figure 1 - Chronological order of the development of the main classes of antibiotics (adopted from (JM Conly 2005).

1.1 Modes of action of the different classes of antibiotics

The antibiotics can be classified according to their action, spectrum of activity and chemical structure (Table 1). Antibiotics of the same class have a similar toxicity and spectra of activity (Tenover 2006).

Some classes of antibiotics can act on the bacterial cell, inhibiting their growth or reproduction - bacteriostatic antibiotics; while others may act on the bacterial cell leading to death - bactericidal antibiotics.

Antibiotics can act only against a limited variety of bacterial species – narrow-spectrum antibiotics, or can be effective against a wide range of pathogens of different species – broad-spectrum antibiotics (Clewell 2005; Murray 2009).

The bacterial sensitivity depend of selective toxicity of the antibiotic and varies between different bacterial species and strains within the same bacterial population (B. Henriques Normark 2002).

The antibiotic activity can be measured *in vitro* by determining the minimal inhibitory concentration (MIC). For a reliable result of the tests, one must work with a standardized methodology, which is currently recommended by the Clinical Laboratory Standards Institute, formerly National Committee for Clinical Laboratory Standards (NCCLS). The procedures to determine the inhibitory activity can be performed by broth dilution or agar techniques. The antibiotic agents are usually tested in successive dilutions, and the lowest concentration able to inhibit the growth of an organism is considered as the MIC (CLSI 2006).

The mode of action of most antibiotics is well known, and is generally similar in antibiotics of the same group, giving rise to a pattern of cross-resistance. Knowledge of these mechanisms is important to understand the basis of the combined action of the antibiotics, since a synergistic effect only occurs when there is a pattern in the combination and they have different sites of action (Mc Dermott, Walker et al. 2003)

Thus, there are four mains modes of action distributed for all classes of antibiotics, which are efficacious against different Gram-negative bacteria species, as summarized in Table 1.

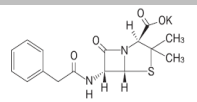
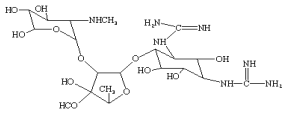
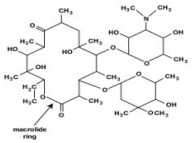
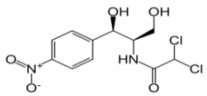
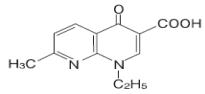
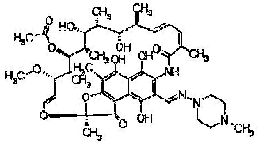
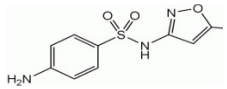
i. Disruption of cell wall due to ligation between the β-lactam antibiotics and penicillin binding proteins (PBPs), present in cell wall, inhibiting the peptidoglycan synthesis (Mc Dermott, Walker et al. 2003; Sousa 2006; Senka Dzidic 2008; Jayaraman 2009).

ii. Inhibition of nucleic acids synthesis due to ligation of the antibiotics to DNA topoisomerase type II (gyrase) or topoisomerase type IV enzymes, inhibiting the replication and the transcription (Tenover 2006; Murray 2009).

iii. Inhibition of protein synthesis and translation due to irreversible ligation of the antibiotics to ribosomal subunits (Mc Dermott, Walker et al. 2003; Senka Dzidic 2008; Murray 2009).

iv. Inhibition of folic acid synthesis and nucleotides biosynthesis by ligation of the antibiotics to dihydropteroate synthase (DHPS) and dihydrofolate reductase (DHFR) (Mc Dermott, Walker et al. 2003; Tenover 2006; Senka Dzidic 2008).

Table 1 - Modes of action of the main classes of antibiotics that are effective against most Gram-negative bacteria species (adapted by Murray 2009).

Classes of antibiotics	Activity spectrum	Action	Mode of action	Chemical structure
β-lactams	Broad/Narrow	Bactericidal	Bind PBPs and enzymes responsible for peptidoglycan synthesis	
β-lactam / β-lactamase inhibitor			Bind β-lactamases and prevents enzymatic inactivation of β-lactam	
Aminoglycosides	Broad	Bactericidal	Produce premature release of aberrant peptide chains from 30S ribosome	
Macrolides, Ketolides, Clindamycin, Streptogramins	Broad	Bacteriostatic	Prevent polypeptide elongation at 50S ribosome	
Chloramphenicol	Broad	Bacteriostatic	Binds to the 50S ribosomal subunit blocking peptidyltransferase reaction	
Quinolones	Broad	Bactericidal	Bind α-subunit of DNA gyrase	
Rifampicin	Broad	Bactericidal	Prevent transcription by binding DNA-dependent RNA polymerase	
Sulfonamides and Trimethoprim	Broad	Bacteriostatic	Inhibit DHFR and DHPS enzymes, leading to disruption of the folic acid synthesis	

2. DEVELOPMENT OF ANTIBIOTIC RESISTANCE

All classes of antibiotics are efficacious against different bacterial species, since they inhibit specific physiological and metabolic essential functions (Smith and Romesberg 2007). However, this potential has been lost due to the ability of bacteria to develop strategies that permit to acquire adaptive characteristics to survive in the presence of these compounds. Several studies show that the exposure to drugs is the factor that has most contributed to emergence of resistance in microorganisms, such as in fungi and bacteria (Levy and Marshall 2004).

Thus, antibiotic resistance can be defined as a result of classical Darwinian hypothesis, "survival of the fittest", since the selective pressure exerted by inappropriate and indiscriminate use of the antibiotics, allows the adaptation of the microorganisms more resistant (Figure 2) (Smith and Romesberg 2007). Indeed, the whole period of antibiotic use can be considered as one large ongoing experiment designed to test this hypothesis, and nowadays, a set of arguments, suggest that the hypothesis of Darwin was surprisingly confirmed (Bennett 2008).

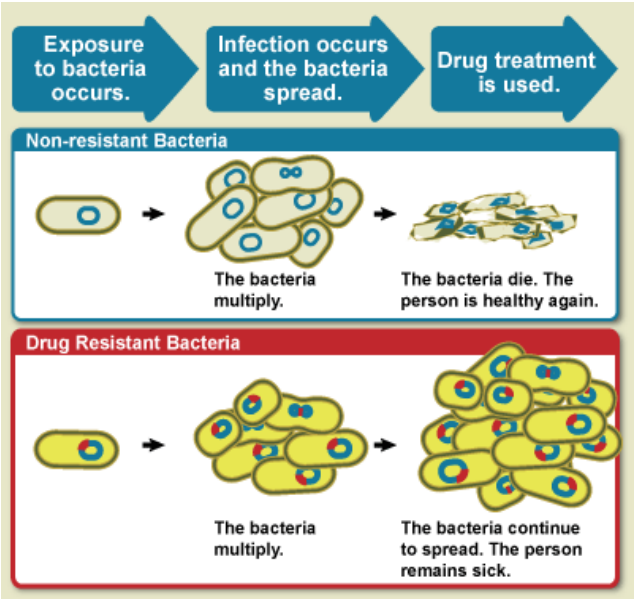


Figure 2 - In the presence of antibiotic selective pressure, some bacteria make no improvement and are lost from the population over time – susceptible bacteria. However, those that confer an adaptive advantage are conserved and multiply, becoming more common and developing an infection – resistant bacteria (Jacoby 2009).

The ability of microorganisms to acquire resistance to antibiotics has surpassed our imagination. Currently, the bacterial evolution culminate in emergence of resistance to a range of antibiotic compounds (Harbottle, Thakur et al. 2006), designated by multidrug-resistance (MDR) bacteria (Smith and Romesberg 2007), which is characterized by the ability of microorganisms to be resistant to more than three classes of these compounds (Tenover, et al. 2006; Mulvey and Simor 2009). Moreover, it seems that there is a directly proportional relationship between the introduction of a new class of antibiotics in the therapeutics and the arising of bacterial resistance (Figure 3), a phenomenon referred as co-evolution (Mulvey and Simor 2009).

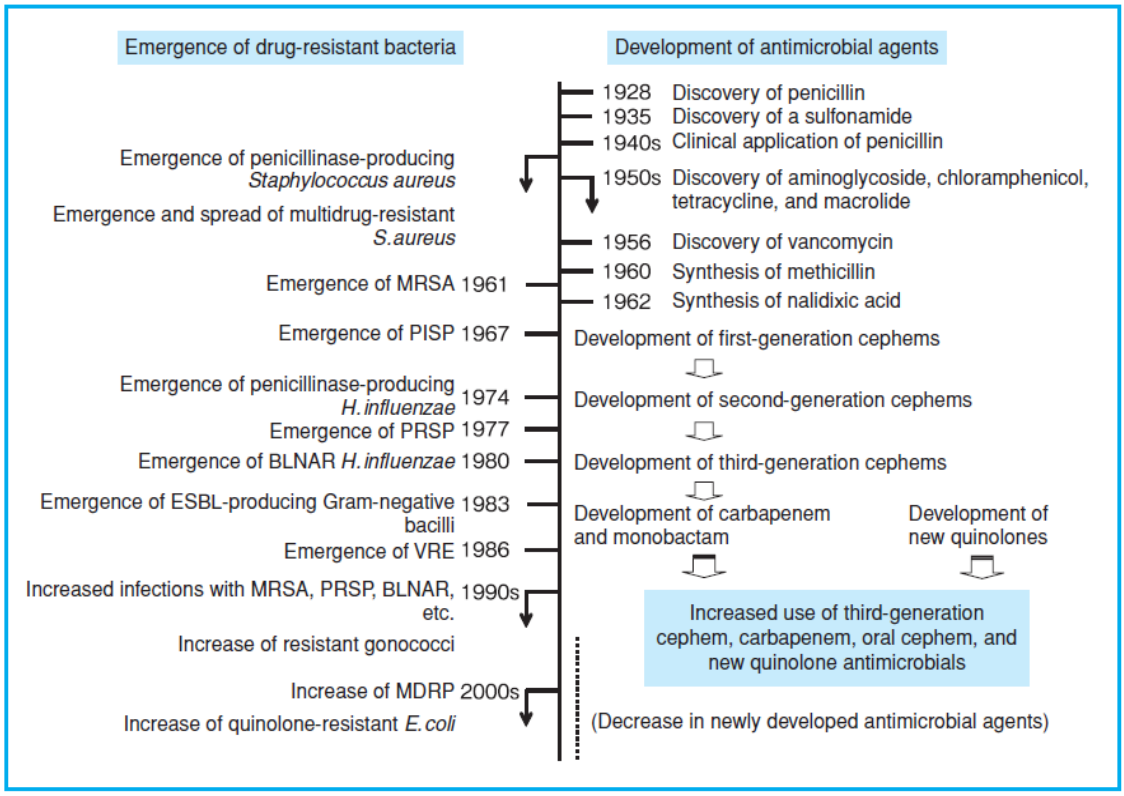


Figure 3 - Emergence of drug-resistant bacteria in response to development of antibiotics (Yamaguchi 2009).

The resistant microorganisms have mainly a clinical origin, since it is in hospitals that antibiotics are used in large scale (Mc Dermott, Walker et al. 2003; Sousa 2006). In fact, in a short period of time, clinical strains resistance has been observed for almost all classes of antibiotics available (Mc Dermott, Walker et al. 2003). Moreover, MDR strains are not only confined to the hospital setting and can be widely spread in the environment (Tenover 2006; Mulvey and Simor 2009), since some antibiotics are stable chemical compounds and remain active in the natural environments for a few years (Alekshun and Levy 2007).

The outcome of the excessive use of different classes of antibiotics in diverse areas, as agriculture, livestock and veterinary medicine, have also contributed to the emergence of resistance bacteria (Stefan and Elisabeth 2001; Harbottle, Thakur et al. 2006; Summers 2006; Sipahi 2008), and their entry in the food chain allow the dissemination in environment (Figure 4) (Shea 2003; Senka Dzidic 2008).

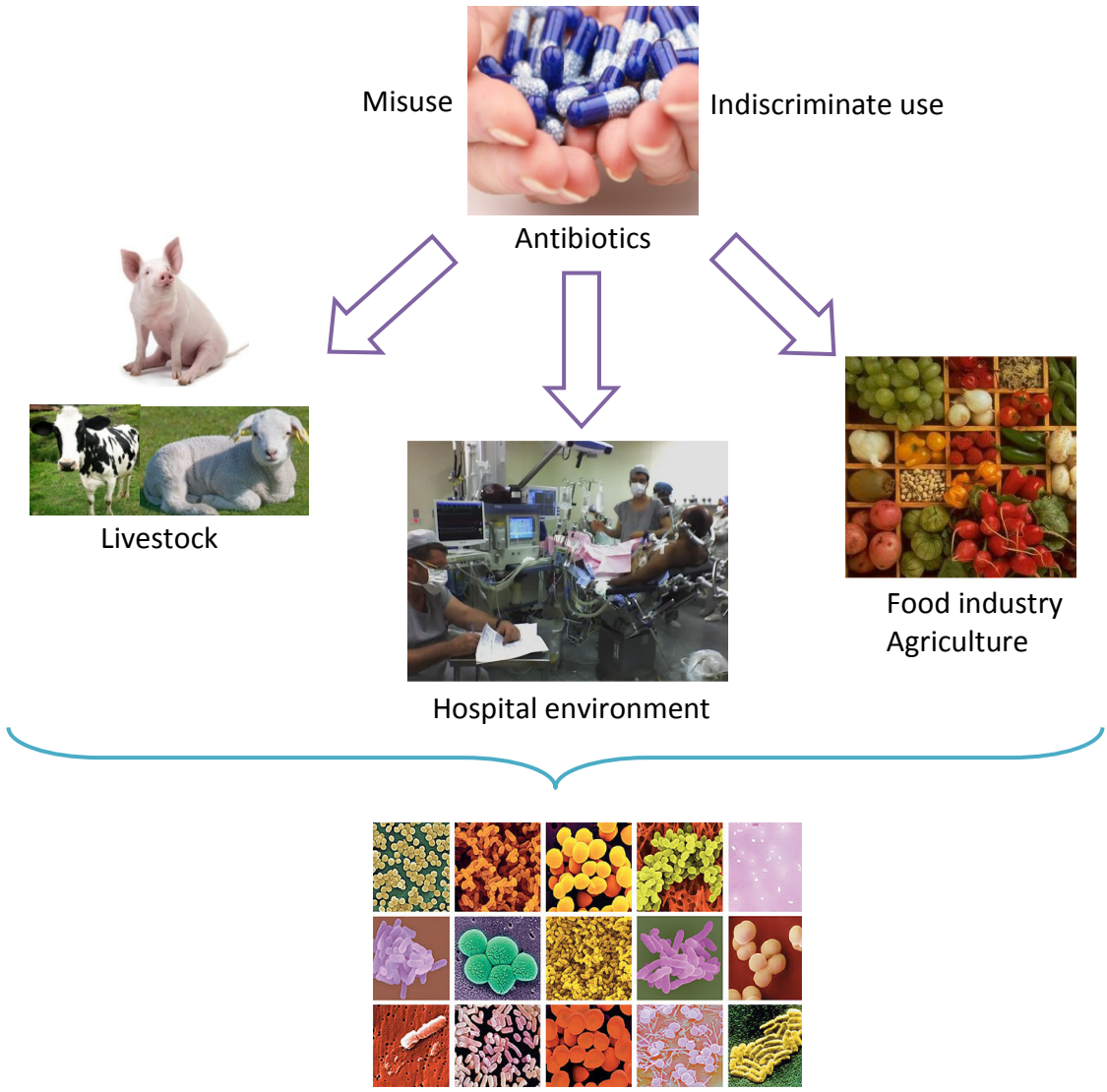


Figure 4 - The food chain can be a vehicle to the dissemination of bacteria that cause infections. The excessive and misuse of antibiotics in different areas leads to the emergence of MDR bacteria (http://images.google.com/).

In resume, the rapid development and use of different classes of antibiotics and the success of adaptive bacteria contributed to the MDR, which currently is one of the largest and more worrying clinical problems worldwide, threatening the well-being of Man, which needs to be well understood in order to be overcome (JM Conly 2005; Mulvey and Simor 2009). So, the use of antibiotics is not a free act, but an act of intelligence (Sousa 2006).

2.1 Antibiotic resistance mechanisms

Bacteria are better genetic engineers than Man and will continue the escape of the antibiotics effects, since they have the ability to develop different adaptive solutions (Barlow, Pemberton et al. 2004).

In order to survive to antibacterial chemotherapy, most Gram-negative bacteria have developed five main antibiotic resistance mechanisms, as shown in Table 3:

i. Inactivation and/or degradation of the antibiotics by specific enzymes;
ii. Reduce antibiotic accumulation by efflux pumps;
iii. Alteration or loss of target site by mutations;
iv. Changes in the cell permeability due to porins;
v. By-pass of metabolic pathway.

Table 2 - Mechanisms of resistance developed by a majority of Gram-negative bacteria, in response to different classes of antibiotics (Senka Dzidic 2008; Jayaraman 2009; Mulvey and Simor 2009).

Resistance mechanism	**Antibiotic affected by bacterial resistance**	**Resistant bacteria**
Antibiotic inactivation		
β-lactamases enzymes (ESBLs and MBLs)	Penicillins, cephalosporins, cefotaximes, carbapenems	*Escherichia coli*, *Klebsiella* spp., *Proteus* spp., *Enterobacter* spp., *Serratia* spp., *Pseudomonas aeruginosa*
Aminoglycoside inactivating Enzymes (*aac* / *aad* genes)	Gentamicin, tobramycin, amikacin, streptomycin	Enterobacteriaceae
Chloramphenicol acetyltransferase (*cat* gene)	Cloramphenicol	
Dihydrofolate reductase (*dfr* gene)	Trimethoprim	
Dihydropteroate synthase (*sul* gene)	Sulfonamides	
Altered target site		
Altered PBPs	Penicillins	Enterobacteriaceae
Mutations in the DNA gyrase or topoisomerase	Ciprofloxacin, norfloxacin, levofloxacin	Enterobacteriaceae, *P. aeruginosa*
Decreased access to the target site		
Change in outer membrane proteins or porins (OprD and OMP)	Tobramycin, gentamicin	Enterobacteriaceae *P. aeruginosa*
Efflux pump (AcrAB and MEX systems)	Erythromycin, tetracycline, ciprofloxacin	*E. coli, P. aeruginosa*
Target by-pass		
By-pass of metabolic pathway	Sulfonamides and Trimethoprim	*E. coli*

These mechanisms may be present in various bacterial species or only in some strains of a species (Harbottle, Thakur et al. 2006), and frequently there is more than one type of resistance mechanism to one antibiotic (Levy and Marshall 2004) (Figure 5).

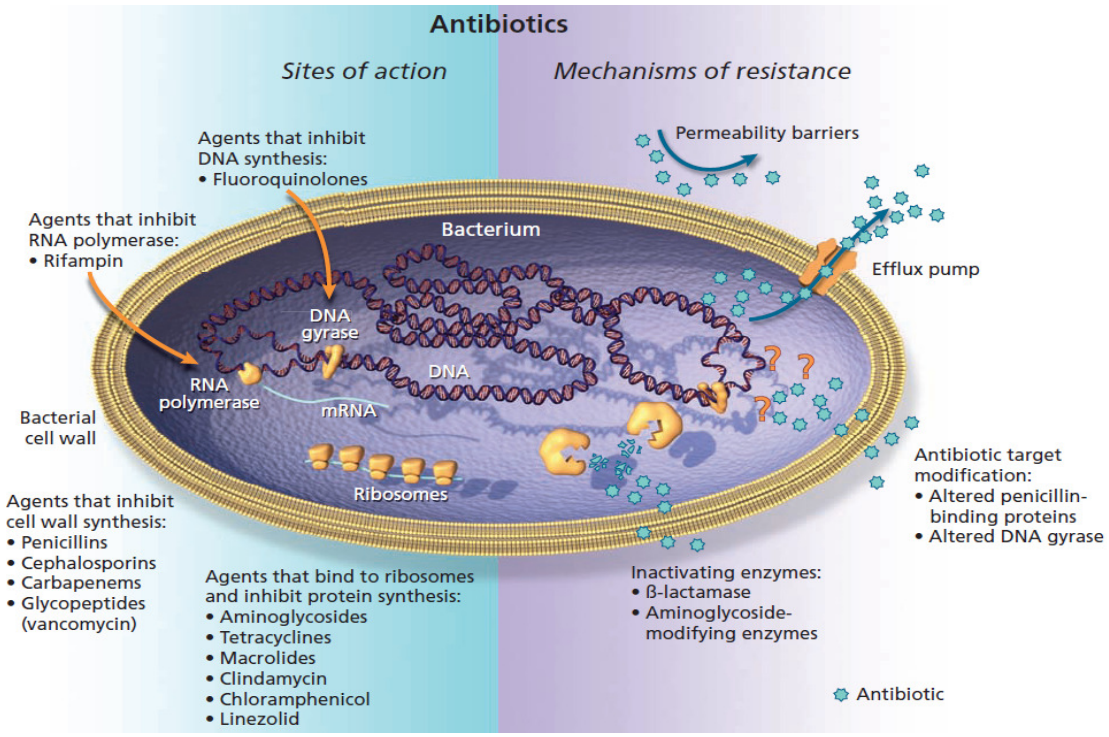


Figure 5 – Sites of action of the main classes of antibiotics and potential bacterial mechanisms of resistance to antibiotics (Mulvey and Simor 2009).

2.2 Acquisition and dissemination of resistance genes

Several published studies showed that bacterial resistance is caused by the articulation of intrinsic or acquired genetic resistance mechanisms (Barlow, Pemberton et al. 2004).

2.2.1 Intrinsic resistance

Intrinsic resistance occurs naturally in the bacterial cell, which does not suffer any genetic or biochemical change in the genome, and therefore developed their own mechanisms of resistance without addition of new genetic information (Harbottle, Thakur et al. 2006; Senka Dzidic 2008). The production of enzymes, such β-lactamases that inactivate the antibiotic is the main intrinsic mechanism of resistance, and arises in many species of Gram-negative bacteria (Mulvey and Simor 2009). Intrinsic resistance is also associated with efflux pumps, present in *P. aeruginosa* isolates, which show a low degree of susceptibility to hydrophobic antibiotics such as macrolides, because they had difficulties in crossing the outer cell membrane, and both are driven by the activity of efflux pumps (B. Henriques Normark 2002; Mc Dermott, et al. 2003).

2.2.2 Acquired resistance

Acquired resistance consist in the capture and insertion of new genetic elements, as well as in the occurrence of “adaptive mutations” (deletions, insertions or inversions of bases) in structural or regulatory genes. These processes happen randomly and change of the genetic makeup of the microorganism (Bennett 2008), since they can change the capacity of bacteria to survive the antibiotics action, by preventing their access or binding to the target (B. Henriques Normark 2002; Alekshun and Levy 2007; Senka Dzidic 2008; Mulvey and Simor 2009).

Resistance determinants can be inherited by vertical gene transfer (VGT), when they pass through generations as the cells divide, or they can be acquired by horizontal gene transfer (HGT). The latter assumes the movement of genetic material between bacteria other than by descent (Harbottle, Thakur et al. 2006; Mulvey and Simor 2009). The HGT is associated with mobile genetic elements (MGEs), and occurs through three different processes (Figure 6).

i. Transformation

The transformation was the first mechanism of HGT to be discovered among prokaryotes (Harbottle, Thakur et al. 2006). This process involves the transfer of cellular DNA between closely related bacteria and is mediated by chromosomally encoded proteins that are found in some naturally transformable bacteria (Frost, Leplae et al. 2005). Initially there is release of DNA from a donor dead or lysed cell (Alekshun and Levy 2007), or in some bacteria, at a specific point in the growth cycle. This DNA is degraded and broken into fragments that are uptaken and incorporated into the competent recipient chromosome (Clewell 2005; Tenover 2006). Transformation is thus limited to moving variants (alleles) of shared chromosomal genes around among strains of a bacterial species. Heterologous genes would not be able to recombine and would be degraded by the recipient's nucleases (Summers 2006).

ii. Conjugation

The conjugation is mediated by self-replicating double stranded DNA molecules, typically circular called conjugative plasmids or chromosomally integrated conjugative, which included conjugative transposons (Alekshun and Levy 2007). This process involves the mobilization of resistance genes, through cell-to-cell contact by a long protein structure, designated sex pilli or fimbriae, which facilitate the transfer of cellular DNA from the donor plasmid-carrying cell to a recipient cell that lacks the plasmid or integrated conjugative elements (Frost, Leplae et al. 2005; Summers 2006). During this mechanism, occurs the transfer of only a single strand of the plasmid from the donor cell to receptor cell, and a second strand is synthesized. So, the conjugation is a replicative process, since both bacteria keep a copy of the plasmid transferred with the resistance genes, but additional copies can be produced (Tenover 2006).

iii. Transdution

The transduction is mediated by independently replicating bacterial viruses called bacteriophages or phages that use the transposition to replicate (Frost, Leplae et al. 2005; Tenover 2006; Bennett 2008). At low frequency, bacteriophages can accidentally package segments of host DNA in their capsule and can inject this DNA into a new host, in which it can recombine with the cellular chromosome and be inherited (Frost, Leplae et al. 2005). Some of these fragments of the host DNA will contain resistance determinants (Mc Dermott, Walker et al. 2003). Genetic exchange via transduction involves bacteriophage infection of a bacterium, phage replication, and packaging of some of the bacterial DNA with the phage DNA (which may include resistance determinants), lyses of that bacterium, and infection of subsequent bacteria. Upon subsequent infection, those resistance determinants may be transferred to the infected bacterium (Harbottle, Thakur et al. 2006).

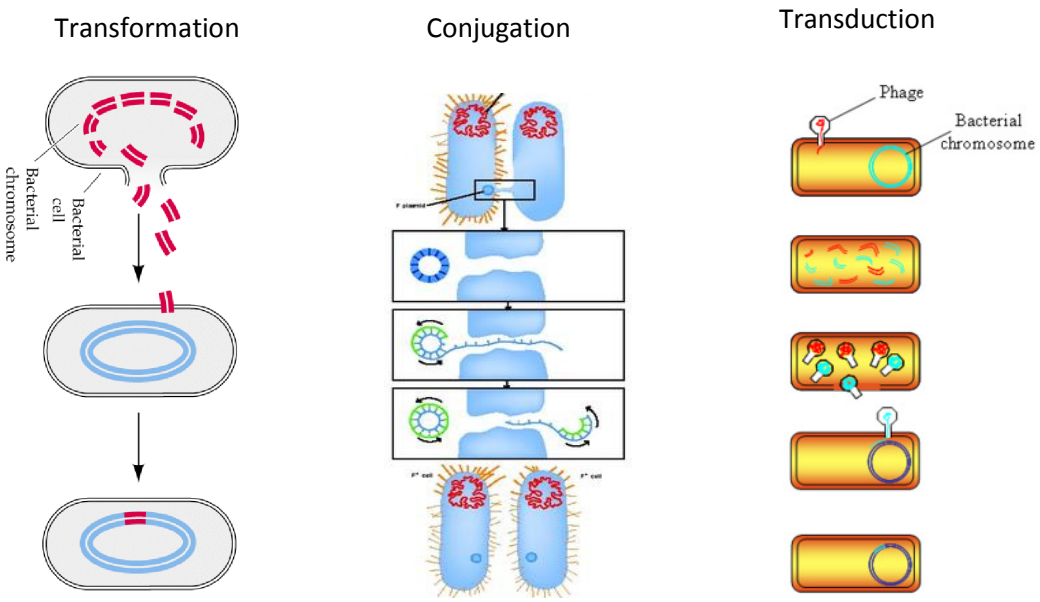


Figure 6 – Schematic representation of the processes that contribute to acquisition of resistance genes: conjugation, transformation and transduction (http://images.google.com).

All these processes of the acquisition and dissemination of resistance genes allow that any opportunistic or susceptible bacteria could become MDR and potentially pathogenic when presenting virulence factors (Clewell 2005; Tenover 2006; Alekshun and Levy 2007; Bennett 2008). Moreover, these mechanisms contribute to the rapid dissemination of resistance determinants among several bacterial genera (Harbottle, Thakur et al. 2006), through MGEs (Bennett 2008; Tetu and Holmes 2008; Mulvey and Simor 2009).

3. MOBILE GENETIC ELEMENTS ASSOCIATED WITH ANTIBIOTIC RESISTANCE

As referred above, the bacterial respond to antibiotics, antiseptics, disinfectants and heavy metals used in clinical medicine depends of factors such as mutations in physiological genes and selective pressure, but also of movement of different resistance genes by HGT and VGT. These are the predominant factors for the escalation of antibiotic resistance in more than half a century (Toleman, Bennett et al. 2006).

The majority of resistance genes are associated to MGEs such as plasmids, transposons, insertion sequences (IS), integrons and bacteriophages (Bennett 2008; Tetu and Holmes 2008; Mulvey and Simor 2009). These structures catalyze the DNA movement and play an important role in the dissemination of antibacterial resistance genes. Therefore, they contribute to bacterial genetics evolution, since they allow the rapid bacterial adaptation to new classes of antibiotics in the environment (Tenover 2006), increasing the rate of MDR and potential pathogenic strains (Tetu and Holmes 2008). Consequently, it is of extreme importance the understanding of MGEs genomics, in order to take advantage and avoid the dangers of these ‘natural genetic engineers’ (Frost, Leplae et al. 2005).

3.1 Plasmids

The plasmids are described as DNA extra-chromosomal molecules, since replication happens independently of replication of the bacterial chromosome (Mc Dermott, Walker et al. 2003; Summers 2006), and can be distinguished by their origins of replication (Alekshun and Levy 2007; Bennett 2008). These structures are not essential for the survival of the bacterial cell (Mc Dermott, Walker et al. 2003), but when present carry genes that confer a selective and adaptive advantage for bacterial cell, namely resistance to antibiotics (Harbottle, Thakur et al. 2006; Mulvey and Simor 2009).

The classical plasmids are circular double-stranded DNA molecules, smaller than the cellular chromosome (the size is variable and depend of the plasmid type), and usually does not accommodate any of the set of core genes needed by the cell for basic growth and multiplication (Clewell 2005; Frost, Leplae et al. 2005; Summers 2006; Bennett 2008).

The plasmids may serve as vehicles for transposons and integron (Mc Dermott, Walker et al. 2003; Summers 2006; Bennett 2008), which are transferred within the same specie or between different bacterial species (B. Henriques Normark 2002; Alekshun and Levy 2007). This movement is random and independent of the use of antibiotics. However these compounds supply a powerful selective pressure that contribute to the MDR, which have increased in both pathogens and opportunists Gram-positive and Gram-negative bacteria (Levy and Marshall 2004; Harbottle, Thakur et al. 2006).

Resistance to almost all classes of clinically important antibiotics can be mediated by R plasmids (initially R factors) (Bennett 2008), which were first discovered in penicillin-resistant bacteria in the 1950s. Given their nature, R-plasmids are mobile and carry one or more resistance genes (Li 2005), such as for toxic heavy metals, certain antiseptic compounds and disinfectants among different Gram-negative (Bennett 2008) (Figure 7).

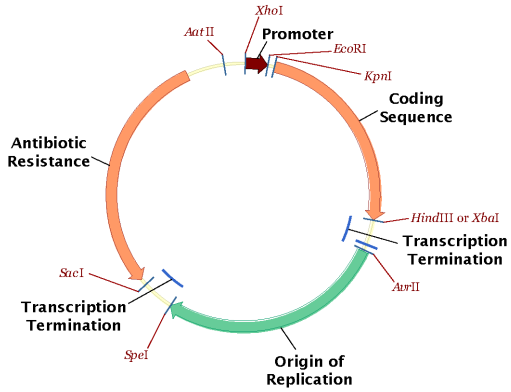


Figure 7 – Schematic representation of a resistance plasmid, with three main regions: origin of replication, antibiotic resistance genes and promoter associated to a coding sequence (http://images.google.com).

Conjugative plasmids can exhibit broad or narrow host range. Some broad host range plasmids from Gram-negative bacteria appear to have no host limitation within the division and, 'in vitro' experiments have shown that they are able to transfer, but not survive in Gram-positive bacteria. One example of a broad host range plasmid is the resistance plasmid RP1 (also known as RP4 and RK4), first identified in a clinical strain of *P. aeruginosa* that is able to transfer productively to most, if not all, Gram-negative bacteria (Bennett 2008).

Plasmids must replicate, control their copy number, and ensure their inheritance at each cell-division. Plasmids of different modes of replication are able to reside in the same bacterium, while two different plasmids with the same replication mechanism are mutually incompatible and are unable to coexist in the same bacterium for prolonged periods (Harbottle, Thakur et al. 2006). This phenomenon is termed by 'incompatibility' (Inc) (Frost, Leplae et al. 2005). They are categorized into Inc groups according to their mode of replication and maintenance in a bacteria cell (Clewell 2005). Examples of Inc groups have been defined for plasmids of the Enterobacteriaceae, *Pseudomonas* spp. and some Gram-positive isolates (Frost, Leplae et al. 2005).

Other plasmid types lack the genes that encode the functions that allow the transfer of DNA, but may still be mobilized by conjugative elements by the formation of transient or stable fusions, called co-integrates, that occur as part of a homologous or specifically directed recombination process (Bennett 2008).

3.2 Transposons

Transposons belong to the set of transposable elements and they are characterized as MGEs of DNA, since they mediate the movement of the DNA due to their ability to "jump" and to recombine randomly between replicons (Harbottle, Thakur et al. 2006), and therefore they are important motors of genetic variability (Frost, Leplae et al. 2005). They can "jump" from one site to another within a DNA molecule – intracellular movement, or from one DNA molecule to another – intercellular movement (Mc Dermott, Walker et al. 2003; Harbottle, Thakur et al. 2006). Due to their location in phages or more typically in plasmids, these structures carry resistance genes to different classes of antibiotics, allowing their dissemination (Summers 2006; Senka Dzidic 2008).

In general, these MGEs are made up of insertion sequences (IS) of DNA and encode a site-specific enzyme responsible for the transposition, called transposase, that participate in recombination and facilitates the incorporation into specific genomic regions (Harbottle, Thakur et al. 2006; Alekshun and Levy 2007). The IS are usually flanked by inverted repeated sequences corresponding roughly to 25 to 50 nucleotides, which are recognized by the transposase (Summers 2006) (Figure 8). Target sites for IS often have little specificity and therefore insertion and recombination can occur almost anywhere in genome, where genetic mutations are commonly generated (Bennett 2008).

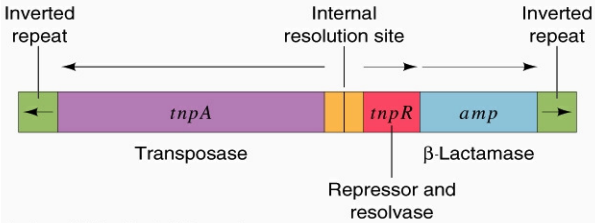


Figure 8 – General structure of a transposon (http://images.google.com).

Bacteria contain a large number of transposable elements that can be categorized in groups according to their mechanisms of transposition (B. Henriques Normark 2002; Clewell 2005; Summers 2006). Thus, the transposons can be mainly grouped in IS elements, complex and conjugative transposons (Harbottle, Thakur et al. 2006; Alekshun and Levy 2007; Bennett 2008; Tetu and Holmes 2008).

3.3 Integrons

Integrons were discovered in 1989, and initially were defined as DNA elements, potentially mobile and located in independents sites (H. W. Stokes 1989). But nowadays, these genetic systems are defined as DNA elements that cannot move by themselves, but have the ability to capture genes, that confer antibiotic resistance, being designated as natural reservoirs (Bennett 2008; Senka Dzidic 2008; Mulvey and Simor 2009).

The feature that defines these integrons is a site-specific recombination system (instead of transposition), capable of integrate and express open reading frames (ORFs) and convert them to functional genes (Rowe-Magnus and Mazel 1999). These ORFs are contained in modular structures called mobile genes cassette and have contributed greatly to the mobilization, rearrangement and dissemination of antibiotic resistance among bacteria (Harbottle, Thakur et al. 2006; Labbate, Case et al. 2009).

These gene capture systems act as natural expression vectors for any gene cassette, and are located on the bacterial chromosome or on plasmids, frequently associated to transposons. So, as they are intimately associated to antibiotic resistance genes and to MGEs, the dissemination of these "genetic tool box" is facilitated (Summers 2006; Senka Dzidic 2008). For this reason, they also contribute greatly to the problem of the emergence of MDR bacteria and nosocomial infections, due to HGT that have become generally widespread as a result of human activities (Labbate, Chowdhury et al. 2008; Sally, Guy et al. 2009).

Although it was once believed that integrons were mainly limited to Gram-negative bacteria, recently they have been found in many other closely and distantly related bacteria such as Salmonella, Enterococci and Staphylococci, not only associated with the hospital environment but also with the food (Summers 2006). In fact, their diversity is greater in environmental isolates than in clinical isolates, suggesting that integrons are ancestor structures in genomes of bacteria from the environment (Labbate, Case et al. 2009).

3.3.1 Integron's structure

As referred previously, integrons have a specific and distinctive structure, making them unique (Figure 9). These DNA elements are usually characterized by three regions: two conserved segments (CS) flanking a central region, designated as variable region (VR), in which "genes cassette" can be inserted (Mc Dermott, Walker et al. 2003; Harbottle, Thakur et al. 2006). The VR, as the name indicates, presents a sequence and a length variable. This variability is due to the constitution of this region, depending of the number and identities of antibiotic resistance genes (Bennett 2008). The VR constitution is only possible due to the presence of a specialized recombination system consisting of a gene, *intI*, which encodes a site-specific recombination enzyme called an integrase. This gene is located in the integron's 5'-CS (Bennett 2008).

Each part of the integron's genetic structure will be discussed in the following sections.

Figure 9 – Schematic representation of the general integron's structure.

3.3.1.1 5'-Conserved regions (5'-CS)

The 5'-CS consists of a site-specific integrase enzyme (*intI* gene) oriented opposite to the direction of its associated cassettes, followed by the attachment site *attI,* in which DNA sequences are inserted. The presence of a promoter (*Pc*) has not been functionally demonstrated for all integron classes (Labbate, Case et al. 2009), but when present permits the expression and transcription of the genes cassette integrated in the VR (Summers 2006).

IntI belongs to super-family of site-specific recombinases, known as tyrosine recombinases or integrases (Clewell 2005; Senka Dzidic 2008). This super-family presents a number of short regions of similar and conserved residues that are part of the catalytic site, and these features are all located in a large C-terminal domain of a 180 amino acids. These conserved residues are not found in other tyrosine recombinases, and therefore these enzymes can be identified on basis of this motif. Any deletion or substitution in this region can abolishes the integrase activity (Christina M. Collis 2002). Thus, IntI enzyme facilitates the capture, integration and excision of gene cassettes in VR.

Recombination site *attI* site is located adjacent to the *intI* gene in the integron. The *attI* site presents a simpler structure and conserved than *attC* (Karin, Ola et al. 1997; Sally, Guy et al. 2009). The general structure is GTTRRRY/RYYYAAC (R = Purina and Y = pirimide) (Recchia, Stokes et al. 1994).

The promoter *Pc* (formerly P_{ant}) does not make part of the site-specific recombination system, but it permits the expression and transcription of the genes cassette inserted in the VR, and is located within the coding sequence of *intI1* (Labbate, Case et al. 2009). In some integrons, a second promoter (*P2*) complement the *Pc* and is located 119 bp downstream from *Pc*, and is formed by an insertion of three G bases optimizing the spacing (17 bp) between the -35 and -10 sites (*P2* active) (Costas C. Papagiannitsis 2009; Sally, Guy et al. 2009). Several *Pc* variants have been identified ("weak", "strong", "hybrid 1" and "hybrid 2") due to differences in the -35 and/or -10 sequences, and different combinations have been characterized in some integrons (Costas C. Papagiannitsis 2009).

The integration process has been shown experimentally for the class 1 and class 3 integrons, where the cassettes insertion reaction preferentially occurs by IntI-catalysed homologous recombination between two architecturally distinct sites, *attI* and *attC* sites (Figure 10). Insertion occurs from the 5' end to the 3'end, and cassettes initially in a free and inactive circular form, when inserted in VR acquire a linear and active form. In the case of excision of cassettes, this process occurs also via homologous recombination between *attI* and *attC* sites or between two *attC* sites, and cassettes again acquired a circular form (Bennett 2008; Labbate, Case et al. 2009).

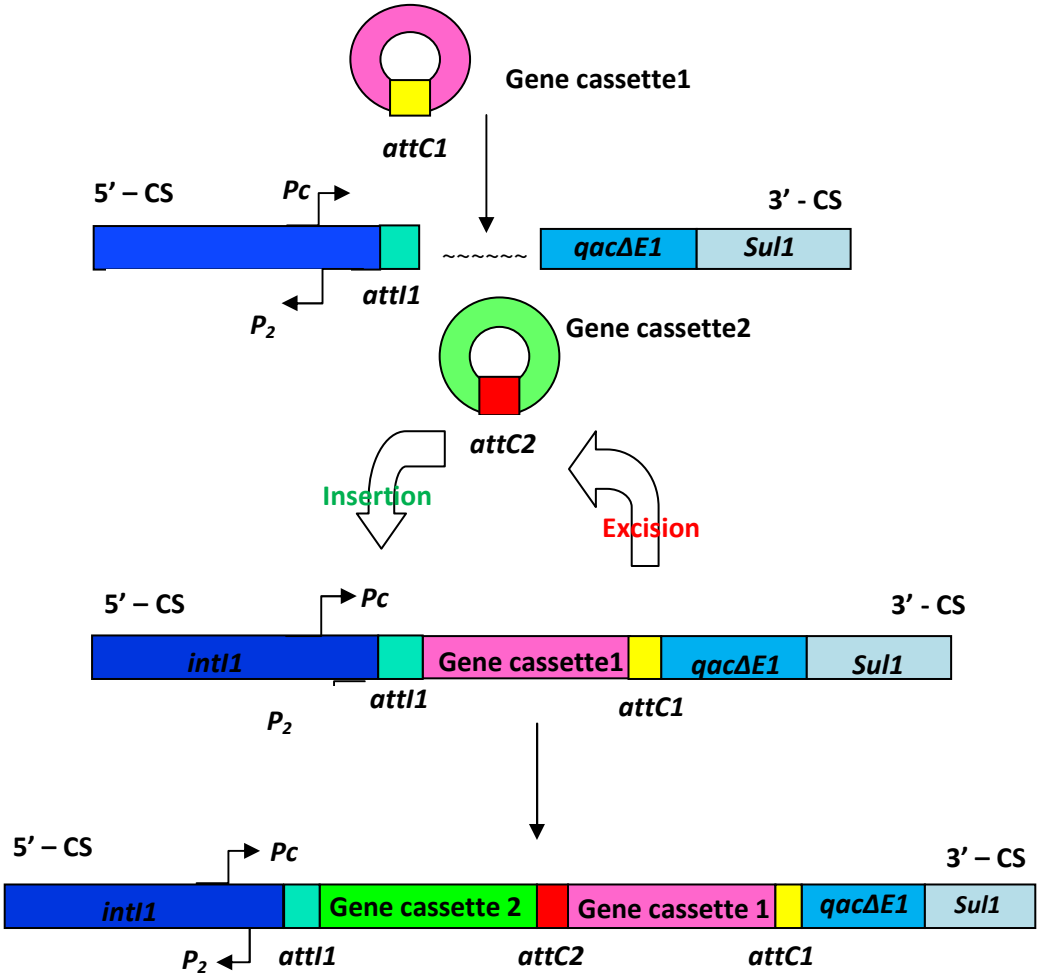


Figure 10 – Schematic representation of the recombination and integration of the genes cassette in the VR, catalyzed by integrase enzyme.

Recognition and integration processes of cassettes are mediated by IntI enzyme that use a tyrosine residue as the nucleophile to catalyze strand exchange reactions in an ordered fashion and recombine with the core sites of the *attC* (Christina M. Collis 2002). Both the core sites of the *attC* are directly oriented to IntI binding sites and are essential for efficient activity of *attI* when an *attC* site is present (Sally, Guy et al. 2009). The current model for IntI1-catalysed site-specific recombination, described by Mazel and their colleagues (Mazel 2006), involves the bottom strand of the *attC* site only folded into a bulged hairpin structure. The folded *attC* recombines with a double-stranded *attI* or

another *attC* site and a subsequent replication step is required to resolve the Holliday junction intermediate (Figure 11). The analysis of transcripts originating from *Pc* suggests that the stem-loop structures formed by *attC* sites might be acting as transcription terminators (Sally, Guy et al. 2009).

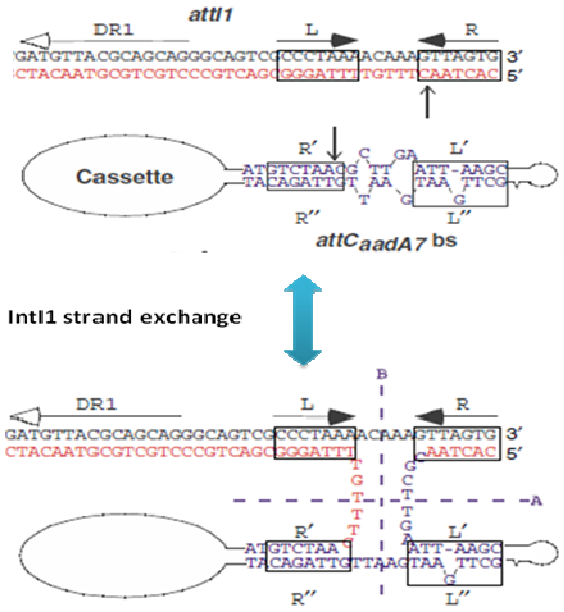


Figure 11 – Model of the recombination molecular mechanism using an *attC* and an *attI* site (Bouvier, Demarre et al. 2005).

Genes cassette can be deleted and newly inserted in another order, and therefore a promoter-distal cassette can become a promoter-proximal cassette and a new resistance phenotype can appear (Summers 2006).

The expression and transcription of genes cassette inserted in the VR may terminate before all cassettes transcribed. This processes depend not only on the strength of promoter but is also influenced by the position of the cassettes in the array in relation to promoter (Toleman, Bennett et al. 2006), and by the sequence of the respective *attC* (Rowe-Magnus and Mazel 2002; Labbate, Case et al. 2009). These factors explain different bacterial resistance phenotypes (Costas C. Papagiannitsis 2009).

3.3.1.2 Variable region (VR)

The discrete units that compose the VR are known as mobile genes cassette and are located downstream of the resident promoter (Rowe-Magnus and Mazel 1999). The high level of unknown proteins encoded by the genes cassette and their origin has made it difficult to understand the adaptive roles of the cassettes in bacterial evolution. Nevertheless, some cassettes have been shown to include ORFs encoding specific advantageous functions (Labbate, Case et al. 2009).

Genes cassette are defined as the smallest mobile genetic entities that are independently mobilizable and can carry resistance determinants, but are not expressed on their own due to the lack of the promoter region (Senka Dzidic 2008). They are composed by a single gene and an IntI-recognizable recombination site called 59-base element (59-be) or *attC* site located downstream of the region of the gene cassette (Toleman, Bennett et al. 2006; Sally, Guy et al. 2009).

The analysis of the gene sequences allows grouping the cassettes according to their role and identity. Nowadays there are approximately 132 different cassettes carrying known antibiotic resistance genes (or homologous assumed to confer similar resistance phenotypes) and 62 different ORFs of unknown function, recently designated by *gcu* (Sally, Guy et al. 2009). The nomenclature of the new cassettes is not formally regulated, and several authors gave the same name to two different cassettes, or to the same gene different names have been given, using the roman and arabic numerals, as for example *dhfrVII* and *dfr7*, for a dihydrofolate reductase (Sally, Guy et al. 2009). Other problems in the nomenclature of the cassettes are the emergence of the 'hybrids' formed by homologous recombination between two closely related cassettes (89% identical) (Sally, Guy et al. 2009), or the fusion of cassettes due to deletions in the ends of two adjacent cassettes (Recchia and Hall 1995). These changes can be associated with the reduction of the resistance level (Levings, Lightfoot et al. 2006). Examples of hybrid cassettes appear in bla_{vim-12} gene (Pournaras, Ikonomidis et al. 2005), which was automatically annotated as a partial bla_{vim-1} followed by a partial bla_{vim-2} cassettes (Sally, Guy et al. 2009), and *aadA1*

and *aadA2* hybrid genes and several variants of these cassettes are known (Partridge, Collis et al. 2002; Gestal, Stokes et al. 2005).

Several minor variants have been identified in some cassettes, and may have implications in the movement and expression of cassettes, dramatically changing the resistance phenotype. These resistance genes can be useful as epidemiological markers (Sally, Guy et al. 2009).

Formerly, the recombination site of genes cassette was designed by *59-be*, due to the length of sequence, but in fact, very few elements have this size, although the term continues to be found in the literature. In recent years, the term *attC* has been introduced and became favored, because it is more consistent with the terminology used to describe site-specific recombination sites in general (Labbate, Case et al. 2009).

attC sites are highly conserved in some species, but varies in length and sequence depending of the gene cassette (from 55 to 141 bp), and closely related cassettes may be associated with similar or different *attC* sites, suggesting divergence from a common ancestral cassette or different origins, respectively (Poirel, Naas et al. 2000; He Yan 2007; Sally, Guy et al. 2009). These sites are composed by simple sites (also called core sites) that have approximately 20 bp and are associated to regions, called imperfect inverted repeat sequences (Clewell 2005; Labbate, Case et al. 2009). These regions referred as L'/L'' and R''/R' are separated by a spacer of 6–8 bp (also called central region) that varies in length and sequence between *attC* sites. Analysis of some *attC* site sequences in 1997 (H. W. Stokes 1997) suggested the consensus sequence GTTAGSC/GYTCTAAC for R'/L', where GTT nucleotides (completely conserved residues of the R') are the crossover point with IntI. The recombination happens between the G and first T of R' (Recchia, Stokes et al. 1994) (Figure 12). L''/R'' are quite variable and difficult to identify conclusively for some *attC* sites (Sally, Guy et al. 2009).

All these sites are important for *attC* sites activity, but sometimes deletions or changes occur that interfere with the integration and expression of the genes cassette.

Figure 12 – Integron recombination sites. **a.** The sequence of the double-stranded *attC* site. **b.** The proposed secondary structure for the *attC* bottom strand. The asterisk shows the position of the protruding G base present in L'' relative to L'. The putative IntI1-binding domains are marked with boxes (Mazel 2006).

3.3.1.3 3' Conserved region (3'-CS)

The 3'-CS, consists of a truncated copy of *qacE* gene, which when intact confers resistance to quaternary ammonium compounds, followed by a *sul1* gene that confers resistance to sulfonamides (He Yan 2007; Labbate, Case et al. 2009). Initially, downstream of *sul1,* an *orf513* gene of unknown function was present (Bennett 2008), but nowadays, this gene is referred as *ISCR*1 (Toleman, Bennett et al. 2006; Labbate, Chowdhury et al. 2008).

The majority of the cassettes arrays are flanked by 5'-CS and 3'-CS (approximately 300 different cassette arrays are described in the GenBank), and are composed of a few genes cassette. This type of arrays is present mainly in *E. coli*, *P. aeruginosa*, *A. baumanii* and *K. pneumoniae*, and recently has also been reported in some Gram-positive bacteria (Sally, Guy et al. 2009).

In other cases, the 3'-CS is absent, and the cassette arrays can be flanked by a 5'-CS and a complete *tni* region downstream of the inserted cassettes that is present in Tn402 (also called Tn5090), found in plasmid R751 and pTB11 (Labbate, Case et al. 2009; Sally, Guy et al. 2009). The outer boundary of the 5'-CS is defined by a 25-bp inverted repeat

sequence called IRi and IRt, and when present is found beyond the 3'-CS. The IRi/IRt inverted repeats are a feature of transposon and define their limits, and are required for transposition using a suite of four transposition genes designed by *tni* module (*tniR, -Q, -B, -A*) (figure 13) (Labbate, Case et al. 2009).

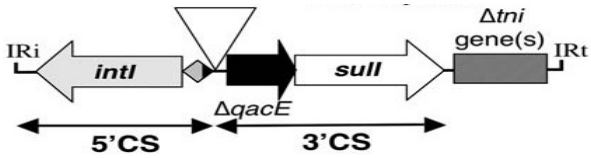


Figure 13 – Evolution of the class 1 integrons. The origin of class 1 integrons is believed to have come from a chromosomal location. A class 1 integrons eventually became associated with *tni* functions represented by the Tn402 structure. Partial deletion of the *qacE* cassette and insertion of *sul1* resulted in formation of the 3'-CS commonly found in clinical class 1 integrons (Labbate, Case et al. 2009).

Recent publications suggest that the Tn*402*-like transposon is the ancestral mobile form of the clinical class 1 integron, and that deletions, insertions, and recombination events resulted in the genesis of the 3'-CS and the partial loss of *tni* functions. This is supported by the presence of a *qacE* gene cassette in Tn*402* that is 100% identical to the equivalent region present in the truncated *qacE* gene of the 3'-CS, suggesting a partial deletion event had occurred possibly as a result of the *sul1* insertion (Labbate, Case et al. 2009). Class 1 integrons with *tni* in place of the 3'-CS are less frequent, but structures with the 3'-CS are likely to be at a selective advantage due to the presence of *sul1* gene (Sally, Guy et al. 2009).

Another type of structure has emerged, where cassette arrays are flanked by the usual 5'-CS, but lack the 3'-CS and/or *tni* region. Recently, a region containing the *sul3* sulfonamide resistance gene has been found in *E. coli* and *Salmonella* sequences deposited in the GenBank (Sally, Guy et al. 2009). Moreover, the analysis of other sequences that contain a cassette array but lack the 3'-CS revealed several other possible explanations involving various IS associated with resistance plasmids, such as IS6100 and IS1353 (Partridge, Brown et al. 2001).

3.3.2 Integrons classification

Comparative analysis of the amino acid sequences of integrase enzymes have been used as a basis for dividing integrons into "classes", with those carrying *intI1* defined as "class 1", *intI2* as "class 2", *intI3* as "class 3" and so on (Mazel 2006; Sally, Guy et al. 2009). However, other studies made at level of insertion and excision reactions, showed that *attI* and *attC* sites differ between classes, but yet there is no reason to assume that the two reactions are equivalent in relation to recombination efficiency (Labbate, Case et al. 2009).

To date, different cassette arrays of integrons are divided into nine classes (He Yan 2007), which can be divided in two main types: resistance integrons (RI), which are mainly present in the clinical environment and can be divided in three classes (class 1, 2 and 3 integrons), and 'super-integrons' (SI), which are present mainly in the community associated to class 4 integrons (Tetu and Holmes 2008; Labbate, Case et al. 2009). RIs contains few genes cassette (Sally, Guy et al. 2009), while SIs have a complex structure typically with long arrays (can incorporate one hundred genes cassette), encoding a multiplicity of functions (Mazel, Dychinco et al. 1998; Harbottle, Thakur et al. 2006).

Although the phylogenetic origin of these classes of integrons continue unknown, there is a close relationship between RIs with SIs from environmental microorganisms that has implications in the origin and emergence of the systems in clinical environment. An example of this is some *attC* sites found in cassettes carried by RI that are strongly related to those in SIs (Sally, Guy et al. 2009).

In resume, the integrons are ubiquitous and play an important evolutionary role in the emergence of MDR between different species (Clewell 2005).

3.3.2.1 Resistance integrons

The term RI refer to integrons that carry mainly antibiotic resistance genes conferring a resistance phenotype, and that are associated with mobile or potentially mobile elements (transposons, plasmids and IS).

RI generally contains few cassettes (usually one to six cassettes) but the associated *attC* sites may be quite varied (Sally, Guy et al. 2009). The degree of homology between RI classes (45-58%) suggests that their evolutionary divergence has extended over a longer period for more than the 50 years of the antibiotic era (Rowe-Magnus and Mazel 2002). In a comparative study with *V. cholerae* and class 1 integrases, it was possible to verify that the *intI1* gene recognizes a larger spectrum of *attC* sites and consequently is able to access the gene cassette metagenome. This feature show the mobilization of integrons of these classes in *Vibrio* spp. strains, and an important selective advantage (Labbate, Case et al. 2009).

i. Class 1 integrons

Class 1 integrons are genetic elements that possess a specific recombination site, *attI1*, into which many combinations of resistance genes can be inserted by site-specific recombination (Toleman, Bennett et al. 2006), as previously described in 3.3.1.1 section. They are also the most clinically important and broadly distributed integrons in a large variety of Gram-negative clinical isolates (22–59 %), but in recent times have been reports in some Gram-positive bacteria as *Mycobacterium* spp. (A. C. Fluit 2004; Harbottle, Thakur et al. 2006; Labbate, Case et al. 2009). Thus, this class can be the biggest contributor to the global resistance problem.

The ancestor of mobile class 1 integrons may have been generated by acquisition of *intI1* and *attI1* by a transposon of the Tn5053 family to give a structure related to Tn402, as previously described in 3.3.1.3 section. Therefore may present a new evolutionary advancement in the class 1 integron, and new clinical concerns (Harbottle, Thakur et al. 2006). This class is usually associated with many different plasmids and transposons, such as Tn*402*-like transposons and Tn*3* family (e.g. Tn*21* and Tn*1696*) (Labbate, Case et al. 2009). The IntI1 enzyme is related with IntI3 enzyme (59%) (Rowe-Magnus and Mazel 1999; Mazel 2006).

ii. Class 2 integrons

The *intI2* gene was first described as part of the approximately 14 Kb transposon Tn7, which includes the *tns* transposition region and is bounded by short segments, containing transposase-binding sites, called Tn7-L (approximately 150 bp) and Tn-R (approximately 90 bp), which are necessary for transposition (Labbate, Case et al. 2009).

Mobility of class 2 integrons is due to usually to association with Tn*7*, a transposon that contains five transposition genes (*tnsA, -B, -C, -D*, and -*E*), and preferentially inserts at high frequency in a unique specific site within of the bacterial chromosomes, or also

able to transpose to other sites, such as conjugative plasmids, but with lower frequency (Hansson, Sundstrom et al. 2002; Labbate, Case et al. 2009).

Examples of the *intI2* gene described to date contain an internal stop codon at amino acid 179 that renders IntI2 inactive, but natural suppression of this codon or the action of other IntI in trans may allow occasional acquisition of new cassettes by capture or recombination. This mutation has been attributed to the low diversity of integrated genes cassette observed within class 2 integrons (Labbate, Case et al. 2009).

Cassette boundaries in class 2 integrons can be identified, TAATAAAATG is found adjacent to the start of the first cassette and TTAGAG defines the start of the *ybeA* cassette usually found at the end of arrays. The most commonly identified array flanked by *intI2* and *ybeA* was |*dfrA1*|*sat2*|*aadA1a*|, as seen in Tn7 itself, followed by others arrays (Sally, Guy et al. 2009).

This class of integron is low frequent in clinical environment (Labbate, Case et al. 2009), and has been commonly found in *Acinetobacter, E. coli*, *Shigella* and *Salmonella* (A. C. Fluit 2004).

3.4 Insertion sequences common regions (*ISCR*s)

Recently a recombination system that contributes to the assembly of banks of resistance genes, mainly on bacterial plasmids, has been described. However, some studies show that they also appear associated with the chromosome (Bennett 2008). These MGEs were discovered in 1990´s and are linked to sequences termed “common regions” (CRs), because the same sequence was commonly found in it. A comparative analysis of these CRs has revealed that they are related to each other and resemble a family of unusual ISs, designated IS*91*-like (Toleman, Bennett et al. 2006; Bennett 2008).

*ISCR*s are small cryptic sequences of sizes similar to those of many IS elements, which lack the typical terminal inverted repeats of most IS elements, and therefore use the common 3'-CS sequence as point of crossover (L. Poirel 2008). Therefore the mobilization of any gene from any location is different and designed by rolling-circle (RC) transposition (Toleman, Bennett et al. 2006), which permits genetic rearrangements by homologous recombination into another class 1 integron (Harbottle, Thakur et al. 2006). Rather they possess distinct terminal sequences designated oriIS (origin of replication) and terIS (replication terminator) of the RC replication stage, which are relevant to the spread of antibiotic resistance genes (Bennett 2008).

The first CR element (now *ISCR*1) was discovered downstream of the *sul1* gene in 3'-CS of the class 1 integron. Initially these integrons presented the *orf513* gene, but recently this region gives rise to the *ISCR*1. Downstream of the *ISCR*1, there is another VR associated to the resistance genes, mainly all classes of β-lactamases, fluoroquinolones, trimethoprim, aminoglycosides and chloramphenicol, followed by a duplication of the 3'-CS (*qacEΔ1* and *Sul1* genes). This structure is classified as complex class 1 integron (Toleman, Bennett et al. 2006; Bennett 2008; Labbate, Chowdhury et al. 2008).

The particularly striking aspect of these CR-associated resistance genes is the fact that are not associated to *attC* sites, and therefore cannot have been acquired as genes cassette, but have the ability to create free circular species that may be transposition intermediates (Toleman, Bennett et al. 2006; Toleman, Bennett et al. 2006; Bennett 2008).

Since the first CR sequence was identified, more than a dozen related elements have been discovered worldwide, on plasmids and bacterial chromosomes in both Gram-negative and Gram-positive bacteria of clinical importance. So, *ISCR*2 is linked to genes encoding resistance to trimethoprim, tetracycline, cloramphenicol and sulfonamides; *ISCR*3 is linked to *qac*, *dfrA10*, *ereB* and *yie*; *ISCR*4 to *blaSPM-1*; and *ISCR*5 to both *blaOXA-45* and *ant4'IIb* (Toleman, Bennett et al. 2006; Toleman, Bennett et al. 2006).

The most worrying aspect is that *ISCR* elements are increasingly responsible for mobilization and dissemination of resistance genes and are implicated in the construction of the MDR strains being a potential clinical problem that, with time, can get worse (Bennett 2008).

4. MOLECULAR TECHNIQUES EMPLOYED IN THE EPIDEMIOLOGY STUDIES

Currently, almost all Gram-negative bacteria are classified as MDR, because they present resistance to more than three antibiotic classes. Over time, the antibiotics most frequently used were penicillins, cephalosporins, aminoglycosides and, more recently, carbapenems and quinolones (Mulvey and Simor 2009).

Commensal and opportunistic bacteria can contribute to acquisition of nosocomial infections. This type of infections is becoming common since they are acquired in the hospital environment, and their treatment is compromised due to emergency of MDR bacteria. Comparing different studies from around the world, the existence of clonal outbreaks of a common “epidemic” strain can be observed, since the resistance genes and similar resistance profiles have been observed within the same species in different geographic regions (MacGowan and on behalf of the 2008). The spreading of resistance determinants is not only due to their mobilization by MGEs, but also by vehicles of dissemination, such as hands, surgical materials, surfaces of inanimate hospital environment and transfer of patients between hospitals (Levy and Marshall 2004; Tenover 2006; Mulvey and Simor 2009).

To reduce the level of nosocomial infections and also resistance, mainly in the hospital environment, genetics and functional genomics tools are now being implemented in order to give a quicker response and allow a better choice of appropriate treatment to the patient (Sipahi 2008). Knowledge in detail of the sites of action of the antibiotics and the mechanisms of resistance exhibited by the bacteria cell, the

dissemination of resistance genes by HGT and utilization of synergistic of antibiotics is necessary (Sousa 2006; Bennett 2008; Senka Dzidic 2008). With this information new programs of effective infection control, prevention and epidemiological surveillance can be implemented. Consequently, a decrease of the mortality and morbidity rates will be observed (Zwar, Wolk et al. 1999; Levy and Marshall 2004; Senka Dzidic 2008; Mulvey and Simor 2009).

Studies have been carried out and discussed about the regional and global epidemiology and epidemiological relationships between bacteria. To investigate this problem, the use of a proper methodology is essential for comparing isolates which are temporal and geographically distant and handled by different laboratories. Molecular techniques as polymerase chain reaction (PCR), macrorestriction profiling of genomic DNA by pulsed-field gel electrophoresis (PFGE), random amplification of polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP) and multilocus sequence typing (MLST) allow to identify the bacteria and to study their resistance profile, permitting a bacterial phylogenetic classification (Figure 14), and the characterization and location of the integrons structure (Giske, Libisch et al. 2006). Thus, these molecular techniques are important in the study of the bacterial infections, since they can be helpful on the identification of outbreaks.

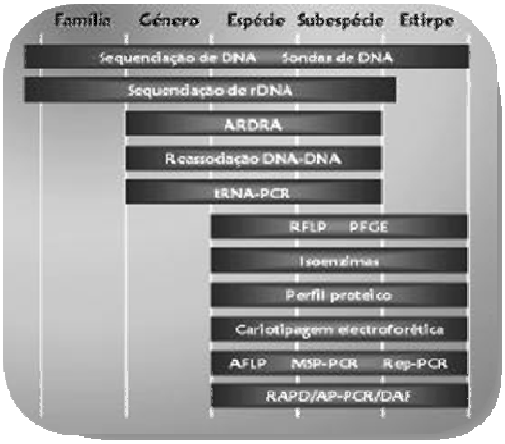


Figure 14 – Relative resolution of various fingerprinting and DNA techniques (BRUIJN 1997).

PCR technique is very sensitive and can detect target sequences that are in extremely low copy number in a sample. The exponential amplification from a single copy gene is quick and possible through the use of a temperature-resistant DNA Taq polymerase, and DNA primers corresponding to known sequences of the gene to be synthesized. The annealing temperature and extension period is important to the pairing of the primers and amplification of respectively gene.

PCR is generally useful in DNA diagnostics to check for the presence of a gene or the mutational state of a specific gene, to detect hereditary diseases and to study the resistance profile of the microorganisms. Moreover, provide information about the evolution of the microorganisms and prevalence of resistance genes and mechanisms of resistance (Jonas, Spitzmüller et al. 2003).

rep-PCR is a typing technique that permits to amplify highly conserved, repetitive DNA sequences, present in multiple copies in the genomes of majority of the Gram-negative isolates. Three families of repetitive sequences have been identified, including the 35-40 bp repetitive extragenic palindromic (REP) sequence, the 124-127 bp enterobacterial repetitive intergenic consensus (ERIC) sequence, and the 154 bp BOX element. REP-, ERIC- and BOX-PCR genomic fingerprints generated from bacterial isolates permit differentiation to the species until strain level, as schematized in Figure 14 (BRUIJN 1997).

These techniques permit to obtain a distinct banding pattern that help to know and study the phylogenetic relationship between different bacterial species. Using computer programs it is possible to construct the phylogenetic tree determining different clonal groups into the specie (Tenover, Arbeit et al. 1995).

Electrophoresis permits to separate shorter DNA fragments or proteins according to molecular size, on gels under the influence of strong electric field. PFGE is more reliable and conclusive, and therefore is used to separate the larger DNA molecules, since is considered to have both the reproducibility and resolving power of a standard technique for the epidemiological typing of bacterial isolates. The DNA fragments are viewed under U. V. light, after ethidium bromide (EtBr) staining (Tenover, Arbeit et al. 1995).

4.1 Clinic relevant pathogenic bacteria

Treatment of nosocomial infections is worldwide compromised due to emergency of MDR bacteria. Thus, molecular and typing techniques are important to study bacteria causing infections, since they are very helpful on the identification important outbreaks.

The more relevant and more problematic Gram-negative MDR bacteria are, for example, *E. coli*, *K. pneumoniae*, *P. aeruginosa* and *A. baumanii*, which will be discussed in more detail in the following sections (Alekshun and Levy 2007; Sipahi 2008).

i. *E. coli*

E. coli is a commensal of the human intestine, but can cause a variety of important infections, including those of the gastrointestinal, urinary, biliary, and lower respiratory tract septicemia, neonatal meningis, etc. These strains are producers of β-lactamases mainly ESBL types (TEM, SHV, OXA and CTX-M), and are therefore highly resistance to β-lactams antibiotics. Recently, *E. coli* isolates are becoming resistant to fluoroquinolones as result of the mutations in the topoisomerases that increase the expression of membrane proteins that extrudes the antibiotic (Levy and Marshall 2004).

Class 1 integrons are frequent in these strains, and the VR is characterized mainly by the presence of genes that confer resistance to aminoglycosides and trimethoprim. Since these bacteria are commonly resistant to penicillin and ampicillin, the antibiotics often used to treat *E. coli* infections include the cephalosporins, trimethoprim, ciprofloxacin, and aminoglycosides (Martin 2009).

ii. *Klebsiella pneumoniae*

K. pneumoniae strains are widespread in the environment and in the intestine flora of humans and other mammals. Infections are often opportunistic and associated with hospitalization, and include pneumonia, urinary tract and wound infections and neonatal

meningis. These strains often produce ESBLs enzymes, mainly TEM, CTX-M and more recently KPC (Thomson and Bonomo 2005).

The presence of class 1 integrons that transport cassettes conferring resistance mainly to aminoglycosides, trimethoprim and quinolones and more recently, a second VR containing *ISCR*1 has been identified, that contains also resistance genes (Bennett 2008).

This specie often appears associated with plasmids, and therefore contributes quickly to the dissemination of genes cassette.

Cephalosporins (cefotaxime and ceftriaxone) and aminoglycosides (gentamicin and amikacin) are commonly used to treat Klebsiellae infections, however multiple MDR strains may limit antibiotic choice (Martin 2009).

iii. ***Pseudomonas* spp.**

Pseudomonas spp. is one of the groups with the higher number of MDR bacteria species. These bacteria are normal commensal in the human gastrointestinal tract, but may colonize other sites when host defenses are compromised, including burns and leg ulcers, the respiratory tract of patients with cystic fibrosis or bronchiectasis, urinary tract and urethral catheters. *P. aeruginosa* strains are the most pathogenic, and cause a large diversity of infections, because have the ability to grow in the presence of some disinfectants (Martin 2009).

Different studies show that these isolates are resistant to carbapenems due to the presence of cassettes of the VIM and IMP types in class 1 integrons, and they are resistant to quinolones due to the active transport by efflux pumps (Livermore and Woodford 2006). A less frequent mechanism is the formation of biofilms, which these strains adopt as a sessile lifestyle, and has been frequently observed in lung infections in patients with cystic fibrosis (Thomson and Bonomo 2005).

The colistin, aminoglycosides (gentamicin), broad-spectrum penicillins (piperacillin), third-generation cephalosporins (ceftazidime) and quinolones (ciprofloxacin) are the antibiotics of choice to treat *Pseudomonas* spp. infections (Mulvey and Simor 2009).

iv. ***Acinetobacter baumanii***

A. baumannii is an important pathogenic and nosocomial bacteria acquired in the clinical environment. This bacteria cause infections of different types, such as pneumonia, urinary tract infections, wound and bloodstream. Isolates of this genus are usually MDR, and the glycylcyclines and carbapenems are the options to treatment (Mulvey and Simor 2009). It frequently expresses MDR patterns to quinolones, aminoglycosides and β-lactams (Martin 2009).

A. baumannii has emerged as an important nosocomial pathogen due to production of ESBL, mainly TEM, OXA and *ampC* (Livermore and Woodford 2006). Most infections are caused by limited clones or strains with the propensity to cause outbreaks that often involve multiple facilities or countries. This suggests that clones have a greater epidemic potential (Lee 2009).

5. OBJECTIVES OF THIS THESIS

The continued and imprudent therapeutic use of different classes of antibiotics has resulted in the increase of MDR bacteria, due to development or acquisition of antibiotic resistance mechanisms.

Recently, the presence of class 1 integrons has been studied in different Gram-negative bacteria, and since these structures are associated with MGEs, the dissemination of resistance genes in the hospital environment is facilitated. Thus, this is a problem of concern and requires a permanent monitoring.

This work has one main objective:

Evaluate the prevalence of determinants of resistance in bacteria collected from inanimate surfaces within a hospital environment associated to class 1 integrons.

To achieve this main goal the workflow was divided in the following tasks:

1) Isolate Gram-negative bacteria from inanimate surfaces of a female ward sanitary facility.
2) Screen for the presence of class 1 integrons and β-lactamase genes.
3) Characterize the class 1 integrons found, determine their genetic location to evaluate the probably of the dissemination through HGT.

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II. Inanimate surfaces as reservoirs for Gram-negative bacteria possessing antibiotic resistance genes contained in class 1 integrons

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Chapter to submit as original article.

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Inanimate surfaces as reservoirs for Gram-negative bacteria possessing antibiotic resistance genes contained in class 1 integrons.

1. ABSTRACT

Objective: In the hospital environment, antibiotic resistant strains are not only confined to patients but also to the surrounding environment. This is a worldwide problem of concern given the selective pressure exerted by overuse of antibiotics in this particular environment. With the present study we aimed to assess the prevalence of antibiotic resistance genes in bacterial strains collected from inanimate surfaces.

Methods: Samples were collected with sterile cotton swabs rubbed in the surfaces of the toilet, lavatory and door knob of a female ward facility, that were placed in rich medium o/n at 37°C. Dilutions were plated in MacConkey agar and incubated as previously. Phenotypically different colonies were selected and their clonal relationship was determined by rep-PCR. Individual clones were identified using VITEK2 system and VITEK2 AES (BioMérieux, Marcy L´Étoile, France). Antimicrobial susceptibilities were determined according to guidelines of CLSI standards. Class 1, class 2 integrons and presence of β–lactamases gene sequences were screened by PCR, using specific primers. Nucleotide and deduced aminoacid sequences were analyzed with Blast and ClustalW programs and compared to others in the database.

Results: Forty-five genetically different strains belonging to genera Escherichia, Pseudomonas, Enterobacter, Klebsiella, Citrobacter, Acinetobacter, Serratia, Morganella, Proteus, Alcaligenes and Stenotrophomonas, were identified. The *intI1* gene was amplified in 33 % of the isolates and *intI2* gene was not detected. A total of 15 different arrays were identified: 47% correspond to novel combinations of genes cassette, never described before; 53% were already described in different countries and species, but in biological samples from patients or animals. Screening for β-lactamases and metallo-β-lactamases enzymes revealed the presence of CTX-M (3%), OXA (6%), SHV (16%), TEM (66%), VIM (7%) and IMP (2%) variants. The most prevalent β-lactamases were TEM-1 and SHV-12 variants, followed by metallo-β-lactamase of the VIM-2 type, which appeared always associated with class 1 integron.

Conclusions: Class 1 integrons and β-lactamases enzymes are widely spread in the hospital environment, particularly among opportunistic strains. These results are worrisome, since all the isolates were collected from inanimate surfaces, and possess different antibiotic resistance determinants. Moreover, given the selective pressure of this environment it seems that these surfaces can act as a reservoir of bacterial antibiotic resistant strains that can be acquired by compromised patients, and cause difficult to treat infections.

Keywords

Class 1 integrons, β-lactamases enzymes, inanimate hospital surfaces, Gram-negative bacteria

2. INTRODUCTION

Opportunistic bacteria can cause nosocomial infections due to their ability to survive in the presence of antibiotics. The selective pressure caused by use and overuse of antibiotics, can help the bacteria to develop resistance mechanisms, such as inactivation and efflux of the antibiotics, mutations in the ribosomal subunits and altered membrane permeability (Mulvey and Simor 2009). Horizontal gene transfer is the main mechanism that permits the acquisition and dissemination of antibiotic resistance genes. Thus, the rapid emergence of antibiotic resistance genes occur due to mobile genetics structures, such as plasmids, transposons and insertion sequence common regions (*ISCR*), which permit that opportunists bacteria become multi-drug resistance (MDR) (Bennett 2008).

Class 1 integrons were the first type integrons to be described (H. W. Stokes 1989), and constitute the most intensively studied group to date. These elements are found in 40 to 70% of Gram-negative pathogens isolated from clinical contexts, and at similar frequencies in pathogens and commensals isolated from livestock. The rapid emergence of these structures in Gram-negative and, more recently in Gram-positive bacteria, have been facilitated by their location on mobile DNA elements, such as plasmid and transposons (Gillings, Boucher et al. 2008). Integrons are defined as systems capable of capture and express exogenous resistance genes. Usually, they are composed by two conserved segments (5'-CS and 3'-CS) that limit a variable region (VR) containing genes cassette, which confer resistance to different classes of antibiotics. The integration or excision of these cassettes is catalyzed by integrase enzyme (IntI) and by a primary recombination site, *attI*, which recombine with the corresponding *attC* site present in the genes cassette (Labbate, Case et al. 2009). The expression of these genes is mediated by a promoter (Bogaerts, Huang et al.; Simon A. Hardwick 2008). In some integrons, the genes that compose the 3'-CS were deleted and downstream of the VR a complete transposition of a *tni* module of the transposon to which the integron found is associated (Gillings, Boucher et al. 2008).

Recently, a new type of class 1 integron has arisen, and is designed complex class 1 integrons. This structure is similar to the class 1 integron, but downstream of the *sul1* gene in the 3'-CS, an *ISCR*1 is present followed by a second VR that terminate in a repeated copy of 3'-CS (Quiroga, Andres et al. 2007).

β–lactamases enzymes are associated to the inactivation or hydrolysis of the β-lactams antibiotics. These enzymes can be divided in extended spectrum β – lactamases (ESBLs) (mainly TEM, SHV, CTX and OXA types), which confer resistance to penicillins, the majority of the cephalosporins and aztreonam (T. Naas 2008); or metallo-β-lactamases (MBLs) (mainly VIM and IMP types), which confer resistance to majority of β-lactam antibiotics, including carbapenems. ESBLs are present mainly in plasmids and transposons, although OXA and CTX-M can arise in complex class 1 integrons, integrated in a second VR (Sacha, Wieczorek et al. 2008). VIM and IMP types emerged mainly associated to integrons that can be embedded in transposons. Due to their location, these enzymes are quickly disseminated between different bacterial species worldwide (Libisch, Watine et al. 2008).

Molecular methods are essential to study the epidemiology of bacterial isolates, and to determine the resistance determinants associated to mobile genetic elements such as integrons and β-lactamases enzymes. Polimerase chain reaction (PCR) is usually employed to amplify the genetically important resistance genes. Pulsed-field gel electrophoresis (PFGE) was employed to locate the most important integrons. PCR-associated to typing methods such as BOX-PCR, was used to distinguish between different strains of the same species, since it is based in repetitive and conserved sequences that are present in the bacterial genome.

Here, we investigate the prevalence of the antibiotic resistant determinants, such as class 1 integrons and β–lactamases enzymes in Gram-negative isolates collected from inanimate surfaces of a female ward sanitary facility (doorknob, sink and toilet) in the Hospital Infante D. Pedro.

3. MATERIALS AND METHODS

3.1 Bacterial strain selection and antibiotic susceptibility determination

Sterile cotton swabs were rubbed in the surfaces (doorknob, sink and toilet) of a female ward sanitary facility and incubated in the liquid rich medium tryptone soya broth (TSB) at 37°C for 24 hours. Serial dilutions were made and inoculated in a selective medium, MacConkey agar, for Gram-negative isolates. rep-PCR was performed to distinguish bacteria by clonality. Gram-negative isolates were selected for further characterization studies. Those strains were identified using the automatic VITEK 2 system and Advanced Expert System (VITEK 2 AES) (BioMérieux, Marcy L´Étoile, France) and confirmed by 16S rDNA sequence (Martinez-Freijo, Fluit et al. 1998). Susceptibility testing was carried out according to guidelines of CLSI standards (2006) and different antibiotics were used, such as aztreonam, amoxicillin/clavulanic acid, ampicillin, piperacillin/tazobactam, cefotaxime, ceftazidime, cefuroxime, imipenem, meropenem, gentamicin, ciprofloxacin, norfloxacin, tobramycin, trimethoprim-sulfamethoxazole.

Species found included *Escherichia coli, Pseudomonas aeruginosa*, *Pseudomonas putida*, *Pseudomonas mendocina, Enterobacter cloacae*, *Citrobacter freudii*, *Citrobacter braakii*, *Klebsiella pneumonia*, *Proeteus mirabilis*, *Stenotrophomonas maltophilia*, *Serratia liquefaciens*, *Morganella morganii*, *Alcaligenes xylosoxidans* and *Acinetobacter baumannii*.

3.2 Molecular typing: rep-PCR technique

rep-PCR was employed to evaluate the clonal relationship between the isolates, decreasing the initial study population. Genomic DNA profiles were obtained by BOX-PCR using the specific primer, BOXA1R (BRUIJN 1997).

After electrophoresis, distinct banding pattern was analyzed with the Gel Compar II software (Applied Maths, Kortrijk, Belgium), which allowed the construction of a phylogenetic tree to distinguish different clonal group.

3.3 Screening of integrase class 1 and class 2 integron and amplification of variable region

Screening for the *intI*1 gene was performed using the specific primers HS464 and HS463a and for the *intI*2 were used RB201 and RB202 (Barlow, Pemberton et al. 2004). In the strains that were positive for this gene, their VR was further amplified with specific primers (Barlow, Pemberton et al. 2004). In a *P. putida* strain, the VR was not amplified with the usual primers, but could be amplified with the primers RB317 and TniC (Toleman, Vinodh et al. 2007). By primer walking all the genes cassette present were characterized.

PCR amplifications were carried out in 12,5 – 50 µl volumes containing 1 µl of template DNA and a mix with $MgCl^{2+}$ (3.0 mM), buffer NH^{4+} (10X), dNTPs (0,4 mM) and Taq polimerase (0.02 U).

In general, PCR conditions consisted of an initial cycle of 4’ at 94°C; followed 30 cycles of 30’’ at 94°C; 30’’ at an annealing temperature of 65°C and 59°C for *intI1* gene and for VR, respectively; and 72°C, 45’’ for integrase and 3’30’’ for VR; followed a final extension cycle at 72°C during 5’ and 10’, for integrase and VR, respectively. Annealing temperature and extension time depended on the length of the genes to amplify.

3.4 Screening of ESBLs and MBLs enzymes

ESBLs (CTX-M, SHV, TEM and OXA) and MBLs (VIM and IMP) genes were amplified with specific primers (Henriques, Fonseca et al. 2006).

The PCR programme was similar to that employed for the amplification of the *intI1* gene, but the annealing temperature varied according to the enzyme type (TEM: 44°C, SHV: 62°C, CTX-M: 55°C, OXA: 53°C, VIM: 58°C and IMP: 55°C). The period of extension was 45'' for all enzymes.

3.5 DNA sequencing of the antibiotic resistant determinants

The amplicons were purified with the QIAquick PCR purification kit (QIAGEN, Germany) and both DNA strands were sequenced by StabVida company. Subsequently nucleotide and amino acid sequences were analyzed and compared with those available in the GenBank database using the basic local alignment search tool (Blast and ClustalW computer programs).

4. RESULTS

4.1 Molecular typing

By BOX-PCR technique is was possible to reduce the bacterial population to be studied. The analysis of the banding pattern profiles permitted the construction of a phylogenetic tree, using the GelCompar II software (Applied Maths, Kortrijk, Belgium). Analysis of the phylogenetic tree allowed distinguishing between the different clonal groups (data not shown). From those, only forty-five isolates of a total of eighty-five were selected for the present study.

4.2 MICs profiles

The MICs profiles were determined by antibiotic disks. The forty-five isolates revealed a higher resistance level to ampicillin and amoxicillin/clavulanic acid (β-lactam antibiotics), which belong to penicillins family.

Since they were the first antibiotics to be introduced and therefore have been intensively used for the treatment of bacterial infections, the majority of bacteria developed resistance to them. Only two species, *A. xylosoxidans* and *S. maltophilia*, are sensitive to these antibiotics, while *E. coli*, *E. cloacae* and *K. pneumoniae* strains show a high percentage of resistance to these compounds. Moreover, resistance to the carbapenems (imipenem and meropenem) and monobactams (aztreonam) was only observed in *Pseudomonas* spp. (Figure 15).

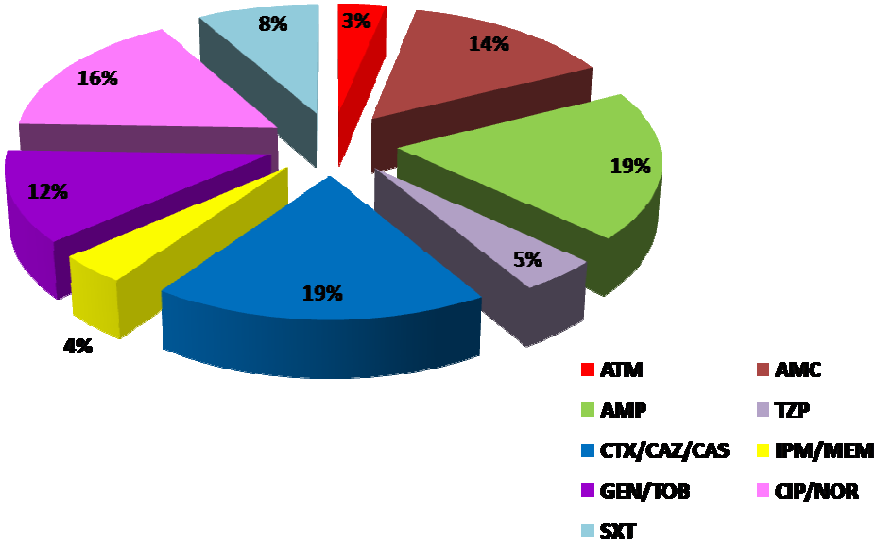

Figure 15 – Percentage of resistance profiles present in the eighty-five isolates. β-lactams antibiotics: ATM, aztreonam; AMC, amoxicillin/clavulanic acid; AMP, ampicillin; TZP, piperacillin/tazobactam CTX, cefotaxime; CAZ, ceftazidime; CAS, cefuroxime; IPM, imipenem; MEM, meropenem; and non β-lactams antibiotics: GEN, gentamicin; CIP, ciprofloxacin; NOR, norfloxacin; TOB, tobramycin; SXT, trimethoprim-sulfamethoxazole.

4.3 Detection of class 1 integrons

PCR specific for the *intI*1 gene revealed the presence of class 1 integrons in 33,33% of the isolates (15 different arrays in 45 isolates). These structures were detected in 20% of *Escherichia coli* (3/15), 13% of *P. aeruginosa* (2/15), 20% of *P. putida* (3/15), 7% of *P. mendocina* (1/15), 14% of *K. pneumonia* (2/15), 13% of *M. morganii* (2/15) and 20% of *P. mirabilis* (2/15) isolates (Figure 16).

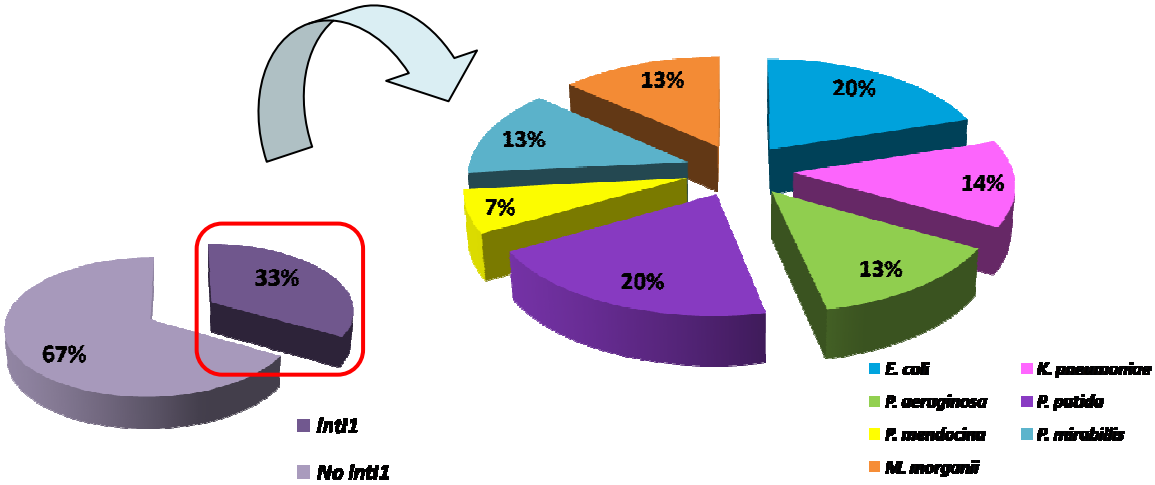

Figure 16 – Percentage of isolates that carry class 1 integrons, and the percentage of these structures present in each bacterial specie.

4.4 Characterization of the variable region

VRs were characterized in all the *intI1* gene positive strains. The amplicon lengths corresponding to the sizes of the VRs varied from 900 bp to approximately 4200 bp, indicating heterogeneity of the genes cassette (Table 4). No class 2 integrons were detected.

In the present work were found 15 different arrays, of which 47% correspond to novel combinations of genes cassette (7/15), never described before; and 53% were already described in different countries and species (8/15), but in biological samples from patients or animals.

All class 1 integrons found in *E. coli* isolates have been previously described, in different geographic locations (Peters, Leverstein-van Hall et al. 2001; Ahmed, Miyoshi et al. 2005; Bae, Lee et al. 2007; Osman Birol Ozgumus 2007; Il Kwon, Yong Hwan et al. 2008; Kadlec and Schwarz 2008; Ajiboye, Solberg et al. 2009).

In99 described by Caetano *et al.* (Caetano, Ferreira et al. 2007) was found in *P. aeruginosa*; in *M. morganii* a new array was found in Portugal (EU430601), however it has already been described in the same species from Malaysia (unpublished); in *P. putida* an In58 was found consisting in the first description of this integron in Portugal and in the species (FJ548856). Nevertheless, this array was described in a *P. aeruginosa* from France (AF263520). All the other arrays found and described in this study are novel and gene sequences were deposited in GenBank (Table 4).

In a *P. putida* isolate, a *tniC* gene was found in place of the 3'-CS. Downstream of the VR a gene corresponding to the transposon where the integron is associated was found. In this case the fusion *qacEΔ1/sul1* was deleted, maybe due to the discontinued use of quaternary ammonium compounds and sulphonamides, respectively (submitted).

Table 3 – Gene cassette arrays found in class 1 integron-positive bacterial strains and respective length and accession number.

Bacterial specie	Cassette arrays	Approximate length (bp)	GenBank no.
E. coli	*aadA2*	1200	Described in different countries
	dhfr1 / aadA1	1900	
	dhfr12 / ORF F / aadA2	2100	
K. pneumoniae	*arr-3 / dfrA27 / aadA16* *aac(6')-Ib / blaOXA-30 / catB3 / arr-3*	VR1: 2600 VR2: 7000	FJ459817
	dhfrA15 / aadA1	1900	FJ232919
P. aeruginosa	*aacA4 / blaP1b / aadA2* **(In99)**	3000	Portugal
	aacA4 / VIM-2 / aacA4 / aadA1	3500	FJ532358
P. putida	*aacA4 / VIM-2 / aac(6')-IIc/tnpA/aac(6')-IIc*	4200	FJ715942
	aacA7 / VIM-2 / aacC1 / aacA4 **(In58)**	3200	FJ548856 (France)
	VIM-2 / ORF-1 (tniC)	2000	FJ237530
P. mendocina	*IMP-8 / aacA4 / aadA1 / tnpA*	4100	FJ594416
P. mirabillis	*aacC1*	900	EU851865
	dhfrVII	1000	EU860402
M. morganii	*blaP1b / aadA2*	2200	EU430602
	aadB / catB3	1700	Malaysia

Overall, the VR present found are composed mainly by genes cassette that confer resistance to aminoglycosides (54%) (aminoglycoside-acetyltransferase – *aac* gene and aminoglycoside-adenylyltransferase – *aad* gene), followed by genes cassette that confer resistance to trimethoprim (dihydrofolate reductase – *dhfr* gene), present in 14% of the arrays. Other genes cassette such as oxacillinase (bla_{OXA-30} gene), is present only in one array in *K. pneumoniae* (2%), while a putative transposase (*tnpA* gene), proteins unknown function (ORFs), rifampin ADP-ribosylating transferase (*arr* gene) and cloramphenicol acetyltransferase (*cat* gene) appear in only 3 % of the arrays (Figure 17).

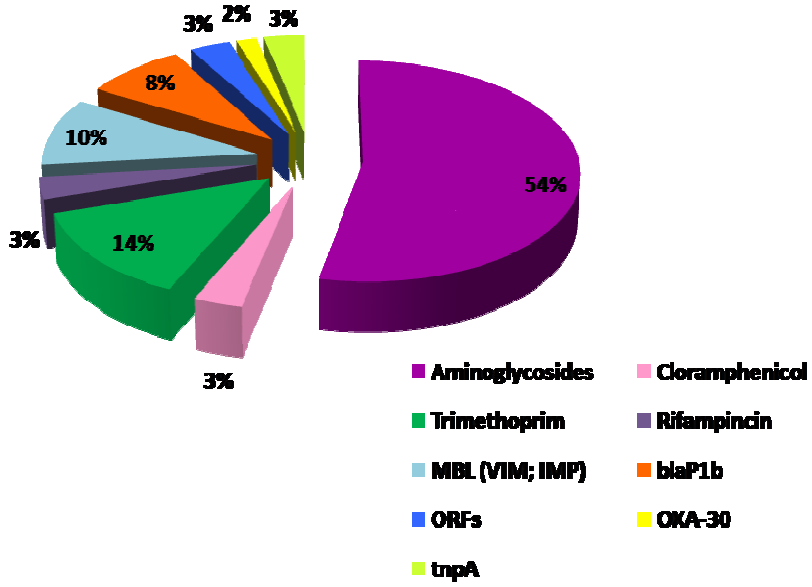

Figure 17 – Representation of all genes cassette found in the different arrays of class 1 integrons amplified.

4.5 Detection and analysis of β – lactamases enzymes gene sequences

Although many ESBL-encoding genes have been described in the literature, only a few of these (namely bla_{TEM}, bla_{SHV}, bla_{OXA} and bla_{CTX}) were detected by PCR and confirmed by nucleotide sequencing. The sequences of the amplicons, when compared with others deposited in the database, revealed that TEM-1, SHV-12, OXA-30, CTX-M-15, VIM-2 and IMP-8 were predominant in the different strains.

According to the Figure 18, TEM type was the most prevalent gene (66%), and was present in all the studied species, except *S. liquefaciens*, followed by SHV type (16%) that was present in *E. coli, K. pneumonia* and *E. cloacae* isolates. MBLs (VIM and IMP types), appeared only in some *Pseudomonas* spp. in a lower percentage, and are associated to the VR of the class 1 integrons.

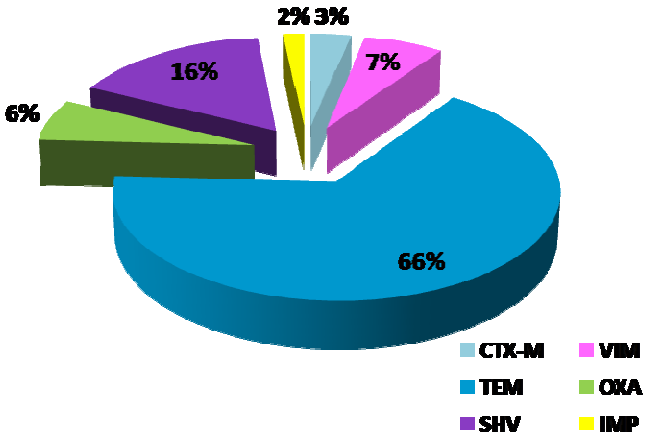

Figure 18 – Percentages of the β-lactamases enzymes found in the forty-five studied isolates.

4.6 Relationship between MICs profile, class 1 integron and β-lactamases enzymes

In order to confirm the effect of class 1 integron carriage (Table 4) and β-lactamases enzymes exhibited by the bacteria, we analysed the MIC data of integron positive bacteria with the cassette arrays. Thus, according to Figure 15, almost all *intI*1 positive isolates show high resistance to ampicillin (19%) and cephalosporins (19%), that can be justified by presence of TEM-1 or SHV-12 (in some isolates) and CTX-M-15 and OXA-30 (in *E. coli* and *K. pneumoniae* isolates), respectively. The *Pseudomonas* spp. isolates are the unique that possess gene sequences that produce MBLs enzymes (VIM-2 and IMP-8) (4%), which confer resistance to carbapenems. The resistance to aminoglycosides (12%) predominate in class 1 integrons-positive isolates, and could be related to the presence of *aad* and *aac* genes cassette present in the VR. Resistance to trimethoprim and sulphonamides is present in 8% of the cassettes, and can be due to *dhfr* and *sul1* genes, respectively. Resistance to quinolones (16%) can not be associated to any gene present in the VR. However it can probably due to the presence efflux pumps or mutations in *gyr* or *par* genes.

Analysing of all the cassette arrays here described (Table 4). It is possible to identify only one MDR isolate, a *K. pneumoniae* strain with a complex class 1 integron. This isolate shows resistance to rifampicin (conferred by *arr-3* gene), trimethoprim (conferred by *dfrA27* gene), aminoglycosides (conferred by *aadA27* gene), some β-lactams antibiotics (conferred by bla_{OXA-30} gene), quinolones (conferred by *aac(6')-Ib-cr* gene) and cloramphenicol (conferred by *catB3* gene). All other arrays relate to resistance for only to one or two classes of antibiotics.

In those isolates where MIC values suggest a multi-resistance profile, this is not only due to the presence of integrons or β-lactamases enzymes but it can be attributed to other resistance mechanisms such as mutations or the presence of efflux pumps.

5. DISCUSSION

All isolates collected in the hospital environment were opportunistic bacteria, since they are known to be able to infect a host and cause a bacterial infection. An analysis of the MIC profiles revealed that 77, 65% of the isolates are MDR (66/85), but the analysis of the cassette arrays, only one isolate is MDR (1.18%). The majority of them are resistant to β-lactams, mainly ampicillin (19%) and some cephalosporins (19%), and also to other classes of antibiotics, such aminoglycosides (12%), quinolones (16%) and trimethoprim-sulfametoxazole (8%). Resistance to aztreonam (3%) and carbapenems (4%) was also observed but only in *Pseudomonas* spp. and *Strenotrophomonas* spp. These percentages were expected, since the bacteria are more resistance to the antibiotics commonly used.

Class 1 integrons were detected in 33% of the strains belonging to *E. coli*, *K. pneumoniae*, *Pseudomonas* spp., *P. mirabilis* and *M. morganii* species. The cassette arrays were mainly constituted by genes associated to resistance to the aminoglycosides (54%), trimethoprim (14%) and MBLs (10%), although other genes were present in a lower percentage. As suggested by other authors, the different size cassettes demonstrates the variable nature of these structures, presumably reflecting differences in the number and type of inserted genes cassette (Stokes, Nesbo et al. 2006). All the integrons carried at least one cassette that conferred resistance to the aminoglycosides (*aad* or *aac* genes). Similar results were reported by Poirel and their collaborators (Poirel, Lambert et al. 2001). Concerning the MBLs, VIM-2 and IMP-8 always appeared associated with the VR of the *Pseudomonas* spp. strains. VIM-2 is the most prevalent MBL present worldwide in *Pseudomonas* spp. isolates (Poirel, Naas et al. 2000; Lee, Lim et al. 2002; Guerin, Henegar et al. 2005; Lolans, Queenan et al. 2005; Quinteira, Ferreira et al. 2005; Fiett, Baraniak et al. 2006; Villegas, Lolans et al. 2006; Gutierrez, Juan et al. 2007; Huang, Chang et al. 2007; Walsh and Rogers 2008). Thus, in all isolates, the presence of these genes cassette may be responsible by the resistance profile observed as revealed by the MICs values.

Most of the class 1 integrons here reported are novel, and were never described before. Some of them are described for the first time to be detected in a certain species and others were detected on Portugal for the first time. However, all the arrays found in *E. coli* isolates have been previously described in different countries, including Portugal locations (Peters, Leverstein-van Hall et al. 2001; Ahmed, Miyoshi et al. 2005; Bae, Lee et al. 2007; Osman Birol Ozgumus 2007; Il Kwon, Yong Hwan et al. 2008; Kadlec and Schwarz 2008; Ajiboye, Solberg et al. 2009). These arrays have frequently emerged in different species collected from different biological samples (Dalsgaard, Forslund et al. 2000; L'AbÃ©e-Lund and SÃ¸rum 2001; Norskov-Lauritsen, Sandvang et al. 2001; Gu, Tong et al. 2007; Wiesner, Zaidi et al. 2009)

In99 was found in *P. aeruginosa*. This same integron was previously described in the same species and it was also isolated from an inpatient of the same hospital in 2003 (Caetano, Ferreira et al. 2007) showing its prevalence and dissemination within this environment or in the community. In58 was found in *P. putida*, however this integron was previously described in a *P. aeruginosa* in France (Poirel, Lambert et al. 2001).

In a *P. putida* strain a *tniC* gene appeared in the place of the usual 3'-CS. Recent studies also report these findings (Toleman, Vinodh et al. 2007; Gillings, Boucher et al. 2008). This type of structure was only described in *P. aeruginosa* from different countries (Corvec, Poirel et al. 2008; Samuelsen, Buaro et al. 2009), suggesting the recent evolution of these structures. The same structure was also found in a *P. aeruginosa* strain in Spain (unpublished, accession number: GQ422829).

Other interesting array was found in a *K. pneumoniae* isolate containing a complex class 1 integron. This structure is novel in the specie and geographic distribution. Nevertheless, the first VR was found in *E. coli* (Wei Q 2009) and *V. cholerae* (unpublished; accession number: EU678897) isolates from China. The different genes cassette present in this complex class 1 integron may be responsible for the MDR profile of this isolate.

Since the class 1 integrons are normally associated to MGEs, their dissemination between different species within the hospital environment is facilitated, and can cause bacterial infections in debilitated patients, compromising the treatment.

A screening for presence of some β-lactamases genes was also performed revealing that ESBLs are the most prevalent enzymes (91%) and are present in all species. Most of them were of the TEM-1 type (66%). With the exception of $bla_{CTX\text{-}M\text{-}9}$ and $bla_{OXA\text{-}30}$ that can be found in class 1 integrons (Poirel, Lambert et al. 2001), frequently ESBLs appear associated with plasmids therefore its high dissemination is not directly related to class 1 integrons (Machado, Ferreira et al. 2007). These genes are responsible mainly for the resistance to penicillins and their derivates, and some cephalosporins. MBLs are less abundant (9%) and were only present in class 1 integrons found in *Pseudomonas* spp. isolates. Since these genes are placed in integrons that are associated with other MGE, the rate of dissemination of these genes is greater.

Overall the results show that, as expected given the high selective pressure, the emergence and prevalence of antibiotic resistance among bacteria colonizing the hospital environment is high, and it appears to be disseminated by different opportunistic species that become MDR and are able to cause infections in inpatients. This hypothesis is supported by other studies performed worldwide, in which different Gram-negative bacteria isolated from various biological samples contain similar class 1 integrons and β-lactamases enzymes (Poirel, Lambert et al. 2001; Quinteira, Sousa et al. 2005; Gu, Tong et al. 2007; Kadlec and Schwarz 2008; Ajiboye, Solberg et al. 2009; Wei Q 2009).

Thus, this surveillance studies are very important since the information provided constitute an alert and can contribute to the implementation of new guidelines within the hospital environment.

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III. Tn*5090*-like class 1 integron carrying bla_{VIM-2} in a *Pseudomonas putida* strain from Portugal

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Chapter submitted as research note:

Cátia Santos, Tânia Caetano, Sónia Ferreira and Sónia Mendo. 2009. Tn5090-like class 1 integron carrying bla_{VIM-2} in a *Pseudomonas putida* strain from Portugal.

Clinical microbiology and infection

(Accepted)

1. ABSTRACT

Three *Pseudomonas putida* strains containing $bla_{VIM\text{-}2}$ gene were isolated from an inanimate surface of a female ward sanitary facility in the Hospital Infante D. Pedro, Aveiro. Novel class 1 integron was found in strain Pp2. Strain Pp1 carries a class 1 integron firstly described in this species. Those integrons are located on plasmids as revealed by Southern blot hybridization with *intI1* and 16S rRNA DIG labeled probes. PFi strain carries a class 1 integron associated with a *Tn5090*-like transposon, constituting the first report of this type of arrangement in a strain from Portugal.

Keywords

Class 1 integrons, TniC-like transposon, *Pseudomonas putida*, inanimate surfaces

2. DESCRIPTION

Pseudomonas putida is a Gram negative opportunistic pathogen that is rarely involved in human infections, (Poirel, Cabanne et al. 2006) and is therefore considered as a low-grade pathogen (Corvec, Poirel et al. 2008; Sacha, Wieczorek et al. 2008; Walsh and Rogers 2008).

Carbapenems are broad spectrum antibiotics that are frequently used in the treatment of *Pseudomonas* spp. infections (Walsh and Rogers 2008). Consequently, the emergence of metallo-β-lactamases (MBLs) of IMP or VIM type among non-fermenting Gram-negative bacteria is becoming frequent and represents an epidemiological risk since these enzymes confer resistance not only to carbapenems, but virtually to all β-lactams (Bebrone 2007; Sacha, Wieczorek et al. 2008). Moreover, bla_{IMP} and bla_{VIM} genes are usually carried on integrons, in association with aminoglycosides cassettes. These mobile elements are easily spread horizontally between different species due to their association with transposons or plasmids (Walsh 2005; Bebrone 2007; Libisch, Watine et al. 2008; Sacha, Wieczorek et al. 2008).

bla_{VIM-2} was first described in a *Pseudomonas aeruginosa* (Poirel, Naas et al. 2000) isolated in France. Presently, the VIM type enzymes are the second dominant group of β-lactamases, and it has been reported in different species from 23 countries worldwide (Walsh, Toleman et al. 2005; Sacha, Wieczorek et al. 2008), with the alleged "index" strain being a Portuguese *P. aeruginosa* isolate recovered in 1995 (Toleman, Vinodh et al. 2007; Cardoso, Alves et al. 2008). The presence of VIM-2 enzymes in this species was originally restricted to East Asia, but recently it has been found in Latin America (Buenos Aires) (Almuzara, Radice et al. 2007) and Europe (Belgium) (Bogaerts, Huang et al. 2008).

Here we report and characterize three novel class 1 integrons found in *P. putida* strains from Portugal. Particular emphasis is given to the integron found associated to the *Tn5090*-like transposon.

During an infection control study in 2005, three strains of *P. putida* (PFi, Pp1 and Pp2) were isolated from different surfaces within a female ward sanitary facility (doorknob, sink and toilet: Pp1, Pp2 and PFi isolates, respectively) in the Hospital Infante D. Pedro, Aveiro. The strains were identified by 16S rDNA sequencing. Antibiotic susceptibilities (Table 5) were determined according to guidelines of CLSI standards (CLSI 2006). MBL production was investigated by Etest MBL® with Imipenem/Imipenem+EDTA strips according to manufacturer's instructions (AB BioMérieux, Solna, Sweden).

Table 4 – MICs profiles for *P. putida* Pp1, *P. putida* Pp2 and *P. putida* PFi, determined according to guidelines of CLSI standards.

Antibiotic	**MIC (µg/mL)**		
	Pp1	**Pp2**	**PFi**
Ceftazidime	64	16	64
Cefepime	64	8	16
Cefpirome	64	32	64
Aztreonam	64	16	64
Imipenem	16	16	16
Meropenem	16	16	16
Amikacin	16	2	4
Gentamicin	16	1	1
Isepamicin	64	1	1
Netilmicin	32	1	2
Tobramycin	16	1	1
Colistin	0.5	0.5	0.5
Trimetoprim/Sulfametoxazol	320	320	320

The presence of bla_{VIM} and bla_{IMP} genes was confirmed by PCR as previously described by Henriques *et al.* (Henriques, Fonseca et al. 2006); only bla_{VIM} gene could be amplified; subsequently nucleotide sequencing analysis confirmed the bla_{VIM-2} variant in the three strains. In Pp1 and Pp2 isolates, a variable region of 3800 bp and 3000 bp, respectively, was amplified corresponding to a unique and novel array of genes cassette, never described before in *P. putida* (Figure 19).

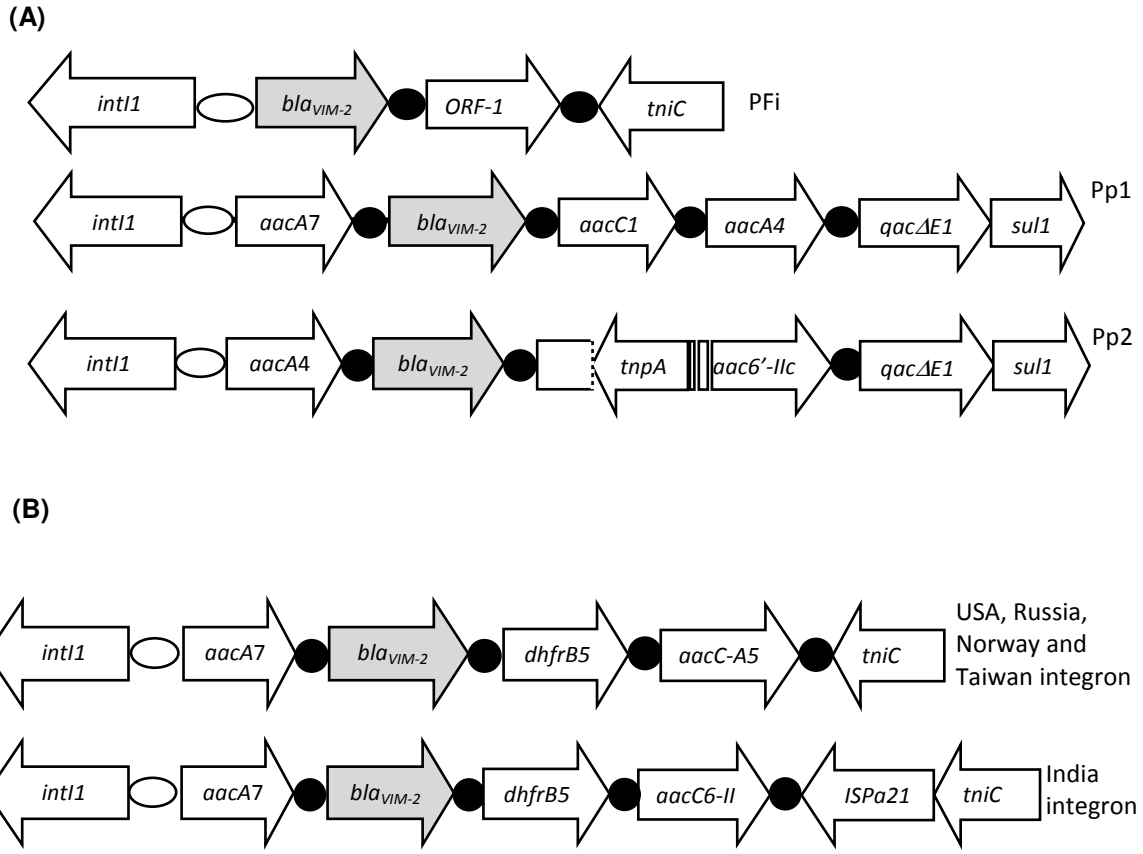

Figure 19: (A) Schematic representation of *P. putida* integrons reported in this study; (B) Schematic representation of *P. aeruginosa tniC*-integrons previously found (adapted from Toleman *et al.* (Toleman, Vinodh et al. 2007). The genes cassette are represented by boxed arrows indicating the transcriptional orientation. A white circle represents the *attI1* site and the black circles represent the genes cassette recombination site, *attC* or 59-be.

In Pp1 isolate three aminoglycosides resistance genes were found (*aacA7*, *aacC1* and *aacA4*) in addition to the bla_{VIM-2} (Figure 19). This integron is identical to In58, originally described in a *P. aeruginosa* collected in 1998 from Paris (Poirel, Lambert et al. 2001), and have been found worldwide, including in Portugal, but it was never described in *P. putida*. A similar structure was previously described in *P. putida* isolates from Korea, however the order of the two first genes cassette is inverted, and the third gene is different (Lee, Lim et al. 2002).

Pp2 integron contains four genes within the variable region (Figure 19). The first position is occupied by an aminoglycoside (6') acetyltransferase gene (*aacA4*), followed by the MBL gene bla_{VIM-2}. These genes are frequently found associated in several class 1 integrons. In the third position an aminoglycoside 6'-N-acetyltransferase gene (*aac(6')-IIc*) is found disrupted by an insertion sequence *IS1382*. This insertion sequence when associated with different antibiotic resistance genes can play an important role in their dissemination. *IS1382* is composed by a putative transposase gene (*tnpA*) flanked by two 35 bp inverted repeats; it was detected in other *P. putida* strains and it is located either in a plasmid or in the chromosome (AF052750 and CP000949, respectively).

Amplification of a variable region of the integron found in PFi strain was unsuccessful. Therefore the substitution of the *qacEΔ1/sul1* by *tniC* gene was investigated. A variable region of approximately 2000 bp was amplified using 5CS/tniCF primers previously described (Toleman, Vinodh et al. 2007), confirming the presence of *tniC* gene. This structure has never been described before in *P. putida*. Nucleotide sequence determination revealed the presence of the bla_{VIM-2} gene, followed by an *ORF1* of unknown function. Moreover, the predicted arrangement of genes is different from previously described *tniC*-like integrons found in *P. aeruginosa* strains isolated from India, Russia, United states (Toleman, Vinodh et al. 2007), Norway (Samuelsen, Buaro et al. 2009) and Taiwan (Yan, Hsueh et al. 2006).

The bla_{VIM-2} gene cassette found in these integrons may be responsible for the resistance to imipenem (≥ 16 µg/ml) and meropenem (≥ 16 µg/ml) in all isolates. According MIC profile of the Pp1 isolate, resistance to aminoglycosides (≥ 16 µg/ml) can be due to *aacA7*, *aacC1* and *aacA4* gene cassettes. However, in Pp2 isolate gentamicin (≥ 1 µg/ml) resistance is not observed despite the presence of *aacA4* gene cassette, and netilmicin and tobramycin (≥ 1 µg/ml) resistance was not expected since *aac(6')-IIc* gene is disrupted by the *tnpA* gene, rendering this gene inactivate. As suggested by other authors in similar studies where the same results were observed, other mechanisms, such as antibiotic target modification or over-expression of efflux systems can contribute for the multi-resistant phenotype of these isolates (Tenover 2006; Mulvey and Simor 2009).

The location of the integrons was investigated by pulsed-field gel electrophoresis (PFGE). Genomic DNA plugs were digested by S1 (New England biolabs, Beverly, Mass.), separated on an 1% agarose gel with a CHEF-DRIII system (Bio-Rad, Hemel Hempstead, UK), and hybridized to labeled 16S rDNA and *intI1* probes. The results obtained for Pp1 and Pp2 strains suggest that both integrons are located on plasmids (Figure 20). The location of the PFi integron could not be determined by both hybridization of S1-PFGE digests and plasmid alkaline lysis extraction.

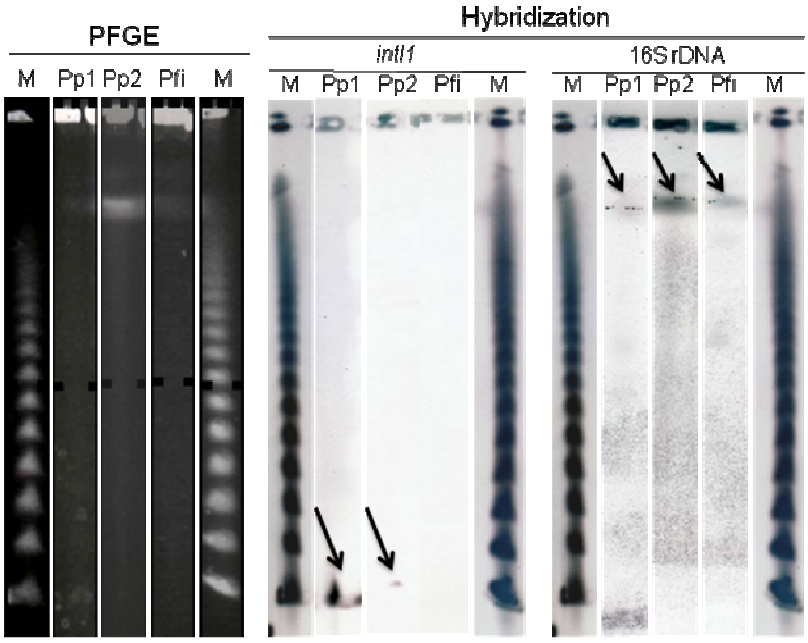

Figure 20: Hybridization with DIG labelled *intI1* (A) and 16S rDNA (B) probes of S1 digested genomic DNA of three *P. putida* isolates (Pp1, PFi and Pp2) DNA after separation in PFGE. Lane M: DNA size marker (λ concactamers); arrows indicate hybridizion signals.

Recently multi-resistant *P. putida* have been associated with difficult-to-treat infections (Almuzara, Radice et al. 2007; Bogaerts, Huang et al. 2008; Bennett, Herrera et al. 2009). This study demonstrates that despite the uncommon association of multi-resistant *P. putida* with human infections within the hospital environment this bacterium can act as a reservoir for antibiotic resistance genes, highlighting its potential as an emerging nosocomial pathogen. More worrying is the fact that these genes are placed in mobile elements, namely the bla_{VIM-2} that was found associated with the *TniC*-like transposon, promoting their dissemination among other pathogens within this highly selective environment.

The nucleotide sequences reported in this paper are deposited in the GenBank under the accession numbers FJ237530, FJ548856 and FJ715942.

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IV. First description of a *bla*$_{IMP-8}$ in a *Pseudomonas mendocina* isolated in Hospital Infante D. Pedro, Aveiro, Portugal

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Chapter submitted as short note:

Cátia Santos, Tânia Caetano, Sónia Ferreira and Sónia Mendo. 2009. First description of a *bla*$_{IMP-8}$ in a *Pseudomonas mendocina* isolated in Hospital Infante D. Pedro, Aveiro, Portugal.

Research in Microbiology.

1. ABSTRACT

A *Pseudomonas mendocina* carrying a novel class 1 integron containing an IMP-8 metallo-β-lactamase was isolated from an inanimate surface of a female ward sanitary facility in the Hospital Infante D. Pedro, Aveiro, Portugal. The integron is chromosomally located as revealed by Southern blotting hybridization with integrase gene (*intI1*) and 16S rDNA.

Here we report for the first time the presence of an IMP-8 metallo-β-lactamase gene in the *Pseudomonas* genera.

Keywords

Class 1 integron, IMP-8 metallo-β-lactamase, Hospital environment

2. INTRODUCTION

Pseudomonas mendocina is a Gram-negative environmental bacterium that can cause opportunistic nosocomial infections, such as infective endocarditis and spondylodiscitis (Mert, Yilmaz et al. 2007).

The ability of bacteria to develop resistance is impressive as observed by the arising of multiresistant bacteria. For instance, the emergence of metallo-β-lactamases (MBLs), mainly VIM and IMP types, conferring resistance to the carbapenems, except monobactams is increasing worldwide. Consequently, carbapenems are becoming less effective in the treatment of Gram-negative rods infections carrying these enzymes (Bebrone 2007).

IMP-1 was the first mobile MBL isolated and characterized from a Japanese *P. aeruginosa* strain in 1988 (Docquier, Riccio et al. 2003; Sacha, Wieczorek et al. 2008). The bla_{IMP} gene is worldwide distributed (Sacha, Zórawski et al. 2007) and is commonly found associated with class 1 or class 3 integrons (Toleman, Biedenbach et al. 2003), that are located in large plasmids (Shibata, Doi et al. 2003), contributing to its rapid dissemination through horizontal gene transfer both from cell to cell and between DNA molecules. IMP producers, usually Gram-negative pathogens, have been reported in nosocomial outbreaks during the last few years (Pagani, Colinon et al. 2005). bla_{IMP-8} is a variant of the bla_{IMP-2} gene; it was firstly described in a *Klebsiella pneumoniae* strain from Taiwan in 1998 (Yan, Ko et al. 2001). Both genes differ in four nucleotides, resulting in two amino acid changes in the final enzymes (Weldhagen 2004; Sacha, Wieczorek et al. 2008).

Recently, bla_{IMP-8} was found in some Gram-negative isolates, such as *Acinetobacter phenon* (Wang, Guo et al. 2007), *Klebsiella pneumoniae* (Yan, Ko et al. 2001; Yan, Ko et al. 2001), *Serratia marcescens* and *Enterobacter cloacae* (Lee, Peng et al. 2008), but it has never been identified in *Pseudomonas* spp.

Here we report and characterize a class 1 integron containing a bla_{IMP-8} as part of a new gene array found in a *P. mendocina* strain, isolated within the hospital environment.

3. MATERIALS and METHODS

a. Bacterial strains and antibiotic susceptibilities

During an infection control study carried out in 2005, a *P. mendocina* (Pm1) strain was collected from an inanimate surface from a female ward sanitary facility in the Hospital Infante D. Pedro, Aveiro. The strain was identified by the automatic VITEK 2 and Advanced Expert System (VITEK 2 AES) (BioMérieux, Marcy L´Étoile, France). Antibiotic susceptibilities were determined according to guidelines of CLSI standards (CLSI 2006). Further confirmation at species level was performed by 16S rDNA sequencing.

b. Detection and characterization of class 1 integrons

Screening for antibiotic resistance determinants associated with integrons using the primers for *intI1* gene (Barlow, Pemberton et al. 2004). The corresponding variable region was amplified with the primers for *intI1* and *qacEΔ* genes. Resulting in an amplicon, that was sequenced. Nucleotide and deduced aminoacid sequences were analyzed with Blast and ClustalW programs and compared with others deposited in databases.

c. Location of the class 1 integron

The genetic location of the class 1 integron was investigated by S1-Pulsed-Field Gel Electrophoresis (PFGE). Briefly, the agarose plugs were prepared as described by Samuelsen (Samuelsen, Naseer et al. 2009), and digested with S1 enzyme (New England biolabs, Beverly, Mass.). The fragments were separated during 12h using a CHEF-DRIII system (Bio-Rad, Hemel Hempstead, UK), transferred to nylon membranes and hybridized with 16S rDNA and *intI1* DIG labeled probes (Roche, Germany).

4. RESULTS and DISCUSSION

Screening for integrons in *P. mendocina* (Pm1) revealed the presence of a class 1 integron with a variable region of 4000 bp. By primer walking a bla_{IMP-8} gene was found located near the *intI1* followed by *aac(6')-Ib/aacA4* gene (encoding for the aminoglycoside-(6')-N-acetyltransferase) and by an *aadA1* gene (encoding for a 3''-adenyltransferase) conferring resistance to the aminoglycosides. The last position, was occupied by a putative transposase (*tnpA*) that usually appears upstream of integrase gene, and is usually associated with different transposons (Tseng, Hsueh et al. 2007) (Figure 21).

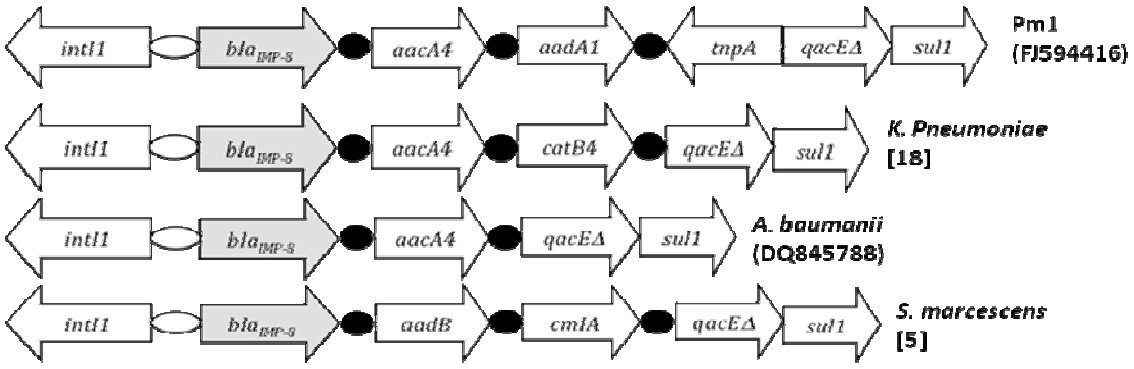

Figure 21 – Structure of *P. mendocina* (Pm1) integron and genetic context of other bla_{IMP-8}-containing class 1 integrons found worldwide.

All the class 1 integrons described so far containing bla_{IMP-8} (Figure 21) possess the MBL gene in the first position of the array followed by an aminoglycoside resistance gene, usually the *aacA4* gene (Yan, Ko et al. 2001; Pagani, Colinon et al. 2005; Lee, Peng et al. 2008). In Pm1 strain, after the *aacA4* gene an *aadA1* gene is found, therefore it is not possible to establish a possible evolutionary relationship between the integron found in Pm1 and the other previously described structures harboring this bla_{IMP} allele. Moreover, this is the first study reporting the occurrence of bla_{IMP-8} in the *Pseudomonas* genera.

Analysis of Pm1 resistance profile (Table 6) shows a reduced susceptibility to imipenem (MIC ≤ 4 µg/ml), and susceptibility to meropenem (MIC ≤ 1 µg/ml). The presence of the bla_{IMP-8} gene in this strain, might contribute to the MIC value observed for imipenem. However, the evaluation of MBL production by Etest® with Imipenem/Imipenem + EDTA strips (AB BioMérieux, Solna, Sweden) was negative. These results highlight the difficulty to detect IMP-8 enzymes by routine susceptibility tests, a problem also referred by others authors (Yan, Ko et al. 2001).

Consequently, a combination of phenotypic and molecular biology techniques applied to determination of carbapenemase activity could be employed to provide more reliable results for epidemiological studies and also for patients treatment (Yan, Ko et al. 2001).

Table 5 - MICs profile of *P. mendocina* Pm1 determined according to guidelines of CLSI standards.

Antibiotic	**MICs** (µg/ml)
Ceftazidime	64
Cefepime	64
Cefpiroma	64
Aztreonam	4
Imipenem	4
Meropenem	1
Amicacin	2
Gentamicin	4
Colistin	0.5
Trimetoprim/Sulfametoxazol	320

According to the S1-PFGE southern blot, identical results were obtained with 16S rDNA and *intI1* probes (Figure 22), suggesting a chromosomal location of the integron, and consequently of the bla_{IMP-8} gene. The same location was reported for other MBL determinants often present in *P. aeruginosa* (Nordmann and Poirel 2002), and also with a Taiwanese *Serratia marcescens* clinical strain carrying the bla_{IMP-8} gene (Lee, Peng et al. 2008) and an *Acinetobacter baumanii* possessing a bla_{IMP-2} as part of a class 1 integron (Riccio, Franceschini et al. 2000; Yan, Ko et al. 2001).

Plasmids are the primary vehicle for horizontal transfer of integron-associated MBL responsible for much of the movement of carbapenem resistance genes among Enterobacteriaceae and other Gram-negative bacteria (Lee, Peng et al. 2008). Therefore, the genomic location of Pm1 integron suggests that its dissemination process will probably involve integration catalyzed by transposition, mainly through transduction and transformation. Since this isolate is part of the hospital environment, it can play a role of donor of such resistance genes to potential pathogenic strains.

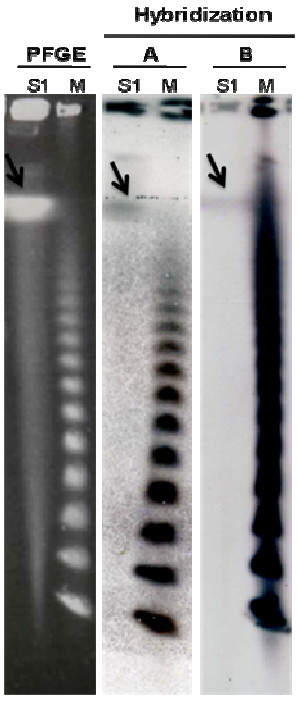

Figure 22 – Hybridization with DIG labelled *intI1* (A) and 16S rDNA (B) probes of S1 digested genomic DNA of *P. mendocina* Pm1 after separation by PFGE. Lane M: DNA size markers (λ concactamers).

P. mendocina was collected from the surface of a toilet and can therefore easily infect debilitated patients, despite the fact that infections caused by this species are rare. Still, given its resistance profile, it may pose a risk for patients mainly when responsible for infections that are difficult to treat.

The nucleotide sequence reported in this paper is deposited in the GenBank under the accession number FJ594416.

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V. Novel complex class 1 integron found in a *Klebsiella pneumoniae* isolate from Portugal

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Chapter to submit as short note:

Cátia Santos, Tânia Caetano, Sónia Ferreira and Sónia Mendo. 2009. Novel complex class 1 integron found in a *Klebsiella pneumoniae* isolate from Portugal.

1. ABSTRACT

A *klebsiella pneumoniae* isolate (KP1) that was isolated from an inanimate surface of a female ward sanitary facility in the Hospital Infante D. Pedro, Aveiro-Portugal, carries a novel complex class 1 integron never described before. It contains two variable regions, the first was previously described in *Escherichia coli* and *Vibrio cholera*; the second variable region has an In37-like structure and it is located downstream of the *ISCR*1. The integron is located on a plasmid of 225 Kb, as revealed by Southern blotting hybridization with *intI1* and 16S rRNA DIG labeled probes. *qnrB10* gene is present although not associated with the class 1 integron.

Keywords

Complex class 1 integron, plasmid location, inanimate surface, *Klebsiella pneumoniae*

2. DESCRIPTION

Klebsiella pneumoniae is an important opportunistic pathogen that causes urinary tract and intra-abdominal infections, neonatal meningitis and pneumonia in immune-compromised individuals (Martin 2007; Thong, Lim et al. 2008). Klebsiellae are widespread in the environment and in the intestinal flora of humans and other mammals (Martin 2007).

The extensive use of broad-spectrum antibiotics in hospitalized patients led to the development of multi-drug resistance (MDR) strains (Martin 2007). In *Klebsiella pneumoniae* isolates the resistance is due to the production of β-lactamases enzymes, mainly extended spectrum β-lactamases (ESBLs) (L. Poirel 2008; T. Naas 2008), but more recently metallo-β-lactamases (MBLs) have also been detected (Yan, Ko et al. 2001; Bebrone 2007; Lee 2007; Martin 2007).

Nowadays *Klebsiella pneumoniae* strains complex class 1 integrons have emerged. This new structure is composed by the typical structure of a class 1 integron, associated with a new element, designed insertion sequence common region (*ISCR*1), located downstream of the *sul*1 gene. Immediately after the *ISCR*1 a second variable region (VR2) appears containing resistance genes (β-lactamase, fluoroquinolone, trimethoprim, aminoglycoside and cloramphenicol). This region is followed by a repetition of 3'-conserved segment (3'-CS) (Toleman, Bennett et al. 2006; Bennett 2008; Labbate, Chowdhury et al. 2008). Antibiotics such as cephalosporins, β-lactamase-stable penicillins and aminoglycosides are commonly used to treat *Klebsiella* infections, but MDR strains may limit the antibiotic choice or compromise the treatment of the infections (Martin 2007; Chang, Fang et al. 2009).

Here we report and characterize a novel complex class 1 integron in a *Klebsiella pneumoniae* strain collected within the hospital environment in Aveiro, Portugal.

During an infection control study in 2005, a *K. pneumoniae* (Kp1) strain was collected from a sink in a female ward sanitary facility in the Hospital Infante D. Pedro, Aveiro. The strain was identified by the automatic VITEK 2 and Advanced Expert System (VITEK 2 AES) (BioMérieux, Marcy L´Étoile, France), and 16S rDNA sequencing. Additionally antibiotic susceptibilities were determined according to guidelines of CLSI standards Table 7 (CLSI 2006).

Table 6 - MICs for *K. pneumoniae* KP1, determined according to guidelines of CLSI standards.

Antibiotic	**MIC** (μg/ml)
Amikacin	2
Amoxicillin/CA	32
Ampicillin	32
Cefotaxime	4
Ceftazidime	8
Cephalotin	32
Ciprofloxacin	4
Imipenem	4
Meropenem	2
Norfloxacin	16
Piperacillin/Tazobactam	8
Tobramycin	16
Trimetoprim/Sulfametoxazol	320

The presence of class 1 integrons was investigated using specific primers for *intI1* gene and subsequently to 5'-CS and 3'-CS (Barlow, Pemberton et al. 2004). The first VR (VR1) amplified was approximately 3200 bp. By primer walking the complete array of genes cassette was determined. Nucleotide sequence analysis revealed a new integron

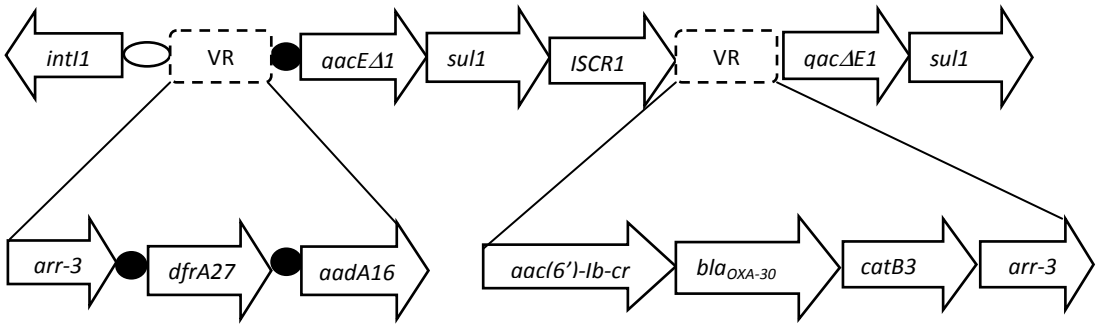
structure, never described before in *K. pneumoniae*, but previously described in *Escherichia coli* (Wei Q 2009) and in a *Vibrio cholerae* in China (unpublished - EU678897).

The VR of Kp1 isolate was composed by three genes. In the first position, near the *intI1* gene, was occupied by a rifampin ADP-ribosyl transferase-encoding gene (*arr-3*), followed by a dihydrofolate reductase-encoding gene (*dfrA27*). The last position was occupied by an aminoglycoside-(3'')(9)-adenylyltransferase (*aadA16*). An *ISCR*1 element was also amplified that was linked to the class 1 integron. To amplify the VR2, specific primers to the *ISCR*1 and *qacEΔ*1 were employed resulting in an amplicon of approximately 6000 bp. Further sequencing reactions revealed the presence of an array consisting of four genes cassette, which constitute the VR of In37 (Figure 23A).

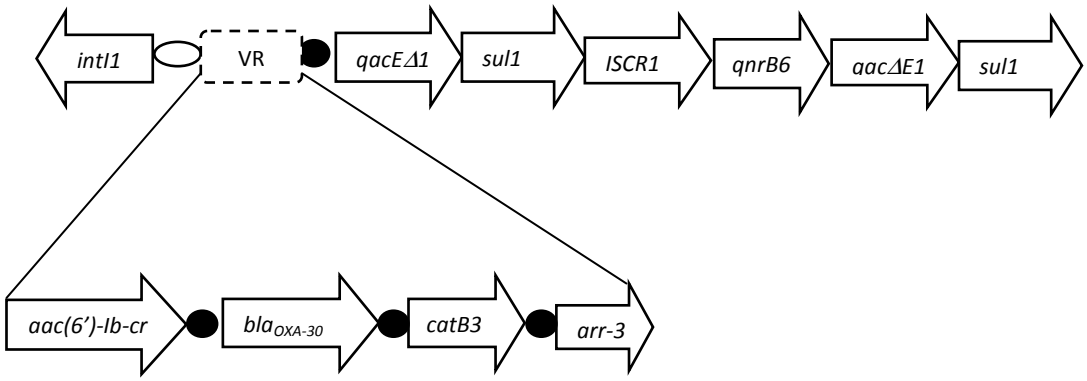
In37 was firstly discovered in a clinical *K. pneumoniae* isolate from Argentina (Quiroga, Andres et al. 2007), and ever since it is described in association with *ISCR*1 and *qnr* genes (Figure 23B). However, its presence was always detected in the first VR (VR1) and never in the VR2, moreover, without an *intI1* gene associated.

The resistance observed to sulfamethoxazole, tobramycin, amoxicillin/clavulanic acid and cephalotin, could therefore be justified by the genes found in this complex structure. Moreover, decreased susceptibility to ciprofloxacin and norfloxacin can be due to other mechanisms such as the presence of the *qnr* genes and efflux pumps. In fact, *qnrB10* gene was identified in this strain but is not associated with the class 1 integron. *qnrB10* gene is frequently found linked to the *ISCR*1 in different class 1 integrons. However in this study the attempt to link that association was unsuccessfull, suggesting a new genetic environment for this gene (Figure 23C).

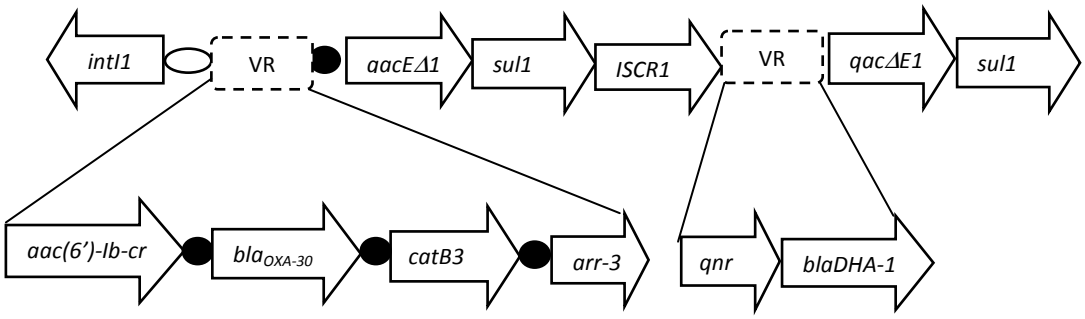
(A) *K. pneumoniae* KP1



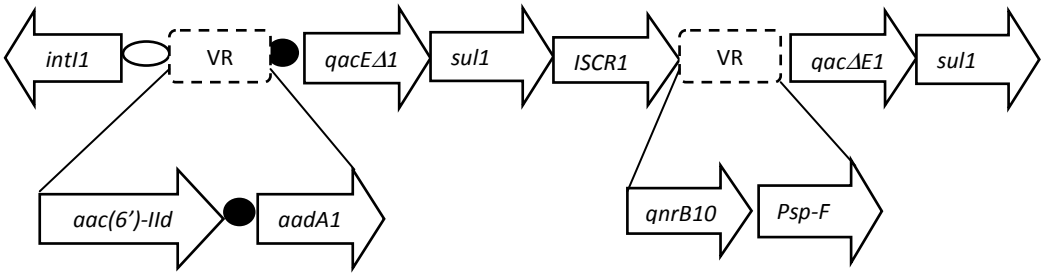
(B) *K. pneumoniae* strain M7943 (Argentina) In37::*ISCR*1::*qnrB6* (Quiroga, Andres et al. 2007)



E. coli plasmid pHSH2 (China) In37::*ISCR*1::*qnr blaDHA-1* (Wang, Tran et al. 2003)



(C) *E. cloacae* strain E705 In131::*ISCR*1::*qnrB*10 (Argentina) (Quiroga, Andres et al. 2007)



E. cloacae strain E701 In132::*ISCR*1::*qnrB*10 (Quiroga, Andres et al. 2007)

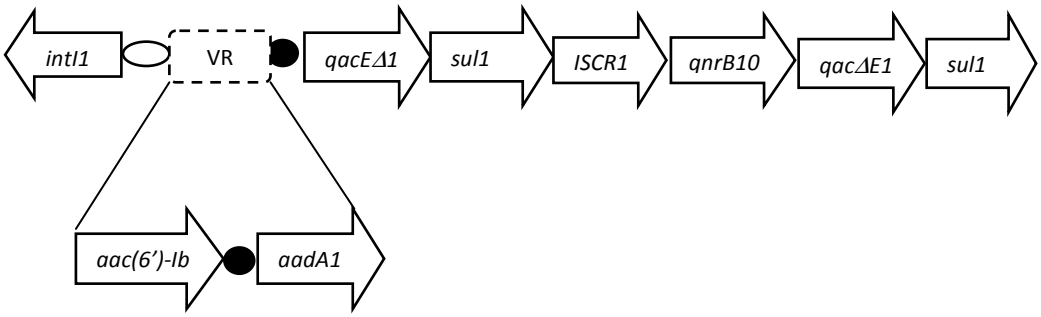


Figure 23 - (A) Schematic representation of *K. pneumoniae* KP1 complex class 1 integron reported in this study; (B) Schematic representation of others *K. pneumoniae* complex class 1 integrons previously found worldwide; (C) Genetic environment of the *qnrB*10 gene in different species.
The genes cassette are represented by boxed arrows indicating the transcriptional orientation. A white circle represents the *attI1* site and the black circles represent the genes cassette recombination site, *attC* element.

The integron location was investigated by Pulsed-Field Gel Electrophoresis (PFGE). Genomic DNA was digested with S1 (New England Biolabs, Beverly, Mass.), separated by electrophoresis with a CHEF-DRIII system (Bio-Rad, Hemel Hempstead, UK), and hybridized to 16S rDNA and *intI1* DIG labeled probes. A positive signal was obtained with both probes in different regions of the genomic DNA digested (Figure 24). This result

reveals that the complex class 1 integron is located in a plasmid with approximately 225 Kb. Plasmids are the primary vehicles for horizontal resistance gene transfer among Gram-negative bacteria (Lee, Peng et al. 2008).

Transfers of resistance genes by conjugation assays were attempted using *E. coli J53* Az^R strain as recipient. Selective media (Mueller-Hinton agar) contained 100 µg/ml sodium azide and 0.125 µg/ml ciprofloxacin were used to select *E. coli* transconjugants (Wang, Yang et al. 2008). Recipient strain was resistance to sodium azide and susceptible to ciprofloxacin, and Kp1 isolate was resistant to ciprofloxacin (≥ 4 µg/ml). Under the laboratory experimental conditions tested, no transconjugants were obtained.

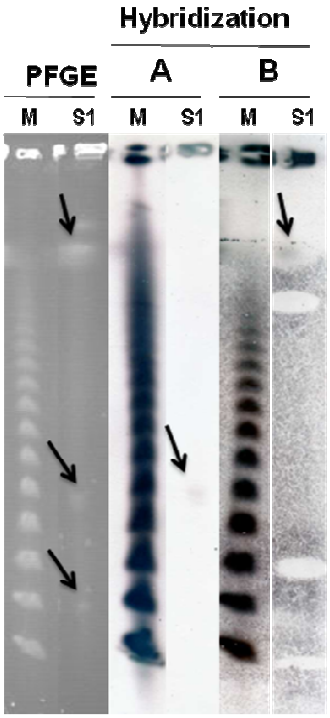


Figure 24 - Hybridization with DIG labelled *intI1* (A) and 16S rDNA (B) probes of S1 digested genomic DNA of *K. pneumoniae* Kp1 after separation by PFGE. Lane M: DNA size markers (λ concactamers).

This study demonstrates that *K. pneumoniae* Kp1 can act as a reservoir for antibiotic resistance within the hospital environment. The plasmid location of the integron represents a threat for the possible dissemination to other species. Since this isolate was collected from an inanimate surface of a toilet, and given its resistance profile it can pose some risk for debilitated patients, mainly because the infections caused by this microorganism are difficult to treat.

The nucleotide sequences reported in this paper is deposited in the GenBank under the accession number FJ459817.

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VI. General discussion

1. DISCUSSION

Integrons are natural expression vectors that permit the insertion and deletion of antibiotic resistance genes by a site-specific recombination mechanism. In the present study these structures were found in Enterobacteriaceae (*E. coli*, *K. penumoniae*, *P. mirabilis* and *M. morganii*) and in *Pseudomonas* spp. strains within the hospital environment. Gene cassettes contained within the class 1 integrons, demonstrates an association between integron carriage and a reduced susceptibility to some antibiotics such as aminoglycosides, trimethoprim and β-lactams. As reported by various authors, class 1 integrons have few gene cassettes inserted in the VR (Sally, Guy et al. 2009). In this study, this feature is also observed, since the class 1 integrons found in the isolates studied contained at the maximum four cassettes.

β-lactamases enzymes were detected in the majority of the isolates; however ESBLs are present at a higher percentage and in almost all of the species, while MBLs are only present in *Pseudomonas* spp. strains. According to various authors, this is frequently observed since ESBLs inactivate the most frequently used β-lactams, such as penicillins, first and second generation cephalosporin (L. Poirel 2008). MBLs are recent enzymes that inactivate the carbapenems, which are compounds less used in treatment of bacterial diseases. Our results show that in fact there is a relation between antibiotic usage and development of resistance mechanisms against that antibiotic as reported before (in page 10). High levels of resistance where observed to aminoglycosides, penicillins and to the first generation cephalosporins. Resistance observed to the other classes of antibiotics can be due to different resistance mechanisms such as efflux pumps, mutations and alteration in the membrane permeability.

The present work is clinically relevant, since it describes the presence of a high diversity of resistance determinants in Gram-negative bacteria collected in inanimate surfaces that are associated with integrons and β-lactamases. Among these determinants, novel arrays were found in different species that encode for enzymes involved in the inactivation / degradation of antibiotics. These structures contribute to the selection of resistant strains. Moreover these results show that the bacteria can survive and colonize different surfaces, remaining in the hospital environment for long periods (Caetano, Ferreira et al. 2007). Some of the resistance determinants described in this work are already deposited in the Genbank and were described before in isolates collected from biological samples of patients and animals. Thus, it may also be suggested that the bacteria present in inanimate surfaces can contribute for some of the nosocomial infections found, since some genes cassette found in this work were described before in biological samples.

In order to evaluate the potential of dissemination of these genetic determinants it is important to investigate their genetic location within the genome of the organism. Usually, class 1 integrons are associated with other mobile genetic elements, such as plasmids and transposons, and therefore can easily disseminate the genes cassette among different bacterial species, by HGT (Summers 2006). Moreover, MBLs usually is associated to the VR of the class 1 integrons, and therefore their dissemination is potentially faster than that of ESBLs, which can be plasmids or chromosomally encoded. Given the worldwide flow of people between cities, countries and even continents, bacterial clonal dissemination is possible and it became quite frequent to find the similar resistance gene array in different geographic locations. Integrons become the major contributors to this global problem of spreading antibiotic resistance genes at the clinical and environmental level. By these means opportunistic and commensals bacteria can become MDR and pathogenic (Stokes, Nesbo et al. 2006). Thus, according other studies (Martinez-Freijo, Fluit et al. 1998), the accumulation of resistance genes by integrons and the arise of β-lactamases enzymes is one explanation for the emergence of MDR strains of *Enterobacteriaceae* and *Pseudomonas* spp..

PCR mapping of genes inserted as cassettes into integrons will provide valuable information for studies of gene expression as it relates to the position of these genes within the integrons (Levesque, Piche et al. 1995). These studies as well as others previously described, can be useful epidemiological tools to study the evolution of multi-resistance plasmids and transposons, dissemination of these antibiotic resistance genes and molecular epidemiology of β-lactamases enzymes (Fiett, Baraniak et al. 2006).

This work constitutes a starting point for the future monitoring studies of resistance cassettes dissemination through integrons and also of β-lactamases enzymes in bacteria within this environment. It also shows the presence of opportunistic bacteria possessing a plethora of antibiotic resistance genes that can be passed to patients compromising the treatment of infections caused by these microorganisms. Results also showed the percentage of species and resistance genes that were found in this particular environment alerting for the implementation of measures to reduce the dissemination of bacteria and consequently resistance genes through inanimate surfaces within the hospital settings.

2. CONCLUSION

According to the main goal of the present work and based on the results obtained it is possible to conclude that:

Inanimate surfaces are colonized by different species of Gram-negative bacteria, which carried different resistance determinants.

Furthermore, the results showed:

. That the prevalence of the class 1 integrons is 33% and β-lactamases enzymes are ESBLs, 91% and MBLs, 9%, among the different species;

. That the 15 different arrays identified, 47% possess novel gene arrays never described before in the specie / worldwide; 53% were already described in different countries and species, but in biological samples from patients or animals; and 73% were found for the first time in Portugal.

. That the genes encoding resistance to the aminoglycosides were the most frequent (54%).

. That the resistance to most recently introduced antibiotics is increasing, namely to the carbapenems (7% VIM; and 2% IMP)

. That ESBL enzymes, mainly TEM (66%) and SHV (16%), were the most prevalent.

These results suggest that precautions should be taken to prevent the dissemination of these resistance determinants within this selective environment. The implementation of simple measures, such as regular disinfection of the surfaces combined with periodic surveillance control, will certainly contribute to avoid the dissemination of these bacteria among hospitalized patients.

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VII. Appendix

Appendix 1

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19th ECCMID

Occurrence and characterization of class 1 integrons from isolates collected in a hospital's sanitary facility in Aveiro, Portugal

Aim

The aim of this study was to investigate the presence of class 1 integrons in bacterial strains collected in a hospital female ward sanitary facility from Hospital Infante D. Pedro, Aveiro.

Introduction

In a hospital environment, arising of antibiotic resistant strains is not only confined to the patients but they can also colonize/survive in the surrounding environment. We investigated the presence of antibiotic resistance mobile elements, such as class 1 integrons in bacterial strains collected in a hospital female ward sanitary facility.

Methods

- Sterile swabs were rubbed in various sanitary facility surfaces and placed in rich medium (TSB); after incubation (37ºC-18 h) serial dilutions were inoculated in MacConkey agar and incubated (37ºC-18 h). Phenotypically different colonies were selected and their clonal relationship was evaluated by Box-PCR.
- Identification to the species level and antibiotic susceptibilities were determined using the automatic VITEK2 system and Advanced Expert System (VITEK2 AES) (BioMérieux, Marcy L´Étoile, France).
- Presence and characterization Class 1 integron was done by PCR followed by nucleotide sequencing. Nucleotide and deduced aminoacid sequences were analyzed with Blast and ClustalW programs.

Results

- 82 genetically different strains were identified, belonging to the following genera: *Acinetobacter*, *Alcaligenes*, *Citrobacter*, *Escherichia*, *Enterobacter*, *Klebsiella*, *Morganella*, *Pseudomonas*, *Proteus*, *Serratia* and *Stenotrophomonas*.
- Class 1 integrons were amplified from 24% of the isolates and 17 different gene arrays were identified. 35% of these arrays contained novel combinations of gene cassette and 41% were described for the first time in the specie.
- *aacA4* was the most abundant gene identified, followed by *aadA1* and *aadA2*. *dhfr*, bla_{PSE-1}, *arr-3*, *catB3*, *ORF* and *tnpA* were also detected, but at lower rates
- bla_{IMP-8} and bla_{VIM-2} genes were found exclusively in *Pseudomonas* spp.
- *tniC*-containing integrons were identified in a *P. putida* isolate.

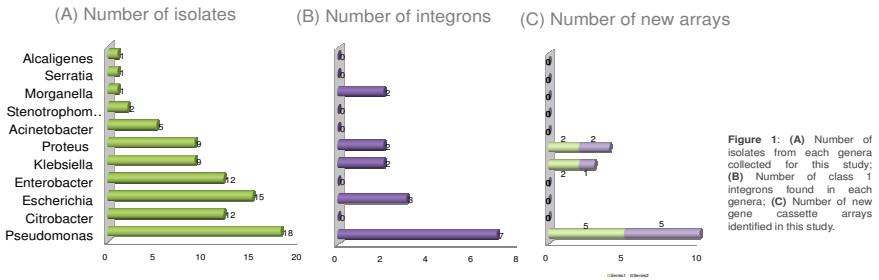

Figure 1: **(A)** Number of isolates from each genera collected for this study; **(B)** Number of class 1 integrons found in each genera; **(C)** Number of new gene cassette arrays identified in this study.

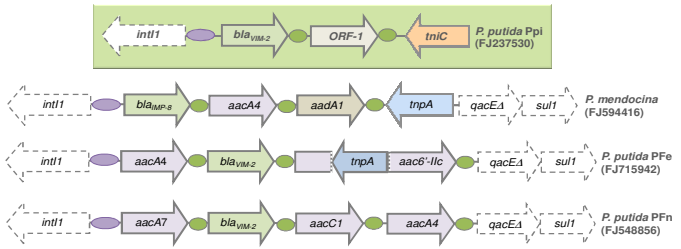

Figure 2: Schematic structure of some of new gene cassette arrays identified in *Pseudomonas* spp. strains. Emphasis is given to the first *tniC*-like integron found in Portugal .

Conclusions

- Class 1 integrons are widely distributed among opportunistic strains examined in this study.
- New integrons containing new gene cassette arrays were found.
- This is the first description of a *tniC*-containing integron in Portugal.
- Results suggest that strains harbouring class 1 integrons constitute part of the normal human flora, that are able to survive in hospital surfaces, constituting a possible source for the dissemination of antibacterial resistant strains between patients.

References

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